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Mutagenesis of Murine Cytomegalovirus Using a Tn3-Based Transposon

Xiaoyan Zhan,* Manfred Lee,* Gerardo Abenes,* Ilse Von Reis,* Chonticha Kittinunvorakoon,* Petra Ross-Macdonald,† Michael Snyder,† and Fenyong Liu*,[1]

*Program in Infectious Diseases and Immunity, School of Public Health, University of California, 140 Warren Hall, Berkeley, California 94720; and †Department of Molecular, Cell, and Developmental Biology, Yale University, 266 Whitney Avenue, New Haven, Connecticut 06511

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A transposon derived from *Escherichia coli* Tn3 was introduced into the genome of murine cytomegalovirus (MCMV) to generate a pool of viral mutants. We analyzed three of the constructed recombinant viruses that contained the transposon within the M25, M27, and m155 open reading frames. Our studies provide the first direct evidence to suggest that M25 and M27 are not essential for viral replication in mouse NIH 3T3 cells. Studies in cultured cells and Balb/c 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 M25 exhibited a titer similar to that of the wild-type virus in the salivary glands, lungs, livers, spleens, and kidneys of the Balb/c mice that were intraperitoneally infected with these viruses. These results suggest that M25 is dispensable for viral growth in these organs and the presence of the transposon sequence in the viral genome does not significantly affect viral replication *in vivo.* The Tn3-based system can be used as a mutagenesis approach for studying the function of MCMV genes in both tissue culture and in animals.

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that causes mild or subclinical diseases in immunocompetent adults but may lead to severe morbidity or mortality in neonates and immunocompromised individuals (Britt and Alford, 1996; Mocarski, 1996). Its DNA genome of 230 kb is the largest among all human herpesviruses and has the capacity to encode 200 open reading frames (McGeoch, 1989; Chee *et al.,* 1990). Unlike the well-studied herpes simplex virus (HSV) and Epstein–Barr virus (EBV), HCMV is a β-herpesvirus that only propagates in human cells and grows slowly due to a long lytic replication cycle (Mocarski, 1996). There are currently no suitable animal models for HCMV infection. These properties of HCMV have hampered the studies of HCMV replication and pathogenesis.

Murine cytomegalovirus (MCMV) provides an excellent model to study the biology of CMV infection. MCMV offers several advantages including rapid viral growth, the availability of a reliable animal model, and the fact that it has sequence homology with HCMV (Hudson, 1979; Jordan, 1983; Britt and Alford, 1996; Mocarski, 1996). Moreover, 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 (Hudson, 1979; Jordan, 1983; Britt and Alford, 1996; Mocarski, 1996). The entire genome of MCMV has recently been sequenced (Rawlinson *et al.,* 1996). Its genome of 230 kb is predicted to encode 170 open reading frames, 78 of which have extensive homology to those of HCMV(Chee *et al.,* 1990; Rawlinson *et al.,* 1996). However, many of these MCMV genes have not been characterized and their functions in viral pathogenesis have not been investigated.

One of the most powerful approaches to identify the function of viral-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, such as those from the herpes simplex viruses, was first reported using site-directed homologous recombination (Mocarski *et al.,* 1980; Post and Roizman, 1981). Moreover the systematic construction of viral mutants has been facilitated by transposon-mediated insertional mutagenesis (Jenkins *et al.,* 1985; Weber *et al.,* 1987 Brune *et al.,* 1999; Smith and Enquist, 1999). The methods to generate CMV mutants by site-directed homologous recombination have been well established (Spaete and Mocarski, 1987; Vieira *et al.,* 1994; Kemble *et al.,* 1996). Methods using overlapping cosmid DNA fragments to generate mutants of HCMV and other herpesviruses have also been reported (van Zijl *et al.,* 1988; Cohen and Seidel, 1993; Cunningham and Davison, 1993; Tomkinson *et al.,* 1993;Kemble *et al.,* 1996). More recently, the MCMV genome as well as the genomes of other herpeviruses have been cloned into a bacterial artificial chromosome (BAC), and MCMV mutants were

[1] To whom reprint requests should be addressed. Fax: (510) 642-6350. E-mail: liu_fy@uclink4.berkeley.edu.

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successfully generated from the BAC-based viral genome by both site-directed homologous recombination and transposon-mediated insertional mutagenesis (Messerle *et al.,* 1997; Delecluse *et al.,* 1998; Saeki *et al.,* 1998; Stavropoulos and Strathdee, 1998; Brune *et al.,* 1999; Smith and Enquist, 1999; Wagner *et al.,* 1999). The BAC-based mutagenesis approach certainly provides a powerful and convenient strategy to generate viral mutants and facilitates the studies of the functions of viral genes in tissue culture and in animals.

In this study, we have applied a Tn3 transposon-mediated shuttle mutagenesis system, employed previously for studies of gene function in *Salmonella typhi* and *Saccharomyces cerevisiae* (Seifert *et al.,* 1986; Hoekstra *et al.,* 1991; Burns *et al.,* 1994; Ross-Macdonald *et al.,* 1997), to generate MCMV mutants. 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. We show that the transposon is randomly inserted into the MCMV genomic sequence. Specifically we have successfully generated mutants containing transpositional insertions in open reading frames M25, M27, and m155. Our results provide the first direct evidence to suggest that the M25 and M27 open reading frames, which are conserved between HCMV and MCMV, are not essential for viral replication in NIH3T3 cells. Studies in Balb/c mice indicate that M25 is dispensable for viral growth in the salivary glands, lungs, livers, spleens, and kidneys of the animals. Moreover, our data suggest that the presence of the transposon sequence in the viral genome does not significantly affect viral pathogenesis *in vivo*. These results demonstrate the feasibility of using this Tn3-based system to study the functions of CMV genes both *in vitro* and *in vivo*.

