A shutoff and exonuclease mutant of murine gammaherpesvirus-68 yields infectious virus and causes RNA loss in type I interferon receptor knockout cells

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Significant loss of RNA followed by severely reduced cellular protein pool, a phenomenon termed host shutoff, is associated with a number of lytic virus infections and is a critical player in viral pathogenesis. Until recently, viral DNA exonucleases were associated only with processing of viral genomic DNA and its encapsidation. However, recent observations have identified host shutoff and exonuclease function for the highly conserved viral exonucleases in γ-herpesviruses, which include Kaposi’s sarcoma-associated herpesvirus, Epstein–Barr virus and the mouse model murine gammaherpesvirus-68, also referred to as MHV-68. In this study, we show that although ablation of the MHV-68 exonuclease ORF37 caused a restrictive phenotype in WT IFN-α/β receptor-positive cells such as NIH 3T3, lack of ORF37 was tolerated in cells lacking the IFN-α/β receptor: the ORF37Stop virus was capable of forming infectious particles and caused loss of mRNA in IFN-α/β receptor knockout cells. Moreover, ORF37Stop virus was able to establish lytic infection in the lungs of mice lacking the IFN-α/β receptor. These observations provide evidence that lytic MHV-68 infection and subsequent loss of mRNA can take place independently of ORF37. Moreover, efficient growth of ORF37Stop virus also identifies a role for this family of viral nucleases in providing a window of opportunity for virus growth by overcoming type I IFN-dependent responses.

INTRODUCTION

RNA turnover is a key regulatory mechanism closely associated with gene expression and takes place in the nucleus as well as in the cytoplasm. Viruses have evolved a number of novel mechanisms to manipulate host-gene expression, including transcription. They can cause global loss of host transcripts, leading to a significant reduction in the cellular protein pool. This phenomenon is called host shutoff. For example, the 3C protease of poliovirus causes cleavage of TBP and inhibition of RNA POL II processivity (Yalamanchili et al., 1996). Other examples of host shutoff include influenza A virus inhibition of 3′ processing of pre-mRNA, and herpes simplex virus-1 (HSV-1) redistribution of snRNPs mediated by viral ICP27; poliovirus-encoded protease 2A can cleave eIF4G, rendering cap-dependent translation inactive (Phelan et al., 1993; Chen et al., 1999).

In HSV, the virus host shutoff (vhs) protein, encoded by UL41, mediates degradation of both cellular and viral mRNA (Fenwick & McMenamin, 1984; Schek & Bachenheimer, 1985; Kwong & Frenkel, 1987; Oroskar & Read, 1987, 1989; Strom & Frenkel, 1987; Zelus et al., 1996). Genetic and biochemical studies have shown that vhs acts as an endoribonuclease, degrading mRNA in the absence of other cellular or viral proteins and with substrate specificity similar to that of RNase A (Taddeo & Roizman, 2006). There is evidence that vhs can target different parts of mRNA molecules and that there may be an element of substrate specificity with respect to mRNA degradation by vhs (Karr & Read, 1999; Taddeo et al., 2006). The direct nuclease activity of vhs and inhibition of pre-mRNA splicing mediated by viral protein ICP27 cause a shift in favour of viral protein synthesis (Hardwicke & Sandri-Goldin, 1994; Hardy & Sandri-Goldin, 1994; Sandri-Goldin, 2011). More recent observations indicate that vhs can also associate with the translation initiation complex eIF4F, which binds the 5′ cap
structure of mRNAs, thereby targeting vhs to actively translating mRNAs (Page & Read, 2010; Dauber et al., 2011).

