



Murine Cytomegalovirus Protein pM91 Interacts with pM79 and Is Critical for Viral Late Gene Expression

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ABSTRACT Viral gene expression is tightly regulated during cytomegalovirus (CMV) lytic replication, but the detailed mechanism of late gene transcription remains to be fully understood. Previous studies reported that six viral proteins (named viral transactivation factors [vTFs]) supporting late gene expression were conserved in beta- and gammaherpesviruses but not in alphaherpesviruses. Here, we performed coimmunoprecipitation experiments to elucidate the organization of these six proteins in murine CMV. Our results showed that these proteins formed a complex by both direct and indirect interactions. Specifically, pM91 strongly bound to pM79 even in the absence of other vTFs. Similar to pM79, pM91 exhibited early-late expression kinetics and localized within nuclear viral replication compartments during infection. Functional analysis was also performed using the pM91-deficient virus. Real-time PCR results revealed that abrogation of M91 expression markedly reduced viral late gene expression and progeny virus production without affecting viral DNA synthesis. Using mutagenesis, we found that residues E61, D62, D89, and D96 in pM91 were required for the pM91-pM79 interaction. Disruption of the interaction via E61A/D62A or D89A/D96A double mutation in the context of virus infection inhibited progeny virus production. Our data indicate that pM91 is a component of the viral late gene transcription factor complex and that the pM91-pM79 interaction is essential for viral late gene expression.

IMPORTANCE Cytomegalovirus (CMV) infection is the leading cause of birth defects and causes morbidity and mortality in immunocompromised patients. The regulation of viral late gene transcription is not well elucidated, and understanding of this process benefits the development of novel therapeutics against CMV infection. This study (i) identified that six viral transactivation factors encoded by murine CMV form a complex, (ii) demonstrated that pM91 interacts with pM79 and that pM91 and pM79 colocalize in the nuclear viral replication compartments, (iii) confirmed that pM91 is critical for viral late gene expression but dispensable for viral DNA replication, and (iv) revealed that the pM91-pM79 interaction is required for progeny virus production. These findings give an explanation of how CMV regulates late gene expression and have important implications for the design of antiviral strategies.

KEYWORDS cytomegalovirus, pM91, pM79, late gene, transcriptional regulation

Human cytomegalovirus (HCMV), which is also known as human herpesvirus 5, belongs to the *Betaherpesvirinae* subfamily (1). Following primary infection, HCMV establishes a lifelong latent infection in the host (2). In normal immunocompetent hosts, HCMV infection is usually asymptomatic. However, in immunocompromised hosts, such as transplant recipients, AIDS patients, or neonates, it can cause severe and even life-threatening disease (3, 4). Currently, no licensed vaccine for HCMV is available (5), and the clinical utility of the anti-CMV agents is limited by associated toxicities, poor

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bioavailability and efficacy, and the risk of resistance with extended use (6–8). Further study is necessary to dissect the roles of viral genes in HCMV infection and will facilitate the effort to develop safer and more effective antiviral therapeutics.

Like all members of the betaherpesviruses, HCMV infection is species specific, and therefore, murine CMV (MCMV) is commonly used in the mouse model to study CMV biology. MCMV shares a series of features with HCMV, including virion structure, genome organization, gene expression program, tissue tropism, and pathogenesis (9, 10). Understanding the functions of conserved viral genes in MCMV will help to analyze the roles of their homologs in HCMV. This may provide novel therapeutic antiviral targets and allow for future testing in mouse models.

Similar to other herpesviruses, CMV lytic replication proceeds through a temporal cascade of gene expression, which can be categorized into three major kinetic classes: immediate early (IE), early, and late (3, 11, 12). IE genes are expressed following viral infection and do not require *de novo* viral protein synthesis (13). The transcription of early genes requires viral IE proteins but is independent of viral DNA replication (14). Late genes are transcribed following viral DNA synthesis, and their transcription is inhibited by viral DNA synthesis inhibitors, such as phosphonoacetic acid (PAA) (15, 16). These genes mainly encode proteins required for virus assembly and egress (17). In addition, some genes, classified as early-late, are initially expressed prior to viral DNA synthesis but accumulate later in a DNA synthesis-dependent manner. However, although the regulation of IE and early gene transcription has been extensively investigated, the regulation of viral late gene expression is still poorly understood.

