The M2 gene product of murine gammaherpesvirus 68 is required for efficient colonization of splenic follicles but is not necessary for expansion of latently infected germinal centre B cells

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Infection of mice with murine gammaherpesvirus 68 is characterized by a marked transient expansion of latently infected splenic germinal centre (GC) B cells, which is followed by lower levels of persistent infection in GC and memory B cells. Virus transcription within GC B cells is restricted to a number of latency-associated open reading frames, including M2. This gene encodes a structurally unique protein of unknown function, which has been shown to be essential for the transient peak of virus latency during the establishment of latent infection in the spleen. This study shows that upon infection of mice with M2-defective viruses, at 14 days post-infection during the establishment of latency in the spleen, there was a reduction in the number of latently infected follicles when compared with wild-type virus. However, the mean number of latently infected cells within each follicle was equivalent between wild-type and M2-defective viruses. Late in infection, disruption of M2 resulted in sustained and abnormally high levels of virus persistence in splenic GC B cells but not memory B cells. These data indicate that during the establishment of latency in the spleen, the M2 gene product is required for efficient colonization of splenic follicles but is dispensable for the expansion of latently infected GC B cells and that M2 might be a critical modulator of B-cell function.

INTRODUCTION

Herpesviruses have the ability to establish latent infections leading to life-long virus persistence in their hosts. In the case of gammaherpesviruses, such as Epstein–Barr virus (EBV) and Kaposi’s sarcoma associated herpesvirus (KSHV), latency is preferentially established in lymphocytes, and persistent infection can be associated with a number of lymphoproliferative disorders and malignancies. Crucial for an understanding of the pathogenesis of these infections is the identification of viral genes expressed during latent infection and the determination of their function. For EBV, a gamma-1-herpesvirus, the differential expression of nine viral genes results in distinct programmes of latent gene expression within B lymphocytes. The functions of five of these proteins, namely the EBV nuclear antigens, EBNA1, 2 and 3C, and the latent membrane proteins, LMP1 and 2A, are well defined (Thorley-Lawson, 2001). In contrast, comparatively less is known about the viral genes and molecular mechanisms leading to latent infection in gamma-2-herpesviruses such as KSHV. Gamma-2-herpesviruses lack the same set of latency-associated genes encoded by the gamma-1-subgroup. Moreover, with the exception of ORF73, which similarly to EBNA1 expresses a multifunctional protein that mediates stable episome maintenance during latent infection (Ballestas et al., 1999; Collins & Medveczky, 2002), analysis of the genetic content within gamma-2-herpesviruses does not reveal a common set of structurally related latency-associated genes. Thus, we used a laboratory mouse model of infection, which utilizes a virus designated murine herpesvirus 68 (MHV-68), to identify latency-associated genes and study mechanisms involved in latent infection of B cells by this representative gamma-2-herpesvirus (Flano et al., 2002b; Nash et al., 2001; Simas & Efstathiou, 1998).

Following intranasal inoculation of mice with MHV-68, B lymphocytes constitute the principal target of latent
infection (Flano et al., 2002a, 2003; Marques et al., 2003; Willer & Speck, 2003). The establishment of latent infection is characterized by a marked transient expansion of infected germinal centre (GC) B cells in the spleen that peaks at 14 days post-infection (Flano et al., 2002a; Marques et al., 2003; Simas et al., 1999) followed by the development of an infectious mononucleosis-like disease (Tripp et al., 1997). Later, long-term latent infection is preferentially maintained in GC B cells (Flano et al., 2002a; Marques et al., 2003; Willer & Speck, 2003) and memory B cells (Flano et al., 2002a; Willer & Speck, 2003). In a manner similar to EBV (Thorley-Lawson, 2001), infection of GC B cells by MHV-68 might reflect a strategy to gain access to long-lived memory B cells. Hence, central for an understanding of MHV-68 pathogenesis is the identification of genes expressed during the establishment of latent infection in GC B cells. A recent study has revealed a restricted pattern of virus transcription within GC B cells during the establishment of latency in the spleen, which includes transcription of ORF M2 (Marques et al., 2003). The M2 gene product encoded by MHV-68 represents a unique protein of unknown function (Virgin et al., 1997). It has been shown that M2 contains a CD8+ T-cell epitope that is actively recognized, constituting an important target for controlling the establishment of latent infection (Husain et al., 1999; Usherwood et al., 2000, 2001). Although, the function of M2 still remains elusive, two independent studies have investigated the role of this latency-associated gene in replication and pathogenesis (Jacoby et al., 2002; Macrae et al., 2003). These studies demonstrated that M2-deficient mutants replicate normally in tissue culture and display normal acute phase replication kinetics in vivo. Nonetheless, consistent with the expression of this gene during the establishment of latency, mutants deficient for M2 are compromised for the transient rise of latency in the spleen, which is normally observed at 14 days post-infection. Despite this failure in expansion of the latently infected cell pool at early times post-infection, long-term latency is relatively unaffected (Jacoby et al., 2002; Macrae et al., 2003) and surprisingly a higher number of latently infected splenocytes are detected at late times after infection (Macrae et al., 2003). These data imply that M2 plays a major role during the establishment phase of latency in the spleen.

