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Elimination of *ie*1 Significantly Attenuates Murine Cytomegalovirus Virulence but Does Not Alter Replicative Capacity in Cell Culture

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The major immediate-early (MIE) genes of cytomegaloviruses (CMV) are broadly thought to be decisive regulators of lytic replication and reactivation from latency. To directly assess the role of the MIE protein IE1 during the infection of murine CMV (MCMV), we constructed an MCMV with exon 4 of the *ie*1 gene deleted. We found that, independent of the multiplicity of infection, the resulting recombinant virus, MCMVdie1, which fails to express the IE1 protein, was fully competent for early gene expression and replicated in different cultured cell types with identical kinetics to those of parental or revertant virus. Immunofluorescence microscopy studies revealed that MCMVdie1 was greatly impaired in its capacity to disrupt promyelocytic leukemia bodies in NIH 3T3 cells early after infection, a process that has been proposed to increase viral transcription efficiency. We examined MCMVdie1 in the murine model using both immunocompetent BALB/c and severe combined immunodeficient (SCID) mice. When MCMVdie1 was inoculated into these two types of mice, significantly lower viral titers were detected in infected organs than in those of the wild-type virus-infected animals. Moreover, the *ie*1-deficient MCMV exhibited a markedly reduced virulence. While all animals infected with 5×10^4 PFU of parental virus died by 30 days postinfection, SCID mice infected with a similar dose of MCMVdie1 did not succumb before 60 days postinfection. The in vivo defective growth phenotype of MCMVdie1 was abrogated upon rescue of *ie*1. These results demonstrate the significance of the *ie*1 gene for promoting an acute MCMV infection and virulence yet indicate that MCMV is able to grow in vivo, although impaired, in the absence of the *ie*1 gene.

Similar to other herpesviruses, the transcription of the cytomegalovirus (CMV) genome during the lytic infection is temporarily regulated (for a review, see reference 47). The immediate-early (IE or α) genes are the first ones to be expressed in the replicative cycle, and their expression does not depend on prior viral protein synthesis. Together with some virion proteins, the IE products activate viral genes and alter the infected cell to generate an appropriate milieu that favors viral replication. Transcription of early (E or β) genes requires the expression of at least one of the IE proteins, and only after viral replication has started, the transcription of late (L or γ) genes proceeds. The majority of the CMV IE transcripts originate from the major IE (MIE) locus. This locus is structurally similar between human CMV (HCMV) and the closely related mouse CMV (MCMV) (14, 53, 59). The primary transcript from the MIE region is under the control of the strong MIE enhancer-promoter and is differentially spliced to generate two predominant transcripts, the *ie*1 transcript that consists of exons 1 to 4, and the *ie*2 transcript that is composed of exons 1 to 3 and 5. In HCMV, the *ie*1 and *ie*2 transcripts are translated into the acidic 72-kDa IE1 and the 86-kDa IE2 nuclear phosphoproteins, respectively (for a review, see reference 59). The corresponding IE transcripts of MCMV encode the acidic 89-kDa IE1 phosphoprotein and the 88-kDa IE3 protein (33, 34, 45).

The MIE proteins of HCMV display multiple functions (for reviews, see references 19 and 47). Mostly based on data from transient transfection assays and in vitro analysis, these viral products have been shown to be promiscuous regulatory proteins. In particular, HCMV IE2 is capable of down-regulating transcription from its own promoter by binding to the *cis* repression signals and exhibits strong transactivating properties on HCMV early promoters as well as on heterologous viral promoters. This gene product has been also shown to transactivate a number of host genes, such as the thymidine kinase gene or the dihydrofolate reductase gene. A number of components of the basal transcription machinery (i.e., TBP, TFIIB, TFIID) and cellular transcription factors (i.e., CREB, CBP, c-Jun) have been reported to directly interact with HCMV IE2 (11, 25, 31, 39, 57). HCMV IE1, the most abundant IE product, autostimulates the MIE enhancer-promoter (15, 59, 60, 62), plays an accessory role in the IE2-mediated activation of HCMV early and late genes (40, 62), and increases transcription of the long terminal repeat of human immunodeficiency virus (67). It can also transactivate a limited number of cellular promoters, including the ones corresponding to the DNA polymerase α (26), dihydrofolate reductase (65), and prointerleukin-1β (29). Interaction of HCMV IE1 with a number of cel-

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lular regulatory proteins (i.e., CTF-1, p107) has also been described previously (51). In addition to their regulatory activities, HCMV IE1 and IE2 are involved in perturbing a variety of other cellular processes, including cell cycle regulation (10, 69), apoptosis (71), and cell architecture. In this connection, IE1 has been shown to interact with chromatin and to cause the dispersion of the promyelocytic leukemia (PML) protein-associated nuclear bodies known as PML oncogenic domains, nuclear domain 10, or PML bodies (1, 2, 30, 35, 70). Though the precise role of these structures has not been defined, it has been hypothesized that they are part of a host cell repression system for viral infections.

In recent years, advances in the methodology that permits the manipulation of the CMV genome, in particular the application of the bacterial artificial chromosome (BAC) technology has enormously facilitated the generation of CMV mutants (6, 46). This has made it possible to begin elucidating the biological significance of the MIE locus during the course of CMV infection. In agreement with the multifunctional nature assigned to the MIE proteins, it has been found that HCMVs with deletions in either the *ie*1 or the *ie*2 gene exhibit severe phenotypes in cultured cells. Marchini and coworkers (41) reported that a recombinant virus lacking the majority of the *ie*2 gene was blocked at the early phase of gene expression and did not produce infection progeny. A number of other HCMV with defects in the *ie*2 gene have been generated and essentially have corroborated the importance of IE2 in the control of viral gene expression (27, 56, 68). Deletion of the *ie*1 gene of HCMV causes a drastic growth defect under conditions of low multiplicity of infection of primary fibroblasts (24, 48). This growth deficiency appears to be the result of a broad reduction of delayed-early gene transcription in the absence of functional IE1 and can be circumvented at a high viral input (20).

Due to the species specificity of HCMV, MCMV replication in mice has been extensively employed as a well-established model system for the study of different aspects of HCMV pathogenesis, latency, and reactivation. However, a limited number of studies have addressed MCMV IE activities. Similar to its HCMV IE2 homologue, MCMV IE3 represents the master switch that determines the transition from IE to E expression, and accordingly, MCMV *ie*3-deficient mutants are completely replication defective (3). The fact that the IE1 proteins of human and murine CMV exhibit a very similar global molecular structure, despite sharing little homology between their nucleic acid or amino acid sequences, has led to the assumption that they might have analogous functions during the course of the infection (33, 34, 60, 61). In this respect, MCMV IE1 has been reported to activate heterologous promoters (36) and to cooperate with IE3 protein in the activation of MCMV early gene promoters (45). In a recent study, Tang and Maul (63) have reported that MCMV IE1, as in the HCMV system, localizes to PML bodies and disperses them. Moreover, these studies reported on the ability of MCMV IE1 to bind to host cell repressors and proposed that the levels of IE1 expression might determine the number of repressed/activated viral genomes and, hence, the efficiency of a productive MCMV infection. An MCMV with a frameshift in exon 4 of the *ie*1 gene that leads to a truncated IE1 protein has been constructed (46) and was shown to have some growth impairment on cultured fibroblasts. However, the phenotype of this recombinant virus has not been further analyzed, and the presence of BAC sequences replacing genomic regions required for the in vivo infection has restricted its use in mice. Thus, the precise roles exerted by this protein during the MCMV life cycle still remain elusive. Moreover, the contribution that IE1 makes to in vivo MCMV growth and pathogenesis is unknown.

In the present study, we have directly addressed the relevance of IE1 on the replicative strategies of MCMV in vitro and in the natural host. Using a parental full-length MCMV genome, we have created and characterized an MCMV devoid of exon 4 of the *ie*1 gene and, hence, unable to synthesize the IE1 protein. We found that this recombinant virus replicated as efficiently as the parental or revertant MCMV in different cell types in culture. In contrast, the *ie*1-deficient MCMV showed a significantly attenuated in vivo replication capacity and virulence. Thus, the MCMV IE1 protein is not essential but promotes efficient viral growth in mice.

MATERIALS AND METHODS

Cells and viruses. The murine fibroblast cell line NIH 3T3 (ATCC CRL-1658), the mouse epithelial tumor cell line C127I (ATCC CRL-1616), and the mouse endothelial cell line SVEC4 (ATCC CRL-2161) were obtained from the American Type Culture Collection (Manassas, VA.). Primary mouse embryonic fibroblasts (MEFS) were prepared from the embryos of pregnant BALB/c.ByJ mice on day 19 of gestation. Primary macrophages were elicited from peritoneal exudate cells following intraperitoneal injection of 1 ml thioglycolate (Becton Dickinson, Cockeysville, MD) into BALB/c.ByJ mice. Peritoneal exudate cells were removed by peritoneal lavage, plated out at 1×10^6 to 5×10^6/ml, and incubated for 24 to 48 h at 37°C, 5% CO_2, after which nonadherent cells were washed away with phosphate-buffered saline (PBS). Cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of gentamicin per ml, and 10% fetal bovine serum, except for the NIH 3T3 cells, where calf serum was used instead. The parental BAC-derived MCMV strain MW97.01 (named MCMV in this study) (66) and the recombinant MCMVs generated in the course of the study were propagated on NIH 3T3 cells. The *ie*3-deficient mutant MCMVdie3 was described by Angulo et al. (3).

