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Construction and Characterization of Murine Cytomegaloviruses That Contain Transposon Insertions at Open Reading Frames m09 and M83

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A transposon derived from *Escherichia coli* Tn*3* was introduced into the genome of murine cytomegalovirus (MCMV) to generate a pool of viral mutants, including two recombinant viruses that contained the transposon sequence within open reading frames m09 and M83. Our studies provide the first direct evidence to suggest that m09 is not essential for viral replication in mouse NIH 3T3 cells. Studies in cultured cells and in both BALB/c-Byj and CB17 severe combined immunodeficient (SCID) mice indicated that the transposon insertion is stable during viral propagation both in vitro and in vivo. Moreover, the virus that contained the insertion mutation in m09 exhibited a titer similar to that of the wild-type virus in the salivary glands, lungs, livers, spleens, and kidneys of both the BALB/c and SCID mice and was as virulent as the wild-type virus in killing the SCID mice when these animals were intraperitoneally infected with these viruses. These results suggest that m09 is dispensable for viral growth in these organs and that the presence of the transposon sequence in the viral genome does not significantly affect viral replication in vivo. In contrast, the virus that contained the insertion mutation in M83 exhibited a titer of at least 60-fold lower than that of the wild-type virus in the organs of the SCID mice and was attenuated in killing the SCID mice. These results demonstrate the utility of using the Tn*3*-based system as a mutagenesis approach for studying the function of MCMV genes in both immunocompetent and immunodeficient animals.

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus which causes mild or subclinical diseases in immunocompetent adults but may lead to severe morbidity or mortality in neonates and immunocompromised individuals (2, 24). Disseminated HCMV infection, common in AIDS patients and organ transplant recipients, is usually associated with gastroenteritis, pneumonia, and retinitis (12, 29). Studies on the functions of viral genes in HCMV replication in vivo are essential for understanding viral pathogenesis and developing new strategies to combat the viral infection. However, there are currently no suitable animal models for HCMV infection. HCMV only propagates in human cells and grows slowly due to a long lytic replication cycle (24). These properties of HCMV have hampered the studies of HCMV pathogenesis and gene function.

Infection of the mouse with murine CMV (MCMV) provides a valuable in vivo model for studying the biology of CMV infection. This is because infection of mice by MCMV resembles in many ways its human counterpart with respect to pathogenesis during acute infection, establishment of latency, and reactivation after immunosuppression, transfusion, or transplantation (2, 15, 17, 24). Its genome of 230 kb is predicted to encode more than 170 open reading frames, 78 of which have extensive homology to those of HCMV (5, 32). However, many of these MCMV genes remained uncharacterized and their functions in viral pathogenesis have not been investigated.

One of the most powerful approaches to study the function of virus-encoded genes is to introduce mutations into the viral genome and to screen viral mutants in both tissue culture and animals for possible growth defects in vitro and in vivo. The construction of herpesvirus mutants was first reported using site-directed homologous recombination and then using transposon-mediated insertional mutagenesis (16, 25, 31, 34, 48). Methods using overlapping cosmid DNA fragments to generate mutants of HCMV and other herpesviruses have also been reported (7, 9, 18, 44, 45). More recently, the MCMV genome as well as the genomes of other herpesviruses have been cloned into a bacterial artificial chromosome (BAC), and MCMV mutants were successfully generated from the BAC-based viral genome by both site-directed homologous recombination and transposon-mediated insertional mutagenesis (3, 11, 23, 37, 41–43, 47). It has been shown that the BAC sequence in a BAC-based pseudorabies virus does not affect viral pathogenesis in vivo in animals (41, 42). Moreover, two different approaches to excise the BAC sequence from the viral genome have been described (42, 47). These studies have greatly facilitated the identification of the functions of viral genes in tissue culture and in animals.

We have recently applied a Tn*3* transposon-mediated shuttle mutagenesis system to generate MCMV mutants (49). In this approach, the transposon is inserted into a plasmid library of MCMV genomic DNA in *Escherichia coli*. Regions bearing an insertion mutation are then transferred to the MCMV genome by homologous recombination. In the study reported here, we have successfully used this approach to generate MCMV mutants containing transpositional insertions in open reading frames m09 and M83. Our results provide the first direct evidence to suggest that the m09 open reading frame is not essential for viral replication in NIH 3T3 cells. Studies in BALB/c-Byj mice and CB17 severe combined immunodeficient (SCID) mice indicated that m09 is dispensable for viral growth in the salivary glands, lungs, livers, spleens, and kidneys of these animals that were infected intraperitoneally with the

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viral mutant. Moreover, our data suggest that the presence of the transposon sequence in the viral genome does not significantly affect viral growth in both the BALB/c and SCID mice. These results demonstrate the feasibility of using this Tn*3*-based system to study the functions of MCMV genes in both immunocompetent and immunodeficient hosts.

MATERIALS AND METHODS

Cells and viruses. The wild-type Smith strain of MCMV was obtained from the American Tissue Culture Collection (ATCC) (Rockville, Md.). NIH 3T3 cells (ATCC CRL1658) were cultured in a humidified incubator at 37°C and in the presence of 5% CO_2. Cells were maintained in complete medium containing Dulbecco modified Eagle medium (DMEM) supplemented with 10% NuSerum (Becton Dickinson, Mass.). The Smith strain and the viral mutants, Rvm09 and RvM83, were propagated in NIH 3T3 cells as described previously (49).

Isolation of viral DNA and construction of a MCMV DNA subclone pool. NIH 3T3 cells were infected at a multiplicity of infection (MOI) of 0.1 PFU per cell. Approximately 5 to 7 days postinfection, almost all of the cells showed cytopathic effects (CPE) and were harvested. Viral particles and DNA were purified as described previously (6, 22). To generate the MCMV genomic pool, the DNA was partially digested with the restriction enzyme *Sau*3A. The first two nucleotides of the 3′ overhang sequence of the digested DNA fragments were then filled in with dGTP and dATP. The digested DNA fragments were separated on 0.8% agarose gels. DNA fragments in the size range of 1.6 to 4 kb were purified and cloned into pHSS6-*Sal*I (a gift from Michael Snyder of Yale University) (4, 14, 40).

Transposon shuttle mutagenesis to generate the MCMV DNA pool that contained the transposon sequence. The MCMV genomic fragment pool was first transformed into B211 {RDP146 [F$^-$ *recAI*(Δ*lac-pro*)*rpsE*; spectinomycin resistant] with plasmid pLB101} and colonies were selected on plates that contained kanamycin and chloramphenicol. These colonies were then mated with strain XZ95 which contained the transposon (Tn*3*-*gpt*) sequence (49). The Tn*3*-*gpt* construct was derived from a *E. coli* Tn*3* transposon and contained the *gpt* gene (49). The mixture was then allowed to grow on plates that contained tetracycline, kanamycin, and chloramphenicol for 2 days at 30°C. At this time, cointegration occurred. Finally, the cointegrates were resolved to generate the plasmid pHSS6::MCMV fragments containing a Tn*3* insertion by mating the bacteria with strain *E. coli* 70 (NG135 [K-12 *recA56 gal*-del*S165 strA*; streptomycin resistant] with plasmid pNG54). The plasmid DNA that contained the viral DNA fragments with a randomly inserted transposon was isolated and used to transform into *E. coli* DH5α for long-term storage.

In order to identify the genes that contained the transposon insertion and the orientation of the insertion relative to the open reading frame, plasmid DNAs that contained the mutated MCMV fragments were isolated. The junctions between the transposon and the viral DNA sequences were sequenced using the Sequenase sequencing system (Amersham, Inc., Arlington Heights, Ill.) with primer FL110PRIM (5′-GCAGGATCCTATCCATATGAC-3′).

Construction of recombinant MCMV. The transposon-MCMV DNA constructs were isolated and digested with *Not*I in order to release the genomic fragments containing the transposon (Fig. 1B). The excised fragments (1 to 3 μg) and full-length intact viral genomic DNA (Smith strain, 8 to 12 μg) were subsequently cotransfected into mouse NIH 3T3 fibroblasts using a calcium phosphate precipitation protocol (Gibco BRL, Grand Island, N.Y.). The recombinant virus was purified by six rounds of amplification and plaque purification in the presence of 25 μg of mycophenolic acid (Gibco BRL) and 50 μg of xanthine (Sigma, St. Louis, Mo.) per ml, as described previously (46, 49). For each cotransfection, several viral plaques were picked and expanded. Virus stocks were prepared by growing the viruses in T150 flasks of NIH 3T3 cells. In order to determine the location of the transposon insertion within the viral genome, the junctions of the transposition in the recombinant viral DNA were directly sequenced with primer FL110PRIM using the fmol Cycle Sequencing Kit (Promega, Inc., Madison, Wis.).

