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Cytomegalovirus replicon-based regulation of gene expression *in vitro* and *in vivo*

vorgelegt von

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`Generation of a virus-resistant cell by dominant-negative genes´

SUMMARY

Conditional gene expression systems are valuable tools for herpesviral research. As yet, all available expression systems base on two features: first, an expression cassette with responsive elements and second, the inducer, i.e. a chemical compound or a protein. In this study, a novel inducible expression system has been developed, that bases on intrinsic features of herpesviruses, thus conditional expression is turned on by wild type virus infection. This has been achieved by exploiting the herpesvirus origin of lytic replication (oriLyt) of the murine Cytomegalovirus (MCMV) to activate the expression of adjacent genes. Cell lines carrying an episomal vector, with a reporter or transgene combined with the oriLyt were constructed. These oriLyt-based replicon vectors were silenced in uninfected cells; however, virus infection liberated the plasmids from histone-deacetylase-induced inactivation. Replication of the episome after infection led to a very strong induction of gene expression by up to 1.000-fold. This virus-inducible expression system opened a wide range of application possibilities.

A variety of diseases attributed to herpesvirus infections in livestock has a high economical impact. Vaccines have been developed; but several disadvantages limit their general application. Intracellular immunization is discussed as an option to vaccination. Hereby a viral dominant–negative protein is used to prevent herpesvirus dissemination in the animal cell. Previous attempts were unsuccessful due to strong side effects of the constitutively expressed transgenic dominant-negative proteins. An important step towards the implementation was achieved by constructing the replicon vector system, as transgene expression is activated by a wild type virus infection itself without using chemicals or genetically modified viruses. The results of the present study show that viral infection specifically activated the expression of a dominant-negative transgene, which in turn inhibited viral growth.

The *trans*-complementation of late herpesviral proteins is very demanding. Incorrect expression timing and insufficient transgene amounts hamper the successful production of *trans*-complemented viruses. Furthermore, isolated and high expression of viral proteins can be toxic for the cell. These problems were overcome with the replicon vector system. Using glycoprotein O and the transmembrane protein M50 it was demonstrated in this study that the system even enables the expression of toxic proteins.

Furthermore, the replicon vector was used to generate a transgenic mouse line (VIOLA). This mouse line expressed the reporter gene only upon infection with MCMV. The expression was inducible in explant cultures of the mouse but not *in vivo*. This interesting property of the VIOLA mice might be used to study herpesvirus-induced chromatin remodeling.

ZUSAMMENFASSUNG

Konditionale Expressionssysteme sind wertvolle Werkzeuge für die Forschung an Herpesviren. Bisher hatten alle erhältlichen Expressionssysteme eine gemeinsame Eigenschaft. Sie basieren auf zwei Bestandteilen: erstens einer Expressionskassette mit schaltbaren Elementen und zweitens einem induzierenden Molekül oder Protein. In dieser Arbeit wurde ein neuartiges induzierbares Expressionssytem entwickelt, dass auf natürlichen Eigenschaften von Herpesviren zurückgreift und damit durch eine Infektion mit Wildtyp-Viren angeschaltet werden kann. Dafür wurde der herpesvirale Ursprung der lytischen DNA-Replikation (oriLyt) des murinen Zytomegalievirus verwendet, um benachbarte Gene zu aktivieren. Zell-Linien wurden erzeugt, die ein Trans- oder Reportergen mit dem oriLyt auf einem episomalen Vektor enthalten. Diese oriLyt-basierenden Replikonvektoren wurden in nichtinfizierten Zellen inaktiviert; aber durch Virusinfektion konnten die Plasmide von der Histone-Deacetylase- abhängigen Stilllegung befreit werden. Die gleichzeitige Replikation des Vektors führte zu einer sehr starken Induktion der Genexpression um das tausendfache. Dieses neue Virus-induzierbare Expressionsystem eröffnet eine weite Reihe von Anwendungsmöglichkeiten.

Herpesviren sind wichtige Pathogene, die sowohl Mensch als Tier befallen. Durch Herpesviren hervorgerufene Erkrankungen verursachen erheblichen ökonomischen Schaden in der Nutztierhaltung. Impfstoffe für Nutztiere wurden entwickelt; mehrere Nachteile schränken jedoch ihre allgemeine Anwendung ein. Als Alternative ist das Konzept der intrazellulären Immunisierung denkbar. Hierbei wird in den tierischen Zellen ein virales, dominant-negatives (DN) Transgen verwendet, das die Ausbreitung der Infektion verhindern soll. Bisherige Versuche schlugen aufgrund der starken Nebeneffekte der konstitutiv exprimierten transgenen DN Proteine fehl. Ein wichtiger Schritt zur Implementierung wurde durch die Konstruktion der Replikonvektoren erreicht, da die Transgene durch die Infektion mit dem Wildtyp-Virus aktiviert werden, ohne die Notwendigkeit chemische Verbindungen oder genetisch modifizierte Viren zu benutzen. Die Ergebnisse dieser Studie zeigen, dass eine Infektion die dominant-negativen Transgene spezifisch anschaltet und dabei die Ausbreitung des Virus verhindert.

Die *Trans*-Komplementierung von späten herpesviralen Proteinen stellt eine Herausforderung dar. Die erfolgreiche Produktion von *trans*-komplementierten Viren wird durch mangelnde Transgene Menge und falsche Expressionszeitpunkte beeinträchtigt. Außerdem kann die isolierte und hohe Expression von viralen Proteinen toxisch für die Zelle sein. Diese Probleme wurden durch das Replikonvektor-System gelöst. Mit Hilfe des Glycoproteins O und des transmembranen Proteins M50 konnte in dieser Arbeit gezeigt werden, dass das Replikonsystem sogar die Expression von toxischen Proteinen ermöglicht.

Schließlich wurde der Vektor zur Erzeugung einer transgenen Mauslinie (VIOLA) verwendet, die das Reportergen Luciferase abhängig von der viralen Infektion mit MCMV exprimiert. Die Expression war in Explant-Kulturen, aber nicht in der lebenden Maus induzierbar. Diese bemerkenswerte Eigenschaft der VIOLA-Maus könnte eventuell zur Untersuchung von Herpesvirus-bedingter Chromatin Umstrukturierungen dienen.

1 INTRODUCTION

1.1 Herpesviruses

Herpesviruses comprise a very large class of double-stranded DNA viruses, with over 200 different species identified so far. They possess a broad host spectrum, as almost if not all vertebrates and even some invertebrates analyzed to date harbor at least one specific herpesvirus [1]. Still, a common feature of herpesviruses is their primary association with one particular host species. The ongoing identification of new virus species led to a re-classification of herpesviruses by the International Committee on Taxonomy of Viruses in 2009. The old family of herpesviridae is now split into three families and incorporated in the new order herpesvirales [2] (Figure 1).

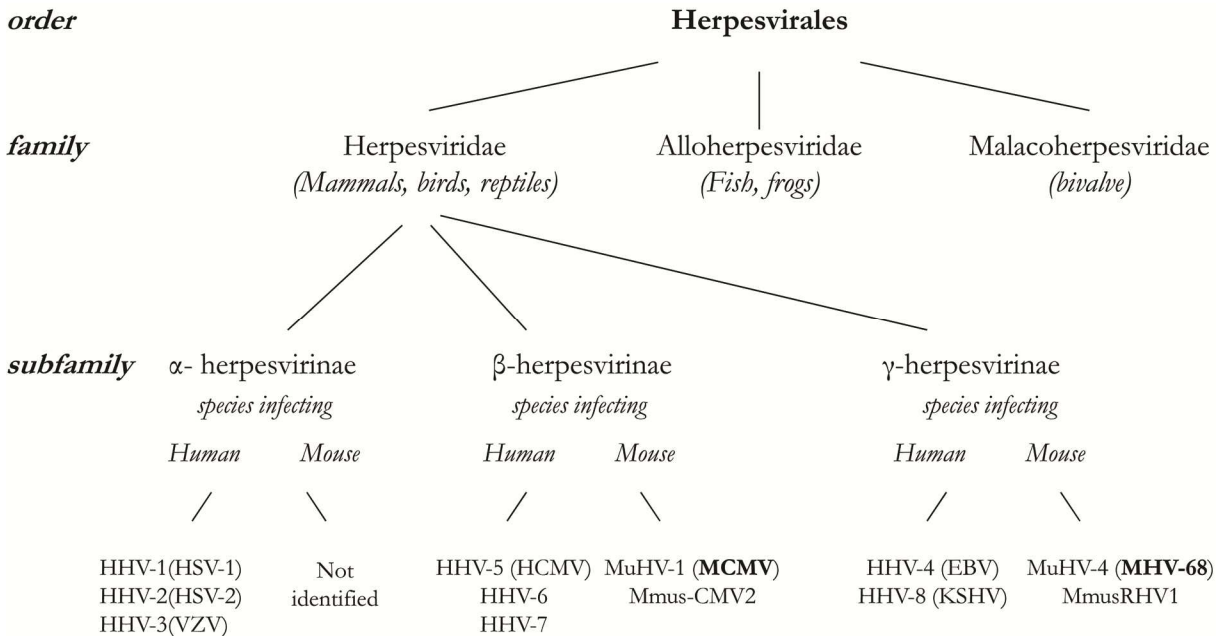


Figure 1: Taxonomy of herpesviruses

Due to newly identified species the herpesvirus taxonomy has been updated in 2009 [2]. The order of herpesvirales is now divided into three families. The most important family, the herpesviridae, is grouped in three distinct subfamilies. For each of the subfamilies viruses infecting humans have been identified. Shown are also species infecting mice with the exception of the species MuHV-3, MuHV-5 and MuHV-6, which are not assigned to any subfamily yet. Up to date no alpha-herpesvirus infecting mice were isolated.

The ability to establish a life-long latency after primary infection is a hallmark of herpesvirus infection [3]. Recurrent infection can emerge by reactivation from this silent state, in which only few herpesviral genes are expressed. Herpesvirus infection, in general, leads to a mild,

asymptomatic course through the long evolutionary adaptation of virus and host immune system [4]. However, severe manifestations of disease can arise in case of primary infection, re-infection or reactivation of immunocompromised hosts, as elderly, during pregnancy or immunosuppression in correlation with solid or bone marrow transplantation. Moreover, some herpesviruses possess also oncogenic potential [5].

The medical and economically most relevant family is the family of herpesviridae that infects mammals, birds and reptiles. The herpesviridae are divided into three major subfamilies, namely α-, β- and γ-herpesvirinae. Herpesviruses infecting and causing disease in humans can be found in any of these subfamilies. These are herpes simplex virus 1(HSV-1; above all herpes labialis, encephalitis, keratoconjunctivitis), herpes simplex virus 2 (HSV-2; herpes genitalis), varicella-zoster virus (VZV; chicken pox, zoster/shingles), human cytomegalovirus (HCMV; hepatitis, pneumonia, leucopenia, gastrointestinal disease, retinitis), human herpesvirus 6 (HHV-6; Roseola infantum), human herpesvirus 7 (HHV-7, Roseola infantum), Epstein-Barr virus (EBV; infectious mononucleosis, Burkitt's lymphoma) and Kaposi's sarcoma-associated virus (KSHV; Kaposi's sarcoma, morbus Castleman). The grouping to one of the subfamilies is largely based on their tissue tropism as well as characteristics of productive infection [5]. The members of the α-herpesvirinae possess a broad cell tropism and reside in their latent stage mainly in sensory neurons of trigeminal ganglia [6]. They can infect several host cell cultures *in vitro* and possess a short and effective replication cycle leading to rapid destruction of infected cells [1]. γ-herpesvirinae infect fewer cell types productively and reside primarily in lymphoid and myeloid cells in their latent stage causing lymphoproliferativ diseases [7]. Characteristic for the β-subfamily is a long replication cycle and relatively broad cell tropism. Latently infected cells are often found in cells derived from the myeloid lineage [8], but many other cell types seem to harbor latent genomes as well [9]. A subgroup of the β-herpesviruses, the cytomegaloviruses (CMV), is of major clinical importance. CMV's are named in accordance with the appearance of greatly enlarged cells with intracellular and intranuclear inclusion bodies. Human CMV (HCMV) plays an important role in infection of immunocompromised individuals such as transplant or AIDS patients, and infection of the fetus or newborn. Congenital infection leads to long-lasting health problems in 50 % of the cases as mental retardation, hearing or vision loss, growth problems and others [10]. Hence, calculating the cost of congenital acquired HCMV disease to the health care system and the threat to human health, the Institute of Medicine selected HCMV to the most needed viral vaccine [11].

1.2 Herpesvirus morphology

All herpesviruses share the same morphology with an icosahedral capsid embedded in a proteinaceous layer called the tegument and are surrounded by a lipid envelope covered by several glycoproteins (Figure 2). The linear double-stranded DNA (ranging from 124-230 kb in length) is packed in form of a torus [12] and encodes between 70 - 230 genes, depending on the subfamily. The icosahedral capsid is about 100 nm in size, and consists of 162 capsomers. Four proteins shape the capsid. The major capsid protein (MCP) forms pentons and hexons that are connected by trimers, which are built by the minor capsid protein and minor capsid binding protein [13]. These are then decorated by the smallest capsid protein (SCP), which is the least conserved of the capsid proteins [14]. While SCP is essential in β-herpesvirinae it is dispensable in other subfamilies.

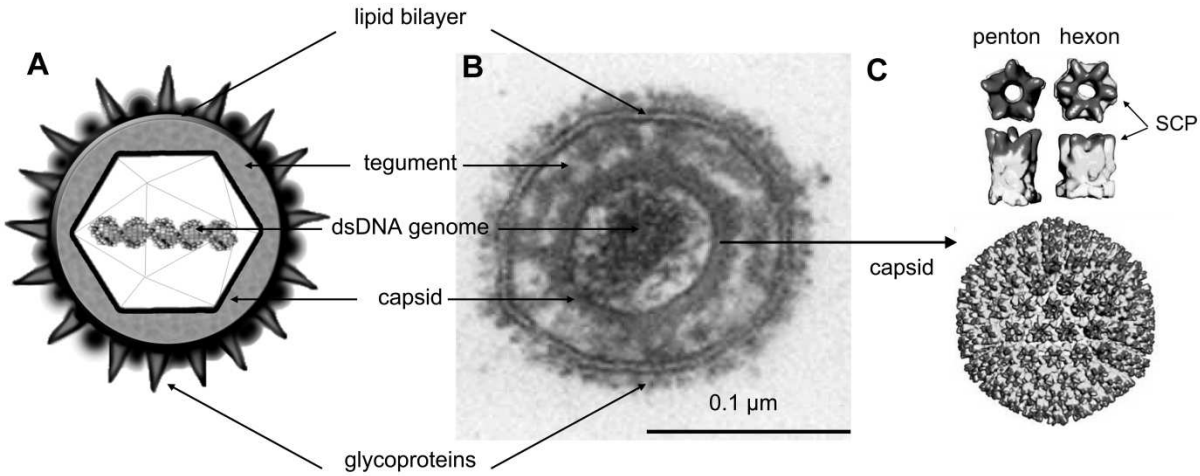


Figure 2: Morphology of a herpesvirus virion

A) Schematic illustration and B) positive stain electron microscopic picture of a herpesvirus virion. (modified from [15]). C) Reconstitution of a herpesvirus capsid from cryo-electromicroscopy. The position of the small capsid protein (SCP) on the tips of hexon and penton bases is depicted in dark grey (modified from [16]).

The capsid is surrounded by the tegument, a protein layer that consists of several proteins (and some RNA), and might possess an ordered layered structure [17]. These proteins modulate host functions without the need of prior viral protein synthesis. They help the virus to evade the immune system and to start its own gene expression [18]. Herpesviruses acquire their final envelope, which is covered with a plethora of different glycoproteins and – complexes [19]. The number, amount and type of glycoproteins varies between different herpesviruses. In total the mature virion reaches a size of 120 – 260 nm.

1.3 Herpesvirus replication cycle

Herpesviruses enter the cell by different ways and therefore not only one specific but many receptors have been identified that might play a role in attachment [20, 21]. Furthermore, fusion of the envelope with the plasma membrane or a membrane of the endosome can be used depending on the cell type to be infected [22, 23]. Right after entry, the tegument proteins hide the virus from the immune system, fend off apoptotic mechanisms and, in some cases, shut off host protein synthesis [4]. Using other tegument proteins, capsids travel along microtubules to the microtubule organizing center (MTOC), from where they are further transported to nuclear pores. There, capsids partially disassemble and inject the viral DNA into the nucleus [24]. Inside the nucleus either latent or lytic replication cycle is started. In the lytic cycle, immediate early proteins are expressed stimulated by proteins brought by the virion [25] or are directly transcribed by the host RNA polymerase II [26]. These immediate early proteins start the cascade of a precisely regulated gene expression [27]. Early gene products are triggered by immediate early viral transcription factors and thus are not or little transcribed without the presence of these viral factors.

Early gene expression leads a. o. to amplification of the viral DNA producing long and branched concatemeric DNA (see chapter 1.4). Recently, the dogma of the mechanism of herpesviral DNA replication has been challenged. At present, it is unclear whether linear or circularized genomes serve as templates. After viral DNA replication has started expression of true-late genes is induced [28, 29]. The concatemeric DNA is cleaved in unit length genomes and is immediately packed into the freshly assembled capsids. Capsids egress from the nucleus by budding through the nuclear membrane — the first envelopment and de-envelopment process [19]. In the cytoplasm, the tegument assembles around the capsids and gain their final glycoprotein covered envelope by budding into the TGN [19]. Mature virions are released by fusion with the plasma membrane (see Figure 3).

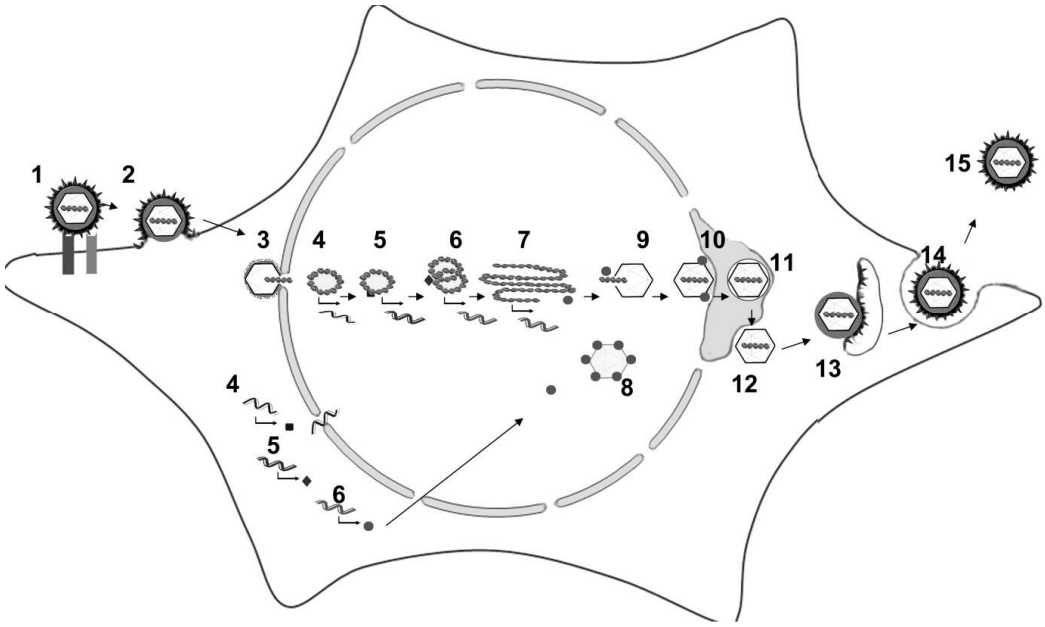


Figure 3: Schematic model of a herpesvirus replication cycle.

(1) Herpesvirus particles bind via glycoproteins to largely undefined receptors at the cell membrane. (2) Entry follows after fusion of the envelope with the plasma membrane (in fibroblasts) or via receptor mediated endocytosis (not depicted here). (3) Capsids travel via microtubules to the MTOC and then to nuclear pores, where the viral genome is released into the nucleus after partial destabilization of the capsid. (4) The viral genome circularizes and the cascade of gene expression starts with immediate early genes being transcribed, (5) which in turn activate early genes. Early gene products drive a. o. (6) the viral DNA replication and subsequently late gene expression is started. At the beginning viral genomes are amplified via a theta-replication or a recombination dependent replication. Replication continues probably by rolling circle amplification. (7) Long branched concatemers are finally produced. (8) Several late proteins build the viral capsids that (9) are packed with linear unique length genomes. (10) Nuclear capsid bud through the inner nuclear membrane, (11) leading to enveloped capsids in the perinuclear space, which are (12) released into the cytoplasm by a de-envelopment step. (13) Tegument proteins assemble around the capsid in the cytoplasm and particles are targeted to the TGN, where they acquire their final envelope covered with viral glycoproteins. (14) Exocytotic vesicles fuse with the plasma membrane and thereby (15) release the mature enveloped virion into the extracellular space.

1.4 DNA replication of herpesviruses

As herpesviruses have two alternative lifecycles (latent and lytic), their DNA replication has adapted to these different needs. During lytic replication a high number of viral genomes is produced. Many viral as well as some cellular proteins are actively involved in the replication of viral DNA, which is initiated from the origin of lytic DNA replication (oriLyt). Most knowledge of the mechanism of herpesviral DNA amplification arises from work on HSV-1, while little information is available about this mechanism in other herpesviruses. In general the DNA replication follows two essential steps. The initiation of DNA replication is the first step. A variety of mechanisms to recognize the origin of replication, to dissociate and open the double helix, and to recruit the replication proteins are exploited by herpesviruses. The second part, the DNA synthesis step however seems to be conserved.

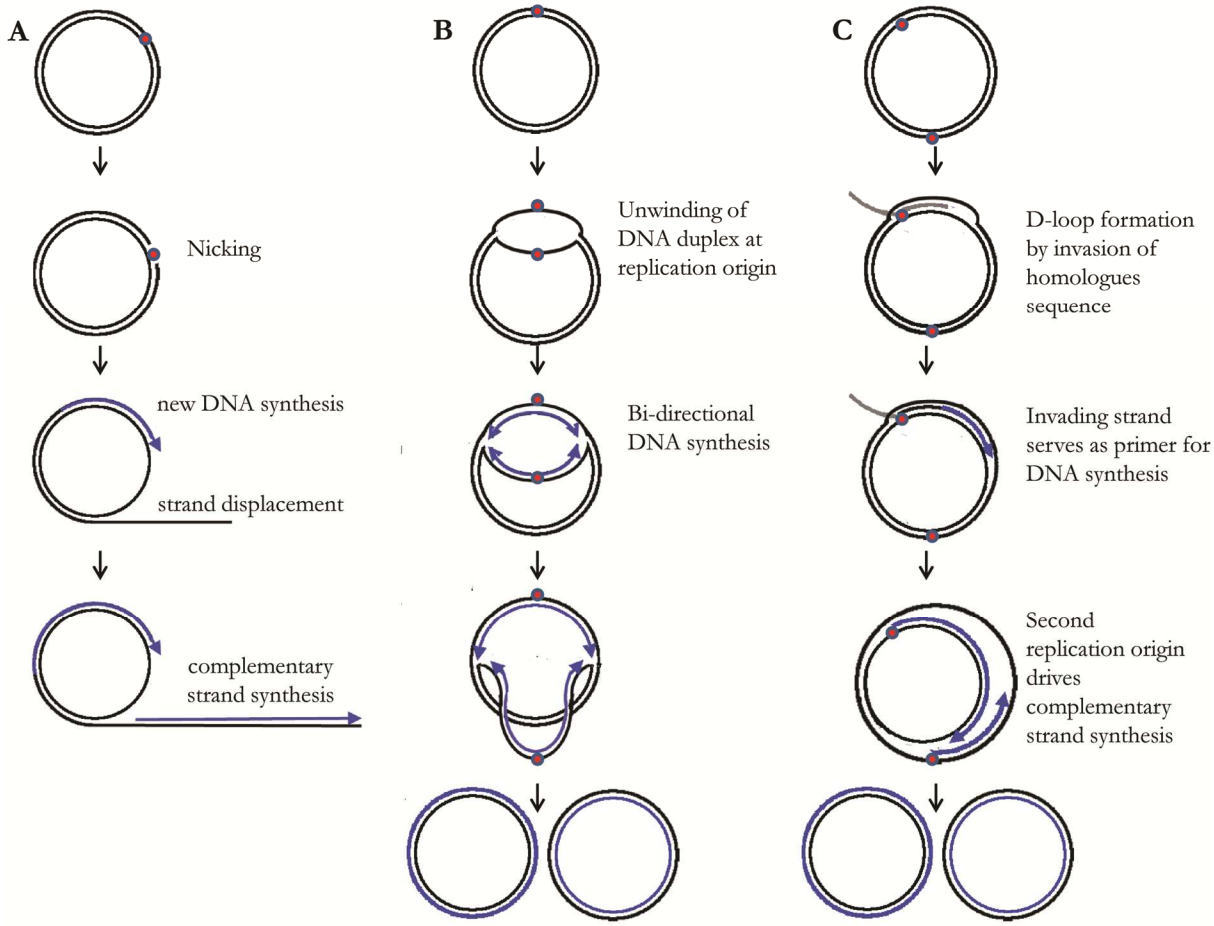


Figure 4: Types of DNA replication

Three types of DNA replication are discussed for herpesvirus genome amplification. A) Rolling Circle Amplification. The DNA strand is nicked and new DNA is synthesized on the 3' end, while the 5' end is displaced from the heteroduplex. The complementary strand is synthesized with the displaced strand as template. This type of replication can produce long concatemeric genomes. B) Theta-replication. At an origin of replication specialized protein initiate unwinding of DNA. Replication of the DNA is bi-directional. Thereby two circular daughter genomes are produced. C) D-loop (or Displacement-loop) Replication. A DNA strand invades the double helix and replaces the complementary strand, resulting in a short stretch of a triple helix. This serves as a primer for DNA synthesis. A second replication origin is activated as the D-loop expands and synthesizes the complementary strand. Two circular genomes are produced by the D-loop like replication.

The commonly accepted model for herpesvirus DNA amplification involves the formation of circular genomes after entry of the genome into the cell. Theta-replication and subsequent rolling circle amplification are thought to generate highly concatemeric and branched genome intermediates [30]. While the existence of head-to tail concatemers and the ability to amplify oriLyt-containing plasmids provide proof for the existence of a rolling circle amplification phase, there is no experimental evidence to support the theta-replication mode [31] (for comparison of the replication types see Figure 4). However, this theory explains the dependence on cellular topoisomerase II at the early amplification stages and the non-linear kinetics [32]. Rolling circle amplification alone cannot explain the highly branched viral

concatemeric genomes. Theoretical explanations could be redundant initiation, recombination intermediates or strand evasion; however unambiguous evidence for these mechanism is lacking [31]. While data are conflicting, most experimental evidence points to circular genomes as templates for DNA replication [32]. Wilkinson and Weller proposed the involvement of homologous recombination and D-loop formation as a model for DNA initiation followed by rolling circle amplification [33], which better explains the observed branched structures then the old model.

1.4.1 Proteins required for herpesviral DNA replication

The core machinery of the lytic DNA replication consisting of six proteins is conserved in all herpesviruses and resembles functional analogues of eukaryotic replication proteins [34]. These are a DNA polymerase with a processivity factor, three proteins building a helicase-primase complex and one single-stranded DNA binding protein. However, the initiation of lytic DNA replication and the sequences and structures of oriLyts are highly diverse between the subfamilies. Furthermore, the mechanisms of initiation of DNA replication seem to be as diverse as the replication origins themselves. Origin binding or origin activating proteins (OBP) are also not conserved — speaking for a co-evolution of activating proteins and origin sequences [35, 36]. Remarkably, the core set of replication proteins can be exchanged by herpesviruses of another class, yet, the origin binding/activating protein and the origin of replication must belong to the same herpesvirus [36].

1.4.2 Structure of herpesviral replication origins and initiation of DNA replication

1.4.2.1 Structure of replication origins of α-herpesviruses

Not only the structure, but also the number of replication origins is variable among the subfamilies. Herpesviruses of the α-subfamily possess three replication origins, according to their positioning in the genomic segment. It is unclear, why α-herpesviruses harbor three origins, as any of these origins suffices for replication and reactivation from latency [37-39]. In contrast, β-herpesvirinae harbor only one oriLyt and γ-herpesviruses encode 2 to 3 replication origins.

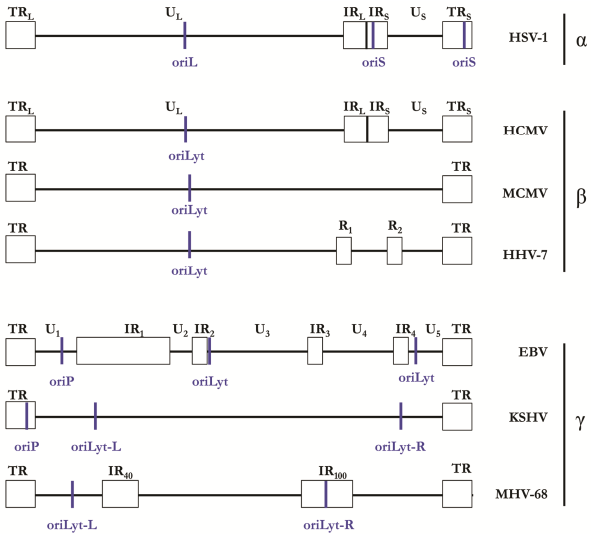


Figure 5: Structure of various herpesviral genomes and position of replication origins

Depicted is the overall organization of various α-, β- and γ- herpesviral genomes. Terminal Repeats (TR), Internal Repeat (IR), Unique Region /long /short (U, U_L, U_S), Repeat (R).

All three origins of α- herpesviruses represent compact short palindromic sequences. The two oriS are identical, whereby one of them might have been acquired due to duplication of the terminal sequence. Moreover, most of the α-herpesviruses use the conserved sequence GTTCGCAC as binding site for the origin binding protein. This motif can be found in high affinity binding sites (Box I) and low affinity binding sites (Box II and Box III). The first step in HSV-1 origin licensing, meaning origin activation, is the binding of protein OBP (UL9) as a dimer to 10 bp motifs in Boxes I,II and III [40]. Although UL9 contains a helicase domain, ATP-dependent unwinding of DNA could not been shown on long double-stranded DNA stretches [34]. UL9 works rather in a corporate fashion with the single-strand DNA binding protein UL29 (alias ICP8) and forms a hairpin structure by complementary base-pairing of Box I and Box III [34, 41]. The binding of UL9 might thereby result in bending, distortion and destabilization of the superhelicity of the origin [41]. Changes in the conformation and the strand separation could then allow binding of the helicase/primase complex UL5/UL52/UL8 to the origin, as well as the recruitment of the other replication enzymes [42](Figure 6). Leading and lagging strand synthesis is then facilitated by the herpesvirus encoded DNA polymerase, which can be specifically blocked with nucleoside analogous like phosphonoacetic acid [43].

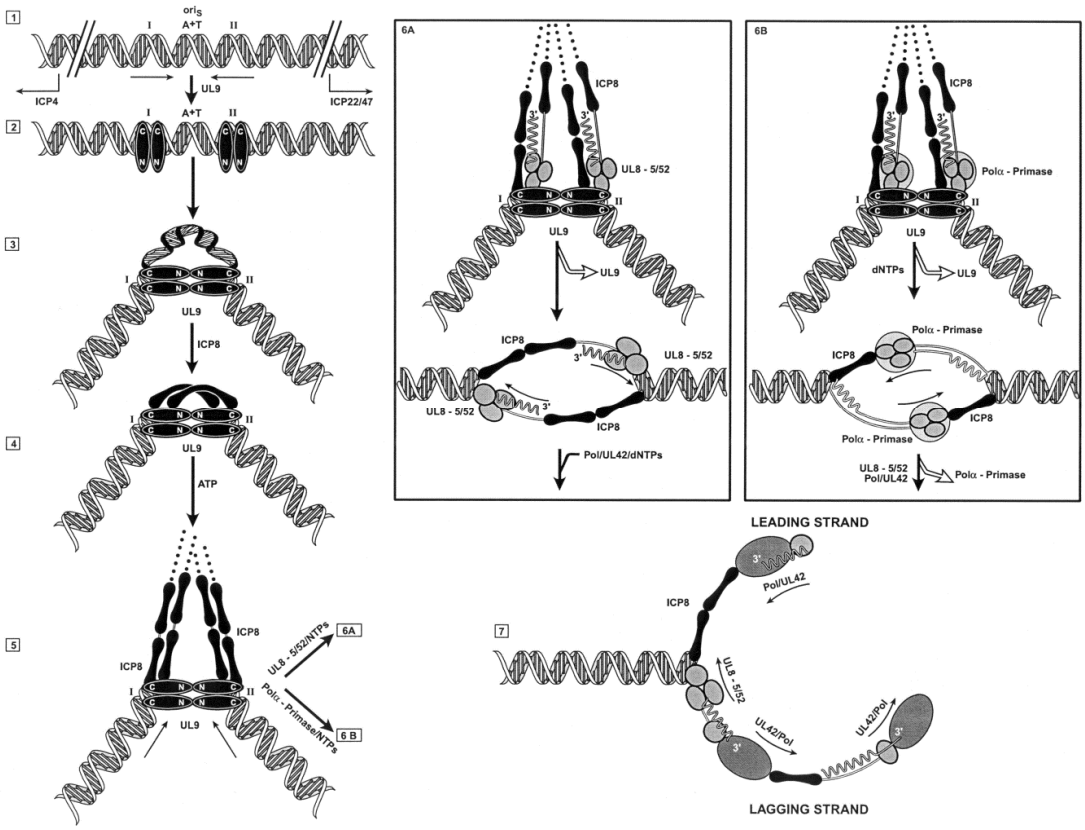


Figure 6: Model of herpes simplex virus type-1 DNA replication (from [44])

1–3: successive binding, looping, and distortion of oriS by the UL9 protein (UL9). Boxes I, II, represent the UL9 protein recognition sites. The converging arrows indicate the relative orientation of Boxes I and II. The diverging arrows indicate transcription from the ICP4 and ICP22/47 promoters. *4*: binding of ICP8 to the UL9 protein and distorted DNA. *5*: ATP-dependent DNA unwinding that generates ICP8-coated DNA strands. *6A*: recruitment of DNA helicase-primase (UL8-5/52) by UL9 protein followed by primer synthesis (*curved line*) and dissociation of UL9 protein. *6B*: recruitment of DNA polymerase -primase (Pol α-Primase) by UL9 protein followed by primer synthesis (*curved line*) and elongation, and dissociation of UL9 protein and DNA polymerase-primase. *7*: Unwinding of the DNA replication fork and lagging-strand priming by the DNA helicase-primase. Leading- and lagging-strand DNA synthesis promoted by the HSV-1 DNA polymerase (Pol/UL42). The arrows indicate the direction of translocation of the DNA replication proteins or that of the DNA.

1.4.2.2 Structure of replication origins of β-herpesviruses

With respect to their replication origins the subfamily of β-herpesviruses is divided into the roseoloviruses (HHV6 and HHV7) and CMVs. While the roseoloviruses encode an OBP and the respective recognition site homologous to the α-subfamily, CMVs harbor a unique replication origin with no homology to any herpesviral replication origin of the other subfamilies. Furthermore, no classical OBP could be identified. Compared to the minimal sequence of the replication origins of HSV-1 of 45 bp or 144 bp respectively, the minimal length for CMVs oriLyt sequences are much larger, with 1.4 kb for HCMV [45], 1.7 kb for murine cytomegalovirus (MCMV) [46] or 1.3 kb for simian cytomegalovirus (SCMV) [47]. In HCMV, additional sequences flanking the oriLyt up to 4 kb in total length increase the replication efficiency *in vitro*

[45-48]. The analysis of sequence requirements of the HCMV oriLyt in the viral context furthermore revealed that besides this quite large minimal oriLyt sequence the accessory regions in the viral genome are absolutely required to produce infectious viruses [49].

The overall organization of the CMV oriLyt sequence is asymmetric and at least two domains can be distinguished. In domain I a high number of direct (DR) and indirect repeats (IR), as well as palindromic sequences and A/T rich regions of varying length can be found. It also contains an unrepeated essential stretch of pyrimidine residues, coined the Y-block [48, 50]. Furthermore, the oriLyt sequences includes several consensus motifs for cellular transcription factor binding sites (Sp1, CRE, MLTF/USF) [48]. The most extensively studied CMV oriLyt, the HCMV oriLyt, was shown to include an essential promoter element that is responsive to the IE2 and UL84 protein [51, 52]. This element can be substituted by an SV40 early promoter, indicating that transcription is important for the functionality of the oriLyt [52]. The promoter element was suggested to be bidirectional as the orientation relative to the second domain was not relevant for activity of the oriLyt. Domain II is less complex and very G/C rich. Moreover, the HCMV oriLyt harbors a stable DNA/RNA hybrid structure with stem loops [53], which are bound by the UL84 protein [54]. Binding of UL84 was proposed to change the conformation of this area [54]. This resembles the UL9 induced hairpin-formation and duplex-distortion of HSV-1. The function and necessity of UL84 is discussed controversially, as in some experimental set ups and virus strains the presence of UL84 was essential [55] and in others not [56].

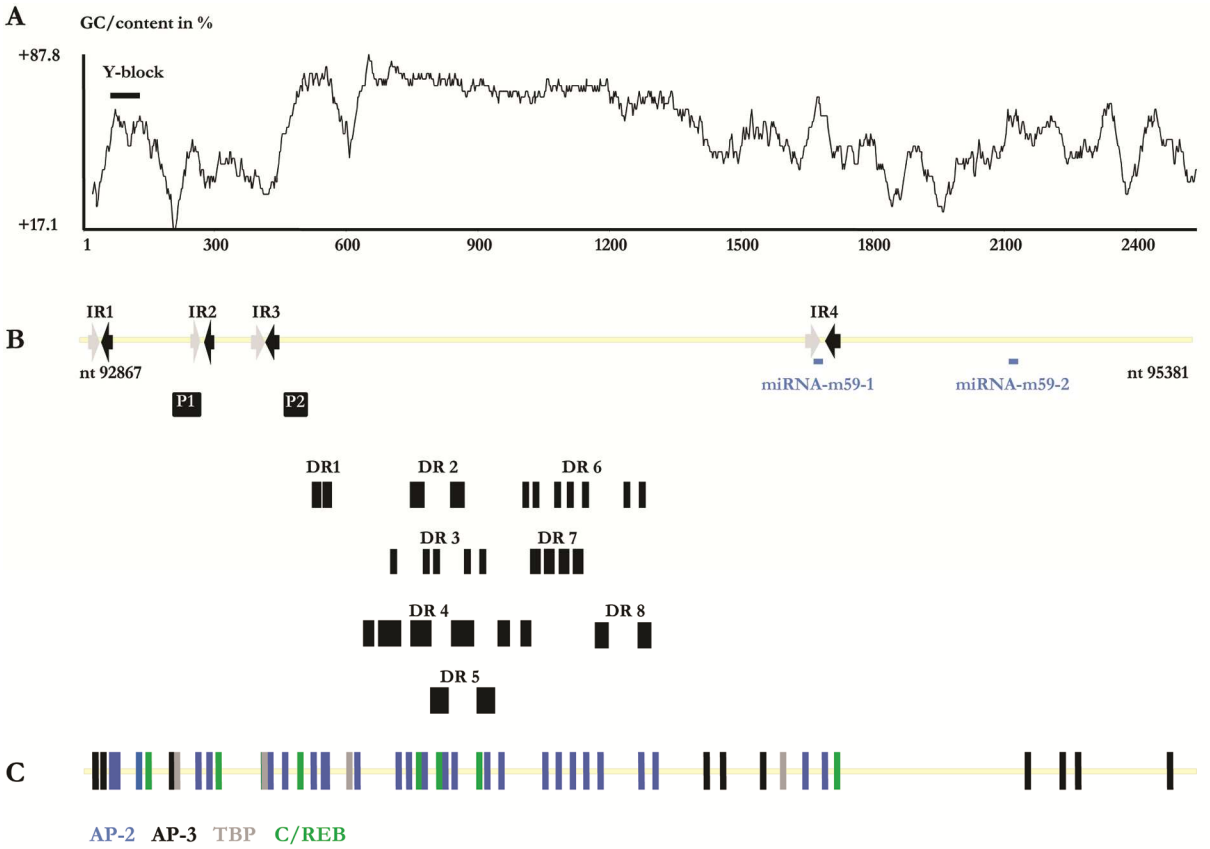


Figure 7: Organization of the MCMV replication origin

Depicted is the overall organization of the MCMV oriLyt. A) GC-content of the minimal oriLyt region (nt position according to pSM3fr).The Y-block has been annotated according to sequence alignment with RCMV and HCMV. B)The oriLyt of MCMC harbors many indirect (IR) and direct repeats (DR) and palindromic sequences (P). C) The oriLyt contains many transcription factor binding sites e.g. for AP2, AP3, C/REB and TBP (predicted by the program PROMO [57].

The oriLyt sequence of MCMV harbors many direct and indirect repeats, palindromic sequences and transcription factor binding sites. These are structural elements which are also present in HCMV, yet there is no sequence homology between MCMV and HCMV oriLyt. The proteins necessary for oriLyt-dependent DNA replication of MCMV are not yet identified. Although it has been proposed that it might be the homologue of HCMV, there is no experimental evidence. However, the core replication components are conserved. Concerns arise, whether the M84 protein of MCMV is a real homologue to UL84 as it is only distantly related and not essential for replication in cell culture [58, 59]. However, no other MCMV protein shares significant homology to UL84 either. The question if the MCMV oriLyt harbors a RNA/DNA hybrid like the human one has not been addressed so far.