In this study, we show that the M2 gene product is required for efficient colonization of splenic follicles but is dispensable for the expansion of latently infected GC B cells at early times post-infection. However, late in infection disruption of M2 resulted in sustained and abnormally high levels of virus persistence in splenic GC B cells but not memory B cells.

**METHODS**

**Viruses and cell culture.** Virus working stocks were prepared by infection of baby hamster kidney (BHK-21) cells grown in Glasgow’s modified Eagle’s medium containing 10% newborn calf serum, 10% tryptose phosphate broth, 2 mM l-glutamine, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (complete-GMEM). Cells were infected at low multiplicity (0·01 p.f.u. per cell) and harvested 4 days after infection as previously described (Simas et al., 1998). Infectious virus titres were determined by suspension assay using BHK-21 cells in complete-GMEM (Simas et al., 1998). Following incubation at 37°C in 5% CO₂ for 4 days, monolayers were fixed in 10% formal saline, counterstained with toluidine blue and plaques counted using a plate microscope. NIH 3T3 cell cultures were grown in Dulbecco’s modified Eagle medium containing 10% fetal calf serum (FCS).

**Recombinant viruses.** An MHV-68 M2 frameshift mutant (M2FS) was generated by a two-step replacement procedure (Adler et al., 2000; Messerle et al., 1997). For that purpose, an additional guanine was introduced via PCR into the HindIII-E fragment of MHV-68 (Efstathiou et al., 1990) between nt 4603 and 4604, immediately downstream from the start ATG-codon (Husain et al., 1999). The PCR fragment was cloned into the HindIII-E fragment using Sサーl (nt 4313) and SpeⅠ (nt 4631). The resulting mutagenized HindIII-E fragment was then cloned into the shuttle plasmid pST76K-SR and electroporated into E. coli strain DH10B containing the MHV-68 wild-type BAC pHA3 (Adler et al., 2000). Insertion of the additional guanine generated a new Apal site, a frameshift and a new stop codon 78 nt downstream from the transcription start site (Fig. 1). For the purpose of constructing a revertant virus, a 3855 kb FseⅠ-HindIII fragment of MHV-68 (nt 2406–6261) was cloned into the shuttle plasmid pSP76K-SR (Adler et al., 2000) and electroporated into E. coli strain DH10B, which contained the M2FS-mutant BAC plasmid. Electroporation of the BAC plasmids into BHK-21 cells resulted in the reconstitution of either M2FS or M2FSR virus expressing green fluorescent protein (GFP). The BAC vector sequence (flanked by loxP sites) was removed by passage through NIH 3T3 Cre cells and limiting dilution to obtain GFP-negative

**Fig. 1.** Targeted disruption of the M2 ORF. (a) Using BAC mutagenesis, a single guanine (bold) was inserted into the genome of MHV-68 generating M2FS recombinant virus with a novel Apal site and a frameshift creating a premature stop codon 26 aa downstream from the start codon. (b) Southern blot of high molecular mass viral DNA that was digested with Apal and probed as indicated in the diagram. The frameshift mutation introduced in M2FS is evidenced by loss of the 11 kb fragment present in Apal digested wild-type viral DNA. The four lanes indicated for M2FS correspond to four independently isolated viruses recovered at 14 days post-infection from spleens of infected mice.
In vivo infections and virus assays. Female BALB/c mice (Charles River UK or Instituto Gulbenkian de Ciência) were inoculated intranasally at 6–8 weeks of age with $4 \times 10^7$ p.f.u. of virus in 20 μl PBS whilst under halothane anaesthesia. At various times after infection, mice were killed by intraperitoneal injection of sodium pentobarbitone or inhalation of CO$_2$, and lungs and spleen removed. For infectious virus assays, tissue homogenates were frozen and thawed twice before suspension assay with BHK-21 cells. Infectious centre assays (Simas et al., 1998) were performed on dissociated spleens. For this assay, homogenized spleens were passed through a 100 μm filter to remove stromal debris. Single-cell suspensions were plated with BHK-21 cells, incubated for 4 days, fixed with 10% formal saline and counterstained with toluidine blue. Plaques were counted with a plate microscope. Animal studies in the UK were conducted under the approval of UK Home Office Project Licence No. 80/1378 or in Portugal at Instituto Gulbenkian de Ciência according to Portuguese legislation Portaria 1005/92 and EU, C.D. EEC86/609.