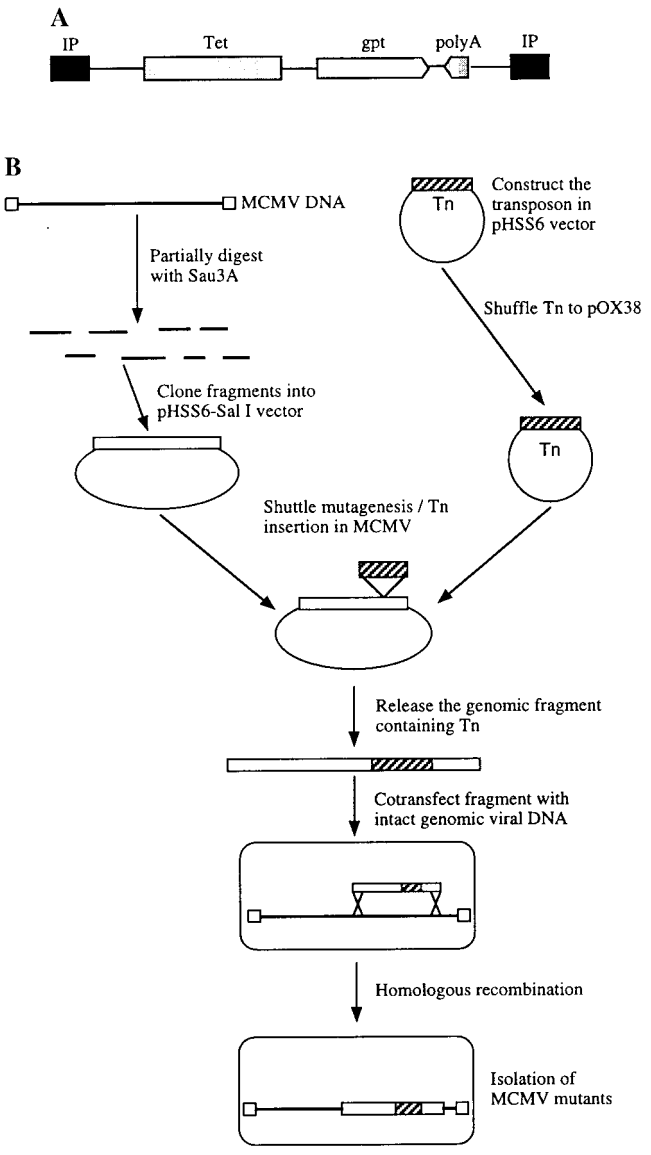


FIG. 1. Schematic representation of the structure of the transposon construct used for mutagenesis (A) and the procedure for the construction of MCMV mutants that contained random transposon insertions (B). IP, inverted repeat; Tet, tetracycline resistance gene; gpt, gene that encodes guanine phosphoribosyltransferase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

RESULTS

Generation of MCMV mutants containing a randomly inserted transposon

The *E. coli* Tn3-based transposon (Fig. 1A) has previously been used for studies of gene functions in *Salmonella typhi* and *Saccharomyces cerevisiae* (Seifert *et al.,* 1986; Hoekstra *et al.,* 1991; Burns *et al.,* 1994; Ross-Macdonald *et al.,* 1997). Our rationale to use this transposon system is twofold. First, the transposon does not have a sequence preference for transpositional insertion, as previously shown in the genomic studies of *Salmonella typhi* and *Saccharomyces cerevisiae* (Hoekstra *et al.,* 1991; Burns *et al.,* 1994). Second, it usually generates only a single insertion on a target DNA due to its unique property of transposon immunity (Lee *et al.,* 1983; Hoekstra *et al.,* 1991). To make the transposon suitable for use in the mutagenesis of the MCMV genome, we have introduced two changes into the transposon cassette first described by Ross-Macdonald *et al.* (1997). First, we cloned into the transposon the gene that encodes guanine phosphoribosyltransferase (*gpt*) (Mulligan and Berg, 1981). The *gpt* gene has successfully been used as a selectable marker in the construction of MCMV recombinant viruses (Vieira *et al.,* 1994). 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 ensure 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. Second, we re-

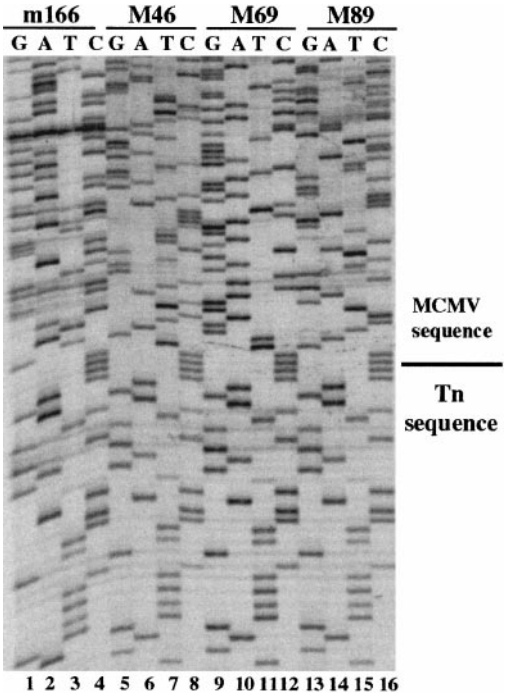


FIG. 2. Sequence analyses of MCMV DNA fragments that contained the transposon insertion. Sequence analyses were carried out using the Sequenase sequencing kit and $\alpha[^{35}S]$dATP (Amersham Inc. Arlington Heights, IL), and the products were separated on 8% polyacrylamide gels that contained 8 M urea.

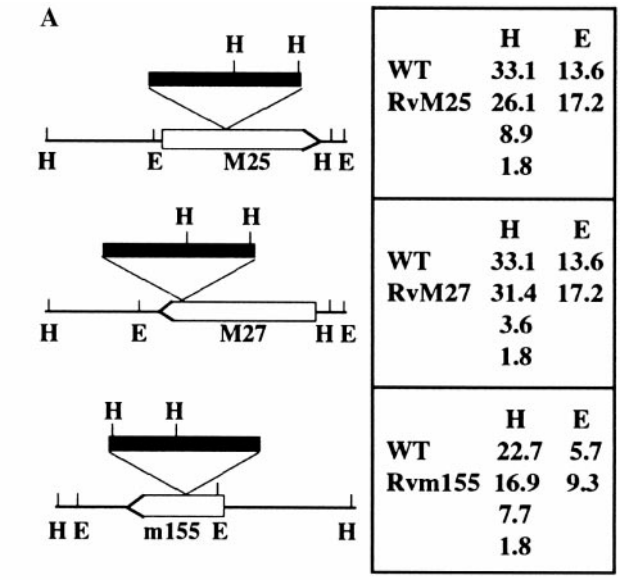
duced the size of the transposon to 3.6 kb by deleting a 2.4-kb DNA fragment that is important for selection in *Saccharomyces cerevisiae* but not essential for selection in mammalian cells. This step is necessary because the MCMV genome is not stable upon introducing a transposon insert of 6 kb and has been found to generate adventitious deletions (X. Zhan, M. Lee, G. Abenes, I. Von Reis, and F. Liu, unpublished results).

