Murine Cytomegalovirus with a Transposon Insertional Mutation at Open Reading Frame M35 Is Defective in Growth In Vivo

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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 (26, 33). For example, disseminated HCMV infection, common in AIDS patients and organ transplant recipients, is usually associated with gastrointestinalitis, pneumonia, and retinitis (10, 32). Moreover, HCMV is one of the leading causes of birth defects and mental retardation in newborns (9). Understanding the biology of CMV infection and developing novel anti-CMV approaches are central in the treatment and prevention of CMV-associated diseases.

HCMV contains a linear 230-kb-long DNA genome that is predicted to encode >200 proteins (4). This virus belongs to the β family of herpesviruses, whose members share the common characteristic of being highly specific species (26, 33). This characteristic essentially precludes the use of experimental animals in studying HCMV infections and pathogenesis. Consequently, other related model systems involving animal CMVs, such as murine CMV (MCMV) or rat, guinea pig, or nonhuman primate CMV, have to be used to provide insight into the tissue tropism, virulence, latency, and reactivation of HCMV (3, 14, 17, 26, 37).

Infection of the mouse with MCMV provides an invaluable in vivo model for studying the biology of CMV infection. This is because infection of mice with MCMV resembles its human counterpart in many ways with respect to pathogenesis during acute infection, establishment of latency, and reactivation after immunosuppression (14, 17, 26, 33). For example, tropism for the salivary gland is believed to be important in infection by both HCMV and MCMV (26, 33). Persistent and recurrent shedding of viral particles from the salivary gland appears to be the principal means by which these viruses spread in the population. MCMV has a genome of 230 kb that is predicted to encode >170 open reading frames, 78 of which have extensive homology with those of HCMV (4, 35). However, many of these MCMV genes remain uncharacterized, and their functions in viral pathogenesis have not been investigated.

One of the most powerful approaches to study the functions of virus-encoded genes is to introduce mutations into the viral genome and to screen viral mutants in both tissue culture and animals for possible growth defects in vitro and in vivo. The construction of herpesvirus mutants was first reported using site-directed homologous recombination and then using transposon-mediated insertional mutagenesis, as well as cosmids libraries of overlapping viral DNA fragments (16, 18, 27, 39, 40, 43). Recently, the MCMV genome, as well as the genomes of other herpesviruses, have been cloned into a bacterial artificial chromosome, and MCMV mutants were successfully generated from the bacterial artificial chromosome-based viral genome by both site-directed homologous recombination and transposon-mediated insertional mutagenesis (2, 7, 25, 38). These studies have greatly facilitated the identification 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 likely needed to perform functions involved only in modulating the interactions between the virus and its respective human or animal hosts. For example, HCMV US11, a nonessential protein, functions to downregulate the expression and presentation of the major histocompatibility
complex class I molecules (44). Meanwhile, MCMV open reading frame m133, which is also called salivary gland gene 1 (sgg1) and is dispensable for viral replication, is a determinant for MCMV replication in the salivary gland in vivo (19, 23). Thus, studies of viral mutants carrying mutations in genes found to be dispensable in tissue culture are valuable for the understanding of gene function in viral pathogenesis and virus-host interactions, including tissue tropism and virulence.

The use of a Tn3-based transpositional-mutagenesis approach to disrupt genes in the MCMV genome and the generation of a pool of recombinant viruses that carry the disrupted genes were previously reported by members of our laboratory (46, 47). In this approach, the transposon is randomly inserted into the MCMV genomic DNA fragments in a plasmid library in Escherichia coli. Regions bearing an insertional mutation are then transferred to the MCMV genome by homologous recombination between the plasmid library and purified MCMV genomic DNA in NIH 3T3 cells. In the present study, we have characterized an MCMV mutant, RvM35, which contains a transposon insertion in open reading frame M35, a homologue of HCMV open reading frame UL35 (4, 35). The function of the M35 open reading frame leads to an attenuation of viral virulence and deficient growth in vivo (when the mutant virus was used to infect immunocompetent Balb/c mice and immunodeficient SCID mice intraperitoneally, the viral titers in the salivary glands, lungs, spleens, livers, and kidneys were significantly lower than those in mice inoculated with the wild-type virus and a revertant virus that rescued the mutation and restored the M35 open reading frame. Moreover, the viral mutant was attenuated in its ability to kill SCID mice. These results suggest that M35 is a viral determinant for MCMV pathogenicity and that it is required for optimal viral virulence and growth in vivo.

