Murine gammaherpesvirus 68 (MHV-68) encodes a set of unique genes, M1, M2, M3 and M4, and eight non-translated tRNA-like molecules that are thought to be important in virus–host interactions and latent infection. The M4 gene is predicted to encode a novel secreted protein. To investigate the role of M4 in viral pathogenesis, a mutant MHV-68 that did not express M4 was constructed and its replication was characterized in vitro and in vivo. Virus replication was identical to the wild type in vitro and no difference could be detected in virus replication in the lung following intranasal infection. However, in the spleen, virus deficient in M4 expression was severely attenuated in the establishment of latency. These results indicate a critical role for M4 in MHV-68 pathogenesis.

The M4 gene of murine gammaherpesvirus 68 modulates latent infection

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Members of the gammaherpesvirus subfamily encode molecules that modify the function of the host immune system and facilitate the establishment and maintenance of latent infection. In murine gammaherpesvirus 68 (MHV-68) infection, genes located at the left-hand end of the viral genome are intimately associated with immune-evasion strategies affecting the establishment and maintenance of latency (Nash et al., 2001; Simas & Efstathiou, 1998; Virgin & Speck, 1999). The genes involved are M1, M2, M3, M4 and tRNA-like sequences (vtRNA), of which M3 has been characterized as a chemokine-binding protein (Bridgeman et al., 2001; Parry et al., 2000; van Berkel et al., 2000) and M2 is involved in B-cell latency (Jacoby et al., 2002; Macrae et al., 2003; Simas et al., 2004). Relatively little is known about the functions of M1 and M4.

Earlier work from our laboratory made use of MHV-76 (a variant of MHV-68 lacking M1–M4 and vtRNAs) to express M4 (Townsley et al., 2004). This MHV-76 recombinant virus (MHV-76inM4) was shown to produce increased titres of virus at early times in the lung and an increased viral DNA load in the spleen compared with MHV-76. In vitro, M4 is transcribed as an immediate-early gene (Ebrahimi et al., 2003; Townsley et al., 2004). In vivo, it is expressed in the lung during lytic infection and during acute-phase latency at day 14 in the spleen (Marques et al., 2003; Townsley et al., 2004). However, it is not consistently detectable during long-term MHV-68 latent infection (Townsley et al., 2004).

These data are consistent with M4 playing a role in modulation of the immune response. In the lymphoid tissue, M4 might delay clearance of virus-infected cells or stimulate cell proliferation, although the latter apparently does not fit with the observation that there is increased replication at early time points in the lung (Townsley et al., 2004). However, interpretation is complicated by the absence of the genes encoding the M1, M2 and M3 proteins, as well as the vtRNA molecules and the recently described small interfering RNAs (Pfeffer et al., 2005). The region also encodes promoter elements for ORF73, a critical gene for latency (Coleman et al., 2005), and other potential ORFs (Dutia et al., 2004). The inserted M4 gene contained 1112 nt of apparently non-coding sequence upstream of the coding region, the function of which is unexplored. Thus, the function of M4 in the context of wild-type MHV-68 gene expression requires investigation. We have constructed and characterized mutants of MHV-68 that lack the ability to express M4. The M4 ORF was mutated by manipulating an MHV-68 genomic clone containing nt 6262–9609 (Efstathiou et al., 1990). A 771 bp BsaAI fragment (nt 8385–9155) was amplified by PCR and cloned, and a 19 bp sequence (DeLuca & Schaffer, 1987) containing three stop codons and a diagnostic HpaI site was inserted at nt 8672. The mutated BsaAI fragment was reinserted into the MHV-68 genomic clone and the mutated sequence was ligated into the shuttle vector pST76_SR. The resultant plasmid was transformed into Escherichia coli DH10B containing the MHV-68 bacterial artificial chromosome (BAC) pHA3 (Adler et al., 2000). Recombinant BAC clones were produced as described by Adler et al. (2000) and Messerle et al. (1997) and screened by restriction analysis. An independent deletion mutant, M4Δ, which lacks nt
8385–9155, was produced by the same method. Revertant BACs were made by transformation of bacteria containing mutant BAC with a wild-type genomic clone and selected by PCR. Virus stocks were produced by transfection of BAC DNA into BHK-21 cells and BAC sequences were excised from the reconstituted virus by passage through mouse NIH 3T3 cells expressing Cre recombinase (Fowler et al., 2003). Virus working stocks were prepared by infection of BHK-21 cells as described previously (Sunil-Chandra et al., 1992a). Viral DNA was then analysed by restriction analysis with Hpal or SmaI and Southern blot hybridization using a probe encompassing nt 6262–9609 (Fig. 1). This confirmed that the mutant and revertant viruses had the expected restriction profile.