Southern and Northern analyses of recombinant viruses. Viral genomic DNA was isolated from NIH 3T3 cells as described previously (20–22). Briefly, cells that exhibited 100% CPE were washed with phosphate-buffered saline (PBS) and then subjected to proteolysis by a solution that contained sodium dodecyl sulfate and proteinase K. The genomic DNA was purified by extraction with phenol-chloroform, followed by precipitation with 2-propanol (22). Southern analyses were carried out to detect the presence of the transposon within the viral genome. Briefly, genomic DNA was digested with either *Hin*dIII, *Not*I, or *Eco*RI; separated on a 0.8% agarose gel; transferred to a Zeta-Probe nylon membrane (Bio-Rad, Hercules, Calif.); hybridized with the ^{32}P-radiolabeled DNA probes that contain the transposon and the MCMV sequences; and finally analyzed with a STORM840 phosphorimager (49). The labeled DNA probes were prepared by random primer synthesis (Boehringer Mannheim, Indianapolis, Ind.).

Cytoplasmic RNAs were isolated from MCMV-infected cells as described previously (21). Cells were infected with virus at an MOI of 10 and harvested at different time points postinfection. In the experiments to assay the expression of immediate-early transcripts, cells were pretreated with 100 μg of cycloheximide (Sigma) per ml and then infected with viruses and harvested 6 h postinfection. Viral RNAs were separated in a 1% agarose gel that contained formaldehyde, were transferred to a nitrocellulose membrane, were hybridized with the ^{32}P-radiolabeled DNA probes that contained the MCMV sequences, and were finally analyzed with a STORM840 phosphorimager. The DNA probes used for Northern analyses were generated by PCR using viral DNA as the templates and radiolabeled with a random primer synthesis kit in the presence of [α-^{32}P]dCTP (Boehringer Mannheim). The 5′ PCR primers used in the construction of DNA probes for the Northern analysis of m09, M83, and M25 transcripts were m09/1 (5′-TTCTGGCACCGTCACACCAG-3′), M83/1 (5′-AGACGTGTACGACGA GCAGG-3′), and M25/1 (5′-AATCCATCTCCGCATCCGAACCCTG-3′), respectively. The 3′ PCR primers used were m09/2 (5′-GGAGTTATCTTATGT GTAAT-3′), M83/2 (5′-AACGTGAAGTTGAACGGTTC-3′), and M25/2 (5′-C CTCAGACGGGATGCTCAATGGCTT-3′), respectively.

Growth kinetics of recombinant viruses. The analyses of the growth of the recombinant viruses in vitro were carried out as described previously (49). In brief, 5×10^5 NIH 3T3 cells were infected at an MOI of either 0.5 or 5.0 PFU per cell. The cells and medium were harvested at 0, 1, 2, 4, and 7 days postinfection, and viral stocks were prepared by adding an equal volume of 10% skim milk, followed by sonication. The titers of the viral stocks were determined by plaque assays in triplicate experiments.

Viral growth studies in animals. Three-week-old male BALB/c-Byj mice (Jackson Laboratory, Bar Harbor, Maine) or six-week-old CB17 SCID mice (National Cancer Institute, Bethesda, Md.) were infected intraperitoneally with 10^3 to 10^4 PFU of each virus. The animals were sacrificed at different time points (e.g., 3 days) postinoculation as specified in Results. For each time point, at least three animals were used as a group and infected with the same virus. The whole salivary gland, about 0.1 to 0.2 g of the liver, one-quarter of the lungs, the whole spleen, and one of the kidneys were collected individually into 3-ml sterile tubes. To avoid cross-contamination of viruses between organs and different recombinants, surgical tools (forceps and scissors) were rinsed once in PBS, rinsed three times in 70% ethanol, and flamed after each rinse in ethanol. Each sample was suspended in a mixture of DMEM and 10% skim milk (50% [vol/vol]) at 0.1 g/ml. The organs were then sonicated on ice using a 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, Pa.) until the organ became homogenized. The samples were stored at −80°C until titers of the viruses in these samples were determined.

Titers of viruses harvested from the mice were determined on NIH 3T3 cells in six-well tissue culture plates (Corning, Inc., Corning, N.Y.). Briefly, cells were first split 1:30 from T150 flasks into six-well plates and cultured overnight (16 to 24 h) and then infected with the viruses at 10-fold serial dilutions. After 2 h of incubation with the homogenates diluted in 1 ml of complete medium at 37°C with 5% CO_2, the cells were overlaid with fresh complete medium containing 1% agarose and cultured for 4 to 5 days before the plaques were counted under an inverted microscope. Virus titers were recorded as the PFU/milliliter of organ homogenates. The titer of each sample was determined in triplicate. The limit of virus detection in the organ homogenates was 10 PFU/ml of the sonicated mixture. Those samples that were negative at a 10^{-1} dilution were given a titer value of 10 (10^1) PFU/ml.

To determine the mortality of the animals infected with the Smith strain, Rvm09, and RvM83, the CB17 SCID mice (five animals per group) were infected intraperitoneally with 10^4 PFU of each virus. The mortality of the infected animals was monitored for at least 46 days postinfection, and the survival rates were determined.

RESULTS

Isolation of MCMV mutants containing the transposon insertions at open reading frames m09 and M83. We recently constructed an *E. coli* Tn*3*-based transposon (Fig. 1A) which contained the expression cassette encoding guanine phosphoribosyltransferase (i.e., *gpt*) and, in addition, a transcription termination site (49). The *gpt* gene was used as a selectable marker in the construction of MCMV recombinant viruses (46). The *gpt* expression cassette was inserted such that its transcription termination site functioned in the opposite direction as the other poly(A) signal presented in the transposon (Fig. 1A). Such a design would assure that the transcription of the targeted gene is truncated without altering the expression of nearby genes that may share a common poly(A) signal with the disrupted gene. To generate a pool of MCMV DNA fragments that contained a randomly inserted transposon, virus DNA was first purified and partially digested with *Sau*3A. Digested fragments in the size range of 1.6 to 4 kb were then cloned into vector pHSS6-*Sal*I (4, 14, 40) to generate a MCMV genomic library. The transposon was introduced into this MCMV DNA library through a shuttle mutagenesis protocol, as described previously (see Materials and Methods) (49), to

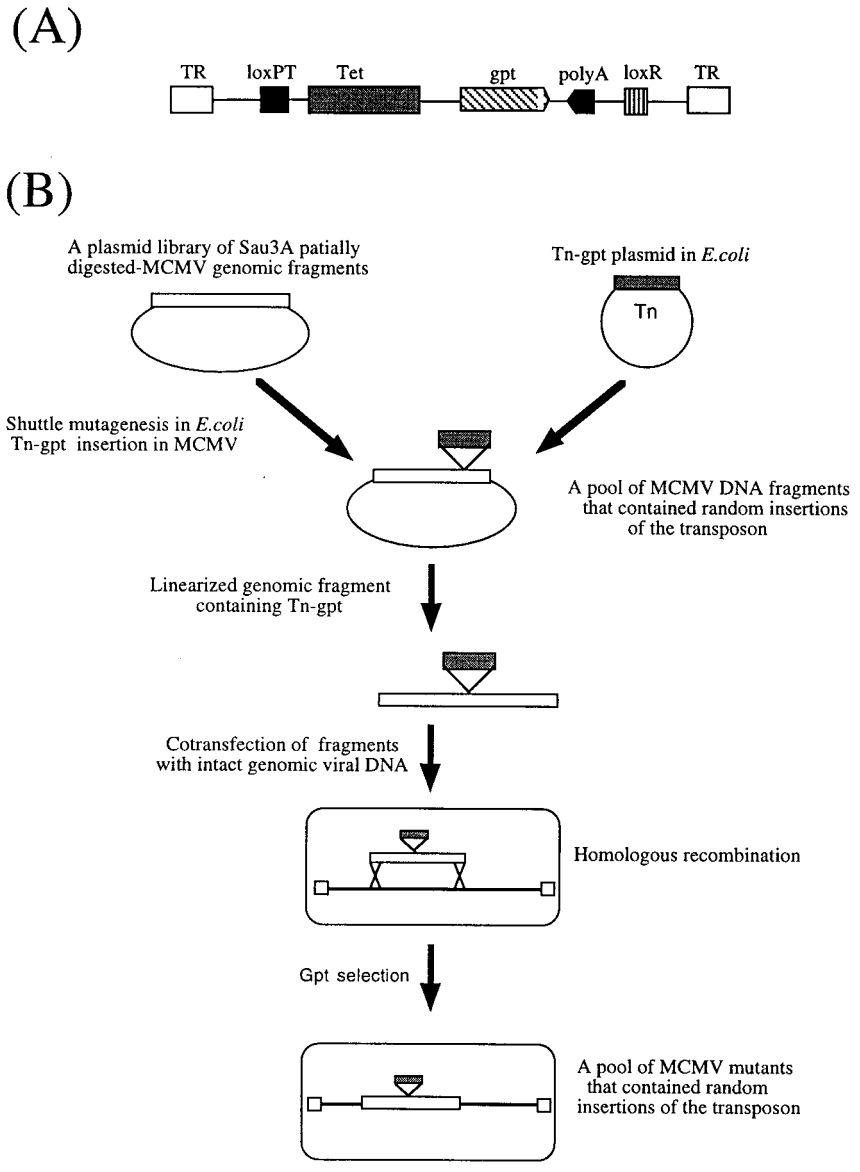

FIG. 1. Schematic representation of the structure of the transposon construct used for mutagenesis (A) and the procedure for the construction of MCMV mutants that contained random transposon insertions (B). TR, terminal repeat; Tet, tetracycline resistance gene; gpt, gene that encodes guanine phosphoribosyltransferase (*gpt*); poly(A), transcription termination signal.

generate a pool of MCMV genomic sequences that contained the transposon sequence (Fig. 1B).