Recent work on γ-herpesviruses has shown that they also cause host shutoff, albeit in the absence of an apparent vhs homologue (Ebrahimi et al., 2003; Glaunsinger & Ganem, 2004a, b; Glaunsinger et al., 2005; Rowe et al., 2007; Covarrubias et al., 2009). Using an in vitro model of Kaposi’s sarcoma-associated herpesvirus (KSHV) reactivation, the viral DNA exonuclease (AE) encoded by ORF37 was shown to mediate host shutoff and that this activity was independent of its DNA exonuclease activity (Glaunsinger & Ganem, 2004a, b). These observations implied that the ORF37 gene product possessed cryptic RNase activity that mediated host shutoff either directly or via a cellular partner, or a combination of both. Interestingly, it was also shown that the two different functions of KSHV SOX (kSOX) protein, DNase activity and host shutoff, were genetically separable (Glaunsinger et al., 2005). Moreover, the host shutoff phenotype of ORF37 is highly conserved in γ-herpesviruses (Covarrubias et al., 2009). Similarly, both DNase and RNase activities have also been reported for the Epstein–Barr virus (EBV) alkaline exonuclease encoded by BGLF5 (Rowe et al., 2007).

The mechanisms by which kSOX and EBV BGLF5 regulate host-gene expression during the lytic virus infection are not entirely clear; kSOX does not have direct RNase activity under physiological conditions, but both kSOX and EBV-encoded BGLF5 can degrade RNA in the presence of high concentrations of metal (Buisson et al., 2009; Bagnéris et al., 2011). Therefore, cofactors (viral and/or cellular) may be required for ORF37-mediated RNase activity or it may activate cellular RNA degradation pathways by proxy (Glaunsinger & Ganem, 2004a, b). Recently, a cellular enzyme, Xrn-1, has been identified as a possible partner in mediating kSOX RNase activity (Covarrubias et al., 2011).

An ideal approach to decipher how a given viral gene product may contribute to host shutoff in the context of a de novo virus infection is to remove completely the gene of interest and then investigate the possible altered phenotype in the context of a successful lytic cycle. Murine γ-herpesvirus-68 (MHV-68) is closely related to human γ-herpesviruses and provides an amenable model to study virus–host interactions in vitro and in vivo. (Nash et al., 2001; Barton et al., 2010). In our present study, we constructed a viable and stable ORF37 mutant of MHV-68, ORF37Stop and its corresponding revertant ORF37StopRev. Although severely restricted in immunocompetent cells in vitro and in vivo, ORF37 virus mutant caused plaque formation in type I IFN receptor knockout (KO) cells at levels comparable to WT virus infection, as well as yielding infectious virus in vivo. Importantly, loss of mRNA was observed in type I IFN receptor KO cells infected with ORF37Stop virus. These observations clearly demonstrate that de novo lytic infection and virion formation can take place in the absence of ORF37 and that a key role of γ-herpesvirus host shutoff proteins is to provide a window of opportunity for lytic infection by overcoming type I IFN-dependent effector mechanisms.

