Published in final edited form as:
*J Gen Virol*. 2008 November ; 89(0 11): 2864–2868. doi:10.1099/vir.0.2008/003137-0.

# Mutagenesis of the murine cytomegalovirus *M56* terminase gene

**Jian Ben Wang** and **Michael A. McVoy***
Department of Pediatrics, Medical College of Virginia campus of Virginia Commonwealth University, 1101 E. Marshall Street, Richmond, Virginia 23298-0163

## SUMMARY

The *M56* gene encodes M56, one of three proteins comprising the murine cytomegalovirus (MCMV) terminase, an enzyme that packages DNA into capsids and cleaves the DNA into progeny genomes. Deletion of *M56* from a bacterial artificial chromosome (BAC) clone of the MCMV genome was lethal, as the mutant BAC failed to reconstitute infectious virus. Reintroduction of *M56* at an ectopic locus complemented the deletion, allowing reconstitution of a virus that replicated with wild type efficiency. Reintroduction of *M56* sequences encoding an N-terminal epitope fusion was lethal, as was a mutation targeting a region in M56 implicated as an ATPase active site. In contrast, a frame shift mutation in *M56a*, an open reading frame that overlaps *M56*, had no effect on viral replication. We conclude that the protein product of *M56a* is dispensable while M56 residues comprising the proposed ATPase active site are critical for terminase function and viral replication.

Herpesviruses replicate their genomes in a manner similar to the large double-stranded DNA bacteriophage. Linear genomic DNA is replicated to form concatemers that are then packaged into preformed capsids and cleaved to produce capsids containing unit length progeny genomes (Brown et al., 2002). This process is carried out by an enzyme called terminase. Phage terminases have two subunits (Black, 1989), while herpesvirus terminases appear to have three. The terminase subunits of human cytomegalovirus (HCMV) are UL51, UL56, and UL89. DNA translocation is believed to be ATP-dependant, and indeed, UL89 contains Walker A and B motifs (Walker et al., 1982) indicative of an ATP binding pocket, but UL89 has not been shown to have ATPase activity in vitro. In contrast, a C-terminal portion of UL56 expressed in *E. coli* has been shown to have ATPase activity (Hwang & Bogner, 2002) and mutations within a putative ATP binding site decreased this activity (Scholz et al., 2003). However, the importance of these residues for viral replication has not been confirmed. Recently, peptides encoded by *UL56a*, an open reading frame (ORF) that overlaps much of *UL56*, have been detected in HCMV virions (Varnum et al., 2004) but the relevance of the *UL56a* gene product to terminase function or viral replication is not known.

To facilitate mutagenesis of the murine cytomegalovirus (MCMV) gene *M56* that encodes M56, the MCMV ortholog of the HCMV terminase subunit UL56, we developed a *cis* complementation system (Hahn et al., 2003) in which *M56* was deleted from a bacterial artificial chromosome (BAC) clone of the MCMV genome, then complemented in *cis* by reintroduction of wild type or mutant sequences at an ectopic location by site-specific Tn7-mediated transposition. BACs thus complemented were assessed for their ability to reconstitute viruses and the growth properties of such viruses were characterized.

*Correspondent footnote: Michael A. McVoy, Department of Pediatrics, Medical College of Virginia campus of Virginia Commonwealth University, P.O. Box 980163, Richmond VA 23298-0163, Telephone: 1-804-828-0132, Fax: 1-804-828-6455, mmcvoy@vcu.edu.

