Identification of a region of the virus genome involved in murine gammaherpesvirus 68-induced splenic pathology

Bernadette M. Dutia,[1] Douglas J. Roy,[1] Bahram Ebrahimi,[1]† Babunilayam Gangadharan,[1] Stacey Efstathiou,[2] James P. Stewart[1]† and Anthony A. Nash[1]

[1]Laboratory for Clinical and Molecular Virology, Division of Veterinary Biomedical Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK

[2]Department of Pathology, University of Cambridge, Cambridge CB2 1QP, UK

Correspondence
Bernadette M. Dutia
B.M.Dutia@ed.ac.uk

Received 16 December 2003
Accepted 1 March 2004

Infection with the murine gammaherpesvirus MHV-68 has profound effects on splenic and mediastinal lymph node pathology in mice which lack the interferon-γ receptor (IFN-γ $R^{-/-}$). In these mice MHV-68 infection causes fibrosis and loss of lymphocytes in the spleen and the mediastinal lymph node as well as interstitial pulmonary fibrosis and fibrotic changes in the liver. The changes are associated with transient elevated latent virus loads in the spleen. Four independent virus mutants with insertions and/or deletions in the left end of the genome fail to induce the pathological changes and establish latency at normal levels in the spleen. The data indicate that the pathology does not correlate with any of the known genes encoded within this region of the genome, genes M1–M4 and the eight vtRNAs. Northern analysis of mRNAs transcribed by wild-type and mutant viruses shows that at least two uncharacterized transcripts are encoded within this region. These transcripts are absent in the mutant viruses and are candidates for the virus genes responsible for the aberrant pathology in IFN-γ $R^{-/-}$ mice.

INTRODUCTION

In immunocompetent mice, the murine gammaherpesvirus MHV-68 causes an acute lung infection which is rapidly cleared, followed by a longer lymphoproliferative phase including lymphadenopathy of the mediastinal lymph nodes, splenomegaly and an infectious mononucleosis like phase involving proliferation of $CD8^+$ T cells (Sarawar *et al.*, 1996; Sunil-Chandra *et al.*, 1992a; Tripp *et al.*, 1997). $CD8^+$ T cells play a major role in clearance of the lung infection and $CD4^+$ T cells are crucial for long-term control of infection, probably via interferon-γ (IFN-γ) (Christensen *et al.*, 1999). The virus establishes a latent infection in lung epithelial cells, B lymphocytes, macrophages and dendritic cells which persists for the lifetime of the animal (Flano *et al.*, 2000; Stewart *et al.*, 1998; Sunil-Chandra *et al.*, 1992b; Weck *et al.*, 1999).

In mice which lack the interferon-γ receptor (IFN-γ $R^{-/-}$ mice), the lymphoproliferative phase is associated with severe pathological changes (Dutia *et al.*, 1997; Ebrahimi *et al.*, 2001; Weck *et al.*, 1997). Following intranasal infection of these mice, the mediastinal lymph nodes draining the lung and the spleen exhibit a drastic drop in cell numbers and become fibrotic (Dutia *et al.*, 1997). These changes are associated with an acute latent load 10–100 times higher than that seen in wild-type mice. After intraperitoneal infection, severe arteritis associated with high mortality occurs (Weck *et al.*, 1997). In this instance splenic pathology is found in 60 % of mice (Clambey *et al.*, 2000). The induction of splenic pathology is dependent on $CD8^+$ and $CD4^+$ T cells and the drop in spleen cell numbers is accompanied by lymphocytosis and the exclusion of circulating T and B cells from the spleen and mediastinal lymph node (Ebrahimi *et al.*, 2001).

The left end of the unique region of the MHV-68 genome is important for virus pathogenesis (Macrae *et al.*, 2001). This region contains the four unique protein-coding genes, M1–M4, as well as genes for eight tRNA-like molecules (vtRNAs) (Bowden *et al.*, 1997; Virgin *et al.*, 1997). In a previous study, Clambey *et al.* (2000) constructed two M1 deletion mutants: M1.LacZ, in which the *lacZ* gene replaced nt 1893–2402; and M1Δ511, which lacks nt 1893–2402. These viruses also lack the first 100 nt of the unique region of the genome. M1.LacZ did not cause splenic pathology or mortality in IFN-γ $R^{-/-}$ mice although it did cause severe vasculitis of the great arteries. The outcome of infection of IFN-γ $R^{-/-}$ mice with M1Δ511 or the marker rescue virus was not determined (Clambey *et al.*, 2000). Thus, mutations in the viral genome

†Present address: Department of Medical Microbiology, University of Liverpool, Duncan Building, Daulby Street, Liverpool L69 3GA, UK.

in the region of M1 appeared to have an effect on splenic pathology in the IFN-γ $R^{-/-}$ system.

This study aimed to extend the observations of Clambey *et al.* utilizing mutants of MHV-68 in order to clarify the potential role of the M1 gene product in splenic pathology in IFN-γ $R^{-/-}$ mice. Wild-type 129/Sv/Ev (WT) and IFN-γ $R^{-/-}$ mice were infected with wild-type MHV-68, with V2 virus (Simas *et al.*, 1998) or LHΔgfp virus both of which lack M1 and 4 or 5 of the vtRNA molecules respectively, with V1 virus which has an insertion in vtRNA3, or with MHV-76 which lacks M1–M4 and the tRNAs (Macrae *et al.*, 2001) and the consequences of infection on the spleen virus latency and/or pathology were examined. The data indicate that the M1 gene product is not involved in the pathological changes which occur in IFN-γ $R^{-/-}$ mice following MHV-68 infection. Northern analysis of transcripts in wild-type and mutant infected cells has identified two candidate transcripts present in wild-type infected cells but not in cells infected with mutant virus.

