INTRODUCTION

Apoptosis is both an ancient and a fundamental final common pathway in the cellular response to stress (Rich et al., 1999). Viral infection stresses cells in many ways and is consequently a strong pro-apoptotic stimulus, leading many, if not all, viruses to evolve specific inhibitors of apoptosis (Hardwick, 1998). Inhibiting apoptosis during lytic-cycle replication extends the window available for virion production. The gammaherpesviruses also inhibit apoptosis in latency, in order to prolong the survival of infected lymphocytes that have been driven to high levels of proliferation. This proliferation is a central component of gammaherpesvirus host colonization (Coleman et al., 2003). It is probably also a major factor in gammaherpesvirus-associated tumorigenesis. Viral genes that inhibit apoptosis in latently infected cells therefore have clinical importance as potential therapeutic targets.

Several Epstein–Barr virus (EBV) latency gene products have been reported to inhibit apoptosis, including EBNA-1 (Kennedy et al., 2003), EBNA-2 (Lee et al., 2002), LMP-1 (Henderson et al., 1991) and LMP-2A (Fukuda & Longnecker, 2004). Anti-apoptotic activity has also been ascribed to the Kaposi’s sarcoma-associated herpesvirus (KSHV) gene products LANA (Friborg et al., 1999), K15 (Sharp et al., 2002), v-FLIP (Thome et al., 1997) and K7 (Feng et al., 2002; Wang et al., 2002). In addition to these relatively poorly conserved genes, all gammaherpesviruses encode a homologue of cellular bcl-2 (Hardwick, 1998; Cuconati & White, 2002). The EBV bcl-2 homologue is a lytic, rather than a latent, gene product (Pearson et al., 1987); in latency, EBV inhibits the mitochondrial pathway of apoptosis by inducing cellular bcl-2 expression (Henderson et al., 1991). The KSHV bcl-2 homologue, encoded by ORF16 (Cheng et al., 1997; Sarid et al., 1997), is similarly induced by tetradecanoyl phorbol acetate treatment of latently infected tumour cells (Sarid et al., 1998; Sun et al., 1999). However, ORF16 transcripts remain detectable in the absence of induction (Sarid et al., 1997), suggesting that KSHV may also use its bcl-2 homologue to inhibit apoptosis in latency.

Murine gammaherpesvirus 68 (MHV-68) is a natural murid pathogen that provides an opportunity to identify the in vivo function of a gammaherpesvirus bcl-2 homologue. This function is not necessarily the same in different gammaherpesviruses, but the fact that some kind of bcl-2 homologue has been maintained over tens of millions of years of viral evolution means that broad parallels are likely. M11 transcripts have been detected in lytically infected fibroblasts by RT-PCR (Virgin et al., 1999; Roy et al., 2000; Marques et al., 2003) and by microarray analysis (Martinez-Guzman et al., 2003), but not by Northern blot (Virgin et al., 1999; Roy et al., 2000), suggesting that lytic-cycle M11 transcription occurs at only a low level. Interestingly, the M11 microarray signal in
MHV-68-infected fibroblasts is resistant to cycloheximide, which, in conjunction with the identification of M11 transcripts in latently infected S11E cells (Martinez-Guzman et al., 2003), suggests that the M11 promoter is active in latency, as well as in the lytic cycle. In vivo, M11 transcripts have been found in peritoneal cells, but not spleens, of B-cell-deficient mice 6 weeks post-infection (Virgin et al., 1999); in mediastinal lymph nodes (Rochford et al., 2001); in the spleens and lungs of persistently infected, immunocompetent mice (Roy et al., 2000); and in macrophages, dendritic cells, newly formed B cells and marginal-zone B cells at the peak of splenic latency (Marques et al., 2003). These data are consistent with M11 being transcribed in at least some forms of in vivo MHV-68 latency.