**RESULTS**

**Construction of ORF37 mutant and ORF37 revertant viruses and their growth characteristics**

There is now ample evidence to support a role for viral exonucleases in mediating host shutoff in infections with γ-herpesviruses (Glaunsinger & Ganem, 2004a, b; Glaunsinger et al., 2005; Rowe et al., 2007; Covarrubias et al., 2009). However, the precise role of these viral proteins in mediating host shutoff in the context of a de novo lytic infection cycle has remained unclear. To address this, we generated an ORF37 stop mutant of MHV-68 (ORF37Stop) and the corresponding revertant virus (ORF37StopRev) (Fig. 1), using a previously described methodology (Geere et al., 2006). This mutant virus has stop codons in all three reading frames inserted 31 nt from the ORF37 start codon and is therefore unable to produce more than the first 10 amino acids of ORF37. The integrity of engineered viruses was confirmed by sequence verification (see Fig. S1, available in the online Supplementary Material), diagnostic real-time PCR (RT-PCR) and Southern blot analysis (Fig. 1). To assess the integrity of transcript generated from this region of the virus genome, we used diagnostic RT-PCR to amplify a 350 bp fragment, which upon digestion with HpaI yielded either two fragments from cells infected with ORF37Stop virus or a single fragment from cells infected with the ORF37StopRev virus (Fig. 2). The region corresponding to the insertion sites was also cloned and its sequence verified (Fig. 1). Having verified the integrity of our engineered viruses, we then investigated the growth characteristics of these viruses in vitro by single-step growth curves in a number of murine cell types, including 129/Sv and NIH 3T3. The one-step growth characteristics with WT MHV-68 and ORF37StopRev were comparable (Fig. 3a). In contrast, reduced plaques were observed in cell monolayers infected with the mutant virus (Fig. 3a). To ascertain whether plaque numbers on monolayer infected with ORF37Stop virus were because of a bona fide infection in these cells rather than from remnants of virus from the inoculum, we used hyperimmune sera against MHV-68 lytic antigens on cell lysates from these cultures, after repeated rinsing of the monolayer post-inoculation. MHV-68 lytic antigens were detected in cells infected with ORF37Stop virus, albeit significantly fewer than in cell lysates from cultures infected with WT virus (Fig. 3b). Importantly, we found no reversion to WT MHV-68 genotype in cultures infected with ORF37Stop in repeated infection cycles as tested by our diagnostic PCR assay. Therefore, ORF37Stop mutant virus was still capable of going through the full lytic cycle and yielding virions in the absence of viral alkaline exonuclease, albeit at significantly reduced levels.

The observations described above prompted us to investigate the impact of infection with ORF37Stop on loss of
cellular RNA. Infection of immunocompetent cells with the WT virus is associated with host shutoff of number of housekeeping genes, e.g. β-actin, at both mRNA and protein levels. Therefore, we used cellular β-actin protein levels as a reliable and amenable indicator of host shutoff because of MHV-68 lytic infection. Reduced levels of β-actin were noticeable in cells infected with WT MHV-68 (Fig. 3c); similar observations were seen in cells infected with ORF37StopRev (data not shown). The loss of β-actin was not due to loss of cells. In contrast, we did not observe detectable loss in β-actin protein levels in cells infected with ORF37Stop virus when compared to cells infected with the WT virus (Fig. 3c). Taken together, these observations suggested that the ORF37 gene product was necessary for efficient plaque formation and for host shutoff in these cells.

Fig. 2. ORF37Stop mutant virus is viable and does not revert to the WT virus genotype. Total RNA was isolated from infected cells and the presence of ORF37 transcript was monitored by RT-PCR using ORF37StopFA and ORF37StopRB primers. PCR products were either run uncut or cut with HpaI. (a) Schematic showing predicted PCR products for ORF37Stop and ORF37StopRev and WT MHV-68 viruses. (b) Gel image of PCR products from mock-infected and infected IFNα/β RK O cultures either uncut or cut with HpaI. The negative control is total RNA isolated from cells without virus infection.
the growth of ORF37Stop mutant virus in cells lacking a functional type I IFN receptor, because low levels of type I IFNs are present in cultured cells, which may interfere with virus growth. In stark contrast to a restricted phenotype of ORF37Stop virus in immunocompetent cells (129/Sv and NIH 3T3), ORF37Stop virus was capable of producing levels of infectious virus comparable to levels seen in infections with both the WT and the ORF37Rev virus in cells lacking a functional type I IFN receptor (Fig. 4a). Previous reports have identified a large number of cellular transcripts that succumb to MHV-68 lytic infection, including c-fos and cyclophilin. Infection of IFNα/β KO cells with WT MHV-68 and ORF37StopRev resulted in loss of both these transcripts (Fig. 4b, c). Similarly, we also observed loss of c-fos and cyclophilin in IFNα/β KO cells infected with either ORF37Stop virus (Fig. 4b, c).