Purification of different splenocyte populations. Single-cell suspensions of at least four pooled spleens were prepared per time point and passed through a 100 μm filter to remove stromal debris. Cell suspensions were washed in PBS-2% FCS and red blood cells lysed in 154 mM ammonium chloride, 14 mM sodium hydrogen carbonate, 1 mM EDTA pH 7.3. Single-cell suspensions were pre-incubated with 2-4 G2 [anti-CD16/CD32 (Fcγ/III/II receptor), rat immunoglobulin G2(κ) culture supernatant; Pharmingen] before staining with the following monoclonal antibodies from Pharmingen: anti-CD19 (clone 1D3), -IgG1 (clone A85-1), -IgG2a/2b (clone R2-40) and -IgM (clone R6-60.2); Southern Biotech anti-IgD (clone 11-26) and -CD38 (clone NIMR.5). The lectin peanut agglutinin (PNA) was from Vector Laboratories. Using a MoFlo cytometer (Cytomation) the following enriched cell subpopulations were obtained: CD19$^+$ for total B cells; CD19$^+$ PNA$^{high}$ or IgG1/ IgG2a/2b IgD/IgM$^{CD38^{low}}$ for GC B cells; and IgG1/IgG2a/2b IgD/IgM$^{CD38^{high}}$ for memory B cells. Sorted populations were analysed in a FACSscan flow cytometer, and the data were processed using CellQuest software (Rector Dickinson Immunocytometry Systems). The purities of sorted populations were usually >95% and always >90% except for memory B cells that ranged between 72 and 76%.

Real-time PCR. Real-time PCR was performed using a LightCycler from Roche Molecular Biochemicals according to manufacturer’s instructions, using sequence-specific fluorescent detection oligonucleotide hybridization probes coupled to suitable fluorophores as described (Marques et al., 2003).

Limiting dilution analysis. FACs purified single-cell suspensions were serially diluted twofold and eight replicates of each cell dilution were lysed overnight (0-45% Tween-20, 0-45% NP-40, 2 mM MgCl$_2$, 50 mM KCl, 10 mM Tris pH 8-3 and 0-5 mg ml$^{-1}$ Proteinase K) at 37°C. Proteinase K was then inactivated (5 min at 95°C) and the samples were analysed by real-time PCR, with primer/probe sets specific for K3, in a final volume of 10 μl per PCR reaction [2 mM MgCl$_2$, 4 ng ml$^{-1}$ each primer, 0-2 μM each internal probe and 1× DNA mix (Roche) and 1 μl cell lysate]. This protocol detected S11 cells (approximately 40 MHV-68 genomes per cell) at a frequency of 1:1×10$^5$ in an uninfected control population. Our data were compatible with the single-hit Poisson model (SHPM) as tested by modelling the limiting dilution data according to a generalized linear log-log model fitting the SHPM ($\log (\frac{1}{\text{log}(\mu_i)}) = \log (f) + \log (\chi_i)$) and checking this model by an appropriate slope test as described by Bonnefoix et al. (2001). A regression plot of input cell number against log-fraction-negative samples was used to estimate the frequency of cells with virus genomes. Estimation of the cell subset frequency of MHV-68 infection, f, consisted of computation by maximal-likelihood estimation. The standard error of f was calculated as the square root of the negative reciprocal of the second derivative of $\log (L)$,

$$SD(f) = \sqrt{\frac{-1}{d^2\log(L)/df^2}}$$

where $\log (L)$ is the natural logarithm of the likelihood function. The 95% confidence interval for f was calculated as 95% CI ($f_0, f_0 +/- 1.96SE (f)$).

RT-PCR analysis of virus transcription. Quantification of virus transcription was performed as described before (Marques et al., 2003). RNA was isolated from splenocytes purified by sorting from pools of five spleens and NIH 3T3 cells using the RNeasy mini kit with the RNase-free DNase set protocol (Qiagen) according to the manufacturer’s instructions. For cDNA synthesis, 2 μg RNA was incubated at 70°C for 10 min with 500 μg pd(T)$_{12-18}$ 5’ P$_4$ sodium salt in a total volume of 23 μl. Samples were then reverse transcribed in a total reaction volume of 40 μl containing 0-5 mM each dNTP, 1× first strand buffer (Gibco-BRL), 1 U RNase OUT ribonuclease inhibitor (Gibco-BRL), and 400 U superscript II RT (Gibco-BRL). Reactions were performed for 50 min at 37°C followed by a step of 5 min at 90°C. Viral cDNAs were quantified using real-time PCR, as described above. Primer and probe genome coordinates used were according to Marques et al. (2003). Tenfold serial dilutions of plasmid template spiked into total splenic cDNA equivalent to a minimum of 10,000 copies of Hprt were used to establish, for each gene, a linear relationship between the input template copy number and the cycle number, at which an arbitrary fluorescence threshold was crossed. This gave the relative quantity of each viral transcript in each sample. The signal in RT-negative controls, indicative of residual viral DNA, was subtracted from the total to give a cDNA-specific signal. In this study, only RT-PCR reactions yielding 10 or more copies of viral transcript per RT-PCR reaction were considered.

