Conditional gene expression systems to study herpesvirus biology in vivo

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Received: 31 January 2008 / Published online: 7 March 2008
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Abstract Cytomegalovirus (CMV), a prototypic β -herpesvirus, is an important human pathogen causing protean clinical manifestations in immature and immunocompromised patients. Mechanisms of infection can be studied in a mouse model. Mouse cytomegalovirus (MCMV) resembles in pathogenesis its human counterpart in many ways. Although MCMV infection is studied extensively on the level of organs, the contribution of specific cell types to viral replication in vivo is still elusive. Here we describe our approach based on the Cre/loxP-system to investigate MCMV infection at the level of cell types in vivo. Using bacterial artificial chromosome (BAC)-technology, we created an MCMV virus containing an *enhanced green fluorescent protein (egfp)* reporter-gene which is not expressed due to a ‘Stop’ cassette flanked by two loxP-sites between promoter and coding sequence. Infection of cre-transgenic mice with this reporter virus results in the deletion of the ‘Stop’ cassette and expression of EGFP in a cell type-specific manner. Using this conditional gene expression system we are able to quantify viral productivity in specific cell types and to determine their contribution to viral dissemination in vivo. Furthermore, the deletion of viral genes can be used to screen for cell type-specificity of viral gene functions. Hence, conditional MCMV mutants allow the study of herpesvirus biology on the level of cell types in vivo.

Keywords Cytomegalovirus · Conditional mutagenesis · Cre-recombinase · Transgenic mice · Cell type-specificity · Endothelial cells · Hepatocytes

Introduction

Herpesvirus infections are widespread in the human population. Cytomegalovirus (CMV), a prototypic β -herpesvirus, is an important human pathogen. Generally, individuals with an immature or compromised immune system, for instance fetuses, premature newborn infants, AIDS patients and transplant recipients frequently develop serious disease [1, 2]. The clinical manifestations of CMV-disease are diverse, since in absence of immune control virus replication occurs in various tissues.

One common characteristic of the β -herpesviridae is their pronounced species specificity [1]. Mouse cytomegalovirus (MCMV) has an extensive sequence homology with human CMV (HCMV) [3] and provides a model to study the biology of CMV infection in the living host [4, 5]. Apart from representing a reliable animal model, MCMV resembles its human counterpart with respect to organ- and cell tropism, pathogenesis during acute infection, establishment of latency, and reactivation after immunosuppression [4, 6, 7]. Thus, MCMV is the model system of choice to get insights into the mechanisms of HCMV pathogenesis. In addition, a wide range of genetically engineered mice offers the opportunity to study the molecular mechanisms of CMV-infection.

Both HCMV and MCMV encompass a sequence of 230 kb, with 165 predicted genes in the case of HCMV [8, 9], while the MCMV genome harbours about 170 ORFs [3]. Although the large genome size complicates the analysis, in recent years new genetic tools simplified mutagenesis.

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sis in the CMV genome to identify and study gene functions. Particularly the cloning of CMV as infectious bacterial artificial chromosomes (BAC) in *E. coli* has made the viral genome accessible for forward and reverse genetic approaches [10–12]. Large deletions in the MCMV genome proved 101 ORFs not to be essential for replication [12]. These genes, however, are probably involved in immune modulation and the establishment of infection in different cell types and thus are relevant virulence factors in vivo. For instance, genes M36 and M45 have been found to be important in the infection of macrophages and endothelial cells in vitro [13, 14]. Yet the analysis of cell types in vitro, even if they were available, can only distantly reflect in vivo conditions. Therefore, it is mandatory to investigate in vivo how MCMV genes regulate virus growth in different cells. To target and to investigate cell type-specificity of viral gene products in vivo, new approaches are required. Here we present our approach based on the Cre/loxP-system.