To generate a pool of MCMV mutants that contained the transposon randomly inserted at the viral genome, the pool of MCMV genomic fragments which contained a transpositional insertion were cotransfected with the full-length genomic DNA of the wild-type virus (Smith strain) into mouse NIH 3T3 cells to allow homologous recombination to occur. The cells that harbored the progeny viruses were allowed to grow in the presence of mycophenolic acid and xanthine, which selects for *gpt* expression (27, 46). The recombinant viruses that contained the transposon and expressed the *gpt* protein were isolated after multiple rounds of selection and plaque purification. Two of the recombinant viruses generated were further characterized and are reported here. These viruses, designated Rvm09 and RvM83, contained the transposon within open reading frames m09 and M83, respectively (Fig. 2). The locations of the transposon sequence in the viral genome were determined by direct sequencing of the genomic DNA of the recombinant viruses. Sequence analyses of the junction between the transposon and the viral sequence revealed that the locations of the transposon in Rvm09 and RvM83 were at nucleotide positions 8683 (m09), and 118237 (M83), respectively, in reference to the genome sequence of the wild-type Smith strain (32) (Fig. 2A, data not shown).

In vitro characterization of MCMV mutants in tissue culture. Southern hybridization analyses with DNA probes containing the transposon and the viral sequence were used to examine the genomic structure of the recombinant viruses and determine if the mutants contained the transposon insertion (Fig. 2B). Analysis of the *Hin*dIII-digested DNA of each recombinant virus clearly showed a small fragment of 1.8 kb

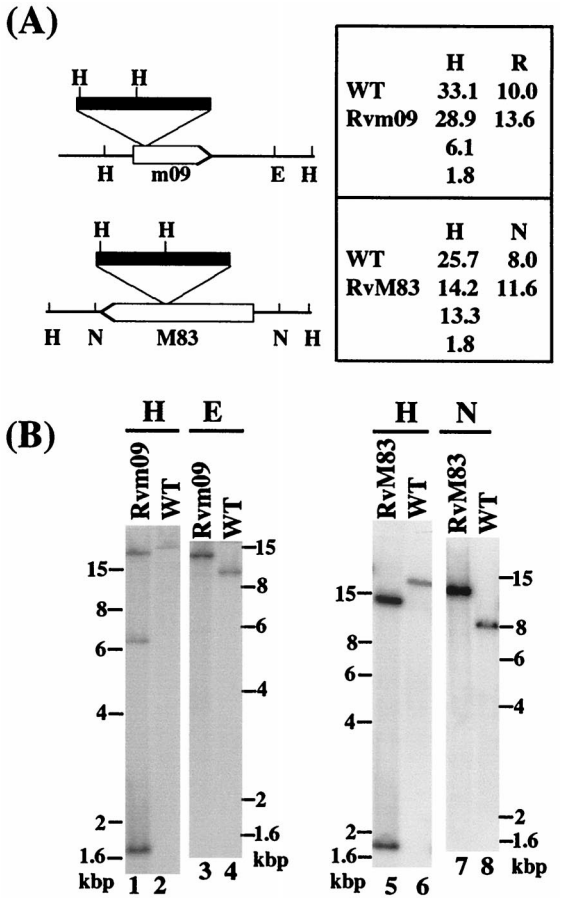

FIG. 2. (A) The locations of the transposon insertions in the recombinant viruses. The transposon sequence is shown as a filled bar, while the coding sequence of each open reading frame is represented by an open arrow. The orientation of the arrow represents the direction of the translation and transcription predicted based on the nucleotide sequence (32). The numbers represent the sizes of the DNA fragments of the mutant viruses that contained the transposon sequence and were generated by digestion with *Hin*dIII (H), *Not*I (N), or *Eco*RI (E). (B) Southern blot analyses of the viral mutants. The DNA fractions were isolated from cells infected with the wild-type (WT) virus and different MCMV mutants. The DNA samples (20 μg) were digested with *Hin*dIII (H), *Not*I (N), or *Eco*RI (E); separated on 0.8% agarose gels; transferred to a Zeta-Probe membrane; and hybridized to a DNA probe. The probes used for the analyses were the plasmids that contained the MCMV DNA fragments inserted with the transposon sequence.

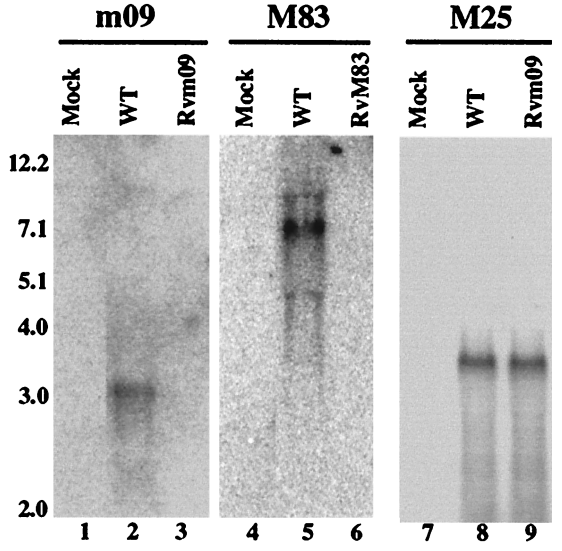

FIG. 3. Northern blot analyses of the RNA fractions isolated from cells that were mock infected (lanes 1, 4, and 7) or infected with the wild-type virus (WT) (lanes 2, 5, and 8) and the mutant viruses (lanes 3, 6, and 9). A total of 10^7 NIH 3T3 cells were infected with each virus at an MOI of 10 PFU/cell, and cells were harvested 24 h postinfection. Equal amounts of RNA samples (30 μg) were separated on agarose gels that contained formaldehyde, transferred to a nitrocellulose membrane, and hybridized to a ^{32}P-radiolabeled probe that contained the sequence of m09 (lanes 1 to 3), M83 (lanes 4 to 6), or M25 (lanes 7 to 9).

representing the *gpt* gene fragment, indicating the presence of the transposon sequence within the viral genome (Fig. 2B, lanes 1 and 5). This conclusion is further supported by the results of Southern analyses of the mutant DNAs digested with another restriction enzyme (i.e., *Eco*RI for Rvm09 and *Not*I for RvM83) (lanes 3 and 4 and lanes 7 and 8). In each case, the genomic fragment from the transposon-containing viruses should be bigger than that of the wild-type virus by 3.6 kb, which is the size of the transposon. The stocks of these recombinant viruses appeared to be pure and free of the wild-type strain, since the hybridizing DNA fragments from the mutants did not comigrate with those of the wild-type Smith strain (Fig. 2B, lanes 1 to 8). For example, the hybridization patterns of the Rvm09 and Smith strain DNAs digested with *Hin*dIII gave rise to three DNA bands (28.9, 6.1, and 1.8 kb) and one DNA band (33.1 kb), respectively (Fig. 2B, lanes 1 and 2). Meanwhile, the hybridized species (13.6 kb) of the *Eco*RI-digested Rvm09 DNA migrated differently from that (10 kb) of the wild-type viral DNA digested with the same enzyme (Fig. 2B, lanes 3 and 4). The sizes of the hybridized DNA fragments (Fig. 2B) were consistent with the predicted digestion patterns of the recombinant viruses based on the MCMV genomic sequence (32) and the location of the transposon insertion in the viral genome as determined by sequence analysis (Fig. 2A). The restriction enzyme digestion patterns of the regions of the mutant genomic DNAs other than the transposon insertional site appeared to be identical to those of the parental Smith strain, as indicated by ethidium bromide staining of the digested DNAs (data not shown). This observation suggested that regions of the viral genome other than those containing the transposon insertion remained intact in these MCMV mutants.