Plasmid construction. A 5.4-kbp EcoRI fragment from plasmid pIE3 (45) was cloned into the shuttle vector pST76ASacB (3) to get the recombination plasmid pST76ASie1. The 5.4-kbp EcoRI fragment comprises the *ie*3 gene with exons 3 and 5 of the M122/123 genes directly fused to each other (nucleotides [nt] 177008 to 179517 and 181372 to 184236 of the MCMV genome) (53). Plasmid pST76KSRie1r, which was used for construction of a revertant virus, was generated by insertion of a 7.2-kbp EcoRI fragment (nt 177008 to 184236) (53) obtained from plasmid pIE111 (45) into shuttle plasmid pST76-KSR, a derivative of pST76K (52) carrying the negative selection marker *sacB* and the *recA* gene. To generate another rescuant virus that could be distinguished from the wild-type MCMV, the 1.5-kbp AvrII fragment (nt 180489 to 182002) in plasmid pIE111 was replaced by an SpeI-AvrII-treated DNA fragment which was obtained by PCR using primers Avr.for (5′-CTG AAT TCC TAG GCC CTG ACA GAA AAA AGG-3′) and AvrSpe.rev (5′-CTG AAT TCA CTA GTC ATG GTG AAG CTA TCA AAG A-3′). Correct insertion of the PCR fragment destroyed the AvrII site at position 180489. The integrity of the insert was checked by sequencing. Finally, the 7.2-kbp EcoRI fragment of the modified pIE111 plasmid was transferred to pST76-KSR as described above, leading to pST76KSRie1r-2.

BAC mutagenesis and reconstitution of recombinant viruses. Recombination between the full-length MCMV BAC pSM3fr (66) and the recombination plasmid pST76ASie1 was performed by a two-step replacement procedure in the *Escherichia coli* strain CBTS essentially as described previously (3) to generate the mutant MCMV BAC pSM3frdie1. To restore the *ie*1 gene, the BAC pSM3frdie1 was transferred into the *E. coli* strain DH10B and recombination was performed using plasmid pST76KSRie1r. A second revertant virus that could be distinguished from the wild-type MCMV by AvrII digestion of the viral genomes was generated by using plasmid pST76KSRie1r-2. Since both rescuants led to equivalent results, only data obtained with one virus (MCMVrev) are discussed in Results. BACs carrying the desired mutation were identified by restriction enzyme analysis and agarose gel electrophoresis as described previously (4). Midi preparations of BAC DNA were obtained from 100-ml *E. coli*

cultures using Nucleobond PC100 columns (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. Recombinant viruses were reconstituted by transfection of the BAC DNA into NIH 3T3 cells using the calcium phosphate transfection method.

In vitro growth of recombinant MCMVs. Cells in 24-well plates were infected with the different recombinant viruses at the indicated multiplicities of infection (MOIs). After a 1-h adsorption period, cells were washed with PBS and incubated in DMEM supplemented with either 3% fetal bovine serum or 3% calf serum (in the case of the NIH 3T3 cells). The supernatants of the infected cells were collected at the different times postinfection, cleared of cellular debris, and frozen at −70°C. Virus titers were subsequently determined by plaque assay on NIH 3T3 cells. In the case of peritoneal macrophages, intracellular viral titers were measured.

Western blotting analyses. NIH 3T3 cells (in six-well dishes) were either mock infected or infected with the different MCMV recombinants at an MOI of 5 PFU/cell. At 6 and 24 h postinfection, samples were lysed in protein sample buffer, vortexed, and boiled for 5 min. For selective expression of IE proteins, the cells were exposed for 30 min before the infection, during viral adsorption, and for 3 h after infection to cycloheximide (CHX, 50 μg/ml), at which time point the CHX was replaced with actinomycin D (5 μg/ml). Actinomycin D was maintained in these cultures until the cells were harvested (7 h postinfection). The polypeptides of cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% polyacrylamide) and transferred to nitrocellulose filters. Equal quantities of total protein were analyzed per lane. Filters were incubated with mouse anti-IE1 or anti-E1 specific monoclonal antibodies, Croma 101 and Croma 103, respectively, or a rabbit IE3-specific antiserum. As secondary antibodies, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Amersham, Buckinghamshire, England) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad Laboratories, CA) were used. Blots were developed by using the Enhancer chemiluminescence system (Amersham) according to the manufacturer's protocol.

Immunochemistry. NIH 3T3 cells were cultured on coverslips and infected with the various MCMV recombinants at an MOI of 0.3 or 1. After 4, 15, or 24 h, cells were rinsed once in PBS and fixed in 2% paraformaldehyde. Cells were stained with primary antibody Croma 101 or Croma 103 and with rabbit anti-PML (kind gift of P. Freemont). For secondary antibodies, fluorescein isothiocyanate-conjugated anti-rabbit and tetramethyl rhodamine isothiocyanate-conjugated anti-mouse (Jackson Laboratories) antibodies were applied. DNA was counterstained with 4′,6′-diamidino-2-phenylindole (DAPI).

Epifluorescence microscopy and quantification. Images were taken with an epifluorescence microscope using applied images software. For quantification purposes, a series of images spanning the nuclei were taken using the 40× lens and a z-step size of 0.8 μm. Z sections were projected to a single image, and the numbers of PML bodies in noninfected and infected cells were counted. To discriminate intranuclear PML from cytoplasmic signal at 24 h after infection, three-dimensional images were taken with a 100× lens and a z-step size of 0.3 μm. Only those PML bodies residing within the DAPI-stained nuclei were counted.

Viral nucleic acid isolation and analysis. Preparation of total DNA from infected cells, restriction enzyme analysis, and gel electrophoresis were essentially done as described previously (3). To examine whether a correct excision of the BAC vector sequences from the recombinant MCMV genomes had occurred, PCRs were performed as described previously (22, 66). Primers b (5′-GCC CGC CTG ATG AAT GCT C-3′) and g (5′-GGA TAC TCA GCG GCA GTT TGC-3′), which bind in the BAC vector sequences and in the EcoRI g fragment, respectively, were used to amplify a 1,950-bp PCR fragment within the BAC-containing genomes. Primers f (5′-GGT TAC TGG ATG GGT ACG AG-3′) and g, which anneal with viral sequences flanking the excision site, were used to detect a 590-bp product after successful excision of the BAC vector segment from the viral genomes. The template used in the PCR was genomic DNA isolated either from cells infected with the recombinant viruses or from a full-length MCMV BAC plasmid. PCRs were performed under the following conditions: 1 cycle at 94°C for 3 min; 30 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C; and 1 cycle at 72°C for 10 min. For Southern blot analysis, the full-length MCMV BAC and DNA isolated from cells infected with the recombinant viruses were digested with MluI, separated on a 0.7% agarose gel, blotted onto a HybondXL nylon membrane (Amersham Pharmacia), and hybridized with a ^{32}P-labeled probe corresponding to nt 216031 (MluI) to 218468 (NotI) of the MCMV BAC genome.

Mouse infections. Six-week-old female BALB/c.ByJ or BALB/cAnNCrl mice were obtained from the Scripps Research Institute breeding colony and Charles River Labs (Lyon, France), respectively. Four-week-old female CB17 SCID mice were obtained from Taconic Farms (N.Y.). Animals were housed in the vivarium (The Scripps Research Institute or The University of Barcelona) under specific-pathogen-free conditions. Mice were inoculated with 1×10^6 PFU of tissue culture-derived MCMV recombinants by the intraperitoneal route. At designated times after infection, mice were sacrificed and organs were removed, weighed, and harvested as a 10% (wt/vol) tissue homogenate. Tissue homogenates were sonicated and centrifuged, and viral titers from the supernatants were determined on NIH 3T3 cells by standard plaque assays. To assess levels of virulence, the lethalities in CB17 SCID mice of mutant, parental, or revertant MCMV (tissue culture derived) were compared. Animals were intraperitoneally inoculated with 5×10^4 PFU of the different viruses, and their survival was monitored daily.