1.4.2.3 Structure of replication origins of γ-herpesviruses

In contrast to α- and β-herpesviruses, most γ-herpesviruses have clearly specialized replication origins for their latent or lytic replication. The most studied γ-herpesvirus is the Epstein-Barr-Virus (EBV). Its latent replication origin oriP leads to a one-per-cell-cycle replication of the circular EBV plasmid, mediated by the viral protein EBNA-1 and the cellular replication machinery. Additionally, the EBV genome encodes two lytic replication origins. Similar to HSV-1 the reason for several lytic replication origins is not clear. The mechanisms of latent and lytic replication have little in common and possess independent *cis*- and *trans*-activating factors pointing to separate mechanisms [60]. There is no sequence homology of the oriLyt of EBV to those of the α-herpesvirus subfamily, but some features resemble structures of the β-herpesvirus subfamily.

The murine herpesvirus 68 (MHV68) serves as a mouse model for γ-herpesvirus infection. MHV68 does not encode a classical oriP but harbors two different lytic replication origins. A 1.25 kbp minimal oriLyt sequence is located at the right end of the linear genome within the M5 - M6 region [61], whereas the second one is 600 bp in length and locates in the M10 locus on the left side of the genome [62, 63]. The homologous elements of both oriLyt sequences revealed several essential CCAAT boxes, as well as auxiliary 40 bp GC-rich repeats at the 3' end and an AT-rich palindrome [62]. Proteins necessary for the activation of MHV68 oriLyts have not been identified, yet.

1.5 Genetic tools to study herpesvirus biology

Cloning the herpesvirus genomes into bacterial artificial chromosomes (BAC) has paved the way for modern herpesvirus genetics [64] (see Figure 8). Thus modifications to the herpesviral genome can be prepared in the bacterial cell and ongoing improvements to the reverse genetic tool set enable now any mutation to be introduced into the herpesviral genome.

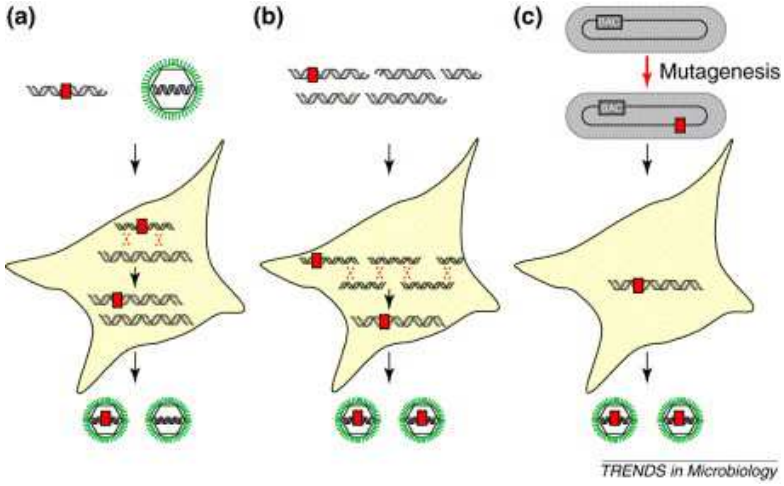


Figure 8: Methods of herpesvirus mutagenesis.

A) Site-directed mutagenesis in eukaryotic cells. A linear DNA fragment containing the mutation and a marker gene (red box) flanked by sequences homologous to the viral target sequence, is transfected into virus-infected cells. By homologous recombination (dashed lines) the marker gene is inserted into the viral genome. Recombinant viruses and wild-type viruses need further separation. B) The cosmid approach. Overlapping fragments spanning the entire virus genome are cloned as cosmids. A mutation is introduced into one fragment (red box). After transfection into permissive cells, the virus genome is reassembled by several homologous recombination events generation the mutant virus progeny. C) The principle of viral bacterial artificial chromosome (BAC) mutagenesis. The viral genome is maintained as a BAC in E.coli. Mutant viral BACs can be generated using various mutagenesis approaches /red arrow). Recombinant viral BAC DNA is transfected into cells and the mutant virus progeny is reconstituted. (from [65])

Observing phenotypes caused by mutation of herpesviral genes and comparison to wt allow delineating their function. To correlate a phenotype to the introduced mutation needs always the controls of reverted mutants to exclude that the observed phenotype is due to unwanted side effects during the cloning procedure. At the moment, routine sequencing of a complete herpesviral genome is not cost-effective and restriction pattern analysis and southern blotting may not allow the detection of all mutations [66]. Therefore, the reversion of the introduced mutation, which should lead to the wt phenotype, is an appropriate method. Various complementation methods have been developed for this purpose (see figure Figure 9). *Cis*-complementation, whether it takes place in bacteria or the host cell, exchanges the mutant allel with the wt allel on its original position. This bears some risks if other genetic elements span the region of interest. Non-annotated overlapping genes, which might have been destroyed by the mutation, will be corrected by this way as well; therefore this procedure does not absolutely allow the correlation of the mutation and the phenotype, but helps to exclude spontaneous mutation on other positions in the genome.

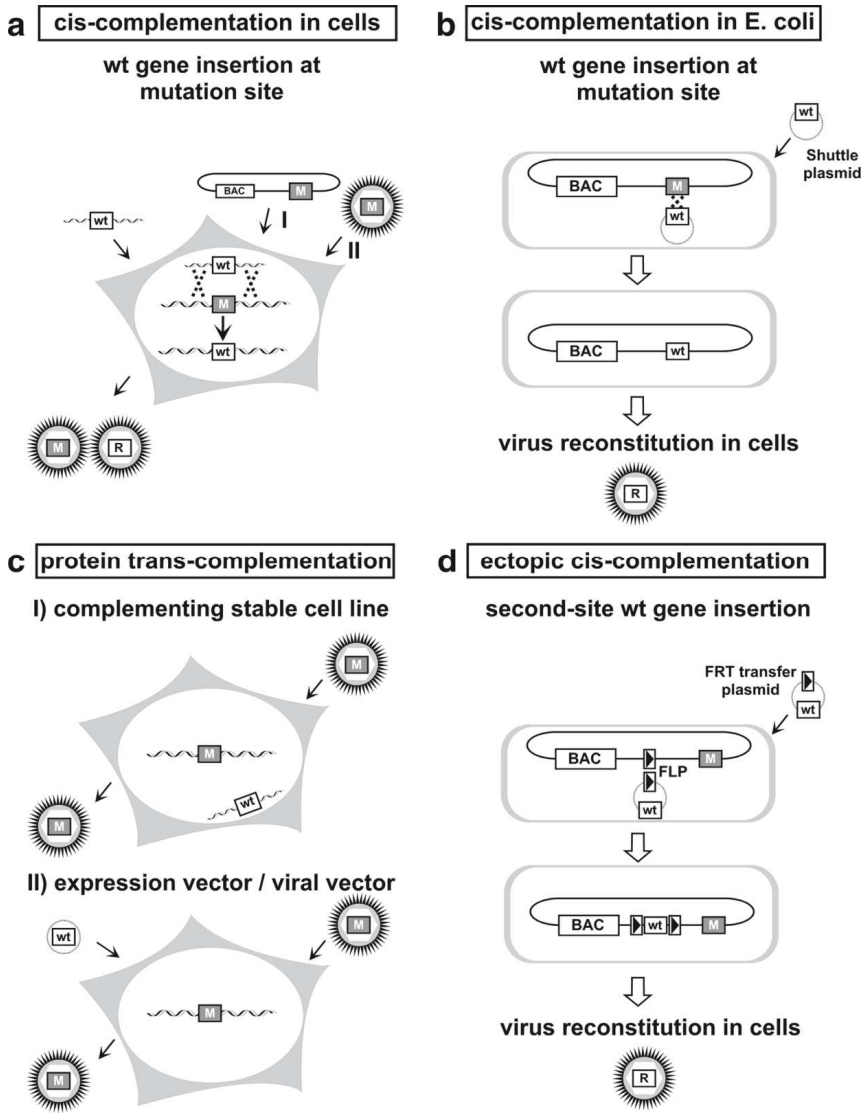


Figure 9: Different approaches for confirmation of the mutation-phenotype connection.

A) *Cis*—complementation in cells allows reversion of the mutation to the wild type (wt) sequence. By transfection of cells with the mutant BAC genome (I) or infection with the mutant virus (II) and co-transfection of a DNA fragment carrying the wt sequence and appropriate viral homologies, the mutation (M) can be reverted to the wt sequence. Since revertant and mutant viruses need further separation, this approach only works efficiently if one can select for the revertant, e.g., if it has a growth advantage over the mutant virus. B) *Cis*-complementation of viral BACs in *E. coli* is best performed by shuttle plasmid mutagenesis. The shuttle plasmid carrying the wt sequence and appropriate homologies is introduced into *E. coli* carrying the mutant BAC plasmid. By RecA-mediated homologous recombination, the wt sequence is inserted at the mutation site without leaving any operational sequences. After transfection of the revertant BAC genome into permissive cells, a homogenous revertant population is gained without any further need for selection against mutant viruses. C) Protein *trans*-complementation in cells. Cells that express the viral wt gene product permanently (I) or transiently by an additional expression vector (II) are superinfected with the mutant virus. This allows transient complementation of the mutant phenotype if the expression times and levels of the wt gene product are appropriate. D) Ectopic cis-complementatin using viral BACS. (from [66])

Ectopic *cis*-complementation, meaning the insertion of the gene on another position in the genome prevents this problem [66]. However, knowledge of the position where this gene should be introduced needs to be collected first. Otherwise, again phenotypes can be falsely attributed. The best option is to *trans*-complement the protein by growth on complementing cell lines. *Trans*-complementation is still a difficult task, especially for late herpesvirus proteins. Incorrect timing, aberrant intracellular distribution due to missing viral interaction partners and incorrect expression levels of the viral protein may explain poor complementation results. Toxicity of the gene products and suitable cell lines limit the approach even more [66].

Several *trans*-complementing cell lines constitutively expressing the viral proteins have been described. Nevertheless, many viral proteins need to be expressed conditionally to circumvent the above mentioned problems. Today, systems for inducible gene expression typically require the use of small chemical compounds such as tetracyclin or doxycyclin (as in the case of Tet-on/Tet-off systems)[67] or rapamycin (for FKBP12-based systems)[68]. In these systems, gene expression is activated synchronously and irrespective of the state of virus replication in all cells [69]. This could again limit the usage, as the protein is expressed at incorrect times regarding the viral life cycle. To adjust the timing problem, transcription activators can be cloned into the herpesviral genome under control of a promoter, which is activated at the desired time point [70]. Similarly, recombination systems like Cre/loxP and FLP/FRT can be used. However, this alternative needs always the manipulation of the viral genome.

Trans-complemented viruses are not only interesting for research purposes, but there is also an increasing market for spread-deficient or so called single-cycle vaccines. In these vaccines an essential gene is deleted, which needs to be compensated by growth on complementing cell lines [71]. Thereby the vaccine can infect the cell only once and thereby induce an immune response. Yet, without the essential protein further virus spread is inhibited. For vaccine development it is not desired that the vector includes bacterial sequences, therefore the above mentioned conditional expression systems cannot be applied. Production of sufficient *trans*-complemented virus is therefore a limiting step in the application of these viruses.

1.6 Vaccination against herpesvirus disease

Studies on human and animal vaccination against herpesvirus infection and disease began in the 1970s. Since then, several vaccination concepts have been tested or realized. These can be classified in five general types: inactivated virus vaccines, subunit vaccines, DNA vaccines, attenuated virus vaccines, and markerless vaccines. Several vaccines against animal-herpesviruses are on the market, but there is only one licensed vaccine against a human herpesviruses, namely against varicella zoster virus.

Inactivated virus vaccines although helpful to contain diverse virus infections, have not been very successful in case of herpesviruses [72, 73]. Subunit vaccines contain isolated antigens of major virus components such as glycoproteins or structural proteins. Some of these vaccines showed good results in the prevention of herpesvirus-associated diseases due to a high neutralizing antibody response [74]. However, subunit vaccines are prone to failure due to different virus serotypes or virus mutations. Furthermore, several doses and adjuvants are typically necessary to obtain sufficient immune responses [75]. Modified live attenuated vaccines, possess usually a good immunogenicity as they express most of the antigen subsets and can replicate in the host, without causing disease. In the classical approaches virus strains were attenuated by several passages in cell culture. The adaptation to the cell culture conditions usually led to loss of gene functions that were necessary for virus pathogenesis *in vivo*. Many licensed vaccines have been generated by this way, as measles, mumps and rubella vaccine[76]. Yet, the mutations that arise are uncontrolled and the cause for attenuation is not always clear. The only herpesvirus vaccine on the market is a life-attenuated vaccine, namely the OKA-strain against varicella-zoster virus [77]. Attempts to create life-attenuated vaccines by similar procedures failed for other human herpesvirus. The right balance between immunogenicity and attenuation is hard to obtain. Thus, several vaccine trials were stopped, as they were either causing disease symptoms or did not prevent infection and disease of wt infection [78]. Thus, recent strategies involve the targeted attenuation of herpesvirus by reverse-genetics methods. The highest safety level coupled with strong immunogenicity is achieved by single-cycle /spread-deficient vaccines. These vaccines lack one or several essential genes for virus replication but possess most of the antigen repertoire of the wild type (wt) virus. As proof of principle the vaccine MCMV-ΔM94 demonstrated that the immune response elicited by the first target cells is sufficient to control MCMV disease [71]. A drawback of these vaccines is the necessity to culture them on *trans*-complementing cell lines and with this the poor efficacy of vaccine production.

1.7 Animal Herpesviruses

1.7.1 Vaccination against animal herpesviruses

The class of herpesviruses is very large (see chapter 1.1) and of course comprises many subtypes infecting domestic and livestock animals. Important pathogens among these are pseudo rabies virus (PRV), which leads to Aujeszky's disease in pigs, bovine herpesvirus 1 (BHV-1), causing e.g. infectious rhinotracheitis and infectious pustular vulvovaginitis, Marek's disease virus (MDV) and gallid herpesvirus 1 infecting poultry, and cyprinide herpesvirus affecting the increasing industry of fish farms. Symptoms of herpesvirus infection of animals share common features with those infecting human reaching from localized vesicular eruptions of surface epithelia, to diffuse and widespread damage of the mucosa of the respiratory, digestive and genital tracts, damage of the vascular epithelium and necrosis of liver, lymphoid and other tissues, as well as specific neuron damage such as diffuse meningo-encephalitis [79]. An exception to the strict species specificity of one herpesviruses to one natural host is seen with PRV. The transmission of PRV to cattle, sheep, goats, dogs, cats, foxes, rats and mice causes neuropathologic diseases and is irretrievably fatal [79, 80]. As in humans, fetuses and newborns are more susceptible to severe or lethal outcomes through herpesvirus infection, while infection of adult animals is typically less severe. Still the infection causes big economic losses to animal husbandry through loss of weight, decrease of milk or egg production and also restrictions in the international livestock trade [72]. As herpesvirus infections can lead to abortion and infertility, animal reproduction is also affected. Due to the nature of herpesvirus infection, latently infected animals can spread the virus in the herds upon reactivation.

For many animal species complete prevention of disease by attenuated herpesvirus vaccines has been reported [72, 81, 82]. Unfortunately, they generally keep some residual virulence and stay latently in the host. Furthermore, they cannot prevent infection with wt virus and establishment of latent wt virus infection [83, 84]. Furthermore, recombination of wt and vaccine virus towards new serotypes has been reported [85]. This is alarming, as in case of MDV the transmission of wt virus in the vaccinated flocks has led to more virulent strains and thus to the failure of vaccines [86]. The general policy in Europe has thus changed to an eradication program for BHV-1 and PRV-1, which includes a test and slaughter policy or test and removal program [87]. Upon these terms sero-positive herds have to be culled. Furthermore, marker vaccines and the so-called DIVA (differentiating infected from vaccinated animal) strategy contributed to the success of the eradication programs and many countries, Germany included, are now free of BHV-1 or PRV-1. As a further safety step, vaccination in these countries is even

prohibited. However, the eradication programs and the related vaccination prohibition keep the risk, that infections of non-immune herds can occur at any time through transmission from wild life animals, like BHV infections of dairy cows by dear, or pigs by PRV infected wild sows [88, 89]. Testing of wild life animals revealed the underestimation of wild life infection and the risk of spill over infections. Also the restructuring of farming procedures towards an animal friendly breeding in outside cages opens the door for re-infection of healthy herds by contacts with wild life.

As vaccination against herpesviruses always resulted in the persistence of the wt virus in vaccinated animals, a better solution to eradicate the virus is certainly needed. The usage of intracellular immunization to protect livestock from infection and disease might be an alternative provided that necessary acceptance among consumers is gained.

1.7.2 Intracellular Immunization against herpesviruses

The term 'intracellular immunization' was coined by Baltimore in 1988 [90] and describes the expression of dominant-negative proteins (DN) in cells to inhibit viral replication. A cell that carries a transgenic DN protein should thus be rendered resistant to virus infection. DN proteins are protein mutants that exert a null-phenotype even in the presence of a functional wt protein (reviewed in [91], see 1.8). The idea of intracellular immunization was postulated in response to the finding of Friedmann and colleagues, that a truncated transcription activator VP16 of HSV-1, which was inserted into the cellular genome can provide DN resistance against HSV-1 infection in cell culture [92].

Based on this concept, other groups have tried to transfer this *in vitro* phenotype to mouse models. A dominant negative ICP4 mutant (X25) of HSV-1 that sequesters functional ICP4 monomers into nonfunctional heterodimeric complexes was used to generate the first transgenic mouse carrying an DN mutant against a herpesvirus [93, 94]. Four transgenic mouse lines were generated by microinjection of linearized DNA fragments encoding the X25 mutant under control of its own viral promoter. Copy numbers of the transgene ranged from 2 to 10 per cell in mouse lines. Although the general principle could be proven, only the mouse line with the highest copy number showed a relevant reduction of viral titer in the range of 5-13 fold [93]. In *in vitro* experiments a copy number of 40 per cell was necessary to inhibit plaque formation by the factor of 38 [94]. A higher copy number of the transgene was proposed to be necessary to achieve resistance. Unfortunately, although X25 expression was driven by the viral ICP4 promoter and therefore should be activated in infected cells, also in uninfected cells expression of

X25 was observed. Furthermore, strong side effects were routinely observed in all the transgenic mouse lines, with one mouse line having only a third of the normal weight of non-transgenic weaning siblings [93]. As ICP4 operates by forming a complex with the TATA-binding protein and TFIIB to activate or repress transcription, it is possible that the DN protein still has some intrinsic potential to interact with cellular proteins and thereby disturbs host cell transcription [91, 95].

Another group implemented intracellular immunization to PRV. In this case the DN protein consisted of a chimeric protein consisting of the DNA binding domain of IE180 of PRV and the tail-truncated VP16 of HSV-1, lacking the transcription activation domain [96]. Infection of transgenic cell lines with PRV-1 and HSV-1 revealed specific inhibition of PRV by the IE180 DN protein [97]. One transgenic mouse line expressing the chimeric DN under control of the interferon inducible Mx-1 promoter could be obtained that transferred the transgene to the F1 generation. This line carried five copies of the transgene and could successfully inhibit lethal PRV infection. In particular 15 of 18 control mice died upon challenge with PRV LD_{50} whereas 16 of 18 transgenic mice survived the challenge [98]. Low constitutive expression of the transgene was observed, although the Mx-1 promoter should drive expression only by induction with interferon α and β [98]. Again, mice exhibited severe side effects as seen with the X25 mutant, namely heavy weight loss, dwarf phenotype, bad reproduction and a high ratio of females at birth. Therefore, intracellular immunization against herpesviruses is possible but seems to need a tight control of the DN protein in the uninfected state as well as an appropriate target protein that does not influence host cell functions. This goal, however, is demanding as the inducible systems generally used today need either administration of small chemical compounds or genetic modification of the virus. Neither system is not appropriate for livestock applications, as the drug would not be administered before the start of disease symptoms and infection occurs with wt virus. An efficient intracellular immunization should inhibit virus spread already in the first infected target cells, so that manifestation of the disease does not even occur. Therefore, a successful and safe implementation of intracellular immunization calls for an inducible system that is tight and in same time is activated by the wild type virus (only).

Thus a suitable method to construct stable cell lines and animals is pivotal in order to test such inducible systems and an appropriate DN protein in cell culture and that furthermore allows the transfer of the technology to livestock.

1.8 Dominant-negative herpesviral proteins

Dominant-negative proteins are mutants capable to inhibit the wt protein in a cell in the way that it causes the cell to be deficient in the function of the gene product [91]. Meaning the wt protein cannot fulfill its full function in the presence of the DN protein. Dominant-negative (DN) proteins can be used for the identification of protein function, identification of pathways and for the inhibition of viral replication by intracellular immunization. Several DN proteins of herpesviruses have been reported, some which were found by chance others which were generated by purpose. The development of a random insertional mutagenesis protocol by transpon based insertions facilitated the screening of any herpesviral gene for DN mutations [99]. Three MCMV genes were subjected to such a DN screen in the past [99-101]. Interestingly in all proteins at least one mutation could be found that possessed DN activity. The strength of individual DN proteins was found to be diverse. The DN mutants of the protein M50 and M94 were in general rather weak in their ability to inhibit viral proliferation, if they were encoded in the viral genome. DN mutants of the protein M53, were found to block viral spread completely.

An attempt to tag the small capsid protein SCP of MCMV with the green fluorescent protein (GFP) led to a fusion protein (GFPSCP) that elicits a strong DN effect on capsid export from the nucleus [102]. Viruses encoding the GFPSCP protein accumulate capsids in nuclear speckles and were unable to spread to neighboring cells *in vitro* [103]. In mice infected with an MCMV mutant, encoding a Tet-regulated GFPSCP, viral titers were markedly reduced e.g. in lungs reaching three orders of magnitude [103]. Thus GFPSCP is very useful as a model DN, as it exhibits a strong DN effect and additionally it can be easily monitored by its fluorescence.

1.9 The non-viral episomal vector pEPI for the generation of stable cell lines and transgenic animal

The construction of transgenic cell lines is a fundamental tool for life science research to characterize proteins, to produce biological compounds like monoclonal antibodies, and also to complement virus deletion mutants. Several techniques have been established over time to manipulate the cell genome to express a foreign gene. In principle two approaches can be differentiated. Either the gene is integrated into the host genome, at a certain position or randomly, or it is maintained extrachromosomally as an episome.

A non-viral episomal self-replicating vector has been described that bases on a surface matrix attachment region (S/MAR) and a functional eukaryotic or viral origin of replication [104]. S/MAR elements are eukaryotic sequences that flexibly anchor the genome in loops to the nuclear matrix [105]. They are typically A/T rich (up to 70 %), enriched in DNA topoisomerase II binding sites, and do not harbor recognizable consensus sequences [106]. They are often associated with chromosomal origins of bidirectional replication [104] and are thought to elicit duplex strand destabilization, a necessary function for DNA replication and transcription [106].

An S/MAR element of the human β-interferon gene [107] was identified being sufficient to allow a stable episomal maintenance and once-per-cell cycle replication of a vector without the need of antibiotic selection pressure [108-111]. Due to its episomal maintenance the vector was termed pEPI-1. Although an SV40 origin of replication is included in this pEPI vector, its episomal persistence is independent of the large T antigen [104] and can be replaced by other replication origins [112]. As a prerequisite for episomal maintenance of pEPI, the direction of transcription needs to run into the S/MAR, most likely to generate an accessible chromatin structure. In the original pEPI vector an *egfp* gene under control of the human CMV immediate early promoter is thus positioned before the S/MAR site, without a transcription termination signal prior to the S/MAR element. The A/T rich S/MAR site itself contains also two AATAA signals that serve as minimal polyA sites. Furthermore a cryptic transcription termination signal is present 1500- 1700 bp inside the S/MAR element [113].

In immunoprecipitation studies pEPI was associated with the cellular scaffold attachment factor A (SAF-A) [114]. SAF-A is typically involved in partitioning eukaryotic genomes into independent chromatin loops by attaching DNA via S/MAR regions to the nuclear scaffold or matrix [115]. The S/MAR element was taken from the human β-interferon gene [107], however artificial MAR elements, containing the SAF-A binding site, can also replace the

eukaryotic element [112]. Vector numbers in transfected cells were usually in the range of 2 to 10 copies per cell [104, 111]. The vector pEPI was found to replicate synchronously once-per-cell cycle in early S-phase and components of the origin recognition complex could be precipitated in CHIP assays, such as Orc1, Orc2 and Mcm3 [116]. Interestingly, replication can start from any position of the plasmid [116]. Fluorescence *in situ* hybridization (FISH) revealed the attachment of pEPI vectors to metaphase chromosomes. However, they are not covalently bound as they can be separated from the chromosomes by higher shear forces during preparation of the metaphase spread [111].

The vector pEPI was used in a variety of different cell lines *in vitro* and supports a constitutive transgene expression in e.g. CHO, K562, HaCat cell lines and others [109, 112, 117]. Remarkably, the transgene expression was silenced in cells of murine origin, such as in the murine erythroleukemia cell line (MEL) [118], murine fibroblasts (NIH3T3) [119], immortalized murine embryonic fibroblasts (IMEF) [120] but also in the human cell lines HEK293 [119] and HCT116 [120]. Interestingly, the episome could also be maintained episomally in the silenced status [118].

The vector attributes can also function *in vivo*. Transgenic pig fetuses were generated by sperm mediated gene transfer expressing GFP from the pEPI vector [121]. In 12 of 18 fetuses episomal pEPI vectors were isolated with less than 10 copies per cell. However, only 9 of 12 fetuses with episomal plasmids expressed the transgene, but then in all analyzed tissues [121].

1.10 Aim of the thesis

In this work a novel gene expression system had to be constructed that had to fulfill several criteria:

1. It should be inducible by viral infection without the need of modifying the virus genome.

2. It should allow a temporally high expression of the transgenic protein.

3. It should be activated under 'late' expression kinetics.

The newly generated replicon vector system had to be tested for several applications.

1. Intracellular Immunization

Previous studies on intracellular immunization revealed the absolute necessity of a strict control of the DN gene, as transgenic mice suffered from uncontrolled transgene expression (see chapter 1.7.2). Using the dominant-negative viral fusion protein GFPSCP, the suitability of the replicon system for the implementation of intracellular immunization should be evaluated.

2. *Trans*-complementation of late herpesviral proteins

Correct expression kinetics and expression strength are important for successful *trans*-complementation of viruses lacking essential late proteins. With two model proteins, the glycoprotein O and the toxic transmembrane protein M50, the efficacy of the replicon system to *trans*-complement the corresponding deletion viruses should be tested.

3. Possibility to use the expression system in vivo

In order to analyze whether the newly generated expression system is compatible with *in vivo* applications, transgenic mouse lines had to be created and analyzed.

2 MATERIAL

2.1 Devices

Bacterial shaker ISF- 1- W	Kühner, Birsfelden, CH
Bio-Photometer	Eppendorf, Hamburg, D
Centrifuges:	
5417 R	Eppendorf, Hamburg, D
Avanti™ J-20xp	Beckman Coulter, Krefeld, D
8-55M ultracentrifuge	Beckman Coulter, Krefeld, D
Multifuge 3 S-R	Heraeus Instruments, Gera, D
Confocal microscope Axiovert 200M	Zeiss, Jena, D
Developing-machine Optimax TR	MS Laborgeräte, Wiesloch, D
Fluorescence microscope 1x71	Olympus, Hamburg, D
Flow cytometer Epics XL-MCL	Beckman Coulter, Krefeld, D
Gene Pulser™	Bio-Rad, Munich, D
Hybridization Oven Unitherm 6/12	Uniequib, Planegg, D
Incubator B5050E	Heraeus Instruments, Hanau, D
Incubator BB16CU	Haraeus Instruments, Hanau, D
Incubator Shaker ISF-1-W	Kühner, Birsfelden, CH
Light microscope Axiovert 25	Zeiss Carl, D
Microplate luminometer LB960	Berthold, Bad-Wildbad, D
Mini-PROTEAN3 Cell	Bio-Rad, Munich, D
ND-1000 Spectrophotometer	Nanodrop, USA
PerfectBlue™, electrophoresis system	Peqlab, Erlangen, D
Photo documentation apparatus EagleEye	Bio-Rad, Munich, D
Roller mixer SRT	Stuart, Staffordshire, UK
Semi-Dry-Transfer Cell Trans-BlotSD	Bio-Rad, Munich, D
Sonifier-bath SONOREX SUPER RK 103H	Bandelin, Berlin, D
TGradient, PCR Machine	Biometra, Göttingen, D
Thermomixer 5436	Eppendorf, Hamburg, D
Tissue cell culture lamina flow	BDK, Sonnenbühl-Genkingen, D
UV-Crosslinker	Vilbour-Lourmat, Eberhardzell, D

Vortex-Mixer	Bender/Hobein, Zürich, CH
Water Bath F10	Julabo, Seelbach D
Shaking water bath GFL 1090	Gesellschaft für Labortechnik, Burgwedel, D

2.2 Consumables

Cell culture dishes (20 cm^2; 55 cm^2; 145 cm^2)	Becton Dickinson, Heidelberg, D
Cell culture plates (6-, 12-, 24-, 48-, 96-well)	Becton Dickinson, Heidelberg, D
Cell culture flasks (25cm^2, 75cm^2)	Becton Dickinson, Heidelberg, D
Cell scrapers (25-, 39 cm)	Costar, Bodenheim, D
Cryotubes	Nunc, Thermo Fisher Scientific, Langenselbold, D
Chemiluminoscence film Hyperfilm^{TM}ECL	GE Healthcare Bioscience, Freiburg, D
Combitips plus (5 mL, 10 mL)	Eppendorf, Hamburg, D
Electroporation cuvettes	Bio-Rad, Munich, D
Falcon conical tubes (15 mL, 50 mL)	Becton Dickinson, Heidelberg, D
Hybond-P membrane	GE Healthcare Bioscience, Freiburg, D
Hybond-N+ membrane	GE Healthcare Bioscience, Freiburg, D
Nylon-Membrane, positively charged	Roche, Mannheim, D
Pipettes (5 mL, 10 mL, 25 mL)	Sarstedt, Nümbrecht, D
Reaction tubes (1.5 mL, 2 mL)	Eppendorf, Hamburg, D
Whatman paper	Macherey-Nagel, Düren, D
Ultracentrifugation tubes	Beckman Coulter, Krefeld, D

2.3 Reagents

All common chemicals were obtained by Roth (Karlsruhe D), Sigma-Aldrich (Deisenhofen, D), Becton Dickinson (Heidelberg, D), Invitrogen (Karlsruhe, D), Merk (Darmstadt, D) or Fluka (Karlsruhe, D) unless otherwise specified.

Restriction cloning enzymes and their buffers were purchased from NEB (Frankfurt/Main, D).

2.4 Commercial Kits

Crimson Taq, PCR system	NEB, Frankfurt/Main, D
DNeasy Blood and Tissue Kit	Qiagen, Hilden, D
DIG Nucleic Acid Detection Kit	Roche Diagnostics, Mannheim, D
Dual-Luciferase assay system	Promega, Madison, USA
ECL plus western blotting detection system	GE Healthcare, Freiburg, D

Expand high fidelity PCR system	Roche Diagnostics, Mannheim, D
Gel extraction kit	Qiagen, Hilden, D
GFX micro plasmid purification kit	GE Healthcare Bioscience, Freiburg, D
Nucleobond PC100	Macherey-Nagel, Düren, D
PCR purification kit	Qiagen, Hilden; D
PCR DIG Probe Synthesis Kit	Roche Diagnostics, Mannheim, D
Taqman 1000 RxN PCR Core Reagents	Applied Biosystems, Foster City, USA

2.5 Antibodies

antibody	**species**	**application**	**source**
A-GFP (ab290)	rabbit polyclonal	WB 1:2500	Abcam, Cambridge, USA
α-dig-Fluorescein-Fab	sheep	FISH	Roche, Mannheim, D
Avidin-Cy3.5	not applicable	FISH	Rockland, Gilbertsville, USA
α-IE1/3 (CHROMA 101)	mouse polyclonal	WB 1:1000 IF: 1:500	Stipan Jonjic, Rijeka, Croatia
α- HA (3F10)	rat	WB: 1:1000	Roche, Mannheim, D
α-actin (20-33)	rabbit	WB: 1:1000	Sigma- Aldrich, Hamburg, D

2.6 Bacterial strains

strain	**genotype**	**application**	**source**
DH10B	F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu) 7697 galU galK rpsL nupG λ– (Strep R)	Maintenance of BACs	Invitrogen Karlsruhe, D
PIR1	F- Δlac169 rpoS(am) robA1 creC510 hsdR514 endA recA1 uidA(ΔMlu I)::pir-116	Maintenance of vectors with R6Kγ origin of replication	Invitrogen Karlsruhe, D
SCS110	rpsL (Strr) thr leu endA thi-1 lacY galK tonA tsx dam dcm supE44 Δ(lac-proAB) [F´ traD36 proAB	Generation of dcm/dam methylation free DNA.	Stratagene, Cedar Creek, USA
XL1-blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F´ proAB lacIqZΔM15 Tn10 (Tetr)]	Routine cloning of plasmid DNA	Stratagene Cedar Creek, USA
NEB10-beta	araD139 Δ(ara-leu)7697 fhuA lacX74 galK (Φ80 Δ(lacZ)M15) mcrA galU recA1 endA1 nupG rpsL (StrR) Δ(mrr-hsdRMS-mcrBC)	Efficient transformation of methylated DNA from eukaryotic sources	NEB, Frankfurt/Main, D

2.7 Eukaryotic cell lines

cell line	type	origin
NIH3T3	murine fibroblasts	(ATCC®: CRL-1658™)
M2-10B4	murine bone marrow stromal cells	(ATCC®: CRL-1972™)
MHEC-5T	murine heart endothelial cells	[122]
SVEC4-10	murine lymphoid endothelial cell line (SV40 transformed)	[123]
mES E14	Murine embryonic stem cell line	[124]

2.8 Viruses

Virus name	reconstituted from	origin
wt-MCMV	pSM3fr-MCK-2fl	[125]
MCMV-gfp	pSM3fr-Δm157-egfp	[126]
MCMV-luc	pSM3fr-Δm157-luc	[126]
MCMV-mCherry	pSM3fr-Δ1-16gfpscpIRESmCherry	kindly provided by Zsolt Ruzsics, Max von Pettenkofer Institut, LMU
MCMV-ΔM50	pSM3fr-Δ1-16ΔM50:gfp/gfpscpIRESmCherry	kindly provided by Zsolt Ruzsics, Max von Pettenkofer Institut, LMU

2.9 Oligonucleotides

primer	sequence	application
H5-MCMV-orilyt-ori6kan-for	5'-GGCGGGAGCGACGGGGGCGAGCTGGAGAGATCGTCGTCCGCCATGCTAGCACGCGTGCCAGTGTTACAACCAATTAACC -3'	pick-up cloning of MCMV-oriLyt
H3-MCMV-oriLyt-ori6kan-rev	5'-GAACGACCCCCGCTCCTGTATAATTTCGATGCCGGGGAGGTCGCCACGCGTCTGAAGATCAGCAGTTCAACCTGTT-3'	pick-up cloning of MCMV-oriLyt
bsr-for-taqman	5'-CCTCATTGAAAGAGCAACGGCTAC-3'	qPCR Detection of bsr
bsr-rev-taqman	5'-GCACCACGAGTTCTGCACAAGGT-3'	qPCR Detection of bsr
LBR-for	5'-GGAAGTTTGTTGAGGGTGAAGTGGT-3'	qPCR Detection of lbr
LBR-rev	5'-CCAGTTCGGTGCCATCTTTGTATTT-3'	qPCR Detection of lbr
M54-for	5'-ATCATCCGTTGCATCTCGTTG-3'	PCR detection of M54
M54-rev	5'-CGCCATCTGTATCCGTCCAT-3'	PCR detection of M54
M74-for	5'-TCCGGACAACGTCTTTCCC-3'	PCR detection of M74

M74-rev	5'-ATCATCCGTTGCATCTCGTTG-3'	PCR detection of M74
P(SV40) probe-for	5'-TACCGAGCTCTTACGCGTGC-3'	PCR detection of luc, VIOLA genotyping, Southern probe
pA(SV40)-probe-rev	5'-TAAGATACATTGATGAGTTTGGA-3'	PCR detection of luc, VIOLA genotyping, Southern probe
P(M143)-HindIII-rev	5'-CCAAGACAAGCTTCGCGCACG-3'	Cloning of Promoter M143 into pEpibo-luc
P(M143)-KpnI-for	5'-CCAAGACAAGCTTCGCGCACG-3'	Cloning of Promoter M143 into pEpibo-luc
P(M53)-KpnI-for	5'-CAGCTGGTACCGACCATGGCG-3'	Cloning of Promoter M53 into pEpibo-luc
P(M53)-HindIII-rev	5'-GGCTCCTAATAAGCTTACTTCTCGACGGTGAAG-3'	Cloning of Promoter M53 into pEpibo-luc
P(CMVie)-KpnI-for	5'-TTACAATTTACGGTACCAGCGCGCGTTG-3'	Cloning of Promoter CMVie into pEpibo-luc
P(CMVie)-HindIII-rev	5'-TTAGCCAAAGCTTGAGAGCTCTGCTTATATAG-3'	Cloning of Promoter CMVie into pEpibo-luc
P(M94)-KpnI-for	5'-TTCGCATCAGGTACCGGTTCGCCGTGATC-3'	Cloning of Promoter M94 into pEpibo-luc
P(M94)-HindIII-rev	5'-GTCGCCATAAGCTTGGTCTACCTGCAGCTG-3'	Cloning of Promoter M94 into pEpibo-luc
bsr-for-FISH	5'-ATGGCCAAGCCTTTGTCTCA-3'	Generation of a bsr FISH probe
bsr-rev-FISH	5'-AGATCGAGAAGCACCTGTCG-3'	Generation of a bsr FISH probe
gfpscp-for-FISH	5'- AGCAAGGGCGAGGAGCTGTT -3'	Generation of a GFPSCP FISH probe
gfpscp-rev-FISH	5'-TAGCGATCGAGAGCATCCGC-3'	Generation of a GFPSCP FISH probe

2.10 Plasmids and bacterial artificial chromosomes

plasmid name	**features**	**origin**
p06kan	Cloning vector with kanamycin cassette and R6K origin of replication	kindly provided by Brigitte Rupp, Max von Pettenkofer-Institute, LMU
pCR3	Cloning vector, encoding for the human CMVie promoter	Invitrogen, Karlsruhe, D
pkD46	L-Arabinose inducible expression of recombinases red α, -β, -γ and temperature sensitive replication origin oriR101	[127]

pGL3-control	Luciferase reporter vector: Firefly luciferase under control of SV40minimal promoter	Promega, Mannheim, D
pTK-RL	Luciferase reporter vector: Renilla luciferase under control of Thymidin Kinase promoter	Promega, Mannheim, D
pHSB5	Hyperactive Sleeping Beauty	kindly provided by A. Erhardt, Max von Pettenkofer- Institute, LMU
pMSB5	Mutated Sleeping Beauty	kindly provided by A. Erhardt, Max von Pettenkofer- Institute, LMU
pTMCS	Transposon vector for Sleeping Beauty	kindly provided by A. Erhardt, Max von Pettenkofer- Institute, LMU
pGPSie1/ie3-ie2	Plasmid encoding entire ie-1/2 and ie3 locus	kindly provided by K. Eisenächer, Medizinische Klinik und Poliklinik, TMU

Table 1: Newly generated plasmids

plasmid name	**features**
pT-mOrange	Transposon for Sleeping Beauty containing Hygromycin resistance gene (*hyg*) and mOrange gene driven by the human CMVie promoter flanked by inverted repeats (IR)
pT-gfpscp	Transposon for Sleeping Beauty containing *hyg* and dominant-negative *gfpscp* driven by the human CMVie promoter gene flanked by IR
B45-gfpscp	Episomal papillomavirus vector with *gfpscp* under control of the methallotheonein promoter, Kan, Neo^R
p06kan-MCMVoriLyt	p06kan harbouring a 3824bp region of the oriLyt of MCMV, Kan^R
pEpibo-luc	Episomal plasmid with firefly luciferase gene (*luc*) under control of the SV40 minimal promoter, BS^R
pEpibo-P(CMVie)-luc	Episomal plasmid with firefly luciferase gene (*luc*) under control of the human CMVie promoter, BS^R
pEpibo-P(M143)-luc	Episomal plasmid with gene under control of the M143 promoter (904 bp; [128]),BS^R
pEpibo-P(M53)-luc	Episomal plasmid with *luc* under control of the M53 promoter (502 bp), BS^R
pEpibo-P(M94)-luc	Episomal plasmid with *luc* under control of the M94 promoter (600bp), BS^R
pEpibo-luc-ori	Episomal plasmid with *luc* under SV40 minimal control and the MCMV oriLyt, BS^R
pEpibo-gfpscp-ori	Episomal plasmid with *gfpscp* under SV40 minimal control and

	the MCMV oriLyt, BS^R
pEpibo-gO-ori	Episomal plasmid with *gO* under SV40 minimal control and the MCMV oriLyt, BS^R
pEpiNo-M50-ori	Episomal plasmid with *M50* under SV40 minimal control and the MCMV oriLyt, BS^R

3 METHODS

3.1 Microbiological methods

3.1.1 Cultivation of *Escherichia coli*

According to the intended application, different *E. coli* strains were used. All *E. coli* cultures were grown in low-salt Luria Broth (LB) medium at 37 °C. Single colonies were achieved by dispersing bacteria on petri dishes on LB-agar medium with appropriate antibiotics. Liquid cultures were grown in LB-medium with appropriate antibiotics under constant shaking at 180 rpm at 37 °C. To conserve *E. coli* strains, equal volumes of an o.n. culture and 50 % (v/v) sterile glycerol were mixed and stored at -80 °C.

LB-medium(low salt)	antibiotics
10 g Bacto Tryptone	100 µg/ml Ampicillin
5 g yeast extract	100 µg/ml Blasticidin S
5 g NaCl	25 µg/ml Chloramphenicol
Add H_2O to 1l	50 µg/ml Kanamycin
	30 µg/ml Zeocin

LB-agar

7.5 g Agar per 500ml LB-Medium

3.1.2 Preparation of electrocompetent *E. coli*

To generate electrocompetent cells, 200 ml pre-warmed LB-medium was inoculated with 5 ml of an o.n. culture and cultivated until bacteria reached an OD_{600} of 0.45 . All subsequent steps were performed at 4 °C with sterile pre-chilled buffers and equipment. After incubation on ice for 30 min, bacteria were centrifuged at 5000 × g for 10 min. Supernatants were completely discarded and the pellet resuspended in 150 ml ddH_2O. Centrifugation steps were repeated three times while washing the bacteria with 150 ml 10 % (v/v) glycerol. After the last centrifugation step the pellet was resuspended in 1 ml 10 % (v/v) glycerol and aliquots of 50 µl were snap-frozen in liquid nitrogen and stored at -80 °C.