We then asked whether our mutation had any detrimental effect on the entire virus transcriptome in IFNα/β KO cells. For this purpose, we used our previously validated MHV-68 microarray platform (Ebrahimi et al., 2003; Towler et al., 2012). We found that insertion of stop codons in the ORF37 encoding sequence had no detrimental effect on other viral gene expression flanking ORF37 nor on the entire virus transcriptome (Fig. 5).

Taken together, these data clearly demonstrate that in the absence of a functional type I IFN receptor, the ORF37 function is non-essential and/or may be carried out by other viral and/or cellular factors, and that host shutoff still takes place in the absence of ORF37.

**MHV-68 ORF37Stop mutant virus establishes lytic infection in lungs of type I IFNα/β KO mice**

Although *in vitro* assays have proved invaluable in understanding the roles of viral gene products, they are far removed from the natural cellular environments found *in vivo* and hence the complex events associated with disease pathogenesis as result of infection with herpes viruses. IFNα/β KO mice and their immunocompetent counterpart 129/Sv mice were infected with WT MHV-68, ORF37Stop and ORF37StopRev viruses. In contrast to WT and ORF37StopRev viruses, ORF37Stop virus did not yield plaques in lung tissues from immunocompetent 129/Sv mice (Fig. 6a). However, ORF37Stop yielded plaques in lungs of IFNα/β KO mice by day 3 and at similar levels to the ORF37StopRev and WT viruses (Fig. 6b). By day 6 post-infection, there was an exponential increase in the titres of WT and ORF37StopRev viruses in lung tissues (Fig. 6b). This was in contrast to titres in mice infected with ORF37Stop virus, which by day 6 had reached a plateau comparable to levels observed on day 3 post-infection (Fig. 6b). These observations are consistent with our *in vitro* data, demonstrating that ORF37 is essential for infection of immunocompetent mice, but not essential if the host is defective in the type I IFN response. However, ORF37 does play a significant role in later stages of infection, which is independent of the type I IFN response. Therefore, lack of

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**MHV-68 ORF37Stop mutant virus mediates loss of mRNA in type I IFNα/β KO cells**

Type I IFNs are potent antiviral cytokines and can effectively control MHV-68 lytic infection (Nash et al., 2001; Barton et al., 2011). The restricted phenotype observed in cells infected with ORF37Stop is reminiscent of cells pretreated with type I IFN prior to infection with WT MHV-68 *in vitro* (Weck et al., 1997; Dutia et al., 1999; Barton et al., 2005). These observations, therefore, prompted us to characterize
DISCUSSION

Recent observations have identified a novel host shutoff activity for highly conserved viral DNA alkaline exonucleases in γ-herpesviruses (Glaunsinger & Ganem, 2004a, b; Glaunsinger et al., 2005; Rowe et al., 2007; Covarrubias et al., 2009). However, the precise mechanism for this virus-mediated host shutoff remains unclear. In this study we aimed to understand a clearer role for ORF37 in the virus lytic cycle and in mediating host shutoff by de novo infection in vitro and in vivo with ORF37Stop, an engineered ORF37 stop mutant of MHV-68.

We demonstrated that host shutoff induced by MHV-68 lytic infection can take place in the absence of ORF37-encoded alkaline exonuclease in cells lacking a functional type I IFN receptor, in vitro and in vivo, providing new insight into the interplay between type I IFN effector mechanisms and virus-initiated host shutoff. Our data fit well with an earlier study that showed ORF37 was not an essential gene for plaque formation in immunocompetent NIH 3T3 cells (Song et al., 2005). This and our observations differ from an attempt by another group in ablating ORF37 of MHV-68 that failed to reconstitute infectious virus from a bacterial artificial chromosome (BAC) construct in vitro (Covarrubias et al., 2009).

