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Latency versus Persistence or Intermittent Recurrences: Evidence for a Latent State of Murine Cytomegalovirus in the Lungs

SABINE KURZ,[1] HANS-PETER STEFFENS,[1] ANNA MAYER,[1] JAMES R. HARRIS,[2] AND MATTHIAS J. REDDEHASE[1]*

Institute for Virology[1] and Institute for Zoology,[2] Johannes Gutenberg University, Mainz, Germany

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The state of cytomegalovirus (CMV) after the resolution of acute infection is an unsolved problem in CMV research. While the term "latency" is in general use to indicate the maintenance of the viral genome, a formal exclusion of low-level persistent productive infection depends on the sensitivity of the assay for detecting infectious virus. We have improved the method for detecting infectivity by combining centrifugal infection of permissive indicator cells in culture, expansion to an infectious focus, and sensitive detection of immediate-early RNA in the infected cells by reverse transcriptase PCR. A limiting-dilution approach defined the sensitivity of this assay. Infectivity was thereby found to require as few as 2 to 9 virion DNA molecules of murine CMV, whereas the standard measure of infectivity, the PFU, is the equivalent of ca. 500 viral genomes. Since murine CMV forms multicapsid virions in most infected tissues, the genome-to-infectivity ratio is necessarily >1. This assay thus sets a new standard for investigating CMV latency. In mice in which acute infection was resolved, the viral DNA load in the lungs, a known organ site of CMV latency and recurrence, was found to be 1 genome per 40 lung cells, or a total of ca. 1 million genomes. Despite this high load of CMV DNA, infectious virus was not detected with the improved assay, but recurrence was inducible. These data provide evidence against a low-level persistent productive infection and also imply that intermittent spontaneous recurrence is not a frequent event in latently infected lungs.

The maintenance of the viral genome after resolution of acute infection and the consequent risk of recurrent infection and disease are hallmarks of herpesvirus biology (35). While the operational term "latency" is used by most authors to indicate the failure to detect infectious virus despite the presence of the viral genome, a formal exclusion of persistent low-level productive infection, as well as of molecular latency interrupted by frequent episodes of recurrence, would require an assay that can detect infectivity with the utmost sensitivity. For α-herpesvirus in neurons (35), a latency-specific pattern of transcription has proven the existence of a molecular latency, whereas for cytomegaloviruses (CMVs), the prototype members of the β-herpesviruses, the question is still open (for reviews see references 16, 28, and 29) and the presence of transcripts of the productive cycle in the absence of detectable infectivity has been interpreted as indicative of persistent infection in organs below the detection limit of currently available assays (11, 39, 40). A first indication of latency-specific transcription of human CMV has been provided by recent work on the infection of hematopoietic progenitors (19, 20), but it still needs to be established whether this applies also to CMV latency in stromal or parenchymal cells of tissues. The discrimination between molecular latency and low-level persistence is important for the understanding of recurrence. In the case of molecular latency, recurrence requires the reactivation of productive transcription, whereas in the case of low-level persistence, virus replication can expand after ablation of immune control without a switch in the transcriptional program. Two recent reports have dealt specifically with the problem of sensitivity in detecting infectious murine CMV in organs and have arrived at controversial conclusions (31, 40). Whereas Yuhasz et al. suggested a chronic infection of the lungs from the presence of an immediate-early 1 (IE1) RNA sequence (40), Pollock and Virgin provided evidence against productive infection during latency in the spleen and kidney with an assay sensitivity of a single PFU per whole organ (31). However, since neither of these two groups employed the method of centrifugal enhancement of infectivity, which is known to improve the efficacy of infection (13, 14), low-level persistence is as yet neither proven nor disproven. Surprisingly, a central question of CMV virology relevant to the sensitivity problem has remained unanswered to date, namely, how many viral genomes are required for infection? We will show here that 1 PFU comprises ca. 500 viral genomes, and we will describe an assay that can detect infectivity in as few as ca. 5 viral genomes. In the lungs, a major predicted site of CMV latency and recurrence (3, 33), infectious virus remained undetectable even with the highly improved assay sensitivity. We therefore conclude that the lungs are an organ site of molecular CMV latency.

* Corresponding author. Mailing address: Institute for Virology, Johannes Gutenberg University, Hochhaus am Augustusplatz, 55101 Mainz, Germany. Phone: 49-6131-173650. Fax: 49-6131-395604. E-mail: REDDEHAS@mzdmza.zdv.uni-mainz.de.

MATERIALS AND METHODS

Infection of mice for the establishment of latency. For syngeneic bone marrow (BM) transplantation, female mice of the inbred strain BALB/c (major histocompatibility complex haplotype *H-2*^{d}) were used at the age of 8 weeks as BM donors and recipients. For hemoablative conditioning, recipients were total-body irradiated with a single sublethal dose of 6 Gy from a ^{137}Cs-γ source (OB58; Buchler, Braunschweig, Germany), delivering a dose rate of ca. 0.7 Gy min^{-1} that was adjusted monthly. Donor femoral and tibial BM cells were obtained as described previously (30), and 5×10^6 BM cells were infused intravenously into the tail veins of recipients at ca. 6 h after the irradiation. Infection with 10^5 PFU of purified murine CMV, strain Smith ATCC VR-194, was performed subcutaneously at the left hind footpad at ca. 2 h after the transplantation. Mortality from CMV disease ranged between 20 and 40% under these conditions. Titers of virus in organs were determined by a plaque assay with centrifugal enhancement

of infectivity as described previously (2, 34). In the case of low titers, all of the respective organ homogenate was tested.

Purification of murine CMV. Mouse embryo fibroblasts (MEF) grown in minimal essential medium (MEM), supplemented as described previously (34), were infected just before reaching confluence in the second culture passage with a multiplicity of infection of 0.02 PFU per cell, which is 200,000 PFU per 14.5-cm-diameter petri dish. After 5 days, that is, after approximately five rounds of replication, when cells began to disintegrate because of the cytolytic infection, culture supernatant was harvested. All further procedures were performed at 4°C. In the first step of virion purification, cell debris, including cell nuclei, were sedimented at 7,000 × *g* for 30 min. In the second step, the pellet was discarded and the supernatant was centrifuged at 25,000 × *g* for 3 h to sediment the virions. In the third step, the pellet was resuspended and the virions were enriched by sedimentation through a 15% (wt/vol) sucrose-PBS-A (phosphate-buffered saline devoid of Ca^{2+} and Mg^{2+}) density cushion at 53,000 × *g* for 1 h in a swing-out Beckman SW27 rotor. The final sediment was resuspended in sucrose-PBS-A and stored at −70°C. The infectious titer was determined by a standard 4-day PFU assay on MEF without centrifugal enhancement and was 2.5×10^8 PFU ml^{-1} in the reported experiments.