RESULTS

Construction of MCMVdie1, an MCMV recombinant with exon 4 of the *ie*1 gene deleted. To start examining the role of IE1 during MCMV replication, we decided to construct an MCMV with a deletion of the entire exon 4 of the *ie*1 gene. An MCMV with a frameshift mutation in exon 4 of the *ie*1 gene was previously reported (46). However, in addition to the *ie*1 alteration, this viral mutant generated using the BAC mutagenesis technique contains BAC vector sequences replacing a set of viral open reading frames (m151 to m158) that have been shown to be required for optimal in vivo growth of MCMV (66). Because a major objective of this study was to analyze the importance of the *ie*1 gene during the acute CMV infection, we undertook the construction of a new *ie*1-defective MCMV based on the MCMV BAC pSM3fr that contains the complete MCMV genome (66). Transfection of pSM3fr into permissive cells results in viral progeny with in vivo growth properties indistinguishable from those of wild-type MCMV. To disrupt the IE1 open reading frame, we introduced a 1.8-kbp deletion (from nt 179517 to nt 181372 of the MCMV genome) (53) into the cloned MCMV genome using the recombination procedures in *E. coli* as described in Materials and Methods. In this BAC recombinant genome, named pSM3frdie1, exons 3 and 5 of the MCMV *ie*1/*ie*3 transcription unit were fused, resulting in the complete removal of the fourth exon (Fig. 1A, line 2). In addition, we made a revertant genome by restoring the IE1 open reading frame. The revertant genome, designated pSM3frdie1-rev, was constructed to corroborate that the phenotype associated with the MCMVdie1 genome was due exclusively to the designed deletion in the *ie*1 gene and not because other alterations were present in the mutant viral genome. In this recombinant BAC, sequences from nt 179517 to nt 181372 of the MCMV genome were reintroduced into the MCMVdie1 genome (Fig. 1A, line 3).

To confirm the genomic structure of the recombinant BACs constructed, their HindIII restriction patterns were examined. As shown in Fig. 1B, the natural 7.6-kbp HindIII K and the 7.2-kbp HindIII L fragments of the parental BAC pSM3fr were replaced by a 12-kbp fragment in pSM3frdie1 (Fig. 1B, compare lanes 1 and 2). Rescue of the *ie*1 gene in the revertant BAC pSM3frdie1-rev led to the reappearance of the natural HindIII K and L fragments (Fig. 1B, compare lanes 1 and 3). Further characterization of the MCMV BACs generated was carried out by digestion with additional restriction enzymes (data not shown). These results indicated that the expected recombination events took place within the *ie*1 region and that no major deletions or rearrangements had occurred elsewhere in the viral genome.

Next, we transfected the three MCMV BAC recombinants

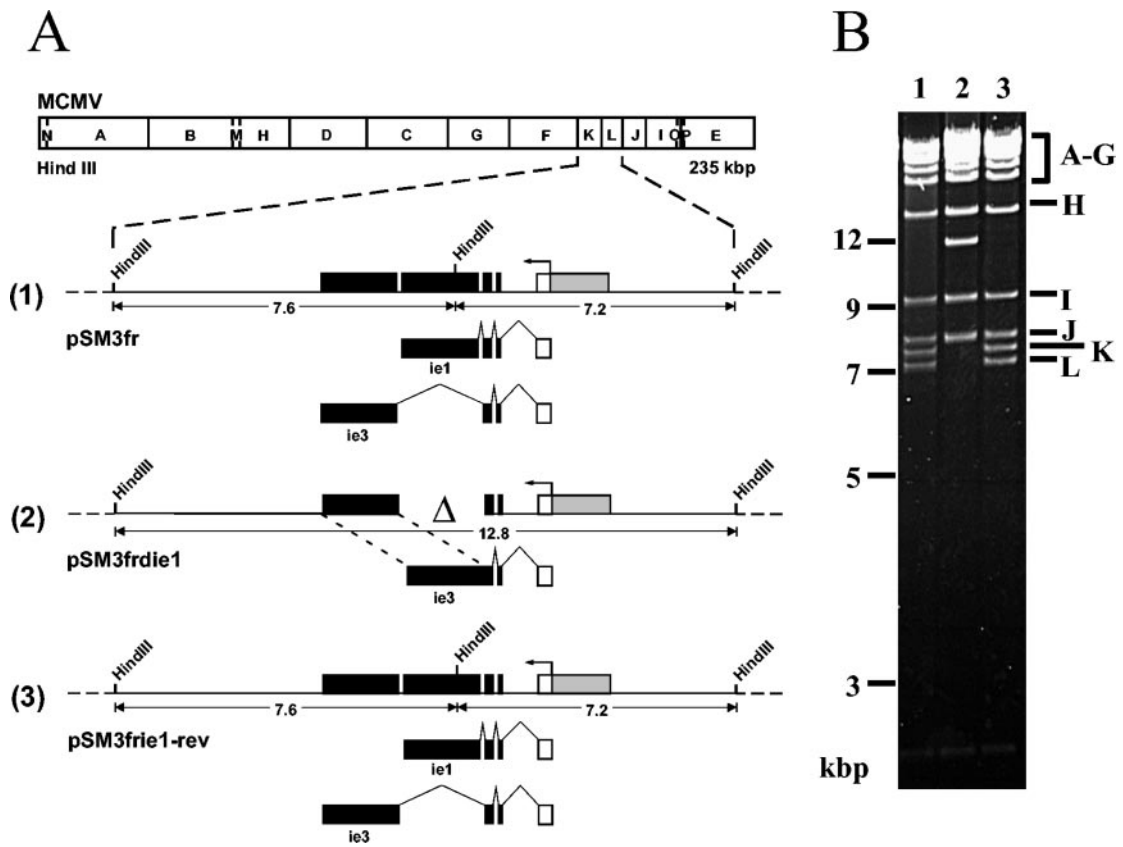

FIG. 1. Construction of an MCMV BAC genome lacking exon 4 of the *ie*1 gene. (A) Schematic representation of the parental pSM3fr, pSM3frdie1, and the revertant pSM3frdie1-rev BAC. The HindIII map of the MCMV genome is shown at the top. The expanded map of the HindIII K and HindIII L fragments represents the MCMV MIE gene region. Coding exons are shown as black rectangles, and the first noncoding exon of the *ie*1/*ie*3 transcription unit is shown as an white rectangle. The gray box represents the MCMV enhancer *ie*1/*ie*3 promoter. The structures of the *ie*1 and *ie*3 transcripts are indicated below line 1 (of the expanded map). Starting with the parental BAC pSM3fr (line 1), the mutant BAC pSM3frdie1 (line 2) and the revertant BAC pSM3frdie1-rev (line 3) were generated by successive rounds of homologous recombination in *E. coli* as described in Materials and Methods. The deletion of the fourth exon of the *ie*1 gene is marked by the delta (Δ). The sizes of the natural HindIII K and L fragments in pSM3fr and pSM3frdie1-rev and that of the new HindIII fragment in pSM3frdie1 are indicated. (B) Genomic structure of pSM3fr, pSM3frdie1, and pSM3frdie1-rev. Ethidium bromide-stained 0.7% agarose gel of HindIII-digested BACs pSM3fr (lane 1), pSM3frdie1 (lane 2), and pSM3frie1-rev (lane 3). Size markers are indicated in the left margin. The names of the MCMV HindIII fragments (16) are shown in the right margin.

into NIH 3T3 cells to reconstitute the corresponding viruses. In all cases, numerous plaques appeared after transfection, spreading quickly through the entire cultures. The progeny viruses were harvested when the cultures reached cytopathic effect, used to infect new cell monolayers, and plaque purified three times. Viral stocks of each of the MCMV recombinants were prepared, and the structure of their genomes was confirmed by restriction digestions. The patterns of restriction obtained for the three genomes were as predicted and identical to those of the corresponding BACs (data not shown). The viruses generated were named MCMV (derived from pSM3fr), MCMVdie1 (derived from pSM3frdie1), and MCMVrev (derived from pSM3frdie1-rev).

Absence of IE1 protein expression in cells infected with MCMVdie1. We next determined whether deletion of exon 4 of the *ie*1 gene in MCMVdie1 had resulted in the complete loss of IE1 protein expression in infected cells. In MCMV-infected cells, the 89-kDa IE1 protein is already detectable 1 h after infection and is expressed during the whole replication program (32, 45). NIH 3T3 cells were infected at a high MOI with either MCMVdie1 or the parental or the revertant MCMV, and cell lysates were prepared at 6 h and 24 h after infection. Since IE proteins can be overexpressed by arresting the MCMV-infected cells in the IE phase, we also infected NIH 3T3 cells in the presence of CHX to allow IE RNA transcription but prevent synthesis of IE proteins. Three hours after infection, the CHX was removed from these cultures, and actinomycin D was added for 4 h to inhibit IE viral RNA transcription, permitting protein synthesis of accumulated RNAs. Samples were analyzed for IE1 expression by Western blotting employing an MCMV IE1-specific monoclonal antibody. As shown in Fig. 2, the 89-kDa IE1 protein was detected in lysates of cells infected with the parental or revertant MCMV under IE conditions and at 6 h and 24 h postinfection. However as expected, the IE1-specific antibody failed to recognize the IE1 polypeptide in lysates of infected cells with MCMVdie1 under any of the conditions tested. Similar conclusions regarding the presence/absence of the IE1 protein in the NIH 3T3 cells infected with the different recombinant viruses could be drawn from immunofluorescence assays (see Fig. 5A, panels c, e, and g).

