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Kinetics of Murine Gammaherpesvirus 68 Gene Expression following Infection of Murine Cells in Culture and in Mice

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A model system to study the pathogenesis of gammaherpesvirus infections is the infection of mice with murine gammaherpesvirus 68 (MHV-68). To define the kinetics of infection, we developed an RNase protection assay to quantitate gene expression from lytic (K3, Rta, M8, DNA polymerase [DNA pol], and gB) and candidate latency (M2, M3, M9, M11, ORF73, and ORF74) genes. All candidate latency genes were expressed during lytic infection of 3T3 cells. Four kinetic classes of transcripts were observed following infection of 3T3 cells: immediate-early (K3, Rta, M8, and ORF73), early (DNA pol), early-late (M3, M11, and ORF74), and late (M2, M9, and gB). To assess the kinetics of viral gene expression in vivo, lungs, spleens, and mediastinal lymph nodes (MLN) were harvested from MHV-68-infected mice. All transcripts were expressed between 3 and 6 days postinfection (dpi) in the lungs. In the spleen, K3, M3, M8, and M9 transcripts were expressed between 10 and 16 dpi when latency is established. The K3, M3, M8, M9, and M11 transcripts were detected in the MLN from 2 through 16 dpi. This is the first demonstration of MHV-68 gene expression in the MLN. Importantly, our data showed that MHV-68 has different kinetics of gene expression at different sites of infection. Furthermore, we demonstrated that K3, a gene recently shown to encode a protein that downregulates major histocompatibility complex class I on the surface of cells, is expressed during latency, which argues for a role of K3 in immune evasion during latent infection.

The human pathogens Epstein-Barr virus (EBV) and human herpesvirus 8 (HHV-8) are members of the *Gammaherpesvirinae* subfamily, which also includes murine gammaherpesvirus 68 (MHV-68) and simian herpesvirus saimiri (HVS). Membership in the *Gammaherpesvirinae* is based on genomic organization as well as the ability to establish latency in lymphoid cells (9). Both EBV and HHV-8 are associated with a number of malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, and Kaposi's sarcoma (9, 10). Because EBV and HHV-8 can replicate only in cells of human origin, studies on the pathogenesis of these viruses have been limited to analysis of biopsies of human tissue, blood samples, and xenotransplantation of human tissue into immunocompromised mice (10, 15). Thus, the natural history of the viral infection is largely unknown due to the lack of animal model systems. In addition, in vitro studies of EBV and HHV-8 are limited to cell lines that are latently infected with these viruses. Analysis of lytic genes can be done only following induction of the lytic cycle in the latently infected cell lines. This limitation has hampered the genetic analysis of EBV and HHV-8.

An alternative model to study the pathogenesis of human gammaherpesviruses is the MHV-68 model system. The MHV-68 genome is sequenced and encodes approximately 80 gene products, 63 of which are colinear and homologous to HVS and HHV-8 gene products (30). In addition, there are several unique open reading frames (ORFs). Like the other gammaherpesviruses, MHV-68 encodes several proteins that have cellular homologs, including homologs of cyclin D, Bcl-2, and the interleukin-8 receptor (30). MHV-68 can lytically infect a variety of cell types in vitro, which has facilitated the generation of recombinant viruses (3, 17). The analysis of patterns of infection with recombinant viruses lacking specific viral genes will help to elucidate the functions of MHV-68 genes. Thus, it is essential to define the kinetics of viral gene expression both in vitro and in vivo.

Infection of mice by the intranasal (i.n.) route of inoculation results in acute viral replication in the epithelial cells of lungs followed by establishment of latency in B cells in the spleen as well as in dendritic cells, macrophages, and lung epithelial cells (4, 23–25, 34). MHV-68 also establishes latency in other lymphoid tissues, including mediastinal lymph nodes (MLN), bone marrow, and peripheral blood cells (1).

A key area of investigation in MHV-68 research is the identification of latency-associated genes. The use of different detection methods with different levels of sensitivity for measuring viral transcripts (e.g., in situ hybridization, cDNA hybridization, Northern blot hybridization, and reverse transcription [RT]-PCR), the use of B-cell-competent mice, B-cell-deficient mice, and persistently infected cell lines, and the use of different routes of inoculation have all contributed to a lack of consensus on sites of MHV-68 latency and transcription patterns during latency. The paradigm of EBV latency suggests that some genes are expressed exclusively during the latent phase of replication (13). Virgin et al. (31) used this paradigm to identify four candidate latency-associated transcripts (M2, M11, ORF73, and ORF74). These transcripts were expressed in peritoneal exudate cells of B-cell-deficient mice 48 days

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following intraperitoneal (i.p.) inoculation but were expressed at low or undetectable levels during lytic infection of murine 3T12 cells in vitro. However, the expression of these candidate latency genes has not been assessed following the lytic phase of replication after i.p. or i.n. infection of mice. M2 was also identified as a candidate latency transcript by Husain et al. (6), based on the expression of M2 in the spleens of B-cell-competent mice following i.n. infection and expression in the S11 B-cell line. The S11 B-cell line, a persistently infected cell line that has both latent and lytic infection within the cell population, is considered to be an in vitro model of MHV-68 latency similar to the lymphoblastoid cell lines latently infected with EBV or HHV-8 (26). Furthermore, M2 expression could not be detected by Northern blot analysis in lytically infected BHK cells.

For many herpesviruses, including EBV, some latency-associated transcripts are also detected during lytic replication. In this regard, Virgin et al. (31) identified the M3 and M9 genes as being abundantly expressed during both lytic infection in vitro and latent infection of B-cell-deficient spleens in vivo. M3 was also identified by Simas et al. (19) as being expressed in latently infected splenic B cells following i.n. infection of mice. In addition, M3 was expressed in the S11 B-cell line. Consistent with these observations, Husain et al. (6) detected expression of M3 transcripts in spleens of latently infected mice. The M8 transcript was expressed early during latency in the spleens following i.n. infection (6) as well as in S11 cells treated with 2′-deoxy-5-ethyl-β-4′-thiouridine (4′-S-EtdU), an inhibitor of lytic replication (19). However, M8 was not identified as a candidate latency transcript by Virgin et al. (31). Thus, there is some discrepancy regarding whether M8 and M3 are latency-associated transcripts. K3 was another transcript detected in the S11 B-cell line treated with 4′-S-EtdU; however, K3 was not detected in the spleens by in situ hybridization following i.n. infection (19).