To generate a pool of MCMV DNA fragments that contained a randomly inserted transposon, viral DNA was purified and partially digested with *Sau*3A. Digested fragments in the size range of 1.6–4 kb were cloned into vector pHSS6-*Sal*I (Seifert *et al.,* 1986; Hoekstra *et al.,* 1991; Burns *et al.,* 1994). Sequence analyses of 50 cloned DNA fragments confirmed that they represented random fragments of the genome (data not shown). The transposon was introduced into a pool of the cloned MCMV genomic fragments through a shuttle mutagenesis protocol (see Materials and Methods; Fig. 1B). Sequence analyses were carried out to determine whether the pool contained MCMV fragments that had random insertions of the transposon. An example of the sequence analyses of four selected clones is shown in Fig. 2. The transposon was found to be inserted into the coding regions of open reading frames M46, M69, M89, or m166, respectively. These results indicate that the transposon was randomly inserted into the sequences of the constructed MCMV DNA pool.

Recombinant viruses that contained the transposon were generated by homologous recombination (Spaete and Mocarski, 1987; Vieira *et al.,* 1994). The pool of MCMV genomic fragments that contained a transpositional insertion were cotransfected with the full-length genomic DNA of the wild-type virus (Smith strain) into mouse NIH3T3 cells. The cells that harbored the progeny viruses were allowed to grow in the presence of mycophenolic acid and xanthine, which selects for *gpt* expression (Mulligan and Berg, 1981; Vieira *et al.,* 1994). The recombinant viruses that contained the transposon and expressed the *gpt* protein were isolated after multiple rounds of selection and plaque purification. Three of the first recombinant viruses generated were further characterized and are reported here. These viruses, designated as RvM25, RvM27, and Rvm155, contained the transposon within open reading frames M25, M27, and m155, respectively (Fig. 3A, see below). 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 RvM25, RvM27, and Rvm155 were at nucleotide positions 27078 (M25), 32363 (M27), and 215103 (m155), respectively, in reference to the genome sequence of the wild-type Smith strain (Rawlinson *et al.,* 1996) (Fig. 3A).

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 whether the mutants contained the transposon insertion (Fig. 3). The analyses of the *Hin*dIII-digested DNA of each recombinant virus clearly showed a small fragment of 1.8 kb representing the *gpt* gene fragment, indicating the presence of the transposon sequence within the viral genome (Fig. 3B, lanes 1, 5, and 9). This conclusion is further supported by the results of Southern analyses of the mutant DNAs digested with another restriction enzyme (i.e., *Eco*RI) (lanes 3 and 4, 7 and 8, and 11 and 12). In each case here, the genomic fragment from the transposon-containing viruses should be 3.6 kb bigger than that of the wild-type virus. The stocks of these recombinant viruses appeared to be pure and free of the wild-type strain because the hybridizing DNA fragments from the mutants did not comigrate with those of the wild-type Smith strain (Fig. 3B, lanes 1–12). For example, the hybridization patterns of the RvM25 and Smith strain DNAs digested with *Hin*dIII gave rise to three (26.1, 8.9, and 1.8 kb) and one DNA bands (33.1 kb), respectively (Fig. 3B, lanes 1 and 2). Meanwhile, the hybridized species (17.2 kb) of the *Eco*RI-digested RvM25 DNA migrated differently from that (13.6



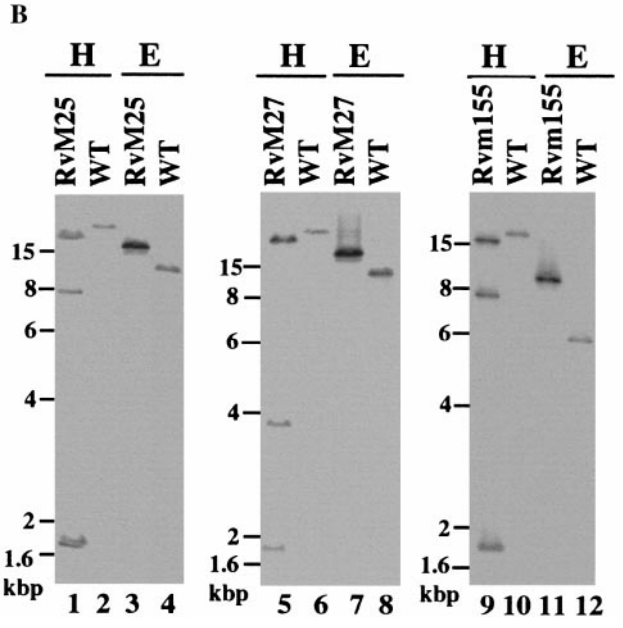


FIG. 3. The locations of the transposon insertions in the recombinant viruses (A) and Southern analyses of these mutants (B). (A) The transposon sequences are shown as a filled bar, whereas the coding sequence of each open reading frame is represented by a hollow arrow. The orientation of the arrow represents the direction of the translation and transcription predicted based on the nucleotide sequence (Rawlinson *et al.,* 1996). The numbers represent the sizes of the DNA fragments of the mutant viruses that contained the transposon sequence and were generated by digestion with either *Hin*dIII (H) or *Eco*RI (E). (B). 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 either *Hin*dIII (H) 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.