Recently, six viral proteins, termed viral transactivation factors (vTFs), have been reported to be essential for late gene expression in gammaherpesviruses (18–24). These vTFs are conserved in beta- and gammaherpesviruses but not in alphaherpesviruses (20, 25). Five, which are expressed in HCMV (pUL79, pUL87, pUL91, pUL92, and pUL95), are found to be involved in regulation of late gene expression (26–29). Specifically, pUL79 participates in RNA polymerase II (RNAP II) elongation (30). The MCMV homologs, pM79 and pM92, are also demonstrated to be key regulators for viral late transcription (16, 31). Recent work in Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) demonstrated that these six vTFs and cellular RNAP II assemble into a viral preinitiation complex (19, 20). Among them, EBV BcRF1 and KSHV ORF24, which are homologs of HCMV pUL87, serve as a TATA box-binding protein (TBP) homolog that binds to RNAP II and TATT sequences in the viral late gene promoter (32–34). In addition, studies in KSHV elucidated clues regarding interactions between vTFs (19, 35, 36). Five homologs (pM49, pM79, pM87, pM92, and pM95) expressed in MCMV were also predicted to form a multicomponent viral complex (37). However, no further experiments were performed to confirm whether the six vTFs in CMV form a complex, and the roles of the vTF complex components require further investigation.

Here, we provide insight into the interactions among the six vTFs expressed in MCMV; we also propose a model of the multicomponent viral complex that regulates late gene transcription. Our findings indicate that four interacting pairs, pM92-pM87, pM87-pM95, pM95-pM49, and pM91-pM79, are involved in forming the complex. In particular, we focused on the pM91-pM79 interaction. The results show that pM91 has an expression pattern similar to that of pM79, localizes with pM79, and is also important for late gene expression but dispensable for viral DNA synthesis. By point mutation analysis, we demonstrate that the pM91-pM79 interaction is essential for infectious virus production. These data suggest that pM91 serves as a component of the viral complex and participates in viral late gene transcription.

RESULTS

Six viral proteins of MCMV form a complex. We initially used coimmunoprecipitation assays to confirm whether a MCMV vTF complex similar to that found in gammaherpesviruses exists and to determine its components. HEK293T cells were transfected with the FLAG-tagged pM87 expression plasmid or the empty vector together with the expression plasmids of the other five vTFs. At 48 h posttransfection,