METHODS

Mice. Wild-type 129/Sv/Ev mice and IFN-γ $R^{-/-}$ 129/Sv/Ev (Huang *et al.*, 1993) were purchased from B & K Universal (Hull, UK) and bred in-house.

Virus and cells. Virus working stocks were prepared by infection of BHK-21 cells as described previously (Sunil-Chandra *et al.*, 1992a). The viruses used are as follows: MHV-68 clone g2.4 (Efstathiou *et al.*, 1990) V1 and V2 (Simas *et al.*, 1998) and MHV-76 (Macrae *et al.*, 2001). LHΔgfp was constructed by co-transfection of viral DNA with DNA containing a human cytomegalovirus (HCMV) immediate-early promoter-driven green fluorescent protein cassette (CMV-GFP) into BHK cells. Resulting recombinant plaques were isolated and purified on the basis of fluorescence under UV illumination. Detailed mapping of the viral genome was performed using Southern analysis, PCR amplification and sequencing. This determined that the viral genome was intact except that the CMV-GFP cassette had inserted by illegitimate recombination at the left end of the unique portion of the genome resulting in the deletion of nt 1–3223 (Fig. 1).

Infection and sampling. Age- and sex-matched mice were anaesthetized with Halothane (Rhone Merieux) and inoculated intranasally with 4×10^5 p.f.u. virus in 40 µl sterile PBS. At various times after infection mice were killed by cervical dislocation and tissues harvested for virus assays or histology.

Latent virus assays. Numbers of latently infected cells were determined by the infective centre assay as described previously (Dutia *et al.*, 1997).

Molecular cloning and sequencing. V1 virus stock was digested with Proteinase K and, after heat inactivation of the protease, the M1 gene coding region (nt 2023–3282, Virgin *et al.*, 1997) was amplified by PCR using the following primers: M1coding for 5′-GCATCATTGAGCGGCGA-3′ (nt 1999–2020); M1coding rev 5′-CAGGCTTAGGACTGCTGCCCA-3′ (nt 3290–3270). PCRs contained 1·5 U recombinant *Taq* DNA polymerase (Invitrogen), manufacturer's buffer, 100 mM dNTPs, 2·0 mM $MgCl_2$ and 50 pmol primer in a total reaction volume of 50 µl. The cycling parameters were as

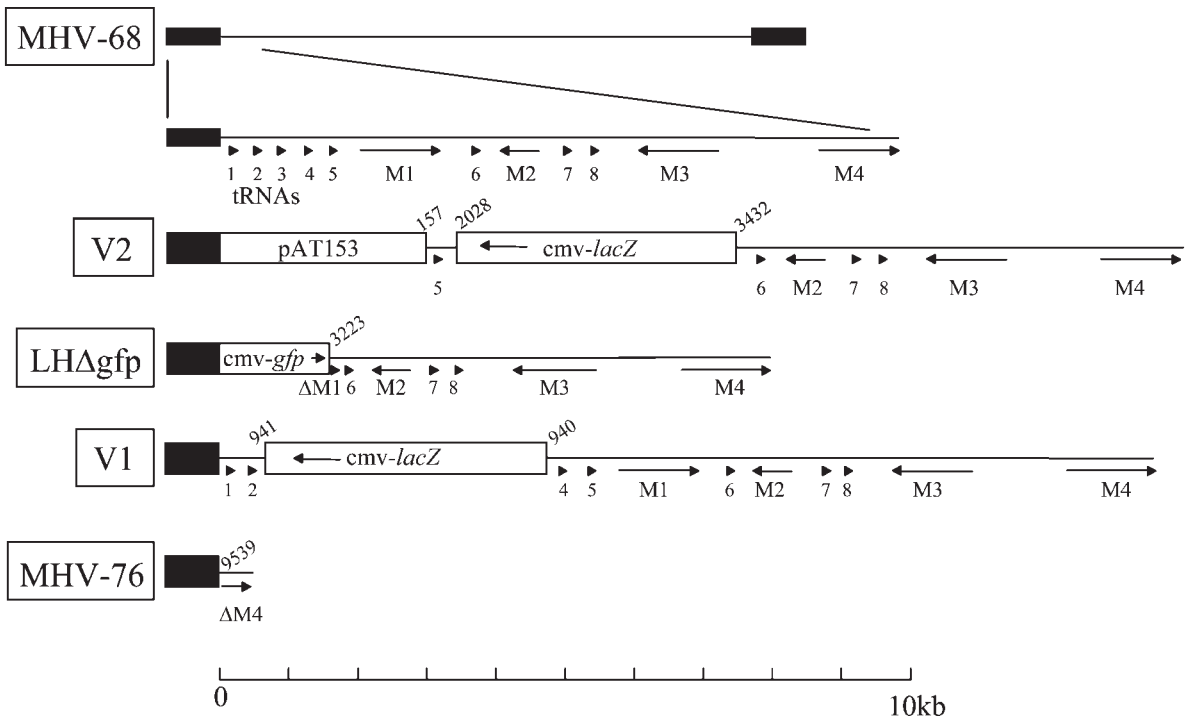

Fig. 1. Genome structures of MHV-68 and the four mutant viruses, V1 and V2 (Simas *et al.*, 1998), LHΔgfp and MHV-76 (Macrae *et al.*, 2001). Solid line represents unique sequence, filled boxes represent repeats. Genome coordinates taken from Virgin *et al.* (1997) given above the line. Approximate positions of genes M1–M4 and vtRNAs given below line.

follows: 94 °C 45 s, 55 °C 45 s, 72 °C 120 s, 35 cycles with a final extension time of 7 min at 72 °C. The PCR product was cloned into pCRII using a TA Cloning Kit (Invitrogen) and three clones were sequenced on a Licor 4000L automated sequencer.

