ORF73 of murine herpesvirus-68 is critical for the establishment and maintenance of latency

Polly Fowler,1 Sofia Marques,2,3 J. Pedro Simas2,3 and Stacey Efstathiou1

1Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1P, UK
2Instituto Gulbenkian de Ciência, 2780-156 Oeiras, Portugal
3Laboratory of Microbiology, Faculty of Medicine, University of Lisbon, 1649-028 Lisbon, Portugal

In vitro studies have established that the latency-associated nuclear antigen encoded by human Kaposi’s sarcoma-associated herpesvirus and the related ORF73 gene product of herpesvirus saimiri interact with virus origins of replication to facilitate maintenance of episomal DNA. Such a function implies a critical role for ORF73 in the establishment and maintenance of latency in vivo. To determine the role of ORF73 in virus pathogenesis, the ORF73 gene product encoded by murine herpesvirus-68 (MHV-68) was disrupted by making an ORF73 deletion mutant, Δ73, and an independent ORF73 frameshift mutant, FS73. The effect of the mutations introduced in ORF73 on MHV-68 pathogenesis was analysed in vivo using a well-characterized murine model system. These studies have revealed that ORF73 is not required for efficient lytic replication either in vitro or in vivo. In contrast, a severe latency deficit is observed in splenocytes of animals infected with an ORF73 mutant, as assessed by infectious centre reactivation assay or by in situ hybridization detection of latent virus. Assessment of viral genome-positive cells in sorted splenocyte populations confirmed the absence of ORF73 mutant virus from splenic latency reservoirs, including germinal centre B cells. These data indicate a crucial role for ORF73 in the establishment of latency and for virus persistence in the host.

INTRODUCTION

Gammaherpesviruses characteristically establish lifelong latency in lymphoid cell populations and are commonly associated with malignant lymphoproliferative disorders. Critical for the maintenance of latent gammaherpesviral genomes in dividing cells are epimrome maintenance functions, which are required for the replication of viral genomes during mitosis and their accurate segregation into daughter cells (Collins & Medveczky, 2002). In the case of Epstein–Barr virus (EBV), the virus-encoded EBNA-1 protein facilitates epimrome maintenance via interaction with the origin of plasmid replication (Yates et al., 1984, 1985, 2000; Lupton & Levine, 1985), and in vitro studies have demonstrated that EBNA-1 is essential for the immortalization of B cells and the establishment of latency (Lee et al., 1999). Similarly, Kaposi’s sarcoma-associated herpesvirus (KSHV) expresses a multifunctional latency-associated nuclear antigen (LANA-1) encoded by ORF73, which binds directly to the terminal repeats of the viral genome to mediate stable episome replication and segregation (Ballestas et al., 1999; Cotter & Robertson, 1999; Ballestas & Kaye, 2001; Grundhoff & Ganem, 2003). A similar function has also been demonstrated for the ORF73 gene product encoded by the related gamma-2 herpesvirus saimiri (HVS) (Smith et al., 2001; Collins et al., 2002).

From in vitro studies, it is clear that the trans-acting plasmid maintenance proteins encoded by gammaherpesviruses are essential for the stable maintenance of viral genomes during latency. However, the contribution of virus-encoded maintenance functions to virus persistence in vivo is not known. To directly address this question, we have focussed our studies on murine gammaherpesvirus-68 (MHV-68) because the use of this virus enables the study of gammaherpesvirus pathogenesis in an amenable murine host (Simas & Efstathiou, 1998; Nash et al., 2001; Flano et al., 2002a). MHV-68 is a natural pathogen of small rodents (Blaskovic et al., 1980; Blasdell et al., 2003). Following intranasal infection, MHV-68 replicates in respiratory epithelial cells (Sunil-Chandra et al., 1992) and establishes latency in lymphoid tissues. Latency is established predominantly in B cells (Marques et al., 2003) but can also be established in macrophages, dendritic and epithelial cells (Stewart et al., 1998; Weck et al., 1999; Flano et al., 2000). Following infection there is a marked expansion of latently infected B cells in lymphoid germinal centres (Simas et al., 1999), which allows MHV-68 to gain access to the long-lived memory B cell pool (Flano et al., 2003). The expansion
of latency also drives an infectious mononucleosis-like illness, which peaks 1 month post-infection (p.i.) and is characterized by splenomegaly and lymphocyte activation (Doherty et al., 2001). Real-time PCR analyses have revealed a restricted pattern of gene expression within B cells during latency (Marques et al., 2003), which includes transcription of ORF73 in the dividing germinal centre B cell fraction. This observation is consistent with the demonstration that the ORF73 gene products encoded by KHSV and HVS have a latency-associated plasmid maintenance function analogous to that of the EBV-encoded EBNA-1 protein (Ballestas et al., 1999; Collins et al., 2002; Grundhoff & Ganem, 2003). Based on positional homology amongst the gamma-2 herpesviruses, ORF73 genes and C-terminal structural similarity shared between the EBV EBNA-1 and a number of gamma-2 herpesvirus ORF73 gene products (Grundhoff & Ganem, 2003), it is predicted that ORF73 of MHV-68 is likely to encode a plasmid maintenance function. On this basis, we have constructed and characterized two MHV-68 mutants containing disruptions of the ORF73 gene product in order to determine its role in gammaherpesvirus pathogenesis and to determine whether a gammaherpesvirus defective for episome maintenance can persist in vivo.