M11 function has been analysed in vitro by overexpressing the isolated gene in fibroblasts. In this setting, M11 inhibits the apoptosis induced by either Fas ligation (Wang et al., 1999), tumour necrosis factor alpha treatment (Wang et al., 1999; Roy et al., 2000) or Sindbis virus infection (Bellows et al., 2000). In vivo, M11-deficient MHV-68 shows normal lytic replication and latency establishment after intraperitoneal infection, but reduced reactivation from latency (Gangappa et al., 2002). This latter conclusion was based on reduced numbers of infectious centres in the presence of normal viral DNA loads. The present analysis, begun prior to the report of Gangappa et al. (2002) and using the perhaps more physiological intranasal route of infection, identifies an M11-associated deficit in MHV-68 latency establishment. Stable, long-term latency levels were unaffected, but the peak latent load was reduced. In our hands, there was no evidence of a deficit in ex vivo reactivation from latency. M11 might yet have a role in in vivo reactivation from latency. However, the main impact of a disrupted bcl-2 homologue following intranasal infection of immunocompetent mice was reduced latency amplification in lymphoid germinal centres.

**METHODS**

**Mice.** Female BALB/c and C57BL/6 mice were purchased from Harlan UK and housed at the Biological Services Unit of the Cambridge University School of Veterinary Sciences, UK, or were bred at the Gulbenkian Institute for Science, Portugal. Fas-deficient mice (Adachi et al., 1995) were obtained from Dr R. Budjosjo (University of Cambridge School of Veterinary Medicine, UK). Mice were infected intranasally at 6–8 weeks of age with 2 × 10⁴ p.f.u. virus in a volume of 30 μl. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986, and protocols were approved by the Home Office project licence 80/1579.

**Cells and viruses.** Baby hamster kidney cells (BHK-21, ATCC CCL-10), COS-7 cells (ATCC CRL-1651), NIH-3T3-CRE cells (Stevenson et al., 2002) and murine embryonic fibroblasts (MEFs) harvested at 13–14 days gestation were all grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 2 mM glutamine, 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ and 10% fetal calf serum (FCS; PAA Laboratories) (complete medium). Medium for MEFs was further supplemented with 50 μM 2-mercaptoethanol. All virus stocks were grown and titrated in BHK-21 cells.

**Virus titres.** Lungs were homogenized in complete medium, frozen, thawed and sonicated. Tissue debris was pelleted by brief centrifugation (1000 g, 1 min). Infectious virus in homogenate supernatants was measured by plaque assay of tenfold dilutions of lung homogenates on MEFs, as described previously (de Lima et al., 2004). After 5 days, monolayers were fixed in 10% formaldehyde and stained with 0.1% toluidine blue. Plaques were counted with a plate microscope. Latent virus was measured by explant culture of single-cell suspensions of spleens, overlaid onto MEF monolayers and cultured in complete medium with 0.3% carboxymethylcellulose. The monolayers were fixed and stained after 6 days.

**Viral genome quantification.** The viral genome load in individual spleens was measured by real-time PCR. DNA was extracted from each spleen (Wizard genomic DNA purification kit; Promega) and a portion of the MK3 ORF (genomic coordinates 24832–25071) was amplified by PCR from 10 ng DNA over 50 cycles (Rotor Gene 3000; Corbett Research). PCR products were quantified by using Sybr green (Invitrogen) and compared with a standard curve of cloned MK3 template, diluted serially in uninfected cellular DNA and amplified in parallel. The MK3 copy number in spleen samples was calculated from the cycle number at which the Sybr green signal crossed a set threshold on the standard curve. Amplified products were distinguished from paired primers by melting-curve analysis. The correct size of the amplified products was confirmed by electrophoresis and staining with ethidium bromide. We determined the frequency of viral genome-positive cells in pooled spleen samples as described previously (Marques et al., 2003). Briefly, cells pooled from five spleens were either used directly or sorted (MoFlo; Cytomation) for germinal-centre B cells (>95% CD19⁺ PNAhi). Twofold dilutions of cells (eight replicates each) were then lysed overnight with SDS/protease K, denatured and used to amplify genomic coordinates 24832–25071 (Lightcycler; Roche Diagnostics). Quantification was based on comparison with a standard curve of MK3 plasmid dilutions, amplified in parallel. Specificity of all amplified products was confirmed by melting-curve analysis using internal, fluorochrome-labelled oligonucleotides. The frequency of genome-positive cells was then calculated from a regression plot of input cell number against the fraction of negative samples.