Preparation of RNA from infected cells and RT-PCR analysis. BHK-21 cells were infected with 5 p.f.u. per cell virus, incubated at 37 °C for 20 h and harvested by addition of RNAzolB (Biogenesis). Total cell RNA was prepared from the RNAzolB lysates according to the manufacturer's instructions and the integrity of the RNA was determined by agarose gel electrophoresis. cDNA was prepared as described previously (Roy *et al.*, 2000). PCRs contained cDNA transcribed from approximately 0·1 μg RNA, 1·5 U recombinant *Taq* DNA polymerase (Invitrogen), manufacturer's buffer, 100 mM dNTPs, 2·0 mM $MgCl_2$ and 50 pmol primer in a total reaction volume of 50 μl. Cycling parameters were as for PCR of the M1 coding region. Primers were as follows: M1for 5′-CAGAACCTTACCAGTCATGTG-3′ (nt 2686–2708), M1rev 5′-GTTACTAGGACATACAGTGG-3′ (nt 2947–2966), product 278 bp; M2for 5′-TAATAGGAAGACGTATCTCAGG-3′ (nt 4077–4098), M2rev 5′-CTGCTTCCTTAGCCAGTCTC-3′ (nt 5856–5875), product 589 bp; M3for 5′-TGGCACTCAAACTTGGTTGTGG-3′ (nt 6566–6587), M3rev 5′-TAACAGGCAGATTGCCATTCCC-3′ (nt 6904–6925), product 359 bp; M4for 5′-CCTGGAGAAGATGATGATATTC-3′ (nt 8616–8637), M4rev 5′-AAAGTCATAAATCTCAATACC-3′ (nt 9739–9759), product 1143 bp; murine *β*-actin for 5′-TGTGATGGTGGGGAATGGGTCA-3′, murine *β*-actin rev 5′-TTTGATGTCACGCACGATTTC-3′, product 514 bp. Control reactions included PCR on samples from cDNA synthesis reactions carried out without addition of reverse transcriptase and PCRs without addition of template. PCR products were separated on a 1 % (w/v) agarose gel and visualized by ethidium bromide staining. In the case of the M2, the PCR product was transferred to charged nylon membrane (Hybond-N; Amersham) and probed with an M2-specific probe as described previously (Usherwood *et al.*, 2000).

Northern analysis. RNA was prepared from BHK cells infected or mock-infected for 20 h at a m.o.i. of 5 p.f.u. per cell as described above. Poly(A)-enriched RNA was prepared from total RNA by Oligotex (Qiagen) and 10 μg of each sample was run on a MOPS/formaldehyde gel (Sambrook, 2001). RNA was transferred to Nylon membrane and probed with ^{32}P-labelled gel-purified PCR product or restriction fragment in Ultrahyb (Ambion) according to the manufacturer's instructions. Size determinations were made with an RNA ladder (Invitrogen). Membranes were exposed to X-ray film at −70 °C.

Histopathology. Spleens were fixed in neutral buffered formaldehyde, processed routinely to 5 μm paraffin wax-embedded sections, stained with haematoxylin and eosin or Masson's trichrome and examined by light microscopy.

RESULTS

Pathological changes in spleens of IFN-γ R$^{-/-}$ mice infected with mutant viruses

In order to assess the role of individual MHV-68 genes in the pathological changes which occur in the spleens of IFN-γ R$^{-/-}$ mice, we infected wild-type 129/Sv/Ev mice and IFN-γ R$^{-/-}$ mice with wild-type MHV-68 or recombinant viruses and monitored the acute latency load, spleen cell numbers and histopathological changes. Fig. 2 shows the kinetics of latency and spleen cell numbers in wild-type and IFN-γ R$^{-/-}$ mice infected with wild-type virus or V2 virus. V2 virus has a deletion of MHV-68 nt 1–1571 and an insertion of the *lacZ* gene leftwards under control of the HCMV immediate-early promoter replacing MHV-68 nt 2028–3432. It thus retains the viral tRNA gene vtRNA5 and the first 6 nt of the M1 open reading frame but lacks vtRNAs1–4 and the major part of the M1 gene (Fig. 1). On days 13, 16, 21 and 29 post-infection spleens were removed and assessed for pathological changes. As described previously, MHV-68 infection resulted in a significantly higher latent load in IFN-γ R$^{-/-}$ mice than in wild-type mice and caused a drastic drop in spleen cell numbers (Fig. 2a, b). V2, on the other hand, did not cause an increase in latent virus load or a drop in cell numbers. These data indicate that a gene or genes at the left end of MHV-68 is required for the pathological changes which occur in the lymphoid tissue of IFN-γ R$^{-/-}$ mice.

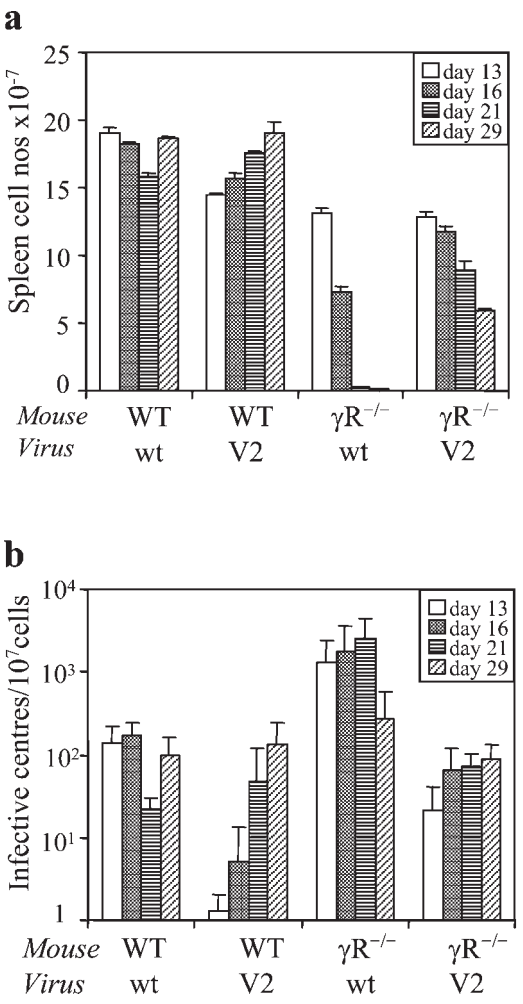

Fig. 2. Spleen cell numbers and latently infected cells (infective centres) in wild-type 129/Sv/Ev (WT) and IFN-γ R$^{-/-}$ 129/Sv/Ev (γR$^{-/-}$) mice infected with 4×10^5 p.f.u. wild-type MHV-68 (wt) or V2 virus (V2) at various times post-infection. Data are representative of three experiments.