Conditional gene expression to study gene activation and silencing in vivo

The Cre/loxP-system was the first established recombination system and is currently the most frequently used one [15, 16]. Cre is the 38 kDa product of the *cre* (cyclisation recombination) gene of bacteriophage P1. The Cre enzyme recognizes a 34 bp sequence called *loxP* (locus of X-over P1) and efficiently catalyzes DNA recombination between pairs of directly repeated *loxP* sites, resulting in the excision of the DNA in-between as a covalently closed circle [17, 18].

About 15 years ago the first *cre*-transgenic mouse was generated [19]. Since then Cre-mediated recombination of genomic DNA has been proved to be a powerful tool in the study of gene functions in vivo [20]. Both the activation and silencing of gene expression can be regulated in a temporally and spatially controlled manner [15]. To silence genes of interest, an essential region of the gene is flanked by *loxP* sites (i.e. a so-called floxed allele). Mating mice that carry a floxed allele with mice expressing Cre under the control of cell type specific promoters will result in progeny in which the floxed allele is deleted in selected tissues (Fig. 1a). These conditional knockout mice made it possible to circumvent early lethal phenotypes. In contrast, tissue-specific gene activation can be achieved if a floxed transcriptional ‘Stop’ sequence (i.e. stop codons and a polyadenylation sequence) is placed between the promoter and the coding sequence keeping the gene dormant. Cell type-specific Cre expression leads to the cleavage of the ‘Stop’ sequence and activation of gene transcription [20, 21].

Fig. 1 Building blocks of the Cre/loxP-system in virology. **a** The ‘standard approach’ for tissue-specific silencing or activation of mouse genes comprises the mating of *cre*-transgenic mice with mice harbouring floxed sequences in their genome [15, 16]. **b** Using engineered viruses, Cre-expressing viruses mediate recombination of floxed sequences in the genome of infected cells [22, 23]. **c** In co-infection experiments, mice are infected with both a virus containing the *cre*-gene and a second virus harbouring floxed sequences [24, 25]. **d** To regulate expression of virally delivered genes in a cell type-specific manner, *cre*-transgenic mice are infected with viruses containing floxed sequences [25, 26]. *cre* = *cre*-gene, $\rightarrow\rightarrow$ = floxed sequences

Usage of the Cre/loxP-system in virology

Both the recombination of floxed cellular genes [22, 23] or viral genes [24, 25] after infection with a virus that carried the *cre* gene has been shown. First, an engineered adenovirus delivered Cre in vivo (Fig. 1b) [22]. After infection of mice harbouring floxed sequences in their genome, spleen and liver, the organs with the highest infection load, also showed the highest levels of recombination. Basing on this principle, Moser and colleagues used conditional knockout mice with a floxed *DNA methyltransferase 1* (Dnmt 1) gene, an essential factor for the survival of proliferating cells, expressed Cre from a recombinant γ MHV68 virus [23] and were able to show that proliferation of B-cells is necessary for the establishment of chronic γ MHV68 infection. A Cre expressing adenovirus was also used in co-infection experiments with a second adenovirus mutant containing a floxed ‘Stop’ cassette followed by the sequence of the murine Fas ligand leading to its ectopic expression in the liver after recombination (Fig. 1c) [24]. More recently, in vivo co-infection of individual cells by different CMV variants could be demonstrated by using a

Cre expressing virus that activates a reporter gene present in the genome of a second virus mutant [25].

Apart from the opportunity to express Cre from a viral vector, however, it is possible to benefit from the steadily growing range of mouse mutants that express the recombinase in different host tissues. Infecting these mouse lines with conditional virus mutants should result in tissue-specific activation or silencing of virally transduced genes (Fig. 1d). Adenovirus containing a floxed reporter gene provided the proof of concept. Mice expressing Cre under the control of a mammary gland-specific promoter accumulated the reporter in the mammary gland [26]. In an elegant approach, deFalco et al. [27] were able to identify neuronal networks by utilizing mice that express Cre specifically in neurons and a pseudorabies virus that was dependent on Cre-mediated recombination for replication and expression of a reporter. After infection, Cre expressing neurons were labelled. Furthermore, since pseudorabies virus is able to cross multiple synapses, neurons connected to the originally infected cells could be identified. This retrograde tracing of conditional pseudorabies virus from specific neurons offers the opportunity to uncover polysynaptic circuits.