It is of interest that our observation that the MHV-68 ORF37 mutant was capable of forming plaques in immunocompetent cells, albeit at a much reduced level, is also similar to the observation made with HSV-1 AE mutants, where it was shown that HSV-1 AE mutants could grow in Vero cells (Weller et al., 1990). This and other studies also showed viral AE is not essential for viral DNA synthesis (Weller et al., 1990; Smiley, 2004). This raises the issue of how nuclease mutant viruses process their genomic DNA, for example in resolution of viral DNA intermediates. It was shown that plasmid DNA recombination can occur with very high efficiency in cells infected with an AE mutant of HSV-1, suggesting a role for cellular nucleases in resolution of Holliday junctions, which exist during herpesviral genomic DNA replication (Martinez et al., 1996). A number of cellular nucleases have since been shown to share significant amino acid and structural similarities to viral nucleases, for example flap endo/exonucleases (Lieber 1997; Tomlinson et al., 2010). Of relevance here is the observation that HSV-1 compared with mock-infected samples. The $P$ values shown on graphs compare loss of mRNA between cultures infected with ORF37Stop and ORF37StopRev viruses. Data were obtained from three independent experiments (mean ± SD, n=3). Other cellular genes tested by RT-PCR also showing loss of mRNA were Hsp70, DHFR, eEF-Tu 1α, β-actin and histone 2A (data not shown).

ORF37 is tolerated in the complex in vivo environment when type I IFN-mediated effector mechanisms are not functional.
vhs (but not HSV-1 AE) shares a significant amino acid sequence homology to a flap endo/exonuclease, FEN-1 (Smiley, 2004). Therefore, in some circumstances, cell-derived nucleases can compensate for loss of viral nuclease activity, which may explain, at least in part, plaque formation by exonuclease-deficient viruses. It is also not clear whether the complement of cellular nucleases that may compensate for lack of viral nuclease activity in vivo may be different to those found in vitro.

While in the last stages of our work, another study by Richner et al. (2011) reported the generation of an MHV-68 ORF37 single non-silent mutation resulting in an amino acid substitution (R443M to R443I) that was selectively defective in host shutoff activity, MHV-68. Both the mutant virus could replicate to levels comparable to the WT virus in NIH 3T3 cells in vitro and in vivo. Interestingly, the random PCR mutation that resulted in generation of MHV-68.ΔHS virus was outside the previously identified motifs associated with host shutoff. The ability of MHV-68.ΔHS to cause host shutoff suggested that virus host shutoff did not play an important role in lytic infection (Richner et al., 2011). This differs significantly from our findings. One explanation for this difference is the fact that the host shutoff mutant virus engineered by Richner and colleagues, as acknowledged by the authors, was still capable of causing mRNA degradation when compared with mock-infected cells, suggesting incomplete inactivation of the host shutoff function (Richner et al., 2011).
et al., 2011). Therefore, it is likely that this residual host shutoff activity could still provide a window of opportunity for the virus to overcome antiviral effector mechanisms.

The critical role of type I IFN in MHV-68 lytic infection was clearly demonstrated by infecting type I IFNα/βR KO mice with MHV-68, which resulted in an uncontrolled systemic infection with increased morbidity and mortality, although some of these mice survived the lytic phase of infection (Weck et al., 1997; Dutia et al., 1999; Barton et al., 2005). Our in vivo data clearly demonstrated that ORF37Stop virus was capable of establishing lytic infection in lungs of mice at levels comparable with WT MHV-68 (Fig. 6). However, by day 6 post-infection, the lytic virus load in mice infected with ORF37Stop virus had stabilized in contrast to lytic virus loads in mice infected with WT MHV-68. Recently, it was shown that in EBV BGLF5 (homologue of ORF37) caused downregulation of HLA class I expression (Zuo et al., 2008). Therefore, it is likely that other components of the innate and acquired immune systems are able to control the expansion of ORF37Stop virus in vivo. These possibilities are currently under investigation.