Negative-staining electron microscopy. Negative staining of specimens from the purified virion suspension was performed by the single droplet procedure (9, 10), with carbon support films that were glow discharged for 20 s. After adsorption of the sample to the carbon film, the stabilization buffer was removed by repeated washing with distilled water; the negative stain, consisting of 5% (wt/vol) ammonium heptamolybdate (pH 7.0) containing 1% (wt/vol) trehalose, was then added. Transmission electron microscopy was performed with a Zeiss EM 900 at 80 kV, and images were recorded on Kodak EM film, type 4489, at an instrumental magnification of ×30,000.

Virion DNA purification and quantitation of viral DNA. DNA was extracted from 2.5×10^7 PFU, that is, from 100 μl of the purified virion suspension, by standard procedures of RNase A digestion followed by proteinase K digestion, phenol-chloroform-isoamyl alcohol extraction, and precipitation with ethanol. In order to increase the solubility, DNA was sonicated by using a Branson cup ultrasonicator (Branson Ultrasonics, Danbury, Conn.). The contamination with cellular DNA was estimated from parallel titrations of virion DNA and pure cellular DNA by Southern dot blot hybridization specific for the single-copy gene encoding tumor necrosis factor alpha (36, 37); the γ-^{32}P-end-labeled oligonucleotide 5′-n6159-6183 (GenBank accession number M38296) was used as the probe. Cellular DNA was present in the virion DNA preparation at ca. 20% of the total amount of DNA, which was quantitated by standard determination of optical density at 260 nm. The corrected yield of virion DNA was then ca. 3.5 μg total. Viral genome was quantitated by Southern dot blot hybridization using the murine CMV *ie1* exon 4-specific γ-^{32}P-end-labeled oligonucleotide IE1.2135 (3) as the probe. The 12.2-kbp plasmid pIE111, which encompasses genes *ie1* and *ie3* of murine CMV (17, 26), was titrated in parallel as a standard. Radioactivity per dot was measured with a digital phosphoimaging system (Phospho-Imager model GS 250; Bio-rad). The genome-to-PFU ratio was calculated from the log-log plot of phosphodisintegrations (ordinate) versus the PFU and the plasmid numbers (abscissa). For comparison, viral DNA was also quantitated by end point dilution followed by a PCR specific for a 363-bp sequence within exon 4 of the murine CMV *ie1* gene, with oligonucleotide IE1.2135 as the internal probe (3).

Isolation of DNA and messenger RNA from mouse tissue. Pieces of tissue or, specifically, separate lobes of the perfused lungs, were aseptically dissected and transferred immediately to liquid nitrogen for storage. Frozen pieces were transferred to Eppendorf tubes containing prewarmed (37°C) standard extraction buffer with guanidinium thiocyanate. The tissue was subjected to Dounce homogenization followed by vortexing. Twice the volume of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA buffer was added, and DNA was sedimented for 10 min at 15,000 × *g*. The DNA was processed as described above. For the PCR, the DNA concentration was adjusted in most experiments to 600 ng per 20 μl, which corresponds to the DNA content of 10^5 diploid mouse cells. From the supernatant of the centrifugation, poly(A)$^+$ RNA was extracted by an oligo(dT)-cellulose affinity method as described previously (1). For the preparation of blood cell DNA, tail vein blood pooled from 10 individual mice was transferred immediately into PBS-A containing 0.1% (vol/vol) EDTA to prevent coagulation. Note that it is important not to use heparin. The samples were further processed as described above.

Quantitation of viral DNA in organs and blood cells. Viral genome in organ or blood cell DNA was quantitated by end point dilution followed by an *ie1* exon 4-specific PCR (3). Plasmid pIE111 was titrated for comparison. Amplification products were separated on a 1.2% (wt/vol) agarose gel and visualized by autoradiography after Southern blot hybridization with the γ-^{32}P-end-labeled internal oligonucleotide probe IE1.2135 (3).

Assay of infectivity in tissue by focus expansion in culture (focus expansion assay [FEA]). Mouse organs or, specifically, separate lobes of the perfused lungs were dissected aseptically and were frozen immediately at −70°C. The tissue was subjected to Dounce homogenization at 4°C, and the homogenate was used in an appropriate dilution in supplemented MEM for the infection of MEF at a centrifugal force of 1,000 × *g* for 30 min at 20°C. Specifically, in the case of the lungs, the homogenate was 36 ml for the whole organ, and 2 ml thereof, that is, a 1/18 aliquot of the whole organ, was used to infect ca. 5×10^6 MEF per 10-cm-diameter petri dish. For the exclusion of productive infection, all of the material was tested in a respective number of dishes. In cases when lobes were tested separately, the left lung (LL) was taken up in 14 ml (7 of 18 parts), the postcaval lobe (PCL) was taken up in 4 ml (2 of 18 parts), and the superior lobe (SL), the middle lobe (ML), and the inferior lobe (IL) were taken up in 6 ml (3 of 18 parts) each. After the centrifugation, the supernatant was discarded, the cells were washed twice with PBS-A, and 10 ml of fresh culture medium was added. Viral replication was allowed to proceed for up to 72 h at 37°C in a 5% CO_2 humidified atmosphere.

Purification of messenger RNA from cells in culture. After the period of incubation, the indicator MEF were detached by trypsinization (PBS [pH 7.2] containing 0.05% [wt/vol] trypsin and 0.02% [wt/vol] EDTA), which was terminated by addition of MEM containing 10% (vol/vol) fetal calf serum. Cells were sedimented at 500 × *g* for 5 min, taken up in PBS-A, sedimented again, and dissolved in the extraction buffer of the QuickPrepMicro mRNA purification kit (Pharmacia Biotech). The average yield of poly(A)$^+$ RNA was ca. 2 μg per dish, and usually, 1/20 of the yield was subjected to reverse transcriptase PCR (RT PCR).