Because construction of MCMVdie1 involved the fusion of

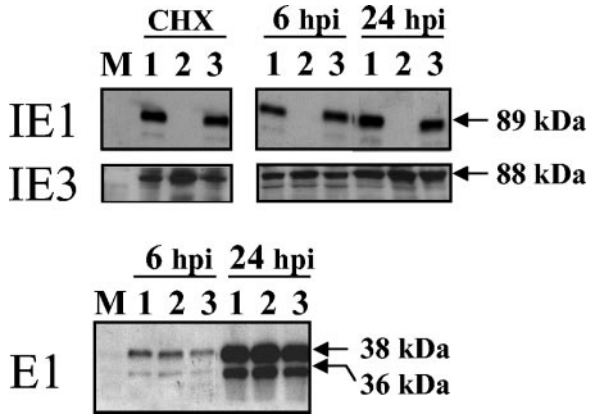

FIG. 2. Absence of IE1 protein in cells infected with MCMVdie1. NIH 3T3 cells were either mock infected (M) or infected with the parental MCMV (lanes 1), MCMVdie1 (lanes 2), or MCMVrev (lanes 3) at an MOI of 5 PFU/cell in the absence or presence of cycloheximide (CHX) as indicated in Materials and Methods. CHX-treated samples were subsequently exposed to CHX for the 3 h after the adsorption period, at which time the CHX was removed and actinomycin D was added for 4 h before the cells were harvested at 7 hpi. At the time points after infection indicated (7 h for the CHX samples), cells were lysed, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis on 7.5% polyacrylamide gels and Western blots performed with anti-IE1 (IE1)- or anti-E1 (E1)-specific monoclonal antibodies or an IE3-specific antiserum (IE3).

exons 3 and 5 of the MCMV *ie*1/*ie*3 transcription unit, we next examined IE3 expression levels in MCMVdie1-infected NIH 3T3 cells by Western blotting using an IE3-specific antiserum. As shown in Fig. 2, levels of the 88-kDa IE3 protein (45) were comparable for MCMVdie1, parental, and revertant virus throughout the three stages of the viral infection analyzed, although we must note that in some experiments a slightly higher amount (below 1.6-fold) of this viral product was detected at the IE phase in cells infected with MCMVdie1 than in cells infected with the corresponding control viruses. Finally, we determined the expression of the MCMV early gene *e*1 in these samples utilizing an E1-specific monoclonal antibody (8). Accordingly, the 36- to 38-kDa E1 protein was expressed at similar levels in cells infected for 6 h and 24 h by the *ie*1-deficient MCMV than in parental and revertant MCMV-infected cells (Fig. 2). Altogether, these results demonstrate that deletion of exon 4 of the *ie*1 gene in MCMVdie1 resulted in an IE1 protein-deficient virus and did not significantly alter IE3 or early (E1) gene expression.

Growth phenotype of MCMVdie1 in cell culture. Having confirmed the identity of the recombinant MCMVdie1, we studied its growth properties in more detail. First, we compared the growth kinetics of the mutant virus in NIH 3T3 cells with that of the parental and revertant MCMVs. Because it has been previously shown that, in the absence of *ie*1, the replication ability of both HCMV and MCMV on fibroblasts was significantly impaired at low MOIs (24, 46, 48), we infected NIH 3T3 cells with a reduced level of the recombinant viruses (MOI, 0.01 PFU/cell). At various times postinfection, viral titers from cultured supernatants were determined. Surprisingly, the growth characteristics of MCMVdie1 were indistinguishable from those of the parental or revertant MCMV (Fig. 3, panel A). Equivalent results were obtained when the infections were performed under high-MOI conditions (Fig. 3, panel B).

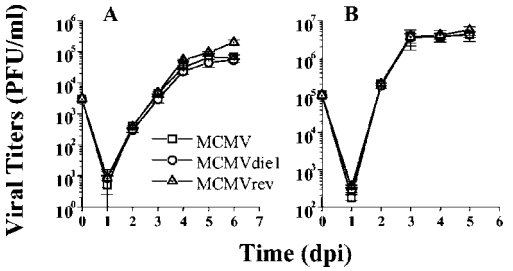

FIG. 3. Growth of MCMVdie1 in NIH 3T3 cells. NIH 3T3 cells were infected at an MOI of 0.01 (A) or 2 (B) PFU/cell with the parental MCMV, MCMVdie1, or MCMVrev. Culture supernatants were collected at the indicated times after infection (days postinfection [dpi]) and titrated by standard plaque assays on NIH 3T3 cells. Each data point represents the average and standard deviation of results from three separate cultures.

To assess whether the absence of phenotype of MCMVdie1 was specific of NIH 3T3 cells, we examined the growth kinetics of this mutant virus on various cell lines known to play a role in CMV biology in vivo. To this end, we infected mouse embryo fibroblasts, peritoneal macrophages, a murine endothelial cell line (SVEC4), and an epithelial cell line (C127I) with the *ie*1-deficient MCMV at a low MOI. As shown in Fig. 4, multistep growth curves demonstrate that the replication kinetics and infectivity yields of the *ie*1-deficient MCMV were very similar to the ones exhibited by its parental or revertant virus in the different cell types. Although viral titers attained in the peritoneal macrophages were low and subjected to some fluc-

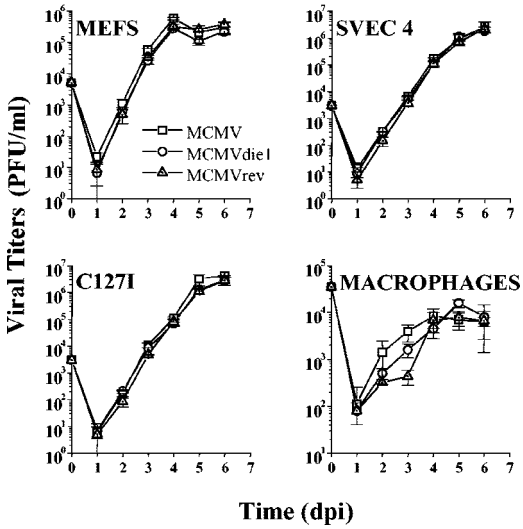

FIG. 4. Multistep growth curves of MCMVdie1 on different cell types. Mouse embryonic fibroblasts, SVEC4 cells, C127I cells, and peritoneal macrophages were infected with 0.05 PFU/cell, except for macrophages, which were infected at an MOI of 0.5 PFU/cell, of parental MCMV, MCMVdie1, or MCMVrev. Culture supernatants were collected at the designated times postinfection (days postinfection [dpi]) and titrated by plaque assays on NIH 3T3 cells. In the case of peritoneal macrophages, intracellular viral titers were determined. Each data point represents the average and standard deviation of results from three separate cultures.

tuations, the data presented here and obtained in an independent experiment generally indicate that mutant, parental, and revertant MCMVs replicate in a comparable manner in these cells. Thus, the IE1 protein is completely dispensable for in vitro MCMV growth.

Reduced capacity of MCMVdie1 to disperse PML bodies at early times after infection. HCMV IE1 has been shown to disrupt PML bodies (1, 2, 30, 35, 70). Recently, Tang and Maul have reported that very early in MCMV infection or after transfection with MCMV IE1, this protein concentrates at PML bodies and then disperses these nuclear structures, similar to its HCMV counterpart (63). We therefore examined the ability of MCMVdie1 to disrupt PML bodies in permissive NIH 3T3 cells. We infected NIH 3T3 monolayers at an MOI of 0.3 with either MCMVdie1 or the parental or revertant MCMV, fixed cells at different times postinfection (4, 15, or 24 h), and stained them for PML and IE1 or the early MCMV gene product E1 (8). We first confirmed that the parental and revertant MCMV had disrupted PML bodies 4 h after infection (Fig. 5A, panels c and d [parental] and g and h [revertant]). Noninfected cells in the same field showed PML bodies similar to mock-infected cells (compare panels c, d, g, and h with panels a and b). As expected, MCMVdie1-infected cells did not express IE1 (panel e in Fig. 5A). Since in this case we could not identify infected cells by the IE1 antibody, we used the E1 antibody that allows the detection of the viral product E1 as a punctate staining within the nucleus at this early time point after infection (Fig. 5A, panels m and o). The number of PML bodies in MCMVdie1- and mock-infected samples showed no difference at 4 h postinfection (hpi) (Fig. 5B; for image, Fig. 5A, panels k, o, and p). They contained the same range of PML bodies per cell (MCMVdie1, 3 to 17; mock, 2 to 20), while MCMV and MCMVrev showed 0 to 3 PML bodies per cell. This clearly shows that MCMVdie1 is not capable of significantly disrupting PML bodies at 4 hpi. Similar results were obtained when cells were infected with the three viruses at an MOI of 1 (number of PML bodies per cell: mock, 2 to 23; MCMV, 0 to 3; MCMVdie1, 2 to 22; MCMVrev, 0 to 3). To ensure that the nondisruption of PML bodies by MCMVdie1 could not be attributed to a general lack of immediate-early proteins, NIH 3T3 cells were infected with MCMVdie3, an *ie*3-deficient mutant (3). In these cells, PML was dispersed in a similar manner as in parental MCMV-infected cells (Fig. 5A, compare panels i and j with panels c and d). This result indicates that PML bodies are efficiently disrupted early after infection by an IE1-dependent mechanism and that IE3 is not involved in this early dispersal. When cultures were analyzed at 15 hpi, surprisingly, a few PML foci were observed in some parental MCMV-infected cells (Fig. 5A, panels q and r; Fig. 5B), while in cells infected with MCMVdie1, a number of PML bodies appeared to be disrupted (Fig. 5A, panels s and t; Fig. 5B). At 24 hpi, we observed that, in the MCMVdie1-infected cells, most PML bodies were also dispersed (Fig. 5A, panels u to x; Fig. 5B). In cells infected either with mutant or parental MCMV, PML antigen could be detected in the viral replication compartments and in foci in the cytoplasm, while in the nuclei of neighboring uninfected cells, we observed scattered foci. These data clearly demonstrate that other viral proteins can disperse PML bodies later during infection independently of IE1. The more complicated pattern observed at these later times suggests that PML bodies are slowly disrupted by an IE1-independent mechanism and that PML is accumulating in viral replication compartments of MCMV-infected cells, while PML foci are reformed.

