

Murine Cytomegalovirus Containing a Mutation at Open Reading Frame M37 Is Severely Attenuated in Growth and Virulence In Vivo

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A pool of murine cytomegalovirus (MCMV) mutants was generated by using a Tn3-based transposon mutagenesis procedure. One of the mutants, RvM37, which contained the transposon sequence at open reading frame M37, was characterized both in tissue culture and in immunocompetent BALB/c and immunodeficient SCID mice. Our results provide the first direct evidence to suggest that M37 is not essential for viral replication in vitro in NIH 3T3 cells. Compared to the wild-type strain and a rescued virus that restored the M37 region, the viral mutant was severely attenuated in growth in both BALB/c and SCID mice after intraperitoneal infection. Specifically, titers of the Smith strain and rescued virus in the salivary glands, lungs, spleens, livers, and kidneys of the SCID mice at 21 days postinfection were about 5×10^5 , 2×10^5 , 5×10^4 , 5×10^3 , and 1×10^4 PFU/ml of organ homogenate, respectively; in contrast, titers of RvM37 in these organs were less than 10^2 PFU/ml of organ homogenate. Moreover, the virulence of the mutant virus appeared to be significantly attenuated because none of the SCID mice infected with RvM37 had died by 120 days postinfection, while all animals infected with the wild-type and rescued viruses had died by 26 days postinfection. Our results suggest that M37 probably encodes a virulence factor and is required for MCMV virulence in SCID mice and for optimal viral growth in vivo.

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that typically causes asymptomatic infections in healthy individuals but may lead to serious complications in newborns and immunodeficient individuals (4, 32). Like other herpesviruses, HCMV causes acute infection that progresses to persistence and latency followed by periodic reactivation. Its 230-kb DNA genome is the largest among all human herpesviruses and has the capacity to encode more than 220 open reading frames, in contrast to only about 85 open reading frames for herpes simplex virus type 1 (7, 30, 32, 39). Unlike the well-studied herpes simplex and Epstein-Barr viruses, HCMV is a betaherpesvirus that propagates only in human cells and grows very slowly due to its long lytic replication cycle (4, 32). There is currently no suitable animal model available for studying HCMV infection in vivo. The functions of many gene products encoded by HCMV in viral pathogenesis, virulence, and latency have not been studied. Consequently, related viruses, such as murine CMV (MCMV), must be used to investigate the tissue tropism, virulence, latency, and reactivation of HCMV (21, 24, 32).

MCMV has proved a useful model for CMV disease. MCMV also causes acute, latent, and persistent infections of the natural hosts (21, 24, 32). MCMV pathogenesis closely resembles that of HCMV, and both viruses cause severe infections in the immunocompromised or immunologically immature hosts, resulting in similar clinical syndromes. Moreover, analysis of the complete nucleotide sequence of MCMV (Smith strain) has revealed that more than 75 open reading frames have significant sequence homology to those of HCMV

(7, 37). These findings have further provided evidence to support the observed biological similarities between these two viruses. In vivo studies of the functions of the genes encoded by MCMV, especially those that are highly conserved with those encoded by HCMV, should allow us to investigate the mechanism of viral pathogenesis and, furthermore, provide insight into the functions of their HCMV counterparts in viral infections in humans.

One of the most powerful approaches to identify 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 via site-directed homologous recombination and transposon-mediated insertional mutagenesis has been reported (5, 22, 42, 51). Methods using overlapping cosmid DNA fragments to generate mutants of HCMV and other herpesviruses have also been reported (8, 13, 25, 47, 48). More recently, the MCMV genome as well as the genomes of other herpesviruses were 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 (5, 15, 31, 41–43, 45, 50). The BAC-based mutagenesis approach provides a powerful and convenient strategy to generate viral mutants and facilitates studies of the functions of viral genes in tissue culture and in animals.

Many of the CMV genes have been found to be dispensable for growth in cultured cells. Their presence in the viral genome indicates that they are probably needed to perform functions involved only in modulating the viral interactions with the respective human or animal hosts. For example, HCMV US2, a nonessential protein, functions to prevent antigen presentation of both major histocompatibility complex class I and II

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pathways (23, 46). Meanwhile, MCMV open reading frame m131, which encodes a β -chemokine homologue and is dispensable for viral replication *in vitro*, functions as a determinant for viral pathogenicity and appears to promote monocyte-associated viremia and dissemination of the virus *in vivo* (16, 27, 28, 40). Thus, studies of viral mutants carrying mutations in genes found to be dispensable in tissue culture are valuable for understanding the function of the genes in viral pathogenesis and virus-host interactions.

We have previously generated a pool of MCMV mutants using a Tn3 transposon-mediated shuttle mutagenesis system (53, 54). In this approach, the transposon is randomly inserted into the MCMV genomic DNA fragments in a plasmid library in *Escherichia coli*. Regions bearing an insertion mutation are then transferred to the MCMV genome by cotransfecting the plasmid library and purified MCMV genomic DNA into NIH 3T3 cells. In the present study, we have characterized an MCMV mutant, RvM37, which contains a transposon insertion in open reading frame M37, a homologue of HCMV UL37 open reading frame (7, 37). The products coded by UL37 are immediate-early membrane proteins localized in mitochondria as well as in the plasma membrane (1, 11, 12). The UL37 proteins have been shown to selectively transactivate the expression of genes under control of both cellular and HCMV promoters (10, 12). More recently, these proteins have also been implicated to have antiapoptotic activities (17). However, the function of UL37 as well as M37 *in vivo* in viral replication and pathogenesis is not known. Indeed, to our knowledge, the M37 open reading frame has not been characterized transcriptionally or translationally. Our results provide the first direct evidence to suggest that M37 is not essential for viral replication *in vitro* in tissue culture. When the viruses were used to infect immunocompetent BALB/c mice and immunodeficient SCID mice intraperitoneally, titers of the viral mutant in the salivary glands, lungs, spleens, livers, and kidneys were significantly lower than those of the wild-type virus and a revertant virus that rescued the mutation and restored the M37 open reading frame. Moreover, the viral mutant is avirulent in SCID mice. These results provide the first direct evidence to suggest that M37 may encode a viral virulence factor required for killing SCID mice and for optimal viral growth *in vivo*.