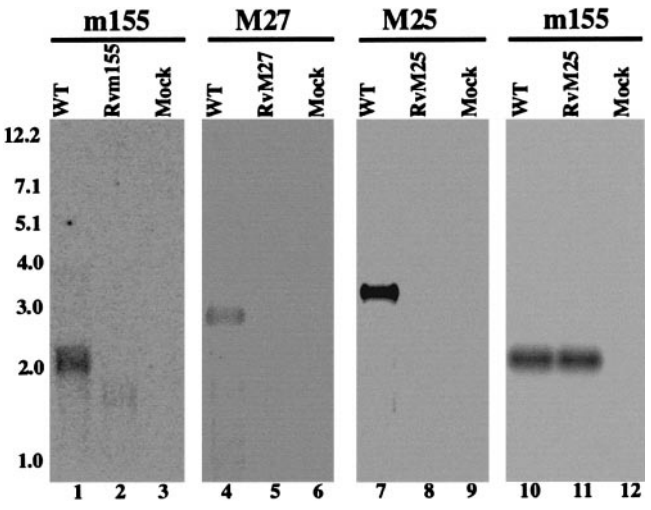


FIG. 4. Northern analyses of the RNA fractions isolated from cells that were mock-infected (lanes 3, 6, 9, and 12), or infected with the wild-type virus (WT) (lanes 1, 4, 7, and 10) and the mutant viruses (lanes 2, 5, 8, and 11). NIH3T3 cells (1×10^7) were infected with each virus at a m.o.i. of 10 PFU per cell, and cells were harvested at 24 h p.i. 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 m155 (lanes 1–3 and 10–12), M27 (lanes 4–6), or M25 (lanes 7–9).

kb) of the wild-type viral DNA digested with the same enzyme (Fig. 3B, lanes 3 and 4). The sizes of the hybridized DNA fragments (Fig. 3B) were consistent with the predicted digestion patterns of the recombinant viruses based on the MCMV genomic sequence (Rawlinson *et al.,* 1996) and the location of the transposon insertion in the viral genome as determined by sequence analysis (Fig. 3A). 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 the viral genome other than the transposon insertion region remained intact in these MCMV mutants.

It is expected that the transcription of the targeted gene would be disrupted due to the presence of the two transcription termination signals within the transposon. To examine the impact of the insertion on the expression of the transcripts encoded by the genes that were disrupted by the transposon, cytoplasmic RNAs were isolated from cells infected with these 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 M25, M27, and m155 regions (Fig. 4). In the three mutant viruses, the transposon was found to insert in the central region of M25 and m155 and the 3′ coding region of M27, respectively (Fig. 3A). The DNA sequences complementary to the 5′ coding region of each of the open reading frame were used as the probes. Substantial amounts of transcripts from

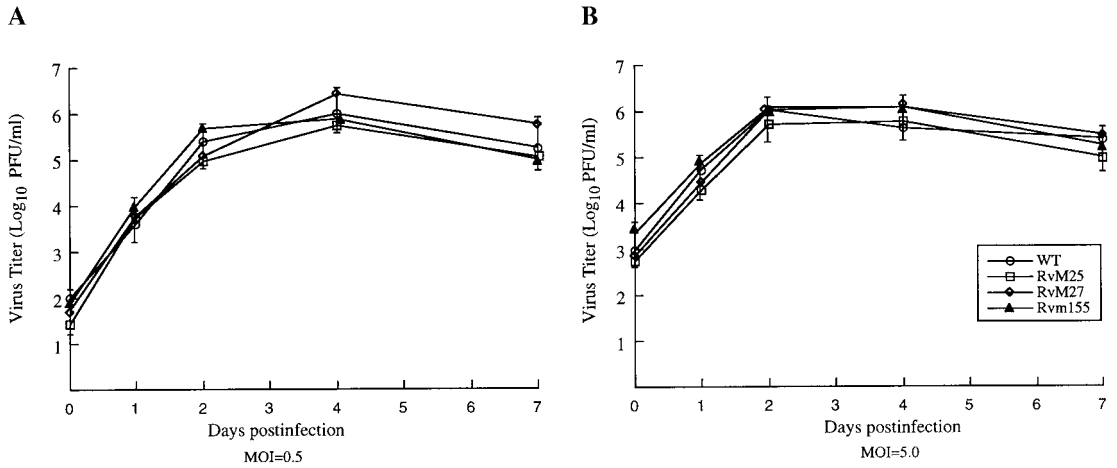


FIG. 5. *In vitro* growth of MCMV mutants in tissue culture. Mouse NIH3T3 cells were infected with each virus at a m.o.i. of either 0.5 PFU (A) or 5 PFU per cell (B). At 0, 1, 2, 4, and 7 days p.i., cells and culture media were harvested and sonicated. The viral titers were determined by plaque assays on NIH3T3 cells. The values of the viral titer represent the average obtained from triplicate experiments. The standard deviation is indicated by the error bars.

these three open reading frames were found in RNA fractions isolated from cells infected with the parental Smith strain (lanes 1, 4, and 7). The sizes of the transcripts for M25, M27, and m155 were ~3.5 (Dallas *et al.,* 1994), 3, and 2 kb (Fig. 4), respectively, and are consistent with the lengths of these three open reading frames (932, 682, and 377 amino acids, respectively). In contrast, no transcripts from the M25 and M27 open reading frames were detected in RNA fractions isolated from cells infected with RvM25 and RvM27 (Fig. 4, lanes 5 and 8) while the m155 transcript detected from Rvm155-infected cells was truncated and its expression was reduced by ≥50-fold (Fig. 4, lane 2). As an internal control, a similar level of mouse RNase P RNA transcript was found in cells infected with either the viral mutants or the Smith strain (data not shown). Moreover, the level of m155 transcript was also used as the internal control for the expression of M25 transcript (Fig. 4, lanes 10–12). The level of m155 transcript detected in cells that were infected with RvM25 was found to be similar to that of m155 transcript in cells infected with the Smith strain (Fig. 4, lanes 10 and 11), suggesting that the low-level expression of M25 transcript from RvM25 (lane 8) is probably due to the insertional mutation. Similar results were also obtained when the levels of the m155 and M25 transcripts were used as the internal controls for the expression of the M27 and m155 transcripts expressed from RvM27 and Rvm155, respectively (data not shown). Thus the insertions truncated or disrupted the transcripts expressed from these open reading frames. Moreover, the insertions abolished or reduced the expression of these transcripts presumably due to the changes of the stability of the transcripts.