METHODS

Virus stocks and cell culture. Virus stocks were prepared using baby hamster kidney (BHK-21) cells. Cells were maintained in GEME supplemented with 5% FCS, 10% tryptose phosphate broth, 2 mM L-glutamine, 100 U penicillin ml$^{-1}$ and 100 μg streptomycin ml$^{-1}$. Cells were infected at an m.o.i. of 0-01 p.f.u. per cell and harvested 4 days p.i. Virus titres were determined by suspension assay using BHK-21 cells (Simas et al., 1998). After incubation at 37°C in 5% CO$_2$ for 4 days, cell monolayers were fixed with 10% formal saline, stained with toluidine blue and plaques counted with a plate microscope. NIH 3T3-Cre cells were used as described previously (Stevenson et al., 2002) and were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin ml$^{-1}$ and 100 μg streptomycin ml$^{-1}$. NS0 cells (a myeloma B cell line) were grown in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin ml$^{-1}$ and 100 μg streptomycin ml$^{-1}$. Acyclovir was added to infected NS0 cells at 2 days p.i. to a concentration of 20 μg ml$^{-1}$.

Recombinant viruses. ORF73 was manipulated from genomic clone BamG (Efstratiou et al., 1990) (genomic co-ordinates, 101654–106903; Virgin et al., 1997) to make two independent mutations. A frameshift mutation was introduced in the BamG fragment in the vector pACYC184 (New England Biolabs) by digestion with BstEII (genomic co-ordinate, 104379). Terminal 5' overhangs were end-repaired and religated, resulting in the introduction of five novel bases, GTGAC. An independent mutation was made by digestion of BamG fragments in the vector pSP73 (Promega) with BstEII/BamHI (New England Biolabs). A 450 bp fragment (genomic co-ordinates, 104379–104830) was removed. The BamG fragments mutated in the ORF73 region were excised from their respective vectors using BamHI digestion and gel purification. The gel-purified inserts were then subcloned into the BamHI site of the shuttle vector pST76k_SR (Adler et al., 2000). BamHI digested plasmid vectors were sequenced across the ORF73 region and the integrity of the mutations confirmed. pST76k_SR BamGF73 and pST76k_SR BamGΔ73 were then transformed into Escherichia coli strain DH10B containing the MHV-68 BAC (bacterial artificial chromosome) pHA3 (Adler et al., 2000). Following a multi-step selection procedure, as described previously (Adler et al., 2000; Messerle et al., 1997), recombinant BAC clones were selected by restriction enzyme digestion. Revertant viruses were made for both independent mutations using wild-type pST76k_SR BamG. This plasmid was then transformed into E. coli strain DH10B containing the MHV-68 ORF73-mutated BACs. The multi-step recombinobation procedure was performed as above and recombinant clones screened for wild-type restriction digestion profiles. Mutant and revertant MHV-68 viruses were reconstituted by transfection of BAC DNA into BHK-21 cells. The BAC cassette (flanked by Lox-P sites) was removed by passage through NIH 3T3-Cre cells and limiting dilution to obtain GFP-negative virus. After excision of BAC vector sequences, virus structures were confirmed by restriction enzyme digestion and Southern blot hybridization.

Mice and in vivo infections. BALB/c mice (Harlan OLAC) were maintained in accordance with UK Home Office guidelines (project licences 80/1378 and 80/1579) at the University of Cambridge, UK, or at the Gulbenkian Institute of Science, Portugal. Mice were infected intranasally with 10$^5$ p.f.u. of virus in 20 μl PBS with 1% FCS after halothane anaesthesia. Intraperitoneal infections used 10$^5$ p.f.u. of virus in a volume of 50 μl PBS with 1% FCS. Infectious virus was assayed on BHK-21 cells following homogenization and freeze–thaw disruption of tissue. Latent virus from the spleen was assessed by infectious centre assay (Sunil-Chandra et al., 1992). For this assay, single-cell suspensions of splenocytes were co-cultured with BHK-21 cells for 4–5 days. Monolayers were then fixed with 10% formal saline and stained with toluidine blue. Plaques were counted using a plate microscope.

In situ hybridization. Viral tRNAs 1–4 were detected by in situ hybridization as a marker for latency (Bowden et al., 1997). As described previously (Arthur et al., 1993), paraformaldehyde-fixed spleen sections were de-waxed in xylene, rehydrated through a graded series of ethanol and treated with 100 μg proteinase K ml$^{-1}$ for 10 min at 37°C, followed by acetylation with 0.25% (v/v) acetic anhydride and 0.1 M triethanolamine. Digoxigenin (DIG, Boehringer Mannheim) labelled riboprobes corresponding to MHV-68 viral tRNAs were made by T7 transcription of pEH1.4 (Bowden et al., 1997). Sections were hybridized with the DIG-labeled riboprobes in 50% formamide and 1 × SSC overnight at 55°C. The stringent wash was carried out at 58°C (0.1 × SSC, 30% formamide and 10 mM Tris, pH 7.5). Hybridized probe was detected with alkaline phosphatase-conjugated anti-DIG Fab fragments (Boehringer Mannheim), according to the manufacturer’s instructions.