RT PCR. All reactions were carried out in an MWG Biotech Hybaid OmniGene thermocycler (Hybaid Limited, Teddington, United Kingdom). The RT reaction was performed in a total volume of 10 μl, containing 60 mM KCl, 15 mM Tris-HCl (pH 8.4), 3 mM $MgCl_2$, 10 mM dithiothreitol, 20% (vol/vol) glycerol, 1 mM each deoxynucleoside triphosphate, 12.5 pmol of RT primer, 10 U of RNasin (Promega), 100 U of Moloney murine leukemia virus RT (GIBCO BRL, Eggenstein, Germany), and the indicated amount of the poly(A)$^+$ RNA test sample. A sample without addition of RT was routinely included to control for DNA contamination. Primer annealing was done for 10 min at 27°C, and reverse transcription was carried out for 30 min at 42°C, followed by denaturation for 5 min at 95°C. The resulting cDNA was amplified in 30 cycles in a total volume of 50 μl, with 25 pmol of each primer and 1.5 U of *Taq* DNA polymerase (Eurobio, Raunheim, Germany) added in 40 μl of a PCR buffer containing 60 mM KCl, 15 mM Tris-HCl (pH 8.4), and 3 mM $MgCl_2$. The reaction temperature profile was as follows. The first cycle consisted of denaturation at 95°C for 2 min, primer annealing at 58°C for 1 min, and elongation at 72°C for 1 min. In the subsequent cycles, the denaturation was performed for 30 s at 96°C, and in the final cycle, elongation was performed at 72°C for 5 min. Transcription of the *ie1* gene was detected by using oligonucleotide 5′-n1690-1671 (exon 4) as the RT primer, 5′-n1361-1380 (exon 3) as the forward primer, 5′-n1670-1651 (exon 4) as the reverse primer, and 5′-n1511-1524 (exon 3) n1647-1655 (exon 4) as the probe (11). Since the primers flank the splicing junction of exons 3 and 4, the RNA amplification product and the DNA amplification product can be distinguished by their sizes of 188 bp and 310 bp, respectively. Further, the probe is directed against the junction. As a cellular transcript control, hypoxanthine phosphoribosyltransferase (HPRT) transcripts were reverse transcribed by using an oligo(dT) primer. Oligonucleotide 5′-n601-625 served as the forward primer, 5′-n763-739 as the reverse primer, and 5′-n649-669 as the probe (21); (fragment length, 163 bp; Swiss-Prot P00493). Amplification products (a 15-μl aliquot) were analyzed by 2% (wt/vol) agarose gel electrophoresis, Southern blot, and hybridization with the respective γ-^{32}P-end-labeled internal oligonucleotide probe, followed by autoradiography.

RESULTS

Molecular equivalent of a PFU. Murine CMV is a cytolytic, plaque-forming virus. Accordingly, the standard measure of its infectivity has always been the in vitro PFU, which by definition forms a plaque within 2 to 4 days in a close-to-confluence monolayer of permissive cells, such as MEF. In order to evaluate the sensitivity of this assay in molecular terms, we estimated the number of virion DNA molecules required for forming a plaque. The infectivity of a virion suspension, purified by ultracentrifugation through sucrose, was first quantitated as PFU and was found to be 2.5×10^8 PFU ml^{-1} in the reported experiments. Negative-staining electron microscopy visualized the particles of which this particular preparation was composed (Fig. 1A). This recalls the fact that murine CMV in vitro (5, 38) as well as in vivo, for example, in the lungs (34), forms multicapsid virions in addition to the monocapsid virions. The exclusive production of monocapsid virions in glandular epithelial cells of the salivary glands (5, 15) is the exception to the rule and reflects a different virion morphogenesis in this specialized cell type. Virion DNA was purified, and the yield of DNA revealed a DNA-to-PFU ratio of 0.14 pg per PFU. For the determination of the genome-to-PFU ratio, the purified virion DNA, expressed as corresponding PFU, was titrated, followed by a Southern dot blot hybridization with an *ie1* exon 4-specific oligonucleotide probe (Fig. 1B). Copies of plasmid