MATERIALS AND METHODS

Cells and viruses. Mouse NIH 3T3 and STO cells and the wild-type Smith strain of MCMV were purchased from the American Type Culture Collection, Manassas, Va. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% NuSerum (Becton Dickinson, Bedford, Mass.), essential and nonessential amino acids, and penicillin-streptomycin (each from a stock solution purchased from Life Technologies Inc. [Gibco BRL, Grand Island, N.Y.]) (53). The wild-type Smith strain, mutant RvM37, and the revertant virus RqM37 were propagated in NIH 3T3 cells as described previously (53).

Analysis of growth of viruses *in vitro*. Growth kinetics of the wild-type virus, mutant RvM37, and rescued virus RqM37 were determined as described previously (54). Briefly, NIH 3T3 cells grown to 50 to 60% confluence were inoculated with virus at a multiplicity of infection (MOI) of either 0.5 or 5. At 0, 1, 2, 4, and 7 days postinfection, the infected cells together with medium were harvested, and an equal volume of 10% skim milk was added before sonication. Virus titers were determined by plaque assays in NIH 3T3 cells; the values reported are averages from triplicate experiments.

Virulence assays. Virulence of the viruses was determined on the basis of mortality of the animals infected with the Smith strain, RvM37, or RqM37. CB17 SCID mice (National Cancer Institute, Bethesda, Md.) (five animals per group) were infected intraperitoneally with 10^4 PFU of each virus. The animals were observed twice daily; mortality of infected animals was monitored for at least 120 days postinfection, and survival rates were determined.

Construction of an MCMV DNA subclone pool, transposon-based shuttle mutagenesis, and generation of MCMV recombinant mutants and rescued viruses. Isolation of viral genomic DNA, construction of a MCMV genomic subclone pool, and transposon-based shuttle mutagenesis to generate a pool of MCMV DNA fragments containing a transposon insertion were performed as described by Zhan et al. (53). To generate a pool of MCMV mutants that

contained the transposon sequence, full-length MCMV genomic DNA and plasmid DNA containing MCMV fragments were cotransfected into NIH 3T3 cells by a calcium phosphate precipitation protocol (Gibco BRL). The recombinant MCMV was selected in the presence of mycophenolic acid (25 μ g/ml; Gibco BRL) and xanthine (50 μ g/ml; Sigma, St. Louis, Mo.) and plaque purified six times as described by Zhan et al. (53). To confirm integration of the transposon in the viral genome and identify the genes that contained the transposon insertion, viral DNA was purified and directly sequenced using the primer FL110PRIM (5'-GCAGGATCCTATCCATATGAC-3') and an fmol cycle sequencing kit (Promega, Inc., Madison, Wis.).

To construct the rescued virus RqM37, the full-length genomic DNA of RvM37 was isolated from virus-infected cells as described previously (53). The DNA sequence that contained the coding sequence of M37 was generated by PCR using MCMV genomic DNA as the template and the 5' primer M37-sense (5'-ACCTGGAGTGGCACTTGCCG-3') and the 3' primer M37-antisense (5'-CGCGAGCCTCTGTATCGATA-3'). The PCR product that contained the M37 coding sequence (1 to 3 μ g) and the full-length intact RvM37 viral genomic DNA (8 to 12 μ g) were subsequently cotransfected into mouse STO fibroblasts by using a calcium phosphate precipitation protocol (Gibco BRL). The recombinant virus was selected in the presence of 6-thioguanine (25 μ g/ml; Sigma) and purified by six rounds of amplification and plaque purification as described previously (18). For each cotransfection, several viral plaques were picked and expanded. Viral stocks were prepared by growing the viruses in T-150 flasks of NIH 3T3 cells.

Analysis of growth of viruses in animals. Four-week-old male BALB/c-Byj mice (Jackson Laboratory, Bar Harbor, Maine) or 6-week-old CB17 SCID mice (National Cancer Institute) were infected intraperitoneally with 10^4 PFU of each virus. The animals were sacrificed at 1, 3, 7, 10, 14, and 21 days postinoculation. Salivary glands were also collected from the BALB/c mice at 28 days postinfection. 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 and 10% skim milk. The sonicates were stored at -80°C until plaque assays were performed.

Viruses harvested from the mice were titered on NIH 3T3 cells in six-well tissue culture plates (Corning Inc., Corning, N.Y.). Briefly, cells were first infected with the viruses at 10-fold serial dilutions. After 90 min of incubation with the homogenates diluted in 1 ml of complete medium at 37°C , the cells were overlaid with fresh complete medium containing 1% agarose and cultured for 4 to 5 days before the plaques were counted. Viral titers (recorded as PFU per milliliter of organ homogenate) for each sample were 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 assigned a titer value of $10 (10^1)$ PFU/ml.

Southern and Northern analyses of recombinant viruses. Viral genomic DNA was purified from NIH 3T3 cells infected with the viruses as described previously (49, 53). Briefly, cells that exhibited 100% cytopathic effect were washed with phosphate-buffered saline and then lysed with 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. The DNA was then digested with *Hind*III or *Eco*RI, separated on agarose gels (0.8%), transferred to Zeta-Probe nylon membranes (Bio-Rad, Hercules, Calif.), and hybridized with ^{32}P -labeled DNA probes that are specific for both the transposon and MCMV sequences. A STORM 840 PhosphorImager was used to analyze the blots.

For Northern blot analysis, cells were infected with virus at an MOI of 5 and harvested at different time points postinfection. Total cytoplasmic RNA was isolated from NIH 3T3 cells infected with the viruses as described previously (26). Viral RNAs were separated in a 1% agarose gel that contained formaldehyde, transferred to a nitrocellulose membrane, hybridized with the ^{32}P -radio-labeled DNA probes that contained the MCMV sequences, and finally analyzed with a STORM 840 PhosphorImager. The DNA probes used for Northern analyses were generated by PCR using viral DNA as the template and radiolabeled with a random primer synthesis kit in the presence of [^{32}P]dCTP (Boehringer Mannheim, Indianapolis, Ind.). The 5' and 3' PCR primers used to construct the DNA probe for the transcript in the M37 region were M37-5'NDS (5'-AAATCACACCGAGACGGTGC-3') and M37-3'NDS (5'-ATCTTTGAAC AGCGACTCGC-3'), respectively. The 5' and 3' PCR primers used to construct the DNA probe for transcripts in the M25 region were M25-5'NDS (5'-CGAC GACGATGACGACGATG-3') and M25-3'NDS (5'-GTCCTGACCGCTCAC TACAC-3'), respectively.