To construct the rescued virus RqM35, the full-length genomic DNA of RvM35 was isolated from infected cells as described previously (46). The full-length intact RvM35 genomic DNA and the DNA sequence that contained the coding sequence of M35 were subsequently cotransfected into mouse cells using a calcium phosphate precipitation protocol (Gibco BRL). A combination virus was selected in STO cells in the presence of 25 μg of 6-thioguanine (Sigma)/ml and purified by six rounds of amplification and plaque purification, following the protocol described previously (12). 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.

Northern and Southern analyses of recombinant viruses. Cells were infected with viruses at a multiplicity of infection (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 (20). Viral RNAs were separated in 1% agarose gels that contained formaldehyde, transferred to a nitrocellulose membrane, hybridized with the 32P-labeled DNA probes that contained specific MCMV sequences, and finally analyzed with a STORM840 PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). 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 [32P]dCTP (Boehringer Mannheim, Indianapolis, Ind.). The 5′ and 3′ PCR primers used for the construction of the DNA probe to detect the transcription of the M35 region were JM522downp2 (5′-AGAGGTCGAAGGAGGAA-3′), respectively. The 5′ and 3′ PCR primers used in the construction of the DNA probe to detect the transcript in the M25 region were M25-5′NDS (5′-GCGAGCATGACGAGGATGAT-3′) and M25-3′NDS (5′-GTCCTGACGCCTAATCAC-3′), respectively.

For Southern analysis, viral genomic DNA was purified from NIH 3T3 cells infected with the viruses as described previously (41, 46). Briefly, cells that exhibited 100% cytopathic effect were washed with phosphate-buffered saline and then subjected to proteinase K digestion with 0.5% 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 digested with HindIII or EcoRI, separated on agarose gels (0.8%), transferred to Zeta-Probe nylon membranes (Bio-Rad, Hercules, Calif.), and hybridized with 32P-labeled DNA probes specific for both the transposon and the MCMV sequences. The results were analyzed using a STORM840 PhosphorImager.

Growth kinetics of recombinant viruses. The analyses of the growth of the recombinant viruses in vitro were carried out as described previously (46). In brief, 5 × 105 NIH 3T3 cells were infected at an MOI of either 0.5 or 5.0 PFU per cell. The cells and medium were harvested 0, 1, 2, 4, and 7 days postinfection, and viral stocks were prepared by adding an equal volume of 10% skim milk, followed by sonication. The titers of the viral stocks were determined by plaque assays in triplicate experiments.

Viral growth studies in animals. Four-week-old male Balb/c-Byj mice (Jackson Laboratory, Bar Harbor, Maine) or 6-week-old CB17 SCID mice (National Cancer Institute, Bethesda, Md.) were infected intraperitoneally with 106 PFU of each virus. The animals were sacrificed at different time points (e.g., 3 days) postinoculation as specified in Results below. For each time point, at least three animals were used as a group and infected with the same virus. The whole salivary gland, ~0.1 to 0.2 g of the liver, one-fourth of the lungs, the whole spleen, and one of the kidneys were collected individually into 3-ml sterile tubes. To avoid cross-contamination of viruses between organs and different recombinants, surgical tools (forceps and scissors) were rinsed once in phosphate-buffered saline and three times in 70% ethanol and flamed after each rinse in ethanol. Each sample was suspended in a mixture of Dulbecco's modified Eagle medium and 10% skim milk (50% [vol/vol]) at 0.1 g/ml. The organs were then sonicated on ice using a 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, Pa.), until they became homogenized. The samples were stored at −80°C until the titers of the viruses in the samples were determined.