If MHV-68 is to be a useful model system to study gammaherpesvirus pathogenesis, it is essential to characterize the patterns of viral gene expression during lytic infection in vitro and in vivo and during latency in vivo. To this end, we developed a multiprobe RNase protection assay (RPA) to quantitate expression of transcripts from genes known to be expressed during the lytic cycle in vitro (K3, Rta, M8 DNA polymerase [DNA pol], and gB) (7, 8, 22, 31, 35), and candidate latency genes (M2, M3, M9, M11, ORF73, and ORF74) (6, 19, 31). The RPA allows not only a sensitive, quantitative assessment of transcription but also intra-assay comparisons between transcripts. We analyzed patterns of gene expression following infection of mouse 3T3 cells and following i.n. infection of mice. We analyzed expression of the MHV-68 transcripts in lungs and spleens as well as in the MLN, the draining lymph nodes of the lung. All transcripts, including the candidate latency transcripts, were expressed during the lytic cycle following infection of 3T3 cells and in the lungs during the acute phase of replication. Four genes—M3, M8, M9, and K3—were expressed exclusively within the spleen and MLN. Interestingly, the kinetics of expression were strikingly different between lungs, spleens, and MLN. Recent reports that M3 encodes a soluble chemokine binding protein (11, 28) which is essential for the establishment of latency (S. Estafthiou, personal communication) and that K3 can down-regulate major histocompatibility complex (MHC) class I expression in vitro (20) suggest that viral modulation of the immune response is a key mechanism for the establishment of latency. Since the functions of the M8 and M9 proteins are unknown and they appear to be expressed during persistence or latency, it will be of interest to determine if these proteins play a role in immune evasion or establishment of latency.

MATERIALS AND METHODS

Cell lines, viruses, and mice. Owl monkey kidney (OMK) cells (ATCC CRL-1556, American Type Culture Collection, Manassas, Va) were maintained in RPMI 1640 medium (Gibco-BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (Gemini BioProducts, Calabasas, Calif.), 2 mM L-glutamine, and penicillin-streptomycin (Gibco-BRL). NIH 3T3 cells (ATCC CRL-1658) were maintained in Dulbecco's modified Eagle medium (BioWhittaker, Walkersville, Md.) supplemented with glucose (4.5 g/liter), 2 mM L-glutamine (Gibco-BRL), penicillin-streptomycin (Gibco-BRL), and 10% fetal bovine serum (Gemini BioProducts). The MHV-68 sequenced clone G2.4 was used for infections (30). Generation of virus stocks and determination of viral titer by plaque assay was done as described elsewhere (1).

For treatment of cells with cycloheximide (CHX) and phosphonoacetic acid (PAA), 3T3 or OMK cells at 80% confluency were infected at a multiplicity of infection (MOI) of 5 in the presence of CHX (50 μg/ml) and anisomycin (2.7 μg/ml) or PAA (200 μg/ml). RNA was harvested at 8 h postinfection (hpi) (CHX-anisomycin) or at 24 hpi (PAA). RNA extraction was performed as described previously (2).

Four to six-week-old male BALB/c mice were purchased from Harlan Sprague (Indianapolis, Ind.) and maintained in specific-pathogen-free housing. Mice were inoculated under light anesthesia with 4×10^5 PFU i.n. Organs were harvested at indicated times postinfection and snap-frozen in a dry ice-ethanol bath. RNA was extracted from organs by Dounce homogenization in solution D (2) in the absence of Sarkosyl to prevent foaming. After transfer to a polypropylene tube, Sarkosyl was added to a final concentration of 0.5%. The remaining RNA extraction protocol was performed exactly as described elsewhere (2).

Generation of RPA riboprobe templates. RNA extracted from MHV-68-infected OMK cells was used (100 ng/reaction) as the template for cDNA synthesis initiated by random hexamer primers, followed by PCR using a GeneAmp kit (Perkin-Elmer Cetus, Foster City, Calif.) and appropriate primers for the amplification of MHV-68-specific DNA fragments. The RT-PCR conditions, strategies for design of PCR primers and ligation of the amplified DNA fragments into the pGEM-4 vector (Promega, Madison, Wis.) were detailed previously (5, 14). The authenticity of all subclones was verified at the University of Michigan Sequencing Core Facility using an Applied Biosystems DNA sequencer (model 377 or 373). The subclone designations (and nucleotide sequence) from MHV-68 GenBank accession number U97553 (30) were as follows: K3 (nucleotides [nt] 24929 to 25287), Rta (nt 68349 to 68680), M8 (nt 76044 to 76343), DNA Pol (nt 21465 to 21736), gB (nt 18618 to 18857), M2 (nt 4110 to 4331), M3 (6779 to 6977), M9 (nt 94060 to 94241), M11 (nt 103628 to 103786), ORF74 (nt 105539 to 105678), and ORF73 (nt 104446 to 104565). The mouse L32(a) (L32) subclone was described previously (5).

RPA. The RPA was performed exactly as described elsewhere (5). Two MHV-68-specific riboprobe template sets, γ-3 and γ-4, were assembled from *EcoRI*-linearized and purified subclones. The γ-3 template set synthesized riboprobes specific for K3, Rta, M8, DNA Pol, gB, M2, M3, M9, and L32. The γ-4 template set synthesized riboprobes specific for M11, ORF74, ORF73, and L32. All riboprobe syntheses were driven by T7 bacteriophage RNA polymerase with [α-^{32}P]UTP (Amersham, Arlington Heights, Ill.) as the labeling nucleotide. The RPA was done exactly as described elsewhere (5). Probe bands were visualized by autoradiography (XAR film; Kodak, Rochester, N.Y.) and were quantified by using a Storm PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.). For the latter, volume measurement with rectangular objects were used to generate PhosphorImager (PI) counts, which are presented as a percentage of the internal housekeeping signal (i.e., L32) present in each lane.