Taken together, the combination of animals and viruses as building blocks of the Cre/loxP-system offers a constructive and conceptual approach to answer specific questions on virus and cell biology.

The application of conditional MCMV reporter viruses to cytomegalovirus biology

MCMV replication occurs in many different tissues in the infected host. It is unclear, which cell types dominate virus production during acute infection and how these cells are controlled by innate and adaptive immunity. To study the role of cell types during viral infections we used the Cre/loxP-system to identify and to quantify viral productivity of selected cell types in vivo (Sacher T, accepted for publication).

Using BAC-technology, we generated a recombinant MCMV reporter virus conditionally expressing the enhanced green fluorescent protein (EGFP) as a reporter under the control of the HCMV major immediate-early promoter (Fig. 2). A floxed 'Stop' cassette between promoter and transgene prevents the expression of the reporter. When the reporter virus infects a cell type that expresses Cre, the viral genome is recombined, the floxed 'Stop' cassette is cleaved and the reporter gene is expressed. Thus, infection of a Cre-expressing cell results in a green fluorescent signal, which can be monitored, whereas after infection of Cre-negative cells no recombination occurs and the reporter stays silent.

Since the recombined locus is stably maintained in the viral progeny, it is possible to track virus transmission to

Fig. 2 Cre-mediated recombination of viral genomes. The reporter virus contains a floxed 'Stop' cassette between the *egfp* coding sequence and the HCMV major immediate early-promoter. After infection of a Cre expressing cell type, the 'Stop' sequence is excised and the recombined reporter locus is maintained in the viral progeny. In other cell types no recombination of the viral genome occurs and produced virus is not genetically labelled

cells that do not express Cre. Dissemination between different cell types within as well as between organs and the colonization of distant targets can thus be studied.

The amount of infectious progeny produced by a specific cell type can be quantified in a standard plaque assay (Fig. 3). While the total number of plaques allows the calculation of virus load of the organ, the amount of green fluorescent plaques gives an estimate of the contribution of the Cre expressing cells to the overall viral productivity. Depending on the aim of the study, it is important to choose the proper time point post infection before assaying the organs. Only after the first viral replication cycle the number of EGFP positive plaques truly reveals the productivity of the Cre expressing cell type. At later time points, virus with a recombined reporter locus allows the tracking of viral progeny between different cell types and dissemination between organs.

The conceptual basis of the experimental approach is simple. However, several points have to be taken into account for a meaningful application and interpretation of conditional reporter viruses.

Marker virus properties

In principle, the experimental approach is suitable to study not only the biology of MCMV, but of any DNA-virus equipped with a reporter sequence that infects mice and replicates in the nucleus (since in most *cre*-transgenic mouse strains the recombinase is targeted to the cell

Fig. 3 Experimental setup to address viral productivity and dissemination of specific cell types in vivo. 1. Transgenic mice expressing the Cre recombinase under the control of a cell type-specific promoter are infected with the conditional reporter virus. 2. After collecting organs of interest, the amount of EGFP⁺ and EGFP⁺ plaque forming units

(PFU) can be quantified in a standard plaque assay on permissive cells using fluorescent microscopy. 3. Total virus load per organ and mean percentage of EGFP⁺ plaques per experimental group are displayed as red dots and green columns, respectively. Red dashed line detection limit

nucleus), as for instance adenoviruses and herpesviruses (e.g. MHV68 and pseudorabies virus). Clearly, DNA-viruses that replicate in the cytoplasm, for example mouse pox virus (ectromelia virus), would require transgenic mice with the Cre enzyme present in the cytosol.

Additional requirements have to be fulfilled for a meaningful application of the Cre/loxP-system in virology. First, it is essential that the in vivo replication properties of the conditional reporter virus are independent of whether or not the reporter gene locus is recombined. Second, the expression of Cre in the transgenic mouse strain used should be restricted to the cell type under investigation. And third, a high—or at least a stable ratio—of the genomes produced by a Cre expressing cell should be recombined.