**In situ hybridization.** Cells expressing viral tRNAs 1–4 were detected by in situ hybridization of formalin-fixed, paraffin-embedded spleen-cell sections with a digoxigenin-labelled riboprobe transcribed from pEH1.4 as described previously (Bowden et al., 1997). Hybridized probe was detected with alkaline phosphatase-coujugated anti-digoxigenin Fab fragments (Boehringer Ingelheim), according to the manufacturer’s instructions.

**Viral mutagenesis.** We generated two M11 mutations by using the MHV-68 bacterial artificial chromosome (BAC) (Adler et al., 2000). First, we used Rec E/T cloning to interrupt the M11 ORF (genomic coordinates 103418–103933). A kanamycin-resistance gene (kan R) flanked by flip recombinase recognition sites was amplified from the plasmid pCP15 by PCR using the primers 5’-TCTCTACATCAT- CAAACATGACATGA-3’ and 5’-AACGAGCTATGACCATG- TGCAGAGCTTCCATTTCCAGTCTTGAGCCGGCCGAGGTTTTGCCAGTCCAGGT-3’. The underlined sequences are homologous to nt 103401–103930 and 103650–103601 of the MHV-68 genome, respectively (Virgin et al., 1997). The PCR product was electroporated into Escherichia coli JCB679 containing the MHV-68 BAC. Rec E/T-mediated recombination between the viral genome and the 50 bp sequences at each end of the PCR product inserted the kan R gene into the BAC in place of genomic coordinates 103451–103600. DNA was extracted from kanamycin-resistant colonies, screened by restriction-enzyme digestion and electroporated into E. coli DH10B (M11 Δ M11). We then removed the kan R-encoding sequence with the flip recombinase expression plasmid pCP20 (Adler et al., 2000),

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leaving a single flp recognition site plus short flanking plasmid sequences (167 bp in total), in place of the 150 bp genomic deletion (M11“K”). This also introduced a stop codon to terminate M11 translation after 11 aa. Secondly, an oligonucleotide encoding stop codons in all reading frames and BamHI and EcoRI restriction sites (underlined) (5’-CTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGTACG-3’) was ligated into a BamHI site at genomic coordinate 103751 (M11“C”). To do this, the BamHI site in pACYC184 (New England Biolabs) was destroyed by digestion with BspHI, blunting with T4 DNA polymerase (New England Biolabs) and religation back to itself. A BamHI-G genomic fragment (Efstathiou et al., 1990) (genomic coordinates 101653–106902) was then cloned into the modified pACYC184. This vector was digested with BspHI and dephosphorylated (Paenibacillus borealis alkaline phosphatase; Roche Diagnostics). The oligonucleotide was heated, reannealed to itself, phosphorylated with polynucleotide kinase (New England Roche Diagnostics). The oligonucleotide was heated, reannealed to itself, phosphorylated with polynucleotide kinase (New England Biolabs) and ligated into the BspHI site. Thus, the M11 coding sequence was truncated after 111 aa. The mutant BamHI-G fragment was then subcloned into the Smal site of the shuttle plasmid pST76K-SR by using EcoRV, which cleaves at genomic coordinate 106316 within BamHI-G and within pACYC184. The oligonucleotide insertion in M11 was then recombined into the MHV-68 BAC by standard methods (Adler et al., 2000). We also cloned the native BamHI-G fragment into pST76K-SR and reverted each M11 BAC mutant to wild-type in the same way. All BACs were reconstituted to infectious virus by transfecting 5 μg BAC DNA into BHK-21 cells using Fugene-6 (Roche Diagnostics). The loxP-flanked BAC cassette was removed by viral passage through NIH-3T3-CRE cells until cells positive for green fluorescent protein (GFP) were no longer visible.