3.1.3 Transformation of *E. coli* by electroporation

Aliquots of electrocompetent cells were thawed on ice and approx. 25 - 50 ng vector DNA was added directly. The mixture was then transferred to pre-chilled electroporation cuvettes (0.2 mm).

DNA transfer into bacteria was achieved by a short high-voltage electrical discharge of 2.5 kV, 25 µF and 400 Ω. Immediately after the pulse, 1 ml pre-warmed SOC-Medium was added and the culture was transferred to round-bottom culture tubes for 1 h incubation at 37 °C at 180 rpm. Cells were plated on LB-agar with selective antibiotics in appropriate dilutions and cultured o.n. at 37 °C. Typical transformation efficiencies of 10^8-10^9cfu/µg control vector pUC19 were achieved.

SOC-Medium:

2 % Tryptone

0.5 % Yeast Extract

10 mM NaCl

2.5 mM KCl

10 mM MgCl2

10 mM MgSO4

20 mM Glucose (freshly added)

3.2 Molecular biological methods

3.2.1 Isolation of nucleic acid from bacteria

3.2.1.1 Small scale isolation of nucleic acid from bacteria

Plasmids:

Isolation of plasmid DNA from *E. coli* has was performed from 2 ml liquid o.n. culture with the GFX Micro Plasmid Kit according to manufacturer's instructions.

BACs:

Due to the size of BACs coding for the MCMV genome (~ 230 kb), small scale column-based purification with commercial plasmid purification kits was not suitable. Therefore, an alkaline lysis procedure with subsequent phenol-chloroform extraction and DNA precipitation by isopropanol was performed. Briefly, a single clone was picked and cultured in 10 ml of LB-medium with appropriate antibiotics at 37 °C o.n.. Cells were centrifuged at 3500 rpm for 15 min at RT and the bacterial pellet was then resupended in 200 µl cold resuspension buffer. For

alkaline lysis, 200 µl lysis buffer was added and solutions mixed by gentle inversions. Precipitation of proteins and chromosomal DNA was achieved by adding 400 µl neutralization buffer. After 10 min incubation on ice, precipitates were sedimented by centrifugation at 20,000 × g for 10 min. Supernatants were transferred into a fresh 2 ml reaction tube and BAC DNA extracted by addition of 1 ml phenol/chloroform and centrifugation at 20,000 × g for 5 min. BAC DNA, in the upper phase, was than precipitated with 1 ml isopropanol at 20,000 × g for 30 min. Salts were removed by washing the DNA pellet with 70 % (v/v) EtOH and another centrifugation at 20,000 × g for 10 min. The supernatant was discarded, pellet allowed to air-dry and the BAC DNA was then dissolved in 100 µl 10 mM Tris-HCl pH 7.5.

Resuspension Buffer pH 8.0	Lysis Buffer	Neutralization Buffer pH 4.8
25 mM Tris/HCl	200 mM NaOH	3 M KAc
10 mM EDTA	1 % (w/v) SDS	
100 mg/l RNase A,		

3.2.1.2 Large scale isolation of nucleic acid from bacteria

Isolation of plasmids and BACs in large scale volume was performed with Nucleobond PC100 Kit according to manufacturer's instructions.

Concentration and purity of the isolated nucleic acids were determined by UV extinction measurement with the Nanodrop spectrometer and agarose gel electrophoresis. Correctness of mutagenesis on plasmids and BACs were verified by restriction fragment analysis and sequencing.

3.2.2 Isolation of nucleic acid from eukaryotic cells

3.2.2.1 Extraction of genomic DNA for molecular biological assays

Extraction of genomic DNA from cultured cell lines as well as from murine tissues have been performed with the Qiagen DNeasy Blood & Tissue Kit according to manufacturer's instructions, with the exception that vortexing was avoided to minimize shearing of genomic DNA.

3.2.2.2 Extraction of genomic DNA from mouse tails for genotyping

VIOLA mice progeny were bred as heterozygotes and were genotyped for the presence of the *luc* gene. To this end, 0.5 -1 cm mouse tails pieces were digested o.n. with 500 µl proteinase K buffer supplemented with 19,2 µg proteinase K at 55 °C under constant shaking. The next day, samples were vortexed for 30 s and residual debris removed by centrifugation at 20,000 × g for 2 min at

RT. The supernatants were transferred into a fresh tube, mixed with 500 µl isopropanol and DNA was pelleted by centrifugation at 20,000 × g for 20 min at RT. Next, the pellet was washed with 500 µl 70 % EtOH to remove salts by another centrifugation for 10 min. Supernatants were aspired, the genomic DNA dried at 37 °C and finally resuspended in 100 µl 10 mM Tris-HCl, pH 7.5. Typically 2 µl of the digest were used for genotyping via PCR (see 3.2.4.2.1).

Proteinase K-Buffer:

200 mM NaCL

100 mM Tris pH 8

5 mM EDTA

1 % (w/v) SDS

3.2.3 Cloning Techniques

3.2.3.1 Restriction enzyme digest

Restriction fragment analysis as well as the preparation of linear DNA fragments was performed with restriction endonucleases. For analytical analysis 1 µg of plasmid DNA was digested with 5 U restriction enzyme for 1 h. Preparative digests 10 µg DNA were digested with 25 U restriction enzyme for 3 h. Temperature and buffers were adjusted according to manufacturer's instructions. Digestion of genomic DNA was performed with at least 8 U restriction enzyme/µg DNA o.n.

3.2.3.2 DNA precipitation

For concentration or removal of reaction components DNA was precipitated from solution by addition of 1/10 volume 3 M sodium acetate (pH 5.3) and three volumes 100 % ethanol. The solution was mixed and either incubated at -80 °C for 20 min or on ice for 1 h. DNA was subsequently precipitated by centrifugation at 20,000 × g at 4 °C for 30 min. The DNA pellet was washed with 70 % ethanol. After another centrifugation at 20,000 × g at 4 °C for 10 min, the pellet was dried at 45 °C. DNA was subsequently resuspended in 10 mM Tris-HCl, pH 7.5.

3.2.3.3 Blunting of DNA overhangs by Klenow polymerase

For cloning fragments with incompatible restriction sites, fragments were subjected to Klenow Polymerase that fills in 5´ overhangs and removes 3´ overhangs of digested restriction sites to form blunt ends. The reaction was performed in 1 × NEB 2 buffer supplemented with dNTP to a final concentration of 33 µM. One unit Klenow Polymerase per µg DNA was added in a total

volume of 50 µl. After 15 min incubation at RT, the reaction was stopped by addition of 2 µl 0.5 M EDTA prior to heat inactivation at 75 °C for 20 min. Before using the blunted fragment in further cloning steps, DNA was purified via QIAquick PCR Purification Kit according to manufacturer's instructions.

3.2.3.4 Dephosphorylation of DNA fragments

To avoid re-circularization of restriction enzyme digested vector DNA, fragments were dephosphorylated with 1 U/µg Antarctic phosphatase for 1 h at 37 °C. The enzyme was inactivated at 65 °C for 10 min and linearized fragments were purified via QIAquick PCR Purification Kit according to manufacturer's instructions.

3.2.3.5 Purification of DNA from agarose gels

For isolation of DNA fragments from agarose gels (3.2.4.1), fragments were excised on a *trans*-illuminator with longwave UV light and purified with the QIAquick Gel Extraction Kit according to manufacturer's instructions.

3.2.3.6 Ligation of DNA fragments

DNA fragments were ligated using T4 DNA ligase with a molar ratio of 1:3 between vector (100 ng) and insert. Ligation was performed in a total reaction volume of 20 µl with 400 U T4 DNA ligase in 1× T4 DNA Ligase buffer in a water bath at 16 °C o.n. In control reactions, insert fragments were replaced with the same volume water. Typically 50 µl electrocompetent bacteria were transformed with 4 µl of the ligation reaction.

3.2.3.7 Manipulation of bacterial artificial chromosomes by homologous recombination

Due to the big size of BACs coding for the entire herpesviral genomes, standard cloning procedures are not feasible [66]. To generate herpesviral mutants, homologous recombination of linear fragments with the BAC DNA was applied according to the protocol established by Wagner and colleagues [129].

In this work, a kanamycin selection cassette was inserted next to the origin of replication (see Figure 15) by homologous recombination. To this end, a linear fragment (containing a kanamycin selection marker, the bacterial origin of replication R6K and 40 bp sequences homologues to the viral genome on both sites) was amplified from the plasmid p06kan by PCR with the primer H3-MCMVori-ori6kan-for and H5-MCMVori-ori6kan-rev. The fragment was purified by DNA precipitation (see 3.2.3.2) and electrocompetent L-Arabinose induced DH10B bacteria harbouring pSM3fr and pKD46 were transformed with 1.5 µg. By induction with L-

Arabinose, recombinases were expressed in a controlled manner and mediated the recombination of the linear fragment and the homologous region on the BAC during 2 h incubation at 37 °C. Bacteria containing an insertion of the kanamycin cassette were selected by growing on LB-CAM-KAN-agar plates. Correct insertion was confirmed by RFLP analysis of the candidate pSM3fr-ori6kan BAC clones.

3.2.4 Analyzing nucleic acid

3.2.4.1 Agarose gel electrophoresis

For analysis of restriction fragments, digested DNA was separated by agarose gel electrophoresis. Plasmid DNA solutions in 1 × loading dye were analyzed in 1 or 2 % agarose/TAE gels, whereas 0.8 % agarose/TBE gels were used for restriction fragments of BAC DNA. Voltage and duration of electrophoresis were adjusted to the length of the fragments to be analyzed. Before pouring the gels, ethidium bromide was added to a final concentration of 0.005 % (v/v) allowing the visualization of the DNA under UV-light with the Eagle-Eye imaging system.

TAE-buffer (1 ×), pH 7.3	TBE-Buffer (1 ×), pH 8.3	10 × Loading Dye
40 mM Tris-Acetate	90 mM Tris-HCl	15 % (w/v) Ficoll
1 mM EDTA	90 mM boric acid	50 mM Tris-HCl, pH 7.5
	1 mM EDTA	2.5 mg/ml Orange G

3.2.4.2 Polymerase chain reaction

3.2.4.2.1 Touch-down PCR

Polymerase chain reaction (PCR) allows the amplification of DNA and was used for different purposes, like genotyping cell clones and mice strains, subcloning genes by introduction of mutated sequences or restriction sites, creating linear fragments for homologous recombination and labeling of DNA with modified nucleotides. In all cases, the amplification of the target DNA was based on a touch-down PCR having the following program conditions (Figure 10).

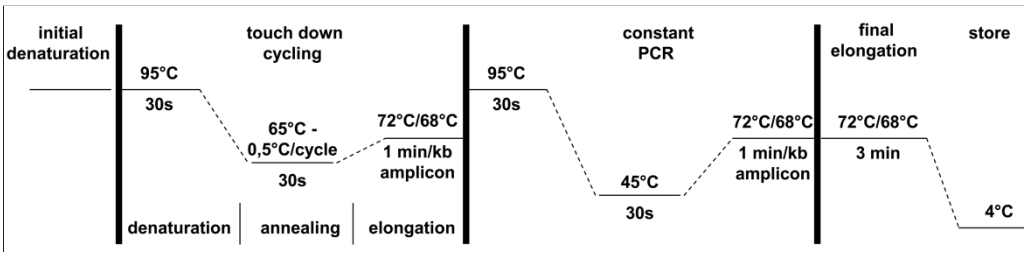


Figure 10: Schematic diagram of a touch-down PCR

An elongation temperature of 72 °C was used to amplify high quality DNA for labeling probes or further subcloning with the Expand High Fidelity PCR System (Roche). Genotyping has been performed with the more robust PCR system Crimson Taq using an elongation temperature of 68 °C. Reaction conditions were used according to manufacturer's instruction. If needed Mg^{2+} concentration has been adjusted or 5 % DMSO added for complex structured templates.

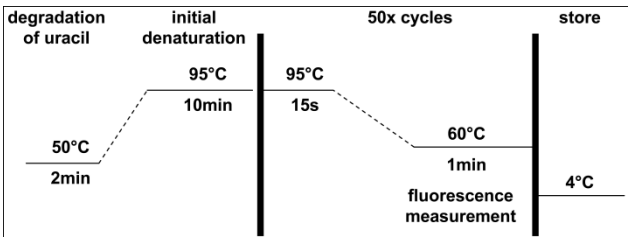
3.2.4.2.2 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed with the TaqMan technology. In this case, amplification of the template was detected by a fluorescence resonance energy transfer (FRET) based probe. Single stranded probes 5'-FAM (Fluorophore) and 3'-TAMRA (Quencher) labeled-were designed complementary to the amplicon of the murine lamin B receptor gene (*lbr*) and the Blasticidin S resistance gene (*bsr)* (Table 2).

Table 2: TaqMan –Probes

probe	sequence	feature
5' FAM-lbr-sense-TAMRA 3'	CTGAGCCACGACAACAAATCCCAGCTCT AC	binds within the sense strand of the *lbr* gene
5'FAM-bsr-sense-TAMRA 3'	CATCTCTGAAGACTACAGCGTCGCCA	binds within the sense strand of the *bsr* gene

Table 3: qPCR Reaction Set up

reagents	volume	program
Forward-Primer [8 µM]	2.5 µl	
Reverse-Primer [8 µM]	2,5 µl	
Taqman-Probe [2 µM]	5 µl	
buffer A [10x]	5 µl	
$MgCl_2$ [25 mM]	8 µl	
dXTP-Mix [2.5 & 5 mM]	4 µl	
UNG [1 U/µl]	0.5 µl	
AmpliTaqGold [5 U/µl]	0.25 µl	
ddH_2O (ROTH)	12.25 µl	
Template	10 µl	

To estimate the copies of transgene in stable cell lines or mice, amplification of the *hsr* gene was correlated relative to the endogenous *lbr* gene. PCR was set up with the Taqman 1000 RXN core reagents in triplicates (Table 3). This includes the labeling of PCR-amplicons with Uracil and its degradation in following PCRs by adding the heat-instable enzyme uracil-DNA-glycosylase to minimize carry over contamination.

3.2.4.3 Southern Blot

Southern blot experiments were performed to analyze the status of transgenes in stable cell lines or mice. The technique bases on the protocol established by Edward Southern [130]. Electrophoretically separated restriction fragments of genomic DNA were transferred onto a nylon membrane and hybridized with labeled probes.

3.2.4.3.1 PCR labeling of probes for Southern Blot analysis

Probes for Southern blot analysis (3.2.4.3) were generated by PCR amplification with digoxigenin-labeled dUTPs with the PCR DIG Probe-Synthesis kit according to manufacturer's instructions. Dot blot analysis and agarose gel electrophoresis was used to verify sufficient incorporation of digoxigenin into the probe. Optimal concentration of probes for hybridization in Southern blot experiments was evaluated empirically.

3.2.4.3.2 Agarose electrophoresis for Southern blot

Genomic DNA (gDNA) was prepared according to the protocol described in section 3.2.2. 10 µg was digested with an appropriate restriction enzyme o.n. DNA was loaded with 1 × GelPilot Loading Dye (Qiagen, Hilden, D) on a 0.5 % TAE-agarose gel and electrophoresis was performed at 80 V for 16 h. The gel was stained afterwards in an ethidium bromide bath and photographed with a fluorescent ruler to determine the running distance of the marker fragments. Subsequently, the gel was destained in water before continuing with capillary transfer.

3.2.4.3.3 Capillary transfer of gDNA

Electrophoretically separated gDNA must be pretreated to be efficiently transferred to a positively charged nylon membrane (Roche). As the target DNA fragments were in general over 10 kb in size, the DNA was depurinated by submerging the gel in 0.25 N HCl until the bromphenol-blue contained in the loading dye turned yellow. Thereby the fragments break into smaller pieces. After a short wash in ddH_2O, DNA fragments were denatured in 0.5 N NaOH/ 1.5 M NaCl until the color of bromphenol changed back to blue. The pH of the gel was neutralized by submerging the gel in 1M Tris /1.5 M NaCl (pH 7.4) for 15 min. In the meantime

the nylon membrane was submerged in ddH_2O and than equilibrated in 20 × SSC. The capillary transfer was set up as depicted in Figure 11.

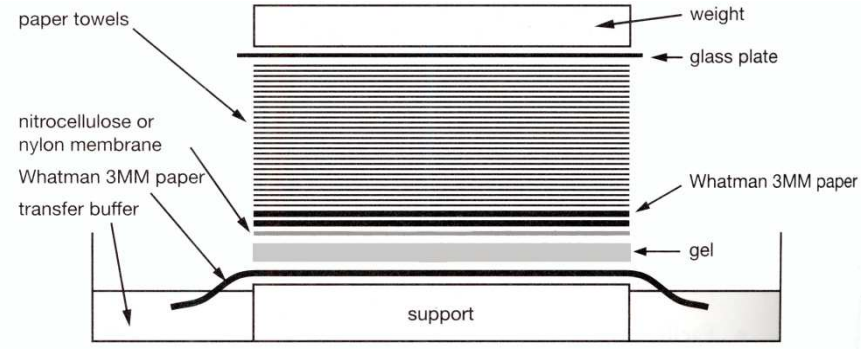


Figure 11: Upward Capillary transfer modified from Sambrook and Russel [131]

Transfer of the DNA was carried out by capillary forces from 20 × SSC transfer buffer that is drawn from the reservoir through the gel onto the membrane. The paper towels and weight help to maintain a constant stream of buffer.

Capillary transfer was performed at RT o.n. Then, the DNA was crosslinked onto the wet nylon membrane in an UV-linker by 0.125 Joule/cm^2. Salt crystals from the 20 × SSC were removed by rinsing the membrane with ddH_2O. Thereupon, the DNA was air-dried before continuing with the hybridization procedure.

20 × SSC pH 7.0

3 M NaCl

0.3 M Na-Citrate

3.2.4.3.4 Vacuum transfer

Vacuum transfer of DNA was performed alternatively to capillary transfer. This method is able to create sharper bands and can be accomplished in a shorter time. To this end, the vacuum blotter was set up with agarose gel, nylone membrane and Whatman paper. Each soaked in 20 × SSC according to manufacturer's instructions. Depurination was performed directly on the vacuum blot, by soaking 0.25 N HCl through the gel while applying constant pressure of 0.2 bar until the indicator bromphenolblue of the loading dye turned yellow. Excess solution was removed before addition of 0.5 N NaOH/ 1.5 M NaCl to denature the genomic DNA until the indicator turned blue. Neutralization solution (1 M Tris /1.5 M NaCl pH 7.4) was added for 10 min. Final transfer of gDNA was achieved by soaking 20 × SSC buffer through the gel for 1.5 h.

Crosslinking of the DNA and preparation of the nylon membrane was performed as described previously (see section 3.2.4.3.3).

3.2.4.3.5 Hybridization of probe

To minimize unspecific binding of probe to gDNA, the nylon membrane was pre-hybridized with DIG-Easy Hyb solution. At least 20 ml of the pre-hybridization solution were added to the membrane in roller-bottles. Incubation was performed at a defined hybridization temperature for 2-3 h. The success of a southern blot analysis is strongly dependent on the right hybridization temperature and probe concentration. The latter one was evaluated empirically — not exceeding 25 ng/ml—. A starting point to find the optimal hybridization conditions can be calculated with following equation:

$T_m = 49.82 + 0.41 \times \%\ GC - (600/l)$

$T_{hyb} = T_m - (20\ °C \text{ to } 25\ °C)$

(with T_m = melting temperatue; T_{hyb} = hybridization temperature, % GC = percent GC-content, l = length of probe)

Table 4: Conditions of Southern blot probes

probe	optimal hybridization temperature	optimal concentration
S2-luc-dig	37 °C	20 ng/ml
S3-gfpscp-dig	47 °C	15 ng/ml

The probe was first diluted in 50 µl ddH_2O, boiled 5 min at 95 °C, immediately put on ice to achieve single stranded DNA and then added to 20 ml pre-warmed DIG-easy hyb solution. The probe was carefully mixed in the solution and then added to the membrane. Hybridization was performed for 16 - 24 h. Unbound probe was removed by two washes with a low-stringency buffer (2 × SSC, 0.1 % SDS) at 37 °C or RT for 30 min and 10 min respectively, followed by two washes with a high-stringency buffer (0.5 x SSC, 0.1 % SDS) at 65 °C for 10 min each.

3.2.4.3.6 Detection of labeled target DNA

For detection of the labeled target DNA an alkaline phosphatase conjugated anti-DIG-antibody that specifically hydrolyzes the chemiluminescent substrate CDP-star was used allowing detection of emitted light on an X-ray film (Hyperfilm ECL, GE Healthcare). The DIG luminescence detection kit and CDP-Star were used according to manufacturer's instruction.

3.2.4.4 Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a technique to visualize the locus of a DNA sequence in the genome. Basic steps involve the preparation of metaphase spreads, generation of specific probes and their hybridization to the chromosomes, as well as detection of the probes by immunofluorescence-labeling.

3.2.4.4.1 Preparation of metaphase chromosome spreads

FISH hybridization was performed on condensed metaphase chromosomes. NIH3T3 were split one day before preparation on a 10 cm^2 plate at a ratio of 1:3. Demecolcin (Sigma-Aldrich) was added to a final concentration of 0.1 µg/ml to the cell culture medium to yield a maximum of metaphase cells. After 2 h incubation found herein to be optimal for NIH3T3, rounded and detached metaphase cells were harvested into a 15 ml falcon tube. After centrifugation at 300 × g for 5 min, cells were washed with pre-warmed PBS (37 °C) and finally resuspended in 0.5 ml PBS. At a minimal vortex speed, 10 ml of 37 °C warm hypotonic solution was added dropwise and cells swelled during the following 15 min incubation in a 37 °C water bath. Than 1 ml fixative was added, the suspension was carefully inverted and incubated at RT for another 15 min. Cells were centrifuged at 300 × g at RT for 5 min, supernatants discarded, carefully resuspended in 1 ml of residual solution and finally resolved in 7 ml fixative. This step was repeated three times at 4 °C. Afterwards, the cells were resuspended in 1 ml fixative and incubated at -20 °C for at least 10 min. Three to four drops of this suspension was dropped from at least 40 cm distance onto ice cold water covered slides that were purified by sonification and pre-chilled to 0 °C. Slides were incubated on a 40 °C hot plate with high humidity and finally dried at RT. After one week of incubation at RT in the dark, metaphase spreads were further processed.

Hypotonic Solution	Fixative
0.91 % (w/v) tri-sodiumcitrate-dihydrate	3 volumes methanol : 1 volume glacial acid
pre-warmed to 37 °C, freshly prepared	pre-chilled to -20 °C, freshly prepared

3.2.4.4.2 Probe preparation

Efficient labeling of probes is very important for successful FISH analysis to achieve a good signal to noise ratio. Probes were either directly labeled by incorporation of Diethylamincoumarin-dUTP (DEAC-dUTP) or were labeled with digoxigenin-dUTP or biotin-dATP allowing indirect detection via fluorescence coupled antibodies, i.e. anti-dig-Fab or anti-

biotin-Fab. The incorporation of the modified nucleotides was performed by PCR or nicktranslation. The optimal length for probes is 300 – 500 bp. Longer PCR fragments were digested with appropriate restriction enzyme or digested with DNase I. Probes were denatured with 1/10 volume of 3 M NaAc, 2.5 volumes of 100 % EtOH and 1 µl salmon sperm DNA, to prevent unspecific binding. The mixture was centrifuged at 20,000 × g for 20 min, supernatant was discarded and the DNA pellet was washed with 150 µl ice-cold 70 % EtOH. After another centrifugation step, the pellet was dried in a vacuum-centrifuge at 65 °C for 5 min. Probes (for one hybridization window with 25 µl) were dissolved in 5 µl 100 % formamide and denatured in a 37 °C waterbath for 30 min. Renaturation of the single stranded probe was inhibited by addition of 5 µl 40 % dextransulfate in 2 × SSC. The mixture was further incubated at 72 °C for 5 min to improve denaturation and subsequently incubated at 37 °C for 30 min. This mix was stored at - 20 °C until use and applied without further treatment.

The commercial available mouse-pancentromeric FISH probe mouse-Pan-Cy3 (BioCat, Heidelberg, D), was used as control and directly applied for hybridization after denaturation at 72 °C for 10 min.

3.2.4.4.3 Hybridization of FISH probes

Before hybridization, metaphase spreads were treated with pepsin and RNAse to remove cell debris. To this end, the previously prepared metaphase slides were washed with 2 × SSC for 5 min at RT. Then 200 µl RNAse solution (0.2 mg/ml RNAse A in 2 × SSC) was added to the coverslide. After 30 min incubation in a dark moist chamber at 37 °C, slides were washed three times with 2 × SSC. For the pepsin digest, prewarmed 50 ml ddH_2O to 37 °C was mixed with 100 µl 5 N HCl and 15 µl 10 % pepsin. Slides were incubated therein for 70 s, to allow the digestion of cytoplasmic proteins without destroying the chromosome structures. Immediately afterwards, slides were washed two times with PBS for 5 min at RT and then with an ascending ethanol row of 70 %, 90 % and 100 % respectively at 4 °C for 3 min each. Subsequently, slides were air-dried in a dark chamber.

To denature the chromosomal DNA, slides were put into a 72 °C hot denaturation solution (70 % Formamide/ 2 × SSC) for 105 s and immediately transferred to -20 °C pre-chilled 70 % EtOH. Slides were dehydrated by putting them into an increasing -20 °C chilled ethanol row for 5 min each (70 %, 90 %, 100 % ethanol). Finally, slides were air-dried in a dark chamber.

For the hybridization, a 10 µl drop of the probe mixture (3.2.4.4.2) was set on the slide and covered by an 18 mm × 18 mm cover slip by sealing the edges with the rubber glue

Fixogum. Slides were heated to 72 °C for 2 min, then transferred to 37 °C water bath in a closed metal chamber and incubated for 2 - 3 days.

3.2.4.4.4 Fluorescence-Immunodetection

Hardened fixogum was removed and coverslides carefully removed by dipping slides into 2 × SSC 0.2 % Tween-20. All following steps were performed under light protection of the slides. Slides were washed three times with 2 × SSC 0.2 % Tween-20 at 42 °C for 5 min, three times with 0.75 × SSC at 60 °C for 5min and once again at 42 °C for 5 min with 2 × SSC 0.2 % Tween-20. Unspecific binding sites for antibodies were blocked with 3 % BSA for 30 min at 37 °C. Slides were washed again at 42 °C with 2 × SSC 0.2 % Tween-20 for 5 min.

Immunolabeling of the probes was achieved by adding anti-biotin-Cy3.5 (1 : 3000 in 1 % BSA) binding to the biotin labeled probes and anti-dig-fluorescein antibody (1 : 150 in 1 % BSA). Incubation was performed for 45 min at 37 °C in a humidified chamber. Excessive antibodies were washed away by incubating slides three times at 42 °C in 2 × SSC for 5 min. DNA was counterstained with 0.05 µg/ml DAPI in 2 × SSC solution for 2 min in a dark moist chamber and washed with ddH_2O for 5 min. Slides were air-dried afterwards. To minimize fading of the fluorescence marker phenylendiamindihydrochlorid was added to the slides and covered by a cover slide.

3.3 Tissue culture techniques

3.3.1 Culturing eukaryotic cell lines

All mammalian primary cells and cell lines were cultured under sterile conditions at 37 °C, 95 % humidity and 7 % CO_2. Cell lines were passaged on a regular basis. To this end, old medium was removed from adherent cells and cells were washed with PBS. After detachment with 0.25 % Trypsin/EDTA (Gibco, Karlsruhe, D), cells were resuspended in 10 ml culture medium and a portion of the cells was transferred on a new culture plate with fresh medium according to the split ratio in shown in Table 5.

Table 5: Culture conditions of used cell lines

cell line	type	medium	split ratio	antibiotics for selection
NIH3T3	continuous murine fibroblast cell line	DMEM, 10 % FCS, 1% Pen/Strep	1:6 every 3-4 days	200 µg/ml G418 10 µg/ml BS 50 µg/ml Hygromycin
MHEC-5T	continuous murine heart endothelial cell	DMEM, 10 % FCS, 1%	1:8	10 µg/ml BS

	line	Pen/Strep	every 3-4 days	
M210-B4	continuous bone marrow stromal cell line	RPMI, 10 % FCS, 1% Pen/Strep	1:4 every 3-4 days	2,5 µg/ml BS
SVEC4-10	continuous murine endothelial cell line, SV40 transformed	DMEM, 10 % FCS,1 % Pen/Strep	1:8 every 3-4 days	10 µg/ml BS
MEF	primary murine embryonic fibroblasts	DMEM, 10 % FCS, 1 % Pen/Strep	1:2, on demand	
mES	murine embryonic stem cells	DMEM, 15 % FCS, 1 % NEAA, 1 % L-glutamine, 1 % Nucleosides, 0.1mM β-mercaptoethanol, 1 % Pen/Strep 1 × 10^3 Units/ml murine LIF (Active Bioscience)	change media daily split 1:6 every 2-3 days	10 µg/ml BS

% in (v/v), 0.6 % (w/v) Penicillin/ 1.3 % (w/v) Streptomycin (Pen/Strep)

3.3.2 Transfection of eukaryotic cells

Lipofection

Transfections of cell lines were routinely performed by lipofection with the reagent Transit3T3 (Mirus) for all mentioned cell lines according to manufacturer's instructions.

Nucleofection

Transfection of mES was performed by nucleofection with the AMAXA nucleofector (Lonza) according to manufacturer's instructions (prog. A-013).

3.3.3 Generation of stable cell lines with the pEPI vectors

For the generation of stable cell lines, cells were seeded in 6-well plates one day before transfection to a confluence of 70 % at maximum. Cells were transfected with 1 µg freshly prepared plasmids via lipofection or mock transfected. The next day, transfection efficiency was controlled via fluorescence microscopy, either by the fluorescence marker encoded on the test plasmid itself or by a fluorescent transfection control. Cells of one 6-well were split onto a 10 cm dish and antibiotics added according to Table 5. Untransfected control cells typically died within one week. Cell lines were either kept as pools or cell clones were subcloned using limiting dilution.

3.3.4 Isolation of cell clones

All cell clones in this work were obtained by limiting dilution with one exception; mES cell clones for blastocyst injections were gained by picking cell clones. For the isolation of cell clones by limiting dilution, cells were trypsinized, resuspended in appropriate medium and counted. Cell suspensions of 0.5 cells, 1 cell or 3 cells per 100 µl respecitively were made. These suspensions were seeded into 96 well plates, with 100 µl per well. Wells were controlled for single clones and grown up to stable cell lines under appropriate antibiotic selection.

3.3.5 Cryoconservation of cell lines

For long term maintenance of cell lines, cells were stored in liquid nitrogen. To this end, cells were incubated to 80 - 90 % confluence on 175 cm^2 dish. Cells were trypsinized and pelleted by centrifugation at 800 × g for 5 min. The pellet was resuspended in 3 ml freezing medium and aliquoted in three cryotubes with 1 ml each. Cells were incubated at -80 °C for one day in special isopropanol-filled container that allow a slow cooling of the cells and then transferred to the gas phase of a liquid nitrogen tank.

Freezing medium

40 % FCS

10 % DMSO

50 % of the respective growth medium

3.3.6 Mouse explants cultures

For the cultivation of mouse explants tissue a protocol was established that combines the needs for simplicity and requirements of the different cell types. As explants cultures were taken from each mouse individually, only a very limited amount of cells was available. Successful explants of heart, kidney, lung, spleen, salivary gland, bone marrow and fat tissue were obtained.

One mouse at a time was sacrificed, fur disinfected with 70 % EtOH and eviscerated. Organs were washed with PBS several times to remove as much blood as possible. Heart, kidney, lung, spleen and salivary gland were minced into small pieces in 2.5 ml freshly prepared dissociation buffer, incubated at 37 °C for 30 - 60 min and dispersed by pipetting up and down with a wide bore pipette tip from time to time until a smooth homogenate was obtained. The homogenate was pressed through a 100 µm strainer and resuspended in 10 ml DMEM. Cells were centrifuged at 300 x g for 5 min at RT. Supernatants removed and resuspended in 5 ml of the appropriate cell culture medium. Cells were seeded on gelatine coated 75 cm^2 tissue flasks. The same procedure was performed for fat tissue, with the exception that it was not minced and

cells containing a high amount of lipids do not pellet by centrifugation but were recovered also from the top of the medium. For the extraction of bone marrow, femure and tibia of one leg was removed and the tips clipped. The bone marrow was rinsed out with PBS, and cells resuspended in 10 ml DMEM. Cells were centrifuged as above and finally resuspended in 5 ml mmES-Medium. Medium was exchanged daily for one week and then every third day.

Dissociation buffer

12.5 mM HEPES in PBS

200 U/ml DNase I

13 Wünsch U/ml Liberase

Table 6: Media for mouse explant tissues

medium	ingredients	organ / cell type
mmES	407.5 ml DMEM 75 ml FCS 5 ml Pen/Strep 5 ml L-Glutamine 5 ml non-essential amino acids (100x) 3.5 µl β-mercaptoethanol 2.5 ml Fungizone (1.25 µg/ml Amphothericin B)	Heart, kidney, salivary gland, bone marrow, fat, muscle
LSGS	402.5 ml DMEM 75 ml FCS 5 ml Pen/Strep 5 ml L-Glutamine 5 ml non-essential amino acids 3.5 µl β-Mercaptoethanol 2.5 ml Fungizone 5 ml 1 × LSGS (Invitrogen) (1 µg/ml hydrocortisone; 10 ng/ml human epidermal growth factor; 3 ng/ml basic fibroblast growth factor; 10 µg/ml heparin)	lung

Spleen - medium	432.5 ml RPMI 1640 50 ml FCS 1 ml 1M HEPES 5 ml Pen/Strep 2.5 ml Fungizone	spleen

3.3.7 Flow cytometry

Fluorescence based flow cytometry assay was performed for the analysis of expression profiles of fluorescent proteins in stable cell lines. To this end, at least 5×10^5 tissue cultured cells were harvested by Trypsin digest, washed with PBS and finally resuspended in 1 ml PBS with 2 % FCS. The parental cell lines were used as negative controls to determine the autofluorescence of the cells. Measurement was performed with the flow cytometer Epics XL-MCL (Beckman Coulter).

3.4 Virological Methods

3.4.1 Reconstitution of viruses from BACs

The cloning of the MCMV genome as a bacterial artificial chromosome (BAC) allows the manipulation of the viral genome, its characterization and control of the modification in bacterial cells [64]. To obtain viruses from BAC encoded genomes, permissive cells were transfected with the corresponding BAC. As a positive control for reconstitution, the BAC pSM3fr-MCK-2fl encoding wt-MCMV genome was used. To this end, MEF cells or transgenic NIH3T3 cells were seeded in 6-well dishes one day before transfection. 1.5 µg freshly prepared BAC-DNA was transfected with the help of Mirus Transit3T3 transfection reagent. Cells were split the next day onto a 10 cm dish, whereby the old supernatant is added back. Plaques from reconstituted viruses arose typically at 4-5 days after. In case of attenuated viruses, plaque formation and lysis was delayed and cells were split with transferring the supernatants until lysis occured. After complete lysis of the cells, supernatants were stored at -80 °C for further processing.

To remove the BAC cassette from the virus genome, which tolerates some oversize, viruses had to be passaged on MEF cells. To this end, a 6 well of confluent MEF cells were infected with the viruses for 1 h and then supernatant was replaced by fresh medium and waited until cells were completely lysed. After four rounds of passaging, the BAC cassette, encoding a

bacterial origin of replication and the chloramphenicol resistance gene, was typically removed from the viral genome and virus stocks were produced.

3.4.2 Production of virus stocks

For production of high titer MCMV virus stocks, six 175 cm^2 dishes of confluent M210-B4 cells were trypsinized, washed and resuspended in 400 ml medium. Cells were infected in suspension with an MOI of about 0.1 and plated on 20 × 175 cm^2 dishes. Typically after 4 – 5 days complete cell lysis occured and supernatant as well as residual cells were harvested in 250 ml beakers. All steps for the preparation were performed on ice with pre-chilled material. The virus/ cell suspension was centrifuged at 6,500 × g for 15 min at 4 °C. The supernatant was collected and stored on ice. The pellet was resuspended and cells cracked by homogenization in a glass douncer to liberate intracellular virions. By centrifugation at 20,000 × g for 10 min, cell debris was removed and the supernatant pooled with the supernatant put on ice. Virions were than pelleted by centrifugation of the supernatants for 3 h at 25,000 × g and again homogenized by douncing in a volume of 4 ml DMEM. This homogenate was loaded on top of 10 ml 15 % sucrose cushion and centrifugation tubes (SW28) completely filled up with virus stock buffer (VSB). By 1h ultracentrifugation at 20,000 × g in a swing bucket rotor (SW28, Beckmann), purified virus particles were pelleted. Supernatants were discarded and the virions resuspended in 1.5 ml VSB by homogenization in a douncer. For virus stocks that were to be used in mouse experiments another centrifugation step was performed to clear the suspension. To this end, the virus stock was two times centrifuged at 1,300 × g at 4 °C for 2 min and the pellet was discarded. The virus stock was then aliquoted in 50 – 100 µl and stored at -80 °C.

VSB (pH 7.8)

50 mM Tris-HCl

12 mM KCl

5 mM EDTA

autoclave

3.4.3 Virus growth analysis

Typically virus growth analysis is performed to compare growth kinetics of two different viruses. In this thesis, virus growth analysis was mainly performed to evaluate the productivity of different stable cell clones infected with the same virus.

Cells of different cell lines were harvested, counted and the viability of the cells was checked. 1×10^5 cells were seeded per well of a 12-well plate, with three wells per timepoint were analyzed for virus titers and a fourth well was used to determine cell density. Cells were allowed to attach for 4 - 5 h before infection with wt-MCMV (or a fluorescence marker containing MCMV) at an MOI of 0.1 in a volume of 1 ml. Virus suspension was washed away after one hour incubation at 37 °C. Subsequently, virus suspension was replaced by 1 ml fresh culture medium. Supernatants from cells were collected at the respective time points of growth analysis and kept at -80 °C until plaque assay was performed to determine virus titer. In addition, uninfected cells were counted daily to monitor the cell density and thus growth properties of the different cell lines.

3.4.4 Plaque assay

Plaque assays were performed to determine virus titer of cell culture supernatants or virus stocks. To this end, serial 1:10 dilutions of virus supernatants were performed in 500 µl MEF-medium. 200 µl of these virus dilutions, typically ranging from 10^{-1}-10^{-7}, were transferred to 48 well plates, seeded previously with a confluent MEF monolayer. After 1 h incubation at 37 °C, virus dilutions were removed and 400 µl carboxymethylcellulose-containing medium added. Due to the high viscosity of this medium, viral particles cannot diffuse through the medium and a local plaque will arise by cell-to-cell spread on the spot where a plaque forming unit (PFU) infected the MEF monolayer. Plaques were counted by a light microscope at day 4 post infection.

The virus titer is calculated by the following equation:

$$virus\ titer\ (PFU/ml) = \frac{number\ of\ counted\ plaques * dilution\ factor}{volume\ of\ plated\ virus\ dilution\ (0.2\ ml)}$$

Carboxymethylcellulose-containing Medium

3.75 g Carboxymethylcellulose

388 ml H_2O

to be autoclaved before supplementation with

25 ml FCS

50 ml 10 × MEM

5 ml L-Glutamine

2.5 ml non essential amino acids

5 ml Pen/Strep

24.7 ml $NaHCO_3$ (7.5 %)

3.5 *In vivo* experiments

All animals were housed at the animal facility of the Max von Pettenkofer- Institute or the animal facility of the department of Molecular Animal Breeding and Biotechnology, LMU, under specified-pathogen-free (SPF) conditions. Animal experiments were approved by the Regierung Oberbayern, the responsible office of the state of Bavaria.

3.5.1 Generation of VIOLA mouse strains

The mES cell line E14 was transfected with pEpibo-luc-ori by nucleofection. Positive cells were selected with 5 µg/ml BS for three days. Appropriate clones were selected after partial differentiation of the clones by removal of LIF and feeder layers to allow productive infection with MCMV [132]. Selected clones were injected into C57BL/6 blastocysts and implanted into foster NMRI mothers (Chair of Molecular animal breeding and biotechnology, LMU). Chimeras were backcrossed to 129X1/SvJ mice (Jackson) and analyzed for presence of the transgene by PCR with the primer P(SV40)-probe-for and pA(SV40)-probe-rev (4.4.2). Two lines were obtained, which were named VIOLA (virus inducible oriLyt-dependent luciferase animal) line A and B.

129-VIOLA-A: derived from mES clone A3, backcrossed to 129X1/SvJ

129-VIOLA-B: derived from mES clone B8, backcrossed to 129X1/SvJ

3.5.2 Invasive bioluminescence detection

As a pre-experiment, invasive analysis of bioluminescence was performed with the VIOLA-A line mice. To this end, two animals of VIOLA-A and two animals of the background strain 129X1/SvJ were infected with 1×10^5 PFU wt-MCMV in a volume of 300 µl in PBS intravenously (i.v). One mouse of the VIOLA line and one of the background strain were not infected and served as background controls. Mice were sacrificed at day 2 or day 5 post infection. Organs and tissues (kidney, spleen, liver, lungs, heart, salivary gland, brain, fat and muscle) were harvested, homogenized by filtring through a cell strainer with a 100 µm pore size and resuspended in 5 ml MEF-Medium. One ml of the homogenates was pelleted by centrifugation at 1,300 × g for 5 min. The supernatant was discarded and cells were washed with PBS. The centrifugation step was repeated and the pellet was finally resuspended in 1 × Passive Lysis Buffer (Promega). After 10 min lysis at 37 °C, FL expression of the lysates was measured with a bioluminescence reader (Berthold).