BAC pSM3-117B, an infectious clone of the MCMV genome, contains a *lacZ*α-mini-*att*Tn7 site that permits insertion of sequences into the *att*Tn7 locus via Tn7-mediated transposition (Luckow et al., 1993). It was made from pSM3-117K by Flip recombinase-mediated removal of a kanamycin-resistance (*kn*) marker (Hahn et al., 2003). BAC pSM3-117ΔM56 was constructed by replacing most of *M56* (nucleotides 86,400 to 88,120; 21716071) with *kn* (Fig. 1a). Briefly, *kn* from pACYC177 was PCR-amplified (Wang et al., 2008) using primers M56-pACYC177-F (GAACATGGGACCGTTGATGAAACGGTAGATCTGCTGCCCGAGCTGGGGCAGCAGCTCGGACGATTTATTCAACGAAGCC) and M56-pACYC177-R (GTCTGTGCGCGGTATGTATAAGCGGAGGGGTAGGGGAGTCGACCTGCTCGCGCGCAACGTGCCAGTGTTACAACCAATT). The product was *Dpn*I-restricted then electroporated into pSM3-117B-containing *E. coli* strain DY380 cells that had been induced at 42 °C for 15 min to express λ recombinases (Yu et al., 2000). Colonies containing recombinant BACs were selected on plates containing 50 μg/ml kanamycin, 50 μg/ml chloramphenicol, and 50 μg/ml tetracycline. One clone was designated pSM3-117ΔM56 after confirmation of correct structure using the five PCR reactions illustrated in Fig. 1a (for primer sequences see Table 1, Supplementary Data). Reaction A was predicted to amplify a 1840-bp product from pSM3-117B or a 1089-bp product from pSM3-117ΔM56. Reactions B and C were predicted to produce 376- and 327-bp products from pSM3-117ΔM56, respectively, but no products from pSM3-117B. Reactions D and E were predicted to produce 476- and 530-bp products from pSM3-117B, respectively, but no products from pSM3-117ΔM56. That each reaction produced the predicted results (Fig. 1b) confirmed that pSM3-117ΔM56 has the predicted structure and lacks a functional *M56* locus. Transfection of pSM3-117ΔM56 DNA into mouse NIH3T3 cells (Wang et al., 2008) failed to reconstitute an infectious virus, consistent with a critical role for M56 in viral replication.

Sequence analysis of the *M56* region revealed an ORF, designated *M56a*, that overlaps *M56* (Fig. 1a) and has homology to *UL56a*. As the importance of *M56a* or its hypothetical protein product M56a were not known, and as the deletion in pSM3-117ΔM56 disrupts both *M56* and *M56a* (Fig. 1a), sequences from the *M56a* start to the *M56* stop were included in efforts to complement the deletion. The Tn7 transposition shuttle pFastBac1 (Invitrogen) was modified to include the HCMV major immediate early promoter (MIEP), a multiple cloning site for insertion of *M56/M56a* sequences, and a 3′ IRES-*gfp* reporter (Fig. 2a). This shuttle, designated pMA178B, was made by ligation of annealed oligonucleotides MOL126/MOL127 into BamHI/HindIII-restricted pFastBac1 (Invitrogen), then restriction of the resulting plasmid with NdeI/MfeI and insertion of a 2.1-kb AseI/MfeI fragment from pIRES2-EGFP (Clontech). Shuttle plasmids containing wild type or mutant *M56/M56a* sequences were subsequently constructed. The sequences of oligonucleotides used for plasmid construction are given in Table 1 (Supplementary Data). Sanger dideoxy sequencing was used to confirm the entire sequence of each *M56/M56a* insertion.

Wild type *M56/M56a* sequences were PCR-amplified using primers MOL129 and MOL153. The product was ligated into pGEM®-T Easy (Promega) then transferred as a 2.8-kb EcoRI fragment into EcoRI-restricted pMA178B to make plasmid pMA219. Sequences between *Tn7R* and *Tn7L* of pMA219 were transposed into pSM3-117ΔM56 to generate pSM3-117ΔM56-t219, as previously described (Hahn et al., 2003). Briefly, pMA219 was transformed into DH10B strain *E. coli* containing pSM3-117ΔM56 and helper plasmid pMON7124 (Luckow et al., 1993). Transformants were selected on plates containing 25 μg/ml chloramphenicol, 50 μg/ml kanamycin, 7 μg/ml gentamycin, 10 μg/ml tetracycline, 200 μg/ml X-gal, and 40 μg/ml isopropylthio-β-D-galactoside and incubated for 48 h at 37°C. White colonies in which *lacZ*α was disrupted by transposition into the *lacZ*α-mini-*att*Tn7 site of pSM3-117ΔM56 were further screened for correct transposition using three PCR reactions as previously described (Wang et al., 2008).

BAC pSM3-117ΔM56-t219 was transfected into mouse NIH3T3 cells. Reconstitution of a virus designated RM219 was evidenced by the appearance of GFP+/CPE+ foci 5 - 10 days post-transfection. The predicted genome structure of RM219 is shown in Fig. 2a. The sequence of the ectopic *M56/M56a* insertion in RM219 was confirmed by Sanger dideoxy sequencing of DNA isolated from virions as previously described (McVoy et al., 1998).