In situ hybridization. Digoxigenin (Boehringer Mannheim)-labelled riboprobes corresponding to MHV-68 vRNAs (tRNA-like transcripts) 1–4 were generated by T7 transcription of pEH1.4 (Simas et al., 1999). In situ hybridization was performed as previously described (Bowden et al., 1997). Briefly, 5 μm paraffin embedded sections were dewaxed in xylene, rehydrated through graded ethanol solutions, treated with 100 mg ml$^{-1}$ proteinase K for 10 min at 37°C and acetylated with 0-25% (v/v) acetic anhydride-0-1 M triethanolamine. Sections were hybridized with labelled riboprobes in 50% formamide, 1× SSC overnight at 55°C. The stringent wash (0-1× SSC, 30% formamide, 10 mM Tris pH 7-5) was carried out at 58°C. Hybridized probe was detected with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer Mannheim) according to the manufacturer’s instructions.

RESULTS

In order to investigate the role of the latency-associated ORF M2 in the pathogenesis of MHV-68, a frameshift mutant, M2FS, was constructed using a MHV-68 BAC pHA3 (Adler et al., 2000). The M2FS mutant contains an insertion of a single guanine creating a novel Apat recognition site at nt position 4604 with only the initial methionine at the N terminus remaining in-frame (Fig. 1a).
Verification of the introduced mutation was carried out by sequencing across the M2FS region in the BAC plasmid. To ascribe phenotypic alterations in M2FS to disruption of M2 rather than spurious mutations introduced during mutagenesis, a frameshift revertant virus, M2FSR, was generated from M2FS. The genomic structure of the mutant M2FS and M2FSR viruses was confirmed by restriction enzyme digestion of viral DNA and Southern blot hybridization (data not shown). The stability of the introduced mutation was checked further in viruses recovered from latently infected spleens (Fig. 1b). This analysis confirmed that insertion of a single guanine into M2FS was intact following in vivo infection.

**M2 is dispensable for lytic replication**

Two independent studies have investigated the role played by M2 in replication (Jacoby et al., 2002; Macrae et al., 2003). These studies demonstrated that recombinant viruses deficient in M2 replicate normally in tissue culture and display normal acute phase replication kinetics in lung tissue upon infection of mice. Consistent with these studies, no significant difference could be detected in the growth kinetics of the M2FS and M2FSR mutant viruses in comparison to wild-type MHV-68, derived from pH43 (Adler et al., 2001) (data not shown). Moreover, in agreement with the in vitro replication kinetics of the M2FS mutant, no significant difference was observed in the ability of this mutant or the M2FSR versus wild-type MHV-68 to productively replicate in lung tissue upon intranasal inoculation of mice (data not shown).

**M2 is required for efficient establishment of latency but dispensable for long-term latency**

To analyse the role played by M2 during the establishment and maintenance of latency, mice were infected with M2FS mutant or wild-type MHV-68 and the latent load in spleen was examined by quantification of reactivation-competent virus by infectious centre assay. The results obtained showed that early during the establishment of latency, 14 and 21 days post-infection, the numbers of infectious centres were reduced by about 10-fold for animals infected with M2FS in comparison with MHV-68 (Fig. 2). In contrast, at later time points, 43 and 71 days post-infection, the mean number of infectious centres was consistently higher for M2FS versus wild-type MHV-68 (Fig. 2a). In a separate experiment the phenotype of the M2FSR virus was examined in the spleen at 14 days post-infection and no difference was detected in the number of infectious centres in comparison to wild-type MHV-68, indicating a reverted phenotype (Fig. 2b).

**Disruption of M2 results in increased levels of virus persistence in GC B cells**

To evaluate further the role of M2 in latency, the frequency of virus genome-positive cells was determined for total B cells and GC B cells, the main targets for virus latency. The frequencies of infection obtained for wild-type MHV-68 and M2FSR were within the range of those previously reported (Marques et al., 2003). During the establishment of latency at 14 days post-infection, however, the frequency of M2FS DNA-positive cells in both cell populations analysed was approximately 10-fold lower in comparison with MHV-68 or M2FSR (Table 1). These data were consistent with the reduced levels of reactivation-competent M2FS mutant recovered from spleen. Notably, in contrast with events during the establishment of latency, late during infection, namely at 50 and 70 days post-infection, the frequency of virus DNA-positive GC B cells in M2FS-infected mice increased significantly (based upon a comparison of the 95% confidence intervals) contrasting with wild-type MHV-68 and M2FSR virus.