3.5.3 Non-invasive bioluminescence imaging

For non-invasive bioluminescence imaging, VIOLA-A mice were infected i.v. with wt-MCMV or mock treated with PBS. As a positive control mice of the background strain 129X1/SvJ were infected with MCMV-luc, which constitutively expresses FL [71]. To measure the background bioluminescence, one measurement was performed before infection of the mice. To this end, 3 mg Na-D-Luciferin in 200 µl PBS were injected intraperitoneal (i.p). After 5 min mice were anesthetized with 2.5 % isofluroan gas in a whole body chamber. Respiration and response to rear foot reflex stimulation was constantly monitored. When mice were fully anaesthetized, they were transferred to the IVIS Lumina measurement chamber onto nosecone inhalators. Isofluran concentration was set down to 1.5 %, a non-irritating eye cream was applied and measurement performed on 37 °C pre-warmed heating plates.

3.6 Biochemical analysis

3.6.1 *In vitro* Luciferase assay

Bioluminescence measurements were performed to quantify induction of luciferase reporter gene expression under various conditions.

3.6.1.1 Transient transfections

Expression studies in transient transfection assays were performed with a dual bioluminenscence reporter measurement, whereby the expression of firefly luciferase (FL) under control of different promoters was normalized to a control vector constitutively expressing Renilla luciferase. To this end, NIH3T3 cells were seeded on a 12-well plate to 70 % confluence. Cells were transfected in duplicates with 1 µg test plasmid (e.g. pEpibo-P(SV40)-luc) and 100 ng control vector pTK-RL using the transfection reagent Transit3T3 (Mirus, Madison, USA) according to manufacturer's instructions. Duplicate transfections were pooled and split on 8 wells of a 24-well plate the day after. Half of the wells were infected with MCMV at an MOI of 0.5. The other half was used as uninfected control. 24 h or 48 h after infection cells were lysed in 100 µl 1 × Passive Lysis Buffer (Promega, Mannheim, D) on 37 °C for 10 min. Bioluminescence measurement was performed with 10 µl of the lysates in duplicates with the Microplate luminometer (LB960, Berthold). 40 µl of luciferin (Promega, Mannheim, D) and 20 µl of colenterazine (Promega, Mannheim, D) were injected into each well and light measured over 10 s.

For the evaluation of FL expression, corresponding relative light units (RLU) of Renilla luciferase was used for normalization.

3.6.1.2 Stable cell lines

Induction of FL expression by MCMV infection was analyzed in cells stably transfected with plasmids pEpibo-luc or pEpibo-luc-ori. Therefore, uninfected and infected cells were compared. To this end, 10^5 cells per well were plated in 12-well plates and allowed to attach for 3 – 4 hours. Half of the wells were infected with MCMV at an MOI of 0.5 and the other half left untreated. Cells were lysed 24 h or 36 h post infection performed as described above.

3.6.2 Bradford Assay

Protein concentration of cell lysates was determined by Bradford assay[133]. The principle of the assay is based on the ability of Coomassie-Brilliant blue to bind to proteins and the resulting absorption shift from 465 nm to 595 nm. Concentration of the cell lysates was correlated to a BSA standard curve. To this end, 1-10 µl cell lysates (harvested with Lysis Buffer B or Passive Lysis Buffer, Promega to be compatible with the assay) were mixed with 1 ml of 1:5 diluted Bradford Reagent (BioRad). After 5 min incubation, solutions where measured in the photometer at OD_{595} and concentration of the protein solution determined.

Lysis Buffer B (pH 6.8):

62.5 mM	Tris
6 M	Urea
10 % (v/v)	Glycerol
2 % (v/v)	SDS

3.6.3 SDS-PAGE

SDS-polyacrylamid gel electrophoresis (SDS-PAGE, [134]) and western blot were performed to analyze the expression of recombinant proteins from cell lines and viruses. For the separation of proteins according to their molecular weight discontinuous SDS-PAGE with the buffer system of Fling & Gregerson [135] was used. For equal loading, protein concentrations were assessed by Bradford assay (see section 3.6.2). Protein samples were denatured prior to loading in total lysis buffer or lysis buffer B and heated for 10 min at 95 °C. For very viscous samples with high gDNA content, 2 µl of Benzonase (Roche) was added to the samples and incubated for 30 min on ice. A prestained protein marker was loaded on each gel to correlate separation of the protein

samples to the size of the marker proteins (NEB, Prestained ColorPlus). Gels were cast as described in Table 7, and run for 15 min at 80 V, to allow a slow running into the stacking gel, and then for 60 min at 175V. In general, separated proteins were further processed by western blot analysis.

Table 7: Composition of gels for SDS-PAGE

reagent	**stacking gel**	**resolving gel**		
	5 %	**10 %**	**12 %**	**15 %**
Acrylamid 30 %	830 µl	3.3 ml	4 ml	5 ml
Gel Buffer 4x	1.25 ml	2.5 ml	2.5 ml	2.5 ml
ddH_2O	3.25 ml	4 ml	3.3 ml	2.3 ml
TEMED	100 µl	100 µl	100 µl	100 µl
10 % APS	20µl	5 µl	5 µl	5 µl

Total Lysis Buffer

62.5 mM Tris

2 % (v/v) SDS

10 % (v/v) Glycerol

6 M urea

0.01 % (w/v) bromphenolblue

0.01 % (w/v) phenolred

5 % (v/v) β-mercaptoethanol

10 × Laemmli buffer

25 mM Tris

10 % SDS

250 mM Glycine

4 x Resolving Gel Buffer (pH 8.8)

1.5 M Tris

0.4 % SDS

4 x Stacking gel buffer (pH 6.8)

0.5 M Tris

0.4 % SDS

5 x SDS loading buffer (pH6.8)

300 mM Tris

10 % SDS

30 % Glycerol

0.01 % (w/v) bromphenolblue

0.01 % (w/v) phenolred

5 % (v/v) ß-mercaptoethanol

3.6.4 Western blot

Western blot analysis is a sensitive method to detect specific proteins by an immunochemical reaction. Proteins separated by SDS-PAGE were transferred by Semi-Dry electroblotting (BioRad, TransBlotSC) onto a activated PVDF-membrane (GE Healthcare, Hybond-P) in western blotting buffer with 19 V for 40-60 min, depending on the designated protein mass. Afterwards membranes were incubated with a suitable blocking buffer (5 % skimmed-milk or 5 % BSA or 5 % FCS in TBS-T 0.1) for 1 h at RT under constant agitation. Primary antibodies diluted in blocking buffer were bound during o.n. incubation at 4 °C on a roller mixer. After five subsequent washes with washing buffer (TBS-T 0.1 or TBS-T 0.2.5) for 10 min each, membranes were incubated with an appropriate secondary antibody coupled to horse radish peroxidase (HRP) dissolved in blocking buffer at RT for 2 h. Unbound antibodies were washed off by five subsequent washes with washing buffer for 10 min each. Chemiluminescence induced by the HRP-tagged proteins was detected with ECL^{PLUS} Western Blot Detection System (GE Healthcare) on an X-ray film (Hyperfilm ECL, GE Healthcare) according to manufacturer's instructions.

Western blotting buffer	Washing buffer (TBS-T 0.1 or TBS-T 0.25)
25 mM Tris	150 mM NaCl
192 mM glycine	10 mM Tris/HCl
20 % (v/v) methanol	0.1 % or 0.25 % Tween-20

3.6.5 Immunofluorescence

For the detection of the immediate early protein 1 (ie-1) of MCMV, immunofluorescence analysis was performed. Cells grown on plates were fixed for 5 min with acetone/methanol (1:1) at RT. After three subsequent washes with PBS, primary antibody CHROMA101 (kindly provided by Stipan Jonjic, Croatia) was added 1:200 diluted in 5 % FCS/PBS to the cells and incubated for 45 min at 37 °C. Cells were washed three times with PBS and incubated with a secondary anti-mouse-Cy3 antibody 1: 5000 diluted in 5 % FCS/PBS at 37 °C for 1 h. After another three subsequent washing steps, nuclei of the cells were counterstained with 5 μg/ml Hoechst 333258 (Invitrogen, Karlsruhe, D) in PBS for 1 min. Finally, cells were again washed three times and analyzed by fluorescence microscopy.

4 RESULTS

4.1 Construction of an virus inducible system on the episomal vector pEPI

Episomal vectors provide interesting features that were thought to be advantageous for the construction of the novel expression system. Most important, episomal vectors do not integrate into the host chromatin, thus are independent from positional effects of the surrounding host chromatin, which in turn results in more reliable expression rates and furthermore they possess a lower risk for insertional mutagenesis that limits their usage *in vivo*.

A non-viral episomal vector, namely pEPI-1 has been originally described by Piechaczek and colleagues [136]. Several follow-up studies underlined the previous findings and highlighted the benefits of this episomal vector.

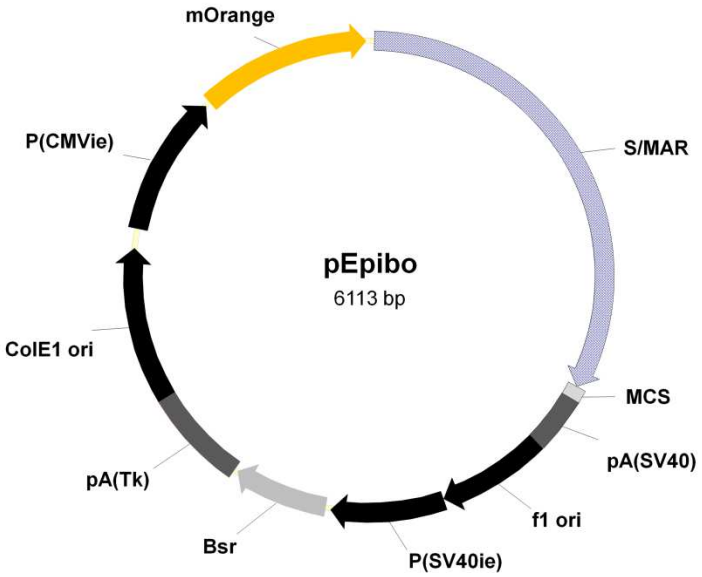


Figure 12: Vector map of pEpibo

The *gfp* gene and the neomycin resistance gene of the original pEPI vector were replaced by mOrange and the blasticidin S resistance gene (*bsr*). The modified vector was termed pEpibo. P(hCMVie) = immediate early HCMV Promoter, S/MAR= surface matrix attachment region, MCS = multiple cloning site, P(SV40ie) = SV40 immediate early promoter/ori, pA(TK) = Thymidin kinase poly A site, f1 ori = phage origin of replication, colE1 ori = bacterial origin of replication.

Fluorescence and antibiotic marker of the original pEPI vector were changed for this project; i.e. the *gfp* gene was replaced by the *mOrange* gene to distinguish its fluorescence from the dominant-negative protein GFPSCP, which was used at later time points of this study. Furthermore, the neomycin resistance was exchanged to a blasticidin S resistance gene (*bsr*), which allows improved selection in NIH3T3 cells. The resulting vector was termed pEpibo (Figure 12).

4.1.1 *Trans*-activation of viral promoters during infection

Using inducible expression systems is a good way to avoid toxic side-effects, often associated with high and constitutive expression levels. It is known that many herpesviral promoters can be induced *in trans*, meaning that viral transcription factors bind and activate promoter sequences inserted into the host chromatin upon infection [137]. However, strength of expression levels as well as tightness of the promoters in the non-induced, meaning in this case the non-infected status, has not been assayed, yet.

As mentioned above (see section 1.3) herpesviruses proteins can be divided in three kinetic classes, according to the time point of protein production in the virus life cycle. The first proteins that can be detected after infection are termed 'immediate-early' proteins. The genes of these proteins are instantly activated by factors provided in the virion or are directly transcribed by the host RNA polymerase II. Some immediate-early proteins are transcription activators that in turn activate early genes and the gene products can be detected around 6 hours post infection. Late proteins arise after about 16 h post infection and can be divided in leaky-late and true-late proteins. True-late genes need in contrast to leaky-late genes DNA replication of the genome for transcription.

Several viral promoters, belonging to different kinetic classes were used to test if the intrinsic feature of the virus to activate its own promoters could be exploited to generate a virus inducible expression system. Thus different promoter sequences of MCMV together with a firefly luciferase open reading frame were cloned into the multiple cloning site of pEpibo. One promoter of an early gene, namely M143 [128], and two of late genes, namely M53 [138] and M94 [100], as well as the strong human CMV immediate-early promoter (P(hCMVie)) and the minimal SV40 promoter were tested. Only for the promoter of M143, namely P(M143), a detailed promoter analysis was available [128] and length of the promoter region was adopted as described therein. For P(M53) and P(M94) a 500 bp long sequence upstream of the start codon was used. P(hCMVie) was derived from the vector pCR3 and the minimal P(SV40) from pGL3-control.

First, induction of FL expression driven by the different promoters was tested. NIH3T3 cells were co-transfected with one of the pEpibo-P(X)-luc plasmids, as well as the control plasmid pTK-RL encoding for Renilla luciferase for transfection normalization. After transfection, cells were split and infected with MCMV or were mock treated. FL expression was measured 24 h and 48 h p.i. and was normalized to Renilla luciferase expression.

The minimal SV40 promoter was not induced by MCMV infection, neither at 24 h nor 48 h p.i. (Figure 13). All other viral promoters were induced in *trans*. Nevertheless, none of the promoters was completely silent prior to infection and the overall induction level was quite low. The P(hCMVie), one of the strongest known promoters, drove the highest FL expression, as expected, and FL expression was enhanced 2.5-fold at 24 h p.i. and 5-fold at 48 h p.i. by MCMV infection. However, the basal constitutive expression in uninfected cells remained high. The highest ratio of induction was seen with the early promoter P(M143), where FL expression was increased 7.5-fold at 24 h p.i., but dropped to 2.5-fold after 48 h p.i. in accordance with the typical early gene expression pattern. While the late P(M94) showed already some response to the MCMV infection at 24 h p.i. and increased to 6.8-fold induction at 48 h p.i., the expression controlled by the second late promoter P(M53) did not increase at 24 h p.i. and only a 2-fold induction was measured at 48 h p.i. Furthermore, P(M53) was the weakest of all tested promoters.

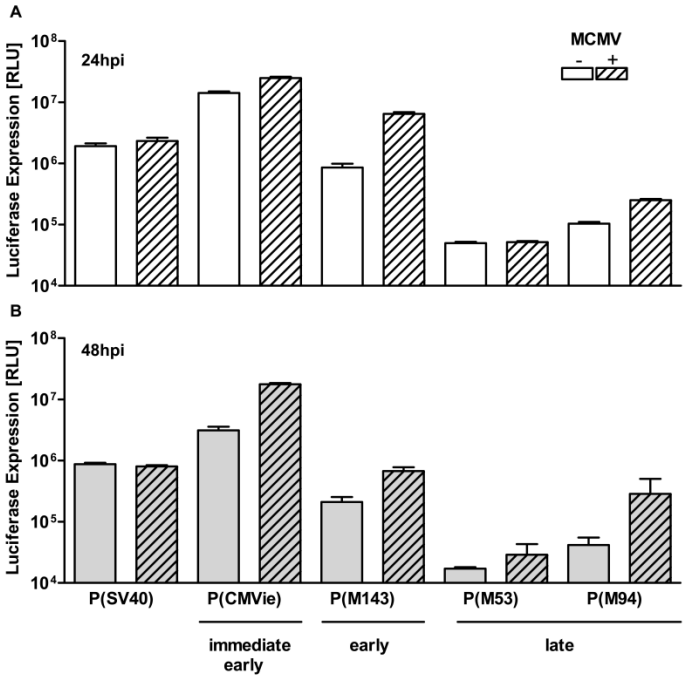


Figure 13: *Trans*-activation of herpesviral promoters by infection.

NIH3T3 cells were transfected with either pEpibo-P(SV40)-luc, or the respective constructs with the human immediate early promoter P(CMVie), the early promoter P(M143) as well as the two late promoters P(M53) and P(M94). Firefly luciferase expression was normalized to the renilla expression by the co-transfection of pTK-RL. Cells were infected with MCMV at an MOI of 0.1 and 24 h (A, white) or 48 h p.i. (B, grey) and a bioluminescence assay was performed.

4.1.2 Construction and characterization of the replicon vector – Induction of gene expression by plasmid replication

As the induction of the viral promoters by *trans*-activation (see section 4.1.1) was low, another possibility to increase gene expression was needed. Herpesviruses can replicate plasmids containing their lytic replication origins (oriLyts) in *trans* [45, 46]. Therefore, we tested if the addition of the MCMV oriLyt to the pEpibo-luc vector would cause vector replication and thus enhance gene expression during infection. Ideally, the transgene should act like a true-late gene and would not be transcribed before infection and replication (Figure 14).

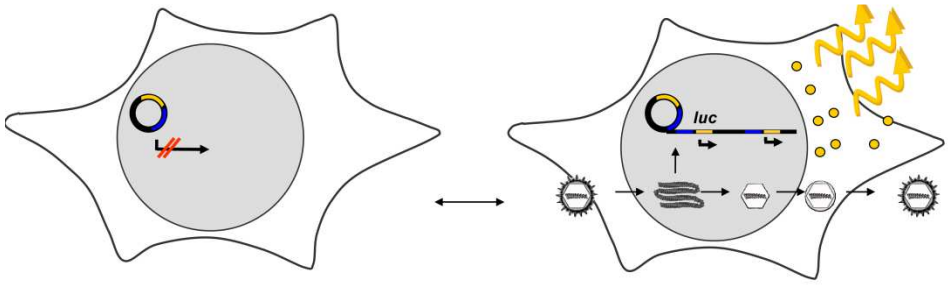


Figure 14: Concept of oriLyt-induced gene expression.

While the gene expression from the episomal plasmid should ideally be silent in uninfected cells, infection with MCMV should activate DNA replication of the episome via activation of the incorporated MCMV oriLyt (blue) and replication of the plasmid, thus inducing and increasing reporter gene (luc) expression.

In order to construct such an episomal vector containing the oriLyt, the oriLyt-sequence had to be obtained from the MCMV genome first. The location of the minimal oriLyt region in the MCMV genome was published by Masse and colleagues in 1997 [46]. The borders of the region were not well defined and no further study of the oriLyt region has been performed. Yet, the oriLyt sequences of the CMVs are known to be complex, containing several inverted and direct repeats as well as several runs of identical nucleotides (see 1.4.2.2). This makes the oriLyt sequence a very difficult template for PCR reactions. To facilitate the cloning of the MCMV oriLyt from the viral BAC pSM3fr into a smaller plasmid, a 'pick-up-strategy' was designed. It has been described for other herpesvirus origins that additional sequences next to the minimal oriLyt can have enhancing effects on replication [139]. Therefore, a 3.9 kb DNA fragment rather than the minimal oriLyt sequence (1.7kb) was cloned. To do so, a PCR fragment containing the selective bacterial replication origin oriR6K together with a kanamycin resistance gene was amplified, which is flanked by *Mlu*I restriction sites as well as one *Nhe*I restriction site on the 5'-site of the construct (Figure 15A). In addition, homologous sequences at both ends allowed the

recombination of the PCR fragment into the MCMV BAC genome into the 5'- site of the oriLyt sequence. The m58 gene partially overlaps with the oriLyt region, therefore the oriR6K-kan fragment was recombined into the ORF behind the start ATG, thereby disrupting the expression unit of m58. Another gene, namely m59, was originally annotated inside the minimal oriLyt region. However, later expression profiling indicated that this gene does not exist [140]. Correct insertion of the PCR fragment was analyzed by fragment length polymorphism of the respective BAC clones (Figure 15 B). As the MCMV genome encodes another *Nhe*I site on the 3'-site of the oriLyt sequence, the replication origin together with the PCR fragment could be excised by *Nhe*I restriction digestion. The DNA fragment was ligated to a functional bacterial plasmid, which can be selected by kanamycin and growth in the oriR6K specialized *E. coli* PIR1. From ten selected clones of the ligation reaction, nine did contain the plasmid p06kan-MCMV-oriLyt (Figure 15 C).

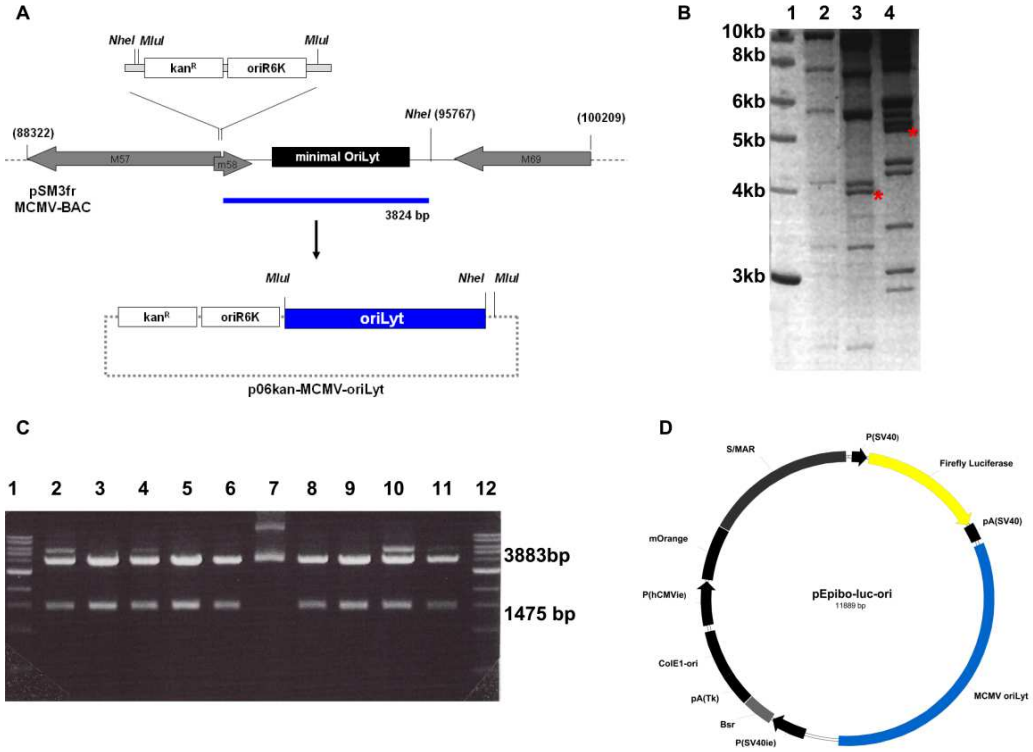


Figure 15: Cloning of pEpibo-luc-ori.

A pick-up-strategy was designed to clone the complex and repetitive oriLyt of MCMV into the transfer vector p06kan-MCMV-oriLyt. Homologous recombination was used to insert a PCR fragment containing a kanamycin resistance gene and a selective bacterial origin oriR6K flanked by *Mlu*I restriction sites and one additional *Nhe*I restriction site into the viral BAC pSM3fr encoding the entire MCMV genome. B) Successful insertion of the PCR-fragment into pSM3fr was analyzed by restriction digest. (lane 1, 1kb Marker, lane 2 native pSM3fr digested with *Ase*I, lane 3 pSM3fr-oriR6kan digested with *Ase*I, lane 4 pSM3fr-oriR6kan with *Nhe*I). Additional bands due to the correct recombination are marked with an asterisk. C) After the recombination the oriLyt can be excised by a *Nhe*I digest together with oriR6K and kanR gene and ligated to the vector p06kan-MCMV-oriLyt. Due to the selection with the antibiotic and the growth in PIR1 *E.coli* that can maintain oriR6K vectors, plasmids obtained contained in 9 of 10 cases the wanted vector. D) By a *Mlu*I digest the oriLyt could then be transferred into the pEpibo vector. Insertion of a FL reporter gene (*luc*) upstream of the oriLyt allowed a sensitive measurement of oriLyt induced gene activation.

The *Mlu*I restriction sites initially flanking the PCR fragment now flanked the oriLyt sequence in the plasmid p06kan-MCMV-oriLyt after ligation. Thus the oriLyt sequence became transferable via these restriction sites into the pEpibo vector, resulting in the replicon vector pEpibo-MCMV-orilyt. To test whether oriLyt-mediated amplification of the replicon vector indeed increased gene expression a FL reporter gene (*luc*) was cloned into the vector (Figure 15 D). This should allow sensitive and quantitative measurements of the transgene expression in a bioluminescence assay. The expression of the FL reporter gene was set under the control of the minimal SV40 promoter, as this promoter is not influenced by MCMV infection (Figure 13). The changes of gene expression in response to MCMV infection should thus depend entirely on the effect of the MCMV oriLyt-induced replication of the vector. The resulting replicon vector was named pEpibo-luc-ori (Figure 15 D).

4.1.2.1 Infection can reactivate silenced gene expression of the replicon vector

To test whether expression of the reporter gene from the replicon vector is enhanced upon infection, NIH3T3 were stably transfected with pEpibo-luc-ori. FL expression was measured in two stable cell pools, NIH3T3: luc-ori t1 and NIH3T3: luc-ori t2 (luc-ori t1, luc-ori t2) over time. In both a strong decline of bioluminescence was found during ongoing culture of the cell pools (Figure 16 A, plain bars). In one pool, i.e. luc-ori t1, the expression of the reporter gene dropped under detection limit after 16 weeks. If the same transfectants, however, were infected with MCMV at an MOI of 0.5 strong reporter gene expression was detected by bioluminescence after 24 h p.i. (Figure 16 A, hatched bars). Reporter gene expression was up to a 1000-fold higher than in non-infected cells. While in the cell pool luc-ori t1 the ability to induce FL after infection declined over time, in the cell pool luc-ori t2 induction strength after 16 weeks was as strong as after the first week.

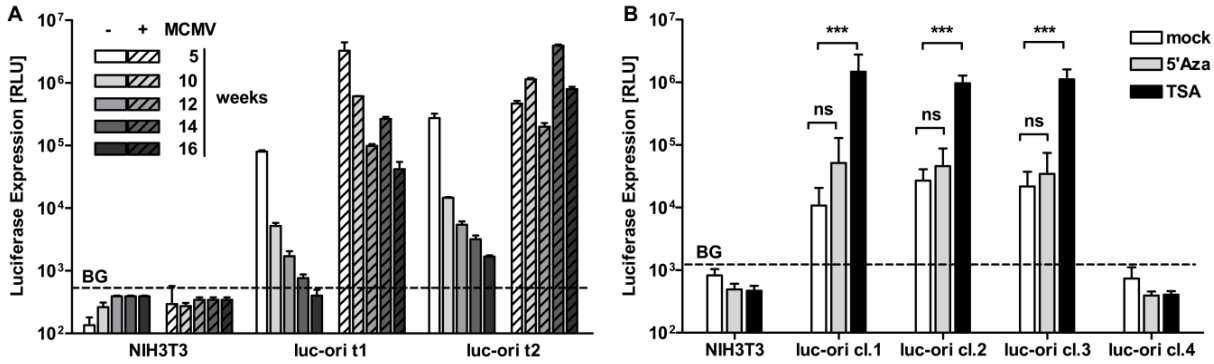


Figure 16: Infection with MCMV reactivates silenced reporter gene expression.

In two stable NIH3T3 cell pools transfected with pEpibo-luc-ori (luc-ori t1, luc-ori t2) expression of FL was measured in relative light units (RLU) over time. Reporter gene expression is lost in uninfected cells over time. Infection of luc-ori t1 and t2 with MCMV (MOI of 0.5) however induced high expression of FL 24 h p.i.. NIH3T3 served as control to determine background (BG) signal. (B) FL expression is inactivated by histone deacetylation. Cell clones derived from subcloning luc-ori t1 were subjected to treatments for 36 h with 25 μM 5'-azacytidine (5'Aza, gray bars), an inhibitor of CpG-methylation or 330 nM trichostatin A (TSA, black bars), an inhibitor of histone deacetylases. FL expression was analyzed in comparison to untreated control (mock, white bars). Recovery of FL expression was significantly enhanced by decondensing histone packaging through TSA treatment in three of four isolated clones. (*** $p < 0.001$, ns $p > 0.05$, Two-Way-Anova, depicted is mean + SD). RLU (relative light units), p.i. post infection, weeks = weeks post transfection of pEpibo-luc-ori

To examine the mechanism of bioluminescence regulation in murine fibroblasts, four isolated cell clones that were derived from luc-ori t1 after 16 weeks of selection were treated with trichostatin A (TSA) or 5'-azacytidine (5'-Aza). TSA is a well known inhibitor of histone deacetylase thus indirectly lifting histone-dependent silencing mechanisms [141]. 5'-Aza is inhibiting methylation of cytosines after incorporation of the modified nucleotide into the genomic DNA [142]. In three of the four isolated cell clones (luc-ori cl. 1 to cl.3) a low FL expression level was detected in the untreated cells (Figure 16 B, white bars). If 330 nM TSA was added for 36 h, FL reporter gene expression increased by approximately 100-fold in the three cell clones (black bars). Bioluminescence in cell clone luc-ori cl.4 could never be detected in any condition. Changes in luciferase expression due to 5'-Aza treatment were not significant (grey bars) in the cell clones luc-ori cl.1 to cl.3. Therefore, the silencing of the transgene expression in the cell lines is most likely due to the inaccessibility of the gene for RNA polymerase due to condensed chromatin packaging, which was lifted upon MCMV infection (Figure 16A, hatched bars).

4.1.2.2 Immediate early proteins cannot remove silencing from pEpibo-luc-ori

The previous experiments showed that the replicon vector was silenced in the stable cell lines by a histone deacetylase-dependent mechanism. For HSV-1 [143], HCMV [144] and MCMV [145] it has been described that viral immediate early proteins can inhibit *de-novo* silencing by histone deacetylase (HDAC), which prevents inactivation of incoming viral genomes. To assess whether the immediate early proteins of MCMV are, in the present case, able to recover reporter gene expression from the silenced cell lines, luc-ori cl. 1 cells were transfected with the plasmid pGPS-ie1/ie3-ie2. This plasmid encodes the entire immediate early locus and should produce all known isoforms (kindly provided by K. Eisenächer). Expression of the ie1 and ie3 protein was controlled by immunofluorescence staining with an anti-ie1/3 antibody (Figure 17 A) and bioluminescence was measured in the treated and the untreated cells. The expression of the immediate early proteins did not result in an increase of reporter gene expression but rather in a slight decrease (Figure 17 B). Thus, the immediate early proteins are not directly involved in the removal of FL silencing on the pEpibo-luc-ori construct.

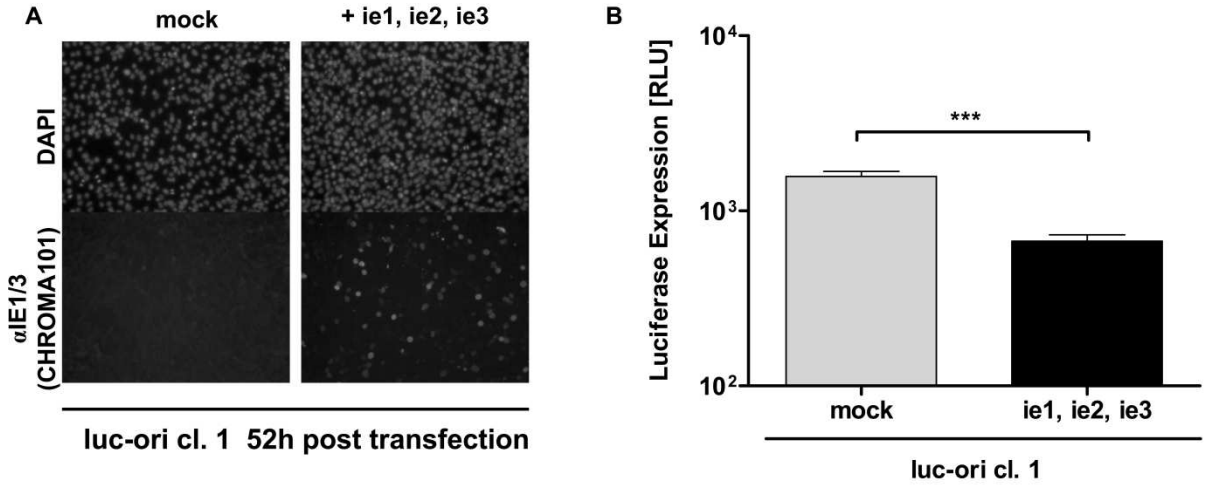


Figure 17: Immediate early proteins do not induce luciferase expression.

luc-ori cl. 1 cell were transfected with a plasmid encoding immediate early proteins ie1, ie2 and ie3 or were mock transfected. 52 h post transfection the presence of the ie1/3 proteins was analyzed by staining with the antibody CHROMA101. Cell nuclei where counterstained with DAPI. (B) Transfection of the ie proteins (black bar) alone cannot induce FL expression in luc-ori cl. 1 cells. Moreover, a significant drop in background expression compared to the mock transfected cells (gray bar) could be detected. (***: $p < 0.001$, ns: $p > 0.05$, Students t-test, depicted is mean + SD)

4.1.2.3 Induction of reporter gene expression requires oriLyt sequence

To test whether reactivation of reporter gene expression is dependent on the oriLyt sequence, NIH3T3 cells were transfected with pEpibo-luc-ori or pEpibo-P(SV40)-luc (see section 4.1.1) and stable cell pools were isolated. Both vectors differ only in the presence or absence of the oriLyt sequence.

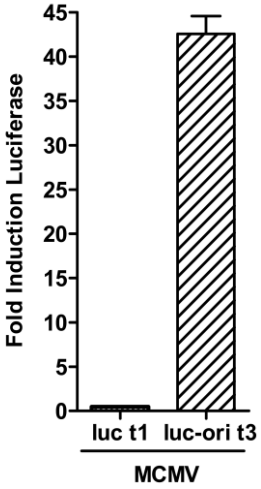


Figure 18: Only construct with oriLyt is activated by viral infection.
Stable cell pools were generated by transfecting NIH3T3 with pEpibo-luc (luc t1) or pEpibo-luc-ori (luc-ori t3). Cells were infected with MCMV at an MOI of 1 and bioluminescence analysis was performed 36 h post infection. Induction of FL expression was calculated as a ratio of RLU of infected and uninfected cells. The pool luc-t1 did not induce FL after MCMV infection, while luc-ori t3 was inducible as previously shown, thus demonstrating that the oriLyt is needed for the activation of reporter gene expression.

After two months of culturing, the transfectants NIH3T3:luc t1 (luc t1, derived from pEpibo-P(SV40)-luc) as well as NIH3T3:luc-ori t3 (luc-ori t3, derived from pEpibo-luc-ori) were analyzed for their ability to induce FL expression upon MCMV infection. In both stable cell pools a low background signal of FL could be detected (data not shown). In the luc-ori t3 pool a > 40-fold increase of luciferase expression could be seen upon infection. Notably, reporter gene expression in the luc t1 cells did not change upon infection (Figure 18). Thus, the reactivation of the replicon vector from silencing was dependent on the presence of the MCMV oriLyt sequence.

The replicon system was tested in different cell types, murine stromal fibroblast cells M2-10B4, murine heart endothelial cells MHEC-5T and SV40 transformed murine endothelial cells SVEC4-10. Cells were transfected with the vector pEpibo-P(SV40)-luc or pEpibo-luc-ori. Stable cell pools were selected and reporter gene expression was measured in infected and non-infected cells. Irrespective of the cell type, only cells transfected with the replicon vector, containing the oriLyt sequence, could be induced upon infection (Figure 19). All cells were infected with an equal virus load. However, the permissivity of the cell types for MCMV is variable, with the MHEC-5T cells being least infectable, which might explain the differences in the induction strengths. In summary, the replicon vector worked in all tested cell types.

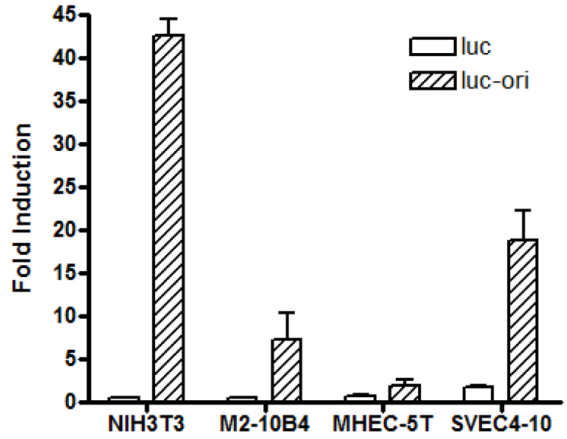


Figure 19: Induction of the replicon vector in different cell types.
NIH3T3, M2-10B4, MHEC-5T and SVEC4-10 were transfected with pEpibo-P(SV40)-luc (luc) or pEpibo-luc-ori (luc-ori). Stable pools of the transfectants were selected. Cells were infected with MCMV at an MOI of 1 and bioluminescence analysis was performed 36 h post infection. Induction of FL expression was calculated as a ratio of the RLU of infected and uninfected cells. In all different cell types, luc-ori cell pools could be induced upon infection with MCMV, while in luc cell pools infection did not influence FL expression.

4.1.2.4 Virus specific activation of the oriLyt-expression system

Herpesviruses usually are species specific and cannot productively infect another animal than their specific host. There are two herpesviruses that naturally infect mice, namely MCMV belonging to the β-herpesviruses and the murine herpesviruses 68 (MHV68), a γ-herpesvirus. No herpesvirus of the α-subfamily could be isolated from mice so far. All herpesviruses share the major six proteins needed for replication (see 1.4.1), but possess different oriLyt sequences and specific proteins for their activation. To examine if the MCMV-based oriLyt expression system can be non specifically activated by a herpesvirus of a different subfamily luc-ori cl. 1 cells were infected with MCMV or MHV68 at an MOI of 0.1 and induction of FL expression was measured 36 h p.i. after (Figure 20).

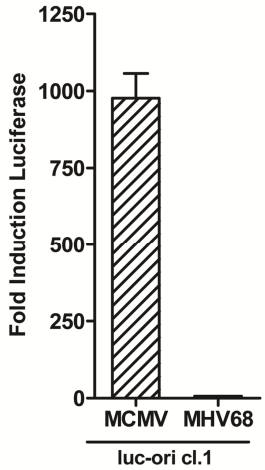


Figure 20 : MHV68 infection cannot activate MCMV orilyt-based expression system
luc-ori cl. 1 cells were infected with MCMV or MHV68 at an MOI of 0.1. 36 h p.i. cells were harvested and bioluminescence was measured. Depicted is the ratio of infected to uninfected cells. While induction was at 1000-fold by infection with MCMV, MHV68 infection resulted only in 5 to 10–fold induction of the MCMV oriLyt-based expression system.

While MCMV induced luciferase expression by ~1000-fold, MHV68 infection resulted only in a 5 to 10-fold increase of reporter gene expression. This is indicative for a highly specific activation

of the system that is dependent on the correct oriLyt sequence and its specific oriLyt activation protein.

4.1.2.5 Activation is dependent on viral DNA amplification

As the induction of reporter gene expression was dependent on the oriLyt sequence of the episomal vector, the necessity of viral DNA replication for the activation of the luciferase was addressed. To do so, the four luc-ori cell clones 1 to 4, as well as NIH3T3 as control were infected with MCMV at an MOI of 0.5 (hatched bars, Figure 21) or left untreated (full bars). Furthermore PAA, a specific inhibitor of the viral DNA polymerase, was added (black bars). Infection with MCMV induced gene expression in a range of 100 to 1000-fold in three of four cell clones (Figure 21), in accordance with the clones that could be reactivated with TSA (Figure 16). Treatment of infected cells with PAA however blocked the induction of the reporter gene expression completely. The presence of the drug did not influence basal luciferase expression in uninfected cells. Therefore, not only the presence of the oriLyt in the sequence but also the functionality of the viral DNA polymerase is needed for the activation of the adjacent reporter gene.

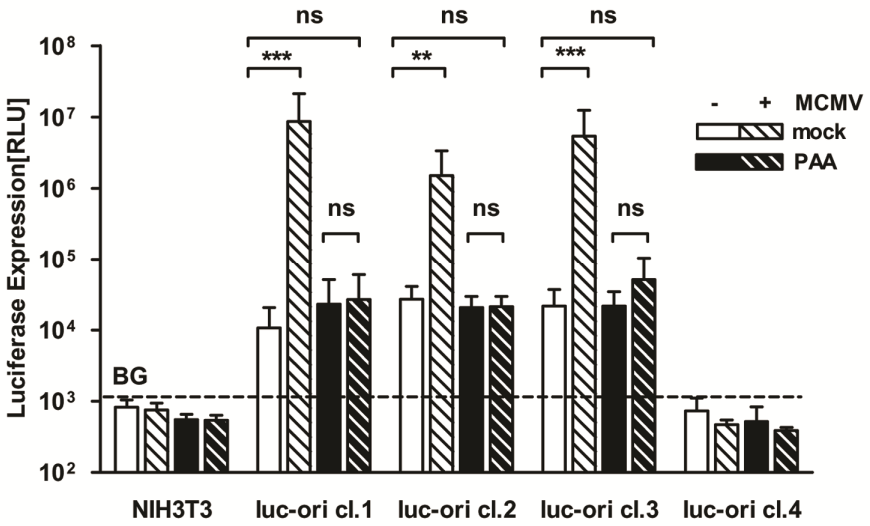


Figure 21: Induction of reporter gene expression depends on viral DNA amplification.