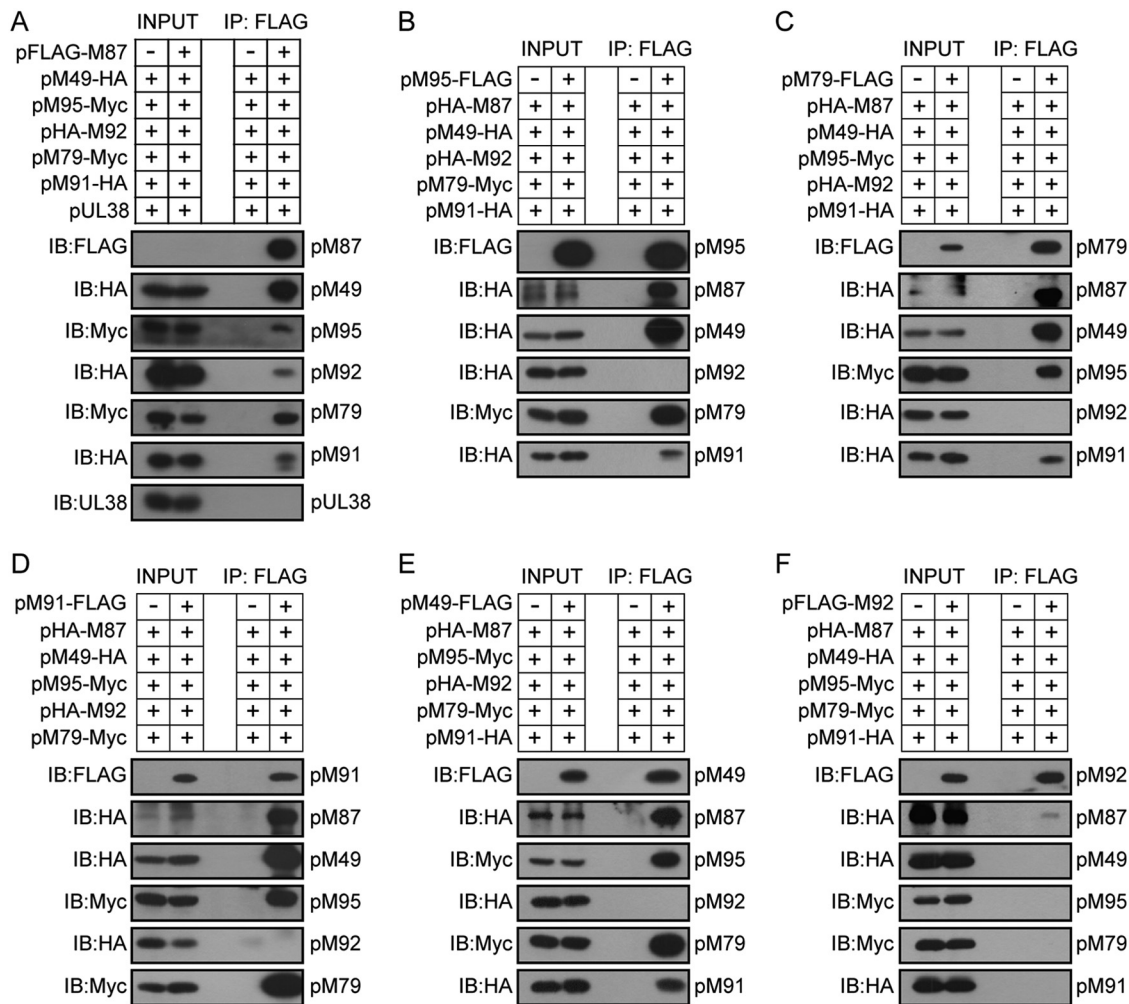


FIG 1 Six MCMV vTFs form a complex. HEK293T cells were cotransfected with the six MCMV vTFs (pM49, pM79, pM87, pM91, pM92, and pM95) with the indicated tags and collected at 48 h posttransfection. HCMV protein pUL38 was used as a negative control. Cell lysates were subjected to immunoprecipitation (IP) using anti-FLAG M2 (mouse monoclonal) magnetic beads, which capture FLAG-tagged pM87 (A), FLAG-tagged pM95 (B), FLAG-tagged pM79 (C), FLAG-tagged pM91 (D), FLAG-tagged pM49 (E), and FLAG-tagged pM92 (F). The whole-cell lysates (INPUT) and the immunoprecipitated fractions were immunoblotted (IB) with the indicated antibodies to detect their interactions.

the cells were harvested, lysed, and subjected to immunoprecipitation using anti-FLAG M2 (mouse monoclonal) magnetic beads. The elution was analyzed by immunoblotting using the antibodies indicated below. All five vTFs were coimmunoprecipitated in the presence of pM87, while HCMV protein pUL38 was not (Fig. 1A). Similarly, when using either pM95, pM79, pM91, or pM49 as the bait, all other vTFs except pM92 were also coimmunoprecipitated (Fig. 1B to E). Consistent with the results presented above, when pM92 was used as the bait, only a small amount of pM87 was coimmunoprecipitated (Fig. 1F), suggesting that the interaction between pM92 and pM87 was primarily weak. The coimmunoprecipitation results are summarized in Table 1. Together, these results indicate that the six vTFs expressed in MCMV, pM49, pM79, pM87, pM91, pM92, and pM95, are involved in a complex.