Replication of MCMVdie1 in BALB/c mice. In the next set of experiments, we sought to evaluate the relevance of IE1 activity during MCMV infection in vivo. First, we tested our viral stocks for the absence of BAC vector sequences that could interfere with the natural course of the infection in mice. Excision of these sequences has been reported to occur after a few virus passages in permissive cells via homologous recombination through short identical viral sequences that flank the BAC vector segment (66). To confirm that the appropriate excision of the BAC vector sequences had occurred after the cloning and amplification steps to which the viral stocks were subjected, PCRs were performed using two different primer sets and genomic DNA from cells infected with the recombinant viral stocks as templates (66) (see Materials and Methods for further details). First, reaction assays were performed with a primer that anneals with the BAC vector sequences and a second primer that binds to the MCMV EcoRI g fragment. This primer set should yield a 1,950-bp PCR fragment if the BAC vector segment was present within the viral genomes or, alternatively, fail to amplify a product in the absence of these BAC vector sequences. As expected, a specific PCR-amplified product was not obtained with this primer set when the DNA from the recombinant viral stocks was used as a template (Fig. 6A, lines 2 to 4). In contrast, a 1,950-bp fragment could be detected in a DNA preparation isolated from a full-length MCMV BAC plasmid (Fig. 6A, line 1). Thus, the data indicated that BAC-containing genomes were not present in the recombinant viral stocks. Next, reaction asays were carried out with a pair of primers that bind to viral sequences flanking the BAC excision site. This second primer set should either fail to detect a product in the recombinant viral genomes if the BAC sequences were present (due to the length of the BAC fragment of approximately 8 kbp) or yield a 590-bp product in the case that the BAC vector sequences had been correctly excised. As shown in Fig. 6A, when DNA isolated from any of the three viral recombinant stocks was used as a template in the reaction assays, the expected 590-bp fragment was obtained (lanes 6 to 8), while no specific amplified product was detected when using DNA from the MCMV BAC plasmid (lane 5). In addition, to further confirm the absence of BAC-containing genomes in our viral stocks, DNA isolated from the three viral recombinants or the full-length MCMV BAC plasmid was digested with the restriction enzyme MluI, and a Southern blot analysis was carried out with a probe (nt 216031 to nt 218468 within the MCMV sequence) (53) (Fig. 6B) which detects a 12.1-kbp band in BAC vector-containing viral genomes and a 3.7-kbp band in BAC-free viral genomes. As shown in Fig. 6C, the 3.7-kbp band, but not the 12.1-kbp band, was present in the DNA corresponding to the viral stocks (lines 6 to 8), while in the DNA corresponding to the full-length MCMV BAC, only the 12.1-kbp band was detected (line 5). Altogether, these results corroborated that our fibroblast-passaged recombinant MCMV stocks were homogeneous and that their genomes were free of BAC vector sequences.

Then we proceeded to analyze the growth properties of MCMVdie1 in immunocompetent mice. Groups of BALB/c

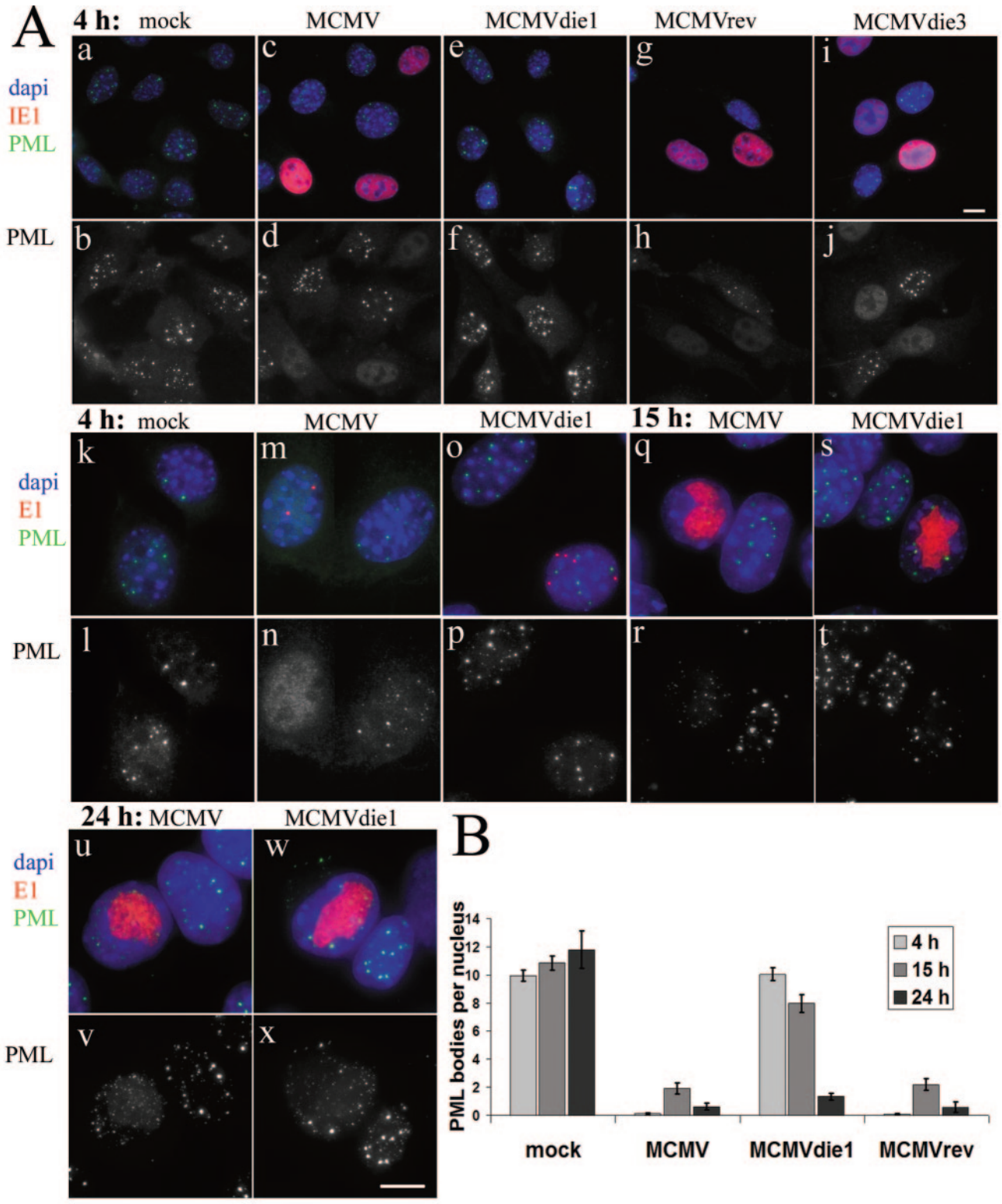

FIG. 5. MCMVdie1 fails to disrupt PML bodies in NIH 3T3 cells at immediate-early times after infection. NIH 3T3 cells were mock infected or infected with MCMV, MCMVdie1, MCMVrev, or MCMVdie3 at an MOI of 0.3. At the different times postinfection indicated, cells were fixed with paraformaldehyde and double labeling was performed for MCMV IE1 or E1 and PML as described in Materials and Methods. DNA was counterstained with DAPI. (A) Shown are photomicrographs from the different cultures. Panels a to p, 4 hpi; panels q to t, 15 hpi; panels u to x, 24 hpi. IE1 is shown in red (a, c, e, g, i), E1 in red (k, m, o, q, s, u, w), PML in green and black/white, and DAPI in blue. Bars, 10 μm. (B) The number of PML bodies in mock-infected cells and cells infected with MCMV, MCMVdie1, or MCMVrev were counted at the various times after infection indicated. For mock-infected samples, 100 cells were considered in the quantitation. For infected samples, only infected cells (E1 positive) were included in the quantitation: at 4 hpi, 100 cells per sample; at 15 hpi, >50 cells per sample; at 24 hpi, 14 to 29 cells per sample.