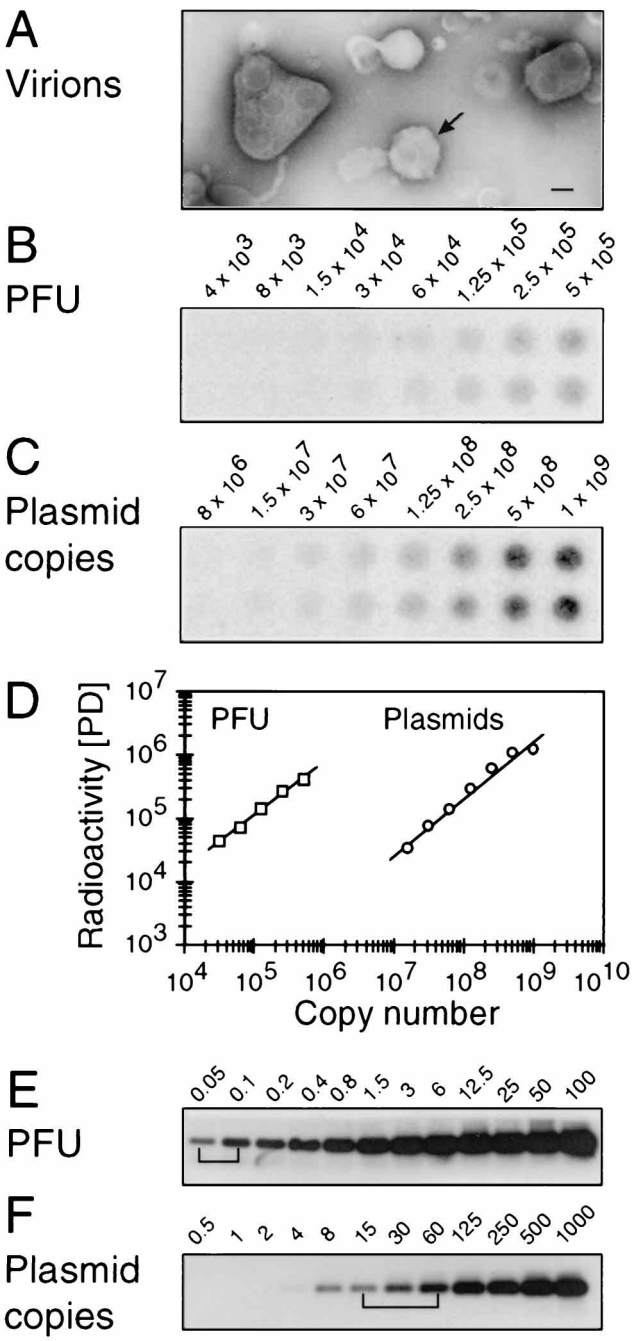

FIG. 1. Molecular equivalent of a PFU. (A) Negative-staining electron microscopy of a purified murine CMV virion suspension, showing a monocapsid virion (arrow), as well as multicapsid virions with four (center left) and two (upper right) visible nucleocapsids. Bar, 100 nm. (B) Dot blot hybridization of purified virion DNA, with the *ie1* exon 4-specific γ-^{32}P-end-labeled oligonucleotide IE1.2135 as the probe. The DNA titration was done in duplicate, starting with 70 ng, which corresponds to 5×10^5 PFU. (C) Dot blot hybridization with probe IE1.2135 of plasmid pIE111, which encompasses gene *ie1*. The plasmid titration was performed in duplicate. (D) Computed phosphoimaging results of the dot blots shown in panels B and C. A log-log plot of phosphodisintegrations (PD; ordinate) versus the numbers of PFU and plasmids (abscissa) is shown. (E and F) PCR amplification of a 363-bp sequence within exon 4 of the *ie1* gene. Shown are the autoradiographs obtained after hybridization with the γ-^{32}P-end-labeled internal oligonucleotide probe IE1.2135. The brackets mark the linear portions of the titrations. (E) Titration of purified virion DNA, starting with 14 pg, which corresponds to 100 PFU. (F) Titration of plasmid pIE111.

TABLE 1. Prediction of the genome-to-infectivity ratio from the particle composition of purified murine CMV[a]

Type of virion	No. of particles	No. of genomes	Particle/ infectivity	Genome/ infectivity
Intact monocapsid	92	92	1.0	1.0[b]
Intact multicapsid	58	198	1.0	3.4
Ruptured monocapsid	29	29	—[c]	—
Ruptured multicapsid	21	69	—	—
Total	200	388	1.3[d]	2.6[e]

[a] Virions were purified by sucrose gradient ultracentrifugation and were visualized by negative-staining electron microscopy (see Fig. 1A). In the electron micrographs, a total of 200 particles were counted.
[b] The number of genomes is represented by the number of capsids.
[c] Visibly ruptured particles, including free capsids, do not contribute an infectivity.
[d] Total number of particles/number of intact virions.
[e] Total number of genomes/number of intact virions.

pIE111, encompassing *ie1*, were titrated in parallel and hybridized accordingly (Fig. 1C). The radioactivity per dot was measured by a digital phosphoimaging system. A comparison between the titrations gives an estimate of ca. 500 viral DNA molecules contained within the amount of virion DNA that corresponds to 1 PFU (Fig. 1D). As a second approach, the genome-to-PFU ratio was also estimated by end point dilution PCR specific for a 363-bp sequence within exon 4 of gene *ie1* (Fig. 1E and F). A comparison between the linear portions of the titrations gives an estimate of 300 to 600 copies of the test sequence within the amount of virion DNA that corresponds to 1 PFU. Both methods have their specific advantages and problems. The direct hybridization in the Southern dot blot is easier to quantitate but includes some risk of overestimation from nonspecifically bound radioactivity. The PCR is less accurate in the quantitation but is much more sensitive and also more specific, since the internal probe detects an amplified fragment of known size. It is therefore important that both methods arrived at essentially the same genome-to-PFU ratio. If precise, this result should give the correct molecular mass of the virion DNA. The ca. 500 copies were contained within ca. 0.14 pg of virion DNA, which gives an estimate of 169×10^6 Da. Considering the confidence limits of all measurements involved, particularly of the determination of the amount of DNA, this calculation is in good accordance with the published molecular mass of 155×10^6 Da (6, 24). In conclusion, the genome-to-PFU ratio is ca. 500; that is, 1 PFU of murine CMV is the equivalent of ca. 500 viral genomes.

Centrifugal enhancement of infectivity. The finding that ca. 500 viral genomes are required for plaque formation could at first glance suggest a high rate of ruptured or defective particles (5) in the preparation. In addition, a multicapsid virion represents one infectious particle but contains several genomes. As a consequence, the genome-to-infectivity ratio for murine CMV is necessarily >1:1. To evaluate the influence of visibly ruptured particles and of multicapsid virions on the genome-to-infectivity ratio, particles in the negative-staining electron micrographs were counted and classified (Table 1). Despite the purification procedure involving high *g* forces, visibly ruptured particles, including some free capsids, represent a minority and can account only for a slight increase in the genome-to-infectivity ratio. Thus, a lower rate of ruptured particles in unpurified virus samples will not be associated with a significantly higher infectivity. Multicapsid virions were found to contain 3.4 capsids on average, with a range from 2 to 8, and they represented ca. 40% of the virions in the purified virion preparation. However, ruptured particles and multicap-

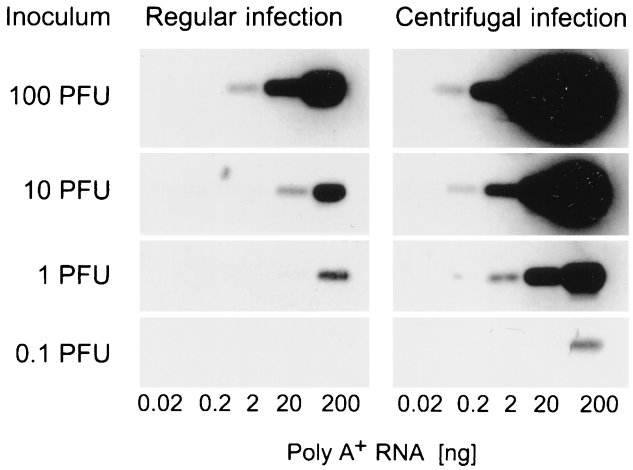

FIG. 2. Enhancement of infectivity by centrifugal infection. MEF were infected with the indicated doses of infectivity, expressed in terms of PFU per culture. After 6 h of viral gene expression in the indicator cultures, poly(A)$^+$ RNA was isolated and titrated, and a sequence encompassing the exon 3/4 splice junction of the *ie1* transcription unit was amplified by RT PCR. Results for infection with no centrifugation (left) and infection in the presence of a 1,000 × *g* centrifugal force (right) are shown. The autoradiographs shown were obtained after hybridization with a γ-^{32}P-end-labeled probe directed against the splice junction.