Limiting dilution analysis on different splenocyte populations. Analysis of virus load in sorted splenocyte populations was carried out as described previously (Marques et al., 2003). Briefly, single-cell suspensions of three to five pooled spleens were prepared per virus, per time-point, and passed through a filter (100 μm). Suspensions were washed in PBS with 2% FCS and red blood cells were lysed (154 mM ammonium chloride, 14 mM sodium hydroxide carbonate and 1 mM EDTA, pH 7.3). Single-cell suspensions were surface-stained with the following monoclonal antibodies (Pharmingen) and lectins (Vector Laboratories): anti-CD19, anti-CD11b, anti-CD11c, anti-B220 (CD45R) and peanut agglutinin (PNA). A MoFlo cytometer (Cytometry) was used to enrich the following populations: CD19$^+$ for total B cells, CD19$^+$ and PNA$^{+}$ for germinal centre B cells, B220$^+$ and CD11c$^+$ for dendritic cells, and B220$^+$, CD11b$^+$ and CD11c$^+$ for macrophages. Sorted populations were analysed using a FACScan flow cytometer and data processed using CellQuest software (Becton Dickinson Immunocytometry Systems). Purities of sorted populations were consistently >95% and predominantly >98%.

FACS-purified cell suspensions were serially diluted twofold and eight replicates of each dilution were lysed overnight (0.45% Tween-20,
0.45% Nonidet P-40, 2 mM MgCl₂, 50 mM KCl, 10 mM Tris, pH 8.3, and 0.5 mg proteinase K ml⁻¹) at 37°C. After proteinase K inactivation at 95°C for 5 min, samples were analysed by real-time PCR for the presence of viral DNA. Real-time PCR was performed using a LightCycler from Roche, according to the manufacturer’s instructions. Primer/probe sets specific for the MHV-68 K3 gene were used (genome co-ordinates: upper primer, 24832–24850; FL-probe, 24973–24998; LC-probe, 25000–25026; lower primer, 25049–25071). The FL-probe is an oligonucleotide labelled at the 3’ end with fluorescin (Roche) and the LC-probe is labelled at the 5’ end with LC-Red fluorophore and modified at the 3’ end by phosphorylation (Roche). Each PCR reaction had a final volume of 10 μl [2 mM MgCl₂, 4 ng each primer μl⁻¹, 0.2 μM each internal probe, 1 × DNA mixture (Roche) and 1 μl cell lysate]. Amplification used the following programme: melting step of 95°C for 10 min followed by 45 cycles of 95°C for 10 s, 55°C for 10 s and 72°C for 20 s with a subsequent melting analysis step of 50–95°C at 0.1°C s⁻¹.

Limiting dilution analysis data were compatible with the single-hit Poisson model (SHPM). This was tested by modelling the data to a generalized linear log-log model fitting the SHPM and checking this model by an appropriate slope test (Bonnefoix et al., 2001). A regression plot of input cell number against log fraction-negative samples was used to estimate the frequency of cells containing viral genomes.

**Gardella gels.** Vertical Gardella gels were performed as described (Gardella et al., 1984). NSO cells (2–5 × 10⁷) were resuspended in 100 μl sample buffer (15% Ficoll in TBE, 0.01 mg RNase A ml⁻¹ and 0.01% bromophenol blue). Samples were loaded into wells of a 0.8% TBE agarose gel. A 100 μl volume of lysis buffer (5% Ficoll, 1% SDS, 100 μg proteinase K ml⁻¹ and 0.05% xylene cyanol green) was then overlaid in the sample wells. Samples were electrophoresed at 12 V for 3–5 h and then overnight at 4°C at 80 V. Gels were stained with ethidium bromide and analysed by Southern blot hybridization.

**Southern blot hybridization.** Viral DNA was analysed by Southern blot analysis as described (Bridgeman et al., 2001). In brief, 10⁷ virus-infected cells were resuspended in 500 μl TE (10 mM Tris and 50 mM EDTA, pH 8) and lysed with 0.5% SDS and 50 μg proteinase K ml⁻¹ at 37°C overnight. DNA was then purified by phenol/chloroform extraction and ethanol precipitation. For Southern analyses, 5 μg of viral DNA was digested with an appropriate restriction enzyme and electrophoresed on a 0.8% agarose Tris/acetate (TAE) gel. DNA was then transferred to a nitrocellulose membrane and probed with a [α-³²P]dCTP random prime-labelled fragment; for analysis of ORF73-mutated MHV-68 clones, an ORF73-specific probe was used and Gardella gels were probed with a 1.2 kb Pst repeat fragment.