The viral genome cannot exceed a certain length. Therefore, the reporter construct has to replace a viral sequence resulting in a deletion mutant. We chose to insert the reporter into gene *m157* yielding the virus MCMV- $\Delta m157$ -flox-*egfp*. The gene product of *m157* is known to be a ligand for the activating NK cell receptor Ly49H [28–30]. Mouse strains expressing Ly49H (e.g. C57BL/6) show a higher resistance to MCMV infection than Ly49H negative mice (e.g. BALB/c). The targeted deletion of *m157* avoids the innate immune control mediated by Ly49H positive NK cells in C57BL/6 mice. The gene function of *m157* is also lost in vivo after passage through Ly49H positive mice and is absent in MCMV strains isolated from wildtype mice. Furthermore, Ly49H is uncommon in laboratory and wildtype mice [31].

Mutant viruses harbouring foreign genes run the risk of not reflecting the physiological reality. Already the mere length of a reporter gene introduced into the viral genome may impair virus fitness and result in attenuation. Also, it is conceivable that the expression of the marker might result in lower titers of the virus with a recombined locus than

with an unrecombined genome, for instance due to immune recognition of the marker. In this case, the virus titer of the recombined derivative MCMV- $\Delta m157$ -rec-*egfp* would not reflect the proportion of virus produced by a certain cell type in comparison to the parental virus MCMV- $\Delta m157$ -flox-*egfp*. We infected immunocompetent C57BL/6 mice with MCMV- $\Delta m157$ -flox-*egfp* and MCMV- $\Delta m157$ -rec-*egfp* and found that both viruses replicated within the first 10 days to comparable titers in different organs. It is not excluded, however, that there exist minor differences as indicated by slightly differing lung titers. Yet, such differences may become important variables in studies over a period of many days. As expected, in the absence of Cre no activation of the reporter gene could be detected. Once Cre-mediated recombination had occurred, the reporter gene was expressed absolutely reliably, as was shown by 100% EGFP positive plaques in C57BL/6 mice infected with MCMV- $\Delta m157$ -rec-*egfp*. Thus, the conditional MCMV reporter viruses proved to be suitable tools to study virus production and dissemination in vivo.

Different conditions for Cre-mediated host and viral genome recombination

Originally, *cre*-transgenic mice were developed to control conditional gene expression in murine tissues. For in vivo gene silencing only one or two floxed alleles per cell have to be cleaved and there is ample time during organogenesis to initiate and accomplish the recombination event. During virus replication, however, the presence of the parental viral genome in the nucleus is a matter of about 12 h; therefore recombination has to occur early and fast. In this situation the Cre enzyme has to be present in sufficient amounts in the nucleus prior to infection. As we find marker expression

already 8 hours after infection, we presume that the recombination of the reporter virus genome takes place before virus DNA replication starts. In addition, only few infected cells are found in most organs by histological examinations. Therefore, we think that each primary target cell is infected probably by only one virus. The recombination occurs only on one single virus genome per infected cell.

There are possibilities to control that the results obtained are independent of Cre expression levels. First, for some cell types different Cre expressing mouse strains are available. For instance, in both *Tie2-cre* and *Tek-cre* mice the same promoter/enhancer combination was used to drive Cre expression specifically in vascular endothelial cells [32, 33]. Since these strains should only differ in the copy number and the insertion site of the *cre* gene, comparative analysis of these independent strains might provide information whether different Cre expression levels have an influence on the experimental outcome. Second, infection of mice either hemi- or homozygous for the *cre* transgene with the reporter viruses reveals whether Cre expression operates at or beyond the critical level for efficient recombination. Third, for some cell types more than one specific promoter has been used to generate *cre*-transgenic mice. For hepatocyte-specific Cre expression, for example, *Alb-cre* as well as *Alfp-cre* mice are available that express Cre under the control of the albumin promoter and albumin regulatory elements plus α -fetoprotein enhancers, respectively [34, 35]. Comparison of both mouse strains might be helpful.