The M4 ORF has been mapped to nt 8538–9785 (Townsley et al., 2004) and is predicted to encode a secreted protein with a secretory cleavage site at nt 8606. Translation of the disrupted gene would produce a secreted peptide of 22 aa rather than 393 aa. This peptide is highly unlikely to be functional. The deletion mutant M4Δ lacks the first 205 aa of M4 and the upstream 153 nt and is therefore also unlikely to produce functional protein.

Deletion of genes M1–M4 and the vtRNAs has been shown to have no effect on growth of MHV-68 in vitro (Clambey et al., 2000; Macrae et al., 2001). Similarly, insertion of M4 into the deletion mutant MHV-76 had no effect on in vitro replication (Townsley et al., 2004). Therefore, it was expected that the mutant viruses would replicate with the same kinetics as wild-type virus. To confirm this, one-step (5 p.f.u. per cell) and multi-step (0–05 p.f.u. per cell) growth curves were carried out in BHK-21 cells on the wild-type BAC-derived virus PHA4, the mutant viruses M4Stop and M4Δ and their revertants. Fig. 2 shows that, in both single and multiple rounds of infection, all viruses replicated with similar kinetics. Thus, functional M4 is not required for efficient replication in vitro.

The M3 ORF lies between nt 6060 and 7277 and the gene is transcribed in a leftward direction. The M3 promoter has not been mapped, but must lie upstream of nt 7277. Whilst it is unlikely that a mutation at nt 8672 within the M4 ORF would disrupt the function of the M3 promoter, transcription of the M3 gene in vitro was confirmed by RT-PCR using RNA extracted from C127 cells 18 h after infection, as described previously (Dutia et al., 2004) (data not shown). Similarly, M4Δ was shown to transcribe the M3 gene.

The M4 insertion virus MHV-76inM4 consistently produces significantly higher lung titres than MHV-76 and MHV-68 on days 1–3 post-infection (p.i.) (Townsley et al., 2004; unpublished data); thus, deletion of M4 might be expected to decrease virus replication. Therefore, we infected BALB/c mice intranasally with 4 x 105 p.f.u. PHA4, M4Stop and M4StopR, harvested the lungs and determined the titre of infectious virus (Sunil-Chandra et al., 1992a). We could not detect consistent differences in the virus titres between mice infected with wild type, M4Stop or M4StopR (Fig. 2c) and concluded that the kinetics of replication of virus lacking M4

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Fig. 1. Construction and verification of the genome structures of M4 mutant viruses. (a) Structure of the region of MHV-68 encoding M4. The diagram shows the site of the stop insertion in M4Stop, the deletion in M4Δ, the restriction fragments generated by digestion with Hpal (H) and SmaI (S) and the position of the probe used for the Southern blot analysis. (b) Southern blot analysis of the wild-type PHA4 virus, mutants and revertants. Purified DNA was digested with Hpal or Smal and probed with a 32P-labelled probe spanning nt 6262–9609. The Southern blot hybridization results are as predicted by structure analysis.
consistent difference in pathological changes at days 2 and 4 p.i. (data not shown).

A recently published analysis of a similar M4 stop mutant in C57Bl/6 mice found no significant difference in lung virus titres at 4 days p.i. and a modest but significant decrease in lung virus titres after 9 days (Evans et al., 2006). In contrast, insertion of M4 into MHV-76 produced elevated titres at days 1 and 3, leading to the hypothesis that deletion of M4 would suppress virus replication at early times (Townesley et al., 2004). The most likely explanation for this discrepancy is that there is interplay between the products of the genes present at the left-hand end of MHV-68 and that the elevated replication observed with the insertion virus is related to the lack of M1, M2, M3 and the vRNAs. Clearly deletion of the entire region increases the ability of the virus to replicate, but it is possible that one or more of the gene products downregulates replication. High levels of virus replication are likely to be disadvantageous to a virus that aims to establish latent infection rather than kill the host. Similarly, virus lacking the potent chemokine-binding molecule M3, which is proposed to function as an immunomodulatory molecule during acute infection, does not produce elevated lung virus titres (Bridgeman et al., 2001; van Berkel et al., 2002).

To investigate the role of M4 in lymphoid-cell proliferation and the establishment of latency, BALB/c mice were infected with 4 × 10^5 p.f.u. virus, spleens were removed and weighed, and infective-centre assays (Sunil-Chandra et al., 1992b) were carried out at various times after infection. Infection with MHV-68 causes an approximate doubling in spleen weight and cell numbers 14 days after intranasal infection. Infection with M4Stop and M4Δ produced similar increases in spleen-cell numbers to those seen with wild-type virus infection (PHA4, 1 ± 0.55 ± 0.21 × 10^8 cells; M4Stop, 1 ± 0.41 ± 0.27 × 10^8 cells; M4StopR, 1 ± 0.63 ± 0.17 × 10^8 cells; M4Δ, 1 ± 0.46 ± 0.18 × 10^8 cells), indicating that M4 is unlikely to play a role in stimulating splenic lymphocytosis. At day 10 p.i., the level of spleen latent virus load in mice infected with M4Stop was similar to that in mice infected with wild-type PHA4 or revertant virus but, by day 14 p.i., there was over 100-fold less latent virus in the M4Stop mouse spleens than in wild type- and revertant virus-infected spleens (Fig. 3a). Similarly, in mice infected with M4Δ, the latent virus load at day 14 was 10-fold less than in wild type-infected mice [PHA4, 415 ± 65 infectious centres (10^7 spleen cells)^−1; M4Δ, 31 ± 2 infectious centres (10^7 spleen cells)^−1; P = 0.0005, Student’s t-test]. Thereafter, the latent virus load in mice infected with M4Stop or M4Δ was consistently lower than in control mice. At no time was infectious preformed virus detectable in the spleen.