**M2 is required for effective colonization of splenic follicles**

We have shown that the small tRNA-like transcripts (vtRNAs) encoded by MHV-68 constitute a marker for latency providing a useful means to monitor the establishment of latent infection in splenic follicles (Bowden et al., 1997; Simas et al., 1999). Thus, we next sought to assess if the reduced virus latent load observed during the establishment of latency was because of an inability of the M2FS mutant to infect B-cell follicles or to a reduced expansion of latently infected GC B cells within B-cell follicles. In situ hybridization using a probe specific for vtRNA1–4 was performed in three randomly selected splenic sections per spleen from three mice infected with either wild-type MHV-68 or M2FS at each time post-infection. Sections were analysed for the number of vtRNA-positive follicles and vtRNA-positive cells within each follicle (Table 2). At
14 days post-infection a mean 35% of follicles were positive for vtRNAs in M2FS-infected animals versus 64% in wild-type MHV-68-infected mice. The *P* value (*P* = 0.056 as determined by the two-tailed unpaired *t*-test) for the hypothesis of equality of means of the mean percentage of vtRNA-positive follicles is close to the nominal level of 0.05.

### Table 1. Frequency of MHV-68 infection

Data obtained from pools of at least five spleens. *p.i.*, Post-infection; *ND*, not done.

<table>
<thead>
<tr>
<th>Cell subpopulation*</th>
<th>Days p.i.</th>
<th>Virus</th>
<th>Reciprocal frequency of viral DNA-positive cells†</th>
<th>Purity (%)‡</th>
<th>Cells Percentage§</th>
<th>Cells No. of viral DNA-positive cells¶</th>
</tr>
</thead>
<tbody>
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<td></td>
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</tr>
<tr>
<td>Total B cells</td>
<td>14</td>
<td>M2FS</td>
<td>2,301 (1,480–5,172)</td>
<td>98:3</td>
<td>52:2</td>
<td>1·0 × 10⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2FSR</td>
<td>489 (303–1,265)</td>
<td>98:4</td>
<td>51:6</td>
<td>1·0 × 10⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MHV-68</td>
<td>311 (178–1,238)</td>
<td>99:7</td>
<td>53:2</td>
<td>1·0 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>M2FS</td>
<td>937 (534–3,798)</td>
<td>99:4</td>
<td>50:4</td>
<td>1·0 × 10⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2FSR</td>
<td>28,342 (17,002–85,101)</td>
<td>99:3</td>
<td>51:8</td>
<td>1·0 × 10⁶</td>
</tr>
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<td></td>
<td></td>
<td>MHV-68</td>
<td>33,847 (19,883–113,737)</td>
<td>99:3</td>
<td>52:1</td>
<td>1·0 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>M2FS</td>
<td>934 (601–2,102)</td>
<td>98:7</td>
<td>51:9</td>
<td>1·0 × 10⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2FSR</td>
<td>&gt; 505,761</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>MHV-68</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC B cells</td>
<td>14</td>
<td>M2FS</td>
<td>625 (403–1,393)</td>
<td>98:2</td>
<td>4:7</td>
<td>9·4 × 10⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2FSR</td>
<td>65 (42–152)</td>
<td>98:5</td>
<td>6:2</td>
<td>1·2 × 10⁷</td>
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<tr>
<td></td>
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<td>MHV-68</td>
<td>100 (63–245)</td>
<td>95:1</td>
<td>6:8</td>
<td>1·3 × 10⁷</td>
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<td>50</td>
<td>M2FS</td>
<td>5 (3–10)</td>
<td>97:8</td>
<td>2:3</td>
<td>4·6 × 10⁶</td>
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<td></td>
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<td>M2FSR</td>
<td>592 (375–1,405)</td>
<td>95:9</td>
<td>2:3</td>
<td>4·6 × 10⁶</td>
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<tr>
<td></td>
<td></td>
<td>MHV-68</td>
<td>510 (325–1,181)</td>
<td>97:2</td>
<td>2:1</td>
<td>4·2 × 10⁶</td>
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<td></td>
<td>70</td>
<td>M2FS</td>
<td>40 (25–106)</td>
<td>94:3</td>
<td>3:7</td>
<td>7·4 × 10⁵</td>
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<td></td>
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<td>M2FSR</td>
<td>1,053 (670–2,452)</td>
<td>96:4</td>
<td>1:6</td>
<td>3·2 × 10⁵</td>
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<td></td>
<td></td>
<td>MHV-68</td>
<td>665 (413–1,713)</td>
<td>93:7</td>
<td>1:4</td>
<td>2·8 × 10⁵</td>
</tr>
</tbody>
</table>