To use the Tn3-based transposon system to generate MCMV mutants and study the phenotypes of the mutants *in vitro* and *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 tissue culture and in animals. To determine whether the recombinant viruses have any growth defects *in vitro*, NIH3T3 cells were infected with these viruses at both low and high multiplicities of infection (m.o.i.). Growth rates of these viruses in mouse NIH3T3 cells were assayed and compared to those of the parental Smith strain. These results, obtained from triplicate experiments, are shown in Fig. 5 and indicate that the peak titers of RvM25, RvM27, and Rvm155 viruses were similar to that of the parental Smith strain. The fact that these viruses did not exhibit significant growth defects are consistent with the previous observations that m155 is dispensable for MCMV replication in NIH3T3 cells (Boname and Chantler, 1992; Thale *et al.,* 1995). Moreover, these results, combined with those from the Southern and Northern analyses, suggest that M25 and M27 are not essential for viral growth in tissue culture.

In vivo characterization of the transposon-containing RvM25 in animals

RvM25 was used as the representative to determine whether the presence of the transposon sequence in the viral genome causes any effects on viral replication in animals *in vivo*. Balb/c mice were injected intraperitoneally with 1×10^3 PFU of RvM25. At 1, 3, 7, and 14 days p.i., salivary glands, lungs, spleens, livers, and kidneys were harvested, and the viral titers in these five organs were determined. These organs are among the major targets for MCMV infection (Hudson, 1979; Jordan, 1983; Britt and Alford, 1996; Mocarski, 1996). At 14 days p.i., the titer of RvM25 in the salivary gland was similar to that of the Smith strain (Fig. 6). At 7 days p.i., the titers of RvM25 in the lungs, spleens, livers, and kidneys were also similar

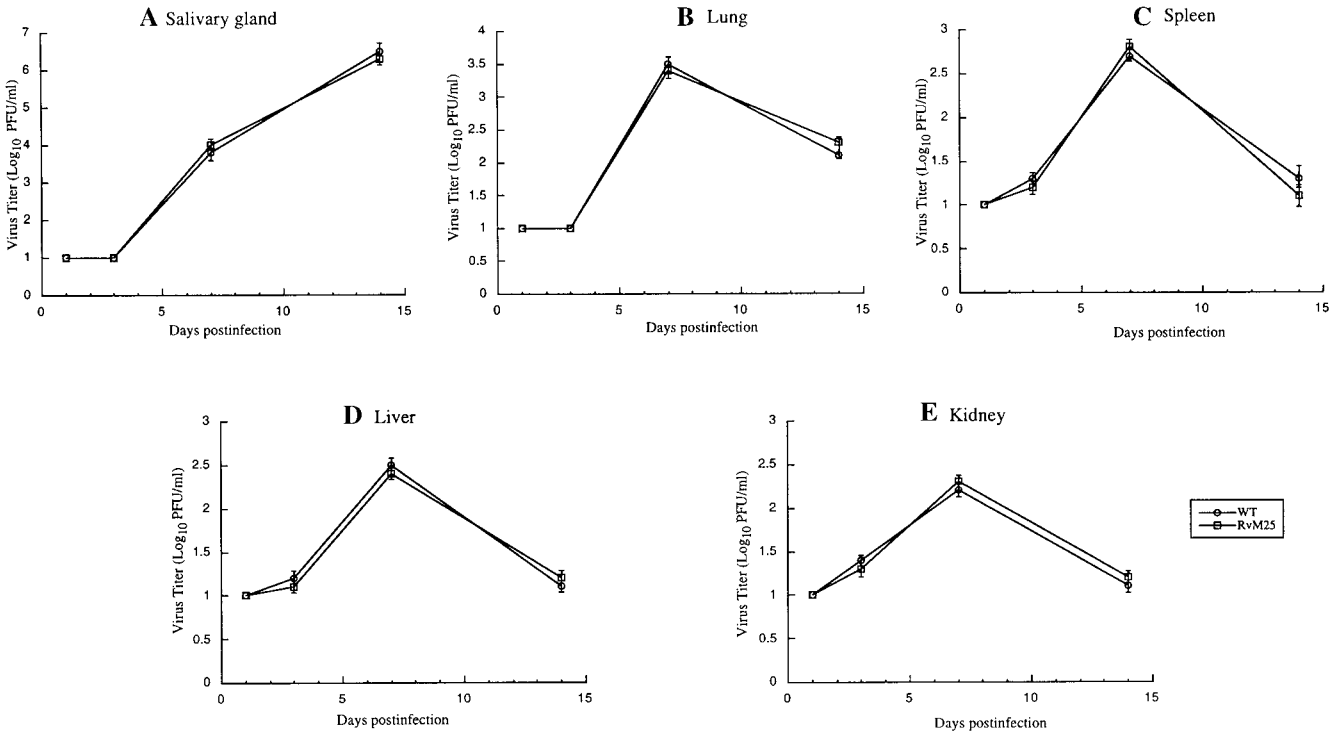


FIG. 6. Titers of MCMV mutants in salivary glands (A), lungs (B), spleens (C), livers (D), and kidneys (E) of the infected mice. Balb/c-Byj mice were infected intraperitoneally with 1×10^3 PFU of each virus. At 1, 3, 7, and 14 days p.i., the animals (three mice per group) were killed. The salivary glands, lungs, spleens, livers, and kidneys were collected and sonicated. The viral titers in the tissue homogenates were determined by standard plaque assays in NIH3T3 cells. The limit of detection was 10 PFU/ml of the tissue homogenate. The viral titers represent the average obtained from triplicate experiments. The standard deviation is indicated by the error bars. Error bars that are not evident indicate that the standard deviation was less or equal to the height of the symbols.

to those of the Smith strain (Fig. 6). Because RvM25 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 vivo* at least in these organs. These results further indicate that M25 open reading frame is dispensable for viral replication in these organs in Balb/c mice.