RESULTS

Development of an RPA to measure MHV-68 gene expression. To characterize the kinetics of MHV-68 gene expression, a multiprobe RPA was developed because of the specificity and quantitative aspects of this assay as well as its greater

TABLE 1. RPA riboprobe templates

Encoding:	Genome coordinates[a]	Probe size (bases)	Comments
K3	25287–24929	360	Down-regulates MHC class I expression (20)
Rta (gene 50)	68349–68680	330	EBV Rta homologue, shown to reactivate latency from S11 cells (7, 35)
M8	76044–76343	300	Unknown function
DNA Pol	21465–21736	270	DNA polymerase (function based on homology) (30)
gB	18618–18857	240	Glycoprotein, homologous to EBV gB (21)
M2	4331–4110	220	Unknown function, target for cytotoxic T lymphocytes (6)
M3	6977–6779	200	Chemokine binding protein (11)
M9	94241–94060	180	Unknown function
M11	103628–103786	160	Bcl-2 homolog (30)
ORF74	105539–105679	140	Interleukin-8 receptor homolog (30)
ORF73	104564–104446	120	HHV-8 LANA homolog (30)

[a] According to GenBank accession no. U97553.

sensitivity relative to Northern blotting. In addition, using the RPA, relative levels of gene expression in an individual sample can be compared. We chose to analyze a panel of genes that were (i) expressed during the lytic cycle and not thought to be associated with latency (the K3, Rta, M8, DNA Pol, and gB genes); (ii) were expressed during the lytic cycle but proposed to be associated with latency in vivo (M3, M9, ORF73, and ORF74); or (iii) had not been detected during the lytic cycle and were identified to be possible latency candidate genes (M2 and M11) (Table 1). Of these, the structures of the Rta (gene 50) (7, 35), M2 (6), gB (22), M8 (8), and M3 (28) genes have previously been determined. The orientation of the other genes was deduced based on identification of ORFs (8).

To verify that protected mRNA of the predicted size was obtained, each riboprobe was tested individually before being included in a multiprobe set (data not shown). To test each riboprobe, 3T3 cells were infected at an MOI of 5, and total RNA was harvested at 8 hpi and analyzed by RPA. Because of the low level expression of the M11, ORF73, and ORF74 transcripts and the intensity of expression from the M3 and M9 genes, we separated the riboprobes into two sets: the γ-3 set, which contains probes for the K3, Rta, M8, DNA Pol, gB, M2, M3, and M9 genes; and the γ-4 set, which contains probes for the M11, ORF73, and ORF74 genes. Also included in each set was a probe for the gene encoding mouse ribosomal protein L32, which serves as a housekeeping gene and corrects for sample variability (5). We observed the expression of all genes including the candidate latency genes in 3T3 cells by 8 hpi (Fig. 1A). To quantitate the levels of expression of the individual transcripts relative to each other, we quantitated the PI units for each protected probe fragment and expressed the PI units as a percentage of the PI units of the housekeeping gene, L32 (Fig. 1B). M11 was the lowest-expressed transcript, while M3 and M9 were the highest (range, $<1\%$ of the L32 PI signal to $\sim 450\%$ of the L32 PI signal, respectively). By measuring expression of the transcripts with the RPA, which uses a single-stranded antisense radiolabeled probe to detect transcripts, we have also confirmed the predicted transcriptional direction of the K3, DNA Pol, M9, ORF73, and ORF74 genes. Our studies also indicated that all candidate latency genes were expressed following lytic infection of cells in culture and thus their expression was not exclusive to latently infected cells.

Assessment of the sensitivity of the RPA. To assess the sensitivity of the RPA, RNA was extracted from MHV-68-infected OMK cells at 16 hpi, twofold serial dilutions of the RNA were done, and the RNA was analyzed with the γ-3 probe set. As shown in Fig. 2A, we could detect viral mRNA in samples derived from as little as 13 ng of total RNA. To examine the linearity of the RPA and to establish the validity of relative comparisons between mRNA species, we used the PhosphorImager to quantify protected fragments in Fig. 2A that were representative of highly expressed (M3) and lowly expressed (Rta) transcripts. Plots of PI counts versus micrograms of RNA (Fig. 2B) show a strong linear relation between PI counts and RNA levels for both mRNA species at amounts 0.025 μg. Importantly, the log-log plots indicate that this difference in expression was consistent throughout the RNA range.

Kinetics of MHV-68 transcription in vitro. To determine the kinetic class of the MHV-68 gene transcripts, 3T3 cells were either infected in the presence of CHX and anisomycin, inhibitors of protein synthesis, infected in the presence of PAA, an inhibitor of viral DNA Pol, or left untreated. Total RNA was harvested at either 8 hpi (CHX-anisomysin treated) or 24 hpi (PAA treated and untreated) and analyzed by RPA (Fig. 3). Transcription from the K3, Rta, M8, M9, and ORF73 genes occurred in the presence of CHX treatment, indicating that these are immediate-early genes. It should be noted however, that M9 and ORF73 transcripts were inhibited in the presence of CHX to a greater degree than the other immediate-early transcripts. MHV-68 DNA Pol, like other herpesvirus polymerases, is encoded by an early gene. Expression of M3, M11, and ORF74 was inhibited but not eliminated in the presence of PAA, suggesting that these are early-late transcripts. M2 and gB have the kinetics of late genes, as their expression is completely inhibited by treatment with PAA. The M9 transcript was strongly inhibited in the presence of PAA, but a faint transcript remains. These results confirm the previous studies identifying the kinetic class of the Rta, DNA Pol, gB, and M3 genes (7, 8, 22, 29, 31, 35). M8 was previously classified as an early transcript (8), but in this study the effects of protein synthesis inhibitors on M8 transcription were not assessed.