**RESULTS**

**Construction of ORF73 mutant viruses**

To analyse the role of ORF73 in the pathogenesis of MHV-68, two ORF73 mutant viruses were constructed, containing either a large deletion (Δ73) or a more subtle frameshift mutation (FS73), using MHV-68 BAC pHA3 (Adler et al., 2000). The deletion mutant removed 450 bp from ORF73 by double digestion with the enzymes BstEII/Bsu36I (Fig. 1a). Only the initial 13 amino acids at the N terminus remain in-frame in the deletion mutant and an additional 19 amino acids before the first stop codon were present (Fig. 1b). A second, independent mutant introduced a frameshift into the open reading frame (FS73) at the BstEII restriction site (Fig. 1a); this encodes for 163 amino acids of the N terminus in-frame and a further 20 amino acids before the first stop codon (Fig. 1b). To confirm the integrity of the ORF73 mutations, both mutants were sequenced across the mutated region in the shuttle plasmid pST76K. In order to ascribe any phenotypic changes seen with these mutants to the specific disruption of ORF73, revertant viruses were made for both mutants, replacing the mutated regions with the wild-type genomic fragment (referred to as Δ73R or FS73R, depending on the mutation reverted). The recombinant viruses were reconstituted by transfection into BHK-21 cells. The viruses were then passaged through Cre recombinase-expressing fibroblasts to remove the BAC vector sequences, as it has been shown that the presence of BAC vector sequences can attenuate virus growth in vivo (Adler et al., 2001). The structures of MHV-68 FS73, Δ73 and respective revertants were verified by restriction enzyme digestion and Southern blot hybridization (Fig. 2).

**ORF73 is not essential for growth in vitro**

The role of ORF73 for growth in vitro was assessed by multi-step growth curves on permissive BHK-21 cells. Replication was compared between ORF73 mutants, wild-type MHV-68 (derived from pHA3) (Adler et al., 2001) and respective revertant viruses (Fig. 3). No significant difference was observed in the growth of ORF73-negative viruses in vitro, suggesting that ORF73 is not required for in vitro replication of MHV-68.

**ORF73 is required for efficient episome maintenance in vitro**

Extensive in vitro studies using EBV, KSHV and HVS have shown that the plasmid maintenance genes of these viruses are essential for episome persistence (reviewed by Collins & Medveczky, 2002). ORF73 of KSHV and HVS encodes the plasmid maintenance protein of these two viruses. Therefore, it is predicted that ORF73 of MHV-68 may also have a role in plasmid maintenance. To address this hypothesis, NSO cells (a B cell line) were utilized: the presence of latent episomes in this cell type can be detected following infection with MHV-68 (Sujil-Chandra et al., 1993). NSO cells were infected with Δ73, Δ73R and wild-type viruses. Acyclovir was added to the cultures at 2 days p.i. to reduce lytic replication and cells were harvested 3 days later. Viral DNA from infected cells was then analysed using Gardella gels to discriminate between the presence of circular (latent) and linear (lytic) DNA. In the absence of a functional ORF73, a significant decrease was seen in the circular fraction of DNA on the Gardella gel compared to wild-type or revertant viruses (Fig. 4). This suggests that MHV-68 requires a functional ORF73 to efficiently maintain virus episomes in cell culture.
MHV-68 replication in the lung following infection with ORF73-deficient mutants

As discussed previously, MHV-68 lacking ORF73 was able to replicate with wild-type kinetics in vitro. To assess the role of ORF73 during the acute phase in vivo, infectious virus in the lung was assayed after intranasal inoculation with D73, D73R, FS73, FS73R and wild-type virus (Fig. 5). Virus titres from the lungs sampled 3–14 days p.i. were not affected by ORF73 disruption; therefore, ORF73 does not play a significant role in acute phase replication.

ORF73 is essential for MHV-68 latency

MHV-68 establishes a long-lived latent infection in mice (Simas et al., 1999). To assess the role of ORF73 in latency, the latent virus load in the spleen was analysed using a reactivation assay after intranasal inoculation. In contrast to the normal replication seen in the lung following infection, a severe deficit in virus reactivation was observed with ORF73-knockout viruses following intranasal inoculation of BALB/c mice (Fig. 6a, b). The deficit in reactivation, as assessed by infectious centre assay, was similar for both ORF73 mutants; the revertant viruses efficiently rescued the phenotype (Fig. 6a, b). To verify that the observed decrease in infectious centres was not due to an inability of the mutant viruses to seed to the spleen, an infectious centre assay was also performed following intraperitoneal infection, which efficiently seeds virus to the spleen (Weck et al., 1996, 1999; unpublished observations). To confirm that virus was efficiently delivered to the spleen, infectious virus