In general, probably the best evidence that the Cre expression level is sufficient to mediate efficient recombination in a certain cell type is provided when at early time points following infection close to 100% of the progeny is recombined. This result, however, is only possible if the cell type under study is also the major producer cell type in a given tissue. Yet low proportions of recombined virus may not be due to unsuccessful recombination but may rather reflect unproductive cell types. Our approach presented so far was aimed at a positive identification of cell types important for virus production and dissemination.

Selectivity and stability of Cre expression in cell types and organs

In order to identify a certain cell type as a virus producer by the *Cre/loxP*-system, the Cre expression has to be restricted to this cell type. Unfortunately, the cell type-specific expression of Cre in transgenic mouse lines is usually examined in the absence of infection and inflammation. A problem may arise if the promoter driving Cre expression is also active in other cell types during inflammation. In this case, an unexpected cell type would contribute to the recombined reporter virus fraction. Therefore, Cre expres-

sion in third party cells should not occur in the presence of infection and inflammation. To date this possibility cannot be entirely excluded. Next, different transgenic mouse lines can vary in the reliability of cell type-specific Cre expression. A just transient Cre expression in multiple cell types during embryogenesis, as for instance observed in *Tie2-cre* mice [32], does not represent an objection to our approach. In our case not the developmental Cre expression but only the actual cell type-specific Cre expression in endothelial cells during infection with the reporter virus is crucial for the experiment. Altogether, the conditions of Cre expression for host gene activation or silencing need not reflect the situation required for viral marker expression.

In the example reported by deFalco and colleagues mentioned above [27] the recombination of any number of virus genomes sufficed to label the infected cell. In contrast to this qualitative approach we aim to use the *Cre/loxP*-system for a quantitative approach. We count the number of viral progeny with a recombined genome and estimate the viral productivity of a defined cell type. Which factors could account for differences in the proportion of recombined reporter virus found in an organ? Differences in the proportion of recombined reporter virus found between different Cre positive cell types constitute the basic read-out of our experimental system. They give an estimate of the contribution of a given cell type to the overall viral load of an organ, provided that the analysis is restricted to a single viral replication cycle. First of all, the level of Cre should suffice to recombine all genomes, which contribute to production of progeny. Besides this, the size of the virus progeny with a recombined genome depends on whether the cell type under investigation is highly or poorly productive. Differences in the proportion of recombined virus between organs do not necessarily reflect physiologic differences in cell type-specific viral productivity, but rather the different numbers of the Cre expressing cells in different tissues. After infection of *Tie2-cre* mice, for instance, the viral genome is replicated and recombined in endothelial cells. Some organs possess more endothelial cells than others, which may result in a higher proportion of virus with a recombined genome. Virus in the blood must cross the endothelial cell barrier to infect parenchymal tissues. If the endothelium is fenestrated or discontinuous, however, the reporter virus might cross the endothelial barrier and enter the parenchyma of the organ even without experiencing recombination. Indeed, in *Tie2-cre* mice organs with fenestrated or discontinuous endothelium like kidney, adrenal glands and liver generally exhibit lower proportions of recombined virus. The same would be true if the virus just passes the endothelial barrier by transcytosis without replication in endothelial cells.

Recombination of the reporter locus in the cell type under investigation should occur with high efficiency, prior