To examine this further, mice were infected with two other recombinant viruses with mutations in the left end of the

genome, V1 and LHΔgfp. V1 has the *lacZ* gene under the control of the HCMV immediate-early promoter inserted leftwards within vtRNA3 (nt 940). LHΔgfp has MHV-68 nt 1–3223 replaced with the green fluorescent protein under the HCMV promoter in the rightwards direction (Fig. 1). Spleens were removed on days 16, 20 and 24 and examined for pathological changes. By day 16 post-infection, wild-type virus infection had produced the expected increase in latent virus load and the decrease in spleen cell numbers in IFN-γ $R^{-/-}$ mice (Fig. 3). However, IFN-γ $R^{-/-}$ mice infected with V1 and LHΔgfp had similar cell numbers and infective centres to wild-type mice.

V1 virus established latency and caused splenomegaly in wild-type mice with kinetics indistinguishable from wild-type MHV-68 (Fig. 3). Both V2 and LHΔgfp consistently showed delayed onset of latent infection in the spleen.

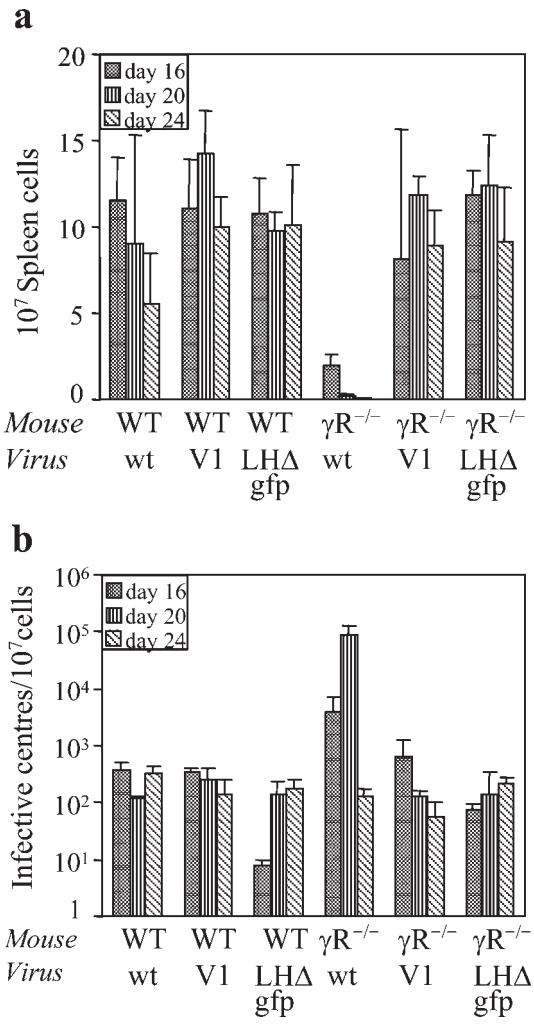

Fig. 3. Spleen cell numbers and latently infected cells (infective centres) in wild-type 129/Sv/Ev (WT) and IFN-γ $R^{-/-}$ 129/Sv/Ev ($\gamma R^{-/-}$) mice infected with 4×10^5 p.f.u. wild-type MHV-68 (wt), V1 virus (V1) or LHΔgfp virus (LHΔgfp) at various times post-infection. Data are representative of three experiments.

However, they both replicate with similar kinetics to wild-type virus in the lung and with identical kinetics of infection to wild-type virus *in vitro* (Simas *et al.*, 1998; S. Selvarajah, personal communication), suggesting that the deleted region plays a role in trafficking of virus to the spleen or in the establishment of latent infection. Interestingly, these changes in kinetics are not evident in IFN-γ $R^{-/-}$ mice.

To address the possibility that there was delayed onset of splenic changes following infection with the mutant viruses infection was monitored up to day 29 for V2 virus and day 24 for V1 and LHΔgfp. No elevated latency load or decrease in cell numbers was observed. It is unlikely therefore that the failure to cause severe splenic pathology is due to delayed kinetics of virus infection.

The lack of splenic pathology following infection of IFN-γ $R^{-/-}$ mice with V1, V2 and LHΔgfp was confirmed by examination of spleen sections stained with Masson's trichrome, which stains collagen. Spleen sections from mice infected with a further mutant virus, MHV-76 (Macrae *et al.*, 2001), which lacks the MHV-68-specific genes M1, M2, M3 and M4 and all eight vtRNAs, were also examined. Fig. 4 shows that while wild-type MHV-68 infection of IFN-γ $R^{-/-}$ produced characteristic changes in the spleen structure, including contraction of the white pulp, destruction of the red pulp and deposition of collagen, these changes did not occur following infection with the three mutant viruses and MHV-76. No evidence of pathological changes was found up to day 35 post-infection.

M1 gene sequence

Because M1 has been implicated in the pathogenesis of splenic atrophy in IFN-γ $R^{-/-}$ mice (Clambey *et al.*, 2000) we considered the possibility that V1 virus had, in addition to the insertion in vtRNA3, a mutation in the M1 gene. Therefore, the coding region of the M1 gene of V1 virus was amplified by PCR, cloned and sequenced. Three independent clones were sequenced, each of which was identical in sequence to the M1 gene of wild-type MHV-68. This established that V1 virus did not have a mutated M1 gene.