It is expected that the transcription of the targeted genes would be disrupted due to the presence of the two transcription termination signals within the transposon. Specifically, the regions of the open reading frames downstream from the transposon insertion site will not be transcribed due to the presence of these termination signals. To determine whether this is the case, cytoplasmic RNAs were isolated from cells infected with the mutant viruses at different time points (e.g., 4, 12, and 24 h) postinfection. Northern analysis was carried out to examine the expression of the transcripts from the m09 and M83 regions downstream from the transposon insertion site (Fig. 3). In the two mutant viruses, the transposon was found to insert in the 5′-coding region of m09 and the central region of M83, respectively (Fig. 2A). The probes used in the Northern analyses contained the DNA sequences complementary to the m09 and M83 coding region about 100 nucleotides downstream from the site of the transposon insertion. Substantial amounts of transcripts from m09 and M83 open reading frames were found in RNA fractions isolated from cells infected with the parental Smith strain (Fig. 3, lanes 2 and 5). The size of the single transcript from m09 was ca. 3 kb (lane 2) and is consistent with the length of this open reading frame (293 amino acids) (32). RNA transcripts of about 5, 7 to 7.5,

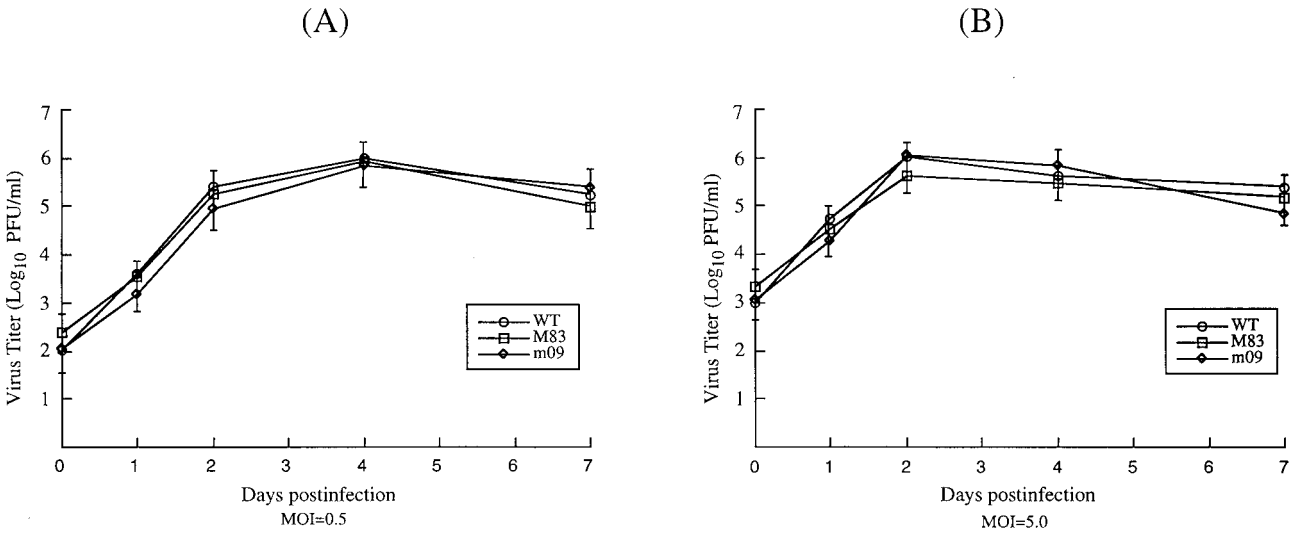

FIG. 4. In vitro growth of MCMV mutants in tissue culture. Mouse NIH 3T3 cells were infected with each virus at an MOI of either 0.5 PFU (A) or 5 PFU (B) per cell. At 0, 1, 2, 4, and 7 days postinfection, cells and culture media were harvested and sonicated. The viral titers were determined by plaque assays on NIH 3T3 cells. The values of the viral titer represent the average obtained from triplicate experiments. The standard deviation is indicated by the error bars.

and 10 kb were detected in the M83 region (lane 5) and are consistent with the previous observations (8). However, these transcripts were not detected in RNA fractions isolated from cells infected with Rvm09 and RvM83 when the same probes were used (Fig. 3, lanes 3 and 6). The level of MCMV M25 transcript (10, 49) was used as the internal control for the expression of these transcripts. As an example shown in Fig. 3 (lanes 7 to 9), the level of M25 transcript detected in cells that were infected with Rvm09 was found to be similar to that of M25 transcript in cells infected with the Smith strain (Fig. 3, lanes 8 and 9). Similar results were also obtained when the level of the M25 transcript was used as the internal control for the expression of the M83 transcripts from RvM83 (data not shown). Thus, the insertions truncated or disrupted the transcripts expressed from these open reading frames.

To determine whether the recombinant viruses had any growth defects in vitro, NIH 3T3 cells were infected with these viruses at both low and high MOIs. The growth rates of these viruses in mouse NIH 3T3 cells were assayed and compared to those of the parental Smith strain. These results, obtained from triplicate experiments, are shown in Fig. 4 and indicate that the peak titers of Rvm09 and RvM83 were similar to that of the parental Smith strain. The fact that these viruses did not exhibit significant growth defects is consistent with the previous observations that M83 is dispensable for MCMV replication in NIH 3T3 cells (26). Moreover, these results, combined with those from the Southern analyses, suggest that m09 is not essential for viral growth in tissue culture.

Replication of the transposon-containing viral mutants in immunocompetent animals. In order to use the Tn*3*-based transposon system to generate MCMV mutants and study the phenotypes of the mutants in vivo, it is necessary to determine whether the presence of the transposon sequence in the viral genome does not significantly affect viral replication in animals. To determine accurately the capability of the viral mutants to grow in animals, a low dose of viruses was used for inoculation. BALB/c-Byj mice were injected intraperitoneally with 10^3 PFU of Rvm09, RvM83, and Smith strain. At 1, 3, 7, and 14 days postinfection, salivary glands, lungs, spleens, livers, and kidneys were harvested, and the virus titers in these five organs were determined. These organs are among the major targets for MCMV infection (2, 15, 17, 24). At 14 days postinfection, the titers of Rvm09 in the salivary glands were similar to those of the Smith strain, while a reduction of 500-fold in the virus titer was found in the salivary glands of the RvM83-infected animals (Fig. 5). At 7 days postinfection, the titers of Rvm09 in the lungs, spleens, livers, and kidneys were also similar to those of the Smith strain. In contrast, a reduction of at least 20-fold in the virus titer was found in the lungs, spleens, livers, and kidneys of the animals infected with Rvm83 (Fig. 5). These results are consistent with previous observations that a MCMV mutant with a deletion at M83 was attenuated in replication in these organs (26). Since Rvm09 replicated as equally well as the Smith strain in all the organs examined, the presence of the transposon sequence per se within the viral genome appears to have no significant effect on viral replication in BALB/c mice, at least in these organs. These results further suggest that the m09 open reading frame is dispensable for viral replication in these organs of BALB/c mice.

Replication of the transposon-containing viral mutants in immunodeficient animals. Previously studies have indicated that immunodeficient animals are extremely susceptible to MCMV infection (13, 28, 30, 33). For example, the CB17 SCID mice, which lacks functional T and B lymphocytes, are sensitive to an extremely low level of viral replication since these animals succumb to as little as 10 PFU of MCMV (28, 30). These animals have served as an excellent model in determining the virulence of different MCMV strains and mutants and in studying the mechanism of how they cause opportunistic infections in immunocompromised hosts. In order to use the Tn*3*-based transposon system to generate MCMV mutants and study the phenotypes of the mutants in vivo in an immunodeficient host, it is also necessary to determine whether the presence of the transposon sequence in the viral genome does not significantly affect viral replication in these animals. Two sets of experiments with the SCID mice were carried out to address this issue. First, the survival rates of the animals infected with Rvm09 and RvM83 were determined and compared with those infected with the Smith strain. The SCID mice (five animals per group) were injected intraperitoneally with 10^4 PFU of