RESULTS

Construction of an MCMV mutant with the transposon insertion at open reading frame M37. Using an *E. coli* Tn3-based transposon mutagenesis system, we recently constructed a pool of MCMV mutants carrying random insertions of the transposon sequence (53). Figure 1A shows the structure of the transposon used to generate MCMV mutants. The transposon, designated Tn3-gpt, contains the expression cassette

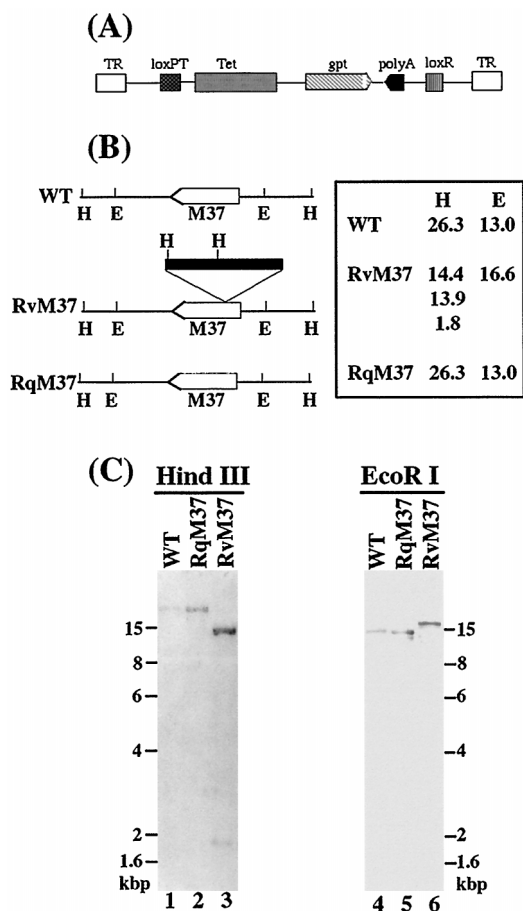


FIG. 1. (A) Schematic representation of the structure of the transposon construct used for mutagenesis. TR, terminal repeat; Tet, tetracycline resistance gene; poly(A), transcription termination signal. (B) Location of the transposon insertion in the recombinant virus. The transposon sequence is shown as a filled bar; the coding sequence of open reading frame M37 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 (37); numbers represent sizes of the DNA fragments of the viruses that were generated by digestion with *Hind*III (H) or *Eco*RI (E). WT, wild type. (C) Southern analyses of recombinant viruses. DNA samples (20 µg) isolated from cells infected with the wild-type (WT) virus, RvM37, or RqM37 were digested with either *Hind*III (H) or *Eco*RI (E), separated on 0.8% agarose gels, transferred to a Zeta-Probe membrane, and hybridized to a DNA probe (the plasmid that contained the MCMV DNA fragment inserted with the transposon sequence).

encoding guanine phosphoribosyltransferase (*gpt*) and an additional transcription termination site, which allow selection of MCMV mutants in mammalian cells and transcription termination of the target gene at the insertion site, respectively (53). The direction of transcription of the *gpt* expression cassette is opposite that of the additional poly(A) signal (Fig. 1A). Such a design should ensure undisrupted expression of nearby genes that may share a poly(A) signal with the targeted gene.

To generate a pool of MCMV mutants containing a transposon sequence, an MCMV genomic library containing a randomly inserted transposon in each fragment was first created by using a shuttle mutagenesis method as described by Zhan et al. (53). Such a library of Tn3-*gpt*-MCMV fragments were then cotransfected with the full-length genomic DNA of the wild-type virus (Smith strain) into mouse NIH 3T3 cells. Homologous recombination between the full-length Smith strain viral DNA and the DNA fragments inserted with the Tn3-*gpt* trans-

poson sequence occurs in the transfected NIH 3T3 cells. Cells that contained the recombinant viruses would express the *gpt* protein and were allowed to grow in culture medium containing mycophenolic acid and xanthine. Under these conditions, only the viruses expressing the *gpt* protein were selected (18, 33, 49). After multiple rounds of selection and plaque purification, we isolated individual recombinant viruses and determined the location of the inserted transposon by directly sequencing the genomic DNA of the recombinants. One of the recombinant viruses that were further characterized is reported here. This viral mutant, designated RvM37, contains the transposon sequence inserted within open reading frame M37 (Fig. 1B). Sequence analyses of the junction between the transposon and the viral sequence revealed that the transposon in RvM37 is located at nucleotide position 50104 (amino acid residue 125 of the 345-amino-acid-long open reading frame) with reference to the genome sequence of the wild-type Smith strain (37) (Fig. 1B and data not shown).

Characterization of the genomic structure of viral mutant RvM37 and rescued virus RqM37. Southern blot hybridization analysis was carried out to examine the genomic structure of RvM37 and to map the location of the transposon insertion in the viral genome, using a DNA probe containing both the transposon and viral sequences (Fig. 1B and C). A small fragment of 1.8 kb representing the *gpt* gene was detected when the viral DNA samples were digested with *Hind*III and subjected to Southern analyses. This observation indicates the presence of the transposon sequence within the viral genome (Fig. 1C, lane 3). This finding was further supported by the results of Southern analyses of the RvM37 DNA samples digested with another restriction enzyme, *Eco*RI (Fig. 1C, lane 6). In these experiments, the genomic fragments containing the transposon were shown to be 3.6 kb larger than the wild-type virus, which is the size of the transposon (Fig. 1B and C).

The Southern blots also showed that stocks of the mutant virus were pure and free of the wild-type strain, as hybridizing DNA fragments from the mutant did not comigrate with those of the wild-type Smith strain (Fig. 1C, lanes 1, 3, 4, and 6). For example, the hybridization patterns of the RvM37 and Smith strain DNAs digested with *Hind*III gave rise to DNA bands of 14.4, 13.9, and 1.8 kb and a single DNA band of 26.3 kb, respectively (Fig. 1C, lanes 1 and 3). Meanwhile, the hybridized species (16.6 kb) of the *Eco*RI-digested RvM37 DNA migrated differently from that (13 kb) of the wild-type viral DNA digested with the same enzyme (Fig. 1C, lanes 4 and 6). The sizes of the hybridized DNA fragments (Fig. 1C) were consistent with the predicted digestion patterns of the recombinant virus, based on the MCMV genomic sequence (37) and the location of the transposon insertion in the viral genome as determined by sequence analysis (Fig. 1B). The restriction enzyme digestion patterns of the regions of the RvM37 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 that containing the transposon insertion remained intact in this MCMV mutant.