Titers of the viruses harvested from the mice were determined on NIH 3T3 cells in six-well tissue culture plates (Corning Inc., Corning, N.Y.). Briefly, cells were first split 1:30 from T-150 flasks into six-well plates, cultured overnight (16 to 24 h), and then infected with the viruses at 10-fold serial dilutions. After 2 h of incubation with the homogenates diluted in 1 ml of complete medium at 37°C with 5% CO2, the cells were overlaid with fresh complete medium containing 1% agarose and cultured for 4 to 5 days before the plaques were counted under an inverted microscope. Viral titers were recorded as PFU per ml of organ homogenates. The titer of each sample was determined in triplicate. The limit of virus detection in the organ homogenates was 10 PFU/ml of the sonicated
mixture. Those samples that were negative at a 10^{-1} dilution were assigned a
titer value of 10 PFU/ml.

**Viral virulence assays in SCID mice.** The virulences of the viruses were
studied by determining the mortality of the animals infected with the Smith
strain, RvM35, or RqM35. The CB17 SCID mice (10 animals per group) were
infected intraperitoneally with 10^5 PFU of each virus. The animals were observed
twice daily, the mortality of the infected animals was monitored for at least 40
days postinfection, and the survival rates were determined.

**RESULTS**

**Construction of an MCMV mutant containing the transposon insertion at open reading frame M35 and the rescued virus that restored the mutation.** We used a previously developed *E. coli* Tn3-based transposon mutagenesis system to construct a pool of MCMV mutants (47). In our mutagenesis procedure, an MCMV genomic library containing a randomly inserted transposon (designated Tn3gpt) in each viral DNA fragment was first constructed using a shuttle mutagenesis method as described previously (46). This pool of MCMV genomic fragments containing randomly inserted Tn3gpt se-
quenve was then cotransfected with the full-length genomic DNA of the wild-type virus (Smith strain) into mouse NIH 3T3 cells, in which homologous recombination occurred between the transposon-containing viral DNA fragment and the Smith DNA sequence. The cells that harbored the progeny viruses expressing the gpt gene were selected for growth in the presence of mycophenolic acid and xanthine (12, 29, 41). Individual recombinant viruses were isolated after multiple rounds of selection and plaque purification. The location of the inserted transposon was determined by directly sequencing the genomic DNAs of the recombinants.

Figure 1A shows the structure of the transposon used to generate the MCMV mutant. The transposon contains (1) the expression cassette, consisting of the gpt gene driven by a promoter and a transcription termination signal, and (2) an additional transcription termination site, which allow the sele-
cion of MCMV mutants in mammalian cells and the truncation of the transcript expressed from the disrupted gene (46). The gpt expression cassette was inserted such that its transcription termination site functioned in the direction opposite that of the other poly(A) signal in the transposon (Fig. 1A). Such a design ensured that the transcription of the targeted gene would be disrupted without altering the expression of nearby genes that might share a common poly(A) signal with the disrupted gene.

One of the recombinant viruses generated from our pool of MCMV mutants, designated RvM35, contained the transposon insertion within open reading frame M35 (Fig. 1B). Sequence analyses of the junction between the transposon and the viral sequence in RvM35 revealed that the location of the transposon is at nucleotide position 46408 (amino acid residue 167 of the 519-amino-acid-long open reading frame) in reference to the genome sequence of the wild-type Smith strain (35) (Fig. 1B and data not shown).

Previous studies have shown that spontaneous mutations within the viral genome, including deletion and rearrange-
ment, can occur during the construction of viral mutants using a homologous-recombination approach (23; X. Zhan, J. Zhu, A. Tam, and F. Liu, unpublished results). To exclude the possibility that the phenotype observed with RvM35 might be
due to some other adventitious mutations in the genome of the viral mutant rather than the disruption of the M35 open reading frame, a rescued virus, RqM35, was derived from RvM35 by restoration of the wild-type M35 sequence in RvM35 (Fig. 1B). Construction of the rescued virus was carried out using a procedure similar to that used for generating the viral mutant. A DNA fragment that contained the M35 coding region was cotransfected with the full-length RvM35 genomic DNA into
mouse cells to allow homologous recombination to occur. The STO cells that harbored the progeny viruses were allowed to grow in the presence of 6-thioguanine, which selects against gpt expression (12, 29). The rescued virus, RqM35, which did not express the gpt protein and no longer contained the transposon, was isolated after multiple rounds of selection and plaque purification.