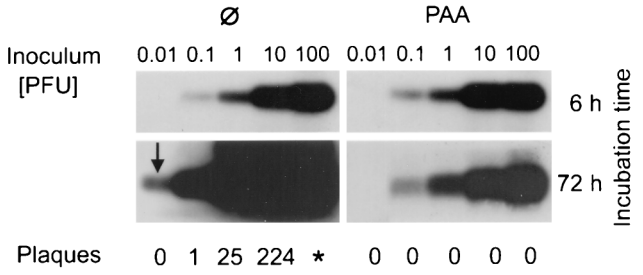

FIG. 3. RT PCR-based FEA of viral infectivity. MEF were infected with the indicated doses of infectivity, expressed in terms of PFU per culture. The efficacy of infection was enhanced by centrifugation. Poly(A)$^+$ RNA was isolated after 6 h and after 72 h of viral gene expression in the indicator cultures, followed by *ie1* exon 3/4-specific RT PCR. Throughout, 100 ng of poly(A)$^+$ RNA were tested. Cultures with no PAA added are shown on the left; the end point of detection is marked by an arrow. Cultures with PAA (250 μg ml^{-1}) added are shown on the right. The number of plaques counted after 72 h is indicated in the bottom line. *, number of fusing plaques too high for an accurate counting.

sid virions together can only explain a genome-to-infectivity ratio of 2.6 (Table 1) and can by no means account for the observed genome-to-PFU ratio of ca. 500. That this low infectivity may be caused by inefficient viral entry rather than by defective genomes is suggested by a phenomenon described two decades ago as "centrifugal enhancement of infectivity" (14). Since two recent reports dealing specifically with the sensitivity of detection of infectious murine CMV (31, 40) did not include this method, we found it instructive and necessary to recall it and to document the enhancement on the molecular level. Close-to-confluence monolayers of MEF were infected with graded declining doses of virions quantitated in terms of PFU, one series according to the standard procedure of a plaque assay (Fig. 2, left panel) and a second series under the influence of a 1,000 × *g* centrifugal force (Fig. 2, right panel). After 6 h, a period that does not allow for viral DNA replication in the indicator cells, poly(A)$^+$ RNA was isolated and a sequence specific for IE1 (*ie1* gene exon 3/4) transcripts was amplified by RT PCR. The result shows at least a 10-fold enhancement in the level of IE1 transcription. Note that 0.1 PFU, corresponding to ca. 50 genomes, failed to induce viral transcription. By contrast, with the same sensitivity of the RT PCR, viral transcripts were clearly detected at this low dose of infection after centrifugal enhancement of infectivity. The effect of the centrifugal force most likely operates on the level of penetration rather than of adsorption. This conclusion is based on the finding that the enhancement is prevented by neutralizing antiserum added after adsorption and before centrifugation, but not after centrifugation (data not shown). It is important to emphasize that any attempts to increase the sensitivity of the RNA detection are in vain if centrifugal enhancement is not employed in the first place to increase the efficacy of viral penetration.

Further improvement of the sensitivity by focus expansion. In a formal sense, IE1 transcription after centrifugal enhancement is not a proof of infectivity, since it could detect genomes that are capable of being transcribed but defective in replication. We therefore prolonged the period in culture after centrifugal infection to 72 h, a time that allows three cycles of replication and infection of the neighboring cells to occur, causing formation of a focus of infected cells in a short-term indicator culture. Fig. 3 (left panel) documents the time-dependent increase in the amount of IE1 transcripts as a function of the inoculum dose of PFU per culture. That this increase does not just represent an accumulation of IE1 RNA, but requires viral DNA replication, is indicated by the fact that phosphonoacetic acid (PAA) prevents it (Fig. 3, right panel). In the absence of PAA, after 72 h, plaques were visible at inoculum doses of ≥0.1 PFU, and fluorescent foci were detectable with fluorochromated antibody to the intranuclear IE1 protein pp89 (data not shown). The yield of plaques reveals at least a 20-fold enhancement of infectivity by centrifugal infection. Most importantly, with the sensitivity of the RT PCR as the read-out, infectivity was detected with an inoculum virus dose of 0.01 PFU, that is, within ca. 5 viral genomes (Fig. 3, left panel), although a plaque was then not visible in the indicator culture. It should be noted that a further extension of the period in culture increased the amount of RNA but did not improve the assay sensitivity (data not shown). Altogether, the experiment shows that there exists an infectivity below the sensitivity of detection of the plaque assay.

Statistical verification of the sensitivity of the RT PCR-based FEA. To verify the end point of the infectivity titration, the assay was performed as a limiting-dilution assay (7) with nine replicate cultures per inoculum virus dose (Fig. 4). If the end point is reached, negative cultures must occur and the fraction of negative cultures must follow the Poisson statistics. These conditions were fulfilled for inoculum virus doses of ≥0.002 and <0.05 PFU. The most probable number for the infectious dose of virus was 0.009 PFU, or 4.5 genomes (0.009 PFU × 500 genomes per PFU), with 95% confidence limits of 2.0 to 9.0 genomes. Is this the end of the road? As shown above (Table 1), the particle composition of the purified virion preparation predicts that the genome-to-infectivity ratio cannot be lower than 2.6. Thus, the observed ratio of 4.5 is very close to the theoretically possible value.

Resolution of acute infection in organs and clearance of viral DNA from the blood. We have previously shown by experimental models of neonatal infection (3, 33), adult infection (33), and infection concurrent with BM transplantation therapy (2, 4) that the load of viral DNA in tissues after resolution of acute infection can differ greatly under various conditions. For the subsequent experiments we have chosen the BM trans-

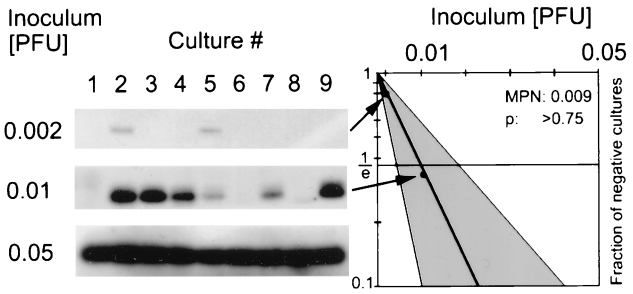

FIG. 4. Verification of FEA sensitivity by limiting-dilution Poisson distribution assay. Nine replicate MEF cultures per indicated inoculum virus dose were infected with centrifugal enhancement. Poly(A)$^+$ RNA was isolated after 72 h of viral gene expression, and 100 ng per replicate was tested by *ie1* exon 3/4-specific RT PCR. The most probable number (MPN) (0.009 PFU) of infectious units was estimated from the fractions of negative cultures by the maximum likelihood method (7). The shaded area represents the 95% confidence interval (0.004 to 0.018 PFU). The null hypothesis is accepted with $P > 0.75$ ($\chi^2 = 0.03$; df = 1).