Expression of M1 gene in mutant virus infections

We then considered the possibility that, while the coding sequence for M1 was intact, either the insertion into vtRNA3 or a mutation in the promoter meant that the M1 gene was not transcribed. A further possibility was that the insertions/deletions upstream of M1 had altered transcription of other genes in the region. The transcription of M1–4 genes from wild-type, V1, V2 and LHΔgfp viruses was examined in lytically infected BHK cells by RT-PCR as described in Methods. Controls were PCR on cDNA reactions incubated without reverse transcriptase, PCR without cDNA and RT-PCR on mock-infected cell RNA. These were negative in all cases. M1, M3 and M4 RT-PCR products were visible as single bands on ethidium bromide-stained gels. The M2

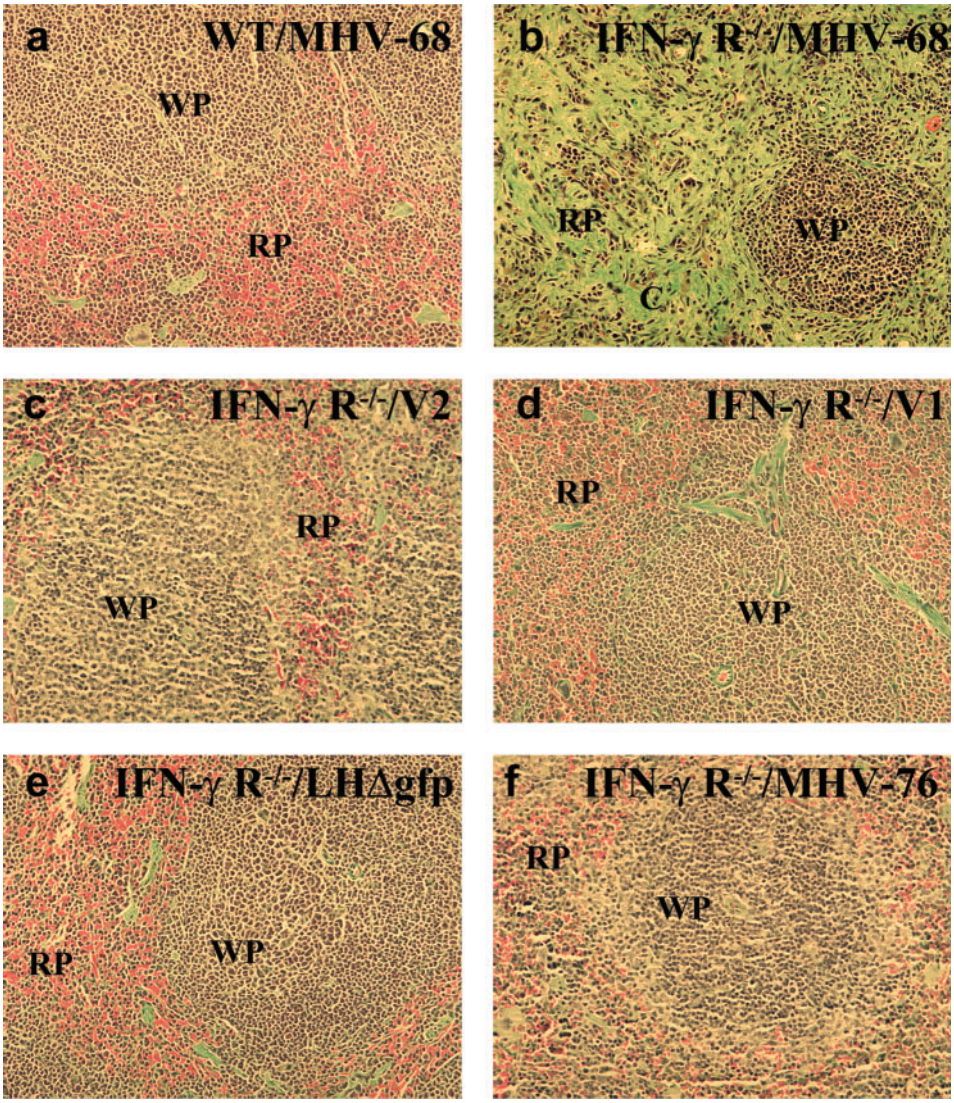

Fig. 4. Histology of wild-type (a) and IFN-γ $R^{-/-}$ (b–f) mice 20 days after infection with wild-type MHV-68 (a, b), V2 (c), V1 (d), LHΔgfp (e) or MHV-76 (f). WP, white pulp; RP, red pulp; C, collagen.

gene is expressed at very low levels in lytically infected cells and hence only a weak signal was detectable on an ethidium bromide-stained gel. The presence of the M2 RT-PCR product was examined by Southern hybridization. Fig. 5(a) shows that the four viruses tested, wild-type, V1, V2 and LHΔgfp, all transcribed M2, M3 and M4. As predicted, V2 and LHΔgfp did not transcribe M1. M1 transcription was readily detected in wild-type virus and V1-infected cells. Thus the insertion in the V1 virus had not resulted in lack of transcription of the M1 gene.

Transcription in BHK cells infected with wild-type virus, V1 virus and LHΔgfp was examined further by Northern blotting. Poly(A) RNA from infected and mock-infected cells was transferred to Nylon membrane and probed with a probe spanning the M1 gene. This showed that transcripts of the expected length were present in V1-infected cells and that the quantity of transcript was similar to that present in wild-type virus (Fig. 5b).