**Characterization of mutant RvM35 and rescued virus RqM35 in tissue culture.** The genomic structures of the recombining viruses were examined by Southern blot hybridization and compared to that of the wild-type Smith strain, using a DNA probe containing both the transposon and the viral sequences (Fig. 1B and C). When the viral DNA samples were digested with HindIII and subjected to Southern analysis, a small fragment of 1.8 kb representing the gpt gene was detected in RvM35 DNA, indicating the presence of the transposon sequence within the genome of the mutant (Fig. 1C, lane 1). This finding was further supported by the results of Southern analyses of the RvM35 DNA samples digested with another restriction enzyme, EcoRI. In these experiments, the genomic fragments containing the transposon were found to be larger than those of the wild-type virus by 3.6 kb, which is the size of the transposon (data not shown) (see Fig. 7).

The Southern blots also showed that the stocks of the mutant virus RvM35 were pure and free of the wild-type strain, since the hybridizing DNA fragments from the mutant did not comigrate with those of the wild-type Smith strain (Fig. 1C, lane 1; also see Fig. 7, lanes 1 and 2). For example, the hybridization patterns of the RvM35 and Smith strain DNAs digested with HindIII gave rise to three (14.8-, 13.6-, and 1.8-kb) and one (26.5-kb) DNA bands, respectively (Fig. 1C, lanes 1 and 2). Meanwhile, the hybridized species (16.6 kb) of the EcoRI-digested RvM35 DNA migrated differently from that (13.0 kb) of the wild-type viral DNA digested with the same enzyme (Fig. 1B; also see Fig. 7, lanes 1 and 2). The sizes of the hybridized DNA fragments (Fig. 1C; also see Fig. 7) were consistent with the predicted digestion patterns of the recombinant virus based on the MCMV genomic sequence (35) 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 mutant genomic DNA other than the transposon insertion 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 suggests that regions of the viral genome other than that containing the transposon insertion remained intact in this MCMV mutant.

Analysis of the RqM35 DNA samples digested with HindIII and EcoRI showed that the sizes of the hybridized DNA fragments for the rescued virus were identical to those of the hybridized fragments for the Smith strain and were different from those for RvM35 (Fig. 1B and C, lane 3). These results indicate that the M35 region was restored and that RqM35 did not contain the transposon sequence (Fig. 1C, lane 3). Moreover, our results suggest that the regions of the RqM35 genome other than the M35 region remained intact and were identical to those of RvM35. This is because the restriction enzyme digestion patterns of the regions of the rescued RqM35 genomic DNA samples other than the M35 region appeared to be identical to those of the parental RvM35, as indicated by ethidium bromide staining of the digested DNAs (data not shown). Thus, RqM35 represents a rescued virus derived from RvM35.

Because of the presence of the two transcription termination signals within the transposon (Fig. 1A), it was anticipated that transcription of the target M35 region would be disrupted. In particular, the region of the M35 open reading frame downstream from the transposon insertion site was not expected to be expressed. To determine whether this was the case, we isolated cytoplasmic RNAs from cells infected with the mutant virus at different time points (e.g., 4, 12, and 24 h) postinfection and carried out Northern analyses to examine the expression of the transcripts from the M35 open reading frame downstream from the transposon insertion site (Fig. 2). The probe (the 3′ probe) used in the Northern analyses contained the DNA sequence complementary to the 3′ M35 coding region that is within 200 nucleotides downstream from the site of the transposon insertion. We were able to detect an RNA species of ~1.8 kb in the RNA fractions isolated from cells that were infected with the wild-type Smith strain (Fig. 2, lane 5). This ~1.8-kb RNA species was also readily detected in cells infected with the Smith strain by using a DNA probe (the 5′ probe) complementary to the 5′-terminal sequence of the M35 open reading frame that is within 600 nucleotides downstream from the M35 translational initiation site (data not shown). These results suggest that the ~1.8-kb RNA species represents the transcript expressed from the M35 open reading frame. However, this transcript was not detected in the RNA fractions isolated from cells infected with RvM35 when the 3′ probe, which is complementary to the M35 coding region downstream from the site of the transposon insertion, was used in the Northern analyses (Fig. 2, lane 7). These observations suggest that the transcription from the M35 region downstream from the transposon insertion site was disrupted in RvM35. Mean-
while, we detected the expression of the ~1.8-kb transcript in the RNA fractions isolated from cells infected with the rescued virus RqM35 (Fig. 2, lane 6). Detection of a single transcript expressed from the M35 region indicates that the transcripts from the adjacent genes do not overlap with M35 and that the transposon insertion does not disrupt the expression of these adjacent genes.