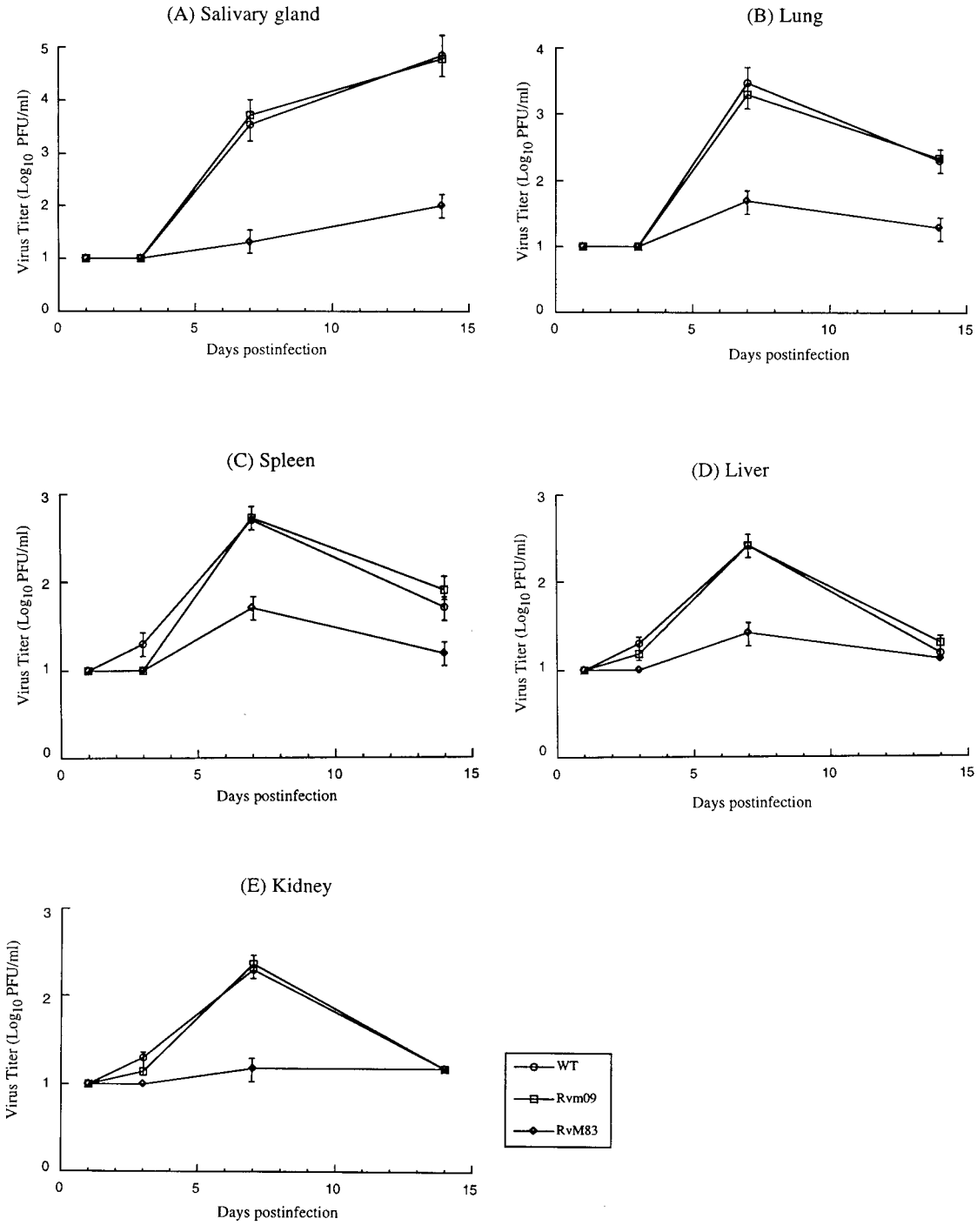

FIG. 5. Titers of MCMV mutants in salivary glands (A), lungs (B), spleens (C), livers (D), and kidneys (E) of the infected BALB/c mice. BALB/c-Byj mice were infected intraperitoneally with 10^3 PFU of each virus. At 1, 3, 7, and 14 days postinfection, the animals (three mice per group) were sacrificed. The salivary glands, lungs, spleens, livers, and kidneys were collected and sonicated. The viral titers in the tissue homogenates were determined by standard plaque assays in NIH 3T3 cells. The limit of detection was 10 PFU/ml of the tissue homogenate. The viral titers represent the average obtained from triplicate experiments. The error bars indicate the standard deviation. Error bars that are not evident indicate that the standard deviation was less than or equal to the height of the symbols.

Rvm09, RvM83, and Smith strain. As shown in Fig. 6, the survival rates of the animals infected with Rvm09 were similar to those of the animals infected with the Smith strain. Half of the animals infected with Rvm09 and the Smith strain died within 22 and 23 days postinfection, respectively. In contrast, no animals infected with RvM83 were found dead until 40 days postinfection.

To further study the pathogenesis of the mutant viruses in these immunodeficient animals, the replication of the viral mutants in different organs of the animals was studied during

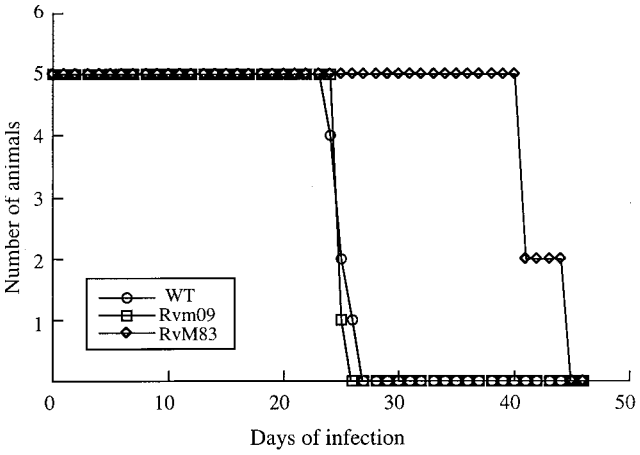

FIG. 6. Mortality of the SCID mice infected with the Smith strain, Rvm09, and RvM83. CB17 SCID mice (five animals per group) were infected intraperitoneally with 10^4 PFU of each virus. Mortality of mice was monitored for 46 days postinfection, and survival rates were determined.

a 21-day infection period before the mortality of the animals infected with Smith strain became apparent. SCID mice (three animals per group) were injected intraperitoneally with 10^4 PFU of Rvm09, RvM83, and Smith strain. At 1, 3, 7, 10, 14, and 21 days postinfection, salivary glands, lungs, spleens, livers, and kidneys were harvested, and the viral titers in these five organs were determined. During the 21-day infection period, the titers of Rvm09 in the five organs were not significantly different from those of the Smith strain (Fig. 7). In contrast, at 21 days postinfection, the titers of RvM83 found in the salivary glands, lungs, spleens, and livers of the infected animals were 5,000-, 500-, 100-, and 60-fold less than the titers of the Smith strain found in the same organs from the infected animals, respectively (Fig. 7). No viruses were detected in the kidneys of the animals infected with RvM83, while a viral titer of about 10^4 PFU/ml was found in those of the animals infected with the Smith strain and Rvm09. The titers of RvM83 in the organs of the infected SCID mice at 39 days postinfection are similar to those of the wild-type virus in the infected animals at 21 days postinfection (data not shown). These results suggest that RvM83 is attenuated and its infection exhibits delayed pathogenesis in SCID mice. It is possible that the observed results with RvM83 are due to a second mutation rather than the actual transposon insertion at M83. To address this issue, another viral mutant with a transposon insertion at M83 was isolated independently from RvM83. The titers of this second mutant in the organs of the infected animals are similar to those of RvM83 (J. Xiao, X. Zhan, E. Haghjoo, and F. Liu, unpublished results). Thus, it is unlikely that a second mutation is responsible for the results with RvM83, although we cannot completely rule out this possibility. Because Rvm09 replicated as well as the Smith strain in all of the organs examined, the presence of the transposon sequence per se within the viral genome appears to have no significant effect on viral replication in vivo in these animals, at least not in these organs. Together, these results suggest that the m09 open reading frame is dispensable for viral replication in these organs of the SCID mice. Moreover, these observations suggest that M83 is important for viral replication in immunodeficient animals.

Stability of the transposon mutations in the recombinant viruses. Our previous studies have indicated that a transposon sequence inserted at the 3′-terminal region of the MCMV genome (i.e., the m155 open reading frame) is stable during viral replication in NIH 3T3 cells and in BALB/c mice (49). However, it is not known whether the transposon sequence inserted at the 5′-terminal sequence of the viral genome is also stable during viral propagation. Equally unclear is whether the insertional mutation is stable during viral propagation in immunodeficient animals, in which the viral replication may achieve a higher level than that in immunocompetent mice. To address these issues, two sets of experiments were carried out. First, recombinant virus Rvm09 and RvM83 were used to infect NIH 3T3 cells at an MOI of <0.01 and allowed to grow for five generations (60 days) in the absence of *gpt* selection. Second, 10^4 PFU of viruses were used to infect both the BALB/c and SCID mice. At 14 days postinfection, salivary glands and lungs were harvested from the infected animals and sonicated to release the virus. Viruses were recovered by infecting NIH 3T3 cells with the sonicated tissue homogenates. Viral DNAs were purified from the infected cells, and their restriction digestion patterns were analyzed in agarose gels. Figure 8 shows a Southern analysis of the Rvm09 viral DNAs with a DNA probe that contained the transposon and m09 open reading frame sequence. These results indicated that no change in the hybridization patterns of Rvm09 occurred as a result of viral growth for five generations (60 days) in cultured cells (Fig. 8, lane 3) or in animals for 14 days (lanes 4 to 7). Moreover, the overall *Hin*dIII-digestion patterns of Rvm09 DNA isolated from either infected cultured cells or animals were identical to those of the original recombinant virus, as visualized by ethidium bromide staining of the viral DNAs (data not shown). Similar results were also observed in experiments with RvM83 (data not shown). Thus, the transposon insertion in Rvm09 and RvM83 appeared to be stable in tissue culture and in both BALB/c and SCID mice.