Stability of the transposon mutations in the recombinant viruses

The MCMV genome is not stable upon introducing an insert of 6 kb and has been found to generate adventitious deletions (X. Zhan, M. Lee, G. Abenes, I. Von Reis, and F. Liu, unpublished results). A viral mutant with a spontaneous deletion in the 3′ terminal region of the genome which includes the m155 sequence has also been reported (Boname and Chantler, 1992). To investigate whether the transposon insertion is stable within the viral genome, two sets of experiments were carried out. First, recombinant viruses were used to infect NIH3T3 cells at a m.o.i. of 0.01 and allowed to grow for five generations (60 days) in the absence of the *gpt* selection. Second, 1×10^3 PFU of viruses were used to infect Balb/c mice. At 14 days p.i., salivary glands were harvested from the infected animals and sonicated to release the virus. Viruses were recovered by infecting NIH3T3 cells with the sonicated tissues. Viral DNAs were purified from the infected cells and their restriction-digest patterns were analyzed in agarose gels. RvM25 as well as Rvm155 was used in these studies. The inclusion of Rvm155 in the study is to determine whether the transposon insertion is stable in the m155 region which has been previously shown to be spontaneously deleted (Boname and Chantler, 1992). An example of the Southern analyses of the Rvm155 viral DNAs with a DNA probe that contained the transposon and m155 open reading frame sequence is shown in Fig. 7. These results indicated that no change in the hybridization patterns of Rvm155 occurred as a result of growth of the virus for five generations (60 days) in cultured cells (Fig. 7, lanes 3 and 7) or in animals for 14 days (lanes 2 and 6). Moreover, the overall *Hin*dIII-digestion patterns of Rvm155 DNA that either replicated in cultured cells or in animals were identical to those of the original recombinant virus, as visualized by ethidium bromide staining of the viral DNAs. Similar results were also observed in experiments with RvM25 (data not shown). Thus the transposon insertion appeared to be stable in both tissue culture and in animals.

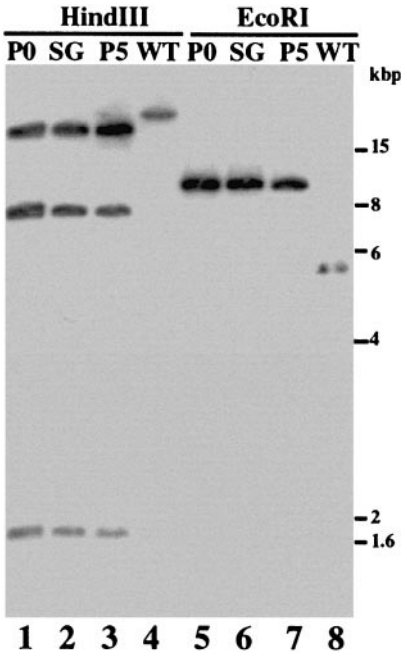


FIG. 7. The stability of the transposon mutations in tissue cultured cells and in mice. Viral DNAs were isolated from either cells that were infected with Rvm155 (m.o.i. 0.01) that have grown in culture for 5 days (P0; lanes 1 and 5) or for five generations (60 days; P5; lanes 3 and 7), or from cells that were infected with the virus collected from the salivary glands of mice 14 days after intraperitoneal inoculation with 1×10^3 PFU of Rvm155 (SG; lanes 2 and 6). Southern analyses of the viral DNA fractions digested with *Hin*dIII (A) and *Eco*RI (B) are shown. The DNA of the wild-type virus (WT) is shown in lanes 4 and 8. The ^{32}P-radiolabeled probe was derived from the same plasmid which was used for Southern analyses of Rvm155 in Fig. 3 and contained the transposon and the m155 open reading frame sequence.

DISCUSSION

Potential function of open reading frames M25, M27, m155 in viral replication

In this study, recombinant viruses that contained the insertional mutations at open reading frames M25, M27, and m155 were generated. All these viruses were able to replicate in NIH3T3 cells. These results are consistent with the previous observation that m155 is not essential for MCMV replication *in vitro* (Boname and Chantler, 1992; Thale *et al.,* 1995). Moreover our results provide the first direct evidence to suggest that M25 and M27 are not essential for viral replication in NIH3T3 cells *in vitro*.

Although 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 coding regions of these open reading frames was inserted with the transposon sequence. Second, the transcripts from these genes were either undetectable in cells infected with the mutant viruses or the expression level was significantly less than that of the transcript from cells infected with the wild-type virus. Moreover the transcript expressed from the disrupted open reading frame (e.g., m155) was truncated. The low level of the transcripts detected from the targeted genes is probably due to the change of the stability of these transcripts, which are now either disrupted or truncated and may be more susceptible to degradation of intracellular RNases .

M25 belongs to the CMV UL25 gene family, which also includes M35 open reading frame (Rawlinson *et al.,* 1996) and has been shown to encode a tegument protein (Dallas *et al.,* 1994). Its human counterpart, the UL25 protein, also encodes a tegument protein (Baldick and Shenk, 1996; Battista *et al.,* 1999; Zini *et al.,* 1999). However, the function of this protein in viral pathogenesis remains elusive. Our results indicate that M25 is not essential for viral replication in NIH3T3 cells. These results are the first to demonstrate the nonessentiality of a member of the UL25 gene family for MCMV replication *in vitro*. RvM25 appeared to replicate as equally well as the wild-type virus in the salivary glands, lungs, spleens, livers, and kidneys of the animals that were infected intraperitoneally. These results indicate that M25 is not essential for viral growth in these organs *in vivo*.

Open reading frame m155 belongs to MCMV m145 gene family and is believed to encode a membrane protein (Rawlinson *et al.,* 1996). However, neither the transcript nor the protein product coded by this open reading frame has been reported. Our results indicate that a transcript of ~2000 nucleotides is expressed from the m155 open reading frame. A previous study has indicated that m155 is not essential for MCMV replication in NIH3T3 cells as a 9-kb DNA fragment that contained the m155 open reading frame was found to be dispensable for viral replication *in vitro* (Boname and Chantler, 1992; Thale *et al.,* 1995). However, the function of m155 is still unknown.