Four luc-ori cell clones were tested for their response to MCMV infection. A low basal level of luciferase signal could be detected in three of four isolated cell clones (white bar). By infection with MCMV with an MOI of 0.5, FL expression was induced up to 1000-fold (white, hatched bar). To determine if the induction is dependent on viral DNA amplification, a specific inhibitor (PAA) was added to either uninfected (black bars) or infected (black, hatched bars) cell clones. While PAA did not have any influence on uninfected cells, it could block the increase of FL in the infected cells completely. Thus, the activation is highly dependent on viral DNA amplification. (p.i., post infection; BG = Background; ***: $p < 0.001$, ns: $p > 0.05$, Two-Way-ANOVA, depicted is mean + SD)

To find out if DNA replication or just the activation of the oriLyt is necessary for the induction, a time course (12, 15, 18, 21, 24 and 26 h p.i.) of reporter gene induction upon infection was performed. The co-translational folding of FL is very fast and lies in the range of 20 min [146] and has a half-life of three to four hours [147], and thus does not contribute much to the shape of time course. The exact onset of MCMV DNA replication has not been analyzed in detail so far, but FACS measurement of total DNA content stained by propidium iodide in MCMV infected NIH3T3 roughly revealed the expected DNA replication onset at about 16 h p.i. [148]. Induction of luciferase expression was assayed under five conditions: either PAA was added directly after the infection, after 12 h p.i., 15 h p.i., 18 h p.i. or the measurements were performed in absence of the drug. If PAA was added prior to the onset of viral DNA replication (0 h p.i., 12 h p.i.) [149] no induction of FL was detected. If PAA was administered after DNA replication had just started, a reduced level of induction could be detected that was dependent on the elapsed time since addition. In infected cells without inhibitor an exponential increase of luciferase expression could be seen after 18 h p.i. Therefore, it can be concluded, that the activation of reporter gene expression in the stable cell lines requires viral DNA replication and increases further with ongoing replication. Furthermore it can be reasoned that the start of viral DNA replication in NIH3T3 probably takes place earlier than reported and begins already between 12 and 15 h p.i.

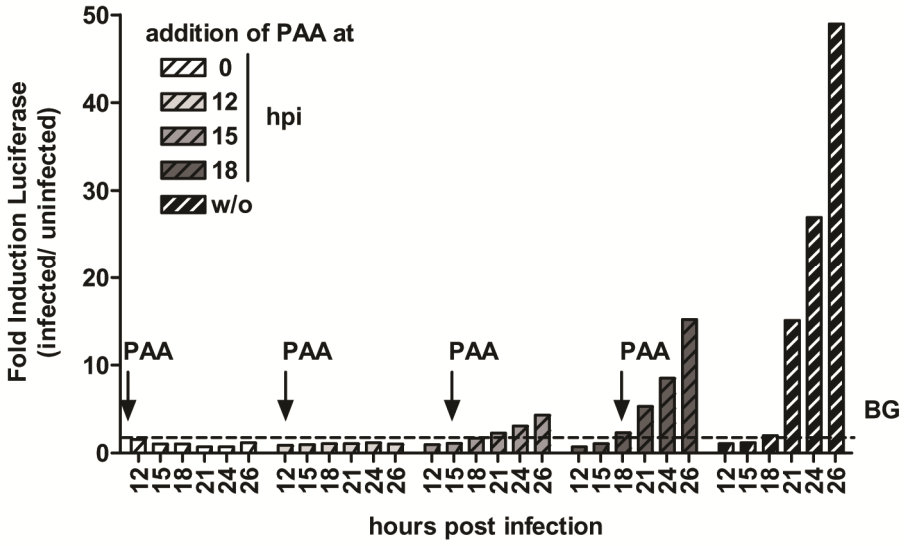


Figure 22: Time course of FL induction.

luc-ori cl. 1 cells were infected with wt-MCMV at an MOI of 0.5. At 12, 15, 18, 21, 24 and 26 h p.i. induction of FL was measured. In addition the replication inhibitor PAA was added after; either immediately at infection, at 12, 15 or 18 h p.i. (indicated by the arrows). Induction without PAA, starts before 15 h p.i. and increases exponentially (black bars). If PAA is added before viral DNA replication takes place, no induction of FL appears (white and light gray). Whereas induction can be blocked in a dose related fashion the later PAA is added to the infected cells (gray and dark gray).

4.1.2.6 MCMV infection amplifies replicon vector

As the induction of reporter gene expression was dependent on viral DNA replication, it was reasonable to ask whether also the episomal plasmid itself was amplified by viral infection. Therefore, quantitative real time PCR was performed on uninfected (plain bars, Figure 23 A) and infected luc-ori cell clones (hatched bars, Figure 23 A). Amplification of the vector was measured by detection of the blasticidin resistance gene (*bsr*) on the vector pEpibo-luc-ori and normalized to the endogenous lamin B receptor gene (*lbr*). Results are presented as relative copies of the pEpibo-luc-ori vector per *lbr*. A significant increase of pEpibo-luc-ori copies of more than 50-fold could be detected in the infected cell lines luc-ori cl. 1-3. Only in the luc-ori cl. 4 clone that was not responsive to viral infection there was no increase in copy numbers.

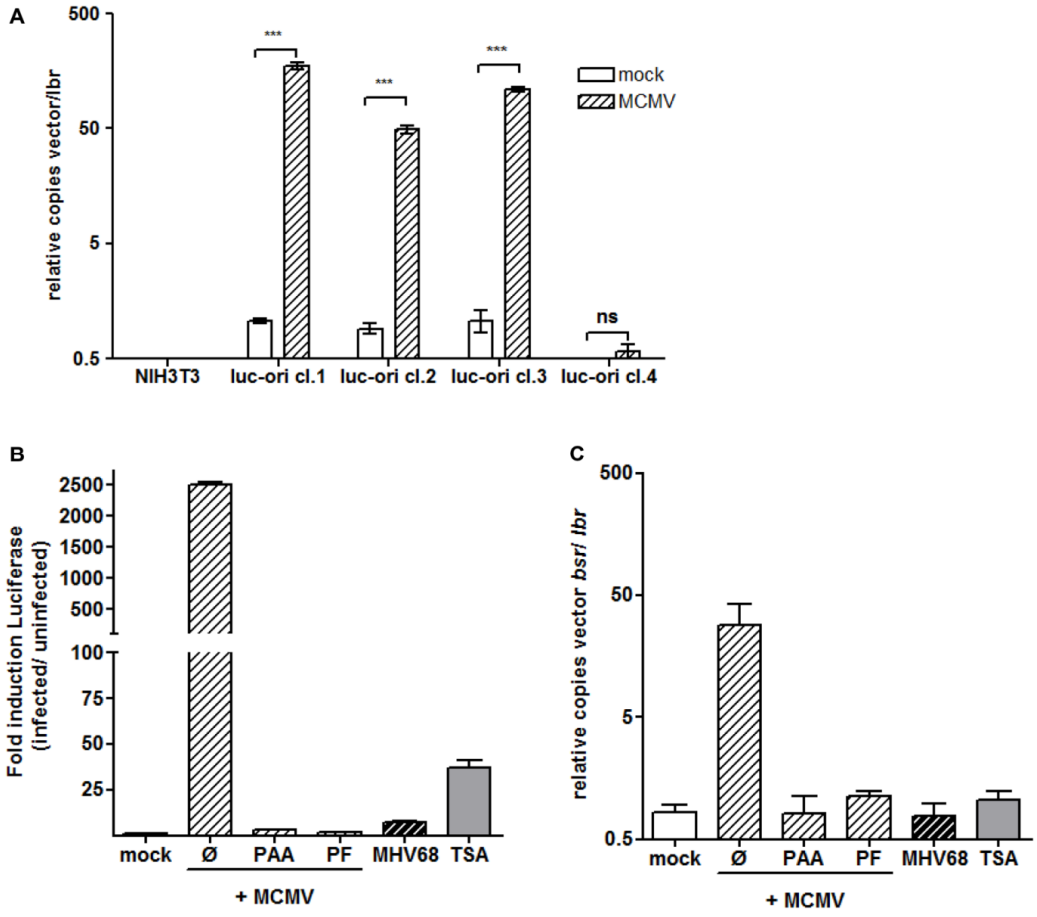


Figure 23: Amplification of pEpibo-luc-ori in infected cells.

A) The vector pEpibo-luc-ori is amplified after infection. NIH3T3 or luc-ori cl. 1 to 4 were infected with wt-MCMV with an MOI of 1 (hatched bar) or left untreated (closed bar, mock). 36 h p.i. quantitative realtime PCR was performed to determine copy numbers of the PCR products *bsr* (Blasticidin resistance gene on vector) and *lbr* (cellular lamin B receptor gene). Shown is the mean of triplicates of *bsr* relative to lbr copy numbers with standard deviation. B) and C) Strong induction of luciferase correlates with amplification of the vector. luc-ori cl. 1 cells were subjected to several treatments; cells were infected with MCMV (white hatched bars) or MHV68 (black hatched) bars at an MOI of 1 or 330 nM TSA was added to the cells. 300 µg/ml phosphonoacetic acid (PAA) or 100 µg/ml phosphonoformic acid (PF) was added to MCMV infected cells. Bioluminescence measurements (B) or qPCR (C) was performed.

To strengthen the correlation between amplification of vector DNA and induction of reporter gene expression qPCR analysis under different conditions was performed. Luc-ori cl. 1 cells were infected with MCMV or MHV68 at an MOI of 1 and additionally treated with the viral replication inhibitors PAA and phosphonoformate (PF) (Figure 23 B and C). Under these conditions, only luc-ori cl. 1 cells infected with MCMV induced strong FL expression of 2500-fold, which correlated with an amplification of the vector DNA of 50-fold. In contrast, treatment with 300 ng/ml TSA partially resolved silencing, but did not lead to vector amplification. Accordingly, FL induction by TSA treatment was 100-fold less effective than infection. Thus, vector DNA replication is a prerequisite for strong expression of the reporter gene.

Southern blot analyses of uninfected and infected cell lines were performed to analyze the status of the pEpibo-luc-ori plasmid DNA before and after replication. Although the pEpibo-luc-ori plasmid could not be detected in the uninfected cells, strong specific signals were found for the three cell lines luc-ori cl. 1-3, which were also responding to MCMV infection with strong FL induction (Figure 16). The infected luc-ori cl.1-3 exhibited band patterns of amplified pEpibo-luc-ori vectors (Figure 24).

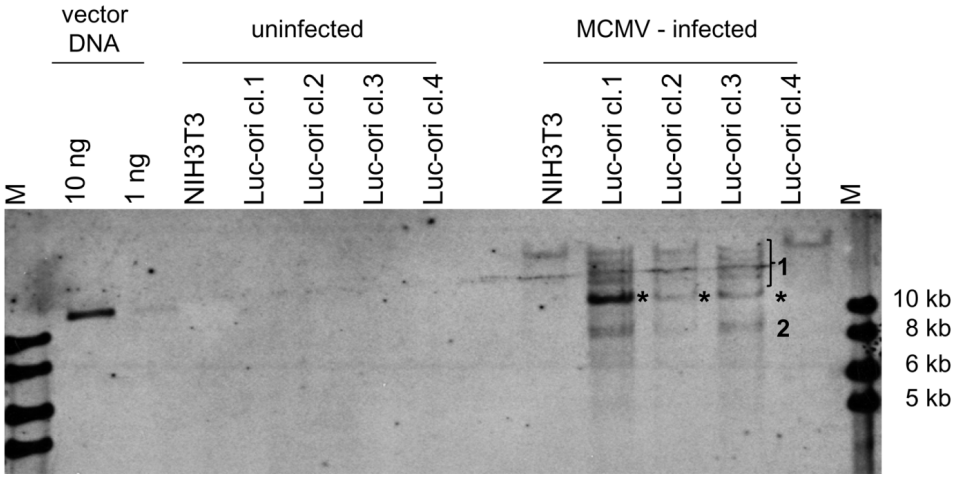


Figure 24: Southern blot of uninfected and infected luc-ori cell clones.

Amplification and conformation of the pEpibo-luc-ori vector were analyzed by Southern blotting. 10 µg genomic DNA of uninfected and infected (MOI of 0.5, 24 h p.i.) luc-ori clones cl.1 to 4 as well as NIH3T3 was digested with *Pst*I. 10 ng and 1 ng of *Pst*I-linearized plasmid DNA was loaded as control. The blot was treated with a dig-labeled probe directed against the *luc* gene. (* = single-unit, episomal plasmid; 1= concatemeric bands, 2 = probably supercoiled plasmid form)

The strongest signal was detected at 11 kb, the same height as the linearized loading control of pEpibo-luc-ori. Yet, additional weaker bands appeared migrating at positions higher as well as lower as the single unit size. This indicated first, that the replicon vector replicates similar to the

MCMV genome and second exists as supercoiled and relaxed plasmid forms, as well as in the form of concatemers typical for herpesvirus genomes during replication. No specific bands could be detected in the non responsive clone luc-ori cl.4 (Figure 24).

4.1.2.7 Induction of FL expression from the oriLyt based system in comparison to virus encoded FL expression

In order to compare the expression strengths of the replicon vector system with direct transgene expression by the virus, luciferase expression encoded by MCMV-luc virus and the ori-luc cells was compared. The *luc* gene in the MCMV-luc virus is expressed by the strong CMVie promoter and starts with early kinetics after viral infection, thus also expressing the FL earlier than the replicon system. The expression of the replicon vector and the MCMV-luc were comparable at very low MOI, but the virus induces a stronger signal at higher infection doses. Thus, although the replicon system induces strong and accumulating gene expression, it cannot completely compete with the FL expression encoded by the virus under early kinetics. Nevertheless, the differences were only 10-fold at an MOI of 0.5, which is quite small given that virus-encoded expression has a head start whereas replicon-derived expression of FL starts 16 h later.

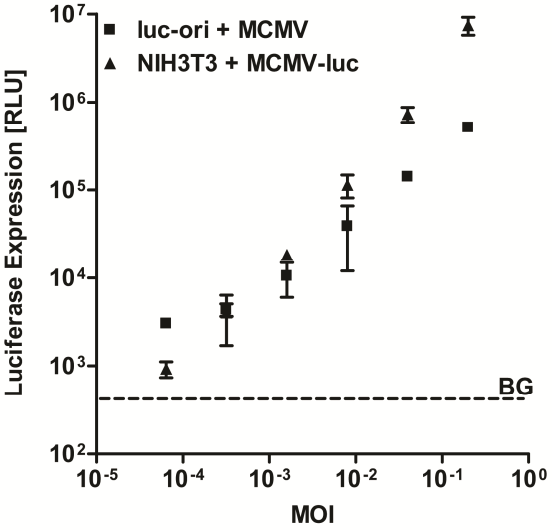


Figure 25: Comparison of expression strength of virus or luc-ori driven luciferase production.
NIH3T3 cells were infected with MCMV-luc, a virus encoding a firefly luciferase under the strong immediate early hCMV Promoter, and luc-ori cl. 1 cells were infected with wt-MCMV at the indicated MOIs. 30 h p.i. cells were harvested and bioluminescence measured.

4.2 Intracellular immunization with the DN GFPSCP in the replicon system

Previous attempts to realize intracellular immunization by the means of several other expression systems (data not shown) indicated that some important prerequisites need to be fulfilled. First of all, a strong expression of the inhibitory protein is necessary to block virus spread. Second, positional effects can be minimized by the usage of extrachromosomal elements. Initial experiments using the luciferase as reporter gene showed that the oriLyt-based replicon system combined both criteria. As a next step, the suitability of the replicon vector with respect to intracellular immunization was evaluated. To do so, the *luc* gene of the pEpibo-luc-ori episome was replaced by the inhibitory gene DN *gfpscp* (see section 1.8). While in permissive host cells MCMV multiplies and spreads to neighboring cells, in cells carrying the pEpibo-gfpscp-ori episome, viral DNA replication should strongly induce the gene expression of the viral dominant-negative fusion protein GFPSCP (see section 1.8) and appropriate expression levels of this inhibitor should block further budding of viral capsids from the nucleus into the cytoplasm resulting in the block of virus maturation and spread to uninfected cells (Figure 26).

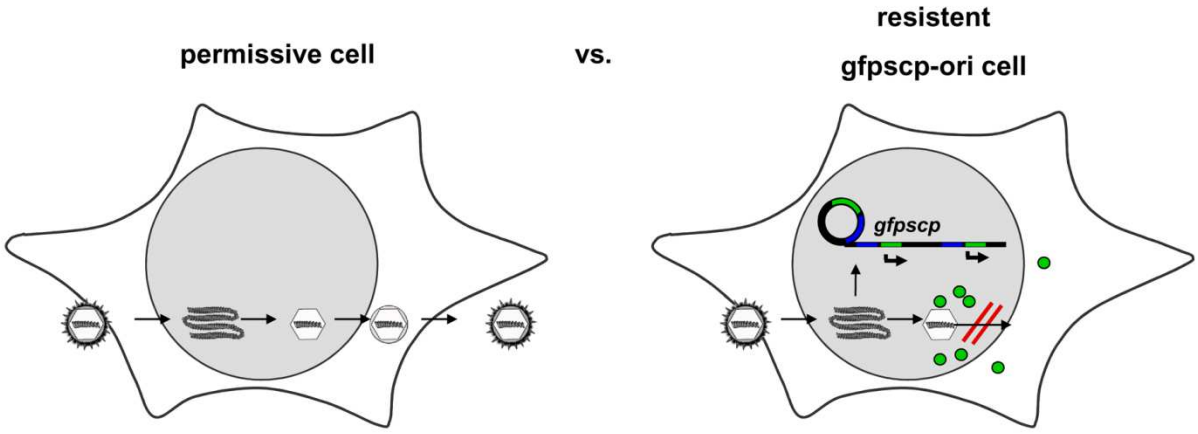


Figure 26: Schema of oriLyt induced expression of the dominant-negative protein GFPSCP.

In MCMV-permissive cells, MCMV enters the cell and replicates its DNA in the nucleus. Viral capsids are filled with the virus genome and bud through the inner nuclear membrane. After acquiring the tegument in the cytoplasma, MCMV exhits by budding through the cell membrane. Gfpscp-ori cell lines, stably carrying the episome pEpibo-gfpscp-ori, encoding a dominant negative version of the small capsid protein (SCP) of MCMV fused to the fluorescent protein GFP should be resistant to MCMV infection. During an infection with MCMV, pEpibo-gfpscp-ori should be replicated and thereby the expression of the inhibitory protein should be induced, which in turn should block egress of viral capsids from the nucleus.

4.2.1 Validation of MCMV infection and spread on cell lines carrying the replicon pEpibo-gfpscp-ori

To test the hypothesis of intracellular immunization on the basis of the oriLyt-based expression system, two MCMV-permissive cell lines NIH3T3 and M210-B4 cells were stably transfected with the plasmid pEpibo-gfpscp-ori and stable cell clones were isolated.

In contrast to NIH3T3 cells, MCMV spread was visibly reduced on gfpscp-ori cells. After infection with wt-MCMV at an MOI of 0.5, full lysis occurred at day 3 p.i. in NIH3T3 cells, while only small plaques were found in the gfpscp-ori cl.3 culture (Figure 27 A). Remarkably, silencing of the *gfpscp* gene happened much faster than the *luc* gene and within two weeks after transfection no fluorescence signal could be detected in uninfected cells (Figure 27 B). However, expression of GFPSCP was strongly induced by infection (Figure 27 B). In infected cells GFPSCP localizes to the cytoplasm and is then transported by the help of the major capsid protein into the nucleus, where it localizes in nuclear speckles. At very late stages of infection the induced GFPSCP production is so strong that the complete cell fluoresces brightly.

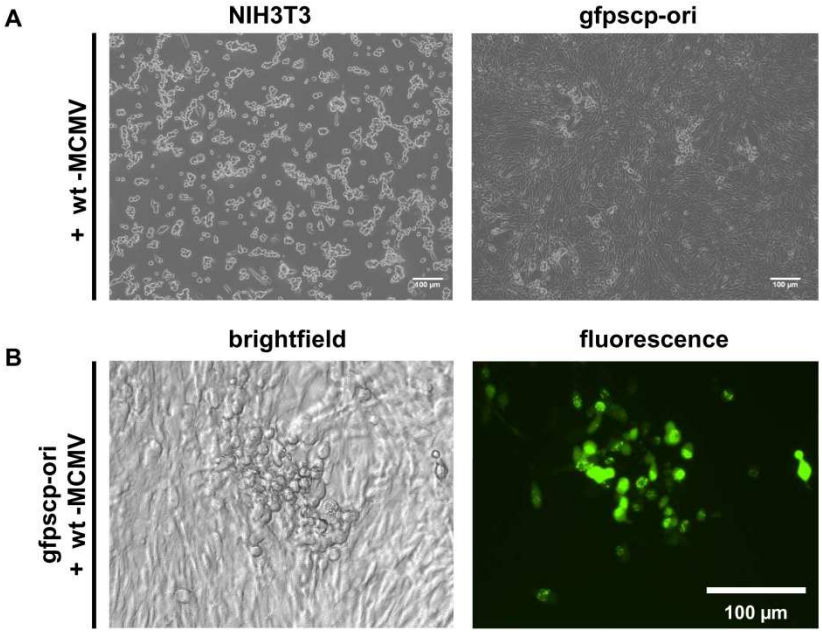


Figure 27: Infection pattern of gfpscp-ori cl.3
A) NIH3T3 and gfpscp-ori cl.3 cells were equally infected with wt-MCMV at an MOI of 0.5. Three days post infection brightfild microscopy pictures were taken. B) gfpscp-ori cl.3 cells were infected with wt-MCMV at an MOI of 0.5. Brigthfield and fluorescence microscopy pictures were taken 5 d p.i. Uninfected cells do not express GFPSCP. After infection GFPSCP is induced.

To check whether and to what extend various gfpscp-ori clones were able to inhibit viral spread, they were infected with MCMV at an MOI of 0.1 and virus growth analysis was performed. In NIH3T3:gfpscp-ori cl. 3 and M210-B4:gfpscp-ori cl. 3 the system was inducible by MCMV infection and GFP fluorescence could be detected. In the other cell clones either a weak constitutive or no GFP expression at all was found.

Only clones producing GFPSCP in response to MCMV infection were able to reduce viral spread. In case of NIH3T3:gfpscp-ori cl. 3 a reduction of ~ 250-fold virus titer could be detected on day 3 and 5 in comparison to the wt-NIH3T3 cells, which represents a 99.5 % inhibition of MCMV. In the M210-B4:gfpscp-ori cl. 3 also a strong reduction of 100-fold could be measured on day 3, which then dropped to a 10-fold reduction on day 5. All other clones possessed either very weak or lacked inhibitory potential (Figure 28).

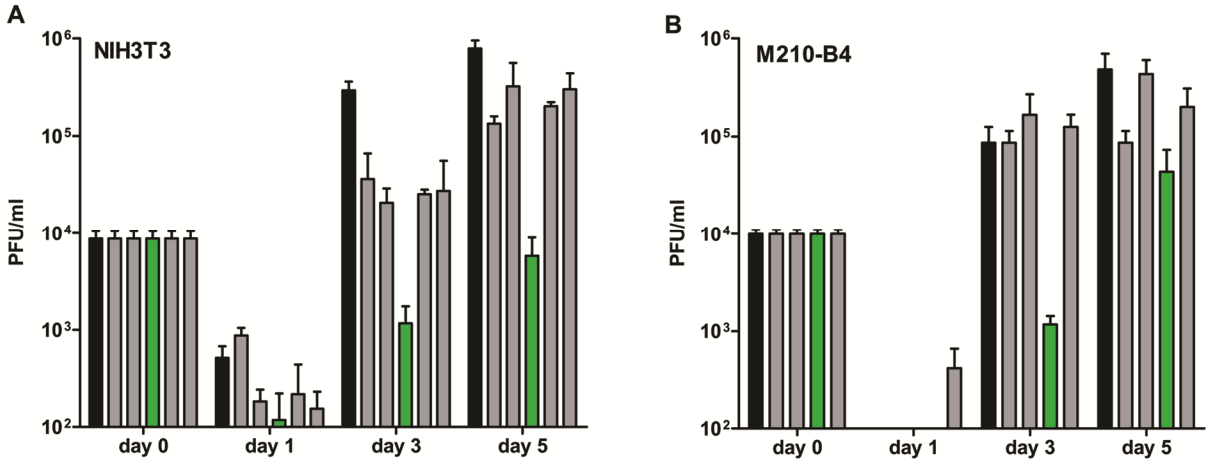


Figure 28: Virus growth analysis of wt-MCMV on NIH3T3:gfpscp-ori and M210B4:gfpscp-ori lines

NIH3T3 or M210-B4 cells respectively were transfected with pEpibo-gfpscp-ori. Stable cell clones were isolated and tested for their potential to inhibit MCMV spread. One NIH3T3:gfpscp-ori clone (cl. 3 green bar, left) out of five (cl. 1 to 5) could reduce MCMV titer up to 250-fold, meaning a 99.5 % inhibition of MCMV spread compared to the parental cell line (black bar). In the M210-B4: gfpscp-ori clones (cl. 1 to 4) also one clone (cl. 3, green bar, right) was able to reduce virus titer by 100-fold on day 3 and 10-fold on day 5.

4.2.2 The vector pEpibo-gfpscp-ori is maintained as an episome

As individual clones of NIH3T3:gfpscp-ori showed a different antiviral potential, the status of the vector DNA pEpibo-gfpscp-ori in cells was analyzed. To this end, Southern blot analysis of the stable cell clones was performed with a dig-labeled DNA probe detecting the *gfpscp* gene. In case of an episomal maintenance one band at the size of the full length plasmid is expected, when the genomic DNA is digested with an restriction enzyme that cuts only once in the plasmid. As control for the Southern blot experiment linearized pEpibo-gfpscp-ori plasmids were loaded. Three specific bands recognized by the *gfpscp* probe could be detected in gfpscp-ori cl. 3 (lane 3, Figure 29), the lowest band possessing the expected size of the linearized plasmid of 11.5 kb and two higher bands (red asterisks).

In another independent clone (gfpscp-ori cl. 8, lane 5) that also responded with GFPSCP expression to infection the same pattern was detected. In cl. 7, however, a weak constitutively expressing clone, two bands at approximately 9 kb and 2.7 kb were marked by the probe (black asterisks, lane 4) indicating that the plasmid has partially integrated into the host genome. In cl. 1 (lane 2) no specific signal was detectable over background pattern of NIH3T3 (lane 1). In this case an integration of only the *bsr* gene seems likely, as this clone did not express GFPSCP at all. Function or failure to inhibit MCMV spread thus seems to be associated with the genomic status of the pEpibo-gfpscp-ori plasmid.

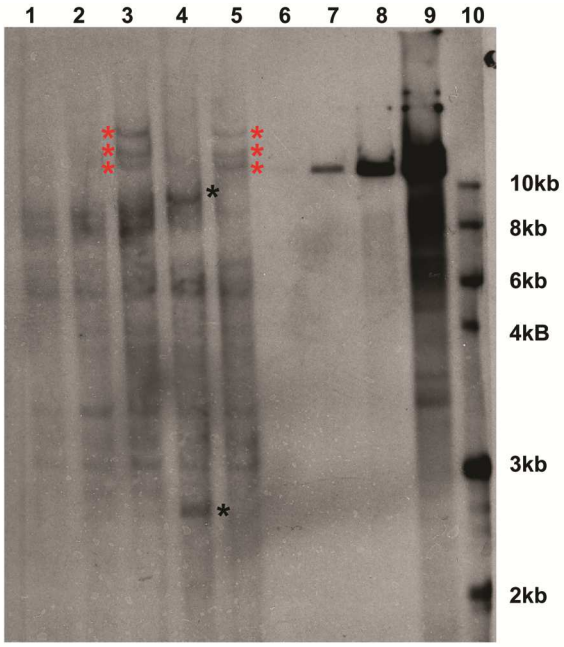


Figure 29: Southern blot of uninfected gfpscp-ori cell lines.
To determine the status of the pEpibo-gfpscp-ori plasmid in the different NIH3T3 cell clones Southern blot experiments were performed. Genomic DNA of NIH3T3 (lane 1), gfpscp-ori cl. 1 (lane 2), gfpscp-ori cl. 3 (lane 3), gfpscp-ori cl. 7 (lane 4), gfpscp-ori cl. 8 (lane 5), 0,5 pg, 5,0 pg, 50 pg and 0,5 ng of pEpibo-gfpscp-ori (lane 6 to 9) was digested with *Ps*I. A 1 kb Marker was loaded as size control (lane 10). Southern blots were hybridized with a dig-labeled probe against the gfpscp gene. Red asterisks mark bands additionally appearing to background of NIH3T3 in the lines that are responsive to MCMV infection. Black asterisks mark the bands that appear in the line NIH3T3:gfpscp-ori cl. 7 that constitutively expresses GFP but does not respond to MCMV infection.

The Southern blot hybridization detected three bands specific for the *gfpscp*-probe, whereas only one band was expected for the episomal state. Thus the plasmid status of pEpibo-gfpscp-ori was further analyzed by fluorescence in situ hybridization (FISH). Thereby it should be clarified if the plasmid might was integrated in tandem repeats or whether bands resembled different conformation forms representing artifacts of incomplete restriction enzyme digest.

To detect different subregions of the plasmids at the same time, a multicolor FISH was performed. Probes were generated against the *gfpscp* gene (FITC-labeled, green), the *bsr* gene (Cy3.5-labeled, pink) and the oriLyt region (DEAC-labeled, red). All probes co-localized to extrachromosomal elements in the metaphase spreads of gfpscp-ori cl. 3 that could not be detected in the NIH3T3 control. In 18 metaphase spreads, on average two extrachromosomal spots were found. Note, that smaller DNA fragments can drift much further than chromosomes and thus might not have been attributed belonging to the spread of the analyzed cell. In many

cases, the spots seemed to be close to centromeres or telomeres of the acrocentric mouse chromosomes. As mouse chromosomes are difficult to distinguish, it could not be completely determined whether the extrachromosomal spots were associated with any specific chromosome. However spots where clearly also found completely free and unconnected. This fits well to the published behavior of pEPI-1 episomes (see section 1.9), which are described to be only loosely linked to chromosomes [111]. In some metaphase spreads, integration events of the plasmid were also found. Although it has been published that pEPI-1 integrations occur with less than 5 % [108], the ratio in the gfpscp-ori cl. 3 line lies higher at approximately 10 % integration events. Still, in the cell line gfpscp-ori cl. 3 that is responding to MCMV infection the bulk of the plasmid is mainly in an episomal form.

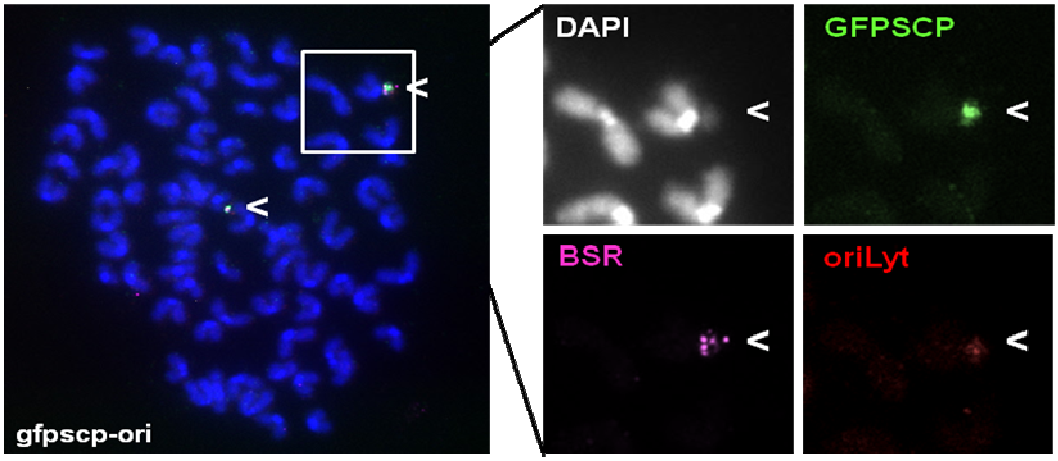


Figure 30: Fluorescence in situ hybridization of gfpscp-ori cl. 3.

Metaphase spread of uninfected NIH3T3:gfpscp-ori cl. 3 (4n = 76). Three different probes complementary to the *gfpscp* gene (green), *bsr* gene (pink) or oriLyt (red) were used. All probes co-localized to DAPI stained extra-chromosomal spots, indicating an episomal persistence of pEpibo-gfpscp-ori.

4.2.3 Fidelity of activation of the oriLyt-expression system by infection

Inhibition of MCMV infection in the NIH3t3:gfpscp-ori cl. 3 cell line was not complete despite a strong GFPSCP expression. Next to the delayed start of the DN expression one reason could be that perhaps not all MCMV-infected cells equally respond to the infection and express the DN fusion protein GFPSCP. Such cells would allow MCMV spread and thus reduce the inhibitory potential of the cell line. To address this question, gfpscp-ori cl. 3 cells were infected with MCMV-mCherry. In this virus the red fluorescence protein is co-expressed together with the wt-SCP with the help of an IRES site placed behind the viral ORF. Thereby expression of the mCherry protein starts under late kinetics after viral replication has taken place. In that way,

mCherry, encoded on the virus and GFPSCP, encoded by the host cell should be expressed at approximately the same time. Thus the infection marker mCherry and the DN GFPSCP expression can be compared in the infected cells. Fluorescence microscopy revealed an extremely high coincidence of GFPSCP and mCherry fluorescence, whereby GFPSCP is found at different cell compartments depending on the time course of infection and mCherry being localized to the whole cell (Figure 31). A correlation of over 95 % could be detected, which might eventually be underestimated as the fluorescence signal of GFPSCP is weaker than the strong mCherry signal. Thus activation of the silenced replicon plasmid is probably reliable in all infected cells and the residual spread is not due to individual cell fates.

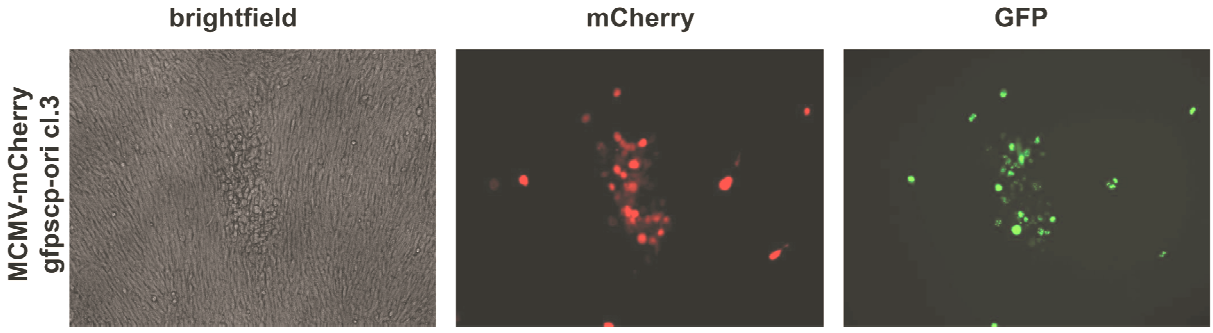


Figure 31: Fluorescence microscopy of NIH3T3:gfpscp-ori cl. 3 infected with MCMV-mCherry

NIH3T3: gfpscp-ori cl. 3 was infected with MCMV-mCherry, expressing the fluorescence marker mCherry under the late promoter of *scp* at an MOI of 0.1. Three days post infection fluorescence microscopy was performed. Infected cells were detected by the red mCherry fluorescence. Induction of GFPSCP was produced only in the infected cells, showing the typical 'speckles' pattern. A very high (> 95 %) correlation of GFP to mCherry fluorescence could be observed, speaking for the specificity as well as the efficiency of reactivation from the silenced status.

4.2.4 Comparison of expression strength of GFPSCP driven by the oriLyt system or by the virus

Considering the results that nearly all infected cells express GFPSCP, the failure to block MCMV entirely in NIH3T3:gfpscp-ori cl. 3 might be rather due to the expression strength of the protein rather than the fidelity of activation. As it was found before that the DN GFPSCP could inhibit viral spread completely when encoded as a second gene copy by the viral genome (MCMV-SVTgfpscp) [103], the expression strength of both systems was compared. To this end, NIH3T3 were infected with wt-MCMV to determine background fluorescence or with the MCMV-SVTgfpscp and NIH3T3:gfpscp-ori cl. 3 cells were infected with wt-MCMV at an MOI of 1. Fluorescence pictures were taken 16 h p.i. (Figure 32).

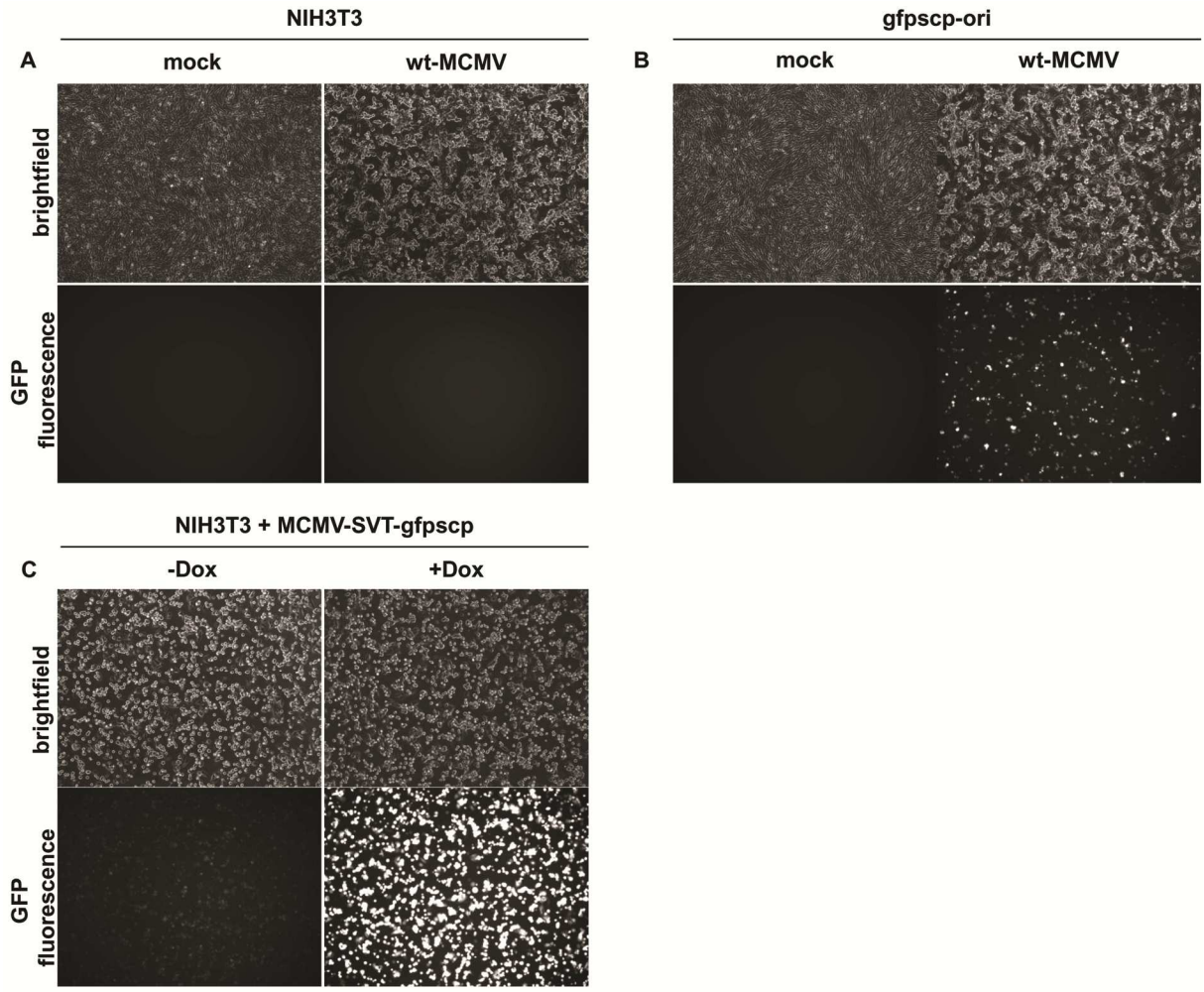


Figure 32: Comparison of GFPSCP expression from the inducible cell line and the inducible virus.
Fluorescence and brightfield microscopy was applied to evaluate the expression strength of the inducible gfpscp-ori cl. 3 cell line in comparison to the infection of NIH3T3 with MCMV-SVTgfpscp, which expresses the inhibitory protein under control of a Tet-ON CMV/SV40enhancer-Promoter. A) As control NIH3T3 were infected with wt-MCMV. B) gfpscp-ori cl.3 does not express GFPSCP in the uninfected state, but GFPSCP is induced upon infection. C) NIH3T3 were infected with an MCMV-SVTgfpscp. Without Doxycyclin (Dox) weak background expression of GFPSCP can be seen, indicating that the system is not absolutely tight. Under induction with Dox, a strong expression of GFPSCP that exceeds the level obtained from the NIH3T3: gfpscp-ori cell line can be seen. All infections were performed with an MOI of 1, fluorescence pictures were taken 16 h p.i..

While a considerable expression of GFPSCP could be already detected from the cells infected with MCMV-SVTgfpscp in absence of doxycyclin needed for gene induction, indicating leaky control of the gene cassette, no expression of GFPSCP was found in uninfected gfpscp-ori cl. 3. However, doxycyclin induction of the viral encoded expression of *gfpscp* produced a much stronger signal than the viral induced gfpscp-ori cl. 3 cells. Similarly to the data obtained with the luc-ori and MCMV-luc comparison (see Figure 25), the cell line cannot cope with the expression strength of the virus at early time points. The expression strength increases during replication in

the oriLyt cells, so does expression of the viral-encoded gene. The GFPSCP DN was selected for proof-of-principle of the replicon vector, yet it may not be the optimal DN for intracellular immunization, which is reflected by the fact that the basal leaky expression of GFPSCP in the viral context was not even inhibitory for virus spread [103]. A protein target with a lower constitutive abundance than the wt MCMV SCP protein, close to 900 copy numbers per virion should be a better target for competitive inhibition [16].

4.3 *Trans*-complementation of MCMV late viral proteins by the replicon vector

True-late herpesviral proteins are expressed only after DNA replication has occurred and true-late promoters need an oriLyt sequence in *cis* for appropriate timing. Thus, late protein complementation has been a difficult task. In order to test, whether the replicon system would be suitable to *trans*-complement late viral transgenes two exemplary genes, coding for MCMV proteins, were cloned into the pEpibo-oriLyt vector. One was the glycoprotein gO of MCMV and the other the transmembrane protein M50, which is involved in the egress of MCMV from the nucleus. No M50 *trans*-complementing cell line could be generated via traditional methods due to toxicity of the protein [150].