DISCUSSION

Analyses of herpesviral genome by transposon-mediated insertional mutagenesis. Transposon-mediated mutagenesis has been widely used to study gene function in viruses, bacteria, yeast, and mammalian cells. Previously, Tn*5* and phage-mu-based transposons have been used in the genetic analysis of herpes simplex virus 1 (34, 48). More recently, transposons derived from Tn*1721* and Tn*5* have also been used in the mutagenesis of the BAC-based genomes of MCMV and pseudorabies virus, respectively (3, 41). The system we used is based on an *E. coli* Tn*3* transposon which has been employed in the mutagenesis studies of the genomes of *Saccharomyces cerevisiae* and other organisms such as *Salmonella enterica* serovar Typhimurium and *Neisseria gonorrhoeae* (35, 36, 39, 40). One of the major advantages of the Tn*3* system is its transposition immunity. A plasmid already containing a copy of Tn*3* is immune to further insertions of the transposon. This immunity is due to the presence of a 38-nucleotide sequence, which is also found in the *E. coli* chromosome (19). Therefore, Tn*3* mutagenesis is simple, is straightforward, and yields little background since most of the transposition occurs in the target sequence (e.g., MCMV DNA) rather than in the *E. coli* chromosome sequence (14). In this study, the constructed Tn*3*-*gpt* transposon efficiently transposed into the MCMV DNA fragments, and viral mutants that contained transposon mutations at open reading frames m09 and M83 were successfully constructed. Moreover, preliminary characterization of 20 different mutants among a library of ca. 500 isolates revealed that most of these mutants contained the transposon insertion at different locations of the genome (data not shown). These results further demonstrate that the Tn*3* system can be used

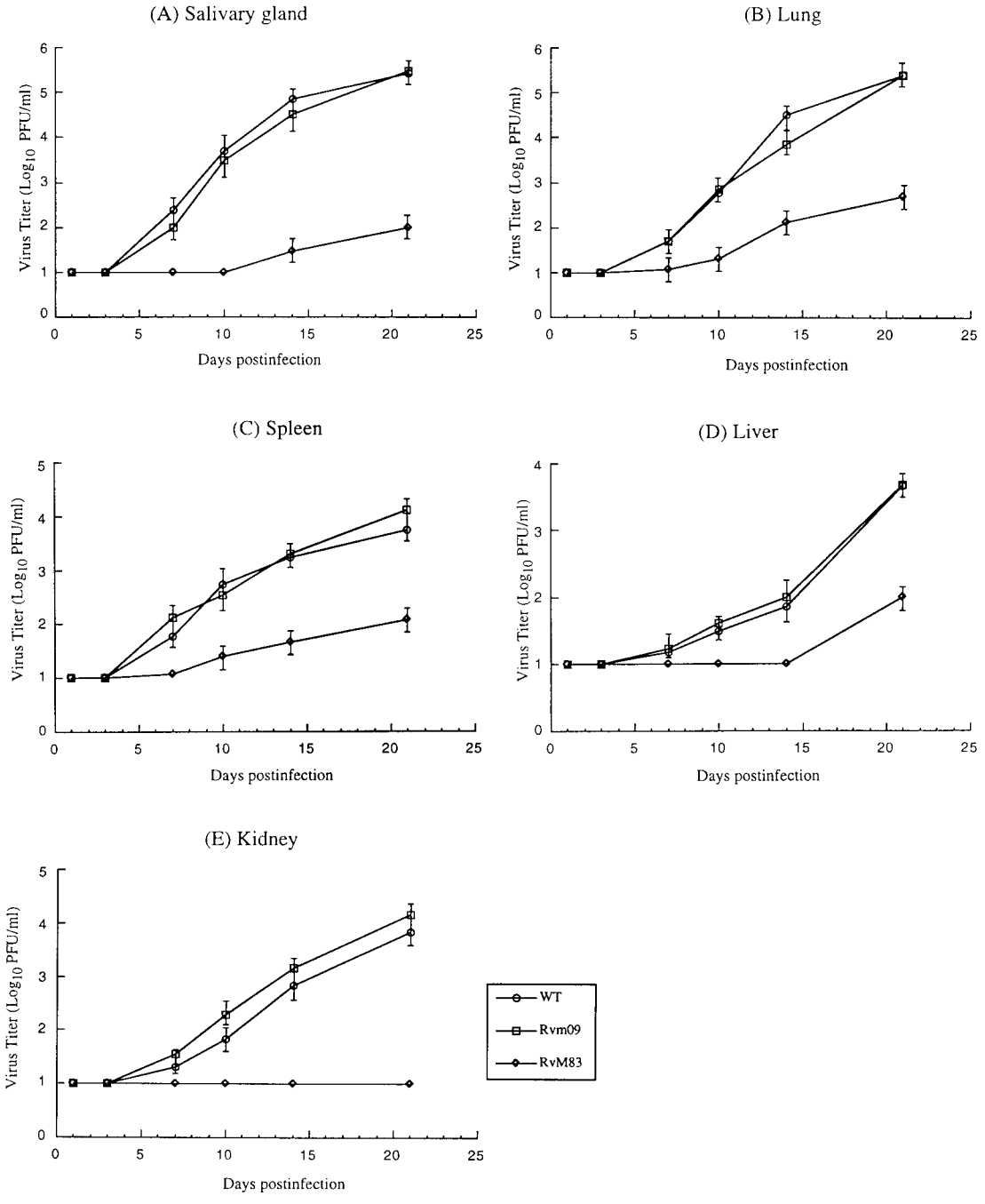

FIG. 7. Titers of MCMV mutants in the salivary glands (A), lungs (B), spleens (C), livers (D), and kidneys (E) of the infected SCID mice. CB17 SCID mice were infected intraperitoneally with 10^4 PFU of each virus. At 1, 3, 7, 10, 14, and 21 days postinfection, the animals (three mice per group) were sacrificed. The salivary glands, lungs, spleens, livers, and kidneys were collected and sonicated. The viral titers in the tissue homogenates were determined by standard plaque assays in NIH 3T3 cells. The limit of detection was 10 PFU/ml of the tissue homogenate. The viral titers represent the average obtained from triplicate experiments. The error bars indicate the standard deviation. Error bars that are not evident indicate that the standard deviation was less than or equal to the height of the symbols.

for simultaneous isolation of multiple viral mutants and may have advantages over traditional homologous recombination system for systematic generation of viral mutants.

In order to identify the function of virus-encoded genes, we intend to introduce mutations into the viral genome using the Tn*3*-based transposon and to screen viral mutants in both tissue culture and animals for possible growth defects in vitro and in vivo. However, several criteria must be satisfied if the Tn*3*-based transposon is to be used as a mutagenesis tool to generate MCMV mutants for studies of viral replication in vitro and in vivo. This includes the stability of the transposon within the viral genome during viral replication in vitro and in vivo and its effect on viral pathogenesis in vivo. In our study, the transposon insertion in Rvm09 and RvM83 was stable during the replication of the viral mutant in vitro in NIH 3T3 cells and in vivo in both immunocompetent and immunodefi-

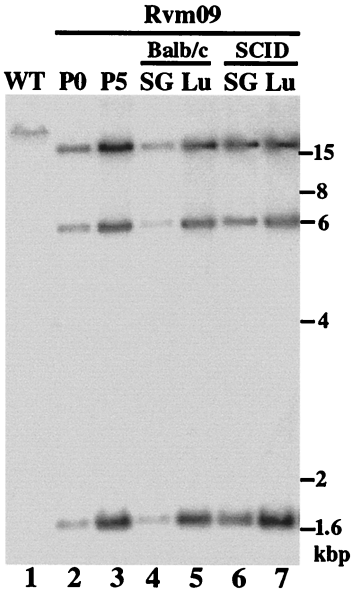

FIG. 8. The stability of the transposon mutations in tissue cultured cells and in BALB/c and SCID mice. Viral DNAs were either isolated from cells that were infected with Rvm09 (MOI = <0.01) and allowed to grow in culture for 5 days (P0) (lane 2) or five generations (60 days) (P5) (lane 3) or from cells that were infected with the virus collected from the salivary glands (SG, lanes 4 and 6) and lungs (LU, lanes 5 and 7) of either BALB/c (BALB/c, lanes 4 and 5) or SCID mice (SCID, lanes 6 and 7) 14 days after intraperitoneal inoculation with 10^4 PFU of Rvm09. Southern blot analyses of the viral DNA fractions digested with *Hin*dIII are shown. The DNA of the wild-type virus (WT) is shown in lane 1. The ^{32}P-radiolabeled probe was derived from the same plasmid which was used for Southern analyses of Rvm09 in Fig. 2 and contained the transposon and the m09 open reading frame sequence.

cient animals (Fig. 8 and data not shown). The viral genome other than the transposon insertion region appeared to be intact in these MCMV mutants, as suggested by the results of the ethidium bromide staining of the digested viral DNAs (data not shown). These results strongly suggest that the transposon insertion in the constructed MCMV mutants is stable during viral replication in vitro and in vivo.