To compare the growth properties of RM219 to wild type virus, NIH3T3 cells were infected at an m.o.i. of 0.1. Cells were washed 3 h post-infection and culture supernatants were collected daily and titrated by limiting-dilution in 96-well plate cultures as described (Cui et al., 2008). The replication kinetics and efficiency of virus RM219 were similar to those of parental of viruses SM3 (derived from pSM3) and RM117B (derived from pSM3-117B) (Fig. 3). Therefore, the ectopic *M56/M56a* sequences were able to complement in *cis* the deletion of native *M56/M56a* sequences without significant loss of viral replication efficiency.

To determine if the putative M56a protein is important for viral replication, two mutations were engineered into *M56a* 5′ of the *M56* start codon. In plasmid pMA247 a 3-bp insertion that added an arginine to M56a was created by digestion of pMA219 with BssHII, blunt-ending with Klenow DNA polymerase, and religation. In plasmid pMA249 a single base insertion created a premature stop in addition to a frame shift in *M56a* (Fig. 2b). This was achieved by PCR amplification of pMA219 with primers MOL210b and MOL211, ligation of the product into pGEM®-T Easy, and transfer of a 0.45-kb BssHII/NdeI fragment to BsHII/NdeI-restricted pMA219.

The sequences in pMA247 and pMA249 were transposed into pSM3-117ΔM56 to produce pSM3-117ΔM56-t247 and pSM3-117ΔM56-t249 respectively. Both BACs reconstituted viruses (RM247 and RM249, respectively) that replicated with wild type kinetics and efficiencies (Fig. 3). Sequencing of virion DNA confirmed that RM247 and RM249 retained their respective mutations within their ectopic *M56/M56a* sequences. Moreover, while PCR reaction A amplified 1089- and 1840-bp products from RM219, RM247, and RM249 DNA, reaction E failed to amplify these DNAs yet produced an abundant 530-bp product from RM117B DNA (data not shown). Thus, the 1840-bp products of reaction A were presumably derived from the ectopic *M56/M56a* insertions while the native *M56/M56a* region remained disrupted in all three viruses. That the frame shift in *M56a* was maintained in virus RM249 yet caused no impairment in its replication demonstrates that *M56a* is fully dispensable for viral replication.

Hwang et al. reported an ATPase activity for a C-terminal portion of HCMV UL56 expressed in *E. coli* (Hwang & Bogner, 2002). Residues 709-716 were proposed as a putative ATP binding pocket and substitutions of residues within this region impaired the in vitro ATPase activity of the *E. coli*-expressed protein (Scholz et al., 2003). UL56 residues 709-716 are identical to M56 residues 674-681. To determine if this region is important for MCMV replication, the complementing *M56* gene was modified to encode two amino acid changes, G679D and K680E (Fig. 2b). Plasmid pMA241 was constructed by PCR overlap extension. DNA from pMA219 was amplified using MOL189 and MOL190 or MOL191 and MOL192 to generate overlapping products containing two nucleotide changes. The two products were then mixed and amplified again using primers MOL189 and MOL191. The resulting product was double-digested with BbvCI/BamHI and ligated into BbvCI/BamHI-restricted pMA219 to produce plasmid pMA241. Sequences from pMA241 were transposed into pSM3-117ΔM56 to generate pSM3-117ΔM56-t241. Repeated attempts to reconstitute virus from pSM3-117ΔM56-t241 failed, suggesting that residues within the proposed ATP binding site are critical for the function of M56 during viral replication.

As antibodies have not been raised to the M56 protein, we sought to construct viruses encoding M56 fused with an N-terminal FLAG epitope tag. Plasmid pMA219 was PCR-amplified with MOL237 and MOL242 and the product ligated into pCR8/GW/TOPO (Invitrogen). A 2.4-kb EcoRI fragment was then excised and ligated into EcoRI-restricted pMA178B to produce plasmid pMA292. Transposition into pSM3-117ΔM56 produced pSM3-117ΔM56-t292 in which the native *M56* AUG and upstream *M56a* sequences were replaced by sequences encoding an N-terminal FLAG epitope (Fig. 2b). Infectious virus cold not be reconstituted from pSM3-117ΔM56-t292, suggesting that the M56 protein was likely rendered nonfunctional by the N-terminal epitope fusion.