4.3.1 *Trans*-complementing MCMVΔgO on NIH3T3:gO-ori

The glycoprotein O governs cell-type specific entry of MCMV, like its homologue in HCMV [22]. In fibroblasts MCMVΔgO is restricted to focal spread, meaning that the virus still infects the neighboring cell by cell-to-cell contact but is not able to spread via the supernatant. Deletion of gO in MCMV leads to a 100 to 500-fold reduction of total virus release into the supernatant. In order to *trans*-complement this growth defect, the cell line NIH3T3:gO-ori (gO-ori) was generated.

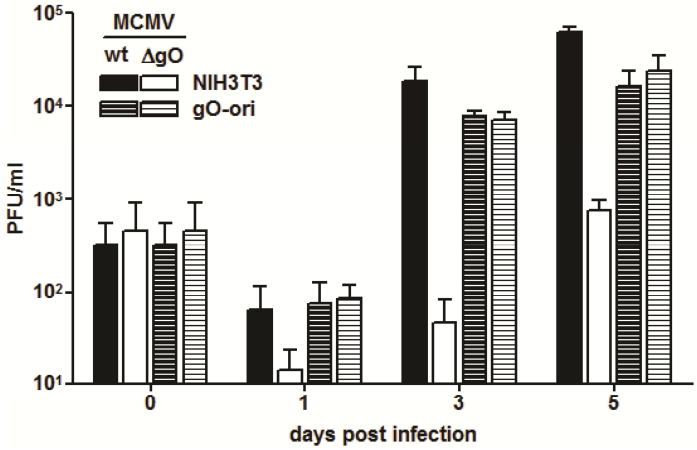


Figure 33: Virus growth analysis of MCMVΔgO on the *trans*-complementing cell line NIH3T3:gO-ori

NIH3T3 (striped bars) or NIH3T3:gO-ori (plain bars) were infected with wt-MCMV (white bars) or MCMVΔgO (black bars) at an MOI of 0.05 with centrifugal enhancement respectively. Supernatants of infected cells were harvested at the indicated time points post infection and infectious virus in the supernatants was analyzed by standard plaque assay. The cell line gO-ori can effectively rescue the deletion of gO of MCMV.

The virus growth analysis was performed on NIH3T3 and NIH3T3:gO-ori to determine the ability to *trans*-complement MCMVΔgO on the replicon-based cell line. MCMVΔgO and wt-MCMV grew to comparable titers on NIH3T3:gO-ori, thus the release defect of infectious virus could be completely rescued (Figure 33). Furthermore, the *trans*-complemented MCMVΔgO/trans gO virus was not restricted to the focal spread pattern as the deletion virus on NIH3T3, but spread like wt virus on gO-ori cells (Figure 34).

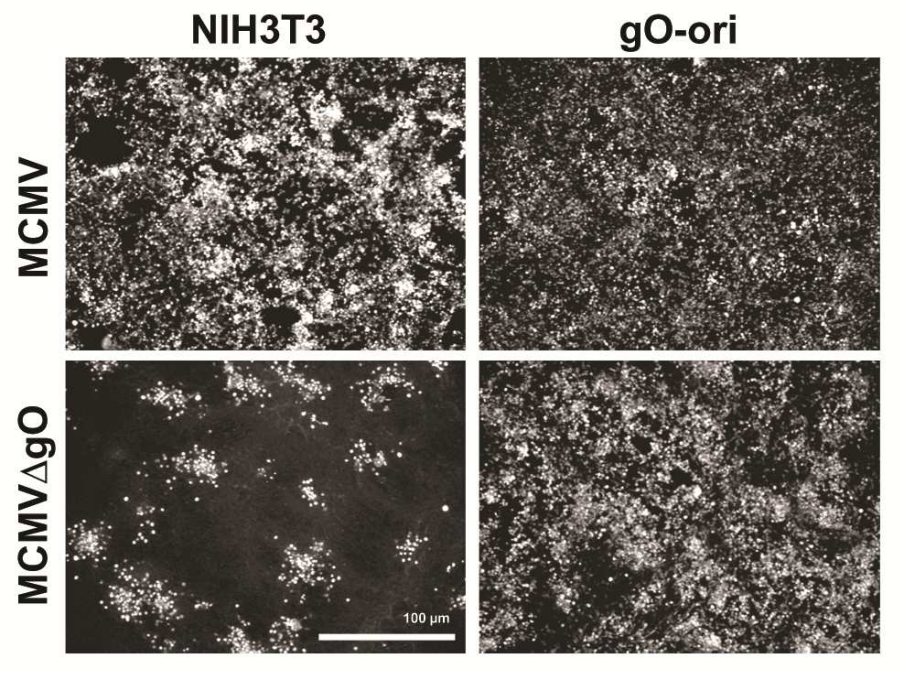


Figure 34: Virus spread pattern of MCMVΔgO on NIH3T3 and gO-ori cells

NIH3T3 and gO-ori cells were infected with MCMVΔgO or wt-MCMV respectively at an MOI of 0.05 with centrifugal enhancement. Five days post infection MCMV infected cells were stained with anti-IE1/3 antibody CHROMA 101. (bar = 100 µm)

As the replicon vector possesses homologous sequences to the viral genome, recombination due to homologous pairing could occur that would lead to reversion of the gO deletion. The *m74* gene coding for gO overlaps with the coding region of *gN*. Thus *m74* could only be partially deleted in MCMVΔgO leaving 782 bp of homologous sequence. To address the question whether recombination occurs, PCR analysis detecting the *m74* gene coding for gO was performed on supernatants harvested from the virus growth analysis experiment on day 5 (Figure 33) of MCMVΔgO on gO-ori and NIH3T3. In order to discriminate between the *m74* gene on the replicon vector in the cells and the virus genome, supernatants were cleared by centrifugation and liberated cellular DNA was digested with Benzonase. Lack of cellular debris was controlled by amplifying the cellular gene *lbr* in the purified supernatants, while integrity of the viral DNA

was controlled by amplification of the *M54* gene, coding for the viral polymerase. All supernatants were positive for the *M54* gene and negative for the *lbr* gene (Figure 35). The *m74* gene however was detected only in wt-MCMV genomes, but not in MCMVΔgO whether or not it descended from NIH3T3 or gO-ori cells. Thus, no recombination within the *m74* gene locus was detected by PCR analysis. Furthermore, a phenotypic reversion of MCMVΔgO, meaning loss of the cell-to-cell spread restriction was never observed (data not shown).

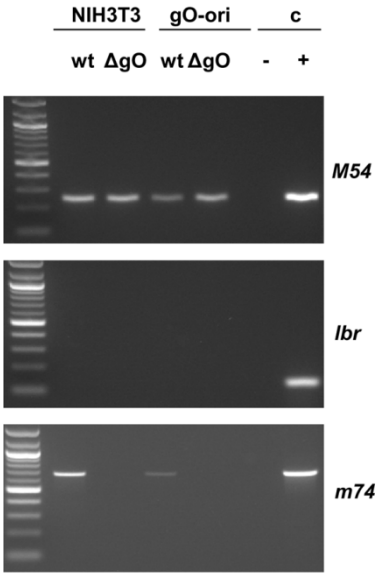


Figure 35: PCR test for recombination of MCMVΔgO with pEpibo-gO-ori
Supernatants of virus harvested from the virus growth experiment (Figure 33) were analyzed for the recombination between the viral genome and the replicon vector. *M54* gene, indicating viral genomes, were amplified with PCR primers M54-for and M54-rev. As indicator for cellular debris PCR detecting lbr was performed with primers LBR-for and LBR-rev. Presence of the *m74* gene in wt-MCMV and deletion viruses was performed with primers m74-for and m74-rev.

4.3.2 *Trans*-complementing MCMVΔM50 on NIH3T3:M50-ori

As a second proof-of-principle, the *trans*-complementation of the viral M50 protein with the replicon system was analyzed. The protein M50 belongs to the essential nuclear egress complex of MCMV. M50 is a type II membrane protein of approximately 35 kDa, which is located in the inner nuclear membrane and recruits viral as well as cellular partners [99]to promote the egress of viral capsids form the nucleus to the cytoplasm. Isolated constitutive expression of M50 was found to be toxic for the cell and thus no *trans*-complementing cell line could be constructed by conventional methods [150].

For the generation of the M50-ori cell line a C-terminal HA-tagged M50 ORF was inserted into the vector pEpiNo-luc-ori, replacing the *luc* gene. NIH3T3 cells were transfected with the resulting vector pEpiNo-M50-ori and two stable cell pools were obtained two weeks post transfection by selection with G418. Western blot experiments were performed with the two cell pools M50-ori t1 and t2 under infection and mock conditions (Figure 36). Interestingly, no M50HA expression was detected in the uninfected state, already at this early stage of analysis,

indicating a very fast silencing process of the M50HA gene. However, infection did induce a strong M50HA signal in both cell pools. Loading of the blots was controlled by detecting actin and the viral infection rate by detecting IE1/3. Both cell pools expressed M50HA at comparable levels after infection.

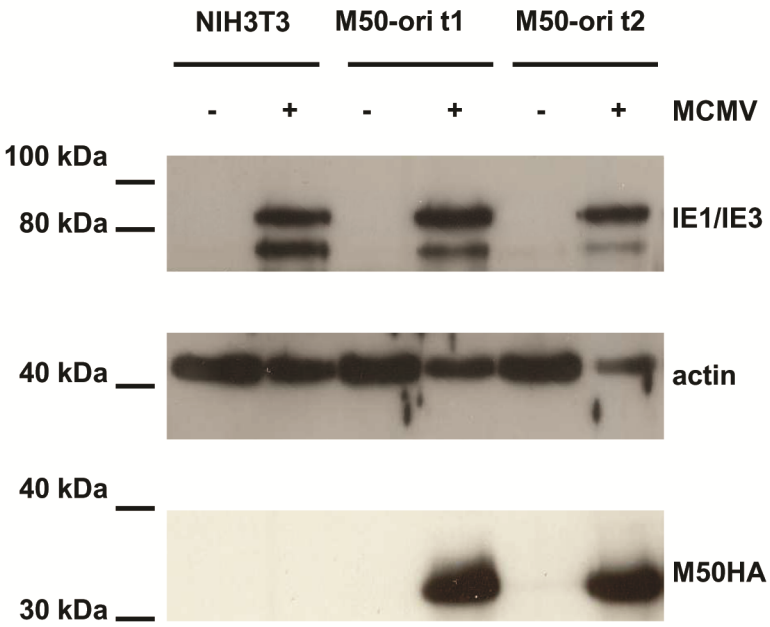


Figure 36: Western blot analysis of M50-ori cell pools

Detection of the M50HA protein (~35 kDa) in cell lysates of NIH3T3, M50-ori t1 and M50-ori t2. To analyze the induction of M50HA, the respective cell lines were infected with wt-MCMV at an MOI of 1 or mock treated and cell lysates harvested 36 h p.i. Viral load was analyzed by detection of IE1/IE3 (76 kDa/89 kDa) and cellular load was analyzed by detecting actin (42 kDa). No expression of M50HA could be detected in uninfected cells, whereas a strong induction of the protein was observed after infection of the M50-ori t1 and t2 cell pools.

M50 is an essential protein for MCMV. To assess the ability to *trans*-complement a M50 deletion virus, the BAC pSM3fr-Δ1-16-ΔM50-F (MCMVΔM50), which encodes a *gfp* gene at the endogenous position of *M50* and as a late fluorescence marker *mCherry* with an IRES site behind the endogenous *scp* ORF, was transfected into M50-ori t1, M50-ori t2 and NIH3T3. The parental BAC without *M50* deletion served as positive control (MCMV-*mCherry*). At three days post transfection first plaques of MCMVΔM50 were detectable in the M50-ori t1 and t2 cell pools but not in NIH3T3. At five days post transfection, full lysis of the cells by the reconstituted viruses occurred. In contrast, MCMVΔM50 could not be reconstituted on NIH3T3 cells, due to the lack of the essential M50 protein, and no plaques were detectable up to the end of the experiment four weeks post transfection. This indicates a very effective reconstitution of the deletion virus in the M50-ori cell pools.

In order to assess the reversion of MCMVΔM50 to a wt-MCMV virus, supernatants of M50HA *trans*-complemented MCMVΔM50 (MCMVΔM50/M50HA) were harvested one day after full lysis occurred in the reconstitution plates and titrated on M50-ori and NIH3T3 cells by TCID50. Remarkably, the viral titer of 2×10^7 TCID50/ml of the *trans*-complemented virus was comparable with viral titers obtained with wt-MCMV. However, genetic reversion did also occur, as few plaques could be detected after infection of NIH3T3 or MEF cells with MCMVΔM50/M50HA at high titers. This recombination could be detected in 1 out of 10^4 to 10^5 PFU when complemented on the M50-ori cell pools.

The subcloning of the cell pools led to a variety of clones, which showed major differences regarding their performance, as already seen with the other replicon constructs. From twelve subcloned M50-ori cell lines, three clones failed to reconstitute pSM3fr-Δ1-16-ΔM50F (MCMVΔM50 BAC). In six cell clones only a slow reconstitution compared to the wt controls was observed, as seven days post transfection only very small plaques were detectable and reconstituted virus spreaded very slowly in these cultures. In three further subclones M50-ori cl 1.7, M50-ori cl 1.8 and M50-ori cl. 2.1 a very efficient and fast reconstitution of the MCMVΔM50 BAC was found. Serial dilution of MCMVΔM50/M50HA derived from M50-ori cl 2.1 on NIH3T3 and M50-ori showed again a very high titer of the reconstituted virus of 5.4×10^7 PFU/ml. Fluorescence microscopy detecting the reporter protein mCherry of MCMVΔM50/M50HA indicated an effective spread on M50-ori cl 2.1 cells, while infection stopped after initial infection on NIH3T3 cells (Figure 37). Thus the rate of recombination of the virus on M50-ori cl 2.1 cells was much lower than recombination rates of viruses reconstituted on the M50-ori cell pools.

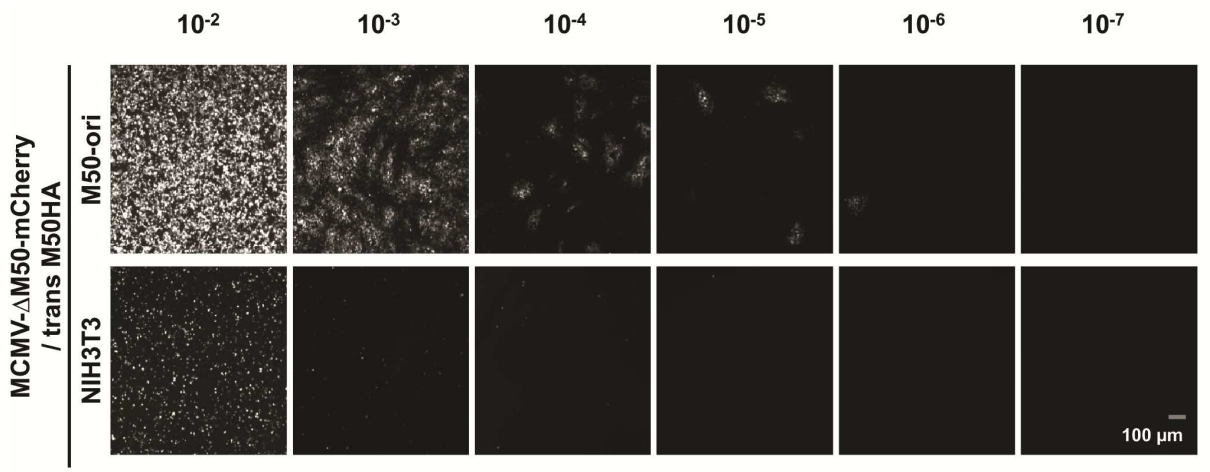


Figure 37: Fluorescence imaging of MCMVΔM50-mCherry/trans M50HA on NIH3T3 and M50-ori cells

Supernatants derived from the reconstitution of MCMVΔM50-mCherry on M50-ori cl.2.1 cells were serially diluted and used to infect NIH3T3 and M50-ori cells. Fluorescence images were performed detecting the mCherry signal derived from the reconstituted virus. While on M50-ori cells, the *trans*-complemented virus spreaded to neighboring cells and formed plaques after 5 days of incubation, the same supernatant only initially infected NIH3T3 cells, but d spread to neighboring cells.

With aging of the cell clones even better performances regarding viral titers and smaller recombination rates were observed. Virus reconstitution on M50-ori 1.7, 1.8 and 2.1 in parallel resulted again in very high titers of reconstituted viruses. Especially the clone 2.1, performed extremely well with a titer of 3.4×10^8 $TCID_{50}$/ml, while recombined viruses titrated on NIH3T3 were under the detection limit of the assay with $8{,}3 \times 10^1$ $TCID_{50}$/ml (Table 8). Although recombination rates were sometimes extremely low, no cell clone was isolated whose progeny did not form any plaque in NIH3T3 cells, meaning that recombination could not be entirely excluded.

Table 8 : Reconstitution of MCMVΔM50 on various M50-ori cell lines

BAC pSM3fr-Δ1-16-ΔM50-F was reconstituted on different M50-ori cell lines. After full cell lysis, supernatants were harvested and titrated on M50-ori (the respective cell line) or NIH3T3 cells via TCID50. (n.d.= not detectable).

experiment	cell line for reconstitution	titer of reconstituted virus		detection limit
		on M50-ori cells	on NIH3T3 cells (reversion rates)	
No. 1	M50-ori t1	5.4×10^6 $TCID_{50}$/ml	1.2×10^3 $TCID_{50}$/ml	4.3×10^2 $TCID_{50}$/ml
	M50-ori t2	2.5×10^7 $TCID_{50}$/ml	1.2×10^3 $TCID_{50}$/ml	4.3×10^2 $TCID_{50}$/ml
No. 2	M50-ori t1	2.5×10^7 $TCID_{50}$/ml	1.2×10^3 $TCID_{50}$/ml	4.3×10^2 $TCID_{50}$/ml
	M50-ori t2	7.9×10^7 $TCID_{50}$/ml	7.9×10^2 $TCID_{50}$/ml	4.3×10^2 $TCID_{50}$/ml
	M50-ori 2.1	5.4×10^7 $TCID_{50}$/ml	7.9×10^2 $TCID_{50}$/ml	4.3×10^2 $TCID_{50}$/ml
No. 3	M50-ori cl. 1.7	1.1×10^7 $TCID_{50}$/ml	n.d.	$8{,}3 \times 10^1$ $TCID_{50}$/ml
	M50-ori cl. 1.8	2.3×10^5 $TCID_{50}$/ml	n.d.	$8{,}3 \times 10^1$ $TCID_{50}$/ml
	M50-ori cl. 2.1	3.4×10^8 $TCID_{50}$/ml	n.d.	$8{,}3 \times 10^1$ $TCID_{50}$/ml

4.4 In vivo analysis of the replicon system- generation of the transgenic mouse line VIOLA

In order to test if the induction principle of the oriLyt-expression system can be eventually used for intracellular immunization principles in transgenic animals, the system had to be evaluated in the *in vivo* situation. So far, the episomal vector pEPI-1 has not been used for the generation of transgenic mice. Successful establishment of episomes was demonstrated in transgenic pig fetuses generated via sperm mediated gene transfer [151]. This technique of gene delivery is not commonly applied for the generation of transgenic mice, where the use of either microinjection of DNA into the pronucleus of fertilized oocytes or the injection of embryonic stem cells (ES) into blastocysts are common procedures. As the latter one allows the pre-selection of suitable transgenic ES cells, generation of transgenic replicon-carrying mice was performed by this technique.

Firefly luciferase (FL) is a non-toxic and very sensitive expression marker. Therefore, FL was preferred over the DN GFPSCP as a transgene for the first generation of the transgenic mice to test functionality *in vivo*. The general outline of the strategy followed the scheme presented in Figure 38. ES cells were transfected and isolated cell clones were selected for virus-induced expression of FL. Responding ES cells were then used for the transfer into blastocysts and generation of chimeras. Progeny of the chimeras were subsequently checked for germ line transmission of the pEpibo-luc-ori plasmid and the resulting mouse lines were evaluated for their ability to express FL upon MCMV infection. The newly generated mouse line was termed VIOLA for *v*irus *i*nduced *o*riLyt-dependent *l*uciferase *a*nimal.

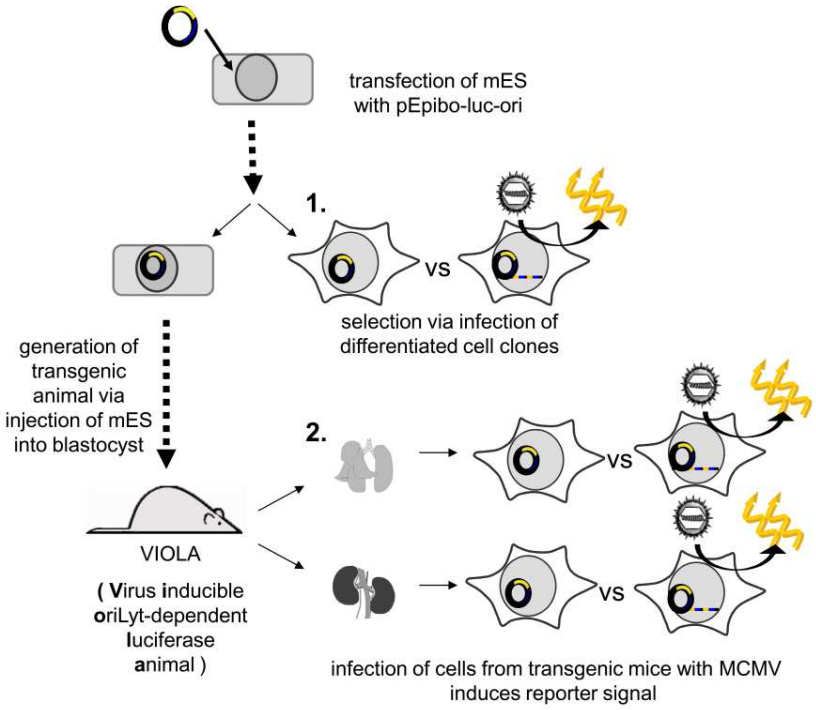


Figure 38: Outline of VIOLA generation and selection
Generation of virus inducible oriLyt-dependent luciferase animal (VIOLA). mES cell clones were transfected with pEpibo-luc-ori and (1.) pre-selected for their induction capacity by MCMV infection *in vitro*. (2.) Mouse lines were analyzed for expression of FL before and after infection in explant cultures of several organs.

4.4.1 Transfection and selection of mES clones for the generation of transgenic mice

Murine embryonic stem cells (mouse line 129 14.1, [124]) were transfected with pEpibo-luc-ori by nucleofection (Amaxa). Two different conditions for further culture and selection were tested. mES cells need to be grown on feeder cells to maintain their pluripotent state. Either mES cells were grown on mitomycin-inactivated luc-ori cl.4 cells (carrying the *bsr* restistance gene, condition A) or mitomycin-inactivated MEF feeder cells (condition B).To select for positively transfected mES cells, Blasticidin S was added to the culture medium. In case of condition B selection of transfected mES cells with Blasticidin S kills also the feeder layer cells, whereas under condition A feeder cells survive due to the integrated *bsr* copy, but might not provide all necessary factors to keep the cells pluripotent.

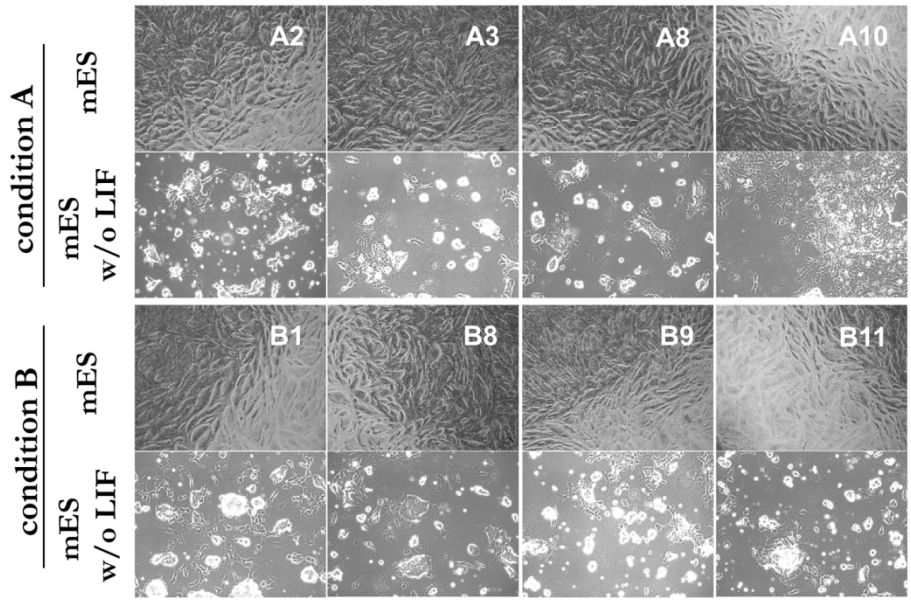


Figure 39: Differentiation of transfected mES clones.
As mES cell clones cannot be productively infected with MCMV, cells were differentiated according to the protocol of Matsukage *et al.* [132]. Undifferentiated mES cell cultures (upper row) are differentiated by withdrawal of the Leukemia inhibitory factor (LIF) and feeder cells. Differentiate cultures formed spikey fibroblast like cells surrounding mES colonies after three weeks. Feeder layers in condition A are NIH3T3:luc-ori cl.4 and MEF in condition B.

Four cell clones of each condition were picked, grown up and splitted in two parts. One part was frozen for later blastocyst injection, the other one expanded for *in vitro*-testing of the clones. MCMV can infect mES cells only after differentiation, therefore cells were differentiated first. Cells were differentiated by removing feeder cells and LIF according to the protocol of Matsukage *et al.* [132]. After three weeks of continuous culturing, morphology of the mES cells changed to a fibroblast-like appearance (Figure 39)

Clones were then infected with MCMV and a bioluminescence assay was performed 36 h post infection. As permissivity to MCMV is dependent on differentiation status of the cells, the results are only semi-quantitive. Nevertheless, cells either responded to MCMV infection (even only in the range of two-fold) or did not express luciferase at all. Two clones, A3 and B8, one from each condition, were selected for the generation of the transgenic animals as they showed a clear FL induction upon infection.

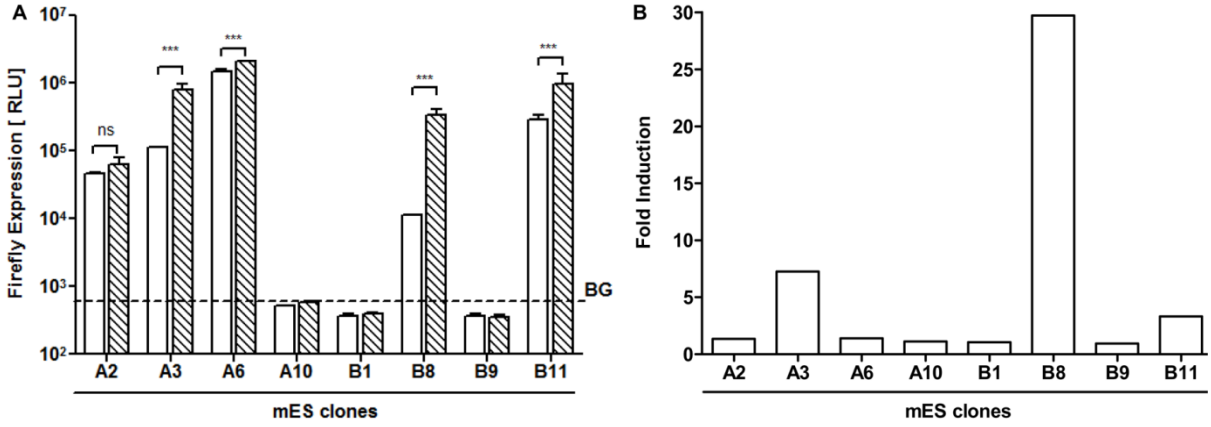


Figure 40: Selection of transfected mES clones by inducibility upon MCMV infection.

A) Differentiated mES clones were mock treated (white) or infected with MCMV (hatched). In three clones A10, B1 and B9 no expression of FL could be detected (BG: Background of substrate luciferin). In all other clones induction of FL could be detected upon MCMV infection. B) The fold induction of luciferase expression was calculated as ratio of the RLU of infected to non-infected cells. Three clones A3, B8 and B11 showed an explicit induction upon MCMV infection. Clones A3 (VIOLA-A) and B8 (VIOLA-B) were chosen for the generation of transgenic mice.

4.4.2 Testing of the generated VIOLA- mouse lines

4.4.2.1 Inheritance of pEpibo-luc-ori in the mouse line VIOLA

Viable chimeras were born from both blastocyst transfers of mES clones A3 and B8. Agouti coat-color indicated a successful germ-line transmission in the C57BL6 blastocyts (black fur) in the first generation (F1), and mice progeny were screened by PCR for the presence of the *luc* transgene (Figure 41). From both transfers one mouse line each could be bred that transferred the *luc* gene to its progeny. The lines were termed VIOLA (virus-inducible oriLyt dependent animal) line A or B respectively.

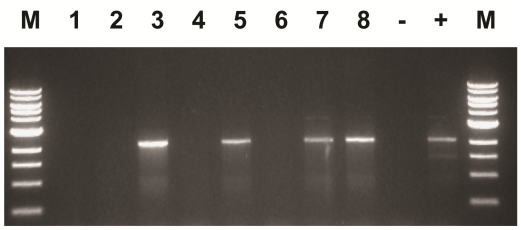


Figure 41: Genotyping PCR of VIOLA mice.

Depicted is a typical result from a genoytyping PCR of the VIOLA-A line. Genomic DNA was extracted from mouse tail pieces and subjected to PCR analysis with the primer P(SV40)-probe-for and pA(SV40)-probe-rev, which amplifies the 2.2 kb *luc* gene. Genomic DNA from luc-ori cl. 1 line was used as positive control (+).

VIOLA lines inherited the *luc* transgene stably over several generations. Surprisingly, it followed a typical mendalian pattern, as around the half of the progeny carried the transgene when crossed to a non-transgenic background mouse (Figure 42).

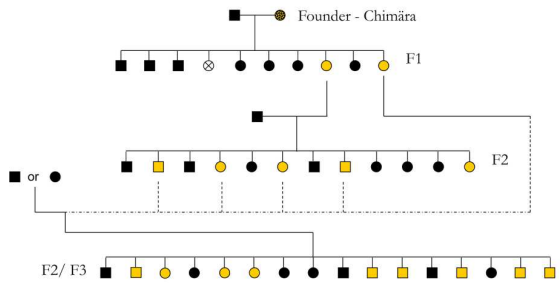


Figure 42: Pedigree of VIOLA-A line.

Yellow symbols represent animals positive for the luciferase transgene. Black symbols represent wt animals. Round symbols indicate female mice, squares male mice. 42.1 % of the progeny of the chimera were positive for the transgene.

4.4.2.2 Southern blot analysis of pEpibo-luc-ori status in VIOLA

In this study, the first transgenic mouse based on the episomal pEPI vector system was described. Therefore, the replicon vector was analyzed regarding its maintenance type in the VIOLA mice genomes. Genomic DNA of uninfected mice was extracted from mouse tails and was subjected to Southern blot hybridization. A probe detecting the *luc* gene was used for the detection of the vector. Linearized pEpibo-luc-ori vector served as positive control and for the estimation of vector copies. Two mice of the VIOLA-B line and five of the VIOLA-A line were analyzed. For episomal persistence of the replicon vector only a single band was expected. Yet, in all mice, two bands with equal and lower size than the linearized vector were obtained after restriction digestion with an enzyme that cuts only once in the pEpibo-luc-ori plasmid. Thus, signals in all mice corresponded to a pattern typical for vector integration (Figure 43). While the signal for unique length was present there was also a band of lower size detectable with the same signal strength. The presence of episomal vector copies cannot be formally excluded but the presence of additional bands with the same intensity as the unique size band, indicate a mainly integrated presence. Hybridization signal of the 100 pg control and the signals obtained from the mice had comparable signal strength, thus the vector copy number can be roughly determined to be between 1-10 copies per cell.

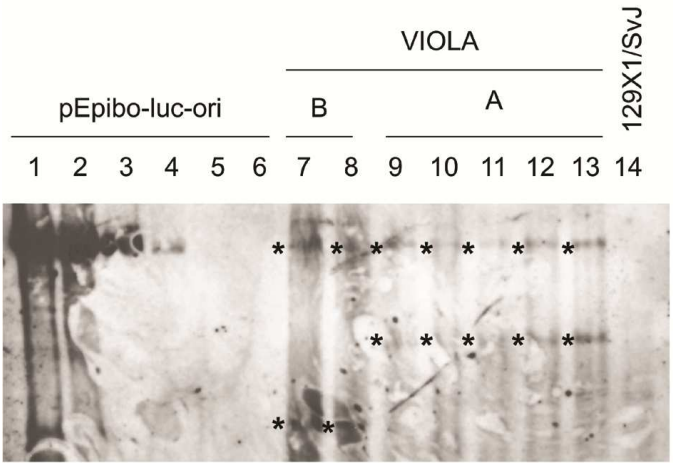


Figure 43: Southern blot analysis of VIOLA lines.

Genomic DNA of 129X1/SvJ or two VIOLA- B and five VIOLA-A mouse tails (of the 3rd and 4th generation) was extracted and digested with *PstI*. As control the vector pEpibo-luc-ori was linearized with *PstI* and loaded at different amounts (100 ng, 10ng, 1 ng, 100 pg, 10 pg, 1 pg). Asterisks mark specific bands probed with an anti-luc-dig probe, indicating an integration of the pEpibo-luc-ori constructs in the VIOLA lines.

4.4.2.3 Non-invasive bioluminescence imaging of VIOLA-lines

As the luciferase gene should be activated by infection in the replicon system, the VIOLA lines were infected with MCMV and analyzed by non-invasive bioluminescence imaging. To determine whether the VIOLA mice would produce bioluminescence signal after infection, three mice of the VIOLA line were infected i.v. with 1×10^6 PFU wt-MCMV into the tail vein. As controls the background line 129X1/SvJ was also infected with wt-MCMV or, for a positive control with MCMV-luc with 1×10^5 PFU, respectively. Bioluminescence signal was measured daily over a period of five days. To determine the background FL signal, bioluminescence images were taken from all mice before infection. No bioluminescence signal could be detected in any of the non infected VIOLA mice (data not shown). Bioluminescence signals in the positive control mice, 129X1/SvJ, infected with MCMV-luc followed a typical infection pattern (Figure 44).

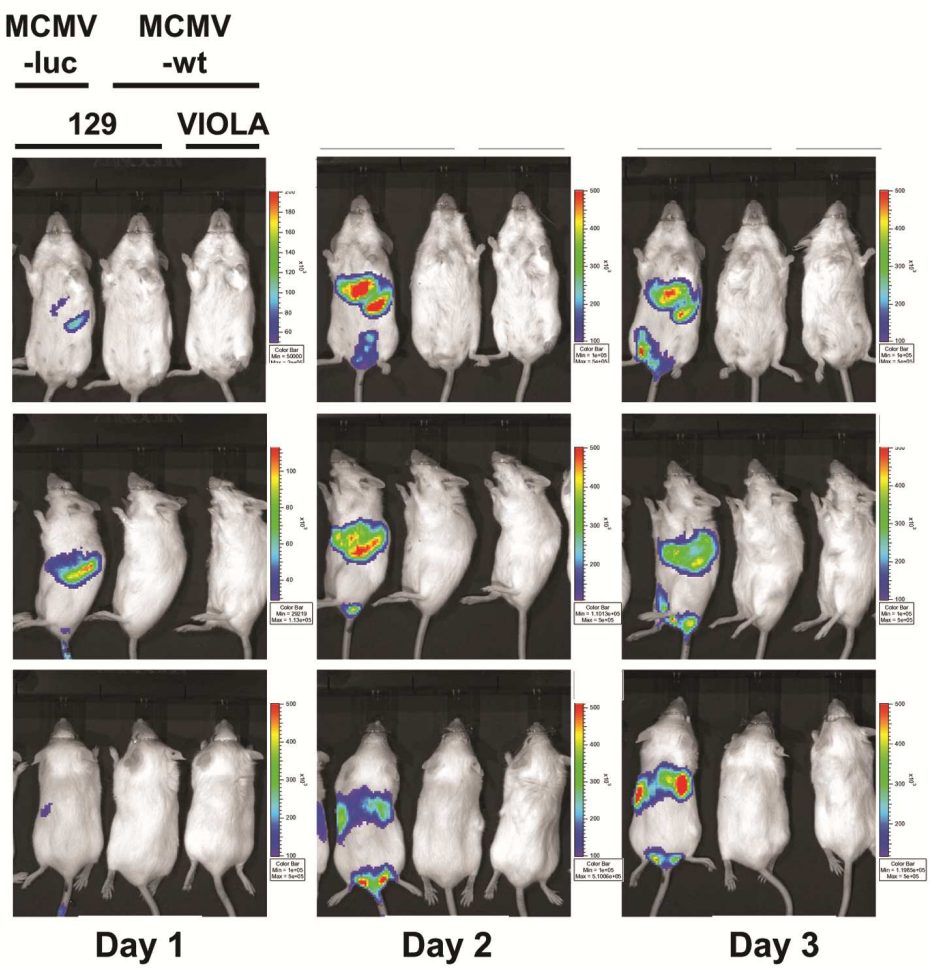


Figure 44: Non-invasive imaging of VIOLA-lines and controls

From left: 129X1/SvJ infected with 1 × 10^5 PFU/ml MCMV-luc, 129X1/SvJ infected with 1 × 10^6 PFU/ml wt-MCMV, or VIOLA infected with 1 × 10^6 PFU/ml wt-MCMV. Depicted is one exemplary VIOLA mouse of three animals in the experiment. Mice were injected with 300 µl 50 mM Luciferin and anesthetized with 2 % isofluran gas. Bioluminescence images were taken with the IVIS Lumina imaging system, with 5 min exposure time and highest binning rate of 8 pixels. 129X1/SvJ mice infected with MCMV-luc served as positive control and followed a typical MCMV infection pattern. Depicted are images taken one, two and three days post infection. VIOLA mice did not produce infection specific bioluminescence signals.

A local infection of the tail and also the liver as well as the spleen was monitored at the first days of infection (Figure 44). At day 3 and 4, infection of kidneys can be seen (data not shown). Infection of the lungs can be monitored from day 3 on. Very weak signals can be also detected in salivary glands at day 5 (data not shown). No bioluminescence signal was detected in the negative control mouse 129X1/SvJ infected with wt-MCMV at any time point. Unexpectedly, also all VIOLA mice did not show any bioluminescence signal as response to infection at any time point.

In order to exclude that the mice were not sufficiently infected, virus titers were determined in several organs of VIOLA mice infected with 1 × 10^6 PFU wt-MCMV and bioluminescence assays were performed in the organ homogenates in parallel. Again, no bioluminescence signal was detectable in infected organs of VIOLA mice. Determination of virus titers in the organ homogenates revealed normal virus titers according to the typical course of dissemination. Thus failure of FL expression is not due to a lack of MCMV infection in the transgenic mice.

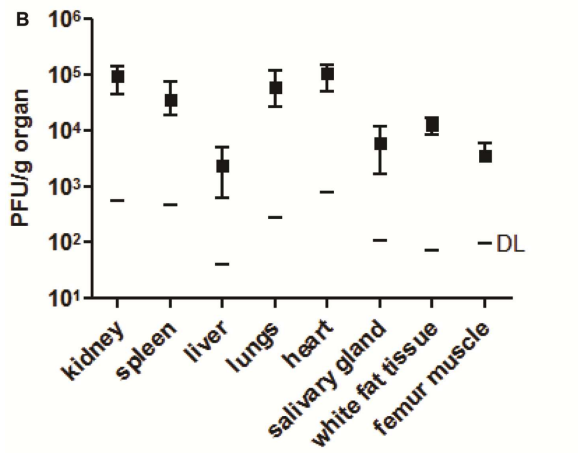


Figure 45: Virus titers in infected VIOLA mice

In order to exclude that failure of bioluminescence induction is due to lacking infection in the transgenic mice, viral titers in organ homogenates were determined. VIOLA mice were infected with 1 × 10^6 PFU/ml wt-MCMV and sacrificed at day 5. Organs were homogenized and virus titers determined with standard plaque assay. Titration of the organs revealed normal viral loads (black squares). (DL = detection limit of each organ respectively; depicted is the standard deviation)

4.4.2.4 Ex vivo analysis of VIOLA explants cultures

To analyze the lack of induction in more detail, explants cultures of mice were made. To this end, a simple and versatile protocol for the extraction and cultivation of different organ and tissues from individual mice had to be developed (see 3.3.6). Lungs, kidney, heart and fat tissue of four VIOLA mice, two of the 2nd and two of the 3rd generation, as well as a 129X1/SvJ were extracted and explanted. Cultures were grown until at least two confluent 6 well dishes could be obtained. Explant cultures of the majority of organs were obtained, however, as the protocol was not optimized for each organ specifically, some extractions failed. Cultures were infected with wt-MCMV at a calculated MOI of 0.5 or left untreated. Infection density was always lower than the calculated dose, as permissive as well as non-permissive cells were extracted. As the proportion of these cells were not equal, infection density could not be equalized. Thus results reflect only semi-quantitative data. In contrast to the bioluminescence assays in infected animals and their organ extracts we found induction of FL in tissue cultures derived from these mice (Figure 46). In one mouse induction of 500-fold could be detected in one organ, and no induction could be seen in cells from another organ of the same mouse, which reflected the different permissivity of

MCMV to certain cell types. FL expression correlated typically with the presence or absence of rounded cells, a marker for CMV infection (data not shown).