Fig. 1. (a) Schematic diagram of the genome of MHV-68 showing the variable number of terminal repeat regions at the left and right ends. The BamG fragment (101654–104379) is enlarged to show restriction sites used for the two ORF73 mutant viruses, Δ73 and FS73. (b) The sequence of ORF73 is represented schematically to show how ORF73 mutations are predicted to effect the ORF73 gene product. Regions of sequence and predicted structural homology to LANA and EBNA-1 are based on those described by Grundhoff & Ganem (2003). Areas shaded grey indicate regions of homology with KSHV LANA. Wide cross-hatching represents regions of predicted structural homology with EBNA-1, within the area of LANA homology. Narrow cross-hatching denotes the amino acid sequence that is out of frame as a result of the mutations introduced before the first stop codon.
was assayed at 5 days p.i. with $10^5$ p.f.u. of virus by the intraperitoneal route. The mean titre of infectious virus after infection with the ORF73 mutant FS73 was $1.71 \pm 0.6 \log_{10} \text{p.f.u. ml}^{-1}$, compared to mean wild-type and revertant (FS73R) virus titres of $2.07 \pm 0.69$ and $1.83 \pm 0.5 \log_{10} \text{p.f.u. ml}^{-1}$, respectively. In contrast, at day 14 p.i., a severe deficit in infectious centres was observed with Δ73 and FS73 viruses (Fig. 6c), implying that the lack of infectious centres occurs despite efficient seeding of virus to the spleen. Therefore, the reduction in infectious centres is due to an absence of a functional ORF73 and is independent of the route of infection.

The reactivation deficit observed in ORF73-deficient mutants is due to a reduction in latent virus load

To distinguish between an establishment versus a reactivation deficit, analysis of latent virus in the spleen was performed using in situ hybridization. In this assay, viral

**Fig. 2.** (a) FS73 and FS73R MHV-68 structure was confirmed by restriction enzyme digestion and Southern blot hybridization. The panel on the left shows an ethidium bromide-stained 0.8% agarose TAE gel of MHV-68 BAC DNA compared to FS73 and FS73R BAC DNA. DNA was digested with BsrEII for 3–5 h. Circles indicate band changes due to the mutation introduced. M, 1 kb PLUS DNA ladder (Gibco). For FS73, end-repairing of the BsrEII site destroyed this restriction site. Therefore, digestion with this enzyme shows the loss of the 4.3 kb and 5.9 kb bands to produce a higher molecular mass band of 10.2 kb. The panel on the right shows a Southern blot of high molecular mass viral DNA that was digested with BsrEII and electrophoresed on a 0.8% agarose gel. The blot was then probed with a random prime-labelled fragment corresponding to ORF73. FS73R shows identical bands compared to wild-type (WT), while the FS73 mutant shows the loss of the 4.3 kb and 5.9 kb fragments and a novel 10.2 kb fragment, as described previously. (b) Δ73 and Δ73R MHV-68 structure confirmed by restriction enzyme digestion and Southern blot hybridization. The panel on the left shows a restriction digestion profile of BsrEII-digested BAC DNA, as described in (a). For Δ73, a 450 bp fragment was deleted from ORF73, deleting the BsrEII restriction site and truncating the ORF by 450 bp. This is shown by loss of the 4.3 kb and 5.9 kb bands (as for FS73) and production of a novel 9.8 kb band, indicated by circles on the ethidium bromide-stained gel. The panel on the right shows a Southern blot of viral DNA, as for (a), with Δ73R showing identical bands to wild-type MHV-68, with the novel 9.8 kb band in the mutated Δ73 viral DNA.
tRNA-positive cells can be used as a marker for latency (Bowden et al., 1997). In the spleens of wild-type- or Δ73R-infected animals, vtRNA-positive cells are detected at day 7 p.i., followed by a large expansion by day 14 (Fig. 7). In contrast, animals infected with Δ73 showed a complete absence of vtRNA-positive cells in spleen sections at either day 7 or day 14 p.i. (Fig. 7). Thus, the latency deficit revealed for this mutant by infectious centre assay was confirmed by in situ hybridization analyses. This result indicates that the failure of virus reactivation from latency was due to an inability of the ORF73-deficient mutants to establish latency and a failure to colonize expanding germinal centres.

A further, more detailed, analysis of virus load in different splenocyte populations was then undertaken to rule out a reactivation deficit. To this end, splenocyte populations were sorted based on the following criteria: total B cells (CD19+), germinal centre B cells (CD19+ and PNA hi), dendritic cells (B220− and CD11c+), and macrophages (B220−, CD11b+ and CD11c−). The frequency of genome-positive cells in these different splenocyte populations was then determined by limiting dilution analysis and real-time PCR to allow comparison of virus load between ORF73-deficient FS73 MHV-68 versus wild-type or revertant (FS73R) viruses (Table 1). In wild-type and FS73R MHV-68 infection, latent virus was detected in all of the different cell types analysed. Consistent with previous data (Marques...
et al., 2003; Flano et al., 2002b), wild-type and FS73R viruses showed a high frequency of genome-positive cells at 14 days p.i. in all of the cell populations sorted. Frequencies were highest in germinal centre B cells; for example, at day 14 p.i., 1 of 131 germinal centre B cells was positive for FS73R and 1 of 128 was positive for wild-type virus. One unexpected observation was that a higher frequency of FS73R versus wild-type virus was detected in germinal centre B cells (1 of 55 for FS73R and 1 of 443 for wild-type) and total B cells (1 of 387 for FS73R and 1 of 4125 for wild-type) at day 21 p.i. The reasons for this discrepancy are unclear and the reproducibility of this result is currently under evaluation. We do, however, note that the frequencies for FS73R and wild-type virus that we determined are still within the range of that described by Marques et al. (2003). In contrast, in FS73-infected mice, no viral DNA-positive cells were found in the sorted splenocytes; for example, there was less than one genome-positive cell in $1 \times 10^6$ germinal centre B cells (based on the limit of detection for the assay). Similarly, despite high frequencies of 1 of 980 or 1 of 1788 for FS73R or wild-type viruses, respectively, in dendritic cells at day 14, no genome-positive dendritic cells were detected in FS73-infected mice. This result shows a huge reduction in virus load in the absence of ORF73 in specific cell populations, in which a high degree of infection is normally established, and is consistent with the lack of infectious centres and the absence of tRNA-positive cells by in situ hybridization. Taken together, these data show that the establishment and maintenance of latency in MHV-68 is critically dependent on ORF73.