As a separate assessment of the temporal pattern of gene expression, we examined expression of the genes in the γ-3 and γ-4 riboprobe sets following infection of mouse 3T3 cells. RNA was harvested at 1, 2, 4, and 8 hpi and analyzed by RPA (Fig. 4). We observed that the transcripts fell into four broad patterns: those expressed by 1 hpi (K3, Rta, M3 and M9), those

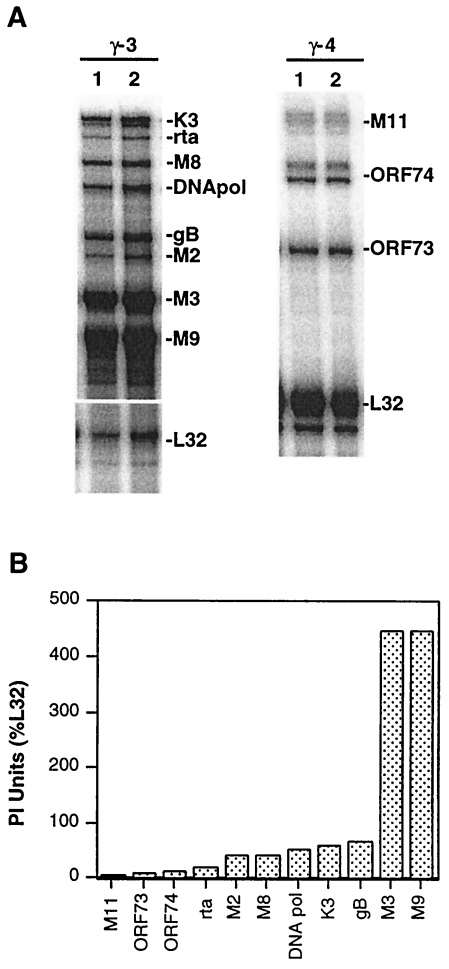

FIG. 1. (A) MHV-68 gene expression in vitro. 3T3 cells were infected with MHV-68 at an MOI of 5. Cells were harvested at 8 hpi, RNA was extracted, and 5 μg of total RNA was subjected to RPA analysis using the γ-3 or γ-4 riboprobe templates. The protected RNA fragments were visualized with a PhosphorImager. RNAs from two separate experiments were analyzed (lanes 1 and 2). (B) The PI counts for each protected probe fragment were obtained, and the data are presented as a percentage of the internal housekeeping (i.e., L32) signal present in each lane. The average values from two experiments are presented.

not detected until 2 hpi (M8 and ORF73), those not expressed until 4 hpi (DNA Pol, M2, M11, and ORF74), and those not expressed until 8 hpi (gB). In addition, the expression of M9 transcripts was expressed at a low level early but a dramatic increase in expression is observed between 4 and 8 hpi. The temporal expression of these transcripts mirrors their kinetic class assignment based on their expression in the presence of CHX or PAA. Levels of all transcripts (excluding gB and M9) increased dramatically between 2 and 4 hpi. The M9 transcript has the kinetics of both an immediate-early transcript (low level detected at 1 hpi) and a late transcript (elevated transcription at late times postinfection and inhibition by PAA). This suggests that there is differential regulation of the M9 transcript or that there are multiple transcripts expressed through the M9 ORF. We also observed the shutoff of host mRNA expression, as indicated by the absence of L32 expression by 24 hpi. These results demonstrates the use of the RPA for elucidating the temporal class of viral transcripts.

Quantitation of MHV-68 transcription in vivo. Following i.n. infection, MHV-68 causes an acute infection in the lungs followed by the establishment of a latent infection in the spleen and other lymphoid tissues including the MLN (1, 12, 23–25). To characterize the pattern of MHV-68 transcription in vivo, BALB/c mice were infected i.n., and RNA was extracted from lungs, spleens, and MLN and analyzed by RPA. We observed that the peak of viral gene expression was at 3 days postinfection (dpi) in the lung and at 10 dpi in the spleen and MLN (data not shown). To compare the levels of each transcript during the peak of viral gene expression in the lungs (3 dpi), spleen (10 dpi), and MLN (10 dpi), we quantitated the relative PI units for each transcript expressed and calculated each as a percentage of the PI units for L32. Three mice per time point were analyzed to give an average value for each transcript (Fig. 5). Both lytic and candidate latent transcripts can be detected in the lungs early in infection. In addition, the relative levels of transcripts are similar following infection of lungs and 3T3 cells (Fig. 1). No expression of the candidate latency transcripts (ORF73, ORF74, and M2) was observed in spleen or MLN. Only the M11, M8, K3, M3, and M9 transcripts were detected in the MLN at 10 dpi. The K3 transcript was expressed to the highest level in vivo in the MLN. Furthermore, K3 was expressed at higher levels than the M9 transcript and to almost the same levels as the M3 transcript. Similar to results for the MLN, the K3, M8, M9, and M3 transcripts were expressed in the spleen at 10 dpi. Expression of the M11 transcript was not detected. In the spleen, the same high level of expression of K3 was observed relative to M3 and M9 transcript levels. The expression of M3 and M9 in the MLN and spleen and M11 in the MLN confirms that these appear to be transcripts that are expressed during latency in these tissues (19, 31). The restricted, higher-level expression of K3 and M8 transcripts in both spleen and MLN suggests that they are not primarily lytic transcripts but are associated with the latent phase of viral replication. The clear differences in their kinetics relative to other lytic transcripts (e.g., DNA Pol and gB) and the differences in the levels relative to other candidate latency transcripts (e.g., M3 and M9) are strongly suggestive that the K3 and M8 genes are differentially regulated during the lytic and latent phases of replication in vivo. This could represent differences in cell type specific transcription of these genes.