to DNA replication. There are factors that should have an effect on the level of Cre expression. First of all, the copy number of the *cre*-transgene harboured by a mouse genome is important. Whereas mice generated by knock-in technology contain one to two *cre* copies, transgenic mouse strains may carry multiple copies of the *cre* gene. In direct comparison, the Cre expression levels of the former should be lower. In initial experiments using conditional reporter viruses, we studied CD11c-*cre* mice and CD45-*cre* mice. CD45 is a pan-leukocyte marker and CD11c is predominantly expressed on dendritic cells. Therefore, if not even higher in CD45-*cre* mice, the recombination levels in both mouse strains should at least be equal. To our surprise, however, we could not find progeny with a recombined reporter locus in CD45-*cre* mice, while in CD11c-*cre* mice a considerable amount of recombined reporter virus was found. The fact that the *cre* gene was introduced into the genome of CD45-*cre* mice by knockin technology, whereas the CD11c-*cre* mouse is a transgenic strain, might explain these apparently contradictory results. We feel that the level of Cre expression in knock-in mice is usually not high enough to mediate efficient recombination of the virus genome. Unfortunately, some mice engineered to express Cre in a cell type-specific manner are only available as knock-in strains.

Even if in a transgenic mouse strain Cre cell type-specific expression is proven, as for instance in endothelial cells in Tie2-*cre* mice, the constitutive Cre expression levels may vary between endothelial cells in different organs. For example, endothelial cells that are found in the sinusoids of the liver are morphologically and phenotypically distinct from endothelial cells that line the large vessels (e.g. the aorta) [36, 37] and this may extend to constitutional promoter activities.

As discussed above, virus induced inflammation can activate Cre expression in unexpected cell types. Similarly, the infection may modulate the activity of promoters which drive Cre expression. This would lead to altered levels of Cre recombinase during infection and inflammation. For instance, usually the albumin promoter is used for cell type-specific Cre expression in hepatocytes [34]. Yet, it was shown that MCMV infected hepatocytes produce less albumin [38], and Cre expression levels during infection may in fact be reduced.

Identification of cell type-specific functions of viral gene products

Although the application of MCMV reporter viruses in the Cre/loxP-system is a powerful tool to identify virus productive cells, there are still apparent difficulties in comparing different transgenic mouse lines with regard to

constitutive and inflammation induced Cre expression and with regard to the number of Cre expressing cells in a particular organ. The prospects are much better when a comparison of different virus mutants in one and the same *cre*-transgenic strain is the objective. Genes M36 and M45 were already found to be important in the infection of macrophages and endothelial cells in vitro [13, 14]. It is conceivable that a proportion of MCMV's 101 ORFs which are not essential for replication play a role in governing virus-cell interactions in the living host [12, 39]. On the basis of the BAC-cloned MCMV- $\Delta m157$ -flox-*egfp* genome, mutants can easily be generated with deletions in additional genes resulting in viruses MCMV- $\Delta m157$ -flox-*egfp*- $\Delta geneX$. Good candidates for screening of cell type-specific functions for example would be individual members of the m145 gene family and gene M27, genes which are involved in NK cell regulation and interferon response modulation, respectively [40–42]. Furthermore, it would be of interest to test published mutants that do not seem to have any phenotype *in vivo*. For example, viruses mutated in genes *m09*, *M25* and *m128* (*ie2*) exhibited a titer similar to that of the wildtype virus in the salivary glands, lungs, liver, spleen and kidneys of BALB/c mice [43–45]. *M43* mutants also did not show any replication difference in most organs, except in the salivary gland [46]. The same total titer, however, could result from the contribution of different producer cells. The inability to produce progeny in one cell type might be compensated by the productivity in another cell type. A cell type might be poor as a virus producer but highly active as a stimulator of the immune response. If this cell type is excluded from productive infection, the indirect effects of the reduced inflammation might surpass the loss of direct productivity.

After infection of *cre*-transgenic mice with a virus mutant MCMV- $\Delta m157$ -flox-*egfp*- $\Delta geneX$ and the parental virus MCMV- $\Delta m157$ -flox-*egfp*, the shift in the proportion of virus with a recombined locus would indicate a function of gene *X*.