**DISCUSSION**

The predicted role of MHV-68 ORF73 in plasmid maintenance would imply a critical role for this gene product during the establishment of latency in vivo. This hypothesis is strengthened by the detection of ORF73 transcription in germinal centre B cells, marginal zone B cells and dendritic cells isolated from latently infected mice (Marques et al., 2003), and the fact that the ORF73 gene products encoded by KSHV and HVS are known to function in episome maintenance (Ballestas et al., 1999; Ballestas & Kaye, 2001; Collins et al., 2002). In addition, there is significant structural similarity of a C-terminal region, predicted to encompass the DNA-binding domain of EBNA-1 (amino acids 461–607) and ORF73 gene products encoded by a number of gamma-2 herpesviruses, including both KSHV and MHV-68 (Grundhoff & Ganem, 2003). In this context, it is of significance that truncation of the KSHV ORF73 gene product after amino acid 1036, which removes the structurally conserved C-terminal domain, renders the protein inactive in episomal DNA replication (Grundhoff & Ganem, 2003). In this manuscript, we describe the construction and characterization of MHV-68 mutants containing disruptions in the ORF73 gene with the aim of defining the role of this gene product in virus pathogenesis. No significant lytic growth deficit was observed in permissive BHK cells, indicating that, at least in this cell type, ORF73 is dispensable for lytic replication. Similarly, no detectable replication deficit was observed during acute phase lung infection in vivo. To directly address the role of ORF73 in the maintenance of virus episomes, the NSO
myeloma B cell line was infected in vitro and viral DNA analysed by Gardella gel electrophoresis in order to discriminate circular versus linear viral DNA (Gardella et al., 1984). In comparison to wild-type or revertant viruses, there was a substantial reduction in the amount of circular DNA following infection with Δ73 – an observation consistent with the predicted role of this gene product in plasmid maintenance.

In contrast to the relatively normal replication kinetics of ORF73-deficient viruses in BHK cells and lungs of acutely infected mice, these mutants were severely disabled for the establishment of latency in the spleen. Following intranasal infection with either wild-type MHV-68 or Δ73R, infectious centres could be recovered from the spleen at 7 days p.i., peaked at 14 days p.i. and reached a stable level of approximately 800 infectious centres per spleen thereafter. Following infection with Δ73, despite the relatively normal seeding of the spleen at 7 days p.i., the characteristic amplification of infectious centres was not observed at 14 days p.i. and by day 21 p.i. infectious centres were not detectable. The critical role of ORF73 in latency was verified using an independently derived frameshift mutant, FS73, administered by the intranasal route and a similar reactivation deficit was observed for both mutants following intraperitoneal injection of virus, a route of infection that efficiently targets virus to the spleen. These data strongly argue that the observed reactivation deficit of the ORF73-deficient

**Fig. 6.** Latent virus in the spleen measured by infectious centre assay. (a, b) BALB/c mice were infected intranasally with 10^4 p.f.u. in 20 μl PBS with 1% FCS. At various times p.i., spleens were removed and single-cell suspensions of splenocytes were assayed by co-cultivation with BHK-21 cells and incubated for 4–5 days to form infectious centres. (a) Time-course of latent virus in the spleen in Δ73-infected mice compared to wild-type (WT) and Δ73R-infected mice. (b) Infectious centres following infection with wild-type, FS73 and FS73 viruses at 14 days p.i. (a, b) Each data-point represents the mean of infectious centres from five spleens and error bars show the SD from the mean. (c) BALB/c mice were infected intraperitoneally with 10^5 p.f.u. in 50 μl PBS with 1% FCS. Spleens were removed 14 days p.i. and virus assayed as described for (a) and (b). Each data-point represents infectious centres from one spleen and the mean is indicated with a grey bar. Error bars show the SD from the mean.
Fig. 7. In situ hybridization detection of tRNAs in spleens sampled at 7 or 14 days p.i., as indicated. BALB/c mice were infected intranasally with 10^4 p.f.u. with Δ73, wild-type (WT) or Δ73R. Spleens were removed at 7 or 14 days p.i., fixed and paraffin-embedded before sectioning and in situ hybridization was performed. Sections were counterstained with haematoxylin. Dark staining indicates cells positive for viral tRNAs, highlighted with arrows. In situ hybridization (viral tRNA) positive cells are seen for wild-type and Δ73R infections, while no positive cells were seen at 7 or 14 days p.i. in any sections from spleens of Δ73-infected mice.