Kinetics of MHV-68 expression in vivo. To compare the temporal patterns of expression of the newly defined candidate latent transcripts (K3 and M8) and the other candidate latent transcripts (ORF73, ORF74, M2, M11, M3, and M9) and their relative levels in the lung, spleen, and MLN, BALB/c mice were infected i.n., and RNA was extracted from lungs, spleens, and MLN harvested every day for days 1 through 10 postinfection and every other day until 16 dpi. Two mice per time point were analyzed. RNA was analyzed by RPA to detect viral transcripts using the γ-3 and γ-4 riboprobe templates (Fig. 6).

Viral gene expression could be detected in the lungs as early as 1 dpi. However, only the candidate latency transcripts M3 and M9 transcripts were detected. This could indicate that there is a functional significance to the expression of these transcripts so early in infection. Alternatively, the detection of only M3 and M9 transcripts could be due to their high-level

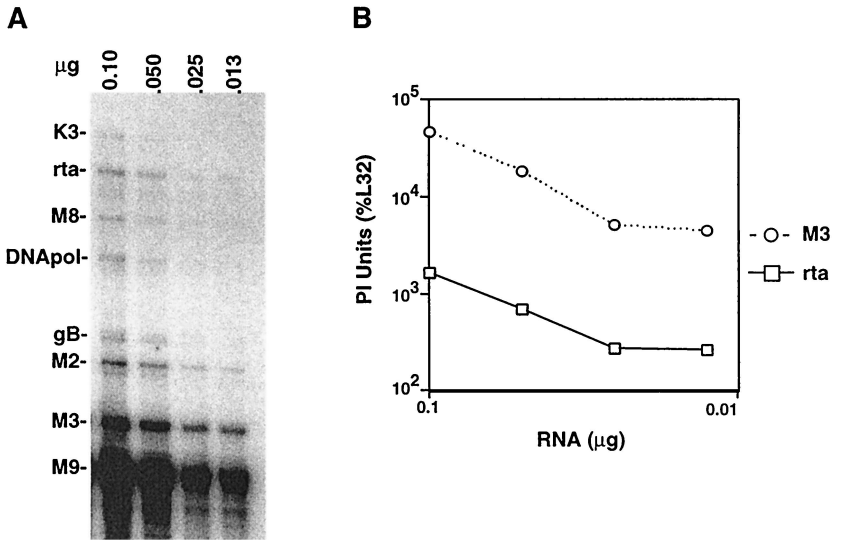

FIG. 2. Assessment of the sensitivity of the RPA. Total RNA was extracted from OMK cells at 24 hpi, serially diluted, and analyzed with the γ-3 probe set. (A) Shown is a phosphorimage following exposure of the dried gel to a PhosphorImager screen. (B) The PhosphorImager was used to quantify the signal present in the Rta and M3 protected bands of the dried gel. Log-log plots are shown for PI counts versus input total RNA amounts.

expression relative to other transcripts (Fig. 5). All transcripts were expressed in the lungs between 3 and 6 dpi. This is in agreement with plaque assay data showing high levels of viral replication in the lungs at these time points (24). By 7 dpi, only low levels of the M3 and M9 transcripts were detected, suggesting that clearance of the viral infection was occurring as previously demonstrated by others (24, 27). Levels of M3 and M9 transcripts are relatively equivalent to each other and are much higher than the level of the K3 or M8 transcripts in the lungs. No viral gene expression was observed in the lung after 10 dpi.

The pattern of gene expression in the spleens is strikingly different from that observed in the lungs. No gene expression was observed in the spleen until 7 dpi. At that time point, we observed expression of the K3, M8, M3, and M9 transcripts. Overall levels of the K3, M3, and M9 transcripts were much lower in the spleen than in the lungs. However, the relative level of K3 to the M3 and M9 transcripts had changed such that K3 transcripts were expressed at levels almost equivalent to those for both the M3 and M9 transcripts. M8 expression was discordant from the K3, M3, and M9 transcripts in that a sharp increase was observed between 12 and 14 dpi. Expression of the M8 transcripts was also significantly lower than that of the M3, M9, and K3 transcripts. Interestingly, no expression of the other candidate latency transcripts M2, M11, ORF73, and ORF74 was observed. Furthermore, expression of the clearly defined lytic transcripts (e.g., Rta, DNA Pol, and gB) was not observed throughout the time course (data not shown).

The kinetics of gene expression in the MLN were distinctly different from those observed in either the lung or spleen. We observed viral gene expression in the MLN as early as 2 dpi. Expression was maintained through 16 dpi. Similar to the patterns of transcription in the spleen, the predominant transcripts in the MLN were the K3, M8, M3, and M9 transcripts. In addition, the candidate latency M11 transcript was also detected. Expression of candidate latency transcripts M2, ORF73, and ORF74 and lytic transcripts Rta, DNA Pol, and gB was not observed. The M3, M8, M9, and M11 transcripts exhibited a biphasic pattern of expression. The first phase of expression had a peak expression of M3 and M9 transcripts at 4 dpi. Interestingly, this corresponded to the peak expression of these transcripts in the lungs. The second phase of expression occurred around 10 dpi, which corresponded to the peak expression of these transcripts in the spleen. In contrast to the discordant pattern of M8 expression observed in the spleen, expression of M8 was similar to that of M3 and M9, perhaps suggesting that different cell types express M8 in the spleen and MLN. The K3 transcript did not show a biphasic pattern of expression. Instead, levels of the K3 transcript increased steadily until 10 dpi (the second peak of expression of the M3, M8, and M9 transcripts). In addition, K3 expression exceeded the level of M9 transcription. Taken together, these data demonstrate that MHV-68 exhibits distinct kinetics of viral gene expression in lung, spleen, and MLN, suggesting cell-type-specific programs of viral gene expression.