plantation model for achieving an extraordinarily high load, a condition that should favor the detection of persistent virus replication as well as of spontaneous intermittent recurrences. In accordance with the previous studies (2, 4), virus replication as defined by a plaque assay performed with centrifugal enhancement of infectivity was cleared in all tested organs (Fig. 5, upper panel), with a typical succession of rapid clearance in the spleen (by ca. 2 months after infection), delayed clearance in the lungs (3 to 4 months), and slow clearance in the salivary glands (5 to 6 months). Acute infection is associated with a high load of viral DNA in blood leukocytes (2–4). Since the selection of some important organs for the infectivity assay entails a risk of missing persistent productive infection at an unknown site that may serve as a source delivering viral DNA to the blood, clearance of viral DNA from the blood provides harder evidence for the establishment of latency in the whole organism. We have therefore tracked the clearance of viral DNA from blood by PCR in a cohort of 10 mice that were bled from the tail vein at 1 month, 2 months, and every second month from then on (Fig. 5, lower panel). The slow but progressive decline in the load of viral DNA in the blood suggests a vanishing source. Notably, the blood still carried viral DNA several months after the resolution of productive infection in the tested organs, but by 1 year after infection the load in the blood was below the detection limit of the PCR. At that time point, the 10 mice were used for the detection of latent CMV in the lungs and for the induction of recurrence.

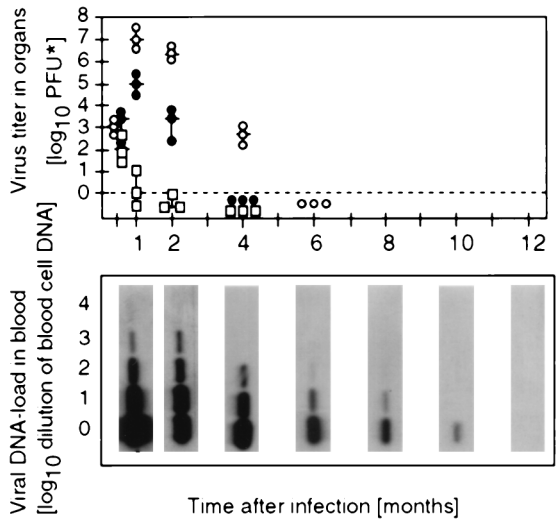

FIG. 5. Kinetics of the resolution of acute infection after syngeneic BM transplantation. (Upper panel) Clearance of productive infection in organs. Titers of virus in organ homogenates were determined by infection of indicator MEF with centrifugal enhancement and are expressed as PFU* to indicate the enhancement. The dashed line marks the detection limit, which was 1 PFU* after testing of the whole organ. Titers are shown for three mice per time point. The median value is marked by a horizontal bar. Cases in which virus titers for an organ were negative for all three tested individuals are depicted only on the first occasion, but the titers then remained negative throughout the kinetics. Symbols: open circles, salivary glands; solid circles, lungs; open squares, spleen. (Lower panel) Clearance of viral DNA from the blood. A cohort of 10 mice were bled from the tail vein at the indicated time points, starting at 1 month postinfection. The viral DNA load in the pooled blood was determined by end point dilution PCR specific for a 363-bp sequence within exon 4 of the *ie1* gene. The titration starts with 3 μg of blood cell DNA, which represents the DNA content of 5×10^5 blood cells. Shown are the autoradiographs obtained after hybridization with the internal probe IE1.2135.

High virus genome load but absence of infectivity in latently infected lungs. The RT PCR-based FEA is the method of choice to reinvestigate the hypothesis of a low-level productive persistent infection in mouse organs after the virtual resolution of acute infection. We focus here on the lungs, because previous work has identified the lungs as a major organ site of murine CMV DNA load and recurrence of infectivity (3, 33), and because low-level persistent infection of the lungs has been proposed from the detection of IE1 transcripts (40). The LL and the PCL were used for detecting infectious virus by RT PCR-based FEA, and the remaining three lobes were used to determine the tissue load of viral DNA (Fig. 6). With only a little variance among five mice tested individually, the DNA load was ca. 200 viral copies per 50 ng of cellular DNA (Fig. 6, left panels). On the basis of the known DNA content of a diploid mouse cell, this gives an estimate of ca. 1 viral copy per 40 lung cells. By the counting of nuclei in standard volumes of lung sections, the average number of lung cells was calculated to be ca. 60×10^6; this matched well with the average yield of lung cell DNA, which was ca. 300 μg. Accordingly, the viral DNA load in the lungs was on the order of magnitude of 10^6 genomes in the whole organ. RT PCR-based FEA was employed to detect infectious virion DNA in this huge pool of viral DNA. In none of the five mice analyzed individually (only the data for animal 1 are given) could infectivity be detected when eight of nine aliquots of the homogenate of the LL and the PCL were tested (Fig. 6, lower right panel). The first aliquot for each animal was reserved for controlling potentially adverse effects of homogenate contents, whatever these may be, on the sensitivity of the assay. In order to avoid a statistically negative result, 0.05 PFU was added to the aliquots selected for the control, as this dose should be invariably positive (Fig. 4). RT PCR-based FEA clearly detected the 0.05 PFU within lung homogenate, and 100 ng (a 1/20 aliquot) of the RNA from the indicator culture could be further diluted 10,000-fold before the detection limit was reached. It should be emphasized that the technical conditions of the assay excluded neutralization of virus by antiviral antibodies. In conclusion, despite a high viral DNA load in the lungs, infectious virus was absent.