In these experiments, we used the level of MCMV M25 transcript (6, 46) as the internal control for the expression of the M35 transcript. As shown in Fig. 2, the levels of the M25 transcript detected in cells that were infected with RvM35 and RqM35 were found to be similar to that of the M25 transcript in cells infected with the Smith strain (Fig. 2, lanes 1 to 4). Thus, the transposon insertion in RvM35 appeared to disrupt the transcript expressed from the M35 open reading frame, whereas the wild-type expression of the transcript was restored in RqM35. In order to determine whether these viruses have any growth defects in vitro, experiments were carried out to study the growth rates of the recombinant viruses in NIH 3T3 cells. Cells were infected with these viruses at both low and high MOIs, and their growth rates were assayed in triplicate experiments. No significant differences were found among the growth rates of RvM35, RqM35, and the Smith strain (Fig. 3). These results, combined with those from the Southern and Northern analyses, provide the first direct evidence to suggest that the M35 open reading frame is dispensable for viral replication in vitro in tissue culture.

Deficient growth of RvM35 in immunocompetent animals. To determine whether disruption of M35 adversely affects viral replication in vivo, Balb/c-ByJ mice were injected intraperitoneally with $10^4$ PFU of RvM35, RqM35, or the wild-type Smith strain. The viral inoculum used for the infection of animals was prepared by growing the viruses in NIH 3T3 cells. At 1, 3, 7, 10, 14, and 21 days postinfection, salivary glands, lungs, spleens, livers, and kidneys were harvested, and the titers of infectious viruses in these five organs were determined on NIH 3T3 cells. Moreover, the titers of viruses from the salivary glands at 28 days postinfection were also determined. It was previously shown that these organs are among the major targets for MCMV infection (14, 17, 26). The mutant appeared to be severely deficient in replication in the salivary glands. For example, at 14 and 21 days postinfection, the titers of RvM35 found in the salivary glands were at least 500-fold lower than those of the Smith strain (Fig. 4A). Moreover, the peak titers of RvM35 found in the lungs, spleens, livers, and kidneys of the infected animals (10 days postinfection) were ~10-4, 8-6, and 4-fold lower than the titers in the same organs from the animals infected with the Smith strain (Fig. 4B to E). In contrast, the titers of the rescued virus RqM35 found in the same 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 Balb/c mice in vivo (47). Thus, these results suggest that the growth deficiency of RvM35 in the organs examined is due to the disruption of M35 and that open reading frame M35 is important for optimal viral growth in vivo, at least in these organs in Balb/c mice.

Attenuated virulence and deficient growth of recombinant virus RvM35 in immunodeficient SCID mice. Immunodeficient animals are extremely susceptible to MCMV infection (13, 31, 34, 36). For example, the CB17 SCID mice, which lack functional T and B lymphocytes, are sensitive to low levels of viral replication, as these animals succumb to as little as 10 PFU of MCMV (31, 34). Analysis of viral replication in these mice serves as an excellent model for comparing the virulences of different MCMV strains and mutants and for studying how they cause opportunistic infections in immunocompromised hosts. To determine whether the M35 open reading frame plays a significant role in MCMV virulence, we compared the survival rates of the animals infected with RvM35 with those of animals infected with RqM35 and the wild-type Smith strain. The viral inoculum used for the infection of animals was prepared by growing the viruses in NIH 3T3 cells. For each virus, 10 SCID mice were injected intraperitoneally with $10^4$ PFU of
RvM35, RqM35, or the Smith strain. All the mice that were infected with either the Smith strain or RqM35 died within 25 to 27 days postinfection (Fig. 5). In contrast, no animals infected with RvM35 died until 35 days postinfection (Fig. 5). This observation indicates that the virulence of RvM35 is attenuated in killing 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 killing SCID mice (47). Thus, these results suggest that disruption of the M35 open reading frame diminishes viral virulence and that M35 plays an important role in MCMV virulence in SCID mice.