Type I IFNs were also found to regulate latent gene expression and suppress reactivation from latency, demonstrating a key role played by type I IFN not only during the initial lytic infection but also during viral persistence and latency (Dutia et al., 1999; Barton et al., 2005). It is known that IFNs can block efficient synthesis of viral DNA and nucleocapsid formation in HSV-1-infected cells (Pierce et al., 2005). Therefore, it is also likely that in IFNα/β KO cells, the block to viral DNA synthesis and nucleocapsid formation is removed, leading to plaque formation as was observed in this study with ORF37 mutant virus. Hence, a picture is emerging whereby ORF37-mediated loss of RNA, which includes IFN-responsive genes, will create a window of opportunity for the virus to overcome blocks to viral DNA synthesis and nucleocapsid formation. One such possibility involves IFN regulatory factor-5 (IRF-5), a key type I IFN effector mechanism also involved in the TLR-MyD88 signalling pathway essential for the induction of pro-inflammatory cytokines such as TNF-α, IL-6 and IL12p40 (Takaoka et al., 2005). Of relevance here is the observation that TNF-α significantly repressed the activity of KSHV RTA promoter (a key virus trans-activator gene), thereby suppressing lytic virus replication (Milligan et al., 2004).

Our results clearly demonstrate that in the absence of type I IFN signalling, host shutoff can proceed in the absence of MHV-68 ORF37, supporting the widely accepted view that additional host shutoff factors must be at work during infection with MHV-68 and related viruses.

**METHODS**

**Cells, tissue culture and virus assays.** Primary mouse embryo fibroblasts from 129/Sv and IFNα/βR KO embryos and NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with FCS (10%, v/v), glutamine (2 mmol l⁻¹), potassium benzylpenicillin (100 U ml⁻¹) and streptomycin sulphate (100 µg ml⁻¹). Cells were infected with MHV-68 at an m.o.i. of 5 p.f.u. per cell. The WT parent virus was MHV-68 strain g2.4. To assay for virus infection in vivo, WT 129/Sv and IFNα/βR KO 129/Sv mice were infected with 4 × 10⁹ of each virus stock via the intra-nasal route as described elsewhere (Sunil-Chandra et al., 1992a, b). To determine virus titres in infected mice, lung tissues were harvested at various times post-infection and frozen immediately. To assay for infectious virus, tissues were processed by three cycles of freezing/thawing and then homogenized to release virions. The homogenates were centrifuged to remove cell debris, and supernatants containing virions were titrated on cell monolayer (Sunil-Chandra et al., 1992a, b).

**Generation of ORF37 mutant and revertant viruses of MHV-68.** Stop mutants of MHV-68 ORF37 were generated by manipulating an MHV-68 genomic clone containing nt 49 951–59 889 (Efstathiou et al., 1999). A 17 bp DNA sequence containing three stop codons and a diagnostic HpaI site (DeLuca & Schaffer, 1987) was inserted at nt 54 157 into a PCR product spanning the Hpy99I site at nt 53 949 and the SacI site at nt 54 270. This product was cloned and sequenced, and then inserted into the genomic clone. The mutated genomic clones were then ligated into the shuttle vector pST76_SR. The resultant plasmids were used to transform Escherichia coli DH10B containing the MHV-68 BAC (Adler et al., 2000). Recombinant BAC clones were produced as described by Merserle et al. (1997) and by Adler et al. (2000), screened by PCR using primers spanning the regionnt 53 949–54 270, followed by HpaI digestion, to identify mutated colonies. A revertant virus of MHV-68 ORF37Stop (termed ORF37StopRev) was made by transformation of bacteria containing mutant BAC with shuttle vector containing the WT genomic clone and selected by PCR in the same way. Virus stocks were produced by transfection of virus BAC DNA into 3T3 cells expressing Cre recombinase to excise the BAC sequences. (Stevenson et al., 2002). Because of highly restricted virion formation, subsequent virus working stocks were prepared by infection of mouse type I IFN receptor KO cells. Viral DNA was then analysed by restriction analysis with Hpal and Southern blot hybridization using a probe encompassing nt 53 949–54 270. Total genomic DNA was harvested from virus stocks as follows. Virus stocks were ultracentrifuged at 20 000 g at 4 ºC for 2 h. The resultant pellets were digested with proteinase K (1 mg ml⁻¹) at 53 ºC overnight in lysis buffer (20 mM Tris/HCl pH 7.5, 2 mM EDTA, 12 % SDS). The DNA was then extracted with phenol/chloroform followed by ethanol precipitation with ammonium acetate. Probes were generated by amplification of a 340 bp fragment using forward (ORF37StopFA, GACATCGACGGAGGAAGCAG) and reverse (ORF37StopRB, GTCTTTGATGTTGCCAGGAG) primers labelled with [α-32P]dCTP (PerkinElmer) using a Ready-To-Go DNA bead labelling kit (GE Healthcare). Blot images were generated using a Storm 860 (Molecular Dynamics) phosphorimager.