Recurrence of viral infectivity from latently infected lungs. Since defective genomes add to the viral DNA load without any biological significance for recurrence, the presence of genomes capable of reactivation has to be demonstrated to prove that recurrence can take place in the absence of low-level persistence. The remaining five mice of the latently infected group were subjected to hemoablative, immunosuppressive treatment by total-body γ-irradiation with a single dose of 6.5 Gy, and their lungs were tested for recurrence of infectious

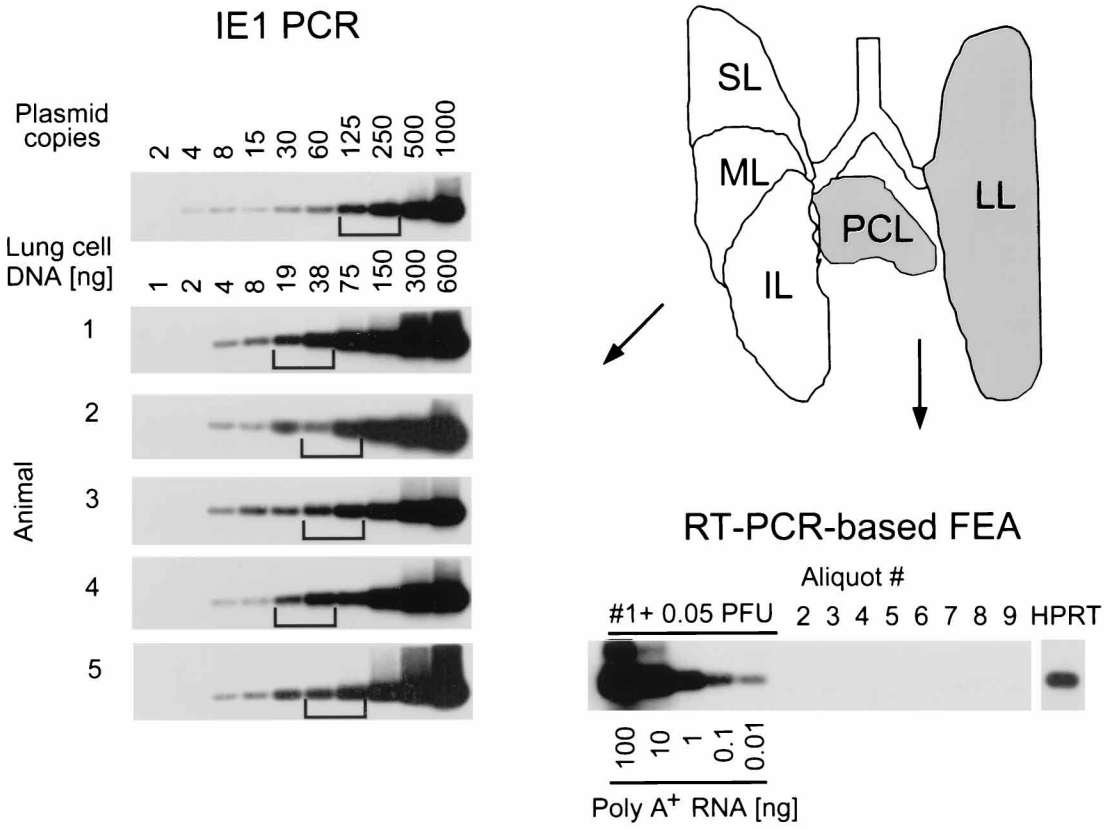

FIG. 6. Molecular latency of murine CMV in the lungs. The scheme of the lobular anatomy of the lungs in ventral view is shown at the upper right. The loads of latent viral DNA in the lungs are shown on the left. Total DNA was isolated from the pool of SL, ML, and IL, and viral DNA was quantitated by end point titration of lung cell DNA followed by *ie1* exon 4-specific PCR. Plasmid pIE111 was titrated in parallel. Five latently infected mice, selected randomly from the cohort of 10 mice (see Fig. 5), were tested individually 12 months after infection. The linear portions of the titrations that were used for the estimation are marked by brackets. Results of an RT PCR-based FEA for exclusion of infectious virus are shown on the lower right. The results were uniformly negative for the five latently infected mice, and therefore, data for only one are given. The homogenate of the LL and the PCL was assayed in total, evenly distributed to nine replicate MEF cultures. As a positive control, 0.05 PFU of purified murine CMV was added to aliquot 1 of the homogenate before centrifugal infection of the culture. Poly(A)$^+$ RNA from this culture was titrated as indicated, whereas for each of the remaining cultures, 100 ng of poly(A)$^+$ RNA was subjected to *ie1* exon 3/4-specific RT PCR, and in the case of culture 9, also to an RT PCR specific for the HPRT housekeeping gene transcript.

virus at day 14 by RT PCR-based FEA. To test whether viral infectivity recurred in all parts of the lungs, the five lobes were analyzed separately. Three of five mice were positive for infectious virus in all five lobes of the lungs, with some variance in quantity, whereas in mice 7 and 9, the IL and the PCL, respectively, were negative (Fig. 7). In conclusion, the lungs contained viral genomes capable of reactivation.

DISCUSSION

The question of whether CMV establishes molecular latency in the lungs has been a matter of recent debate (3, 40). In previous work we had shown that the lungs harbor viral genome after the virtual resolution of acute infection (3) and that the incidence of recurrence observed after ablation of cellular immune control correlates positively with the tissue load of viral DNA (33). In that work, murine CMV latency in tissues had been verified by the absence of infectious virus on the basis of an in vitro plaque assay performed with centrifugal enhancement of infectivity and with a prolonged period of plaque formation. Pollock and Virgin (31) specifically elaborated on the problem of assay sensitivity. By prolonged in vitro culture as well as by limiting-dilution inoculation of highly susceptible SCID mice, they improved the assay sensitivity to the level of a single PFU per whole organ. On the basis of this sensitivity, they concluded that murine CMV DNA in spleen and kidney exists in a state of molecular CMV latency in the absence of low-level persistent infection. By contrast, Yuhasz et al. (40) proposed that, at least in the lungs, murine CMV is maintained in a state of low-level persistent infection, and they suggested that our previous results concerning a high incidence of recurrence in the lungs (3, 33) need to be reinterpreted as an expansion of virus multiplication after ablation of the immune control. Their conclusion was entirely based on the finding that a sequence specific for IE1 RNA could be amplified by RT PCR from lung tissue but not from spleen. That this phenomenon should be specific for the lungs is not in agreement with reports of Henry and Hamilton, who described the detection of IE1 transcripts (11), and of Yu et al., who found an expression of the early-late viral phosphoprotein pp50 (39) in virtually latent spleens. While these authors interpreted their data as indicative of persistent infection, they all failed to positively demonstrate the presence of infectious virus. It should be noted that we also can detect IE1 RNA in latently infected lungs (unpublished data). However, we principally disagree with the view that viral gene expression is indicative of productive infection. The productive cycle can be interrupted at any stage. Specifically, Lucin et al. have demonstrated that gamma interferon and tumor necrosis factor alpha operating in