To restore the M37 open reading frame, a rescued virus, designated RqM37, was derived from RvM37 via a protocol similar to that used for construction of the viral mutants. A DNA fragment that contained the M37 coding region was cotransfected with the full-length RvM37 genomic DNA into mouse STO cells. Homologous recombination between the full-length RvM37 viral DNA and the DNA fragment containing the M37 coding sequence would occur in the transfected cells. The cells that harbored the progeny viruses were allowed

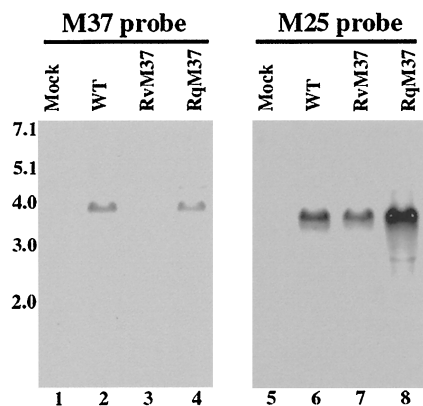


FIG. 2. Northern analyses of RNA fractions isolated from cells that were mock infected (lanes 1 and 5) or infected with the wild-type virus (WT; lanes 2 and 6), RvM37 (lanes 3 and 7), and RqM37 (lanes 4 and 8). NIH 3T3 cells (10^7) were infected with each virus at an MOI of 5 PFU per cell and harvested at 24 h postinfection. RNA samples (20 to 30 μ g) 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 M37 (lanes 1 to 4) or M25 (lanes 5 to 8). Sizes are indicated in kilobase pairs.

to grow in the presence of 6-thioguanine, which selects against *gpt* expression (18, 33). The rescued virus, RqM37, which did not express the *gpt* protein and no longer contained the transposon, was isolated after multiple rounds of selection and plaque purification.

To determine whether the M37 region was restored in RqM37, the genomic structure of the rescued virus was studied by Southern hybridization analyses using DNA probes containing the transposon and the viral sequence (Fig. 1C). Analysis of the RqM37 DNA samples digested with *Hind*III and *Eco*RI showed that the sizes of the hybridized DNA fragments for RqM37 were identical to those of the hybridized fragments for the Smith strain and different from those for RvM37. These results indicate that RqM37 did not contain the transposon sequence and the M37 region was restored (Fig. 1C, lanes 2 and 5). Moreover, the restriction enzyme digestion patterns of the regions of the rescued RqM37 genomic DNA samples other than the M37 region appeared to be identical to those of the parental RvM37, as indicated by ethidium bromide staining of the digested DNAs (data not shown). These observations suggest that regions of the RqM37 genome other than the M37 region remained intact and identical to those of RvM37. Thus, RqM37 may represent a rescued virus for RvM37.

Characterization of transcription from the MCMV M37 region in tissue culture. Whether there is transcription from the M37 open reading frame has not been reported. Transcription from the target M37 region is expected to be disrupted due to the presence of the two transcription termination signals within the transposon (Fig. 1A). In particular, the region of the M37 open reading frame downstream from the transposon insertion site is not expected to be expressed. To determine whether this is the case, cytoplasmic RNAs were isolated from cells infected with the mutant virus at different time points (e.g., 4, 12, and 24 h) postinfection. Northern analysis was carried out to detect expression of the transcripts from the M37 open reading frame downstream from the transposon insertion site (Fig. 2). The probe used in the Northern analyses contained the DNA sequence complementary to the M37 coding region about 100 nucleotides downstream from the site of the transposon insertion. An abundant RNA species of about 4 kb was detected in the RNA fractions isolated from cells that

were infected with the wild-type Smith strain (Fig. 2, lane 2). However, this transcript was not detected in the RNA fractions isolated from cells infected with RvM37 when the same probe was used (Fig. 2, lane 3). Meanwhile, expression of the transcript was found in the RNA fractions from cells infected with the rescued virus RqM37 (Fig. 2, lane 4). The level of MCMV M25 transcript (14, 52, 53) was used as the internal control for expression of the M37 transcript. As shown in Fig. 2 (lanes 5 to 8), substantial amounts of the M25 transcript were detected in cells that were infected with RvM37, RqM37, and the Smith strain (Fig. 2, lanes 5 to 8). Thus, the transposon insertion in RvM37 disrupted the transcript expressed from the M37 open reading frame, whereas expression of the transcript was restored in RqM37. A probe from the 3' end of the M37 open reading frame sequence was also used in the Northern analysis. With this probe, the M37 transcript of \sim 4 kb was readily detected in cells infected with the Smith strain and RqM37. However, the M37 transcript was not detected in cells infected with RvM37. These results further support our conclusion that the region of the M37 open reading frame downstream from the transposon insertion site is not expressed.

Characterization of growth of viruses in vitro in tissue culture. To determine whether the recombinant viruses RvM37 and RqM37 have any growth defects in vitro, NIH 3T3 cells were infected with these viruses at both low and high MOIs. Growth rates of the viruses in mouse NIH 3T3 cells were assayed and compared to those of the parental Smith strain. The results, obtained from triplicate experiments (Fig. 3), indicate that no significant difference was found in growth rates among RvM37, RqM37, and the Smith strain. For example, the peak titers of RvM37 and RqM37 were similar to that of the parental Smith strain (Fig. 3). These results, combined with those from the Southern and Northern analyses, suggest that M37 is not essential for viral growth in tissue culture.