*Total B cells (CD19⁺); GC B cells (CD19⁺ PNAhigh).
†Frequencies of infection were calculated by limiting dilution analysis with 95% confidence intervals (numbers in parentheses).
‡Purity of sorted cells assessed by FACS analysis.
§Percentage of each population of total spleen was determined by FACS analysis.
||Total number of cells were estimated from the percentage of total spleen, based on an estimate of 2 × 10⁸ cells per spleen.
¶Number of latently infected cells based on the frequency of latency within each cell type and the estimated total number of cells.

### Table 2. Quantification of viral load in spleen by in situ hybridization detection of vtRNAs

Data obtained from spleens of three mice. *p.i.*, Post-infection.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Days p.i.</th>
<th>Total no. follicles</th>
<th>vtRNA⁺ follicles (%)</th>
<th>Mean vtRNA⁺ follicles (%)</th>
<th>Mean no. vtRNA⁺ cells*</th>
<th>Mean vtRNA⁺ cells</th>
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<tbody>
<tr>
<td>MHV-68</td>
<td>14</td>
<td>22·5</td>
<td>67</td>
<td>64</td>
<td>18</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35</td>
<td>55</td>
<td>17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>31·5</td>
<td>70</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>92</td>
<td>2·7</td>
<td>1</td>
<td>0·65</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>91</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>84</td>
<td>2·7</td>
<td>1</td>
<td></td>
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</tr>
<tr>
<td>M2FS</td>
<td>14</td>
<td>39</td>
<td>33</td>
<td>10</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>46</td>
<td>19</td>
<td>35</td>
<td>29</td>
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<td>67</td>
<td>96</td>
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<tr>
<td></td>
<td></td>
<td>105</td>
<td>17</td>
<td>6·7</td>
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</table>

*Mean number of vtRNA⁺ cells per follicle.
significance. However, spleens from mice infected with M2FS when compared with spleens from MHV-68-infected mice did not show splenomegaly, which is a hallmark of MHV-68 infection. Thus, although only approximately twofold, a decrease in the number of M2FS-infected follicles correlated with absence of splenomegaly. The mean number of cells positive for vtRNAs within each follicle, however, was not different between spleens from mice infected with wild-type MHV-68 or M2FS. It is worthwhile noting that during the establishment of latency the approximately twofold reduction in the number of vtRNA-positive M2FS-infected follicles did not parallel the 10-fold deficit in infectious centres and frequency of virus genome-positive cells. This apparent discrepancy may reflect the fact that in situ hybridization analysis is restricted to those splenic cells with detectable vtRNA transcripts, thus, the data are not directly comparable.

In contrast to events during the establishment of latency, at 67 days post-infection, the mean percentage of positive follicles was 10-fold higher for spleens from animals infected with M2FS mutant in comparison with spleens from mice infected with wild-type MHV-68 or M2FS. Moreover, the mean number of vtRNA-positive cells per follicle was about 15-fold higher for M2FS-infected animals in comparison with wild-type MHV-68-infected mice. Moreover, the mean number of vtRNA-positive cells per follicle in mice infected with M2FS mutant did not decrease significantly \((P = 0.165\) as determined by the two-tailed unpaired \(t\)-test) between 14 and 67 days post-infection. These data indicate that although M2 is required for efficient colonization of B-cell follicles, it is dispensable for the expansion of latently infected cells within B-cell follicles. Moreover, disruption of M2 did not result in the normal decrease of latently infected cells within B-cell follicles, as observed for wild-type MHV-68, thus leading to abnormal high levels of virus persistence.

**Reduced levels of M2FS mutant in memory B cells**

We next sought to assess if the observed increased frequency of M2FS persistence in GC B cells resulted in a concomitant increase in the levels of infection established in memory B cells. To this end, the frequency of virus genome-positive cells was determined for GC B cells and memory B cells (Table 3). Enriched populations of GC B cells \((\text{IgG1/} \text{IgG}\text{2a/2b}^+ \text{IgD/} \text{IgM}^- \text{CD38}^\text{low})\) and memory B cells \((\text{IgG1/} \text{IgG}\text{2a/2b}^+ \text{IgD/} \text{IgM}^- \text{CD38}^\text{high})\) were obtained (Fig. 3) as previously described by others (Flano et al., 2002a). During the establishment of latency at 14 days post-infection, the ratio of the reciprocal frequencies of viral DNA-positive GC B cells versus memory B cells was 0:5 and 0:3 for wild-type MHV-68 and M2FS, respectively. Later in infection, however, the ratios were 0:9 and 0:08 for wild-type virus and M2FS mutant, respectively. That is, late in infection in comparison with MHV-68, a 20-fold increase in GC B cells harbouring M2FS resulted in only a twofold increase in frequency of infection in memory B cells. These data showed that the sustained high levels of M2FS persistence in GC B cells were not accompanied by a simultaneous increase in the levels of virus persistence in memory B cells indicating that M2 is important for efficient exiting of infected GC B cells into the memory compartment.