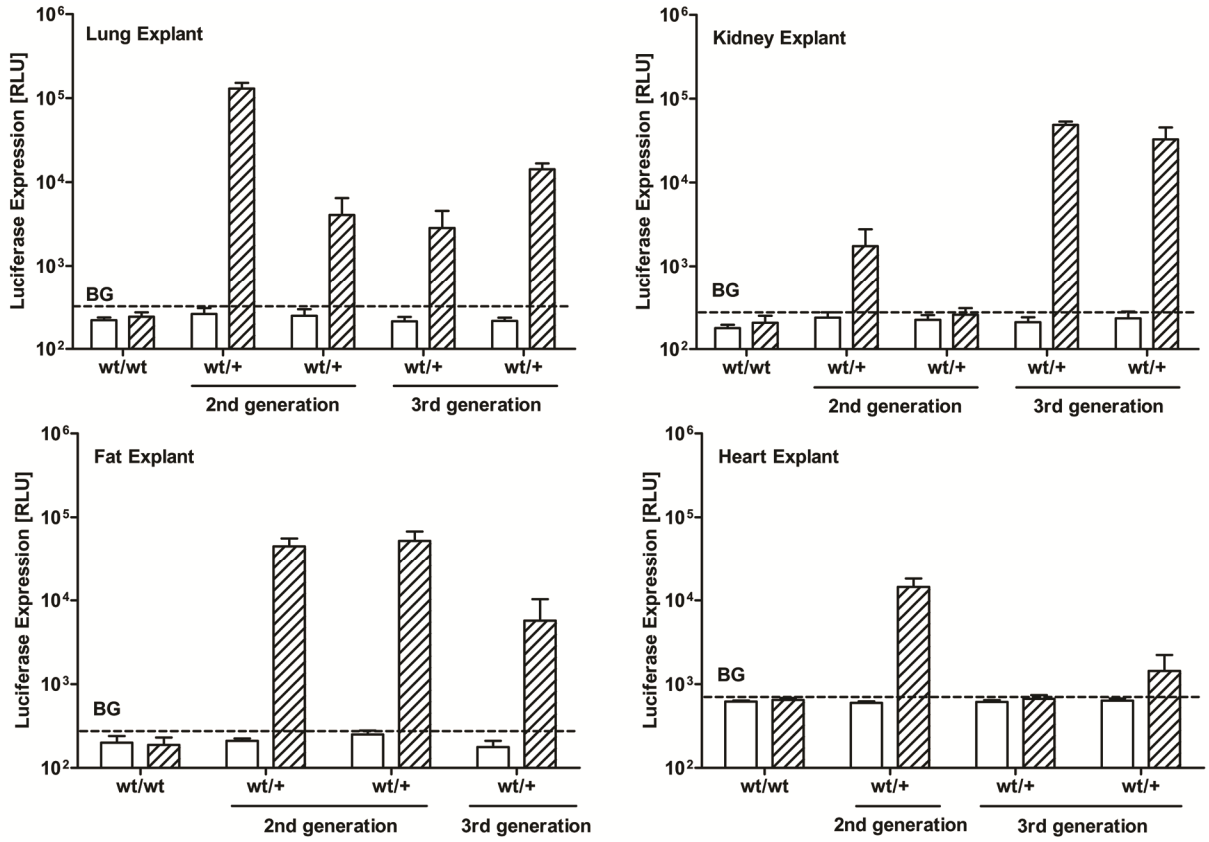


Figure 46: Induction of FL in VIOLA explant cultures upon MCMV infection.

Mice of the second and third generation of VIOLA-A were sacrificed and various organ and tissues were explanted. Shown are explants cultures of lung, kidney, fat and heart that were infected with MCMV or left untreated (not for all animals all organ explants were successful). Due to the inhomogeneity of the explants cultures infection could not be equalized therefore RLU values represent only semi-quantitative results. Notably, in almost all tissues and cultures, FL expression could be induced after infection although no signal could be detected in uninfected cells. (BG: Background of luciferin)

In addition, bone marrow, heart, muscle, fat, spleen and salivary gland tissue were explanted of a mouse of the fourth VIOLA generation. Again, specific signals could be obtained from extracted tissues. However induction strength was altogether very low in all assayed organs (Figure 47), which might be due to the loss of episomal persistence of the replicon vector. Thus, the VIOLA mice could be specifically induced upon infection, meaning that the replicon expression system can be transferred into the mouse model. However, the induction was only detectable in explant cultures but not *in vivo*.

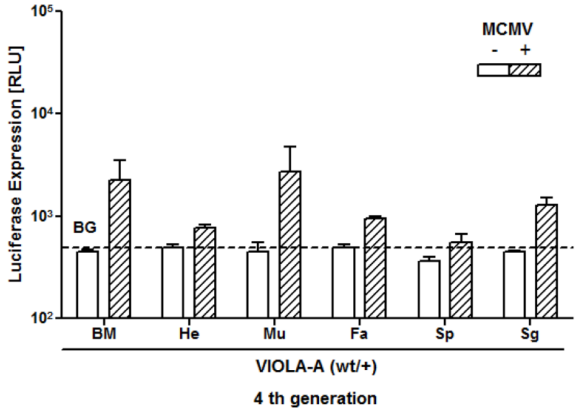


Figure 47 Induction of FL in explant cultures of various organs from the 4th generation of VIOLA

Explant cultures of bone marrow (BM), heart (He), muscle (Mu), fat tissue (Fa), spleen (sp) and salivary gland (Sg) of a VIOLA mouse of the 4th generation was infected with wt-MCMV and bioluminescence assay was performed. Signal strength of the induction was very low, but induction of FL expression could be detected in any of the explanted organ. (BG = background of luciferin)

5 DISCUSSION

The replicon system established herein offers a wide application range: successful implementations were shown for the study of herpesviral DNA replication, intracellular immunization, and *trans*-complementation of late herpesviral genes.

5.1 Advantages and disadvantages of non-viral episomal plasmid pEPI for the generation of stable cell lines

Pre-experiments (data not shown) indicated that episomal maintenance is an interesting feature for the establishment of stable cell lines. Especially, the independence of positional effects appeared favorable for reliable expression rates. The major disadvantage of episomal expression vectors is the unfaithful distribution of vectors during cell division. In contrast to this, the reliable once-per-cell cycle and even distribution to daughter cells is one of the key features of pEPI vectors. The essential element of these vectors is the cellular surface matrix attachment site (S/MAR) sequence that regulates the episomal maintenance of the vector (see chapter 1.9).

While in many human cell types, transgene expression derived from pEPI vectors was stable and not subjected to CpG-dependent silencing [109], transgene expression was always silenced in all cell types tested in this thesis. In accordance with the results from Jenke *et al.* [109] methylation-dependent silencing was not found in the replicon vectors, as cells did not respond to 5'-azacytidine treatment. In contrast, expression of the reporter gene through the replicon vectors was strongly silenced by a HDAC-dependent mechanism. This stands in line with results from Papapetrou *et al.* [118], who found also a HDAC-dependent silencing of a pEPI vector in murine MEL cells. Detailed studies on transgene expression of pEPI vectors in MEL cells revealed strong regulation at the chromatin level [120]. The vector is heavily chromatinized with histone H3 that becomes acetylated after treatment with TSA, leading to transcriptional activation (as also seen with the replicon vector). Therefore, Tessadori *et al.* concluded that pEPI-derived vectors are regulated 'remarkably' similar to host genes and are also responsive to histone modulation. Interestingly, inactivation of reporter gene expression on pEPI in MEL cells or the replicon vectors in the NIH3T3 cells did not result in vector loss although an active transcription unit was reported to be a crucial pre-requisite for vector maintenance [112]. Tessadori *et al.*, however, also found regulation by H3K9 methylation and direct methylation of DNA. The literature regarding methylation of pEPI vectors is, however, contradictory [109, 113, 118]. Differences in chromatin modifications indicate a species-specific or cell type-specific mode of

action of pEPI vectors and S/MAR elements. Interestingly, the kinetic of silencing was dependent on the type of transgene, as the genes for the toxic M50 and the GFPSCP proteins were much faster inactivated than the *luc* gene. This indicates once more the influence of the host chromatin on the regulation of the replicon vector in order to inactivate 'unwanted' genes.

While transgene silencing points to a limited use of the standard pEPI vectors in murine cells, the reliable inactivation of the vector served as tool for viral *trans*-activation of gene expression in the replicon system. Stable cell clones with the replicon vector were kept under continuous antibiotic selection pressure in this work. Failure of the antibiotic in one case did result in loss of the vector (data not shown). This is contradictory to published results in which a silenced pEPI vector remained stable without selection [118]. By transcribing the antibiotic resistance gene, even at low levels during selection, the S/MAR site might promote stronger association to chromatin as proposed by Jenke *et al.* [110]. This would explain the genomic integration of the replicon vector in mice lacking selection pressure. Silencing of the episome would thus result in poor maintenance of the vector, as no transcription would run into the S/MAR element and hold it in an open, meaning active, conformation.

Transgenic mice on the basis of the pEPI vector have not been generated so far. However, transient gene therapy studies with the pEPI vector have been described in mice. Intravenous injection of pEPI-1 into mice did not result in prolonged transgene (luciferase) expression or DNA persistence when compared to a conventional plasmid [152] indicating that the vector is not automatically maintained as an episome in mice. In the original pEPI vector an hCMVie promoter controls the transcription unit running into the S/MAR site. In several studies, including this one, this promoter has been found to be prone to epigenetic silencing [113, 153]. However, change of the hCMVie promoter to cell type-specific promoters, as alpha 1-antitrypsin, or novel synthetic promoters, as CMV-EF1α hybrid promoter, offers the possibility to influence the duration of transgene expression and with it maintenance of the derivative vector [152, 153]. Thus, transgene expression and vector stability are closely linked properties. In this replicon system, however, silencing of the transgene expression was important for transgene regulation. Inhibition of silencing would prevent the usage of cell-toxic transgenes. This leads to a dilemma, as discontinuous expression seems to be detrimental for stability but favorable for expression of toxic proteins. Murine S/MAR sites, as identified in the α-globin gene [154], and constitutive promoters could enhance transgene expression and thereby also the stability of the vector. It needs to be tested, if the second transcription unit downstream of the S/MAR site, which harbors the transgene, might be still silenced in this case or not. As S/MAR sites do also

work as insulators [155], individual transcription units on the replicon vectors could principally be regulated independently. This hypothesis is supported by the fact that the antibiotic resistance gene was always active *ex vivo* in this study by continuous addition of antibiotics to the cell culture while the reporter gene was inactivated. Still, balancing the two effects might be difficult to achieve *in vivo*.

5.2 Induction of transgene expression through DNA replication

The herpesviral expression profile is determined by a cascade of gene induction, with sequential immediate-early, early and late gene expression. This cascade is (besides other elements) achieved by usage of different viral transcription activators that possess specific binding sites in promoters of genes. Therefore, it was reasonable to assume that by using viral promoters the specific induction of a gene can be obtained. Several studies showed the possibility to activate herpesviral promoters *in trans* [137]. Here, five representative viral promoters were tested for their responsiveness to MCMV infection. To this end, the promoters P(M143), P(M53) and P(M94) as well as the control promoters P(hCMVie) and P(SV40) were cloned upstream of an firefly luciferase ORF into the vector pEpibo. The FL expression under control of the minimal P(SV40) was unaffected by MCMV infection and thus was used as control henceforth to dissect additional regulatory effects in further experiments. For the MCMV promoters, induction could be detected, which corresponded to the expected kinetic profile. Nevertheless, the overall induction levels were quite low, limiting their usage for further studies. However, the expression driven by isolated late herpesviral promoters *in trans* does not reflect the expression kinetics in the viral context and the expression from such constructs is started under early kinetics. In the context of viral infection, the dependence on DNA replication *in cis* for true-late gene expression is a well known phenomenon and defines a true-late viral protein. The mechanism of this 'cis-dependent' regulation, however, is not yet understood.

Replicon expression aimed at very strong expression to cope with expression levels reached during natural viral infection. It is known that plasmids containing herpesviral origins of replication-sequences can be replicated *in trans* [156]. However, it was not known whether combining an oriLyt sequence to an expression unit has an enhancing effect on transcription. There are controversial opinions about the cooperativity of replication and transcription. It has been stated that DNA replication and transcription are two exclusive mechanisms, which would hinder each other as the DNA sequence is occupied by the respective binding proteins [157]. The other opinion supports the view that DNA replication enhances adjacent gene transcription

[158]. As herpesvirus true-late gene expression is dependent on DNA replication of the viral genome it was unlikely that amplification of the replicon vector would hinder the expression of the encoded transgenes. Indeed, MCMV infection, more precisely viral DNA replication, induced the expression of the encoded transgenes in replicon vector-transfected cells. Experimental evidence was given by inhibition of the viral DNA polymerase by a drug, which blocked induction of the transgene, while the presence of the oriLyt sequence in the vector was necessary to obtain induction. This supports an obligatory role of DNA amplification of the replicon construct for transgene induction. Due to this feature, the oriLyt-containing pEPI constructs were coined here as replicon vectors.

The induction of gene expression from replicon vectors was generally remarkably high, up to 1,000-fold induction of FL expression at 36 h post infection in independently isolated cell clones. Already the mere amplification of the template could account for this effect. On the other hand, DNA replication and replication forks could change chromatin structures, which would make the gene more accessible to the transcription apparatus. In the present tests, the induction of the transgene upon infection was around 10 to 100-fold higher than by reactivating the construct from silencing by trichostatin A, even if the maximal non-toxic concentration of TSA was added (data not shown). Thus, both aspects account for the induction of the replicon vector, as the vector is released from silencing, and the number of vector copies that means the transcription templates is increased.

In many herpesviral oriLyt sequences functional or cryptic promoters have been identified. Therefore, adjacent ORFs can be principally transcribed by activation of oriLyt promoters. Yet, the influence of an unknown promoter in the MCMV oriLyt sequence on the replicon vector can be excluded, as the oriLyt is positioned downstream of the transgene expression unit. Another possibility for induction of the transgene could be the existence of an enhancer element within the oriLyt sequence. In this case, the enhancer is either directly activated by DNA replication or the *trans*-acting factor that activates the enhancer is a viral late protein in order to explain the inhibitory effect of PAA. Although PAA acts directly on the DNA replication apparatus by mimicking the pyrophosphate leaving group of the nucleotide transit reaction [159], PAA inhibits not only DNA replication but also all subsequent DNA replication-dependent steps like late gene expression. True-late proteins are not expressed under PAA treatment. Therefore, the inhibition of the transgene expression on the replicon vector using PAA can in principle reflect the lack of a late viral protein. Therefore, it cannot be formally

excluded that the presence of certain (late) *trans*-acting transcription factors suffices for induction of gene expression, without the need to replicate the construct.

In the γ-herpesvirus MHV68, viral proteins were identified that are essential for late viral transcription. Deletion of these genes causes inhibition of late gene transcription while DNA replication is not affected [160, 161]. These findings, therefore, partially refute the dogma of late gene transcription and replication forming an inseparable unit. Recently, the deletion of UL79 of HCMV has been found to cause the same phenotype as seen in MHV68 [162]. The HCMV protein UL79 shares 51 % amino-acid sequence identity with the yet uncharacterized M79 protein of MCMV. It is likely, that the proteins present functional homologues. In this case, it might be interesting to see, whether the replicon system could be activated by an MCMVΔM79 virus and how or if the transgene expression might change.

To obtain clarity if DNA amplification or induction of a late gene is the trigger for activation of the reporter gene, the MCMV replication proteins, especially the oriLyt activating protein, need to be identified. If cells carrying the replicon can be induced solely by the replicator proteins, DNA replication and with it the change of the chromatin state should be sufficient to activate adjacent gene transcription. If these proteins cannot activate the transgene, it would be more likely that activation is dependent on, or due to, a late viral transcription factor. CHIP analysis could help in both cases to identify the viral proteins bound to the replicon vector.

Induction of genes adjacent to replication origins is not limited to viral genomes. An interesting observation has changed the former static view of replication origins. In metazoa several classes of replication origins exist, of which some are activated very early during S-phase and others activated later on [163]. Usually early-firing replication origins are found in actively transcribed regions but are not limited thereto. Furthermore, not all potential origins are used during DNA replication as initiators for the replication fork. Some are only passively duplicated like any other DNA sequence [164]. Remarkably, some replication origins are only used during certain stages of development of the organism. This implicates regulatory effects between replication origins on gene expression and effects of transcription on origin usage [165]. A simple model for the activation or desilencing of genes during DNA replication was described by Wolffe in 1991 [166]. Every nucleosome is displaced in front of the replication fork. With ongoing time the newly synthesized DNA is again packed in nucleosomes. In excess of transcription factors that can bind to the promoter elements of the deliberated DNA, further packaging into tighter nucleosome structures is inhibited. Thereby, replication opens a window of opportunity to reorganize epigenetic imprinting and transcription factor binding on the replicated genes [158,

165]. Similarly, incoming herpesviral genomes are rapidly packaged by histones that are again removed during DNA synthesis [167], which might contribute to the DNA replication-dependent expression of true-late genes. Furthermore, this model could also explain the desilencing effect of the replicon vector after DNA replication. (Figure 48)

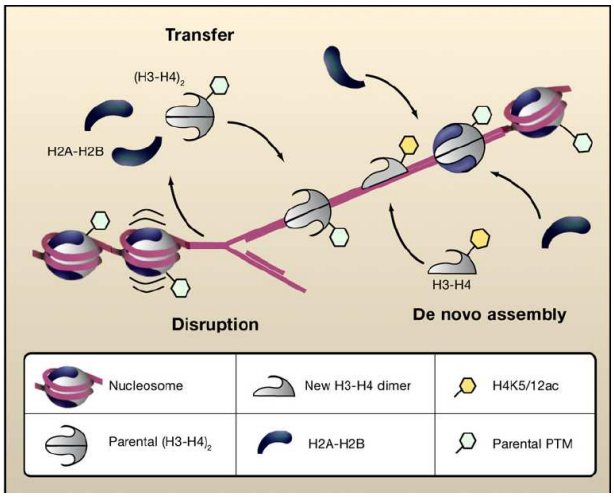


Figure 48: Model of the disruption and reassembly of nucleosomes during DNA replication.
In order to facilitate DNA replication nucleosomes are disrupted in front of the replication fork. First original H3-H4 histones are reassembled in a random fashion and can be exchanged by H3-H4 molecules with different posttranslational modified marks (PTM) (picture taken from [168]). This replication dependent removal of nucleosomes thus opens the opportunity to modify the epigenetic landmark or by access of transcription factors might even inhibit formation of nucleosomes.

A genome-wide study in *Saccharomyces cerevisiae* revealed that most genes are transcribed without being influenced by DNA replication [169]. However, in 3.5% of the genes DNA replication was necessary to start gene expression, e.g. genes coding for histones are highly upregulated by DNA replication. This indicates that DNA replication or origin activation could have an impact on adjacent gene expression even in higher eukaryotes. Still, regulation via this way is apparently only rarely used.

5.3 Intracellular immunization with the replicon vector

Herpesviruses are important pathogens for humans as well as for livestock. For some livestock specific vaccines were successfully constructed, which prevent disease in the animals. However, cases of vaccine failures and emergence of more virulent virus strains have been reported in vaccinated livestock, because such vaccines do not eradicate the virus but rather prevent symptoms of the disease. Herpesviral vaccines stay latent in the host, a basic feature of herpesviral infection, and are able to spread within the flocks [83]. This led to the prohibition of vaccination for some of the livestock species such as pigs and cattle [170]. The use of dominant-negative proteins to block virus infection, also termed intracellular immunization, has been proposed as an alternative to generate resistant animals. Yet, previous attempts to realize this concept have mainly failed due to toxicity of the constitutively expressed viral DN proteins

(summarized in section 1.7.2). In the present study several expression systems were evaluated. To this end, a replicon system was constructed offering for the first time the possibility to induce expression of transgenes, activated by viral wild type infection and concomitantly to produce high amounts of transgenic protein. Besides the advantages of an inducible system regarding health and safety, acceptance for such transgenic animals by consumers is likely to increase as the protein is not expressed in the uninfected animal.

Previous studies with DN proteins of HSV-1 were hampered by the strong toxicity of the transgenes. There were also attempts to generate inducible expression systems, either by usage of herpesviral promoters or by chemically inducible systems. Sheppard *et al.* tried to circumvent the toxicity of an HSV-1 DN mutant by using the ICP4 promoter of HSV-1, which is activated by herpesviral transcription factors [94]. Nevertheless, constitutive expression of the DN was found already in absence of infection and the toxicity of the transgene could not be avoided. Similar results were obtained in the present study, when MCMV promoters of different kinetic classes were used. Although all promoters were inducible by infection and the expression correlated to the expected time of activation, none of the promoters was inactive in uninfected cells. Usually, only short promoter stretches of 500 bp were used to 'preliminary' define the promoter regions, however, there is only little information about regulatory elements in these promoters and adjacent regions. Lack of inhibitory transcription factor binding sites or silencers, in the minimal 500 bp promoters, might result in the uncontrolled leaky expression of the isolated promoters. Due to overlapping coding sequences, borders of genetic elements, especially promoters are hard to define. Usage of longer promoter sequences might help to get tighter regulations, but bears the risk to include genetic elements of overlapping or the complementary strand sequences.

Obviously, herpesviruses are able to strictly control the timing of gene expression and protein production during infection. Recent data from Marcinowski *et al.* (personal communication) point to an even more complex mechanism to regulate gene expression, as the transcription of true-late genes can be found during a short period at immediate-early time points although no protein can be detected at this stage. Viral post-transcriptional regulation, however, has so far not been analyzed at large and might be difficult to translate into an expression cassette.

A major improvement to the regulation of dominant-negative proteins for intracellular immunization was achieved by the replicon vector system. The host-mediated silencing of the expression cassettes was highly reproducible in all assayed cell types in this study, although the

time period, until complete inactivation was reached, was dependent on the individual transgenes. 'Harmful' proteins were, in general, inactivated much faster than non-toxic proteins. For example, the regulation of the GFPSCP was extremely tight, as no fluorescence could be detected in uninfected cells. The infection of the GFPSCP-ori cells with MCMV-mCherry revealed a high correlation of the infection marker mCherry and induced GFP fluorescence. Although the correlation was calculated to be greater than 95 %, this number might even be underestimated as the fluorescence signals of mCherry are typically higher than the GFP signals and 'negative' cells, carrying only one fluorescence signal could be possibly re-evaluated at later stages of infection. The mCherry gene was set under control of the late SCP promoter; still the timing might not be completely identical, although the replicon system is also activated under late kinetics, which might lead to a miscalculation of fluorescence correlation.

Although the replicon expression system drives a very strong gene expression, MCMV spread could not be completely blocked in GFPSCP-ori cell lines. In contrast to the cell lines, a recombinant virus encoding the DN protein in the viral genome was not able to spread [103]. In this case, expression of the DN occurred in early kinetics and thus the inhibitory protein had a headstart before the onset of expression of the wt *scp* gene. The DN was more abundant compared to the wt protein and could occupy binding sites before the wt protein is even present. As the replicon system is activated in late kinetics, it is much harder to out-compete the wt SCP protein. Note that SCP is one of the most abundant proteins in the viral capsid [171]. The GFPSCP protein was only selected to demonstrate proof-of-principle, as the expression of the DN is simple to monitor by the marker. Other DN proteins, which are less abundant, might be better targets to inhibit MCMV spread.

Most remarkable, the replicon system having on average two copies per cell reached the same inhibitory potential as another previously tested episomal papilloma virus-derived vector system B45, which is maintained with 50 to 100 copies per cell and constitutively expresses GFPSCP (data not shown). The low copy number of replicon vectors in cells is probably advantageous to reduce potential side effects. As 2 to 10 copies of pEPI-vector were typically found per cell and in particular 2 copies of the DN replicon vector in the GFPSCP-ori cl. 3 clone, it is possible that cell lines with a higher initial vector load might result in a stronger inhibition of MCMV spread. In this case, it is unlikely that the presence of few more replicon vectors will cause stronger side effects, as the additional DN genes are most likely subjected to silencing as well.

Generation of virus-resistant animals is the aim of intracellular immunization. As a first test of functionality of the replicon vector *in vivo*, a firefly luciferase transgene was used to generate transgenic mice. In the resulting VIOLA mice (standing for virus-inducible oriLyt-dependent luciferase animal) the bioluminescence of the transgene is easier to monitor and to quantify compared to an inhibitory DN protein, which should be used in the end. Testing the replicon vector *in vivo* led, however, to unexpected results. In particular, the expression of the replicon vector encoded transgene was detectable only after infection in explant cultures but not in the living animal. Furthermore, the replicon vector was not stably maintained as an episome in the VIOLA mice. Rather integration of the replicon vector was found. Without having explanations and answers to these findings, it had no point to generate transgenic animals with the GFPSCP containing replicon.

To gain more information on these peculiar results, it has to be completely ruled out whether the total FL signal in VIOLA was too weak to be detected due to a rather low infection density in mice compared to the tissue culture experiments or whether there was really no induction of transgene expression upon infection *in vivo*. If the former assumption is correct, the induction in individual infected cells could still inhibit viral spread. Unfortunately, the vector integrated in the genomes of VIOLA mice. This, however, makes the expression dependent on positional effects of the integrated vector. Positional effects might thus also explain the failure of the VIOLA-B line in contrast to the VIOLA-A line, which showed also different integration patterns in the Southern blot experiments. Large numbers of animals will be needed to screen for expression of the transgene before and after infection using an integrating construct. Therefore optimization of the vector for episomal maintenance is necessary before applying the replicon system. The fact that episomal maintenance was found in transgenic pigs with the pEPI-EGFP vector [151] opens however a window of opportunity to directly translate the replicon system to the pseudorabies virus, which is an important veterinary pathogen (see section 1.7). Therefore, if the pEPI-vector is already suitable for the usage in swine, further adaptation of the replicon vector to mice seems not to be essential. Notably, the sequence of the origin of replication of pseudorabies virus is well defined [172] and should be thus easily transferable to the replicon system.

5.4 Usage of the replicon vector to study MCMV oriLyt function

DNA replication of the β- herpesvirus subfamily is not completely understood yet. While some work has been published on human CMV (HCMV), almost no data is available for any of the

other cytomegaloviruses used as models for HCMV disease, like rat CMV, guinea pig CMV or MCMV. While eleven proteins are necessary to replicate an oriLyt-containing vector in HCMV, no information is available, which proteins are necessary for the DNA replication of MCMV, although the core set of proteins is conserved [30]. The study of DNA replication on MCMV might be of special interest as HCMV, like MCMV, does not harbor a special latent origin of replication. Moreover, only one replication origin has been identified for cytomegaloviruses in contrast to other herpesviruses, which harbor up to three origins of replication. Still, HCMV resides latently in actively replicating cells as for example myeloid stem cells [173]. To prevent the loss of the viral genome during division of the host cell an active DNA replication process appears to be necessary. Therefore, the oriLyt of HCMV, like that of MCMV must provide the factors for latent DNA replication. Although there is no sequence homology between the oriLyt of MCMV and HCMV, the structural composition and functional elements, such as direct and indirect repeats, A/T-rich regions, Y-block and transcription factor-rich binding sites are shared [45, 46]. Studying HCMV latency in humans is extremely difficult: information on time-point and course of infection in patients without pathological findings and availability of tissue-samples is restricted. Therefore a detailed knowledge of MCMV DNA replication and *in vivo* studies might be helpful to elucidate the general mechanism of replication in latency.

Identification of the proteins necessary for DNA replication of HSV-1 has been performed by a method proposed by Challberg [156]. Viral genome fragments were ligated, cells transfected with the plasmids, and super-infected with the respective herpesvirus. Amplification of the plasmid with the potential origin of replication was analyzed by Southern blot hybridization and several oriLyt sequences were identified by this way [45, 46, 63, 174-177]. Later on, plasmids containing oriLyt sequences were co-transfected with several plasmids containing herpesviral genes, to clarify which individual proteins were necessary for the amplification [156]. Although often successful, this strategy is very tedious and time consuming. In this work, a strict correlation of DNA replication and induction of gene expression of the replicon vector was found. Applying the replicon vector principle would simplify the identification all replication proteins. Superinfection of the luc-ori cells and subsequent bioluminescence assays would simplify the procedure. While the Challberg-method relies on Southern Blot analysis as read-out of DNA amplification, the DNA replication of the replicon vector is simply monitored by FL expression. Southern blot experiments extend over a period of four to five days, a bioluminescence assay of the replicon vector system can be performed in less than one hour. Optionally, quantitative PCR, which is also the more precise and direct proof for DNA amplification compared to the bioluminescence experiments can be added to verify findings from

the bioluminescence assay. Yet, the bioluminescence assay is cheaper and less prone to errors compared to qPCR, which makes it very interesting for a fast high-throughput screening.

Notably, even the minimal oriLyt sequence of MCMV has been mapped only roughly and no key elements have been assigned yet. The HCMV oriLyt possesses two important regions, whereby one region can be replaced by an SV40 promoter [52]. The replicon vector contains also a SV40 promoter close to the oriLyt sequence. This could contribute to the connection of replication and gene induction, if the SV40 promoter directing FL expression would mimic part of the oriLyt sequence. Several viral as well as cellular transcription factor binding sites have been mapped to the HCMV oriLyt sequence, but none of these have been analyzed in detail. Mutagenesis of the oriLyt sequence in the replicon vector instead of mutagenesis of the viral genome should give clearer results, as there is no risk of analyzing side effects due to overlapping ORFs or promoters. A RNA/DNA hybrid has been found in the HCMV oriLyt [178], thus it would be interesting to know if MCMV possess also RNA/DNA hybrid regions and how these elements regulate DNA replication. Furthermore, miRNAs have been identified close to the oriLyt [179]. There is no information about their role, yet. Again, these questions can be addressed in the replicon system using the FL expression of the pEpibo-luc-ori vector as a simple read-out.

The induction of DNA replication varies between the herpesvirus subfamilies. While the mode of action is very well analyzed in α-herpesviruses only little knowledge is available for the other subfamilies. The origin binding protein of the α-subfamily and also that of the roseoloviruses initiates DNA replication by forming a cruciform/hairpin structure by complementary intrastrand base pairing, which leads to strand separation and recruitment of the core replication proteins [180]. In γ-herpesviruses a hairpin structure is also a key element to the initiation of DNA replication. There, an imperfect preformed hairpin within a repetitive element is recognized by a viral transcription factor and is an important feature of DNA replication initiation [181]. A common herpesviral mechanism of DNA initiation based on the formation and stabilization of such secondary elements has been proposed [36]. In cytomegaloviruses no hairpin structure has been identified yet, although there are several inverted repeats that would allow such a conformation. DNA conformation analysis of the oriLyt might be more easily accessible in the replicon vector than in the large MCMV genome, with a size of about 230 kb.

5.5 *Trans*-complementation of late viral proteins with the replicon vector

Protein *trans*-complementation is an important tool to study mutant herpesviral genomes carrying deletion of essential or non-essential genes. To analyze and control the effect of a targeted deletion, in order to exclude additional unwanted mutations in the viral genome, revertants of the generated mutants are generated. Genetic reversion is a common procedure, where the original sequence is reintroduced into the deleted region. This bears, however, the risk to 'overlook construction flaws' [182] that originate from overlapping gene or regulatory regions, which will be also corrected by genetic reversion but not by *trans*-complementation. Therefore additional information may be gained by reversion of a virus mutant phenotype through *trans*-complementation. Furthermore, transient complementation of a mutant virus can help to study the function of the protein.

Especially the *trans*-complementation of late herpesviral proteins is a difficult task. Improper timing and expression levels on the one hand can hamper the correct localization of the proteins, while on the other hand isolate expression, i.e. without the co-expression of viral binding partners, can lead to toxicity. Particularly, construction of correctly timed expression is hindered by the very nature of herpesviral late gene expression. Only limited information about the regulation of true-late gene expression is available. As discussed earlier (see section 5.2) one remarkable feature is their dependency on DNA replication for the induction of gene expression [183]. Removal of late gene promoters from the viral genome and their insertion into the cellular genome resulted in wrong, namely early, expression [184]. Correlation of DNA replication and late gene expression was demonstrated by the fact that incoming genomes, which were not replicated yet, cannot serve as template for late gene expression [183]. Moreover, late gene expression could be restored if a late gene promoter or a minimal promoter was present together with a lytic origin *in cis* [185, 186]. Deviations from this principle exist in that some late gene promoters were dependent on DNA replication *in trans* [187]. Although *trans*-complementation of late herpesviral protein is so difficult to achieve with the constitutive expression cassettes, nobody has tried to construct an expression cassette mimicking herpesviral late gene expression to our knowledge.

'Toxic' proteins have to be expressed via conditional systems. The most common inducible expression systems are the Tet-ON/Tet-OFF system [67] or the FKBP12 [68] system, which rely on the administration of small chemical compounds. By the addition of the compound, activation takes place synchronously in all cells in the culture. This activation is

independent from the state of virus replication in all cells. In contrast the oriLyt-based system uses viral DNA replication as signal for the induction. Moreover, the expression of the transgene follows the natural route of late kinetics in the replicon system as the expression increases in proportion with the amplification of the vector DNA. Thus each cell is activated individually upon infection with incoming virus, which leads to appropriate and correct timing of the late transgene. It has to be noted, that due to the replication dependency the system is only suitable for *trans*-complementation of late but not of early viral transgenes.

The TET-regulatory system has been successfully used to *trans*-complement the late protein M94 of MCMV [71]. In this case, the M94 gene in the viral genome was replaced by the gene encoding the tetracycline transcription activator (tTA) and a cell line encoding the M94 gene under control of the tetracycline response element (TRE) was constructed. In this setting the virus lacking M94 induces the expression of M94 upon infection in this cell line. By this elegant way the tTA protein is produced at the time point the endogenous M94 protein would be activated and binds to the TRE element, which leads to the transcription of the transgenic M94. In this case, the protein is produced at the correct time point. However, an increase of transgene expression as it is achieved with the replicon system does not take place here. While the tTA-TRE method allowed *trans*-complementation of the essential M94 protein, the mutant production in large scale was tedious (personal communication, C. Mohr). A further disadvantage of the system is the necessity to modify the viral genome for presence of bacterial elements in the inducing expression cassette within. Especially for vaccine production, bacterial sequences within the viral genome should be avoided. The replicon system, in contrast, is activated by wt virus and thus allows more possibilities for the design of virus mutants. Moreover the system is not marred by the presence of 'foreign' DNA elements.

Usage of the replicon system for protein *trans*-complementation was demonstrated with two viral proteins, namely gO and M50. In case of the non-essential glycoprotein O, the deletion of the gene causes a 2 to 2.5 fold order of magnitude smaller amount of virus in supernatants and a strict cell-associated spread. Growth on the complementing cell line gO-ori of the MCMVΔgO mutant restored the phenotype, meaning that the virus is no longer restricted to a focal spread pattern and releases similar amounts of virus compared to the wt situation. Thus the replicon system was suitable to *trans*-complement even such a difficult transgene like a glycoprotein. Due to the host-mediated silencing of the transgenes, the question whether the system is also suitable to *trans*-complement a toxic protein was addressed. Previous attempts to generate M50-complementing cell lines via common methods failed [150]. In contrast, the creation of M50-

complementing cell line with the replicon system was very successful. No difference to any other non-toxic transgene was detectable regarding efficacy of cell line generation. This was due to the fast host-mediated shut-off of the M50 transgene expression. Western Blot analysis revealed the absence of the protein in uninfected cells and a high induction of the protein in infected cells. Very high titers of MCMVΔM50 virus could be grown on the M50-ori cell lines speaking for the high efficacy of *trans*-complementation. Furthermore, reconstitution of ΔM50 virus from transfected BACs was just as quick as the reconstitution of wt virus.

Trans-complementation bears the risk of reversion of the mutant virus to wt sequences due to recombination of homologous sequences. Viral genes are often organized in an overlapping fashion in the genome. Therefore deletion of an open reading frame is not always possible, as the neighbouring gene would be affected as well. Furthermore, the replicon vector carries the oriLyt-sequence, which of course is present in the viral genome as well. While no recombination of MCMVΔgO could be detected when propagated in the gO-ori cells, reversion of the M50 deletion was found after propagation on the M50-ori cells. In both cases there were complementary sequences in the replicon vector and the viral genome as the genes could not be completely deleted due to overlapping coding sequences. However, the selection pressure on MCMV-ΔM50 is much higher, as the gene is essential for virus spread. The deletion of gO causes only a reduction in viral release and a change in the mode of virus entry. The detection of recombination is however much more sensitive in case of M50 as a few recombined genomes have a growth advantage on non-complementing cells, as these are the only virus mutants that are able to survive. The recombination rate in the cell pools was high with 1 of 10^4 viruses. Yet, the recombination rate in the isolated M50-ori cell clone 2.1 was very low with less than 1 of 10^8 viruses. Maybe, the presence of non-functional cells in the cell pool, i.e. cells only having the resistance marker integrated but not the transgene as seen in the luc-ori cl.4 line, increases the selection pressure towards recombined genomes. In case of gO, recombination via phenotypic assays is much more difficult to detect as the protein is not essential for virus amplification. Yet, even PCR analysis could not detect any recombination of MCMVΔgO with the replicon vector. Furthermore, no recombined viruses where found by immuno-histology in mice infected with *trans*-complemented MCMVΔgO (personal communication B. Adler), where recombination would provide a major growth advantage and selection pressure would be also very high. It needs to be determined what favors recombination with the replicon vector and how it might be prevented.

Single cycle viruses (see section 1.6) are of rising importance for vaccine development [71, 188]. A single cycle virus is a virus lacking an essential gene in its genome but is *trans*-complemented with the respective protein in order to allow the infection of the host. Still, the virus cannot spread further to neighboring cells. The major advantage of this vaccination strategy is the presentation of almost all antigens and the high safety in comparison to attenuated vaccines. A major limitation with single cycle viruses for vaccination is the necessity to *trans*-complement the missing protein. Vaccine production needs to be safe as well as efficient in order to be applicable. The *trans*-complementation of essential genes with the replicon vector system might help to improve the latter point, as the titers that were obtained after *trans*-complementing mutant viruses in replicon cell lines were comparable to wt titers. Yet, the degree of recombination of the vector might limit the usage of the system for vaccine production at this stage.

5.6 Difference between *ex vivo* and *in vivo* performance of the replicon vector system

While the replicon vector could be very successfully used in tissue culture, its performance in mice was disappointing. The question remained why the bioluminescence signal upon infection could be easily detected in explanted tissue culture but not in the living animal. A simple solution would be to hold the detection limit of the non-invasive bioluminescence signal to account for the lack of the FL signal. However, measurements were performed with maximal sensitivity settings. Under such conditions it has been possible to detect even as few as 500 to 1000 bioluminescent cells *in vivo* [189]. Regarding the infection dose of 1×10^6 PFU wt-MCMV, the initial amount of infected cells is much higher as the cell number in the former mentioned reports — without even taking further dissemination in account. The high bioluminescence values in tissue culture were obtained with an MOI of 0.5; this infectious density is not reached *in vivo* in the first place. However, when amplification and spread takes place high titers can be locally obtained, which should have resulted in detectable FL signals. Furthermore, also low MOI infection as 0.01 gave a measurable signal in the luc-ori cells.

Even invasive bioluminescence assays on organ homogenates were not successful either (data not shown), although the sensitivity is here even much higher, as the signal is not blocked by fur or tissue. The induction of gene expression was however readily detectable in explanted tissue cultures of the VIOLA mice. Still, the bioluminescence values were rather low, compared to the data of the luc-ori cell line. One possible explanation for the low induction is the

integration of the replicon vector into the host genome. Although the general opinion of herpesviral replication proposes the initiation of replication from circular genomes, the hypothesis has been challenged as the lytic replication may originate from linear templates [190]. Replication of integrated origin of replication sequences of HSV-1 (oriS) has been reported [191]. Previous experiments with an oriS associated with an expression unit in an integrated vector, proposed that the replication origin had no influence on gene expression [192]. However, as the data obtained in that study was derived from integrated constructs, the two data sets, namely with and without oriS, were not directly comparable as the influence of positional effects was not taken into account. In the line with this, also integrated replicon constructs might be subjected to positional effects. This could cause failure of the system or lower induction strength in general. Yet, in case of the VIOLA mice, this does not explain the difference between *in vivo* and *ex vivo* experiments, as the construct has always the same genomic position even if the integration decreases full replicon strength.

The curious difference of the replicon system in the VIOLA mice is reminiscent to the behavior of herpesviral genomes. The hallmark of herpesvirus infection is establishment of latent infection. In the latent infection almost no gene expression occurs and no viral progeny is produced. A reliable observation is the reactivation of herpesviruses from latently infected cells after explantation of tissue [193, 194]. Interestingly, addition of histone deacetylase inhibitors like TSA can enhance the reactivation rate. Thus, it can be concluded that the chromatin state of the herpesviral genome is involved in the establishment and maintenance of latency. During explantation major epigenetic changes occur, which release the latent genomes from the silenced state. A similar effect might be active with the replicon vector as well. It might be that the vector is in a chromatin state that is inaccessible for the DNA replication machinery of MCMV and therefore the induction of gene expression is hindered *in vivo*. During explantation, a less tight meta-stable chromatin status may be established, which can be than resolved during infection. To address this question, the epigenetic status of uninfected and infected cells from mice and explanted tissue needs to be analyzed. CHIP experiments should show different histone marks to proof this hypothesis.

5.7 Concluding remarks

In this study, a herpesvirus lytic origin of replication was combined with the transcription unit from an episomal vector to generate a novel inducible expression system, namely the replicon vector, which is induced by infection with wt virus. Transgene expression of the replicon vector was reliably silenced in all tested cell types. However, upon viral infection, de-silencing and activation of the replicon vector led to a >1000-fold increase in induced gene expression. The novel and major advantage of the replicon system over other inducible system resides in the usage of fundamental viral processes. Wt virus infection suffices and no chemical compound has to be added in order to achieve induction of transgene expression. Instead, the DNA replication of the infecting virus does also lead to the DNA amplification of the replicon vector, which, in turn, releases the vector from silencing and activates gene expression. Due to the increase in vector templates a remarkably strong induction of transgene expression was achieved. Therefore, the expression of the transgene was much stronger when compared to conventional expression systems and can almost cope with the strength of viral expression itself. Moreover, fluorescence imaging revealed a nearly perfect correlation of infected cells and cells expressing the marker protein. Thus, the transgene is only expressed in the virus infected cell, which undergoes a lytic infection cycle. This, of course, prevents toxic side effect by unwanted expression of the transgene protein and thus solves the major problem that marred previous applications like intracellular immunization and *trans*-complementation of toxic proteins.