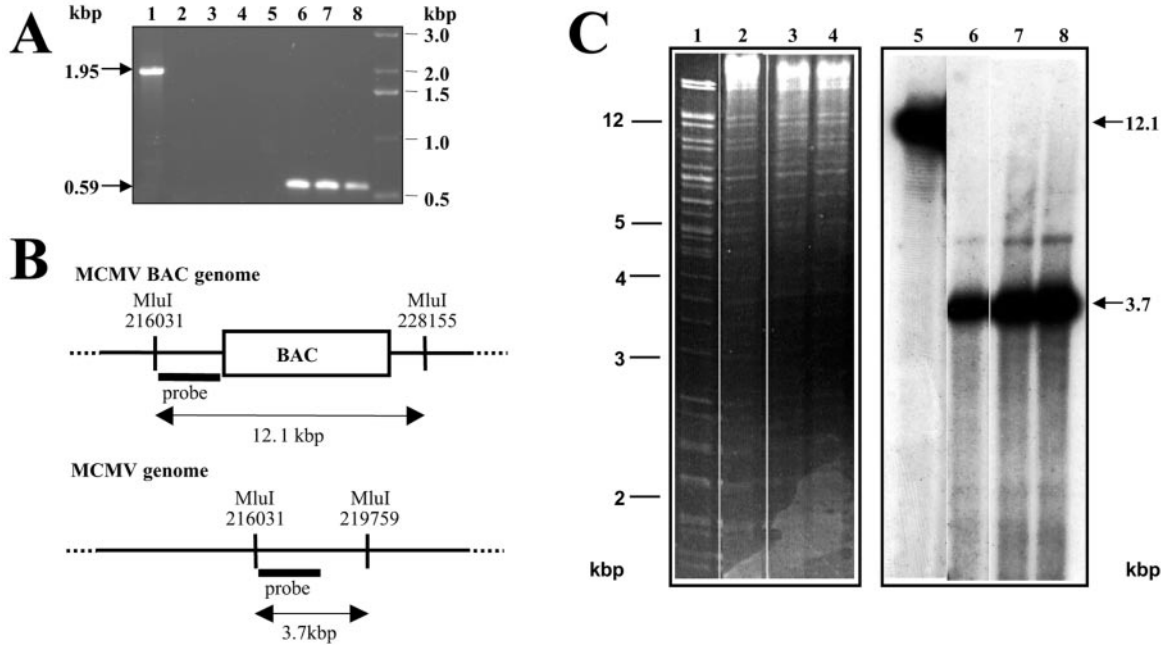

FIG. 6. Excision of BAC vector sequences from the MCMVdie1 genome. (A) PCRs were carried out using genomic DNAs isolated from a full-length MCMV BAC (lanes 1 and 5) or from cells infected with the viral stocks of MCMV (lanes 2 and 6), MCMVdie1 (lanes 3 and 7), and MCMVrev (lanes 4 and 8) as templates. Two distinct primer sets were used to analyze the appropriate excision of the BAC vector sequences (see Materials and Methods for details). The first primer set includes a primer that binds to the BAC sequence and a primer that anneals with a sequence located within the EcoRI g fragment (lanes 1 to 4), and the second primer set contains two primers that anneal with MCMV sequences flanking the excision site (lanes 5 to 8). Amplified products were separated on a 1% agarose gel and visualized by ethidium bromide staining. Products obtained in the PCR are marked by arrows indicating their sizes. The positions of the size markers are shown on the right. (B) Schematic representation of the viral genomes before and after excision of the BAC sequences. The probe detects a fragment of 12.1 kbp (before excision of the BAC sequences) and 3.7 kbp (after excision of the BAC sequences) in MluI-digested viral DNA. Nucleotide positions refer to the MCMV sequence (53). (C) Ethidium bromide-stained 0.7% agarose gel of MluI-digested viral DNAs from recombinant viral stocks (left panel) and corresponding Southern blot analysis (right panel). The sizes of the molecular size markers and detected DNA fragments are indicated in the left and right margins, respectively. Lanes 1 and 5, full-length MCMV BAC; lanes 2 and 6, MCMV; lanes 3 and 7, MCMVdie1; lanes 4 and 8, MCMVrev. The blot was overexposed to clearly show the absence of signals corresponding to BAC sequences in the viral stock DNAs.

mice were injected intraperitoneally with 1×10^6 PFU of MCMVdie1 or the parental or revertant MCMVs. At 3, 7, and 14 days postinfection, 6 mice from the MCMVdie1 group and 4 mice from either the parental or revertant MCMV group were sacrificed, and selected organs were removed to determine levels of viral growth. As seen in Fig. 7, titers of MCMVdie1 in the spleens, livers, and kidneys of mice infected for 3 days were reduced at least 7-, 18-, and 3-fold, respectively, when compared to those in the corresponding organs from animals infected with the parental MCMV. In a few cases, in the group of animals infected with MCMVdie1, infectious virus could not be detected in some of the organs. To calculate the median values of the viral titers in these groups, the titers in these particular organs were assigned to be the limit of detection of the assay (5×10^1 PFU/g). Therefore, the reduction in those MCMVdie1-infected organs probably represents an underestimation. On day 7 postinfection, titers of MCMVdie1 in the spleens and livers were approximately 10- and at least 2-fold lower, respectively, than those of the parental MCMV, while titers in the lungs were similar between mice from the two groups (Fig. 7). Finally, viral titers in the salivary glands of the MCMVdie1-infected mice at 14 days were reduced around fourfold compared to those of the parental MCMV-infected mice. In a separate experiment, similar results were obtained, except that titers in the lungs at day 7 after infection were

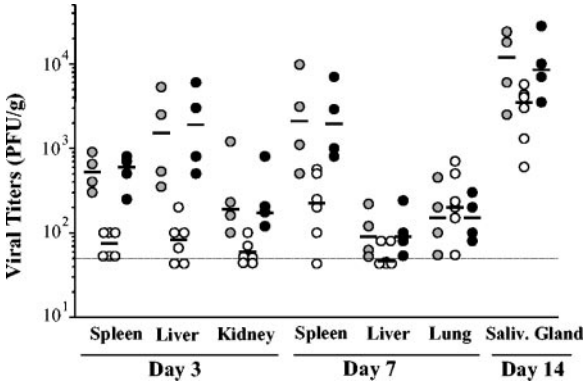

FIG. 7. Growth of MCMVdie1 in different organs of BALB/c mice. Groups of BALB/c mice (four to six per group) were inoculated intraperitoneally with 1×10^6 PFU of tissue culture-propagated parental MCMV (gray circles), MCMVdie1 (white circles), or MCMVrev (black circles). On days 3, 7, and 14 after infection, mice were sacrificed and the indicated organs removed, weighed, and sonicated as a 10% (wt/vol) tissue homogenate in DMEM. Viral titers of the resulting homogenates were determined by standard plaque assays on NIH 3T3 cells. Shown are the titers corresponding to the different organs for each individual mouse within a group. The dashed line shows the detection limit. Horizontal bars, median values; Saliv., salivary.

significantly lower in MCMVdie1-infected animals than in parental MCMV-infected mice (data not shown). As expected, restoration of IE1 expression in the revertant virus resulted in wild-type replication levels in the different infected organs analyzed. Taking all of the data together, the MCMV gene *ie*1 is not essential but influences viral replication in the immunocompetent host.

Replication of MCMVdie1 in SCID mice. It is known that MCMV infection is predominantly controlled by CD8 T cells and that the IE1 protein represents one of the dominant cytotoxic T lymphocyte (CTL) targets. While CTLs specific for peptides derived from MCMV products different than IE1 have been reported to play a prominent role in the control of MCMV replication, the lack of presentation of IE1 could have influenced the overall growth capacity of the MCMVdie1 in the BALB/c animals, thus reducing potential differences that may exist between the replication of the MCMV lacking *ie*1 and control viruses in this animal model. Therefore, we examined the growth properties of MCMVdie1 in CB17 SCID mice, which are devoid of functional T and B lymphocytes and Ly49-NK cells. For this purpose, six mice per group were intraperitoneally inoculated with 1×10^6 PFU of parental MCMV, MCMVdie1, or MCMVrev, and on day 7 postinfection, the spleens, livers, and lungs were extracted and examined for the presence of infectious virus. As shown in Fig. 8A, titers of MCMVdie1 in these organs were reduced between 8- and 27-fold relative to that exhibited by the parental MCMV. In contrast, mice infected with the revertant virus displayed viral titers in the target organs examined equivalent to that of the parental MCMV-infected animals, implying that restoration of the *ie*1 gene rescues virus replication to wild-type levels. We then assessed the replication of the viral mutant lacking the *ie*1 gene at a later time during the animal infection. CB17 SCID mice were infected as indicated above with the parental, *ie*1-defective mutant, or revertant MCMV, and on day 14 after infection, their spleens, livers, lungs, and salivary glands were harvested and assayed for infectious virus. Again, viral titers in the target organs analyzed of the MCMVdie1-infected mice were reduced by 10- to 60-fold compared to that of the parental MCMV- or MCMVrev-infected animals (Fig. 8A). The data demonstrate that MCMVdie1 is markedly attenuated in replication in most of the target organs of the SCID mice in comparison to the parental MCMV.