<table>
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<th>Table 3. Frequency of MHV-68 infection in GC and memory B cells</th>
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*Frequencies of infection were calculated by limiting dilution analysis with 95% confidence intervals (numbers in parentheses). †GC B cells \((\text{IgG1/} \text{IgG}\text{2a/2b}^+ \text{IgD/} \text{IgM}^- \text{CD38}^\text{low})\); memory B cells \((\text{IgG1/} \text{IgG}\text{2a/2b}^+ \text{IgD/} \text{IgM}^- \text{CD38}^\text{high})\).
Absence of M2 does not significantly alter the transcriptional programme of MHV-68 during persistent infection of GC B cells

In the first instance gene transcription of ORFs adjacent to the M2 gene in the M2FS virus was analysed to assess the influence of the introduced frameshift mutation (Fig. 4a). Lytic infection of NIH 3T3 cells showed that there were no significant differences in the transcriptional profile of M2 neighbouring ORFs, namely M1, M3, M4 and 4, between the M2FS, wild-type MHV-68 or M2FSR. Moreover, transcription of M2 in the M2FS mutant was also not changed when compared to its revertant virus or wild-type MHV-68.

Next, we sought to assess if the absence of M2 led to a change in the previously reported virus transcription programme in GC B cells (Marques et al., 2003). To this end, RNA extracted from FACS purified GC B cells derived from mice at 49 days post-infection with M2FS was analysed. The virus ORFs investigated corresponded to those previously analysed by us (Marques et al., 2003) and predicted to encode immune evasion or latency functions (Fig. 4). In order to control for patterns of lytic infection we have included ORF50, an immediate-early gene that has been shown to play a central role both in the initiation of lytic replication and reactivation from latency (Liu et al., 2000; Wu et al., 2000, 2001), and ORFs 6 and M7, which encode for an early single-stranded DNA-binding protein (Virgin et al., 1997) and a late structural glycoprotein (Stewart et al., 1996), respectively. The lack of detectable transcripts for ORFs 50 and M7 was consistent with an overall pattern of M2FS latent infection. This interpretation is further supported by the fact that transcripts corresponding to M9, which is one of the most abundant transcripts present during lytic infection (Ahn et al., 2002; Ebrahimi et al., 2003; Marques et al., 2003; Virgin et al., 1999), were not detected. Thus, despite the high frequency of infection in GC B cells, the M2FS transcription programme was consistent with a pattern of latent infection. Moreover, absence of M2 did not significantly alter the previously reported pattern of wild-type MHV-68 transcription in latently infected GC B cells (Marques et al., 2003). As for M2, the observed selective expression of ORFs M1, M3, M4, K3 and 73 is expressed during latency, unless it plays a role in immune evasion. It is not immediately apparent why ORF6 is expressed during latency, unless it plays a role in replication of viral episomes.

**DISCUSSION**

In this study, we have evaluated the role played by the M2 latency-associated gene following intranasal infection of mice by generating a defective mutant virus. Disruption of M2 was achieved by the insertion of a frameshift mutation in M2 with only the first 8 aa at the N terminus remaining in-frame. This mutation did not alter transcription of M2 or of its neighbouring genes and, as previously reported (Jacoby et al., 2002; Macrae et al., 2003), did not affect the ability of the virus to cause productive lytic infection both in vitro or in vivo in the lungs of mice following intranasal inoculation. The non-essential role of M2 for efficient lytic replication contrasted with its requirement for latent infection. As previously shown by others (Jacoby et al., 2002; Macrae et al., 2003), we confirmed that disruption of M2 resulted in reduced levels of reactivation-competent virus during the establishment of latency in spleen. This deficit paralleled a concomitant decrease in the frequency of M2FS DNA-positive total B cells and GC B cells, indicating that M2 is not required for efficient reactivation from latency. In agreement with our results, a recent study by Macrae et al. (2003) reported a positive correlation between viral DNA-copy number and infectious centre assays during the establishment of latency in