The non-viral episomal vector system pEPI offered an interesting opportunity for the construction of the replicon vector. Previous reports highlighted the stable expression and maintenance of the vector in the absence of antibiotic selection pressure. Silencing of the encoded transgene was, however, found in all cell types tested, while FISH analysis revealed the mainly episomal persistence of the vector *in vitro*. Although, this limits the usage of the pEPI vector for other purposes were a reliable constitutive expression is needed, it was very advantageous for the goal of this study, as an extremely low or no background expression could be detected. The generation of transgenic mouse lines based on the non-viral and episomal pEPI vector background can be reported herein for the first time, although unfortunately, the vector failed to persist in the episomal state *in vivo*. This emphasizes, while pEPI vectors are used for gene therapy in many other studies, which were mainly *in vitro*, that translation of such system to the living transgenic animal certainly needs further (re-) evaluation to consider the demands on the vector for reliable performance and to finally proof the concepts *in vivo*. In other words, the results shown herein demonstrate – not for the first time – that data obtained from *in vitro*

experiments are not directly translatable to the living animal. Thus, further studies and re-evaluation of the *in vivo* findings made herein are needed. It remains open if and how a transgenic mouse stably homing an episomal vector can be generated.

Yet, the data obtained in this study, helped to solve important aspects on the way to implement intracellular immunization to livestock. A major improvement was the lack of toxic side effects seen with constitutively expressed dominant-negative transgenes by using a suitable, i.e. an inducible, system. The trigger to activate the inducible system must rely within the virus itself to achieve a fast inhibition of the infection and, moreover, prevent further spread of the virus. *Trans*-activation of viral promoters was considered as one possibility to obtain a virus-specific induction, however, with respect to the strength of expression, the activation of viral promoters (encoded in the host chromatin) by transcription factors provided by the viral infection in *trans* was rather low. Yet, high expression of the dominant-negative protein is necessary in order to block viral spread. The replicon vector provides here the basis for extremely high expression levels that can partially cope with levels obtained during viral infection. Nevertheless, full inhibition of virus spread could not be achieved with the DN GFPSCP used in this study. Targeting proteins, that have lower abundance than the small capsid protein, with 900 copies per virion, might shift the DN to wt ratio resulting in stronger inhibition. An interesting late protein target could be e.g. the portal protein, which is present only twelve times per capsid.

The expression and especially the *trans*-complementation of late herpesviral protein was a difficult task as true-late viral proteins are only expressed after viral replication has taken place. Out of the viral context, true-late promoters are generally activated with an early kinetic. This wrong expression timing often caused problems in *trans*-complementation resulting in low virus yields. This is an unwanted side effect in vaccine production, for example. With the replicon vector system viruses lacking the glycoprotein O or the toxic transmembrane protein M50 could be successfully *trans*-complemented to high virus titers. Thus, the replicon system closes the gap for production of such virus deletion mutants. This would allow, if transferable to viruses infecting man, to generate spread-deficient vaccines. These should be promising candidates for vaccination against infection and/or reactivation of human herpesvirus infections.

6 REFERENCES

1. Pellett, P.E. and B. Roizman, *The family of Herpesviridae: A brief introduction*, in *Fields Virology*, D.M. Knipe and P.M. Howley, Editors. 2007, Lippincott Williams & Willkins: Philadelphia. p. 2479-2501.

2. Davison, A.J., et al., *The order Herpesvirales.* Arch Virol, 2009. **154**(1): p. 171-177.

3. Gesser, R., *The role of latency in herpesvirus infections.* Seminars in Pediatric Infectious Diseases, 1997. **8**(3): p. 128-135.

4. Roizman, B. and J. Baines, *The Diversity and Unity of Herpes-Viridae.* Comp Immunol Microb, 1991. **14**(2): p. 63-79.

5. Nicholas, J., *Evolutionary aspects of oncogenic herpesviruses.* Journal of Clinical Pathology-Molecular Pathology, 2000. **53**(5): p. 222-237.

6. Croen, K.D., et al., *Latent Herpes-Simplex Virus in Human Trigeminal Ganglia - Detection of An Immediate Early Gene Antisense Transcript by Insitu Hybridization.* New England Journal of Medicine, 1987. **317**(23): p. 1427-1432.

7. Tarakanova, V.L., et al., *Murine gammaherpesvirus 68 genes both induce and suppress lymphoproliferative disease.* Journal of Virology, 2008. **82**(2): p. 1034-1039.

8. Hahn, G., R. Jores, and E.S. Mocarski, *Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells.* Proc.Natl.Acad.Sci.U.S.A, 1998. **95**(7): p. 3937-3942.

9. Koffron, A.J., et al., *Cellular localization of latent murine cytomegalovirus.* J Virol, 1998. **72**(1): p. 95-103.

10. Pass, R.F., et al., *Congenital cytomegalovirus infection following first trimester maternal infection: symptoms at birth and outcome.* J Clin Virol, 2006. **35**(2): p. 216-20.

11. Arvin, A.M., et al., *Vaccine development to prevent cytomegalovirus disease: report from the National Vaccine Advisory Committee.* Clin.Infect.Dis., 2004. **39**(2): p. 233-239.

12. Furlong, D., H. Swift, and B. Roizman, *Arrangement of herpesvirus deoxyribonucleic acid in the core.* J Virol, 1972. **10**(5): p. 1071-1074.

13. Yu, X.K., et al., *Three-dimensional localization of the smallest capsid protein in the human cytomegalovirus capsid.* Journal of Virology, 2005. **79**(2): p. 1327-1332.

14. Wu, L.J., et al., *Three-dimensional structure of the human herpesvirus 8 capsid.* Journal of Virology, 2000. **74**(20): p. 9646-9654.

15. Hazelton, P.R. and H.R. Gelderblom, *Electron microscopy for rapid diagnosis of infectious agents in emergent situations.* Emerging Infectious Diseases, 2003. **9**(3): p. 294-303.

16. Liu, F. and Z.Z. Hong, *Comparative virion structures of human herpesviruses.* 2007.

17. Trus, B.L., et al., *Capsid structure of simian cytomegalovirus from cryoelectron microscopy: Evidence for tegument attachment sites.* Journal of Virology, 1999. **73**(3): p. 2181-2192.

18. Kalejta, R.F., *Tegument proteins of human cytomegalovirus.* Microbiology and Molecular Biology Reviews, 2008. **72**(2): p. 249-265.

19. Mettenleiter, T.C., B.G. Klupp, and H. Granzow, *Herpesvirus assembly: a tale of two membranes.* Current Opinion in Microbiology, 2006. **9**(4): p. 423-429.

20. Feire, A.L., H. Koss, and T. Compton, *Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(43): p. 15470-15475.

21. Campadelli-Fiume, G., et al., *The novel receptors that mediate the entry of herpes simplex viruses and animal alphaherpesviruses into cells.* Reviews in Medical Virology, 2000. **10**(5): p. 305-319.

22. Scrivano, L., et al., *The m74 Gene Product of Murine Cytomegalovirus (MCMV) Is a Functional Homolog of Human CMV gO and Determines the Entry Pathway of MCMV*. Journal of Virology, 2010. **84**(9): p. 4469-4480.

23. Spear, P.G. and R. Longnecker, *Herpesvirus entry: an update.* J Virol, 2003. **77**(19): p. 10179-10185.

24. Miyamoto, K. and C. Morgan, *Structure and Development of Viruses As Observed in Electron Microscope .11. Entry and Uncoating of Herpes Simplex Virus.* Journal of Virology, 1971. **8**(6): p. 910-&.

25. Everett, R.D., *Trans Activation of Transcription by Herpes-Virus Products - Requirement for 2 Hsv-1 Immediate-Early Polypeptides for Maximum Activity.* Embo Journal, 1984. **3**(13): p. 3135-3141.

26. Costanzo, F., et al., *Evidence That Herpes-Simplex Virus-Dna Is Transcribed by Cellular Rna Polymerase-B.* Journal of Virology, 1977. **21**(3): p. 996-1001.

27. Honess, R.W. and B. Roizman, *Regulation of Herpesvirus Macromolecular-Synthesis - Sequential Transition of Polypeptide-Synthesis Requires Functional Viral Polypeptides.* Proceedings of the National Academy of Sciences of the United States of America, 1975. **72**(4): p. 1276-1280.

28. Holland, L.E., et al., *Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species.* Virology, 1980. **101**(1): p. 10-24.

29. Mavromaranazos, P. and B. Roizman, *Delineation of Regulatory Domains of Early (Beta) and Late (Gamma-2) Genes by Construction of Chimeric Genes Expressed in Herpes-Simplex Virus-1 Genomes.* Proceedings of the National Academy of Sciences of the United States of America, 1989. **86**(11): p. 4071-4075.

30. Challberg, M.D., *Herpesvirus DNA Replication*, in *DNA replication in eukaryotic cells*1996, Cold Spring Harbor Laboratory Press p. 721-750.

31. Anders, D.G., J.A. Kerry, and G.S. Pari, *DNA synthesis and late viral gene expression*, in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*, A. Arvin, M.E. Campadelli-Fiume G, and et al., Editors. 2007, Cambridge University Press: Cambridge.

32. Strang, B.L. and N.D. Stow, *Circularization of the herpes simplex virus type 1 genome upon lytic infection.* J.Virol., 2005. **79**(19): p. 12487-12494.

33. Wilkinson, D.E. and S.K. Weller, *The role of DNA recombination in herpes simplex virus DNA replication.* IUBMB.Life, 2003. **55**(8): p. 451-458.

34. Weller, S.K. and D.M. Coen, *Herpes Simplex Virus*, in *DNA Replication and Human Disease*, J. Inglis, Editor 2006, Cold Spring Harbor Laboratory Press. p. 663-686.

35. Olsson, M., et al., *Stepwise Evolution of the Herpes Simplex Virus Origin Binding Protein and Origin of Replication.* Journal of Biological Chemistry, 2009. **284**(24): p. 16246-16255.

36. Rennekamp, A.J. and P.M. Lieberman, *Initiation of lytic DNA replication in Epstein-Barr virus: search for a common family mechanism.* Future Virology, 2010. **5**(1): p. 65-83.

37. Igarashi, K., et al., *Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis.* J.Virol., 1993. **67**(4): p. 2123-2132.

38. Longnecker, R. and B. Roizman, *Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction a sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the alpha 47 gene.* J.Virol., 1986. **58**(2): p. 583-591.

39. Polvino-Bodnar, M., P.K. Orberg, and P.A. Schaffer, *Herpes simplex virus type 1 oriL is not required for virus replication or for the establishment and reactivation of latent infection in mice.* J.Virol., 1987. **61**(11): p. 3528-3535.

40. Elias, P. and I.R. Lehman, *Interaction of Origin Binding-Protein with An Origin of Replication of Herpes-Simplex Virus-1.* Proceedings of the National Academy of Sciences of the United States of America, 1988. **85**(9): p. 2959-2963.

41. Makhov, A.M., et al., *The herpes simplex virus type 1 origin-binding protein carries out origin specific DNA unwinding and forms unwound stem-loop structures.* Embo Journal, 1996. **15**(7): p. 1742-1750.

42. Lee, S.S.K., et al., *Interaction of Herpes-Simplex Virus-1 Origin-Binding Protein with Dna-Polymerase-Alpha.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(17): p. 7882-7886.

43. Hay, J., et al., *The effect of phosphonoacetic acid on herpes viruses.* J.Antimicrob.Chemother., 1977. **3 Suppl A**: p. 63-70.

44. Boehmer, P.E. and I.R. Lehman, *Herpes simplex virus DNA replication.* Annu.Rev.Biochem., 1997. **66**: p. 347-384.

45. Masse, M.J.O., et al., *Human Cytomegalovirus Origin of Dna-Replication (Orilyt) Resides Within A Highly Complex Repetitive Region.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(12): p. 5246-5250.

46. Masse, M.J.O., M. Messerle, and E.S. Mocarski, *The location and sequence composition of the murine cytomegalovirus replicator (oriLyt).* Virology, 1997. **230**(2): p. 350-360.

47. Anders, D.G. and S.M. Punturieri, *Multicomponent Origin of Cytomegalovirus Lytic-Phase Dna-Replication.* Journal of Virology, 1991. **65**(2): p. 931-937.

48. Vink, C., E. Beuken, and C.A. Bruggeman, *Cloning and functional characterization of the origin of lytic-phase DNA replication of rat cytomegalovirus.* Journal of General Virology, 1997. **78**: p. 2963-2973.

49. Borst, E.M. and M. Messerle, *Analysis of human cytomegalovirus oriLyt sequence requirements in the context of the viral genome.* J.Virol., 2005. **79**(6): p. 3615-3626.

50. Huang, L., Y. Zhu, and D.G. Anders, *The variable 3' ends of a human cytomegalovirus oriLyt transcript (SRT) overlap an essential, conserved replicator element.* J.Virol., 1996. **70**(8): p. 5272-5281.

51. Pari, G.S., *Nuts and bolts of lytic DNA replication*, in *Human Cytomegalovirus*, T. Shenk and M.F. Stinski, Editors. 2008, Springer-Verlag: Berlin-Heidelberg. p. 153-166.

52. Xu, Y.Y., et al., *Human cytomegalovirus DNA replication requires transcriptional activation via an IE2- and UL84-responsive bidirectional promoter element within oriLyt.* Journal of Virology, 2004. **78**(21): p. 11664-11677.

53. Prichard, M.N., et al., *Identification of persistent RNA-DNA hybrid structures within the origin of replication of human cytomegalovirus.* J.Virol., 1998. **72**(9): p. 6997-7004.

54. Colletti, K.S., et al., *Human cytomegalovirus UL84 interacts with an RNA stem-loop sequence found within the RNA/DNA hybrid region of oriLyt.* J.Virol., 2007. **81**(13): p. 7077-7085.

55. Xu, Y., et al., *Human cytomegalovirus UL84 insertion mutant defective for viral DNA synthesis and growth.* J.Virol., 2004. **78**(19): p. 10360-10369.

56. Spector, D.J. and K. Yetming, *UL84-independent replication of human cytomegalovirus strain TB40/E.* Virology, 2010. **407**(2): p. 171-177.

57. Messeguer, X., et al., *PROMO: detection of known transcription regulatory elements using species-tailored searches.* Bioinformatics, 2002. **18**(2): p. 333-4.

58. Cranmer, L.D., et al., *Identification, analysis, and evolutionary relationships of the putative murine cytomegalovirus homologs of the human cytomegalovirus UL82 (pp71) and UL83 (pp65) matrix phosphoproteins.* J.Virol., 1996. **70**(11): p. 7929-7939.

59. Morello, C.S., L.D. Cranmer, and D.H. Spector, *In vivo replication, latency, and immunogenicity of murine cytomegalovirus mutants with deletions in the M83 and M84 genes, the putative homologs of human cytomegalovirus pp65 (UL83).* J.Virol., 1999. **73**(9): p. 7678-7693.

60. Hammerschmidt, W. and B. Sugden, *Epstein-Barr Virus*, in *DNA replication and human Disease*, M.L. dePamphelis, Editor 2006, Inglis, J.; Cold Spring Harbor Laboratory Press. p. 687-705.

61. Deng, H., et al., *Identification of cis sequences required for lytic DNA replication and packaging of murine gammaherpesvirus 68.* J.Virol., 2004. **78**(17): p. 9123-9131.

62. Gong, D., et al., *Identification and functional characterization of the left origin of lytic replication of murine gammaherpesvirus 68.* Virology, 2009. **387**(2): p. 285-295.

63. Adler, H., et al., *Murine gammaherpesvirus 68 contains two functional lytic origins of replication.* Journal of Virology, 2007. **81**(13): p. 7300-7305.

64. Messerle, M., et al., *Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14759-14763.

65. Wagner, M., Z. Ruzsics, and U.H. Koszinowski, *Herpesvirus genetics has come of age.* Trends in Microbiology, 2002. **10**(7): p. 318-324.

66. Ruzsics, Z. and U.H. Koszinowski, *Mutagenesis of the cytomegalovirus genome*, in *Human Cytomegalovirus*, T.E. Shenk and M.F. Stinski, Editors. 2008, Springer: Berlin, Germany. p. 41-61.

67. Sprengel, R. and M.T. Hasan, *Tetracycline-controlled genetic switches.* Handb.Exp.Pharmacol., 2007(178): p. 49-72.

68. Saez, E., et al., *Inducible gene expression in mammalian cells and transgenic mice.* Curr.Opin.Biotechnol., 1997. **8**(5): p. 608-616.

69. Mohr, H., et al., *Cytomegalovirus replicon-based regulation of gene expression in vitro and in vivo.* PLoS Pathog, 2012. **8**(6): p. e1002728.

70. Mohr, C.A., *Ein verbreitungsdefizientes murines Cytomegalovirus als Modellvakzine gegen die Infektion mit β-Herpesviren.*, in *Max-von-Pettenkofer Institut*2011, LMU.

71. Mohr, C.A., et al., *A Spread-Deficient Cytomegalovirus for Assessment of First-Target Cells in Vaccination.* Journal of Virology, 2010. **84**(15): p. 7730-7742.

72. Nandi, S., et al., *Bovine herpes virus infections in cattle.* Anim Health Res.Rev., 2009. **10**(1): p. 85-98.

73. Plowright, W., et al., *Immunisation of cattle against the herpesvirus of malignant catarrhal fever: failure of inactivated culture vaccines with adjuvant.* Res.Vet.Sci., 1975. **19**(2): p. 159-166.

74. Vandeputte, J., et al., *Vaccination against pseudorabies with glycoprotein gI+ or glycoprotein gI- vaccine.* Am.J.Vet.Res., 1990. **51**(7): p. 1100-1106.

75. Tsuda, T., T. Sugimura, and Y. Murakami, *Evaluation of glycoprotein gII ISCOMs subunit vaccine for pseudorabies in pig.* Vaccine, 1991. **9**(9): p. 648-652.

76. Carter, H. and H. Campbell, *Rational use of measles, mumps and rubella (MMR) vaccine.* Drugs, 1993. **45**(5): p. 677-83.

77. Takahashi, M., et al., *Live vaccine used to prevent the spread of varicella in children in hospital.* Lancet, 1974. **2**(7892): p. 1288-90.

78. Krause, P.R. and S.E. Straus, *Herpesvirus vaccines. Development, controversies, and applications.* Infect Dis Clin North Am, 1999. **13**(1): p. 61-81, vi.

79. Burrows, R., *Herpes virus infections of animals--a brief review.* J.Antimicrob.Chemother., 1977. **3 Suppl A**: p. 9-14.

80. Wittmann, G. and I. Leitzke, *[Modification of the Aujeszky's virus neutralization test attributable different test conditions].* Dtsch.Tierarztl.Wochenschr., 1985. **92**(7-8): p. 262-266.

81. Kit, S., H. Otsuka, and M. Kit, *Gene-deleted IBRV marker vaccine.* Vet.Rec., 1990. **127**(14): p. 363-364.

82. Kit, S., *Genetically engineered vaccines for control of Aujeszky's disease (pseudorabies).* Vaccine, 1990. **8**(5): p. 420-424.

83. Burki, F., et al., *Viraemia and abortions are not prevented by two commercial equine herpesvirus-1 vaccines after experimental challenge of horses.* Vet.Q., 1990. **12**(2): p. 80-86.

84. Van Oirschot, J.T., et al., *Marker vaccines, virus protein-specific antibody assays and the control of Aujeszky's disease.* Vet.Microbiol., 1990. **23**(1-4): p. 85-101.

85. Schat, K.A. and E. Baranowski, *Animal vaccination and the evolution of viral pathogens.* Rev.Sci.Tech., 2007. **26**(2): p. 327-338.

86. Gimeno, I.M., *Marek's disease vaccines: a solution for today but a worry for tomorrow?* Vaccine, 2008. **26 Suppl 3**: p. C31-C41.

87. Ackermann, M. and M. Engels, *Pro and contra IBR-eradication.* Vet.Microbiol., 2006. **113**(3-4): p. 293-302.

88. Muller, T., et al., *Pseudorabies virus in wild swine: a global perspective.* Arch.Virol., 2011.

89. van Drunen Littel-van den, H., *Rationale and perspectives on the success of vaccination against bovine herpesvirus-1.* Vet.Microbiol., 2006. **113**(3-4): p. 275-282.

90. Baltimore, D., *Intracellular Immunization.* Nature, 1988. **335**(6189): p. 395-396.

91. Muhlbach, H., et al., *Dominant-Negative Proteins in Herpesviruses - From Assigning Gene Function to Intracellular Immunization* viruses, 2009. **1** (3): p. 420 -440.

92. Friedman, A.D., S.J. Triezenberg, and S.L. McKnight, *Expression of A Truncated Viral Trans-Activator Selectively Impedes Lytic Infection by Its Cognate Virus.* Nature, 1988. **335**(6189): p. 452-454.

93. Smith, C.A. and N.A. Deluca, *Transdominant Inhibition of Herpes-Simplex Virus Growth in Transgenic Mice.* Virology, 1992. **191**(2): p. 581-588.

94. Shepard, A.A., P. Tolentino, and N.A. Deluca, *trans-dominant inhibition of herpes simplex virus transcriptional regulatory protein ICP4 by heterodimer formation.* J.Virol., 1990. **64**(8): p. 3916-3926.

95. Smith, C.A., et al., *ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB.* J.Virol., 1993. **67**(8): p. 4676-4687.

96. Ono, E., et al., *Inhibition of Pseudorabies Virus-Replication by A Chimeric Trans-Gene Product Repressing Transcription of the Immediate-Early Gene.* Virology, 1995. **210**(1): p. 128-140.

97. Ono, E., et al., *Suppression of pseudorabies virus replication by a mutant form of immediate-early protein IE180 repressing the viral gene transcription.* Vet Microbiol, 1998. **60**(2-4): p. 107-117.

98. Ono, E., et al., *Resistance to pseudorabies virus infection in transgenic mice expressing the chimeric transgene that represses the immediate-early gene transcription.* Virology, 1999. **262**(1): p. 72-78.

99. Bubeck, A., et al., *Comprehensive mutational analysis of a herpesvirus gene in the viral genome context reveals a region essential for virus replication.* J Virol, 2004. **78**(15): p. 8026-8035.

100. Maninger, S., et al., *M94 is essential for the secondary envelopment of murine cytomegalovirus.* J.Virol., 2011. **85**(18): p. 9254-9267.

101. Popa, M., et al., *Dominant negative mutants of the murine cytomegalovirus M53 gene block nuclear egress and inhibit capsid maturation.* J.Virol., 2010. **84**(18): p. 9035-9046.

102. Borst, E.M., et al., *Genetic evidence of an essential role for cytomegalovirus small capsid protein in viral growth.* Journal of Virology, 2001. **75**(3): p. 1450-1458.

103. Rupp, B., et al., *Conditional cytomegalovirus replication in vitro and in vivo.* Journal of Virology, 2005. **79**(1): p. 486-494.

104. Piechaczek, C., et al., *A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells.* Nucleic Acids Research, 1999. **27**(2): p. 426-428.

105. Heng, H.H., et al., *Chromatin loops are selectively anchored using scaffold/matrix-attachment regions.* J.Cell Sci., 2004. **117**(Pt 7): p. 999-1008.

106. Giannakopoulos, A., et al., *The functional role of S/MARs in episomal vectors as defined by the stress-induced destabilization profile of the vector sequences.* J.Mol.Biol., 2009. **387**(5): p. 1239-1249.

107. Mielke, C., et al., *Hierarchical binding of DNA fragments derived from scaffold-attached regions: correlation of properties in vitro and function in vivo.* Biochemistry, 1990. **29**(32): p. 7475-7485.

108. Jenke, A.C., et al., *The nonviral episomal replicating vector pEPI-1 allows long-term inhibition of bcr-abl expression by shRNA.* Hum.Gene Ther., 2005. **16**(4): p. 533-539.

109. Jenke, A.C., et al., *Expression of a transgene encoded on a non-viral episomal vector is not subject to epigenetic silencing by cytosine methylation.* Mol.Biol.Rep., 2004. **31**(2): p. 85-90.

110. Jenke, A.C., et al., *Nuclear scaffold/matrix attached region modules linked to a transcription unit are sufficient for replication and maintenance of a mammalian episome.* Proc Natl Acad Sci U S A, 2004. **101**(31): p. 11322-11327.

111. Baiker, A., et al., *Mitotic stability of an episomal vector containing a human scaffold/matrix-attached region is provided by association with nuclear matrix.* Nature Cell Biology, 2000. **2**(3): p. 182-184.

112. Jenke, A.C.W., et al., *Nuclear scaffold/matrix attached region modules linked to a transcription unit are sufficient for replication and maintenance of a mammalian episome.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(31): p. 11322-11327.

113. Argyros, O., et al., *Persistent episomal transgene expression in liver following delivery of a scaffold/matrix attachment region containing non-viral vector.* Gene Therapy, 2008. **15**(24): p. 1593-1605.

114. Jenke, B.H.C., et al., *An episomally replicating vector binds to the nuclear matrix protein SAF-A in vivo.* Embo Reports, 2002. **3**(4): p. 349-354.

115. Kipp, M., et al., *SAF-Box, a conserved protein domain that specifically recognizes scaffold attachment region DNA.* Mol.Cell Biol., 2000. **20**(20): p. 7480-7489.

116. Schaarschmidt, D., et al., *An episomal mammalian replicon: sequence-independent binding of the origin recognition complex.* Embo Journal, 2004. **23**(1): p. 191-201.

117. Jenke, A.C.W., et al., *The nonviral episomal replicating vector pEPI-1 allows long-term inhibition of bcr-abl expression by shRNA.* Human Gene Therapy, 2005. **16**(4): p. 533-539.

118. Papapetrou, E.P., et al., *Gene transfer into human hematopoietic progenitor cells with an episomal vector carrying an S/MAR element.* Gene Therapy, 2006. **13**(1): p. 40-51.

119. Nehlsen, K., S. Broll, and J. Bode, *Replicating minicircles: Generation of nonviral episomes for the efficient modification of dividing cells - Research article.* Gene Therapy and Molecular Biology, 2006. **10B**: p. 233-243.

120. Tessadori, F., et al., *Stable S/MAR-based episomal vectors are regulated at the chromatin level.* Chromosome.Res., 2010. **18**(7): p. 757-775.

121. Manzini, S., et al., *Genetically modified pigs produced with a nonviral episomal vector.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(47): p. 17672-17677.

122. Plendl, J., F. Sinowatz, and R. Auerbach, *A Transformed Murine Myocardial Vascular Endothelial-Cell Clone - Characterization of Cells In-Vitro and of Tumors Derived from the Clone In-Situ.* Virchows Archiv, 1995. **426**(6): p. 619-628.

123. O'Connell, K.A. and M. Edidin, *A mouse lymphoid endothelial cell line immortalized by simian virus 40 binds lymphocytes and retains functional characteristics of normal endothelial cells.* J Immunol, 1990. **144**(2): p. 521-525.

124. Kuhn, R., K. Rajewsky, and W. Muller, *Generation and Analysis of Interleukin-4 Deficient Mice.* Science, 1991. **254**(5032): p. 707-710.

125. Jordan, S., et al., *Virus progeny of murine cytomegalovirus bacterial artificial chromosome pSM3fr show reduced growth in salivary Glands due to a fixed mutation of MCK-2.* J Virol, 2011. **85**(19): p. 10346-53.

126. Sacher, T., et al., *Conditional gene expression systems to study herpesvirus biology in vivo.* Med.Microbiol.Immunol., 2008. **197**(2): p. 269-276.

127. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(12): p. 6640-6645.

128. Hanson, L.K., et al., *Characterization and regulation of essential murine cytomegalovirus genes m142 and m143.* Virology, 2005. **334**(2): p. 166-177.

129. Wagner, M. and U.H. Koszinowski, *Mutagenesis of viral BACs with linear PCR fragments (ET recombination).* Methods Mol Biol, 2004. **256**: p. 257-268.

130. Southern, E.M., *Detection of Specific Sequences Among Dna Fragments Separated by Gel-Electrophoresis.* Journal of Molecular Biology, 1975. **98**(3): p. 503-&.

131. Sambrook, J. and D.W. Russel, *Molecular Clonig.* Vol. Third Edition. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

132. Matsukage, S., et al., *Mouse embryonic stem cells are not susceptible to cytomegalovirus but acquire susceptibility during differentiation.* Birth Defects Res A Clin Mol Teratol., 2006. **76**(2): p. 115-125.

133. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.

134. Laemmli, U.K., *Cleavage of Structural Proteins During Assembly of Head of Bacteriophage-T4.* Nature, 1970. **227**(5259): p. 680-&.

135. Fling, S.P. and D.S. Gregerson, *Peptide and Protein Molecular-Weight Determination by Electrophoresis Using A High-Molarity Tris Buffer System Without Urea.* Analytical Biochemistry, 1986. **155**(1): p. 83-88.

136. Piechaczek, C., et al., *A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells.* Nucleic Acids Res, 1999. **27**(2): p. 426-428.

137. Spaete, R.R. and E.S. Mocarski, *Regulation of cytomegalovirus gene expression: alpha and beta promoters are trans activated by viral functions in permissive human fibroblasts.* J Virol, 1985. **56**(1): p. 135-143.

138. Lotzerich, M., Z. Ruzsics, and U.H. Koszinowski, *Functional domains of murine cytomegalovirus nuclear egress protein M53/p38.* J Virol, 2006. **80**(1): p. 73-84.

139. Anders, D.G., et al., *Boundaries and Structure of Human Cytomegalovirus Orilyt, A Complex Origin for Lytic-Phase Dna-Replication.* Journal of Virology, 1992. **66**(6): p. 3373-3384.

140. Smith, L.M., et al., *Laboratory strains of murine cytomegalovirus are genetically similar to but phenotypically distinct from wild strains of virus.* Journal of Virology, 2008. **82**(13): p. 6689-6696.

141. Yoshida, M., et al., *Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A.* J Biol Chem, 1990. **265**(28): p. 17174-17179.

142. Christman, J.K., *5-Azacytidine and 5-aza-2 '-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy.* Oncogene, 2002. **21**(35): p. 5483-5495.

143. Gu, H.D. and B. Roizman, *Herpes simplex virus-infected cell protein 0 blocks the silencing of viral DNA by dissociating histone deacetylases from the CoREST-REST complex.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(43): p. 17134-17139.

144. Nevels, M., C. Paulus, and T. Shenk, *Human cytomegalovirus immediate-early 1 protein facilitates viral replication by antagonizing histone deacetylation.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(49): p. 17234-17239.

145. Tang, Q.Y. and G.G. Maul, *Mouse cytomegalovirus immediate-early protein 1 binds with host cell repressors to relieve suppressive effects on viral transcription and replication during lytic infection.* Journal of Virology, 2003. **77**(2): p. 1357-1367.

146. Frydman, J., et al., *Co-translational domain folding as the structural basis for the rapid de novo folding of firefly luciferase.* Nature Structural Biology, 1999. **6**(7): p. 697-705.

147. Thompson, J.F., L.S. Hayes, and D.B. Lloyd, *Modulation of Firefly Luciferase Stability and Impact on Studies of Gene-Regulation.* Gene, 1991. **103**(2): p. 171-177.

148. Neuwirth, A., *Regulation des Zellzyklus durch das Maus- und Ratten-Zytomegalievirus*, 2005.

149. Misra, V., M.T. Muller, and J.B. Hudson, *The enumeration of viral genomes in murine cytomegalovirus-infected cells.* Virology, 1977. **83**(2): p. 458-461.

150. Muranyi, W., et al., *Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina.* Science, 2002. **297**(5582): p. 854-857.

151. Manzini, S., et al., *Genetically modified pigs produced with a nonviral episomal vector.* Proc Natl Acad Sci U S A, 2006. **103**(47): p. 17672-17677.

152. Conese, M., C. Auriche, and F. Ascenzioni, *Gene therapy progress and prospects: episomally maintained self-replicating systems.* Gene Ther., 2004. **11**(24): p. 1735-1741.

153. Magnusson, T., et al., *Sustained, high transgene expression in liver with plasmid vectors using optimized promoter-enhancer combinations.* J Gene Med, 2011. **13**(7-8): p. 382-91.

154. Avramova, Z. and E. Paneva, *Matrix attachment sites in the murine alpha-globin gene.* Biochem.Biophys.Res.Commun., 1992. **182**(1): p. 78-85.

155. Goetze, S., et al., *Performance of genomic bordering elements at predefined genomic loci.* Mol.Cell Biol., 2005. **25**(6): p. 2260-2272.

156. Challberg, M.D., *A Method for Identifying the Viral Genes Required for Herpesvirus Dna-Replication.* Proceedings of the National Academy of Sciences of the United States of America, 1986. **83**(23): p. 9094-9098.

157. Haase, S.B., S.S. Heinzel, and M.P. Calos, *Transcription inhibits the replication of autonomously replicating plasmids in human cells.* Mol.Cell Biol., 1994. **14**(4): p. 2516-2524.

158. Villarreal, L.P., *Relationship of eukaryotic DNA replication to committed gene expression: general theory for gene control.* Microbiol.Rev., 1991. **55**(3): p. 512-542.

159. Leinbach, S.S., et al., *Mechanism of phosphonoacetate inhibition of herpesvirus-induced DNA polymerase.* Biochemistry, 1976. **15**(2): p. 426-430.

160. Wong, E., et al., *Murine gammaherpesvirus 68 open reading frame 24 is required for late gene expression after DNA replication.* J.Virol., 2007. **81**(12): p. 6761-6764.

161. Wu, T.T., et al., *ORF30 and ORF34 are essential for expression of late genes in murine gammaherpesvirus 68*. J.Virol., 2009. **83**(5): p. 2265-2273.

162. Perng, Y.C., et al., *The human cytomegalovirus gene UL79 is required for the accumulation of late viral transcripts.* J.Virol., 2011. **85**(10): p. 4841-4852.

163. Jeon, Y., et al., *Temporal profile of replication of human chromosomes.* Proc.Natl.Acad.Sci.U.S.A, 2005. **102**(18): p. 6419-6424.

164. Norio, P., *DNA replication: the unbearable lightness of origins.* EMBO Rep., 2006. **7**(8): p. 779-781.

165. Mechali, M., *Eukaryotic DNA replication origins: many choices for appropriate answers.* Nat.Rev.Mol.Cell Biol., 2010. **11**(10): p. 728-738.

166. Wolffe, A.P., *Implications of DNA replication for eukaryotic gene expression.* J.Cell Sci., 1991. **99 (Pt 2)**: p. 201-206.

167. Groves, I.J., M.B. Reeves, and J.H. Sinclair, *Lytic infection of permissive cells with human cytomegalovirus is regulated by an intrinsic 'pre-immediate-early' repression of viral gene expression mediated by histone post-translational modification.* J Gen Virol, 2009. **90**(Pt 10): p. 2364-74.

168. Groth, A., et al., *Chromatin challenges during DNA replication and repair.* Cell, 2007. **128**(4): p. 721-33.

169. Omberg, L., et al., *Global effects of DNA replication and DNA replication origin activity on eukaryotic gene expression.* Molecular Systems Biology, 2009. **5**.

170. *Verordnung zum Schutz gegen die Aujeszky'sche Krankheit BGBI.I S.2720 §3 (1)*, 2005.

171. Desai, P. and S. Person, *Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid.* J.Virol., 1998. **72**(9): p. 7563-7568.

172. Klupp, B.G., et al., *Complete, annotated sequence of the pseudorabies virus genome.* J Virol, 2004. **78**(1): p. 424-40.

173. Sinclair, J., *Human cytomegalovirus: Latency and reactivation in the myeloid lineage.* J.Clin.Virol., 2008. **41**(3): p. 180-185.

174. Aucoin, D.P., et al., *Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) contains two functional lytic origins of DNA replication.* Journal of Virology, 2002. **76**(15): p. 7890-7896.

175. Cui, L.L., et al., *Kaposi's sarcoma-associated herpesvirus lytic origin (oti-Lyt)-dependent DNA replication: Identification of the oti-Lyt and association of K8 bZip protein with the origin.* Journal of Virology, 2003. **77**(10): p. 5578-5588.

176. Dewhurst, S., et al., *Identification of A Lytic-Phase Origin of Dna-Replication in Human Herpesvirus-6B Strain-Z29.* Journal of Virology, 1993. **67**(12): p. 7680-7683.

177. vanLoon, N., et al., *Identification and analysis of a lytic-phase origin of DNA replication in human herpesvirus 7.* Journal of Virology, 1997. **71**(4): p. 3279-3284.

178. Prichard, M.N., et al., *Identification of persistent RNA-DNA hybrid structures within the origin of replication of human cytomegalovirus.* J Virol, 1998. **72**(9): p. 6997-7004.

179. Dolken, L., et al., *Mouse cytomegalovirus MicroRNAs dominate the cellular small RNA profile during lytic infection and show features of Posttranscriptional regulation.* Journal of Virology, 2007. **81**(24): p. 13771-13782.

180. Boehmer, P.E. and A.V. Nimonkar, *Herpes virus replication.* IUBMB.Life, 2003. **55**(1): p. 13-22.

181. Rennekamp, A.J., P. Wang, and P.M. Lieberman, *Evidence for DNA hairpin recognition by Zta at the EBV origin of lytic replication.* J Virol, 2010.

182. Feederle, R., E.J. Bartlett, and H.J. Delecluse, *Epstein-Barr virus genetics: talking about the BAC generation.* Herpesviridae., 2010. **1**(1): p. 6.

183. Mavromara-Nazos, P. and B. Roizman, *Activation of herpes simplex virus 1 gamma 2 genes by viral DNA replication.* Virology, 1987. **161**(2): p. 593-598.

184. Dennis, D. and J.R. Smiley, *Transactivation of A Late Herpes-Simplex Virus Promoter.* Molecular and Cellular Biology, 1984. **4**(3): p. 544-551.

185. Johnson, P.A. and R.D. Everett, *Dna-Replication Is Required for Abundant Expression of A Plasmid-Borne Late Us11 Gene of Herpes-Simplex Virus Type-1.* Nucleic Acids Research, 1986. **14**(9): p. 3609-3625.

186. Johnson, P.A. and R.D. Everett, *The Control of Herpes-Simplex Virus Type-1 Late Gene-Transcription - A 'Tata-Box'/Cap Site Region Is Sufficient for Fully Efficient Regulated Activity.* Nucleic Acids Research, 1986. **14**(21): p. 8247-8264.

187. Serio, T.R., J.L. Kolman, and G. Miller, *Late gene expression from the Epstein-Barr virus BcLF1 and BFRF3 promoters does not require DNA replication in cis.* J.Virol., 1997. **71**(11): p. 8726-8734.

188. Dudek, T. and D.M. Knipe, *Replication-defective viruses as vaccines and vaccine vectors.* Virology, 2006. **344**(1): p. 230-239.

189. Klerk, C.P., et al., *Validity of bioluminescence measurements for noninvasive in vivo imaging of tumor load in small animals.* Biotechniques, 2007. **43**(1 Suppl): p. 7-13, 30.

190. Jackson, S.A. and N.A. Deluca, *Relationship of herpes simplex virus genome configuration to productive and persistent infections.* Proc.Natl.Acad.Sci.U.S.A, 2003. **100**(13): p. 7871-7876.

191. Mocarski, E.S. and B. Roizman, *Herpes-Virus-Dependent Amplification and Inversion of Cell-Associated Viral Thymidine Kinase Gene Flanked by Viral-A Sequences and Linked to An Origin of Viral-Dna Replication.* Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 1982. **79**(18): p. 5626-5630.

192. Mosca, J.D., et al., *Differential activation of hybrid genes containing herpes simplex virus immediate-early or delayed-early promoters after superinfection of stable DNA-transfected cell lines.* J.Virol., 1985. **56**(3): p. 867-878.

193. Ecob-Prince, M. and K. Hassan, *Reactivation of latent herpes simplex virus from explanted dorsal root ganglia.* J.Gen.Virol., 1994. **75 (Pt 8)**: p. 2017-2028.

194. Jordan, M.C. and V.L. Mar, *Spontaneous activation of latent cytomegalovirus from murine spleen explants. Role of lymphocytes and macrophages in release and replication of virus.* J.Clin.Invest, 1982. **70**(4): p. 762-768.

7 APPENDIX

7.1 Abbreviations

BAC	Bacterial artificial chromosome
BHV-1	Bovine herpesvirus -1
Bsr	Blasticidin S resistance gene
BS	Blasticidin S
BSA	Bovine serum albumin
CAM	Chloramphenicol
DEAC	Diethylamincoumarin
DN	Dominant negative
DNA	Deoxyribonucleic acid
DR	Direct repeats
dNTP	Desoxynucleotide triphosphate
EBV	Epstein-Barr virus
FCS	Fetal calf serum
FL	Firefly luciferase
gDNA	Genomic DNA
H	Hour
HCMV	Human cytomegalovirus
h p.i.	Hours post infection
i.p.	Intraperitoneal
i.v.	Intravenous
IR	Inverted repeats
KAN	Kanamycin
KSHV	Kaposi's Sarcoma virus
LB	Luria broth
Lbr	Lamin B receptor gene
LIF	Leukemia inhibitory factor
Luc	Firefly luciferase gene
MCMV	Murine cytomegalovirus
MDV	Marek's disease virus

MHV68	Murine herpesvirus 68
min	Minutes
MOI	Multiplicity of infection
NEAA	Non-essential amino acids
NTP	Nucleoside triphosphates
OBP	Origin binding protein
o.n.	O.n.
Ori	Origin of replication
oriLyt	Origin of lytic replication
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRV	Pseudorabies virus
qPCR	Quantitative PCR
RFLP	Restriction fragment length polymorphism
RT	Room temperature
SB	Sleeping Beauty
TGN	Trans-Golgi network
URR	Upstream regulatory region
Wt	Wild type

7.2 List of Figures

7.3 List of Tables

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