DISCUSSION

In this study, we developed a multiprobe RPA to determine the kinetics of transcription from 11 MHV-68 genes including representative lytic transcripts (K3, Rta, M8, DNA Pol, and gB) and possible latency associated transcripts (M2, M3, M9, M11, ORF73, and ORF74). Expression of these transcripts was assessed following infection of 3T3 cells in vitro and in vivo following i.n. infection of mice. Our results show that all candidate latent transcripts are expressed during the lytic phase both in vitro and in the lungs in vivo. Of the potential latency-associated transcripts, only two (M3 and M9) were detected in the spleen and three (M3, M9 and M11) were detected in the MLN, both sites of MHV-68 latency. In addition, two lytic

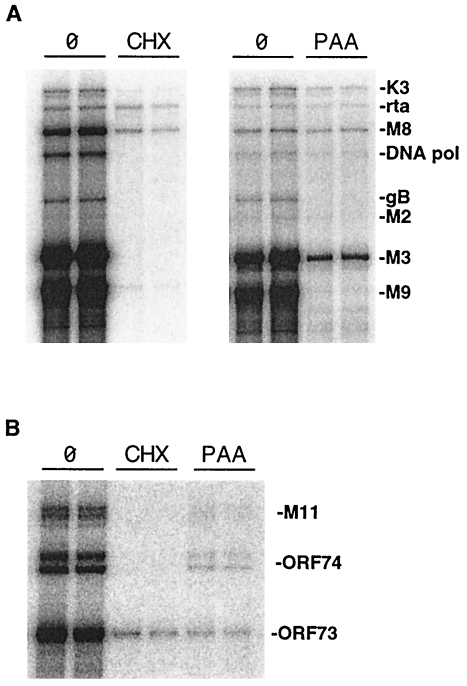

FIG. 3. Determination of kinetic class of MHV-68 transcripts. 3T3 cells were infected with MHV-68 in the presence of CHX or PAA or were left untreated. Total RNA was harvested at 8 hpi (CHX treated) or 24 hpi (PAA treated and untreated), and 2.5 μg of total RNA was analyzed by RPA using the γ-3 probe set (A) or γ-4 probe set (B). Shown are phosphorimages following exposure of the dried gel to a PhosphorImager screen.

transcripts, K3 and M8, were also expressed in the spleen and MLN. No other lytic transcripts were detected. Furthermore, the ratios of the K3 and M8 transcripts relative to the M3 and M9 transcripts were significantly higher than observed in the lungs. In light of these data, we propose that K3 and M8 should also be considered latency-associated transcripts.

Previous studies have shown that MHV-68 latency is established in the MLN (1). Our study is the first demonstration of viral gene expression within the MLN. Although the K3, M8, M3, and M9 genes were expressed in both the spleen and MLN, the kinetics of expression in these organs differed. Notably, viral gene expression was detected in the MLN as early as 2 dpi, whereas expression in the spleen was not detected until 7 dpi. The early detection of these transcripts within the MLN suggests that establishment of the latent infection is concurrent with an ongoing lytic infection in the lungs. The changing pattern of kinetics between lung, MLN, and spleen suggests that the virus is disseminated rapidly from the epithelial cells in the respiratory tract into the lymphoid tissues associated with the upper respiratory tract, and from there, the virus traffics to the spleen. In addition, the continued expression of viral genes at the same time that a strong immune response to the acute infection in the lungs is ongoing (1) suggests that MHV-68 encodes genes that function to evade the host immune response during latent infection. Credence to this argument comes from the recent identification of the function of both the M3 and K3 gene products. K3 was recently shown to down-regulate MHC class I and thus could prevent cytotoxic T-cell killing by immunoevasion (20). M3 is a secreted broad-spectrum chemokine binding protein (11), and deletion of M3 resulted in the inability to establish latency in spleens (Efstathiou, personal communication). In addition, M11, which was expressed in the MLN, has sequence homology to the Bcl-2 family of proteins and can inhibit apoptosis (33). The functions of the M8 and M9 proteins are unknown but may be important for immune evasion or establishment of latency.

Our results are in agreement with the work of others that identified M3 and M9 as candidate latency transcripts based on their expression in latently infected spleens as well as the S11 cell line (6, 17, 31). It is probable that M3 can be expressed in multiple cell types, as Virgin et al. (31) demonstrated expression of M3 in B-cell-deficient spleens after 45 dpi, while Simas et al. (17) identified M3 expression in splenic B cells by in situ hybridization. M9 was also found to be expressed in B-cell-deficient spleens as well as in the latently infected S11 B-cell line (6, 31). However, it was argued that M9 is only a lytic cycle

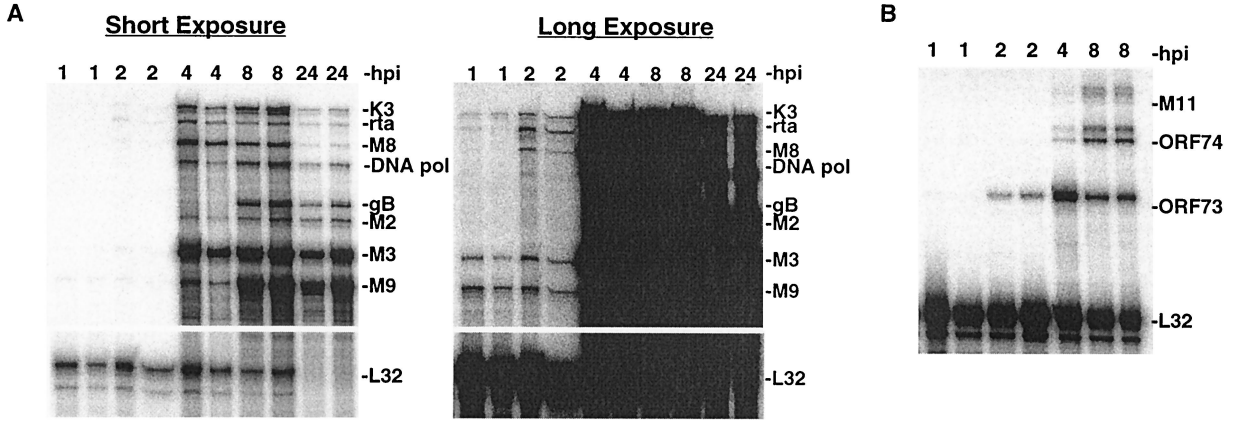

FIG. 4. Time course of MHV-68 transcript expression in vitro. 3T3 cells were infected with MHV-68 in duplicate, RNA was harvested at 1, 2, 4, 8, and 24 hpi, and 2.5 μg of total RNA was analyzed for expression of MHV-68 transcripts by RPA with the γ-3 probe set (A) or γ-4 probe set (B). Shown are phosphorimages following exposure of the dried gel to a PhosphorImager screen. The upper ranges in PI value were 1,000 (A, left panel) and 25 (A, right panel; B).