DNA maturation is an attractive target for the development of novel antivirals, and indeed, several compounds are known to block this process (Hwang et al., 2007, Krosky et al., 2000, Reefschlaeger et al., 2001, Underwood et al., 2004, Underwood et al., 1998, van Zeijl et al., 2000). Future drug discovery efforts would benefit from a better understanding of the protein composition, structure, and biochemical functions of terminase. Progress has been made in expressing terminase subunits and dissecting their biochemical activities in vitro. To confirm the importance of such activities for viral replication we developed a *cis* complementation system that facilitates mutagenic evaluation of the M56 terminase subunit. Deletion of the native *M56/M56a* locus was lethal but could be efficiently complemented by transposition of an ectopic copy of *M56/M56a* sequences. As native *M56* is most probably expressed with late kinetics while ectopic expression from the MIEP likely occurs with early or immediate early kinetics, this result suggests that the kinetics of *M56* expression are not critical.

Bacteriophage terminases use the energy from ATP hydrolysis to translocate DNA into capsids (Catalano, 2000, Feiss & Catalano, 2005, Rao & Black, 2005). The large subunits contain Walker A and B box motifs that form ATP binding pockets (Walker et al., 1982) and many have been shown to possess ATPase activities in vitro (Mitchell et al., 2002). Walker box motifs are highly conserved among the herpesvirus terminase subunits that include HCMV UL89 and its ortholog in herpes simplex virus type 1, UL15. Within these motifs homology even extends to phage terminase large subunits (Davison, 1992, Mitchell et al., 2002, Przech et al., 2003). Although a mutation in the Walker A box of UL15 confirmed its importance for viral replication (Yu & Weller, 1998), ATPase activity has not been demonstrated for UL15, UL89, or their orthologs in other herpesviruses. In contrast, UL56 lacks canonical Walker box motifs or sequence homology with bacteriophage terminase subunits, but a C-terminal region of UL56 has been shown to have ATPase activity when expressed in *E. coli* (Hwang & Bogner, 2002) and mutations within a putative ATP binding pocket affected a decrease in this activity (Scholz et al., 2003). That BAC pSM3-117ΔM56-t241 containing similar mutations in M56 was unable to reconstitute an infectious virus confirms that residues within the proposed ATP binding pocket are necessary for viral replication and supports the hypothesis that an ATPase-activity associated with this region (Scholz et al., 2003) is important for terminase function.

In 2004 Varnum et al. detected peptides within HCMV virions having amino acid sequences encoded by an unannotated ORF that was designated *UL56a* because it overlaps the *UL56* gene (Varnum et al., 2004). We found that similar ORFs are conserved in MCMV, rat CMV, rhesus CMV, chimpanzee CMV, and tupaia herpesvirus, and that they encode hypothetical proteins that are highly conserved at the amino acid level (Fig. 4, Supplementary Data). While this suggests that these ORFs serve an important purpose, other herpesviruses, including guinea pig CMV, a close relative of MCMV and rat CMV (McGeoch et al., 2006), appear to lack *UL56a* homologs. Moreover, the strong nucleotide sequence conservation among the terminase genes that they overlap could account for the amino acid conservation observed in Fig. 4. That virus RM249 replicates with wild type efficiency in vitro clearly

demonstrates that expression of the putative M56a protein is not important for replication of MCMV in cell culture. Why these overlapping ORFs have evolved in some viral genomes and what role the encoded proteins play in vivo remains to be determined.

In summary, amino acids proposed to function as an ATP binding pocket and to mediate an ATPase activity of M56 were confirmed to be of critical importance for viral replication, and an overlapping ORF of unknown function (*M56a*) was shown to be dispensable. In the future this system can provide a rapid genetic approach to complement progress made in defining the in vitro biochemical properties of terminase.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Martin Messerle for pSM3, Don Court for *E. coli* strain DY380, and the National Institutes of Health for their support.