Our results also indicated that the transposon sequence does not significantly affect viral replication in vivo in both immunocompetent and immunodeficient animals. Viral mutant Rvm09, when used to infect BALB/c and SCID mice intraperitoneally, exhibited levels of replication in all five organs examined similar to those of the wild-type virus (Fig. 5 and 7). Moreover, the mortality rates of the SCID mice infected with this viral mutant were similar to those of the animals infected with the Smith strain (Fig. 6). These results suggest that the presence of the transposon sequence does not significantly affect viral virulence and pathogenesis. Thus, the Tn*3-gpt* system is suitable for genetic analyses of the functions of MCMV genes in vivo.

Compared to the BAC-based viral construct technology, the Tn*3-gpt* system requires time-consuming plaque purification of viral mutants and cannot be used for generating mutations in viral genes that are essential for growth in cell culture. The BAC-based mutagenesis approach provides a powerful and convenient strategy to generate MCMV mutants, especially those that contain mutations at the essential genes (3, 41). Meanwhile, the presence of the transposon sequence as well as the BAC sequence inserted at two different locations of the same viral genome may make it difficult to analyze the correlation between the functions of the genes disrupted by the transposon and the phenotypes observed in animals in vivo. Recently, it has been shown that the BAC sequence in a BAC-based pseudorabies virus does not affect viral pathogenesis in vivo in animals (41, 42). Moreover, two different approaches to excise the BAC sequence from the viral genome have been described (42, 47). These studies will further facilitate the development of the BAC-based mutagenesis methodology for the studies of viral gene functions in vivo.

Potential function of open reading frames m09 and M83 in viral replication. In this study, recombinant viruses that contained the insertional mutations at open reading frames m09 and M83 were generated. Both viruses were able to replicate in NIH 3T3 cells. These results are consistent with the previous observation that M83 is not essential for MCMV replication in vitro (26). Moreover, our results provide the first direct evidence to suggest that m09 is not essential for viral replication in NIH 3T3 cells in vitro.

While it is possible that the functional protein products might be synthesized from the transposon-disrupted regions, several lines of evidence strongly suggest that this is not the case. First, the transposon sequence was inserted into the 5′ region and the central region of the coding sequences of the m09 and M83 open reading frame, respectively (Fig. 2A). Second, the transcription from the regions downstream from the transposon insertion site was not detected in cells infected with the mutant viruses (Fig. 3). Thus, the regions of the target open reading frames downstream from the transposon insertion sites were not expressed, and the transcripts expressed from the disrupted open reading frames were truncated.

Open reading frame m09 belongs to MCMV m02 gene family and is believed to encode a membrane protein (32). However, neither the transcript nor the protein product coded by this open reading frame has been reported. Our results indicate that a transcript of about 3,000 nucleotides is expressed from the m09 open reading frame. The growth rate of Rvm09 in NIH 3T3 cells was not significantly different from that of the Smith strain. Since the transposon insertion is at the 5′ terminus of the open reading frame, most of the m09 coding sequence was not transcribed in Rvm09 and it is likely that no functional m09 protein was expressed from the viral mutant. Thus, our results suggest that m09 is not essential for viral replication in NIH 3T3 cells. Rvm09 appeared to replicate as well as the wild-type virus in the salivary glands, lungs, spleens, livers, and kidneys of both BALB/c and CB17 SCID mice that were infected intraperitoneally. These results suggest that m09 is not essential for viral growth in these organs in vivo.

M83 encodes a tegument protein and has been shown to be dispensable for viral replication in NIH 3T3 cells (8, 26). Its human counterpart, open reading frame UL83, encodes pp65, one of the most abundant tegument proteins and is dispensable for HCMV replication in tissue culture in vitro (1, 38). The function of UL83 in HCMV replication in vivo remains unknown. An MCMV mutant with a deletion in the M83 open reading frame was found to be attenuated in replication in the salivary glands, lungs, spleens, and livers of BALB/c mice that were infected intraperitoneally (26). However, whether the viral mutant is also attenuated in immunodeficient host such as the CB17 SCID mice has not been reported. Our results indicated that Rvm83 is attenuated in replication in the salivary glands, lungs, spleens, livers, and kidneys of BALB/c mice. Moreover, Rvm83 is attenuated in replicating in these five organs of the CB17 SCID mice and in killing of these immunodeficient animals. Similar results were also observed with another viral mutant that was isolated independently from RvM83 and also contained a transposon insertion at M83 (data not shown). Thus, it is unlikely that a second mutation rather

than the mutation at M83 is responsible for the observed results with RvM83, although we cannot completely rule out this possibility. Since the transposon sequence per se in the viral genome does not significantly affect viral replication in these organs of SCID mice (as in the case of Rvm09-infected animals), our results with RvM83 suggest that M83 is important for viral growth in the immunodeficient animals. It is possible that M83, an abundant tegument protein, may be important for optimal growth of the virus in vivo by affecting the virion assembly and regulation of gene expression in animals. Meanwhile, these results further demonstrate the feasibility of using the Tn*3*-based mutagenesis approach to study the function of a viral gene by generating viral mutants bearing a transposon insertion at the target gene and studying their replication in vitro and in vivo.

Since the transposon introduces an insertional rather than a deletional mutation, extensive studies are needed to demonstrate that the targeted gene is inactivated after the transposon insertion in order to determine the essentiality of the disrupted gene. In vitro isolation of multiple viral mutants that contain the transposon inserted at the same gene but in different locations should further support the notion that the disrupted gene is not essential for viral replication in tissue culture. Meanwhile, to confirm the assignment of functionality of a particular gene, it is probably necessary to restore the insertional mutation back to the wild-type sequence and determine whether the phenotype of the rescuant viruses is similar to that of the wild-type virus. However, the rescue procedures may also introduce adventitious mutations that occur elsewhere in the genome. Alternatively, another viral mutant that contains a transposon insertion at the same gene but in a different location from the first mutant can be generated using the Tn*3* system. Examination of the phenotype of this second isolate should confirm the results obtained from the first mutant. Further exploitation of the Tn*3* system to analyze the functions of other MCMV genes in animals should lead to the identification of viral determinants important for viral replication and pathogenesis in vivo.