Identification of novel transcripts

In order to investigate the possibility that there are other undefined transcripts encoded within the vtRNA region of the genome, the Northern blot was probed with a probe spanning the vtRNA 1–4 region (nt 106–1517). In wild-type virus-infected cells four major transcripts were detected (Fig. 6). These included the vtRNAs, which were seen as small transcripts of $<0{\cdot}24$ kb, a small transcript of 0·5 kb which is detectable on probing of Northern blots of total RNA with this probe (Bowden *et al.*, 1997; unpublished observations) and two transcripts of approximately 4·3 and 3·2 kb. The 4·3 and 3·2 kb transcripts were present in low abundance and were not evident when total RNA rather

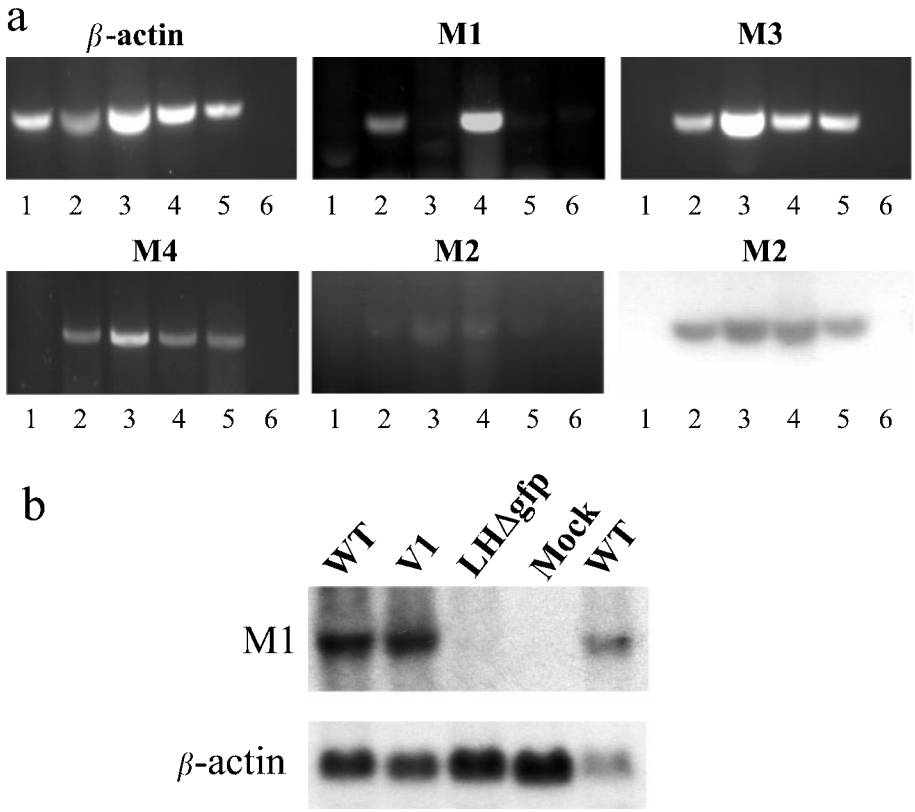

Fig. 5. Transcription of genes M1–M4 of MHV-68 and mutant viruses. (a) RT-PCR analysis of genes M1–M4 and β-actin transcription in BHK cells infected for 20 h with wild-type or mutant MHV-68 viruses. Lane 1, mock-infected; 2, wild-type virus; 3, LHΔgfp; 4, V1; 5, V2; 6, no cDNA. A further control (not shown) in the absence of reverse transcriptase was negative. (b) Northern analysis of M1 and β-actin transcription in BHK cells infected for 20 h with wild-type MHV-68, V1, LHΔgfp or mock-infected.

than mRNA was probed. They were absent in cells infected with V1 virus or LHΔgfp. However, novel transcripts of 10, 6·8, 5·3 and 4·2 kb were detected in cells infected with V1 and a single 5·2 kb transcript was detected in LHΔgfp-infected cells. These transcripts were also found in MHV-68-infected owl monkey kidney cells (data not shown). The vtRNAs were absent from LHΔgfp-infected cells but present in the V1-infected cells. LHΔgfp does not encode vtRNAs 1–5 but the genes for vtRNAs 6–8 are present in the genome. The result indicates that vtRNAs 6–8 are not detected by the vtRNA1–4 probe. V1 lacks vtRNA 3 but expression of vtRNAs 1, 2 and 4 occurs. The 0·5 kb transcript was absent from both V1- and LHΔgfp-infected cells. This transcript may be related to vtRNA 3 or to other changes in the transcription pattern in these mutants.

DISCUSSION

The experiments described here show that the pathological changes that occur in IFN-γ $R^{-/-}$ mice following infection with MHV-68 can be ascribed to genes encoded in the left end of the genome. MHV-76, which lacks nt 1–9538 of MHV-68 encoding genes M1–M4 and the eight vtRNAs does not cause splenic pathology. V2 and LHΔgfp, which also have large deletions in this region of the genome and lack the M1 gene and four or five vtRNAs, do not cause splenic pathology. V1, which has a large insertion at nt 940 disrupting vtRNA3 but encodes the M1–M4 genes, also lacks the ability to cause the pathology. While the data from V2 virus and LHΔgfp, like that described previously for M1 (Clambey *et al.*, 2000), implicate the M1 gene in the process of atrophy, the results from V1 virus suggest that M1 is unlikely to be the primary virus gene involved in the induction of splenic pathology.