Virulence of MCMVdie1 in SCID mice. To further examine the importance of the IE1 protein during the MCMV infection of the immunodeficient animals, we also analyzed the relative virulence of MCMVdie1, parental, and revertant MCMVs by comparing their lethality kinetics for SCID mice. Wild-type MCMV routinely causes death of the SCID mouse when inoculated with a dose as low as 3 PFU (50). We intraperitoneally inoculated groups of 6 SCID mice with 5×10^4 PFU of either MCMVdie1 or the parental or the revertant MCMV, and survival was monitored daily after infection. The survival curves are depicted in Fig. 8B, showing a decreased lethality of MCMVdie1 compared to that of parental MCMV. While mice infected with parental MCMV succumbed to the infection within less than 28 days after viral inoculation, mortality in the MCMVdie1-infected animal group was not detected until 52 days postinfection. As expected, reintroduction of exon 4 of the *ie*1 gene in the revertant virus led to a complete reconstitution of the wild-type MCMV phenotype (Fig. 8B). The results are in good correlation with the in vivo viral growth and show that, in the absence of IE1, MCMV has lost some of its virulence. Thus, IE1 activity is required for efficient MCMV replication and virulence in the immunodeficient mouse.

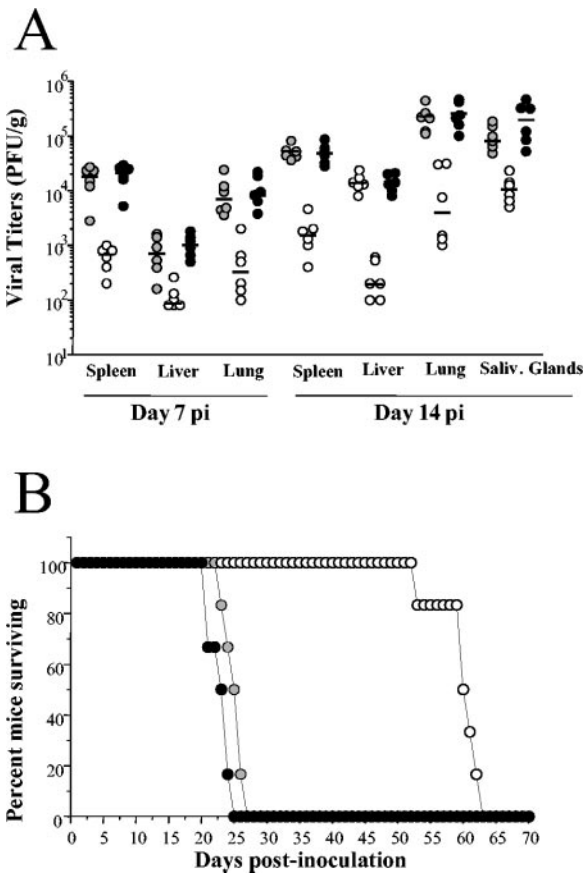

FIG. 8. Growth and virulence of MCMVdie1 in SCID mice. (A) Groups of six SCID mice (CB17) were inoculated intraperitoneally with 1×10^6 PFU of tissue culture-propagated parental MCMV (gray circles), MCMVdie1 (white circles), or MCMVrev (black circles). On days 7 and 14 after infection, mice were sacrificed and the indicated organs removed, weighed, and sonicated as a 10% (wt/vol) tissue homogenate in DMEM. Viral titers of the resulting homogenates were determined by standard plaque assays on NIH 3T3 cells. Shown are the titers corresponding to the different organs for each individual mouse within a group. All values were among the limits of detection of the assay. Horizontal bars, median values. Data shown correspond to results from one of two independently performed experiments in which similar results were obtained. (B) Groups of six SCID mice (CB17) were infected intraperitoneally with 5×10^4 PFU of either parental MCMV (gray circles), MCMVdie1 (white circles), or MCMVrev (black circles) and observed daily for mortality.

DISCUSSION

In the present study, we describe the construction of an MCMV lacking the entire exon 4 of the *ie*1 gene. We found that the mutant virus is indistinguishable from the parental MCMV in its cell culture phenotype in vitro, indicating that IE1 is totally dispensable for MCMV replication in different cell types. In marked contrast, we observed that there is an

important contribution of IE1 to in vivo MCMV pathogenicity and virulence.

Phenotype of MCMVdie1 in tissue culture. We undertook the construction of MCMVdie1, a new MCMV defective in *ie*1, because we were interested in analyzing the impact of *ie*1 on pathogenesis in the natural host in addition to its effect on viral replication in cultured cells. In a previous study, using the BAC technology, an MCMV with a truncation in the *ie*1 gene was constructed, but the presence of BAC sequences replacing genomic sequences in this recombinant virus limited its study in the mouse model (46). For the creation of MCMVdie1, the entire exon 4 of the *ie*1 gene was deleted in the backbone of pSM3fr, an MCMV BAC genome which, after reconstitution and subsequent passages in permissive cells, results in viral progeny free of BAC sequences and a wild-type phenotype in vivo (66). To our surprise, we found that growth of MCMVdie1 was not decreased or delayed compared to that of parental or revertant MCMV on a variety of cell types known to be significant for MCMV infection in vivo. In light of the phenotype exhibited in fibroblasts by the CMV *ie*1 mutants described earlier, we had anticipated that genetic resection of exon 4 of the *ie*1 gene, which accounts for most of the amino acid residues of the IE1 protein, would reduce to some extent the multicyclic replication of MCMV. The reason for the discrepancy between MCMVdie1 and the previously reported MCMV bearing the *ie*1 gene disrupted is not clear at present. The possibility exists that the stop codon introduced after codon 273 of the IE1 open reading frame in the previously reported mutant virus results in an aberrant or defective protein with adverse effects for MCMV growth. Alternatively, this difference could be attributed, although unlikely, to the distinct genetic background in which these two mutants were constructed. It must also be noted that, in the absence of a revertant virus for the *ie*1 mutant previously reported, one cannot completely rule out that the defective phenotype observed might be due to alterations in other regions of the MCMV genome outside the IE locus.

To date, the phenotypes observed for the IE CMV mutants reported so far have been consistent with a model for IE protein action in HCMV that was postulated in the early 1990s (21, 62) mainly based on transient-transfection assays. This model considered the capacity of IE2 to negatively autoregulate its own promoter (the MIE promoter, or MIEP) and activate HCMV early promoters, together with the ability of IE1 to stimulate the MIEP and cooperate with IE2 in the activating effects on early promoters. In this regard, *ie*2-deleted HCMV genomes fail to express early genes and are thereby incapable of making progeny virus (41). Similarly, resection of IE3 also results in an incompetent MCMV unable to progress in the viral life cycle beyond the IE phase (3). An important prediction of this model is that the levels of IE1 provide a key determinant (positive loop) in the switch from IE to E gene expression. The basic tenets of this model seem to certainly hold true for HCMV infection at a low MOI. HCMV *ie*1 mutants display a growth defect under conditions of low viral input that was attributed to the inability of IE1 to efficiently activate HCMV delayed-early gene transcription (20). This IE1 requirement for HCMV growth is circumvented at a high MOI, despite a slight delay in the accumulation of delayed-early genes. The finding reported here that MCMVdie1 grows with the same efficiency as wild-type MCMV in vitro suggests that there must be subtle differences in the functioning of IE1 from HCMV and MCMV in tissue culture cells or that alternative compensating mechanisms to IE1 activities operate during MCMV infection. In this connection, it is possible that IE proteins, virion transactivators, cellular components or other events triggered upon viral attachment or penetration may be sufficient in MCMV to substitute for IE1. We have not analyzed in detail viral gene expression in MCMVdie1-infected cells under low-MOI conditions to ascertain whether a positive feedback and/or the transactivation of early genes are defective. However, when infected with high doses of the *ie*1-deleted virus, at least levels of IE3 and E1 protein do not seem to be significantly altered in NIH 3T3 cells (Fig. 2). Regardless, if differences exist in the extent of immediate-early and early transcription between MCMVdie1 and parental MCMV infections, they do not appear to have an effect on the overall efficiency of MCMV growth. Altogether, the fact that the *ie*1 gene can be deleted without compromising MCMV replication in vitro indicates a higher independency of MCMV on functions of its more predominant IE protein than of HCMV.