Tie2-*cre* mice expressing the Cre recombinase in endothelial cells were infected with MCMV- $\Delta m157$ -flox-*egfp*- $\Delta geneA$ and MCMV- $\Delta m157$ -flox-*egfp* (Fig. 4). The example shows that a deletion of gene *A* results in a reduced virus titer in the spleen. The proportion of gene *A*-deficient virus derived from endothelial cells, however, accounted for more than 90% of the spleen's total virus load compared to only 50% in the case of virus with an intact gene *A*. Thus, in the case of MCMV- $\Delta m157$ -flox-*egfp*- $\Delta geneA$ almost every virus particle found in the spleen now originated from endothelial cells. This demonstrates a clear cell type-specific effect for gene *A*. We suppose that gene *A* is required for virus replication in the major producer cells of the spleen, which still need to be identified. Apparently, gene *A* is not essential for replication in endothelial cells

Fig. 4 Cell type-specific functions of viral gene products in vivo. Cell type-specific effects were found when the parental MCMV reporter virus $\Delta m157$ -flox-egfp (R) was compared with the deletion mutants R Δ geneA and R Δ geneB in **a** the spleen and **b** the liver, respectively. Organs were assayed at day 3 p.i. **a** Tie2-cre mice expressing Cre specifically in endothelial cells were infected with 10^6 PFU of R and R Δ geneA. The green columns represent the proportion of virus originating from endothelial cells. **b** Tie2-cre mice and Alb-cre mice were infected with 10^6 PFU of R and R Δ geneB. The green columns represent the proportion of virus produced by endothelial cells and hepatocytes, respectively. Red dots total virus titer, red dashed line detection limit

and this explains the high proportion of recombined gene A-deficient virus.

A similar experiment was performed in Tie2-cre mice with the deletion mutant MCMV- $\Delta m157$ -flox-egfp- Δ geneB. Here, the total virus titer in the liver remained stable at day 3 post infection, but the fraction of gene B-deficient virus progeny increased more than tenfold indicating that the quantity of virus produced by endothelial cells was significantly augmented. In the complementary experiment, Alb-cre mice were infected with MCMV- $\Delta m157$ -flox-egfp- Δ geneB and MCMV- $\Delta m157$ -flox-egfp. As expected, no changes in the total liver virus titer could be detected. However, the amount of virus produced by hepatocytes decreased. Thus, by deletion of gene B, virus productivity in the liver shifted from hepatocytes to endothelial cells. Clearly, such a phenotype cannot be detected by the use of conventional deletion mutants that do not allow cell type-specific tracing of viral progeny.

Future applications of conditional gene expression to study herpesvirus biology in vivo

Conditional MCMV reporter viruses can be used to trace virus production and dissemination as well as to identify cell type-specific functions of viral gene products in vivo. We hope that the spectrum of applications can be further expanded. Depending on the question, the conditionally expressed marker can be customized. For instance, it is possible to express the firefly luciferase after Cre-mediated

recombination instead of EGFP. Luciferase-dependant bioluminescence can be detected by noninvasive methods and the dynamics of infection can therefore be studied on the level of organs as a single mouse kinetics.

Furthermore, conditional gene expression by MCMV mutants is not just restricted to reporter genes. For instance, MCMV viruses can be engineered to express model antigens after Cre-mediated recombination. Upon infection of transgenic mice with MCMV- $\Delta m157$ -flox-ovalbumin, antigenic markers will be expressed in Cre positive cell types and presented on MHC complexes. If it is possible to restrict antigen expression to the Cre positive cells, questions of direct and cross presentation by different cell types become amenable.

In summary, viral productivity in vivo was so far only accessible on the level of organs. The application of conditional reporter viruses in the Cre/loxP-system makes it now possible to study viral productivity, virus dissemination, antigen presentation, and immune modulation at the level of cell types in vivo.

Acknowledgments S.J. and A.V. were supported by a research fellowship of the Studienstiftung des deutschen Volkes and the Humboldt Stiftung, respectively. U.H.K. and Z.R. were supported by the DFG (SFB 455 A7) and U.H.K. by NGFN grant 01GS0405 and the Bayerische Forschungsförderung. We thank J. Gregor and N. Röder for expert technical assistance and are indebted to S. Schultz for her support on graphic design.

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