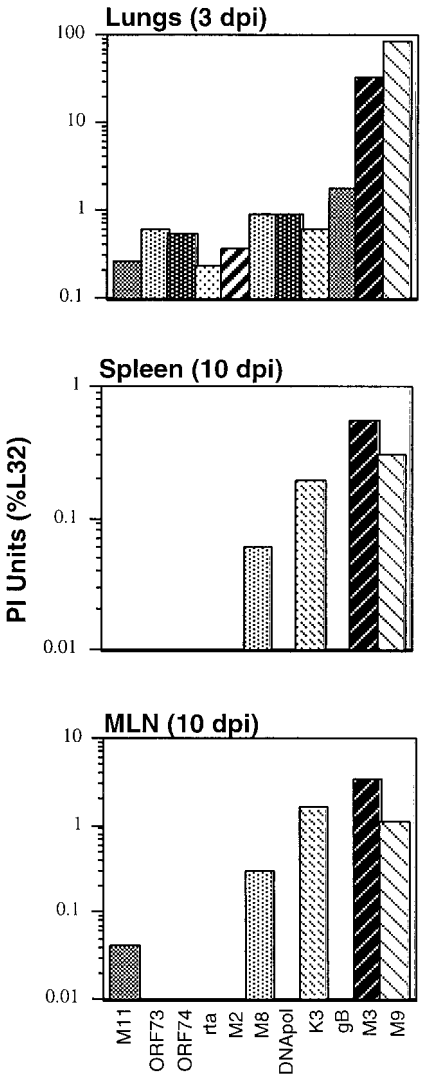

FIG. 5. Comparison of MHV-68 transcript levels following in vivo infection. RNA extracted from lung (3 dpi), spleen (10 dpi), and MLN (10 dpi) was analyzed by RPA using the γ-3 probe set or γ-4 probe set. PI counts were obtained for each protected probe fragment, and the data are presented as a percentage of the L32 signal. Three separate organs were analyzed for both lung and spleen samples, while two organs were assessed for MLN. The average value is shown.

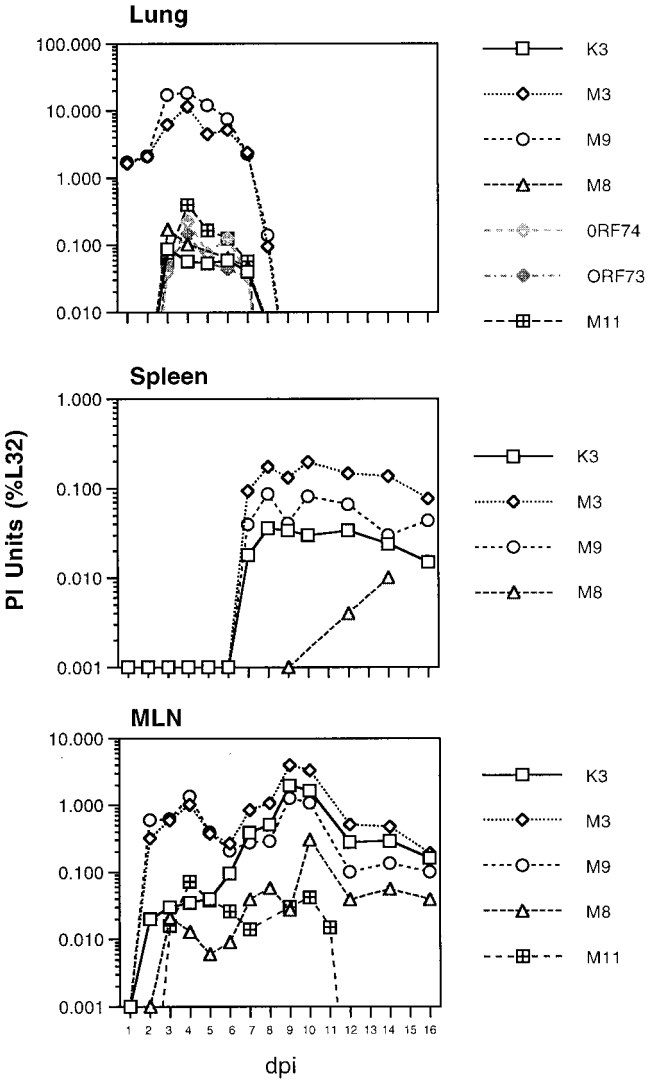

FIG. 6. Kinetics of expression of the candidate latency-associated transcripts following in vivo infection. RNA, extracted from lung, spleen, and MLN for days 1 through 10 postinfection and every other day until 16 dpi, was subjected to RPA analysis using the γ-3 or γ-4 riboprobe templates. PI counts were obtained for the protected probe fragments for the candidate latency-associated transcripts (K3, M3, M9, M8, ORF74, OR73, and M11), and the data are presented as a percentage of the L32 signal.

transcript based on its possible homology to the herpes simplex virus capsid protein gene (6). Simas et al. (17) found that K3 and M8 were transcribed in S11 cells treated with 4′-S-EtdU but did not detect expression of these genes in the spleen by in situ hybridization. However, it is possible that in situ hybridization was not sensitive enough to detect K3 and M8 in the spleens. Husain et al. (6) identified expression of M8 by RT-PCR at 14 dpi in the spleens but not at 28 dpi. They argued, though, that M8 is a lytic cycle transcript based on their inability to detect the transcript in latently infected S11 cells. Here, we detect M8 in MLN and spleen, sites of MHV-68 latency, and assign M8 as a latency-associated gene.