Characterization of growth of viruses in immunocompetent animals. To determine whether disruption of M37 significantly affects viral growth in vivo, BALB/c-Byj mice were injected intraperitoneally with 10^4 PFU of RvM37, RqM37, or the wild-type Smith strain. At 1, 3, 7, 10, 14, and 21 days postinfection, salivary glands, lungs, spleens, livers, and kidneys were harvested, and viral titers from these five organs were determined on NIH 3T3 cells (Fig. 4). The titers of viruses from the salivary glands at 28 days postinfection were also determined (Fig. 4A). These organs are among the major targets for MCMV infection (4, 21, 24, 32). At days 14 and 21 postinfection, the titers of RvM37 found in the salivary glands were about 200- and 2,000-fold, respectively, lower than those of the Smith strain (Fig. 4A). Moreover, the titers of RvM37 found in the lungs, spleens, livers, and kidneys of the infected animals at 10 days postinfection were at least 10-fold lower than the titers of the Smith strain found in the same organs from the infected animals (Fig. 4B to E). In contrast, the titers of the rescued virus RqM37 found in these organs were similar to the titers of the Smith strain. Previous studies have shown that the presence of the transposon sequence per se within the viral genome does not significantly affect viral growth in mice in vivo (54). Thus, these results suggest that the attenuated growth of RvM37 is due to the disruption of M37 and that open reading frame M37 is important for MCMV growth in these five organs in vivo in BALB/c mice.

Characterization of virulence of the viruses in immunodeficient mice. It has been shown that immunodeficient animals are extremely susceptible to MCMV infection (19, 34, 36, 38). For example, CB17 SCID mice, which lacks functional T and B lymphocytes, are extremely sensitive to viral infection (as few as 10 PFU of MCMV could cause serious infection) (34, 36).

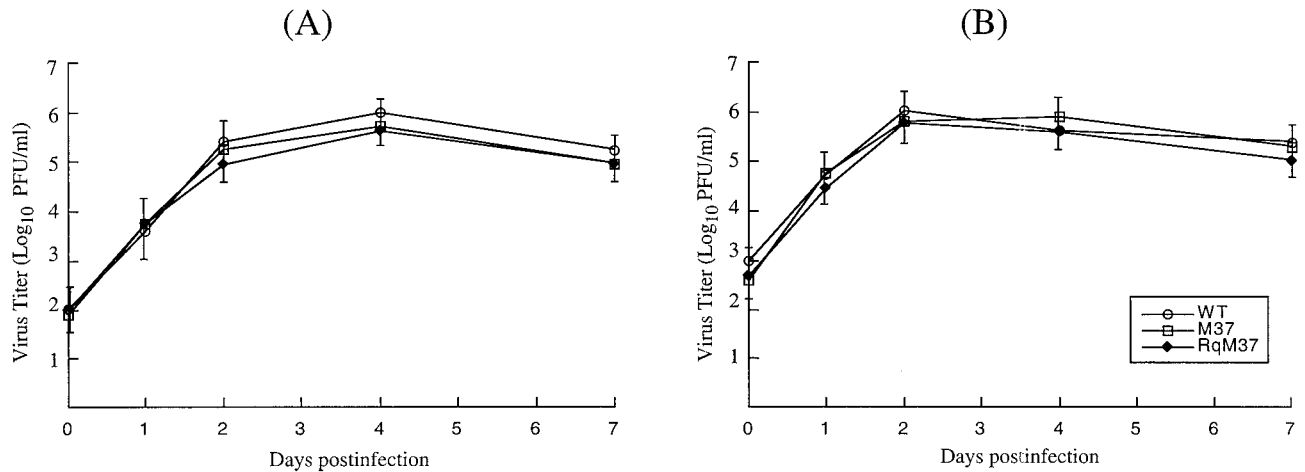


FIG. 3. In vitro growth of MCMV mutants in tissue culture. Mouse NIH 3T3 cells were infected with each virus at an MOI of 0.5 (A) or 5 (B) PFU per cell. At 0, 1, 2, 4, and 7 days postinfection, cells and culture media were harvested and sonicated. Viral titers were determined by plaque assays on NIH 3T3 cells; the values shown represent averages from triplicate experiments; standard deviations are indicated by the error bars. WT, wild type.

Analysis of viral pathogenesis in these mice therefore serves as an excellent model for determining the virulence of different MCMV strains and mutants and studying the mechanism of how they cause opportunistic infections in immunocompromised hosts. To determine whether the M37 open reading frame plays a significant role in CMV virulence, we compared the survival rates of animals infected with RvM37 and those of mice infected with RqM37 and the wild-type Smith strain. For

each virus, five SCID mice were injected intraperitoneally with 10^4 PFU of RvM37, RqM37, or the Smith strain. All mice infected with the wild-type virus or RqM37 died within 23 to 26 days postinfection, whereas none of the RvM37-infected mice had died by 50 days postinfection (Fig. 5). Indeed, no RvM37-infected animals had died by 120 days postinfection, at which time the experiments were terminated because some of the mock-infected mice succumbed to unrelated opportunistic in-