At this stage, we cannot formally discount the possibility that the mutations introduced have influenced cis-acting sequences required for virus latency that overlap ORF73 by coincidence. However, we consider it most likely that the phenotypes observed are due to disruption of ORF73, since similar effects are observed with both a deletion and a subtle frameshift mutation. Furthermore, the phenotypes observed are consistent with the predicted role of this gene product in plasmid maintenance. The role of the neighbouring genes, ORF72 and ORF74, have been studied in vivo and neither gene shows a latency phenotype such as the one described in the present study (van Dyk et al., 2000, 2003; Lee et al., 2003). It is unlikely therefore that the mutations introduced within ORF73 have influenced neighbouring genes that could have contributed to the phenotype observed. The phenotype of the ORF73-deletion mutant was also confirmed by a subtle frameshift mutation and respective revertant viruses had fully restored wild-type phenotypes. It is, therefore, improbable that secondary mutations might have occurred to influence the phenotype of the ORF73-deficient viruses as described here.

KSHV LANA is a multifunctional protein and has been shown to interact with multiple cellular factors as well as having transcription modulatory activity (Garber et al., 2001; Renne et al., 2001; Mattsson et al., 2002; Viejo-Borbolla et al., 2003). Of particular importance to this study, LANA interacts with histone H1 to mediate tethering of the virus genome to host cell chromosomes (Ballestras et al., 1999; Cotter & Robertson, 1999). However, interactions have also been shown between LANA and the tumour suppressor protein p53 (Friborg et al., 1999) as well as the retinoblastoma protein RB1 (Radkov et al., 2000). These studies implicate a role for LANA in virus persistence and cell transformation. To date, interactions of ORF73 of MHV-68 with cellular factors have not been dissected, although ORF73 of MHV-68 does share sequence similarity with KSHV LANA at the C terminus, where multiple functions of the protein have been mapped; these functions include DNA binding, dimer formation, transcriptional repression, localization in the nucleus and protein interactions to mediate chromosome binding (Schwam

Mutants is not due to a failure of these viruses to seed the spleen. Given the proposed episome maintenance function of ORF73, we considered it most likely that the failure to detect infectious centres was a failure to efficiently establish latency rather than a deficit in the ability of the mutants to reactivate from latency. To distinguish between an establishment versus a reactivation deficit, the latent virus load was first evaluated by in situ hybridization detection of viral tRNA-positive cells (Bowden et al., 1997). This measure of latency confirmed that the deficit in recovery of infectious centres from the spleen was due to an inability of the ORF73-deficient viruses to colonize germinal centres and thereby efficiently establish latency. This conclusion was supported by determining the genomic DNA load in FACS-purified splenocyte cell populations. Limiting dilution analysis and real-time PCR were used to determine the frequency of genome-positive cells in the following sorted splenocyte populations: total B cells (CD19^+), germinal centre B cells (CD19^+ and PNA^hi), macrophages (B220^-, CD11b^+ and CD11c^-) and dendritic cells (B220^-, CD11c^+). Wild-type and revertant viruses were able to establish high frequency infection in these cell populations, as described previously (Marques et al., 2003; Flano et al., 2002b; Willer & Speck, 2003). In contrast, no viral genome-positive cells were detected in any of the splenocyte populations derived from ORF73-deficient virus-infected mice. Here, a frequency of less than 1 in 1·5 × 10^6 germinal centre B cells, total B cells or macrophages and less than 1 in 7·5 × 10^5 dendritic cells (based on the maximum number of cells analysed) was determined at 14 days p.i. Viral genome-positive cells were not detected in total B cells or germinal centre B cells sampled at 21 days p.i., suggesting that the latency deficit was not the result of delay in latent virus amplification. These phenotypes are consistent with the predicted role of ORF73 in plasmid maintenance and imply a critical role for this function in the context of an in vivo infection. Using a variety of independent measures of latency, the most consistent picture to emerge from this study is that ORF73 plays an essential role in host colonization by MHV-68.
Table 1. Frequency of MHV-68 infection

Data were obtained from pools of three to five spleens.