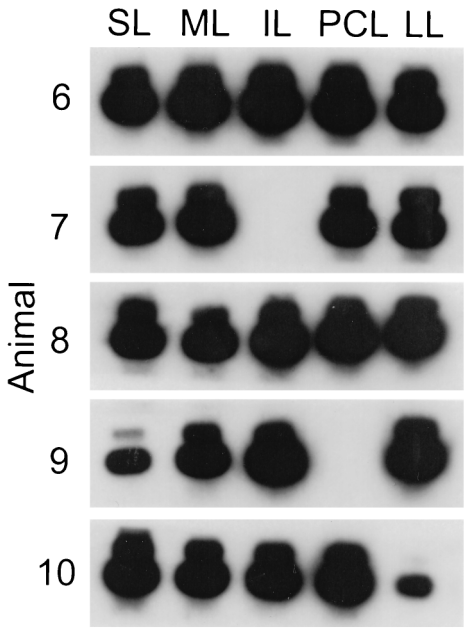

FIG. 7. Recurrence of viral infectivity after hemoablative treatment. The five remaining mice of the latently infected cohort were total-body γ-irradiated with a single dose of 6.5 Gy, and 14 days later the recurrence of infectious virus in separate lobes of the lungs (see the scheme in Fig. 6) was tested by RT PCR-based FEA. For each lobe, the result of one culture infected with one aliquot of the respective homogenate is depicted. The *ie1* exon 3/4-specific RT PCR was performed with 100 ng of poly(A)$^+$ RNA. Negative results were confirmed in a separate experiment (data not shown) by testing the remaining aliquots of the respective homogenates and by verifying the presence of RNA by RT PCR specific for the HPRT transcript.

synergy block murine CMV replication at the stage of nucleocapsid assembly (22). Undoubtedly, the only way to prove persistent infection is to positively demonstrate infectivity.

In this report we have documented that there exists an infectivity below the limit of detection of the plaque assay. While the number of PFU is an appropriate measure for higher doses of infectivity, the plaque assay has its limits at low doses. First, the development of a visible plaque is cell line dependent. Permissive cells with a rapid cell cycle tend to overgrow a developing plaque, and likewise, for MEF, the usual detection system, the number of passages in culture is critical for the plaque assay. Second, and more trivial, there is a risk that single plaques, in particular if they are poorly developed, may escape microscopic detection. We have calculated that a regular PFU represents ca. 500 viral genomes. By combining centrifugal infection of second-passage MEF (14), at least three rounds of viral replication in the indicator culture, and detection of IE1 transcripts in the indicator cells by RT PCR, we have improved the sensitivity of detection to a genome-to-infectivity ratio of ca. 5. This is very close to the theoretically possible sensitivity, since the presence of multicapsid virions in populations of murine CMV from cell culture as well as from the lungs precludes a genome-to-infectivity ratio of 1:1. Altogether, this new assay for infectivity, referred to as RT PCR-based FEA, is highly sensitive, fast, and reliable to document.

Despite a very high tissue load of viral DNA in latently infected lungs, infectivity was not detected by FEA. At least a fraction of this DNA was functional, since recurrence could be induced with high incidence and in all lobes of the lungs. The occasional finding of a negative lobe in the in vivo recurrence assay is in agreement with our previous conclusion that recurrence is based on stochastic events of reactivation that occur independently at different sites (33), and that recurrent virus does not disseminate in a seropositive organism (33), not from organ to organ and not from one lobe of the lungs to the neighboring lobe. It is important to emphasize that the kinetics of the resolution of acute infection differs among organs (Fig. 5) (2, 33). In the spleen, productive infection was cleared after 2 months, whereas in the salivary glands productive infection continued until 4 to 6 months. There is reliable evidence for the establishment of murine CMV latency in the stroma of the spleen (25, 32). As a consequence, apparently, molecular latency can be established in one organ while productive infection continues in another organ. This conclusion is not generally accepted at present. However, why should the establishment of latency in stromal cells of the spleen, the lungs, or any other site of latency wait until virus production discontinues in a distantly located, specialized cell type, namely, the glandular epithelial cell of the salivary glands? Further, there is indirect evidence that the target cells for productive infection are distinct from the cells in which latency is established (2). Hence, for a period of time, molecular latency and productive infection are likely to coexist even in the same organ. Clearly, we have to discriminate between the establishment of molecular latency at specific sites and latency in the whole organism.

It is evident that an analysis of gene expression during latency will give confusing results if productive infection was not absolutely cleared, and this problem may account for some of the discrepant data in the literature. An additional complication in the interpretation of data can result from the fact that the CMV genome is harbored in a nonproductive latency-like state in early hematopoietic progenitor cells in the BM, is exported into the blood and all organs via their progeny, and may switch to a productive state upon cell differentiation. This type of hematopoietic-leukocytic latency is the focus of current research on human CMV latency (19, 20, 23, 27), whereas extrahematopoietic stromal latency has been proposed for murine CMV in sinusoidal lining cells of the spleen (25, 32) and in renal tubule cells (18). It is likely that stromal latency exists also for human CMV, since the virus is effectively transmitted by organ transplantation from CMV-seropositive donors who are PCR negative for CMV DNA in the blood (8, 12). On the other hand, hematopoietic-leukocytic latency exists for murine CMV also. We have shown that murine CMV DNA is maintained in BM (4) and blood for several months until it is eventually cleared (Fig. 5) (2–4). The slow clearance is likely to be related to the limited self-renewal capacity of hematopoietic early progenitor cells, as well as to the half-life of their leukocytic progeny. Since the molecular state of the genome and gene expression during latency are likely to differ among different cell types, an analysis performed before the clearance of viral DNA from BM and blood will result in a mixed pattern that is difficult to interpret. It is therefore important to emphasize that the data shown here refer to stromal murine CMV latency after the clearance of the viral genome from BM and blood.

The exclusion of low-level persistence is the basis for any conclusions on latency. Our data have demonstrated that molecular murine CMV latency does exist in the lungs. The absence of infectious virus also indicates that spontaneous intermittent recurrence is not a frequent event in the lungs. Yet this conclusion should not be mistaken as an argument against frequent spontaneous reactivation of viral gene expression, since immune control could terminate the viral replicative cycle before the recurrence of infectious virus.

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