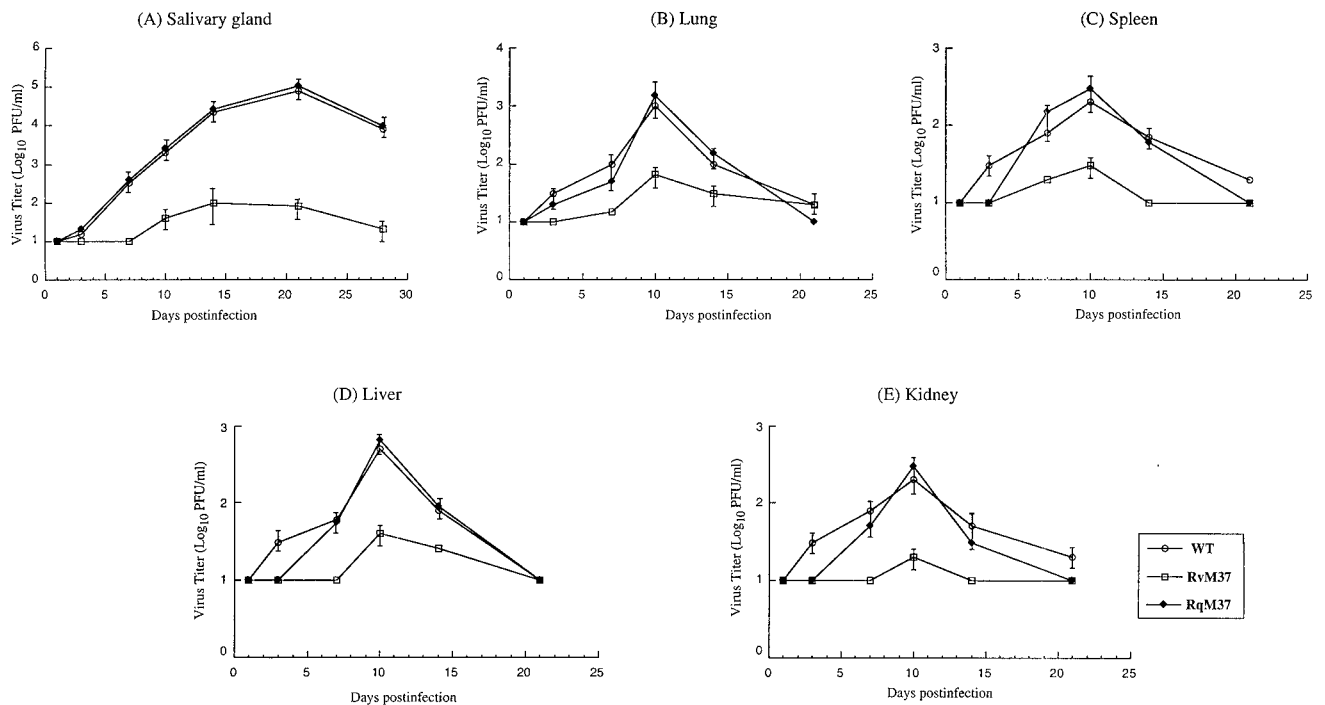


FIG. 4. Titers of MCMV mutants in salivary glands, lungs, spleens, livers, and kidneys of infected BALB/c mice. BALB/c-Byj mice were infected intraperitoneally with 10^4 PFU of each virus. At 1, 3, 7, 10, 14, and 21 days postinfection, the animals (three mice per group) were sacrificed. The salivary glands, lungs, spleens, livers, and kidneys were collected and sonicated. The salivary glands were also collected from animals at 28 days postinfection. 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 values shown represent averages from triplicate experiments; the error bars indicate standard deviations. Error bars that are not evident indicate that the standard deviation was less than or equal to the height of the symbols. WT, wild type.

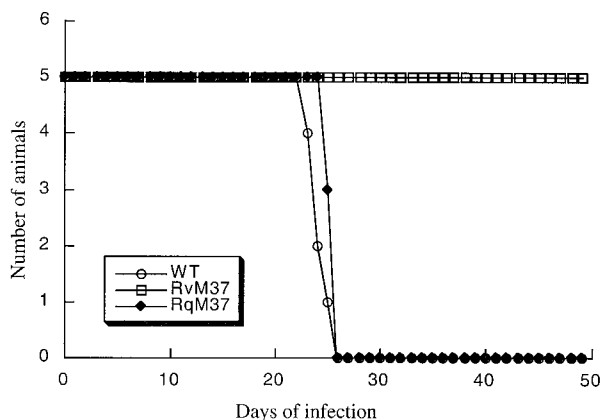


FIG. 5. Mortality of SCID mice infected with the Smith strain (wild type [WT]), RvM37, and RqM37. CB17 SCID mice (five animals per group) were infected intraperitoneally with 10^4 PFU of each virus. Mortality was monitored for at least 120 days postinfection, and survival rates were determined.

fections (data not shown). This observation indicated that RvM37 is avirulent in SCID mice. It has recently been demonstrated in our laboratory that the presence of the transposon sequence per se within the viral genome does not significantly affect MCMV virulence in SCID mice (54). Thus, these results suggest that disruption of the M37 open reading frame abolishes viral virulence and that M37 may be essential for MCMV virulence in SCID mice.

Characterization of growth of the viruses in immunodeficient mice. To further study the pathogenesis of the mutant

virus in SCID mice, replication of RvM37 in different organs of the mice was studied during a 21-day infection period before mortality of the animals infected with the Smith strain and RqM37 became apparent. In these experiments, SCID mice were injected intraperitoneally with 10^4 PFU of each virus. At 1, 3, 7, 10, 14, and 21 days postinfection, we sacrificed three mice from each virus group and harvested the salivary glands, lungs, spleens, livers, and kidneys. The viral titers in these five organs were determined. At 21 days postinfection, titers of the Smith strain and rescued virus RqM37 in the salivary glands, lungs, spleens, livers, and kidneys were about 5×10^5 , 2×10^5 , 5×10^4 , 5×10^3 , and 1×10^4 PFU/ml of organ homogenate, respectively; in contrast, titers of RvM37 in the salivary glands, lungs, spleens, and kidneys were less than 2×10^1 PFU/ml of organ homogenate, while RvM37 titers in the livers were less than 10^2 PFU/ml of organ homogenate (Fig. 6). Therefore, RvM37 appeared to be severely attenuated in growth in the organs from immunodeficient animals. Previous studies have shown that the presence of the transposon sequence per se within the viral genome does not significantly affect viral growth in SCID mice in vivo (54). Thus, these results suggest that the attenuated growth of RvM37 in these organs is probably due to the disruption of M37 and that M37 is essential for optimal growth of MCMV in these organs in immunodeficient hosts.

Characterization of genomic stability of the viral mutant after replication in vivo. Our previous studies indicated that a transposon sequence inserted at several regions (e.g., m09, M25, and M83) of the MCMV genome is stable during viral replication in NIH 3T3 cells and in both BALB/c and SCID mice (53, 54). However, it is not known whether the transposon sequence inserted at the M37 region is stable. It has been