V1 virus, like the other mutant viruses investigated here, does not express vtRNA3. The function of the vtRNAs is unknown but as they are not aminoacylated it is unlikely that they function in translation (Bowden *et al.*, 1997). It is more likely that the vtRNAs have a regulatory function during infection. Moreover, a recombinant MHV-76 virus into which we have inserted vtRNAs 1–5 does not cause splenic pathology in IFN-γ $R^{-/-}$ mice (unpublished data). This recombinant virus expresses vtRNA3 (Anna Cliffe, personal communication). It is therefore unlikely that vtRNA3 is involved in induction of splenic pathology. More likely is the possibility that the insertion into vtRNA3 is

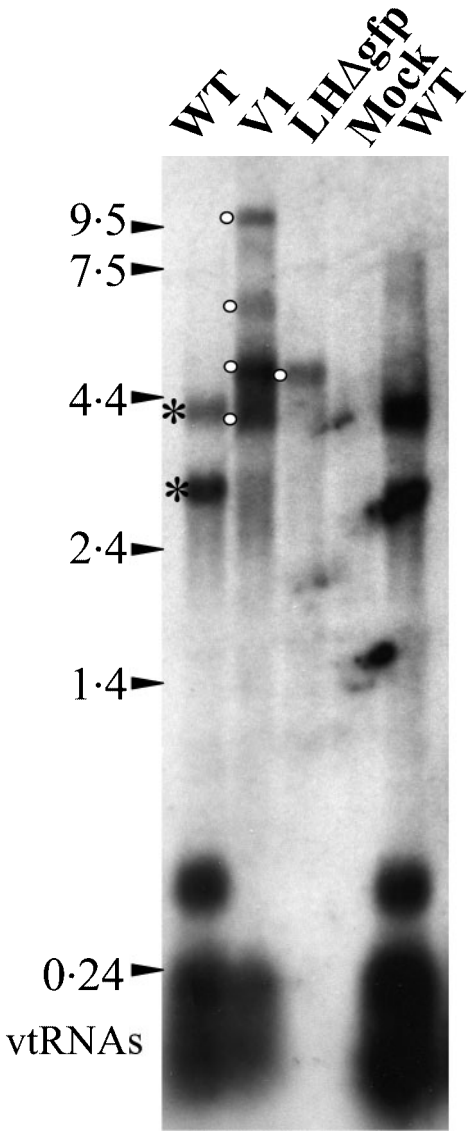

Fig. 6. Northern analysis of transcripts present in BHK cells infected for 20 h with wild-type MHV-68, V1, LHΔgfp or mock-infected. Blot probed with dsDNA probe MHV-68 nt 106–1517. *, Novel transcripts in wild-type virus-infected cells; ○, novel transcripts in V1- and LHΔgfp-infected cells.

disrupting transcription of an unidentified mRNA. Both Epstein–Barr virus and Kaposi's sarcoma-associated herpesvirus encode multi-exon mRNAs which are transcribed across the terminal repeats (Choi *et al.*, 2000; Laux *et al.*, 1988; Sample *et al.*, 1989) and there is evidence for an equivalent transcript in MHV-68 (Husain *et al.*, 1999). We have identified two novel transcripts in wild-type MHV-68-infected BHK cells. These transcripts are expressed at a low level and are not detected when total RNA is probed (Bowden *et al.*, 1997, unpublished data), but can be detected when 10 μg of poly(A) RNA is used for analysis. Although MHV-68 infection of BHK cells is lytic, some transcription of latency-associated genes may occur. The M2 gene, for example, is latency-associated but can be detected at low levels in lytically infected cells. Hence these novel transcripts may be latency-associated genes and are candidates for virus genes involved in the induction of splenic pathology which is associated with latent infection. Cells infected with mutant viruses contain novel transcripts which may be the result of aberrant processing.

A third possibility is that the insertion of the HCMV immediate-early promoter is altering virus gene transcription. There is evidence for both MHV-68 and mouse CMV that insertion of the *lacZ* gene under control of the HCMV promoter-enhancer can alter the ability of the virus to replicate (Clambey *et al.*, 2000; Stoddart *et al.*, 1994). However, the fact that MHV-76 also fails to induce atrophy argues that a gene or genes involved in the induction of atrophy are encoded at least in part within the first 9538 bp of MHV-68.

The replication of V1 and V2 has been characterized previously in BALB/c mice. In these mice no differences between the mutants and wild-type MHV-68 were detected. In contrast, the experiments reported here show a clear difference from wild-type virus phenotype in IFN-γ $R^{-/-}$ mice, highlighting the importance of host phenotype in studies of virus mutants. V2 and LHΔgfp have a distinct phenotype in 129/Sv/Ev mice consistently showing delayed kinetics of latent virus infection in the spleen. The viruses lack four or five vtRNA molecules respectively and the M1 gene product. Studies with an M1 deletion mutant have implicated the M1 gene product in suppression of virus reactivation. These studies are not directly comparable to those described here as virus was inoculated via a different route. No evidence of increased reactivation was found following infection with V2 and LHΔgfp; however, the data are consistent with a role for M1 or a closely related gene in latency.

The data presented here provide evidence for transcripts across the left region of the genome which encodes the vtRNAs. These genes are candidates for novel latency-associated genes and for viral mediators of splenic pathology. Studies to characterize these genes are under way and will clarify these issues further.

ACKNOWLEDGEMENTS

This work was supported by the Biotechnology and Biological Sciences Research Council and the Cancer Research Campaign (UK). J. P. S. is a Royal Society University Research Fellow.

REFERENCES

Bowden, R. J., Simas, J. P., Davis, A. J. & Efstathiou, S. (1997). Murine gammaherpesvirus 68 encodes tRNA-like sequences which are expressed during latency. *J Gen Virol* **78**, 1675–1687.

Choi, J. K., Lee, B. S., Shim, S. N., Li, M. & Jung, J. U. (2000). Identification of the novel K15 gene at the rightmost end of the Kaposi's sarcoma-associated herpesvirus genome. *J Virol* **74**, 436–446.

Christensen, J. P., Cardin, R. D., Branum, K. C. & Doherty, P. C. (1999). CD4(+) T cell-mediated control of a gamma-herpesvirus in B cell-deficient mice is mediated by IFN-gamma. *Proc Natl Acad Sci U S A* **96**, 5135–5140.