**Protein analysis.** Total protein lysate was obtained in cell extraction buffer (Biosource), and quantified using the RC/DC protein assay (Bio-Rad) and spectrophotometry. Twenty micrograms of protein were carried out at room temperature. Gels were incubated in transfer buffer (2000), screened by PCR using primers spanning the regionnt 53 949–54 270. Total genomic DNA was harvested from virus stocks as follows. Virus stocks were ultracentrifuged at 20 000 g at 4 ºC for 2 h. The resultant pellets were digested with proteinase K (1 mg ml⁻¹) at 53 ºC overnight in lysis buffer (20 mM Tris/HCl pH 7.5, 2 mM EDTA, 12 % SDS). The DNA was then extracted with phenol/chloroform followed by ethanol precipitation with ammonium acetate. Probes were generated by amplification of a 340 bp fragment using forward (ORF37StopFA, GACATCGACGGAGGAAGCAG) and reverse (ORF37StopRB, GTCTTTGATGTTGCCAGGAG) primers labelled with [α-32P]dCTP (PerkinElmer) using a Ready-To-Go DNA bead labelling kit (GE Healthcare). Blot images were generated using a Storm 860 (Molecular Dynamics) phosphorimager.
(Vector Laboratories) and with NBT substrate (Vector Laboratories) following the manufacturer’s instructions.

**RT-PCR primer and probe design and assays.** Total RNA was reverse-transcribed using Superscript II reverse transcriptase according to manufacturer’s recommendations (Invitrogen). PCR mixture was based on a previously published protocol (Karsai et al., 2002) with platinum Taq Pol (Invitrogen) in a final reaction volume of 20 μl. Amplifications were performed using the Opticon 2 thermal cycle (Bio-Rad). PCR conditions consisted of an initial denaturing step at 95 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 20 s and polymerization at 72 °C for 1 s to melt any potential primer-dimers, followed by fluorescence reading. Absolute quantification was based on gene-specific standard curves, constructed by amplification of serially diluted synthetic oligonucleotide templates of less than 120 bases (MWG-biotech and Operon). To ensure specificity of amplifications, all PCRs were subjected to melting curve analysis.

Investigations of the murine gammaherpesvirus 68 genome as an infectious bacterial artificial chromosome. Cloning and mutagenesis of the murine gammaherpesvirus 68 genome was based on gene-specific standard curves, constructed by amplification of serially diluted synthetic oligonucleotide templates of less than 120 bases (MWG-biotech and Operon). To ensure specificity of amplifications, all PCRs were subjected to melting curve analysis.

**RNA isolation, cDNA synthesis and MHV-68 microarray hybridization.** Total cellular RNA (20 μg) was isolated from cells with RNeasy columns (Qiagen). Hybridization of labelled cDNA on MHV-68 microarrays and subsequent image and data analyses were carried out as described previously (Ebrahimi et al., 2003; Towler et al., 2012).

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