To further study the pathogenesis of the mutant virus in these immunodeficient animals, the replication of RvM35 in different organs of the animals was studied during a 21-day infection period before the onset of mortality in the infected animals. In these experiments, SCID mice were infected intraperitoneally with 10^4 PFU of each virus (RvM35, RqM35, or the wild-type Smith strain). At 1, 3, 7, 10, 14, and 21 days postinfection, three mice from each virus group were sacrificed and the salivary glands, lungs, spleens, livers, and kidneys were harvested. The levels of viral growth in these five organs were determined by assaying the viral titers in the organs. The titers of RqM35 in all of the organs examined were similar to those of the Smith strain (Fig. 6). In contrast, the titers of the mutant virus RvM35 were consistently lower than those of the wild-type virus at every time point examined. In particular, RvM35 replication appeared to be severely attenuated in the salivary glands, as the viral titers were found to be below 5 × 10^3 PFU/ml of tissue homogenate (Fig. 6A). At 21 days postinfection, the titers of RvM35 in the salivary glands, lungs, spleens, livers, and kidneys of the infected animals were lower than the titers of the wild-type virus by 50,000-, 100-, 10-, 100-, and
50-fold, respectively (Fig. 6). Therefore, RvM35 appears to be deficient in growth in the organs of the 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 (47). Thus, these results suggest that the attenuated growth of RvM35 in these organs is probably due to the disruption of M35 and that open reading frame M35 may be required for optimal growth of MCMV in these organs in immunodeficient hosts, such as in the salivary glands.

Stability of transposon mutations in recombinant viruses in vitro and in vivo. It has been shown that MCMV mutants with an insertional sequence are not stable and generate spontaneous mutations during replication in vitro and in vivo (1, 23; X. Zhan, A. Tam, J. Zhu, M. Lee, and F. Liu, unpublished results). It is possible that the transposon sequence in RvM35 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 the stability of the inserted transposon sequence within the viral genome, two sets of experiments were carried out. First, recombinant viruses were used to infect NIH 3T3 cells at an MOI of <0.01 and allowed to grow for five generations (60 days) in the absence of gpt selection. Second, 10^6 PFU of viruses was used to infect SCID mice. At 21 days postinfection, the livers and spleens were harvested from the RvM35-infected animals and sonicated to release the virus. Viruses were recovered by infecting NIH 3T3 cells with the sonicated tissues. Viral DNAs were purified from the infected cells, and their restriction digest patterns were analyzed in agarose gels. An example of the Southern analyses of the RvM35 viral DNAs with a DNA probe that contained the transposon and M35 open reading frame sequence is shown in Fig. 7. These results indicate that no change in the hybridization patterns of RvM35 occurred as a result of growth of the virus for five generations (60 days) in cultured cells (Fig. 7, lanes 2 and 3) or in animals for 21 days (lanes 4 and 5). Moreover, the overall EcoRI digestion patterns of RvM35 DNA that replicated either 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. Thus, the transposon insertion in RvM35 appeared to be stable and the genome of RvM35 remained intact during replication in both tissue culture and animals.

DISCUSSION

We report here the characterization of an MCMV mutant that contains a transposon insertional mutation in open reading frame M35. Our results provide the first direct evidence to suggest that M35 is not essential for viral replication in vitro in NIH 3T3 cells. Moreover, a disruption of the M35 open reading frame results in reduced growth of the virus in both immunocompetent and immunodeficient hosts and diminishes viral virulence in killing SCID mice. These observations strongly suggest that M35 probably encodes a viral factor important for viral pathogenesis and for optimal viral growth in vivo.
Our results indicate that the transposon sequence was inserted into the M35 region and disrupted the coding sequence of the open reading frame (Fig. 1B). Moreover, transcription from the region downstream from the transposon insertion site was not detected in cells infected with the mutant virus (Fig. 2). These results indicate that the region of the target open reading frame downstream from the transposon insertion site, which includes >65% of the M35 coding sequence, was not expressed. Therefore, it is likely that no functional M35 protein was expressed from the viral mutant. Our results also show that RvM35 replicated in vitro in NIH 3T3 cells as well as the wild-type Smith strain and the rescued virus RqM35 (Fig. 3). These observations suggest that M35, or at least the carboxyl-terminal sequence of the open reading frame, is not essential for viral replication in NIH 3T3 cells.