Clambey, E. T., Virgin, H. W., IV & Speck, S. H. (2000). Disruption of the murine gammaherpesvirus 68 M1 open reading frame leads to enhanced reactivation from latency. *J Virol* **74**, 1973–1984.

Dutia, B. M., Clarke, C. J., Allen, D. J. & Nash, A. A. (1997). Pathological changes in the spleens of gamma interferon receptor-deficient mice infected with murine gammaherpesvirus: a role for CD8 T cells. *J Virol* **71**, 4278–4283.

Ebrahimi, B., Dutia, B. M., Brownstein, D. G. & Nash, A. A. (2001). Murine gammaherpesvirus-68 infection causes multi-organ fibrosis and alters leukocyte trafficking in interferon-gamma receptor knockout mice. *Am J Pathol* **158**, 2117–2125.

Efstathiou, S., Ho, Y. M., Hall, S., Styles, C. J., Scott, S. D. & Gompels, U. A. (1990). Murine herpesvirus 68 is genetically related to the gammaherpesviruses Epstein–Barr virus and herpesvirus saimiri. *J Gen Virol* **71**, 1365–1372.

Flano, E., Husain, S. M., Sample, J. T., Woodland, D. L. & Blackman, M. A. (2000). Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. *J Immunol* **165**, 1074–1081.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R. M. & Aguet, M. (1993). Immune response in mice that lack the interferon-γ receptor. *Science* **259**, 1742–1745.

Husain, S. M., Usherwood, E. J., Dyson, H., Coleclough, C., Coppola, M. A., Woodland, D. L., Blackman, M. A., Stewart, J. P. & Sample, J. T. (1999). Murine gammaherpesvirus M2 gene is latency-associated and its protein a target for $CD8^+$ T lymphocytes. *Proc Natl Acad Sci U S A* **96**, 7508–7513.

Laux, G., Perricaudet, M. & Farrell, P. J. (1988). A spliced Epstein–Barr virus gene expressed in immortalized lymphocytes is created by circularization of the linear viral genome. *EMBO J* **7**, 769–774.

Macrae, A. I., Dutia, B. M., Milligan, S., Brownstein, D. G., Allen, D. J., Mistrikova, J., Davison, A. J., Nash, A. A. & Stewart, J. P. (2001). Analysis of a novel strain of murine gammaherpesvirus reveals a genomic locus important for acute pathogenesis. *J Virol* **75**, 5315–5327.

Roy, D. J., Ebrahimi, B. C., Dutia, B. M., Nash, A. A. & Stewart, J. P. (2000). Murine gammaherpesvirus M11 gene product inhibits apoptosis and is expressed during virus persistence. *Arch Virol* **145**, 2411–2420.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning. a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sample, J., Liebowitz, D. & Kieff, E. (1989). Two related Epstein–Barr virus membrane proteins are encoded by separate genes. *J Virol* **63**, 933–937.

Sarawar, S. R., Cardin, R. D., Brooks, J. W., Mehrpooya, M., Tripp, R. A. & Doherty, P. C. (1996). Cytokine production in the immune response to murine gammaherpesvirus 68. *J Virol* **70**, 3264–3268.

Simas, J. P., Bowden, R. J., Paige, V. & Efstathiou, S. (1998). Four tRNA-like sequences and a serpin homologue encoded by murine gammaherpesvirus 68 are dispensable for lytic replication *in vitro* and latency *in vivo*. *J Gen Virol* **79**, 149–153.

Stewart, J. P., Usherwood, E. J., Ross, A., Dyson, H. & Nash, T. (1998). Lung epithelial cells are a major site of murine gammaherpesvirus persistence. *J Exp Med* **187**, 1941–1951.

Stoddart, C. A., Cardin, R. D., Boname, J. M., Manning, W. C., Abenes, G. B. & Mocarski, E. S. (1994). Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. *J Virol* **68**, 6243–6253.

Sunil-Chandra, N. P., Efstathiou, S., Arno, J. & Nash, A. A. (1992a). Virological and pathological features of mice infected with murine gamma-herpesvirus 68. *J Gen Virol* **73**, 2347–2356.

Sunil-Chandra, N. P., Efstathiou, S. & Nash, A. A. (1992b). Murine gammaherpesvirus 68 establishes a latent infection in mouse B lymphocytes in vivo. *J Gen Virol* **73**, 3275–3279.

Tripp, R. A., Hamilton-Easton, A. M., Cardin, R. D., Nguyen, P., Behm, F. G., Woodland, D. L., Doherty, P. C. & Blackman, M. A. (1997). Pathogenesis of an infectious mononucleosis-like disease induced by a murine gamma-herpesvirus: role for a viral superantigen? *J Exp Med* **185**, 1641–1650.

Usherwood, E. J., Roy, D. J., Ward, K., Surman, S. L., Dutia, B. M., Blackman, M. A., Stewart, J. P. & Woodland, D. L. (2000). Control of gammaherpesvirus latency by latent antigen-specific $CD8^+$ T cells. *J Exp Med* **192**, 943–952.

Virgin, H. W., Latreille, P., Wamsley, P., Hallsworth, K., Weck, K. E., Dal Canto, A. J. & Speck, S. H. (1997). Complete sequence and genomic analysis of murine gammaherpesvirus 68. *J Virol* **71**, 5894–5904.

Weck, K. E., Dal Canto, A. J., Gould, J. D., O'Guin, A. K., Roth, K. A., Saffitz, J. E., Speck, S. H. & Virgin, H. W. (1997). Murine gammaherpesvirus 68 causes severe large-vessel arteritis in mice lacking interferon-gamma responsiveness: a new model for virus-induced vascular disease. *Nat Med* **3**, 1346–1353.

Weck, K. E., Kim, S. S., Virgin, H. I. & Speck, S. H. (1999). Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells. *J Virol* **73**, 3273–3283.