**Plasmids.** Full-length M11 was amplified by PCR from wild-type MHV-68 BAC DNA, including EcoRI and XhoI sites in the respective 5’ and 3’ primers. The PCR product was cloned into pEGFP-N3 (Becton Dickinson) such that M11 was in-frame with the upstream

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**Fig. 1.** Generation of M11-deficient MHV-68. (a) The M11“C” mutant was made by inserting an oligonucleotide with multiple stop codons and an EcoRI restriction site into a BspHI site in the M11 coding sequence. An M11“K”+ mutant was made by recombining a 1.4 kb prokaryotic kanamycin-resistance cassette flanked by flp recombinase recognition sites into the M11 coding sequence. The cassette was then excised with flp recombinase (M11“K”+) to leave a small (167 bp) insert in M11 that included EcoRI, SacI and XhoI restriction sites and in-frame stop codons. (b) Southern blot analysis of viral DNA from wild-type (WT), M11“K” (K), M11“K”+ (K+) and revertant (REV) MHV-68. The probe was generated by random-prime labelling of the BamHI-G genomic fragment (genomic coordinates 101653–106902). The predicted bands for the WT and REV viruses are: EcoRI, 10 831, 5186 and 949 bp; SacI, 12 808 and 3581 bp; and XhoI, 9314, 2019 and 424 bp. EcoRI digestion of the M11“FRT-K” mutant cuts the WT 5186 bp band into 4750 and 437 bp fragments (4750 and 1777 bp with M11“FRT-K”). SacI digestion of the M11“FRT-K” mutant cuts the WT 12 808 bp band into 11 260 and 1556 bp fragments (11 260 and 2696 bp with M11“FRT-K”). XhoI digestion of either the M11“FRT-K” or M11“FRT-K”+ mutants cuts the WT 9314 bp band into 6761 and 2571 bp – an additional 1390 bp band with the K+ virus is not visible, as it is derived entirely from the kanR cassette. (c) Southern blot analysis of viral DNA from wild-type (WT), M11“C” and revertant (REV) viruses, using a BamHI-G probe as in (b). In the M11“C” virus, the predicted EcoRI digestion products are 10 831, 4600, 949 and 586 bp.
enhanced GFP (eGFP)-encoding sequence. M11 with stop codons after aa 111 was amplified from M11−C BAC DNA by using the same primers and cloned in parallel into pEGFP-N3. All constructs were confirmed as being correct by DNA sequence analysis.

Flow cytometry. For assays of apoptosis, MEFs were trypsinized, washed in PBS/0.1% BSA and stained for 1 h on ice with fluorescent isothiocyanate (FITC)-coupled Annexin-V. Cells were washed twice and propidium iodide was added (1 μg ml−1) before analysis. For assays of B-cell activation, splenocytes were disrupted into single-cell suspensions, washed once in PBS/0.1% BSA and incubated for 30 min on ice with 5% mouse serum, 5% rat serum and anti-CD16/32 mAb (Becton Dickinson). Specific staining was done with phycoerythrin-conjugated anti-CD19 and FITC-coupled anti-CD69 (Becton Dickinson). After 1 h incubation on ice, cells were washed twice in PBS/BSA (0-1%) and analysed on a FACScalibur using CellQuest software (Becton Dickinson). Data were analysed with FCSPress v1.3 (www.fcspress.com).

RESULTS

Generation of M11-deficient viruses

We disrupted the M11 coding sequence in two ways (Fig. 1a). Firstly, a kanamycin-resistance gene (M11−K+) or a small non-coding insert (M11−K−) was used to terminate M11 translation after 11 aa. Secondly, a more subtle mutation was made in a different site, inserting an oligonucleotide to terminate M11 translation after 111 aa (M11−C). The correct genomic structures of both mutant viruses and revertants of each mutant were confirmed by restriction-enzyme digestion (Fig. 1b and c).

Deletion of the M11 C-terminal domain alters its localization in the cell

The M11−C mutation left the conserved BH1 domain of M11 intact, but removed its hydrophobic C-terminal domain (aa 151–171). The equivalent C-terminal domain of the myxoma virus anti-apoptotic protein M11L is required for localization to the mitochondrial membrane and, consequently, for protein function (Everett et al., 2000). To test this for the M11−C mutant, we fused the mutant and wild-type forms of M11 to N-terminal eGFP and transfected them into COS-7 cells (Fig. 2). The eGFP–M11 fusion protein showed punctate, cytoplasmic fluorescence, consistent with localization to mitochondrial membranes. In contrast, the eGFP–M11−C protein showed fairly homogeneous fluorescence throughout the nucleus and cytoplasm, in a pattern equivalent to eGFP alone. Truncating M11 upstream of its C-terminal membrane anchor was therefore sufficient to disrupt its cellular localization.