## REFERENCES

Black LW. DNA packaging in dsDNA bacteriophages. Annu Rev Microbiol. 1989; 43:267–92. [PubMed: 2679356]

Brown, JC.; McVoy, MA.; Homa, FL. Packaging DNA into Herpesvirus Capsids. In Structure-Function Relationships of Human Pathogenic Viruses. Bogner, E.; Holzenburg, A., editors. Kluwer Academic/Plenum Publishers; London: 2002.

Catalano CE. The terminase enzyme from bacteriophage lambda: a DNA-packaging machine. Cell Mol Life Sci. 2000; 57:128–48. [PubMed: 10949585]

Cui X, McGregor A, Schleiss MR, McVoy MA. Cloning the complete guinea pig cytomegalovirus genome as an infectious bacterial artificial chromosome with excisable origin of replication. J Virol Methods. 2008; 149:231–9. [PubMed: 18359520]

Davison AJ. Channel catfish virus: a new type of herpesvirus. Virology. 1992; 186:9–14. [PubMed: 1727613]

Feiss, M.; Catalano, CE. Bacteriophage lambda terminase and the mechanisms of viral DNA packaging. In: Catalano, CE., editor. Viral Genome Packaging Machines: Genetics, Structure, and Mechanism. Landes Biosciences; Georgetown, TX: 2005. p. 5-39.

Hahn G, Jarosch M, Wang JB, Berbes C, McVoy MA. Tn7-mediated introduction of DNA sequences into bacmid-cloned cytomegalovirus genomes for rapid recombinant virus construction. J Virol Methods. 2003; 107:185–94. [PubMed: 12505633]

Hwang JS, Bogner E. ATPase activity of the terminase subunit pUL56 of human cytomegalovirus. J Biol Chem. 2002; 277:6943–8. [PubMed: 11744697]

Hwang JS, Kregler O, Schilf R, Bannert N, Drach JC, Townsend LB, Bogner E. Identification of acetylated, tetrahalogenated benzimidazole D-ribonucleosides with enhanced activity against human cytomegalovirus. J Virol. 2007; 81:11604–11. [PubMed: 17728228]

Krosky PM, Ptak RG, Underwood MR, Biron KK, Townsend LB, Drach JC. Differences in DNA packaging genes and sensitivity to benzimidazole ribonucleosides between human cytomegalovirus strains AD169 and Towne. Antivir Chem Chemother. 2000; 11:349–52. [PubMed: 11142634]

Luckow VA, Lee SC, Barry GF, Olins PO. Efficient generation of infectious recombinant baculoviruses by site- specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in Escherichia coli. J Virol. 1993; 67:4566–79. [PubMed: 8392598]

McGeoch DJ, Rixon FJ, Davison AJ. Topics in herpesvirus genomics and evolution. Virus Res. 2006; 117:90–104. [PubMed: 16490275]

McVoy MA, Nixon DE, Adler SP, Mocarski ES. Sequences within the herpesvirus-conserved pac1 and pac2 motifs are required for cleavage and packaging of the murine cytomegalovirus genome. J Virol. 1998; 72:48–56. [PubMed: 9420199]

Mitchell MS, Matsuzaki S, Imai S, Rao VB. Sequence analysis of bacteriophage T4 DNA packaging/terminase genes 16 and 17 reveals a common ATPase center in the large subunit of viral terminases. Nucleic Acids Res. 2002; 30:4009–21. [PubMed: 12235385]

Przech AJ, Yu D, Weller SK. Point mutations in exon I of the herpes simplex virus putative terminase subunit, UL15, indicate that the most conserved residues are essential for cleavage and packaging. J Virol. 2003; 77:9613–21. [PubMed: 12915573]

Rao, VB.; Black, LW. DNA Packaing in Bacteriophage T4. In: Catalano, CE., editor. Viral Genome Packaging Machines: Genetics, Structure, and Mechanism. Landes Biosciences; Gerogetown, TX: 2005. p. 40-58.