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**Fig. 4.** (a) Insertion of a frameshift mutation in M2 does not affect transcription of neighbouring genes. RNA was extracted from NIH 3T3 cells infected with MHV-68, M2FS and M2FSR at an m.o.i. of 5 p.f.u. per cell and virus transcription analysed at 12 h post-infection. The data are presented as the number of viral transcripts normalized for one copy of the housekeeping gene Hprt. (b) Selective M2FS gene transcription in persistently infected GC B cells. RNA was extracted from GC B cells, FACS purified from pools of five spleens at 49 post-infection and M2FS transcription quantified from two independently synthesized cDNA samples. Data are expressed as the mean number of viral transcripts per infected cell normalized to one copy of Hprt.
the spleen. Using in situ hybridization to monitor virus latency within splenic follicles during the establishment of latency, we showed that disruption of M2 resulted in a reduced number of infected follicles. Notably, the mean number of latently infected cells per infected follicle was equivalent between M2FS and wild-type MHV-68. Hence, these data demonstrated that during the establishment of latency, M2 is required for efficient colonization of splenic follicles but is not necessary for expansion of latently infected GC B cells, a hallmark of MHV-68 infection.

The role played by M2 following the establishment of latency in the spleen was evaluated next. Remarkably, at late times post-infection disruption of M2 resulted in an increase in the frequency of virus-positive total B cells and GC B cells. In agreement, and consistent with a non-reactivation deficit phenotype, the levels of M2FS infectious centres, the percentage of infected follicles and the mean number of infected cells per follicle late during infection were higher than those obtained for M2FSR and MHV-68. The finding that disruption of M2 leads to increased levels of infectious centres at late times post-infection has been previously reported (Macrae et al., 2003). Notably, the sustained high levels of M2FS persistent infection of GC B cells did not result in a concomitant increase in the frequency of infection in memory B cells. This finding suggests that M2 might facilitate entry of infected cells into the memory B-cell pool.

In the absence of M2, the high levels of infection of GC B cells could in part reflect an overall cessation of GC reaction or be a consequence of an increased number of follicles infected. However, in situ hybridization analysis revealed that the mean number of infected follicles and the mean number of infected cells within each follicle remained stable throughout infection. These data were further evidence that absence of M2 does not impair expansion of latently infected GC B cells.

Analysis of M2FS transcription during persistent infection of GC B cells revealed a selective pattern of virus transcription coherent with an overall state of latent infection. Amongst those genes transcribed during M2FS persistent infection, ORF73 showed the highest number of detectable transcripts. This result is consistent with previous data showing that during the establishment of MHV-68 latency in GC B cells, transcripts corresponding to ORF73 were also the most abundant (Marques et al., 2003). Recently it has been shown that in the absence of ORF73, MHV-68 is unable to establish latent infection in the spleen (Fowler et al., 2003; Moorman et al., 2003). Thus, ORF73 transcription during persistent M2FS infection of GC B cells is further evidence that, like for ORF73 of KSHV and herpesvirus saimiri, ORF73 of MHV-68 is involved in genome episome maintenance in proliferating cells, namely latently infected GC B cells.

During long-term persistent infections, herpesviruses typically show low levels of transcription activity. In the case of EBV it has been proposed that infected cells enter the long-lived memory pool by shutting down transcription of all the viral latent-proteins, probably a pre-requisite for evasion from immune system elimination (Thorley-Lawson, 2001). Therefore, it is somehow surprising that in the absence of M2, MHV-68 could persist at high levels in GC B cells, although in a restricted fashion, in nevertheless a transcriptionally active state. It is possible that immune evasion activity afforded by MHV-68 is sufficient to avoid immune clearance; namely that mediated by the viral-encoded M3 chemokine-binding protein and MK3 protein that has been implicated in CD8+ CTL evasion (Bridgeman et al., 2001; Stevenson et al., 2002). Another factor that might be involved in facilitating M2FS persistence could be attributed to the absence of M2, itself an important target for CD8+ CTL control of infection (Usherwood et al., 2000, 2001).

It is not immediately apparent why disruption of M2 results in the observed phenotype. It is possible that M2 is required for infected B cells to enter splenic follicles. Once inside follicles, however, M2 is dispensable for the expansion of latently infected cells. The combined observation of sustained high levels of persistent M2FS infection in GCs and the lower frequency of infection of memory B cells relative to GC B cells may be indicative that M2 is thereafter required for the transition of latently infected cells into the long-lived memory B-cell compartment and eventual cessation of virus driven expansion of infected GC B cells. Future experiments directly addressing the molecular function of M2 and analysing further the kinetics and tropism of infection of M2-deficient recombinant viruses will elucidate further the role played by this gene product in infection. In particular, they will reveal if M2, in a manner functionally similar to the EBV LMP1 and LMP2a, can modulate B-cell function by providing surrogate signals necessary for antigen-independent activation of naïve B cells.

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**REFERENCES**


M2 and MHV-68 latency