M11 is dispensable for MHV-68 lytic replication

As established cell lines tend to be inherently resistant to apoptosis, we tested virus replication in primary MEFs (Fig. 3). No reduction was seen in the replication of M11−K−, M11−K+ (Fig. 3a) or M11−C (Fig. 3b) viruses compared to wild-type or revertant. In order to lower the threshold for cellular apoptosis, we also assayed virus replication with cells cultured in reduced (0.1%) FCS (Fig. 3c). Virus replication was impaired, as evident from the lower titres that were attained but, again, no difference was observed between M11− and M11+ viruses.

The implication of normal virus replication in vitro was that, in this setting, M11 is not required to prevent premature cellular apoptosis. This was confirmed by Annexin-V staining of infected cells (Fig. 4). MEFs were stained with Annexin-V and propidium iodide 8 h after infection (5 p.f.u. per cell) with wild-type, M11−K− or revertant viruses. This time point corresponds to the start of infectious virus release after high-multiplicity infection (de Lima et al., 2004). As a positive control, MEFs were cultured for 8 h in serum-free medium. There was little sign of apoptosis in any of the infected cells. We also found no increased apoptosis in the absence of M11 after overnight infection of MEFs (data not shown). MHV-68 can therefore inhibit any pro-apoptotic effects of lytic infection in MEFs, independently of M11. This was
consistent with the M11 mutant viruses showing no in vitro growth deficit. The M11 gene product was also dispensable for lytic-cycle replication after intranasal inoculation of C57BL/6 mice (Fig. 5a).

**MHV-68 latency amplification is impaired in the absence of M11**

After lytic virus replication in alveolar epithelial cells, intranasal MHV-68 seeds to lymphoid tissue. Then follows the virus-driven proliferation of latently infected B cells that results in latency amplification, which is quantitatively the most important event in host colonization (Coleman et al., 2003; Fowler et al., 2003; Moorman et al., 2003; May et al., 2004). In contrast to lytic replication, latency amplification was impaired in the absence of M11 (Fig. 5). This manifested as a reduced peak number of infectious centres with the M11 K- mutant in both C57BL/6 (Fig. 5a) and BALB/c (Fig. 5b) mice. The M11-truncation mutant similarly showed lower peak levels of infectious centres, despite normal seeding to the spleen (Fig. 5c). There was no difference in splenic latency between M11- and M11+ viruses at later times (Fig. 5b). Thus, there was a selective deficit in latency amplification, rather than in the maintenance of latency or in reactivation, which would give a consistently lower number of infectious centres.
In addition to lower infectious-centre titres in the spleens of mice infected with the M11 mutant viruses, there was less B-cell activation (Fig. 6a) and lower viral genome loads (Fig. 6b). B-cell activation correlates with MHV-68 infection both in vitro and in vivo (Stevenson & Doherty, 1999; Flanó et al., 2000) and so provides an indication of the extent of host colonization, independently of any requirement for ex vivo viral gene expression or for further manipulation, such as DNA extraction. There was a clear correlation between these three assays (Fig. 6c), arguing that the reduced infectious-centre titres were not due to a reactivation deficit, but to a reduced amount of latent virus. Flow-cytometric sorting of cells prior to viral genome quantification (Fig. 6d) indicated that the major deficit was in B-cell colonization.

As a further measure of viral latency, we assayed viral tRNA expression in spleen sections by in situ hybridization (Fig. 7). The function of the viral tRNA homologues (vtRNAs) is unknown, but they are expressed in the absence of known lytic transcripts in lymphoid germinal centres (Bowden et al., 1997) and therefore provide a means of tracking latent virus. M11-deficient viruses showed a significant reduction in the number of vtRNA + splenic germinal centres at 14 days post-infection, consistent with the lower latent viral loads. There were both fewer vtRNA + follicles as a proportion of the total counted and reduced levels of colonization of the individual follicles that were vtRNA +.