The availability of an MCMV unable to express IE1 permitted further investigation of IE1 activities in the context of the viral infection. A number of DNA viruses, including adenoviruses and herpesviruses, are known to perturb the speckled macromolecular nuclear domains, named PML bodies during their life cycle (reviewed in references 17 and 42). These PML bodies consist of many proteins, but their structural cohesion as a nuclear body is dependent on the presence of sumoylated PML (43, 49). Viral proteins expressed very early in infection target to PML bodies and disaggregate them either by degrading PML or by depleting sumoylated forms of PML. PML bodies are preferential sites for the beginning of viral transcription and DNA replication (17, 42). A recent observation with HSV suggests that PML bodies may also be formed de novo near entering viruses (58). While the precise role that their disruption plays in viral infection remains to be elucidated, it has been proposed that these processes are of crucial importance for the establishment of loci of efficient viral transcription and DNA replication, in particular under low-MOI conditions. IE1 is the HCMV component to which this PML dispersal activity has been assigned, and in agreement, HCMV-defective *ie*1 mutants fail to disrupt PML bodies (1, 2, 30, 35, 70). Tang and Maul (63) recently showed that transfected MCMV IE1 had the capacity to disperse PML bodies in fibroblasts. The results presented in the present study are in agreement with these observations and show, using the mutant virus, that essentially no loss of PMLs could be detected at early times (4 hpi) after infection with MCMVdie1. Experiments using HCMV or herpes simplex virus (HSV) with disruptions of the corresponding PML body dispersal products (IE1 and ICP0, respectively) together with experiments that employ cell lines that either do not express or overexpress PML suggest that active IE1/ICP0 is needed to counteract a repressive action of PML bodies, particularly under low-MOI conditions (references 2, 9, 18, and 48 and references therein). Thus, the MOI-dependent phenotype of the IE1-defective HCMV was interpreted as a threshold effect resulting from the titration of the cellular repression mechanism by an increasing viral genome load. In the case of MCMV, our findings that a

mutant MCMV lacking IE1 has replication kinetics similar to wild-type MCMV independently of the viral input indicates that, regardless of IE1 levels, MCMV overcomes the lack of PML disruption early during infection. A quantitative analysis of the number of PML bodies per nucleus at 15 h and, in particular, at 24 h after MCMVdie1 infection indicates that indeed a significant number of these are dispersed. This observation suggests that when MCMV infection progresses, a second, IE1-independent, mechanism becomes available that is capable of disrupting PML bodies. At these late times, immunofluorescence staining showed increased levels of PML distributed over the cytoplasm, in small scattered foci in the nucleus, and accumulating in the viral replication compartments outlined by E1 staining. These increased PML levels are in agreement with previous observations (63) shown by Western blotting that PML is not destroyed by MCMV infection as it is in HSV infections but is instead present in increasing levels during MCMV infection. Altogether, our findings with cultured cells confirm those of Tang and Maul (63) in that MCMV IE1 plays a primary role in the dispersion of PML bodies, extending them to the context of the viral infection and identifying an IE1-independent mechanism for MCMV-induced PML disruption. This mechanism does not degrade PML but prevents the formation of PML bodies, probably by affecting the sumoylation status of PML (49). Furthermore, we demonstrate that lack of IE1 and immediate disruption of PML bodies after infection does not limit the replication potential of MCMVdie1 in cultured cells.

We cannot discard the possibility that the processes discussed above might have a role in vivo and may determine in part the attenuated phenotype linked to MCMVdie1 in the mouse model. It is known that interferon increases the size and number of PML bodies in cultured cells. Under low-MOI conditions, HSV infection does not progress after interferon treatment, except in those rare cells in which the PML bodies are dispersed (55, 64). In fact, interferon has been shown to repress HSV infection in part via a PML-dependent pathway (12, 13). It may be that in an in vivo situation, initial MCMV infection induces an interferon response that increases the levels of PML in surrounding cells. When these cells are then infected, the increased PML levels may take longer to be overcome by the IE1-independent mechanism, thus delaying the effect of the infection.

Whether MCMV IE1 is involved in other activities that have been attributed to HCMV IE1, such as altering the cell cycle or preventing apoptosis in infected cells, remains a topic for future studies. However, along the course of our experiments, we did not detect major alterations in cells infected with MCMVdie1 that could be related to these processes. In this sense, it must be noted that other CMV products have been described as having antiapoptotic properties and disturbing the cell cycle under different settings (7, 23, 44, 69, 71). This functional redundancy most likely reflects the various strategies developed by the virus to adapt to the different conditions encountered in vivo.

Phenotype of MCMVdie1 during infection in vivo. Despite the absence of a phenotype for MCMVdie1 in tissue culture cells, we found that IE1 functions are important for viral replication in a natural setting. First, we detected markedly reduced viral titers during the acute phase of infection of the *ie*1-defective virus in most of the organs examined in immunocompetent and immunodeficient mice compared to animals infected with parental MCMV. Secondly, in the SCID model of virulence, we observed a substantial delay in mortality of approximately 35 days among animals infected with MCMVdie1 compared to those inoculated with control viruses. The fact that a revertant from MCMVdie1 and the parental wild type grew equally well in both types of mice and displayed similar virulence degrees in the SCID mice excludes the possibility that alterations in MCMVdie1 other than deletion of exon 4 of the *ie*1 gene could account for the debilitated phenotype of this virus in vivo. Thus, loss of IE1 clearly leads to reduced MCMV disease, but IE1 is not absolutely required for MCMV multiplication in vivo. Beyond question, the *ie*1-defective mutant was still virulent in that virus could be detected in all of the organs analyzed from the infected mice and no SCID mice survived longer than 63 days after infection with MCMVdie1. This is in marked contrast to the completely avirulent phenotype found for the enhancerless MCMV (22), which most likely reflects the results of low levels of both IE1 and IE3, a gene product shown to be absolutely essential for early and late MCMV protein synthesis, in functionally significant amounts.

The reason for the attenuated phenotype of MCMV in the absence of IE1 is unclear at present. It must be noted that in MCMV, the IE1 protein provides the major immunodominant CTL target, with the ^{168}YPHFMPTNL^{176} nonapeptide being the dominant CTL epitope. Thus, a plausible outcome of removing the IE1 epitope could have been that MCMVdie1 would replicate more robustly in the BALB/c mice. However, in this connection, studies with different viral systems have shown that, in the absence of an immunodominant viral epitope, subdominant epitopes are unveiled, thereby maintaining an effective and complete immune control. As an example, a peptide derived from the m164 protein has been reported to play an immunodominant role during the early and late phases of the MCMV replication cycle (28). Importantly, the reduced growth of MCMVdie1 was detected shortly after infection in primary target organs, such as the liver and spleen. It is possible that the mutant virus is partially impaired per se to enter or replicate in certain cell types at primary sites in infected animals, thereby limiting the levels of progeny virus available for infection of secondary target cells. Although the growth kinetics of MCMVdie1 are not altered in a variety of cultured cells, we cannot exclude a defect in replication in other cell types not analyzed in vitro (i.e., hepatocytes) or subjected to specific conditions, such as in different growth, activation, or differentiation states. A decreased ability of this mutant to spread from the site of inoculation to the visceral organs or from those to other secondary organs or tissues might also contribute to the MCMVdie1 phenotype observed in the mouse. However, in this sense, the mutant virus exhibited kinetics similar to and reached titers identical to those of the corresponding controls in peritoneal macrophages extracted from BALB/c mice. Alternatively or simultaneously, a specific virus-host interaction-related phenomenon could be influencing the reduced viral growth of MCMVdie1. IE1 could be interfering with some early immune innate response. The defective growth and significant attenuation of MCMVdie1 even in the absence of B, CTL, and NK (Ly49H) responses in the

SCID mouse model might indicate that IE1 targets the host's interferon responses. Thus, MCMV could be interfering with the interferon-induced antiviral state by means of its more predominantly expressed IE protein and favoring in this manner its replication.

Whether, HCMV IE1 behaves in a fashion similar to that of MCMV IE1 during the infection of its host remains an open question. But based on the poor growth displayed by the defective *ie*1 mutant at low multiplicities in vitro, one should predict that, in the absence of IE1, HCMV would be more severely affected than MCMV, resulting in a highly attenuated or even completely avirulent phenotype in its host.

Latency and reactivation from latency are key aspects of CMV pathogenesis. At the molecular level, these events have been suggested to be determined by the activity of IE gene products. Importantly, Kurz and Reddehase (37) have reported that reactivation is controlled at several checkpoints, the first of which is at the level of the MIEP. Moreover, they show that, in the lungs of latently infected mice, the *ie*1 gene is transcribed, while *ie*3-specific transcripts are not detected (38). The viability of MCMVdie1 in the mouse model allows an assessment of the role of IE1 in the latency reactivation cycle. It should be noted, however, that the generalized defect in replication of MCMVdie1 in the mouse might influence the final load of latent viral genomes in target organs (54). While it has been reported that there is not a straight correlation between the extent of CMV replication in organs during the primary infection and the levels to which the virus is able to establish latency and reactivate (5), the attenuated phenotype of MCMVdie1 must be taken into consideration when drawing conclusions about rates of reactivation.

In summary, the work presented here formally demonstrates that, without being an essential player, IE1 is pivotal in the replicative strategies of MCMV in the natural host. Further studies with this *ie*1-defective MCMV together with the generation of new recombinant viruses bearing specific mutations in regions of the IE1 protein and its subsequent evaluation in the mouse model will contribute in future work to a more complete understanding of IE1 activities in vivo.

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