Reefschlaeger J, Bender W, Hallenberger S, Weber O, Eckenberg P, Goldmann S, Haerter M, Buerger I, Trappe J, Herrington JA, Haebich D, Ruebsamen-Waigmann H. Novel non-nucleoside inhibitors of cytomegaloviruses (BAY 38-4766): in vitro and in vivo antiviral activity and mechanism of action. J Antimicrob Chemother. 2001; 48:757–67. [PubMed: 11733458]

Scholz B, Rechter S, Drach JC, Townsend LB, Bogner E. Identification of the ATP-binding site in the terminase subunit pUL56 of human cytomegalovirus. Nucleic Acids Res. 2003; 31:1426–33. [PubMed: 12595550]

Underwood MR, Ferris RG, Selleseth DW, Davis MG, Drach JC, Townsend LB, Biron KK, Boyd FL. Mechanism of action of the ribopyranoside benzimidazole GW275175X against human cytomegalovirus. Antimicrob Agents Chemother. 2004; 48:1647–51. [PubMed: 15105116]

Underwood MR, Harvey RJ, Stanat SC, Hemphill ML, Miller T, Drach JC, Townsend LB, Biron KK. Inhibition of human cytomegalovirus DNA maturation by a benzimidazole ribonucleoside is mediated through the UL89 gene product. J Virol. 1998; 72:717–25. [PubMed: 9420278]

van Zeijl M, Fairhurst J, Jones TR, Vernon SK, Morin J, LaRocque J, Feld B, O'Hara B, Bloom JD, Johann SV. Novel class of thiourea compounds that inhibit herpes simplex virus type 1 DNA cleavage and encapsidation: resistance maps to the UL6 gene. J Virol. 2000; 74:9054–61. [PubMed: 10982350]

Varnum SM, Streblow DN, Monroe ME, Smith P, Auberry KJ, Pasa-Tolic L, Wang D, Camp DG 2nd, Rodland K, Wiley S, Britt W, Shenk T, Smith RD, Nelson JA. Identification of Proteins in Human Cytomegalovirus (HCMV) Particles: the HCMV Proteome. J Virol. 2004; 78:10960–6. [PubMed: 15452216]

Walker JE, Saraste M, Runswick MJ, Gay NJ. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. Embo J. 1982; 1:945–51. [PubMed: 6329717]

Wang JB, Nixon DE, McVoy MA. Definition of the minimal cis-acting sequences necessary for genome maturation of the herpesvirus murine cytomegalovirus. J Virol. 2008; 82:2394–404. [PubMed: 18094152]

Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL. An efficient recombination system for chromosome engineering in Escherichia coli. Proc Natl Acad Sci U S A. 2000; 97:5978–83. [PubMed: 10811905]

Yu D, Weller SK. Genetic analysis of the UL 15 gene locus for the putative terminase of herpes simplex virus type 1. Virology. 1998; 243:32–44. [PubMed: 9527913]

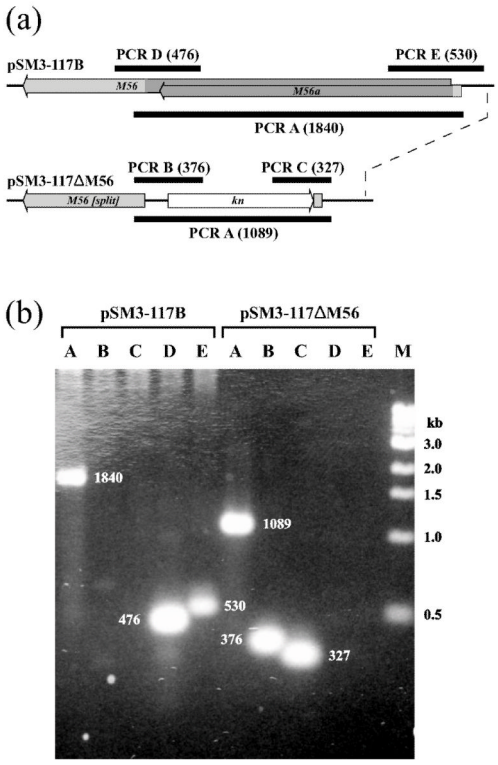


**Fig. 1.**

Deletion of the *M56/M56a* locus. (a) The native *M56/M56a* locus in pSM3-117B is compared to the predicted structure of pSM3-117ΔM56. *M56* and *M56a* ORFs are shown as gray shaded arrows with dark gray indicating the region that is deleted and replaced by *kn* (white arrow) in pSM3-117ΔM56. Black bars represent the products of diagnostic PCR reactions (A-E) with their predicted sizes (bp) shown in parentheses. (b) The products of PCR reactions using pSM3-117B or pSM3-117ΔM56 as templates were separated on 1% agarose, stained with ethidium bromide, and visualized with UV light. The predicted sizes (bp) of each product are indicated. Sizes (kb) of markers (lane M) are indicated to the right.