**Fas deficiency does not rescue M11-deficient MHV-68**

As cells transfected with M11 are resistant to Fas-mediated apoptosis in vitro (Wang et al., 1999), we tested whether M11-deficient MHV-68 would show the same deficit in Fas-deficient mice (Fig. 8). A significant defect in peak latency titres remained, arguing that inhibition of Fas-mediated apoptosis was not the major in vivo function of M11. It is more likely that M11 contributed to the survival of latently infected, activated B cells in lymphoid germinal centres by inhibiting the mitochondrial apoptosis pathway.

**DISCUSSION**

Relating the biochemical functions of genes to their roles in natural infection continues to provide a major challenge in the understanding of complex pathogens. That all viral genes contribute to the fitness of gammaherpesviruses is not in doubt; the compactness of viral genomes implies a strong selective pressure to squeeze the maximum function out of a limited coding capacity. The key questions are...
where each gene acts in the viral life cycle and how important that function is for normal host colonization. The answers to these questions should identify suitable targets for antiviral therapy.

In agreement with Gangappa et al. (2002), we found no evidence for M11 contributing to MHV-68 lytic replication in vitro. This also applied to lytic replication in infected lungs. Although M11 can inhibit the apoptosis of fibroblasts when expressed from a plasmid (Wang et al., 1999; Roy et al., 2000; Bellows et al., 2000), this did not appear to be important in the context of viral infection, either because M11 is not expressed at a sufficient level or because MHV-68 relies mainly on other inhibitors of apoptosis during lytic-cycle replication. No MHV-68 apoptosis inhibitors, apart from M11, have been identified, but the abundant precedents in other herpesviruses would argue that additional inhibitors are inevitable.

In contrast to Gangappa et al. (2002), we found no evidence

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**Fig. 6.** Reduced M11<sup>−</sup> infectious-centre titres correlate with reduced B-cell activation and reduced viral genome loads. (a) Splenic B-cell activation was assessed by CD69 expression 14 days after intranasal infection of BALB/c mice with wild-type (WT), M11<sup>−K</sup> or revertant (REV) viruses. The dashed line shows the level of CD69 expression in age-matched naive mice. CD69 expression was significantly reduced with the M11<sup>−K</sup> virus, compared to wild-type (P < 0.001) or the revertant (P < 0.02). (b) Viral genome loads in the spleens of BALB/c mice 14 days after infection were relatively variable between individual mice, but still showed a significant reduction with the M11<sup>−K</sup> mutant compared to wild-type (WT) or revertant (REV) (P < 0.001). (c) We measured infectious centres, viral genome load and B-cell CD69 expression in spleens of C57BL/6 mice 14 days after intranasal infection with wild-type virus, the M11 mutants (M11<sup>−K</sup>, M11<sup>−C</sup>) or their respective revertants (K-REV, C-REV). All measures of latent viral load were reduced when M11 was disrupted. Mean ± SD values for five individual mice in each group are shown. (d) Spleen cells were pooled from five BALB/c mice 14 days after intranasal infection with the M11<sup>−K</sup> virus or its revertant. These cells were assayed either unfractinated or after flow-cytometric sorting to purify (> 95%) CD19<sup>+</sup>PNA<sup>+</sup> germinal-centre (GC) B cells. The frequency of viral genome-positive cells was measured by limiting dilution and a Scatchard plot of negative wells (%) against input cell number.
This discrepancy presumably reflects different in vitro assay conditions. It is important to note that explanted murine B cells generally show poor viability, which may be why they reactivate MHV-68 much less efficiently than dendritic cells or macrophages (Marques et al., 2003). Clearly, any gene that prolongs the survival of latently infected cells could potentially increase the efficiency of ex vivo reactivation. The question is whether the B-cell apoptosis that would otherwise occur is relevant to viral reactivation in vivo. Our data do not rule out an important role for M11 in reactivation in vivo – no current assays measure this process directly – but, instead, they identify an M11-associated deficit in latency amplification. Such a deficit would presumably also reduce the capacity of MHV-68 to cause chronic disease (Gangappa et al., 2002).

The lymphoproliferative amplification of MHV-68 latency is proving to be highly complex. Key roles have been identified for the viral ORF73 episome-maintenance protein (Fowler et al., 2003; Moorman et al., 2003), M2 [a protein of unknown function (Jacoby et al., 2002; Macrae et al., 2003; Simas et al., 2004)] and MK3, a CD8+ T-cell evasion protein (Stevenson et al., 2002). There may also be a role for the M3 chemokine-binding protein (Bridgeman et al., 2001), although this remains controversial (van Berkel et al., 2002). Our data indicate that normal latency amplification also requires M11.