M2, M11, ORF73, and ORF74 were previously identified as candidate latent genes (6, 31). The criteria used by Virgin et al. (31) to identify M2, M11, ORF73, and ORF74 as latency-associated genes was based on their low or absent expression following infection of fibroblasts in vitro but their restricted expression in peritoneal exudate cells following i.p. inoculation of B-cell-deficient mice (31). Similar to our studies, they did not observe expression in spleens. The recent report that MHV-68 can establish latency in macrophages and dendritic cells as well as in B cells (4) suggests that perhaps these candidate latent genes (e.g. M2, M11, ORF73, and ORF74) are more likely expressed in dendritic cells or macrophages rather than B cells, the predominant cell population in the spleen. The potential to have different programs of latent gene expression in different cell types is similar to EBV in which the

latency programs are expressed not only in different states of B-cell differentiation but also in different cell types (13).

We observed the expression of M11 in the MLN but not in the spleen. It is possible that M11 expression is exclusive to cells in the lymph node relative to the spleen. More likely, given that levels of B cells are equivalent between MLN and spleen at 10 and 15 dpi (16) and that MHV-68 latently infects dendritic cells to a high frequency (4), it is possible that dendritic cells or macrophages are more numerous in the MLN than to the spleen and these are the cells that are the site of M11 expression. Alternatively, since expression of M11 is low in the MLN, it is possibly expressed to even lower levels in the spleen and not detected with our assay. Studies are ongoing to assess this possibility.

We observed that the peak of viral gene expression in the lungs (Fig. 6) correlated with the peak of infectious virus production as reported by others and our own studies (24; R. Rochford and M. L. Lutzke, unpublished observations). This suggests the RPA can be used as an alternative and sensitive measurement of viral replication. Furthermore, we did not detected lytic transcripts in the spleen or MLN at times when the numbers of latently infected cells in these organs are highest (1). This suggests that there is a strong concordance between the techniques assessing gene expression and techniques measuring biologic measures of latency (e.g. infectious center assays). Reports that the MHV-68 genome can be detected in the lungs several months postinfection (23) but the absence of detectable viral gene expression in the lungs after 7 dpi suggest that MHV-68 latently infected cells in the lungs are not expressing any of the candidate latency transcripts but are expressing other previously unidentified transcripts. Alternatively, it is possible that the viral genome during MHV-68 latency in the lungs is silent and no latent transcripts are expressed.

Given that all of the transcripts we assayed are detected during the lytic infection of both fibroblasts and lungs, we suggest that it may be more appropriate that the definition of a latency-associated transcript should take into account not only the detection of the transcript in a particular tissue but also the relative ratio of the candidate transcript levels to clearly defined lytic cycle transcripts such as the DNA Pol or gB gene. For example, while gB transcripts are readily detectable by RPA following infection of 3T3 cells or in the lung during the acute phase of replication, gB transcripts are not detected in the spleen by RPA. In contrast, relative levels of K3 transcripts to gB transcripts are always lower than gB transcripts following infection of 3T3 cells or in the lung during the acute phase of replication (Fig. 1 and 5). However, K3 transcripts are readily detectable and highly expressed in the spleen and in the MLN by RPA even though gB transcription is not detected. Furthermore, levels of K3 transcripts become almost equivalent to those of M3 and M9 transcripts in both spleen and MLN. Thus, different ratios of transcripts are observed in different organs. This could reflect the numbers of cells undergoing either lytic or latent infection or could be due to differences in the types of cells infected or differences in the state of cell differentiation. The detection of a transcript depends on the level of sensitivity of the assay used, and the absence of detection does not always mean that the gene is not transcribed. Although the RPA is a very sensitive assay, it is not as sensitive as nested RT-PCR, and thus we cannot rule out that transcription of lytic transcripts occurs in the spleen because of the limits of sensitivity of our assay. It is likely that similar to other herpesviruses (32), there is always some ongoing lytic replication in vivo, thus, the question becomes, how do we distinguish the low-level lytic transcription from latent transcription? If we define latency in MHV-68 based on analogy to EBV or HHV-8, we are limiting ourselves to the very problem with studying those systems. The elegance of studying MHV-68 is that we can look at early events in the establishment of latency and can clearly identify the roles of individual genes in the context of the complex host-virus dynamics that occur in vivo with multiple tissue types and a vigorous immune response.

We observed the expression of all 11 transcripts by 8 hpi following infection of 3T3 cells. The use of the RPA to define the kinetic class of MHV-68 transcripts allows us to assign an earlier time of expression than previously reported for Rta, gB, and M9 (21, 35). The detection of M3 at 1 hpi is striking and suggests that subversion of host immunity is an important early step in viral infection. Inerestingly, we observed the shutoff of host mRNA synthesis. This is the first demonstration of MHV-68 infection inducing shutoff of cellular transcription. Recently, MHV-68 was also shown to shut off selective host protein synthesis (20). The identification of four classes of MHV-68 transcripts opens the way to analyze class-specific promoters and determine how these promoters function in vivo. For example, the promoter of K3 was shown to have an Rta-responsive element in transient transfection assays (20), yet both K3 and Rta are expressed with similar kinetics following infection of 3T3 cells. In addition, we observed expression of K3 in the absence of detectable Rta expression in the spleen and MLN. This would argue that the K3 promoter is not dependent on Rta for expression following viral infection in vivo at predominantly latent sites of infection.

In summary, our data support a model for MHV-68 infection that includes latency in multiple cell types as proposed by others (4, 18, 31). The differences in kinetics between expression of the candidate latency transcripts in the spleen and MLN suggest organ-specific patterns of expression. Furthermore, our data argue that the lymph nodes associated with the upper respiratory tract are a target site of viral latency and that studies of MHV-68 latency should include analysis of both lymph nodes and spleen. Furthermore, our data clearly show that latency in the MLN is established prior to latency in the spleen, in agreement with earlier studies (1). The differences in kinetics of gene expression between MLN and spleen suggest that the MLN is the site of viral dissemination through the host. Studies are under way to assess the cell types essential for viral dissemination.

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