Homologues of M35 have been found in animal and human betaherpesviruses (e.g., rat CMV and HCMV) but not in alpha- and gammaherpesviruses (e.g., Epstein-Barr virus) (4, 8, 11, 15, 24, 30, 35, 42). For example, M35 (35) shows sequence homology with UL35 of HCMV (4), R35 of rat CMV (42), and U14 of human herpesvirus 6 and 7 (8, 11, 15, 24, 30). The high degree of conservation of this open reading frame among animal and human CMVs suggests that the functions of M35 and its homologues are important in the pathogenesis and virulence of these viruses in vivo (4, 35, 42). Meanwhile, the low degree of sequence homology of these M35 homologues with genes found in other herpesviruses, as well as in other organisms and hosts in the database, suggests that their functions are unique in infections of these betaherpesviruses (4, 35, 42; M. Lee, A. Tam, R. Hai, G. Abenes, and F. Liu, unpublished results). In our present study, RvM35 was found to be deficient in replication in the salivary glands, lungs, spleens, livers, and kidneys of both the Balb/c and SCID mice that were intraperitoneally infected. For example, 21 days postinfection, the titers of RvM35 in the salivary glands, lungs, spleens, livers, and kidneys of the infected SCID mice were lower than the titers of the wild-type virus by 50,000-, 100-, 10-, 100-, and 50-fold, respectively (Fig. 6). The mutant RvM35 appears to be severely defective in growth in the salivary glands, since the viral titers from the organs isolated from both Balb/c and SCID mice were found to be ≤10^3 PFU/ml of tissue homogenate (Fig. 4A and 6A). Moreover, no death occurred among SCID mice infected with RvM35 up to 35 days postinfection while all mice infected with the Smith strain or RqM35 died within 27 days postinfection (Fig. 5). Thus, the mutation at M35 diminishes but does not completely eliminate viral virulence in the SCID mice. These results strongly suggest that M35 is a viral determinant for MCMV growth in vivo in these animals and for viral virulence in killing 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 observed in RqM35 upon restoration of the wild-type sequence in RvM35 (Fig. 1, 4, 5, and 6). Furthermore, the restoration of the wild-type phenotypes in RqM35 occurred together with the restoration of M35 expression (Fig. 2). These observations suggest that the transposon insertion rather than an adventitious mutation is responsible for the observed attenuation of RvM35 replication and virulence in the Balb/c and SCID mice. Second, previous studies indicated that a virus mutant (i.e., Rvm09) with a transposon insertion at the m09 open reading frame replicated as well in both Balb/c and SCID mice as the wild-type virus (47). Moreover, mutant Rvm09 exhibited a level of virulence in killing SCID mice similar to that of the wild-type virus. These observations indicate that the transposon sequence per se in the viral genome does not significantly affect viral replication and virulence in these animals (47). 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 different organs of the infected animals after 21 days of infection (Fig. 7 and data not shown). Moreover, the EcoRI digestion patterns of the RvM35 mutant DNAs, other than the transposon insertion region, appeared to be identical to those of the wild-type virus DNA (data not shown). Fourth, our results show that a single transcript is expressed from the M35 region. These observations indicate that the transcripts from the adjacent genes do not overlap with M35. It is unlikely that the transposon insertion disrupts or affects the transcription and expression of M35.
that a single transcript of reading frame M35 has been reported. Our results indicate that open reading frame M35 has been shown to encode a tegument protein and is dispensable for viral replication in vitro (6, 46). The results presented in this study and in a previous study of a mutant with a mutation at M25 (6, 46) suggest that all members of the M25 gene family, which include M25 and M35, are dispensable for viral replication in vitro. To our knowledge, neither the transcript nor the protein product coded by open reading frame M35 has been reported. Our results indicate that a single transcript of ~1,800 nucleotides is expressed from the M35 open reading frame. The size of the M35 transcript is consistent with the predicted length of the open reading frame, 519 amino acids, and the presence of the polyadenylation sites upstream and downstream of the M35 coding sequence (35). In contrast, a cluster of multiple transcripts has been found to be expressed in the sequences of HCMV and guinea pig CMV that are homologous to M35 (21, 22). HCMV UL35 encodes two proteins, ppUL35 and ppUL35A, which share the common carboxyl-terminal sequence (22). This is because the transcript of ppUL35A initiates within the ppUL35 coding sequence and terminates at the same polyadenylation site as the ppUL35 mRNA, and ppUL35A shares the same open reading frame as ppUL35. ppUL35 has been shown to be a tegument protein that may function to enhance the activity of HCMV UL82 (pp71) in transactivation of viral gene expression, while ppUL35A, which is not found in the tegument or virion, appears to inhibit the transactivating activity of pp71 (22). However, whether ppUL35 or ppUL35A is essential for viral replication in vitro remains unknown, since the construction and characterization of HCMV mutants with mutations at the UL35 sequence have not been reported. Meanwhile, little is known about the functions of ppUL35 and ppUL35A in HCMV pathogenesis and virulence in vivo. Our results provide the first direct evidence to suggest that M35 is probably required for optimal viral growth in vivo in both immunocompetent and immunodeficient hosts and is important for viral virulence in killing SCID mice. It will be interesting to determine whether M35, like UL35, is localized in the tegument and also possesses transactivation activity. Moreover, further studies will reveal how M35 functions as a viral virulence factor and may provide insight into the function of UL35 in HCMV pathogenesis and virulence in humans. Meanwhile, our present study does not address whether the function of M35 is analogous to that of UL35. Future studies are needed to investigate whether UL35 is functionally equivalent to M35.