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Day p.i.</th>
<th>Virus</th>
<th>Reciprocal frequency of viral DNA-positive cells*</th>
<th>% of cells†</th>
<th>Total no. of cells‡</th>
<th>No. of viral DNA-positive cells§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total B cells</td>
<td>14</td>
<td>FS73</td>
<td>(&lt;1.5 \times 10^6)</td>
<td>47-8</td>
<td>9.6 \times 10^7</td>
<td>&lt;64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FS73R</td>
<td>1565 (957–1284)</td>
<td>53-5</td>
<td>1.1 \times 10^8</td>
<td>70288</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wild-type</td>
<td>1852 (1168–4464)</td>
<td>52-6</td>
<td>1.1 \times 10^8</td>
<td>59395</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>FS73</td>
<td>(&lt;1.1 \times 10^6)</td>
<td>53-4</td>
<td>1.3 \times 10^8</td>
<td>&lt;98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FS73R</td>
<td>387 (246–906)</td>
<td>63-3</td>
<td>1.3 \times 10^8</td>
<td>335917</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wild-type</td>
<td>4125 (2564–10536)</td>
<td>55-3</td>
<td>1.1 \times 10^8</td>
<td>26667</td>
</tr>
<tr>
<td>Germinal centre B cells</td>
<td>14</td>
<td>73FS</td>
<td>(&lt;1.5 \times 10^6)</td>
<td>6-2</td>
<td>1.2 \times 10^7</td>
<td>&lt;8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>73FSR</td>
<td>131 (84–306)</td>
<td>7-7</td>
<td>1.5 \times 10^7</td>
<td>114504</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wild-type</td>
<td>128 (75–435)</td>
<td>5-2</td>
<td>1.0 \times 10^7</td>
<td>78125</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>73FS</td>
<td>(&lt;1.0 \times 10^6)</td>
<td>7-9</td>
<td>1.6 \times 10^7</td>
<td>&lt;15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>73FSR</td>
<td>55 (35–126)</td>
<td>9-7</td>
<td>1.9 \times 10^7</td>
<td>345455</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wild-type</td>
<td>443 (265–1329)</td>
<td>5-6</td>
<td>1.1 \times 10^7</td>
<td>24831</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>14</td>
<td>73FS</td>
<td>(&lt;7.5 \times 10^6)</td>
<td>2-1</td>
<td>4.2 \times 10^8</td>
<td>&lt;5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>73FSR</td>
<td>980 (585–3019)</td>
<td>2-8</td>
<td>5.6 \times 10^8</td>
<td>5714</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WT</td>
<td>1788 (1124–4367)</td>
<td>2-7</td>
<td>5.4 \times 10^8</td>
<td>3020</td>
</tr>
<tr>
<td>Macrophages</td>
<td>14</td>
<td>73FS</td>
<td>(&lt;1.5 \times 10^6)</td>
<td>7-8</td>
<td>1.6 \times 10^7</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>73FSR</td>
<td>11314 (7114–27630)</td>
<td>9-5</td>
<td>1.9 \times 10^7</td>
<td>1679</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wild-type</td>
<td>12824 (7693–38508)</td>
<td>7-7</td>
<td>1.5 \times 10^7</td>
<td>1170</td>
</tr>
</tbody>
</table>

*Frequencies of infection calculated by limiting dilution analysis; 95% confidence intervals are shown in parentheses.
†Percentage of each population of total spleen was determined by FACS analysis.
‡Total number of cells as an estimate of the percentage of total spleen, based on 2 \times 10^8 cells per spleen.
§Number of latently infected cells based on the frequency of positive cells within each cell type and the estimated total number of cells.

et al., 2000; Krithivas et al., 2002; Garber et al., 2002; Grundhoff & Ganem, 2003). Further confirmation of the importance of the C terminus is shown in this study; the FS73 mutant encodes 163 amino acids of the N terminus in-frame (approximately the entire N-terminal half of the 314 amino acid protein). This mutant has the same phenotype as the deletion mutant, which only encodes the first 13 amino acids of the N terminus in-frame, suggesting that it is the common deletion of the C terminus that is responsible for the phenotype of the mutant viruses. While the present study suggests that the phenotype observed is consistent with a plasmid maintenance function of ORF73, other functions of the protein that may contribute to the latency phenotype cannot be ruled out. Further analysis of particular functional domains of ORF73 are needed to ascribe particular roles to regions of the protein and this would enable more specific mutations to be made and their contributions to the pathogenesis of MHV-68 assessed. Additionally, for EBV, KSHV and HVS, it has been shown that EBNA-1 and LANA interact with virus origins to support latent replication, and in HVS and KSHV these virus origins map to the terminal repeats (Reisman et al., 1985; Ballestas & Kaye, 2001; Collins et al., 2002). KSHV, HVS and MHV-68 are collinear (Virgin et al., 1997) and based on such homology it is predicted that the origin of plasmid replication of MHV-68 could also be found in the terminal repeats. Thus, further studies are needed to define the elements of MHV-68 that are both necessary and sufficient for plasmid maintenance.

The data presented in this study are consistent with the prediction that ORF73 of MHV-68 is involved in plasmid maintenance. ORF73 appears to be dispensable for acute phase replication of MHV-68, while, conversely, it is critical for the establishment of latency and hence efficient colonization of the host. As such, it is possible that by abrogating the function of this latency-associated antigen, this latency-deficient virus may provide a novel and safe basis for a gammaherpesvirus vaccine.

NOTE ADDED IN PROOF

While this paper was under review, Moorman et al. (2003) (J Virol 77, 10295–10303, 2003) reported the construction of an ORF73 mutant that exhibited a severe defect in the establishment of latency in the spleen of C57BL/6 mice.

ACKNOWLEDGEMENTS

We would like to thank Philip Stevenson for valuable discussions. This work was supported by the Wellcome Trust (grant no. 059601 to S.E.), an MRC Co-operative Group grant (grant no. G980943 to S.E.) and funded in part by the Ministry of Science and Technology of
Portugal (grant no. PRAXIS/10265/98 to J. P. S.). P. F. is supported by a studentship from the Medical Research Council, UK, and S. M. is supported by an FCT scholarship.

REFERENCES


retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells.