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REFERENCES

1. **Baldick, C. J., Jr., and T. Shenk.** 1996. Proteins associated with purified human cytomegalovirus particles. J. Virol. **70:**6097–6105.
2. **Britt, W. J., and C. A. Alford.** 1996. Cytomegalovirus, p. 2493–2523. *In* B. N. Fields et al. (ed.), Virology, 3rd ed. Raven Press, New York, N.Y.
3. **Brune, W., C. Menard, U. Hobom, S. Odenbreit, M. Messerle, and U. H. Koszinowski.** 1999. Rapid identification of essential and nonessential herpesvirus genes by direct transposon mutagenesis. Nat. Biotechnol. **17:**360–364.
4. **Burns, N., B. Grimwade, P. B. Ross-Macdonald, E. Y. Choi, K. Finberg, G. S. Roeder, and M. Snyder.** 1994. Large-scale analysis of gene expression, protein localization, and gene disruption in *Saccharomyces cerevisiae*. Genes Dev. **8:**1087–1105.
5. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison, T. Kouzarides, and J. A. Martignetti.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154:**125–169.
6. **Chen, D. H., J. Hong, M. Lee, F. Liu, and Z. H. Zhou.** 1999. Three-dimensional visualization of tegument/capsid interactions in the intact human cytomegalovirus. Virology **260:**10–16.
7. **Cohen, J. I., and K. E. Seidel.** 1993. Generation of varicella-zoster virus (VZV) and viral mutants from cosmid DNAs: VZV thymidylate synthetase is not essential for replication in vitro. Proc. Natl. Acad. Sci. USA **90:**7376–7380.
8. **Cranmer, L. D., C. L. Clark, C. S. Morello, H. E. Farrell, W. D. Rawlinson, and D. H. Spector.** 1996. Identification, analysis, and evolutionary relationships of the putative murine cytomegalovirus homologs of the human cytomegalovirus UL82 (pp71) and UL83 (pp65) matrix phosphoproteins. J. Virol. **70:**7929–7939.
9. **Cunningham, C., and A. J. Davison.** 1993. A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology **197:**116–124.
10. **Dallas, P. B., P. A. Lyons, J. B. Hudson, A. A. Scalzo, and G. R. Shellam.** 1994. Identification and characterization of a murine cytomegalovirus gene with homology to the UL25 open reading frame of human cytomegalovirus. Virology **200:**643–650.
11. **Delecluse, H. J., T. Hilsendegen, D. Pich, R. Zeidler, and W. Hammerschmidt.** 1998. Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. Proc. Natl. Acad. Sci. USA **95:**8245–8250.
12. **Gallant, J. E., R. D. Moore, D. D. Richman, J. Keruly, and R. E. Chaisson.** 1992. Incidence and natural history of cytomegalovirus disease in patients with advanced human immunodeficiency virus disease treated with zidovudine. J. Infect. Dis. **166:**1223–1227.
13. **Grundy, J. E., and C. J. Melief.** 1982. Effect of Nu/Nu gene on genetically determined resistance to murine cytomegalovirus. J. Gen. Virol. **61:**133–136.
14. **Hoekstra, M. F., H. S. Seifert, J. Nickoloff, and F. Heffron.** 1991. Shuttle mutagenesis: bacterial transposons for genetic manipulations in yeast. Methods Enzymol. **194:**329–342.
15. **Hudson, J. B.** 1979. The murine cytomegalovirus as a model for the study of viral pathogenesis and persistent infections. Arch. Virol. **62:**1–29.
16. **Jenkins, F. J., M. J. Casadaban, and B. Roizman.** 1985. Application of the mini-Mu-phage for target-sequence-specific insertional mutagenesis of the herpes simplex virus genome. Proc. Natl. Acad. Sci. USA **82:**4773–4777.
17. **Jordan, M. C.** 1983. Latent infection and the elusive cytomegalovirus. Rev. Infect. Dis. **5:**205–215.
18. **Kemble, G., G. Duke, R. Winter, and R. Spaete.** 1996. Defined large-scale alterations of the human cytomegalovirus genome constructed by cotransfection of overlapping cosmids. J. Virol. **70:**2044–2048.
19. **Lee, C. H., A. Bhagwat, and F. Heffron.** 1983. Identification of a transposon Tn*3* sequence required for transposition immunity. Proc. Natl. Acad. Sci. USA **80:**6765–6769.
20. **Liu, F., and B. Roizman.** 1993. Characterization of the protease and other products of amino-terminus-proximal cleavage of the herpes simplex virus 1 UL26 protein. J. Virol. **67:**1300–1309.
21. **Liu, F., and B. Roizman.** 1991. The herpes simplex virus 1 gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. J. Virol. **65:**5149–5156.
22. **Manning, W. C., C. A. Stoddart, L. A. Lagenaur, G. B. Abenes, and E. S. Mocarski.** 1992. Cytomegalovirus determinant of replication in salivary glands. J. Virol. **66:**3794–3802.
23. **Messerle, M., I. Crnkovic, W. Hammerschmidt, H. Ziegler, and U. H. Koszinowski.** 1997. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. Proc. Natl. Acad. Sci. USA **94:**14759–14763.
24. **Mocarski, E. S.** 1996. Cytomegaloviruses and their replication, p. 2447–2492. *In* B. N. Fields et al. (ed.), Virology, 3rd ed. Raven Press, New York, N.Y.
25. **Mocarski, E. S., L. E. Post, and B. Roizman.** 1980. Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. Cell **22:**243–255.
26. **Morrello, C. S., L. D. Cranmer, and D. H. Spector.** 1999. In vivo replication, latency, and immunogenicity of murine cytomegalovirus mutants with deletions in the M83 and M84 genes, the putative homologs of human cytomegalovirus pp65 (UL83). J. Virol. **73:**7678–7693.
27. **Mulligan, R. C., and P. Berg.** 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA **78:**2072–2076.
28. **Okada, M., and Y. Minamishima.** 1987. The efficacy of biological response modifiers against murine cytomegalovirus infection in normal and immunodeficient mice. Microbiol. Immunol. **31:**45–57.
29. **Palella, F. J., Jr., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, and S. D. Holmberg.** 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N. Engl. J. Med. **338:**853–860.
30. **Pollock, J. L., and H. W. T. Virgin.** 1995. Latency, without persistence, of murine cytomegalovirus in the spleen and kidney. J. Virol. **69:**1762–1768.
31. **Post, L. E., and B. Roizman.** 1981. A generalized technique for deletion of specific genes in large genomes: alpha gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25:**227–232.
32. **Rawlinson, W. D., H. E. Farrell, and B. G. Barrell.** 1996. Analysis of the

complete DNA sequence of murine cytomegalovirus. J. Virol. **70:**8833–8849.
33. **Reynolds, R. P., R. J. Rahija, D. I. Schenkman, and C. B. Richter.** 1993. Experimental murine cytomegalovirus infection in severe combined immunodeficient mice. Lab. Anim. Sci. **43:**291–295.
34. **Roizman, B., and F. J. Jenkins.** 1985. Genetic engineering of novel genomes of large DNA viruses. Science **229:**1208–1214.
35. **Ross-Macdonald, P., P. S. Coelho, T. Roemer, S. Agarwal, A. Kumar, R. Jansen, K. H. Cheung, A. Sheehan, D. Symoniatis, L. Umansky, M. Heidtman, F. K. Nelson, H. Iwasaki, K. Hager, M. Gerstein, P. Miller, G. S. Roeder, and M. Snyder.** 1999. Large-scale analysis of the yeast genome by transposon tagging and gene disruption. Nature **402:**413–418.
36. **Ross-Macdonald, P., A. Sheehan, G. S. Roeder, and M. Snyder.** 1997. A multipurpose transposon system for analyzing protein production, localization, and function in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **94:**190–195.
37. **Saeki, Y., T. Ichikawa, A. Saeki, E. A. Chiocca, K. Tobler, M. Ackermann, X. O. Breakefield, and C. Fraefel.** 1998. Herpes simplex virus type 1 DNA amplified as bacterial artificial chromosome in *Escherichia coli*: rescue of replication-competent virus progeny and packaging of amplicon vectors. Hum. Gene Ther. **9:**2787–2794.
38. **Schmolke, S., H. F. Kern, P. Drescher, G. Jahn, and B. Plachter.** 1995. The dominant phosphoprotein pp65 (UL83) of human cytomegalovirus is dispensable for growth in cell culture. J. Virol. **69:**5959–5968.
39. **Seifert, H. S., R. S. Ajioka, D. Paruchuri, F. Heffron, and M. So.** 1990. Shuttle mutagenesis of *Neisseria gonorrhoeae*: pilin null mutations lower DNA transformation competence. J. Bacteriol. **172:**40–46.
40. **Seifert, H. S., E. Y. Chen, M. So, and F. Heffron.** 1986. Shuttle mutagenesis: a method of transposon mutagenesis for *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **83:**735–739.
41. **Smith, G. A., and L. W. Enquist.** 1999. Construction and transposon mutagenesis in *Escherichia coli* of a full-length infectious clone of pseudorabies virus, an alphaherpesvirus. J. Virol. **73:**6405–6414.
42. **Smith, G. A., and L. W. Enquist.** 2000. A self-recombining bacterial artificial chromosome and its application for analysis of herpesvirus pathogenesis. Proc. Natl. Acad. Sci. USA **97:**4873–4878.
43. **Stavropoulos, T. A., and C. A. Strathdee.** 1998. An enhanced packaging system for helper-dependent herpes simplex virus vectors. J. Virol. **72:**7137–7143.
44. **Tomkinson, B., E. Robertson, R. Yalamanchili, R. Longnecker, and E. Kieff.** 1993. Epstein-Barr virus recombinants from overlapping cosmid fragments. J. Virol. **67:**7298–7306.
45. **van Zijl, M., W. Quint, J. Briaire, T. de Rover, A. Gielkens, and A. Berns.** 1988. Regeneration of herpesviruses from molecularly cloned subgenomic fragments. J. Virol. **62:**2191–2195.
46. **Vieira, J., H. E. Farrell, W. D. Rawlinson, and E. S. Mocarski.** 1994. Genes in the *Hin*dIII J fragment of the murine cytomegalovirus genome are dispensable for growth in cultured cells: insertion mutagenesis with a *lacZ/gpt* cassette. J. Virol. **68:**4837–4846.
47. **Wagner, M., S. Jonjic, U. H. Koszinowski, and M. Messerle.** 1999. Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution. J. Virol. **73:**7056–7060.
48. **Weber, P. C., M. Levine, and J. C. Glorioso.** 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn*5* mutagenesis. Science **236:**576–579.
49. **Zhan, X., G. Abenes, M. Lee, I. VonReis, C. Kittinunvorakoon, P. Ross-Macdonald, M. Snyder, and F. Liu.** 2000. Mutagenesis of murine cytomegalovirus using a Tn3-based transposon. Virology **266:**264–274.