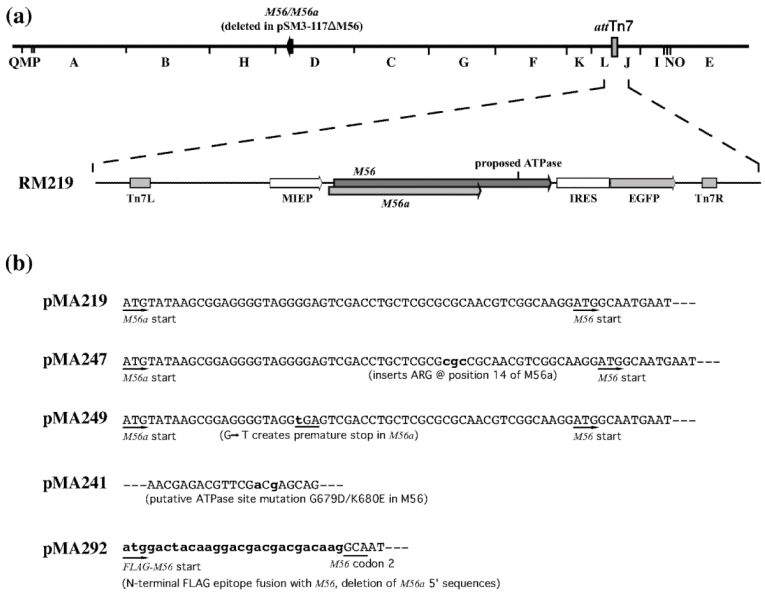


**Fig. 2.**

Complementation in *cis* of the *M56/M56a* deletion. (a) A HindIII map of the MCMV genome illustrating the positions of the *M56/M56a* locus and the *att*Tn7 site. Expanded below is the transposed region (between *Tn7L* and *Tn7R*) of RM219 showing the MIEP (open arrow), *M56* and *M56a* (gray arrows), and the IRES-*gfp* marker cassette. (b) Wild type (pMA219) and mutations in *M56/M56a* sequences cloned in the indicated shuttle plasmids are shown. Nonviral sequences or nucleotide changes are shown in lowercase and bold.

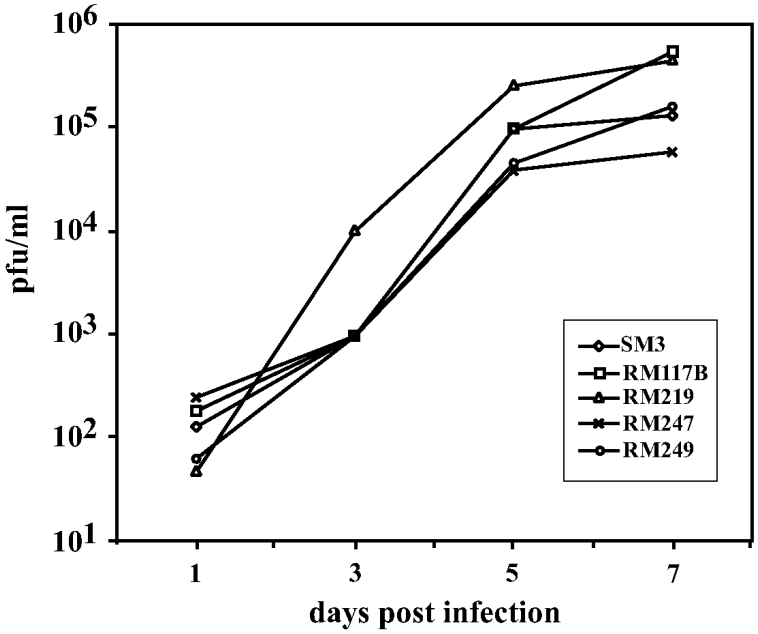


**Fig. 3.**

Growth of wild type and *cis*-complemented viruses. NIH3T3 cells were infected with the indicated viruses at an m.o.i. of 0.1. Viral titers in the culture supernatants were determined on the days post infection indicated.