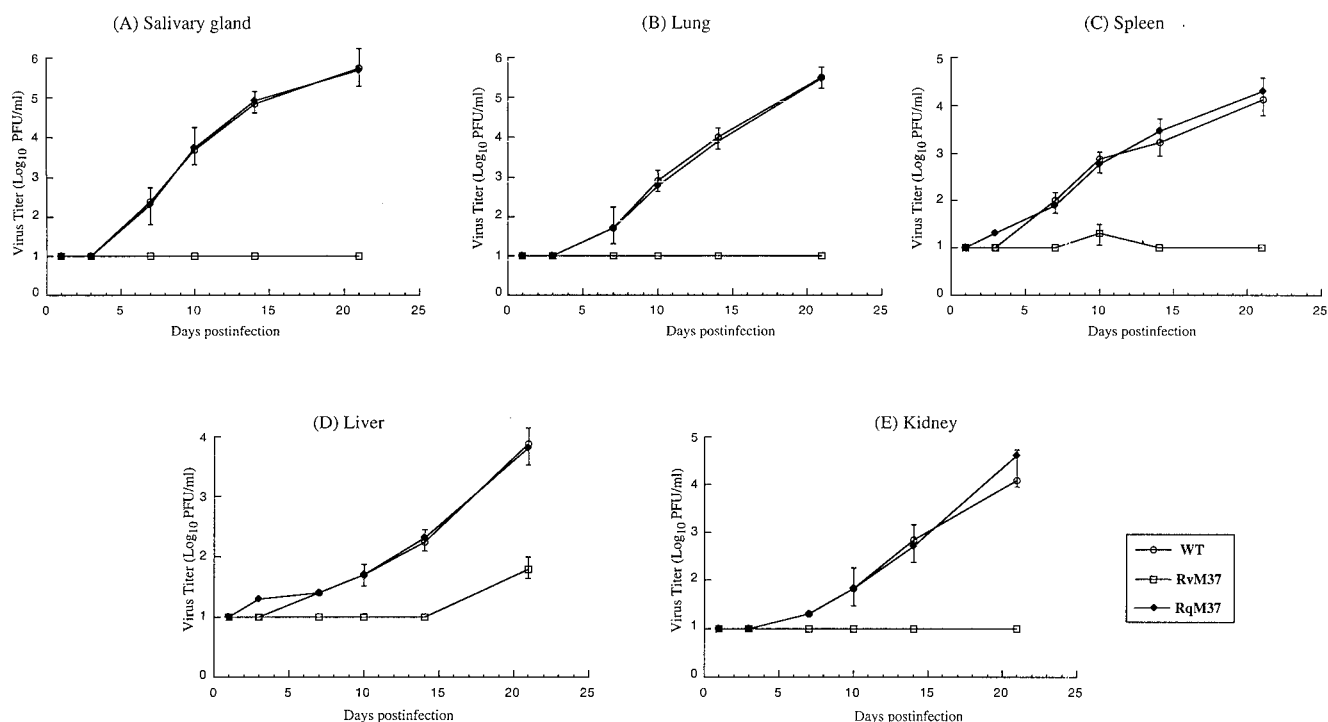


FIG. 6. Titers of MCMV mutants in salivary glands, lungs, spleens, livers, and kidneys of infected SCID mice. CB17 SCID mice were infected intraperitoneally with 10^4 PFU of each virus. At 1, 3, 7, 10, 14, and 21 days postinfection, the animals (three mice per group) were sacrificed. The salivary glands, lungs, spleens, livers, and kidneys were collected and sonicated. 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 values shown represent averages obtained from triplicate experiments; the error bars indicate standard deviations. Error bars that are not evident indicate that the standard deviation was less than or equal to the height of the symbols. WT, wild type.

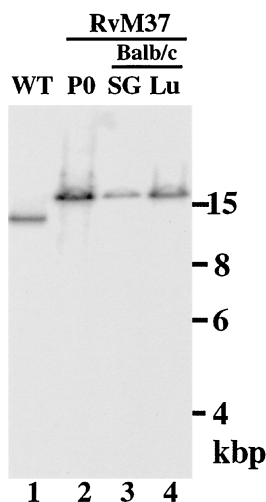


FIG. 7. Stability of the transposon mutation of RvM37 in BALB/c mice. Viral DNAs were isolated either from cells that were infected with RvM37 (MOI < 0.01) and allowed to grow in culture for 5 days (P0; lane 2) or from cells that were infected with the virus collected from the salivary glands (SG; lanes 3) or lungs (LU; lane 4) of BALB/c mice 10 (lane 4) or 21 (lane 3) days after intraperitoneal inoculation with 10^4 PFU of RvM37. Southern analyses of the viral DNA fractions digested with *EcoRI* are shown. 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 RvM37 in Fig. 1 and contained the transposon and the M37 open reading frame sequence.

shown that viral mutants with an additional insertion sequence are not stable and generate spontaneous mutations during replication in vitro and in vivo (2, 29; X. Zhan, M. Lee, G. Abenes, and F. Liu, unpublished results). It is possible that the transposon sequence in RvM37 is not stable during viral replication in vivo, and the introduction of an adventitious mutation may be responsible for the observed phenotypes of the virus in animals. To investigate whether the genome of RvM37 is stable during replication in vivo, the viruses were recovered from salivary glands of the RvM37-infected BALB/c mice as well as livers of the RvM37-infected SCID mice at 21 days postinfection. Viruses were also recovered from the lungs of infected BALB/c mice at 10 days postinfection. Viral DNAs were purified, and their restriction digestion patterns were analyzed in agarose gels. Figure 7 shows a Southern analysis of the RvM37 viral DNAs isolated from the salivary glands and lungs of the infected BALB/c mice with a DNA probe that contained the transposon and M37 sequence. These results indicated that no change in the hybridization patterns of RvM37 occurred as a result of viral growth in the animals for either 10 or 21 days (lanes 2 to 4). Moreover, the overall *EcoRI* digestion patterns of RvM37 DNA isolated from either infected cultured cells or animals were identical to those of the original recombinant virus RvM37, as visualized by ethidium bromide staining of the viral DNAs (data not shown). Similar results were observed for viruses isolated from livers of the infected SCID mice (data not shown). Thus, the transposon insertion in RvM37 appeared to be stable, and the genome of RvM37 remained intact during replication in the animals.

DISCUSSION

In this study, we have characterized an MCMV mutant that contained a transposon insertional mutation in open reading frame M37. Our results provide the first direct evidence to suggest that M37 is not essential for viral replication in vitro in

NIH 3T3 cells. Moreover, disruption of the M37 open reading frame results in severely reduced growth of the virus in both immunocompetent and immunodeficient hosts and abolishes viral virulence in killing SCID mice. These observations strongly suggest that M37 probably encodes a virulence factor and is required for MCMV virulence in killing SCID mice and for optimal viral growth in vivo.

While it is possible that the functional protein product might be synthesized from the transposon-disrupted region, the results presented here suggest that this may not be the case. First, the transposon sequence was inserted into the 5' region of the M37 coding sequence (Fig. 1B). Second, the transcription from the region downstream from the transposon insertion site was not detected in cells infected with the mutant virus (Fig. 2). Thus, the region of the target open reading frame downstream from the transposon insertion site, which includes about 65% of the M37 coding sequence, was not expressed. Therefore, it is likely that no functional M37 protein was expressed from the viral mutant. Our results indicate that the growth rate of RvM37 in NIH 3T3 cells was not significantly different from that of the Smith strain. These observations suggest that M37, or at least the carboxyl-terminal sequence of the open reading frame, is not essential for viral replication in NIH 3T3 cells.