The difference in spleen virus load for M4Stop was confirmed by quantitative PCR. DNA was extracted from splenocytes at days 10, 14 and 17, and 100 ng DNA was amplified in a Corbett Rotorgene using the intercalating dye SYBR Green and M4 gene-specific primers (M4RTfor, 5′-CACCTGAGATCAAGTCTATCG-3′; nt 8869–8889;
M4RTrev, 5'–GTCGCATAACCATGTCACGG–3', nt 9155–9174). All products from the quantitative PCR were analysed by using a melting curve to confirm specificity and the results were normalized against β-actin. Fig. 3(b) shows that the number of genome copies in M4Stop-infected spleen was similar to wild type- or revertant-infected spleen at day 10, but by days 14 and 17, the number of genomes had decreased 100-fold. Latent infection was also monitored by in situ hybridization to detect the vtRNAs, which have been shown to be expressed in latently infected cells (Bowden et al., 1997). Hybridization using a probe transcribed from pEH1.4 (Bowden et al., 1997) showed that, whilst vtRNA expression was present in numerous germinal centres in the spleens of mice infected with wild-type or M4StopR virus at day 14 p.i. (PHA4, 16 out of 29 follicles positive; M4StopR, 21 out of 58 follicles positive), few vtRNA-positive follicles were present in the spleens of mice infected with M4Stop or M4A (M4Stop, two out of 27 follicles positive; M4A, one out of 71 follicles positive). Whilst in the spleens of mice infected with PHA4 or M4StopR, vtRNA-positive cells were detected in every section examined, this was not the case for M4Stop or M4A. Moreover, the positive follicles present in spleens from mice infected with M4Stop or M4A had fewer positive cells than those infected with PHA4 or M4StopR (Fig. 3c).

In contrast to the lack of phenotype in the lung, virus lacking M4 produced a clearly defined phenotype in the lymphoid tissue. Infection with MHV-68 causes an expansion in both B- and T-cell subsets in the spleen, resulting in splenic lymphocytosis. This process has been shown to be dependent on the presence of an intact left-hand end of the genome (Macrae et al., 2001). Deletion of M4 did not affect the ability of the virus to cause splenic lymphocytosis, indicating that the M4 protein is not required to drive this cellular proliferation. Similarly, splenic lymphocytosis has been shown to be independent of M2 expression (Macrae et al., 2003).

Thus, cellular proliferation can be separated from establishment of latency and is under the control of other genes encoded in this region. Proliferation may be driven by a secreted product or by signalling from low numbers of latently infected cells. In contrast to our conclusions, the M4 disruption described by Evans et al. (2006) resulted in spleens of significantly lower weight at day 16 in C57Bl/6 mice. It is not clear why the different mutants led to different results unless this is related to the strain of mice. Whilst the mean spleen cell numbers in mice infected with mutant virus were lower than those in wild type- or revertant-infected mice, this was not significant and was always higher than in uninfected mice. Disruption of M4 resulted in a virus that was severely attenuated in establishment of latency. The virus trafficked to the spleen with similar kinetics to wild-type and revertant viruses and produced comparable levels of latency to wild-type virus and revertant virus on day 10, but thereafter, there was a rapid decline in latent virus infection. These results reflect directly those found with the MHV-76 insertion virus, which did not cause splenocytosis, but caused an increase in the number of latently infected cells, and are in agreement with those published recently (Evans et al., 2006).

At present the function of M4 is unclear, but the results described here are consistent with M4 modulating the immune response and preventing rapid clearance of latently infected cells from the spleen. M4 has been shown to be a secreted glycoprotein (Evans et al., 2006; B. M. Dutia, F. Wan & A. A. Nash, unpublished results). It is likely, therefore, that M4 acts to downregulate the immune response, either by binding directly to cells involved in the immune response and inhibiting their function or by binding to extracellular immune modulators, such as chemokine or cytokine molecules, and preventing their appropriate function in the response to virus infection. It is clear that M4 is a critical factor in the establishment of latency.
determinant of MHV-68 pathogenesis and that understanding its function has important implications for elucidating virus–host interactions. Experiments are currently under way to elucidate its function.

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References