M27 open reading frame has not been characterized transcriptionally or translationally. Our results indicate that a viral transcript of ~3000 nucleotides was expressed from the M27 region in cells infected with the wild-type virus (Fig. 4). In contrast, no transcript was detected from the transposon-targeted region in cells infected with RvM27 (Fig. 4). Because this open reading frame is highly conserved between HCMV and MCMV, it will be interesting to determine whether its HCMV counterpart is also not essential for viral growth *in vitro*.

Genetic analyses of CMV genome by transposon-mediated insertional mutagenesis

Transposon-mediated mutagenesis has been widely used to study gene functions in viruses, bacteria, yeast, and mammalian cells. Previously, Tn5 and phage-μ-based transposons have been used in the genetic analysis of herpes simplex virus 1 (HSV-1) (Roizman and Jenkins, 1985; Weber *et al.,* 1987). More recently, transposons derived from Tn1721 and Tn5 have also been

used in the mutagenesis of the BAC-based genomes of MCMV and pseudorabies virus, respectively (Brune *et al.,* 1999; Smith and Enquist, 1999). Although the BAC-based mutagenesis approach provides a powerful and convenient strategy to generate MCMV mutants, 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, an approach to excise the BAC sequence from the viral genome has been described (Brune *et al.,* 1999) and will further facilitate the development of the BAC-based mutagenesis methodology for the studies of viral gene functions *in vivo*.

The system we used is based on an *E. coli* Tn3 transposon that has been used in the mutagenesis studies of the genomes of *Saccharomyces cerevisiae* and other organisms such as *Salmonella typhimurium* and *Neisseria gonorrhoeae* (Seifert *et al.,* 1986, 1990; Burns *et al.,* 1994; Ross-Macdonald *et al.,* 1997). One of the major advantages of the Tn3 system is its transposition immunity. A plasmid already containing a copy of Tn3 is immune to further insertions of the transposon. This immunity is due to the presence of a 38-nucleotides-long sequence, which is also found in the *E. coli* chromosome (Lee *et al.,* 1983). Therefore, Tn3 mutagenesis is simple, straightforward, and yields little background as most of the transposition occurs in the target sequence (e.g., MCMV DNA) rather than the *E. coli* chromosome sequence (Hoekstra *et al.,* 1991). Inclusion of a *gpt* marker in the transposon provides a powerful tool for the selection of mutant viruses. In our study, the constructed Tn3-*gpt* transposon efficiently transposed into the MCMV DNA fragments. Moreover, the transposon insertions were stable during the replication of the viral mutants in both NIH3T3 cells and in animals. The viral genome other than the transposon insertion region appeared to be intact in these MCMV mutants. Furthermore a virus mutant that contained the transposon (i.e., RvM25), when used to infect animals intraperitoneally, exhibited similar level of replication in the all five organs examined as the wild-type virus (Fig. 6). These results indicate that the presence of the transposon sequence does not significantly affect the pathogenesis of the virus. Thus the Tn3-gpt system is suitable for genetic analyses of the functions of MCMV genes *in vivo*.

To confirm the assignment of functionality of a particular gene, it is important to restore the insertional mutation back to the wild-type sequence and determine whether the phenotype of the rescuant viruses can be reverted 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 a different location from the first mutant can be generated using the Tn3 system. Examination of the phenotype of this second isolate should confirm the results obtained from the first mutant. Meanwhile, because the transposon introduces an insertional rather than deletional mutation, extensive studies are needed to demonstrate that the targeted gene is inactivated after the transposon insertion 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 different locations should strongly suggest that the disrupted gene is dispensable for viral replication in tissue culture. Further exploitation of the Tn3 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*.

MATERIALS AND METHODS

Cells and virus

MCMV (Smith strain), obtained from American Tissue Culture Collection (Rockville, MD), was propagated in NIH3T3 cells (ATCC CRL1658) in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% NuSerum (Collaborative Biomedical Products, Bedford, MA).

Isolation of viral DNA and construction of a MCMV DNA subclone pool

NIH3T3 cells were infected at a m.o.i. of 1 PFU per cell. Approximately 4–5 days p.i., almost all of the cells showed cytopathic effects (CPE) and were harvested. Viral particles and DNA were purified as described previously (Manning *et al.,* 1992; Chen *et al.,* 1999). To generate the MCMV genomic pool, the DNA was partially digested with 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 1.6–4 kb were purified and cloned into pHSS6-*Sal*I (Seifert *et al.,* 1986; Hoekstra *et al.,* 1991; Burns *et al.,* 1994)

Construction of the transposon and shuffling onto the F′ plasmid pOX38 to form pOX38::m-Tn3. The original mini-Tn3 transposon used for previous studies (Seifert *et al.,* 1986; Hoekstra *et al.,* 1991; Burns *et al.,* 1994; Ross-Macdonaldet al., 1997) contained two 38 nucleotides terminal inverted repeats (IP), a sequence of the loxP and loxR, the RES element from *E. coli* Tn3, and the sequences that encode the tetracycline resistant gene (Tet), the yeast URA3, and the lacZ protein (Fig. 1A). To reduce the size of the transposon and include a marker for selection in mammalian cells, we replaced the URA3 and the majority of the lacZ gene sequences with the *E. coli* xanthine-guanine phosphoribosyltransferase (gpt)

gene that is driven by a SV40 promoter. The *gpt* expression cassette was inserted in a way that its transcription termination site functioned in the opposite direction as the other poly(A) signal presented in the transposon (Fig. 1A). The final product is a transposon that contains both the tetracycline resistance gene for selection in bacterial culture and the *gpt* gene for selection in mammalian cell culture (Fig. 1A).