An M11-associated latency deficit was possibly not observed by Gangappa et al. (2002) because they used intraperitoneal infection, which bypasses normal mucosal barriers. Crucially, intraperitoneal virus reaches the spleen when there is little immunity to lytic antigens, allowing lytic viral spread between B cells (Weck et al., 1996) and less dependence on latency amplification. After intranasal infection, the initial epithelial infection triggers an immune response and infectious virus remains essentially undetectable in lymphoid tissue (Sunil-Chandra et al., 1992). The natural route of MHV-68 infection is unknown, but the intranasal route does at least reproduce the requirement for mucosal

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**Fig. 7.** Detection of viral tRNA expression in spleens by in situ hybridization. Mice were infected intranasally with M11− and M11+ viruses as indicated. Fourteen days later, spleens were removed and processed for in situ hybridization using an antisense riboprobe for viral tRNAs 1–4. Representative pictures of infected germinal centres are shown. The table gives mean ± SD counts of tRNA+ germinal centres from four mice in each group. At least four separate spleen sections (>20 follicles per section) were counted for each mouse.

**Fig. 8.** Colonization of Fas-deficient mice by M11− and M11+ viruses. Fas-deficient mice were infected intranasally as indicated. Latent virus was titrated by infectious-centre (IC) assay 14 days later. M11−K− virus titres remained significantly below those of the wild-type (WT; P < 0.001) and revertant (REV; P < 0.03) viruses. Circles show individual mice and bars show the means of each group.
infection to precede lymphoid infection. Notably, M2-deficient MHV-68 shows a defect in latency amplification after intranasal, but not after intraperitoneal, infection (Jacoby et al., 2002).

An obvious function for M11 would be to promote germinal-centre B-cell survival, as germinal-centre B cells are both a major site of MHV-68 latency (Bowden et al., 1997; Flávio et al., 2002; Marques et al., 2003; Willer & Speck, 2003) and a site of action of pro-apoptotic and anti-apoptotic cellular bcl-2 family members (Marsden & Strasser, 2003). The marked reduction in the prevalence of viral genome-positive germinal-centre B cells in the absence of M11 (Figs 6d and 7) was consistent with such a function. However, evidence for M11 transcription in germinal-centre B cells is limited (Marques et al., 2003). M11 could act instead in latently infected B cells before they acquire a germinal-centre phenotype. Germinal-centre B cells themselves may no longer require M11 if MHV-68, like EBV, induces cellular bcl-2 expression. M11 has an advantage of over cellular bcl-2 in having no pro-apoptotic advantage of over cellular bcl-2 in having no pro-apoptotic caspase-cleavage products (Bellows et al., 2000), but in germinal-centre B cells, there may be a strong, counterbalancing selective pressure to maintain immunological silence by minimizing viral gene expression.

Fas is also a major mediator of germinal-centre B-cell apoptosis (Guzman-Rojas et al., 2002). Despite the predictions of in vitro data (Wang et al., 1999), M11 did not appear to act by Fas inhibition in vivo (Fig. 8), which may be consistent with the lack of M11 mRNA in germinal-centre B cells. MHV-68 lacks a v-FLIP, but may encode an as-yet-unidentified inhibitor of Fas. Alternatively, MHV-68 may act in dendritic cells and macrophages (Marques et al., 2003) to indirectly enhance the survival of MHV-68-infected germinal-centre B cells.

Despite the normal long-term level of splenic latency that is seen in the absence of M11, it seems unlikely that defects in latency amplification have no impact on viral fitness. For example, latency amplification may promote viral seeding to biologically important sites of persistence – perhaps submucosal lymphoid tissue or bone marrow – that have not yet been identified. Primary infection represents a unique opportunity for herpesviruses to establish a large latent pool for subsequent reactivation and transmission, and one that they evidently exploit to the full. The data presented here indicate that the MHV-68 viral bcl-2 homologue plays an important role in this process.

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