Specific tropism for the host tissues, particularly for the salivary glands, is an important determinant of CMV biology. Tropism for the salivary gland and persistent and recurrent viral shedding from this organ are believed to constitute one of the main routes for human CMV transmission in normally healthy individuals (26, 33). A key question from our results is how the lack of M35 leads to a change in the level of virulence and growth. Attempts have been made to compare the in vivo phenotypes of RvM35 with the phenotypes of other viral mutants, including those that were generated in our laboratory by transposon insertion at different loci of the viral genome (A. Tam, J. Zhu, R. Hai, X. Zhan, and F. Liu, unpublished results). Like RvM35, a viral mutant with a deletion in sgg1 was also defective in growth in the salivary glands of Balb/c mice (23). Meanwhile, this mutant did not exhibit any growth defects in other organs examined and was as virulent as the wild-type virus in killing Balb/c mice (19, 23). These results have implied that open reading frame m133 (sgg1) is a viral determinant for MCMV replication in the salivary glands (19, 23). Although there is little sequence homology between M35 and sgg1, it is conceivable that the function of the M35 gene is related to that of sgg1 in supporting CMV infection in the salivary glands. The levels of attenuation in the growth and virulence of RvM35 in the infected animals are also found to be similar to those of the viral mutants that contain a transposon mutation or a deletion in the M83 open reading frame, which encodes one of the most abundant viral tegument proteins (5, 28, 47). However, the function of M83 in vivo is not completely understood. Equally elusive is the mechanism through which the mutations at M83 diminished the growth and virulence of viral mutants in vivo. Given the fact that both UL35 and UL83 (the HCMV homologue of M83) are tegument proteins and may potentially interact with HCMV UL82 (pp71) for transactivation of viral gene expression (22, 26), it is conceivable that a viral mutant with disruption of M35, while it replicates normally in NIH 3T3 fibroblasts, exhibits a defect in certain steps of viral replication, such as viral entry, gene expression, and spread, in vivo in particular organs or tissues (e.g., the salivary glands). This defect may lead to slow growth of the viral mutant in the organs and consequently may contribute to severe reduction in growth of the viral mutant and attenuated virulence in vivo. Alternatively, M35 may be involved in virus-host interactions and may play an important role in modulating the host cells for optimal viral replication. More detailed studies of the in vitro and in vivo growth of these mutants will reveal whether M35 functions in a way similar to that of M83 and sgg1 in supporting optimal growth and virulence of MCMV in vivo. These studies, along with studies of other viral mutants exhibiting similar phenotypes, will lead to the identification of viral determinants for optimal growth and virulence in vivo and will provide insights into how these determinants function in supporting CMV pathogenesis and infection.

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