The procedures (Fig. 1B) to transfer the Tn3 into the F′ plasmid pOX38, were carried out as described previously (Seifert *et al.,* 1986; Hoekstra *et al.,* 1991; Burns *et al.,* 1994). Briefly, construct Tn3-pHSS6 which contained the transposon sequence was transformed into *E. coli* strain 15 {RDP146 [F^- recAl(Δlac-pro) rpsE; spectinomycin resistant] with F^- derivative pOX38 and plasmid pLB101 (Cm^r, Tn3 transposase)} and grown in the presence of tetracycline and chloramphenicol for 48 h at 30°C. These transformed colonies served as the donor bacteria and were allowed to conjugate with *E. coli* strain 70 [NG135 (K12 recA56 gal-delS165 strA; streptomycin resistant) with plasmid pNG54], which served as the recipient strain. The conjugation was carried out at 37°C without agitation for 20 min. After mating, 20 μl of the culture was spotted onto the plates that contained tetracycline, streptomycin, and chloramphenicol and allowed to grow for 48 h at 37°C. Finally, the constructed pOX38::m-Tn was transferred to a streptomycin/chloramphenicol-sensitive *E. coli* strain B224 {RDP146 [F^- recAl(Δlac-pro) rpsE, spectinomycin resistant]} by a similar conjugation procedure as described above. Those colonies that could only grow on spectinomycin/tetracycline plates contained the transposon sequence and were designated as strain XZ95. These strains were used as the source of the transposon for the mutagenesis of MCMV fragments (see below).

Shuttle mutagenesis of the MCMV genomic fragment pool

The MCMV genomic fragment pool was first transformed into B211 {RDP146 [F^- recAl(Δlac-pro)rpsE; spectinomycin resistant] with plasmid pLB101[rbrac3] and colonies were selected on plates that contained kanamycin and chloramphenicol. These colonies were then mated with strain XZ95 (see above) containing the modified F^- plasmid pOX38::m-Tn3. 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 Tn3 insertion by mating the bacteria with strain *E. coli* 70. The plasmid DNA that contained the viral DNA fragments with a randomly inserted transposon was isolated and used to transform into *E. coli* strain DH5α for long-term storage.

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, IL) with primer FL110PRIM (5′-GCAGGATCCTATCCATATGAC-3′).

Construction of recombinant MCMV

The transposon-MCMV DNA constructs were isolated and digested with *Not*I to release the genomic fragments containing the transposon (Fig. 1B). The excised fragments (1–3 μg) and full-length intact viral genomic DNA (Smith strain, 8–12 μg) were subsequently cotransfected into mouse NIH3T3 fibroblasts using a calcium phosphate precipitation protocol (Gibco BRL, Grand Island, NY). The recombinant virus was purified by six rounds of amplification and plaque purification in the presence of 25 μg/ml mycophenolic acid (GibcoBRL, Grand Island, NY.) and 50 μg/ml xanthine (Sigma, St. Louis, MO), as described previously (Vieira *et al.,* 1994). For each cotransfection, several viral plaques were picked and expanded. Viral stocks were prepared by growing the viruses in T-150 flasks of NIH3T3 cells. To determine the location of the transposon insertion at 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, WI).

Southern and northern analyses of recombinant viruses

Viral genomic DNA was isolated from NIH3T3 cells as described previously (Liu and Roizman, 1991; Manning *et al.,* 1992). Briefly, cells that exhibited 100% CPE were washed with phosphate-buffered saline (PBS) and then subjected to proteolysis by a mixture solution that contained sodium dodecyl sulfate (SDS) and proteinase K. The genomic DNA was purified by extraction with phenol-chloroform followed by precipitation with 2-propanol (Manning *et al.,* 1992).

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 or *Eco*RI, separated on a 0.8% agarose gel, transferred to a Zeta-Probe nylon membrane (BIO-RAD, Hercules, CA), hybridized with the ^{32}P-radiolabeled DNA probes that contain the transposon and the MCMV sequences, and finally analyzed with a STORM840 phosphorimager. The labeled DNA probes were prepared by random primer synthesis (Boehringer Mannheim, Indianapolis, ID).

Cytoplasmic RNAs were isolated from MCMV-infected cells as described previously (Liu and Roizman, 1991). Cells were infected with virus at a m.o.i. of 10 and

harvested at different time points postinfection. In the experiments to assay the expression of immediate early (IE) transcripts, cells were pretreated with 100 μg/ml cycloheximide (Sigma Co., St Louis, MO), then infected with viruses and harvested at 6 h p.i. Viral RNAs were separated in a 1% agarose gel that contained formaldehyde, transferred to a nitrocellulose membrane, hybridized with the ^{32}P-radiolabeled DNA probes that contained the MCMV sequences, and finally analyzed with a STORM840 phosphorimager. The DNA probes used for Northern analyses were generated by PCR using viral DNA as the templates and radiolabled with a random primer synthesis kit in the presence of [^{32}P]α-dCTP (Boehringer Mannheim, Indianapolis, ID). The 5′ PCR primers used in the construction of DNA probes for the Northern analysis of M25, M27, and m155 transcripts were M25/1 (5′-AATCCATCTCCGCATCCGAACCCTG-3′), M27/1 (5′-CGAGCCCGTCCGAGTCTTCCGAGGT-3′), and m155–1 (5′-CGAGTATGTGCTCTCCTGCTCTTGAT-3′), respectively. The 3′ PCR primers used were M25/2 (5′-CCTCAGACGGGATGCTCAATGGCTT-3′), M27/2 (5′-GCGCGTTCTTCAGGTAGCTGTACTT-3′), and m155–2 (5′-TACAGACGACAGGTGTCAGACCAAA-3′), respectively.

Growth kinetics of recombinant viruses

NIH 3T3 cells (5×10^5) were infected at m.o.i. of either 0.5 PFU or 5.0 PFU per cell. The cells and medium were harvested at 0, 1, 2, 4, and 7 days p.i. 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, ME) were infected intraperitoneally with 1×10^3 PFU of each virus. The animals were killed at 1, 3, 7, and 14 days postinoculation. For each time point, at least three animals were used as a group and infected with the same virus. The salivary glands, lungs, spleens, livers, and kidneys were harvested and sonicated as a 10% (wt/vol) suspension in a 1:1 mixture of DMEM medium and skim milk. The sonicates were stored at −80°C until plaque assays were performed. Viral titers were determined by plaque assays on NIH3T3 cells in triplicate experiments. 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 designated a titer value of 10 (10^1) PFU/ml.

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