Our results indicate that RvM37 replicated poorly in the salivary glands, lungs, spleens, livers, and kidneys of both BALB/c and SCID mice that were intraperitoneally infected. For example, at 21 days postinfection, the titers of RvM37 in the salivary glands, lungs, spleens, and kidneys of the SCID mice were less than 2×10^1 PFU/ml of organ homogenate, while the RvM37 titers in the livers were less than 10^2 PFU/ml of organ homogenate. In contrast, titers of the Smith strain as well as of rescued virus RqM37 in the salivary glands, lungs, spleens, livers, and kidneys of the infected animals were about 5×10^5 , 2×10^5 , 5×10^4 , 5×10^3 , and 1×10^4 PFU/ml of organ homogenate, respectively (Fig. 6). Moreover, all SCID mice infected with RvM37 survived up to 120 days postinfection, while all mice infected with the Smith strain or RqM37 died within 26 days postinfection (Fig. 5). These results strongly suggest that M37 is a determinant for MCMV growth in vivo in these animals and for virulence in SCID mice.

It is possible that the observed change in the levels of virulence and growth of the mutant in animals is due to other adventitious mutations introduced during the construction and growth of the recombinant virus in cultured cells or in animals. However, several lines of evidence strongly suggest that this is unlikely. First, the wild-type phenotypes for growth in both BALB/c and SCID mice and virulence in SCID mice were restored in RqM37 upon restoration of the wild-type sequence in RvM37 (Fig. 1, 4, 5, and 6). Furthermore, restoration of the wild-type phenotypes in RqM37 occurred together with the restoration of M37 expression (Fig. 2). These observations suggest that the transposon insertion rather than an adventitious mutation is responsible for the observed attenuation of RvM37 replication and virulence in BALB/c and SCID mice. Second, our previous studies indicated that a virus mutant (i.e., Rvm09) with transposon insertion at the m09 open reading frame replicated as well as the wild-type virus in both BALB/c and SCID mice (54). Moreover, mutant Rvm09 exhibited a similar level of lethality in SCID mice as the wild-type virus. These observations indicated that the transposon sequence per se in the viral genome does not significantly affect viral replication and virulence in these animals (54). Third, the genome and the transposon insertion in the viral mutant were stable during replication in animals. There was no change in the hybridization patterns of the DNAs from the mutant viruses that were recovered from various organs of the infected ani-

mals after either 10 or 21 days of infection (Fig. 7 and data not shown). Moreover, the *EcoRI* digestion patterns of the RvM37 mutant DNAs, other than the transposon insertion region, appeared to be identical to those of the wild-type virus DNA (data not shown). Thus, the observed change in the level of RvM37 replication and virulence in the infected animals is probably due to the disruption of M37 expression as a result of the transposon insertion.

The function of M37 is not known. Indeed, to our knowledge, neither the transcript nor the protein product encoded by this open reading frame has been reported. Our results indicate that a transcript of about 4,000 nucleotides is expressed from the M37 open reading frame. UL37, the HCMV counterpart of M37, encodes at least three immediate-early glycoproteins, UL37x1, gpUL37, and UL37m (9, 17). These three proteins share the amino-terminal sequence because of alternative splicing and polyadenylation of transcripts initiating at the same promoter and have been shown to be localized in the mitochondria as well as in the plasma membrane (9, 11, 17). The UL37-encoded proteins have been shown to transactivate selectively genes under control of both cellular and HCMV promoters and, more recently, have also been found to have antiapoptotic activities (10, 12, 17). It appears that the hydrophobic leader sequence and the acidic domain, both located within the first 120 amino acids at the amino terminus of these proteins, are responsible for the antiapoptotic and transcription regulation activities, respectively (10, 12, 17). Indeed, an HCMV mutant with a deletion of the exon 3 region of gpUL37 grows as well as the wild-type virus in human cultured cells, suggesting that the carboxyl-terminal region of UL37 is not essential for HCMV replication *in vitro* (3). Meanwhile, previous studies suggest that transcripts from the UL36–UL38 region may be essential for viral replication *in vitro* (35, 44). Thus, whether the amino-terminal region of UL37 is essential for HCMV replication *in vitro* is still not clear. Meanwhile, the functions of UL37 in HCMV pathogenesis and virulence *in vivo* are not known. Our results provide the first direct evidence to suggest that M37 is probably required for optimal viral growth *in vivo* in both immunocompetent and immunodeficient hosts and is essential for virulence in SCID mice. It will be interesting to determine whether M37, like UL37, also possesses transactivation and antiapoptotic activities. Extensive homology in amino acid sequence between M37 and UL37 was found at the carboxyl-terminal region (7, 37). However, very limited sequence homology was found between the amino-terminal sequences of these two open reading frames (7, 37; M. Lee, A. McGregor, and F. Liu, unpublished results). These observations raise the possibility that the carboxyl-terminal regions of these open reading frames may be important for CMV pathogenesis. Further studies will reveal how M37 functions as a viral virulence factor and may provide insight into the function of UL37 in HCMV pathogenesis and virulence in humans. Meanwhile, further studies are needed to investigate whether UL37 is functionally equivalent to M37.

Previous studies have shown that MCMV mutants with deletion of a cluster of at least three open reading frames (e.g., m139, m140, and m141) are avirulent in killing SCID mice (6, 20). The results reported in this study, to our knowledge, demonstrate for the first time that disruption of a single MCMV open reading frame leads to complete abolishment of viral virulence in SCID mice. A key question from our results is how the lack of M37 leads to a change in the level of virulence and growth. It is possible that the defect of M37 inhibits the spread of the virus to the target organs, entry into permissive cells, or full replication inside infected cells. Given that UL37 possesses transactivation and antiapoptotic activities (12, 17), it is con-

ceivable that a viral mutant with disruption of M37, while replicating normally in NIH 3T3 fibroblasts, exhibits a defect in certain steps of viral replication, such as viral gene expression, *in vivo* in particular organs or tissues. This defect may lead to slow growth of the viral mutant in the organs and consequently contribute to severe reduction of growth of the viral mutant *in vivo*. Alternatively, M37 may be involved in virus-host interactions and play an important role in modulating the host cells for optimal viral replication. Further studies on *in vitro* and *in vivo* replication of RvM37 and other viral mutants will provide further insight into the functions of viral genes *in vivo* in MCMV virulence and pathogenesis.

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