As the affinities of the different vTFs varied (Fig. 1 and Table 1), we hypothesized that the complex probably had a specific organization. To further dissect the organization of the complex, we performed a set of coimmunoprecipitation experiments with different combinations of vTFs in HEK293T cells. As pM92 could be coimmunoprecipitated only with pM87 (Fig. 1F), we first analyzed the interaction between pM87 and pM92: pM87 was able to be coimmunoprecipitated with pM92 alone, but this interac-

TABLE 1 Summary of protein interactions

Protein	Interaction of the following with the indicated proteins:					
	pFLAG-M87	pM49-FLAG	pM95-FLAG	pFLAG-M92	pM79-FLAG	pM91-FLAG
pM87	NT ^a	++++	++++	+	++++	++++
pM49	+++	NT	++++	–	++++	++++
pM95	+	++++	NT	–	+++	++++
pM92	+	–	–	NT	–	–
pM79	++	+++	+++	–	NT	++++
pM91	+	+++	++	–	+++	NT

^aNT, not tested.

tion was greatly enhanced in the presence of the other vTFs (Fig. 2A, lane 2 versus lane 4). Moreover, the amount of the other vTFs coimmunoprecipitated with pM87 was similar in the presence or absence of pM92, which suggests that depletion of pM92 had little effect on the interactions between pM87 and the other vTFs (Fig. 2A, lanes 2 and 3). Thus, we focused on the organization of the viral complex in the absence of pM92.

To further analyze the organization of the other five vTFs, we performed pairwise interaction screening via coimmunoprecipitation. The results showed three pairs of strong interactions: pM87-pM95, pM95-pM49, and pM91-pM79 (Fig. 2B). As three pairs of interactions did not explain how these six proteins form a complex, we hypothesized that additional interactions could be established after the formation of the protein pairs and we analyzed the interactions between these protein pairs. Although neither pM87, pM95, nor pM49 could be coimmunoprecipitated with pM91 individually (Fig. 2B), they could bind to pM91 weakly when all three were coexpressed (Fig. 2C, lane 3). In addition, this interaction was largely enhanced in the presence of pM79 (Fig. 2C, lane 2). These results suggest that the pM91-pM79 interaction greatly promoted vTF complex formation. When pM49 was absent, the level of pM95 in the immunoprecipitation fraction was greatly reduced and the level of pM87 and pM79 was also relatively decreased (Fig. 2C, lane 4 versus lane 2), suggesting that the pM49-pM95 interaction is also important for vTF complex formation. Moreover, when both pM49 and pM95 were depleted, the level of pM87 in the immunoprecipitation fraction was further decreased (Fig. 2C, lane 6 versus lane 4). It is possible that the formation of pM87-pM95 promoted the interaction between pM91-pM79 and pM87. Interestingly, depletion of pM87 did not impair the level of pM79, pM49, and pM95 in the immunoprecipitation fraction (Fig. 2C, lane 5 versus lane 2), suggesting that pM91-pM79 and pM95-pM49 interacted strongly with each other. A model for the physical interactions between MCMV vTFs is depicted in Fig. 2D.

pM91 shares expression kinetics and localization with pM79. Our results regarding the strong interaction between pM91 and pM79 focused our attention on their functions during infection. pM79 expression was previously reported to be classified as following early-late kinetics, and localization was generally in the nuclear viral replication compartments (16). Therefore, we hypothesized that pM91 had a similar expression pattern and localization. Without available antibodies to pM79 or pM91, we engineered a recombinant virus, *SM79HA91flag*, by inserting a fragment encoding a 3× hemagglutinin (3×HA) or a 3×FLAG tag in frame at the 3' end of the *M79* or *M91* coding sequence, respectively, just upstream of the stop codon (Fig. 3A). We infected mouse embryonic fibroblast 10.1 (MEF10.1) cells with the *SM79HA91flag* virus at a multiplicity of infection (MOI) of 2 and analyzed pM79 and pM91 expression by immunoblotting against the tags (Fig. 3B). pM91 was not detected at 8 h postinfection (hpi) but was expressed after 24 hpi in a pattern similar to that of pM79 (Fig. 3B). In addition, the expressions of both were significantly reduced when the viral DNA synthesis inhibitor PAA (200 μg/ml) was supplemented at 24 hpi (Fig. 3C), suggesting that viral DNA synthesis greatly enhanced the expression of both pM79 and pM91. These data indicate that pM91 expression follows early-late kinetics.

We next examined the intracellular localization of pM91 and pM79 during infection. MEF10.1 cells infected with the *SM79HA91flag* virus were fixed at 24 hpi and subjected

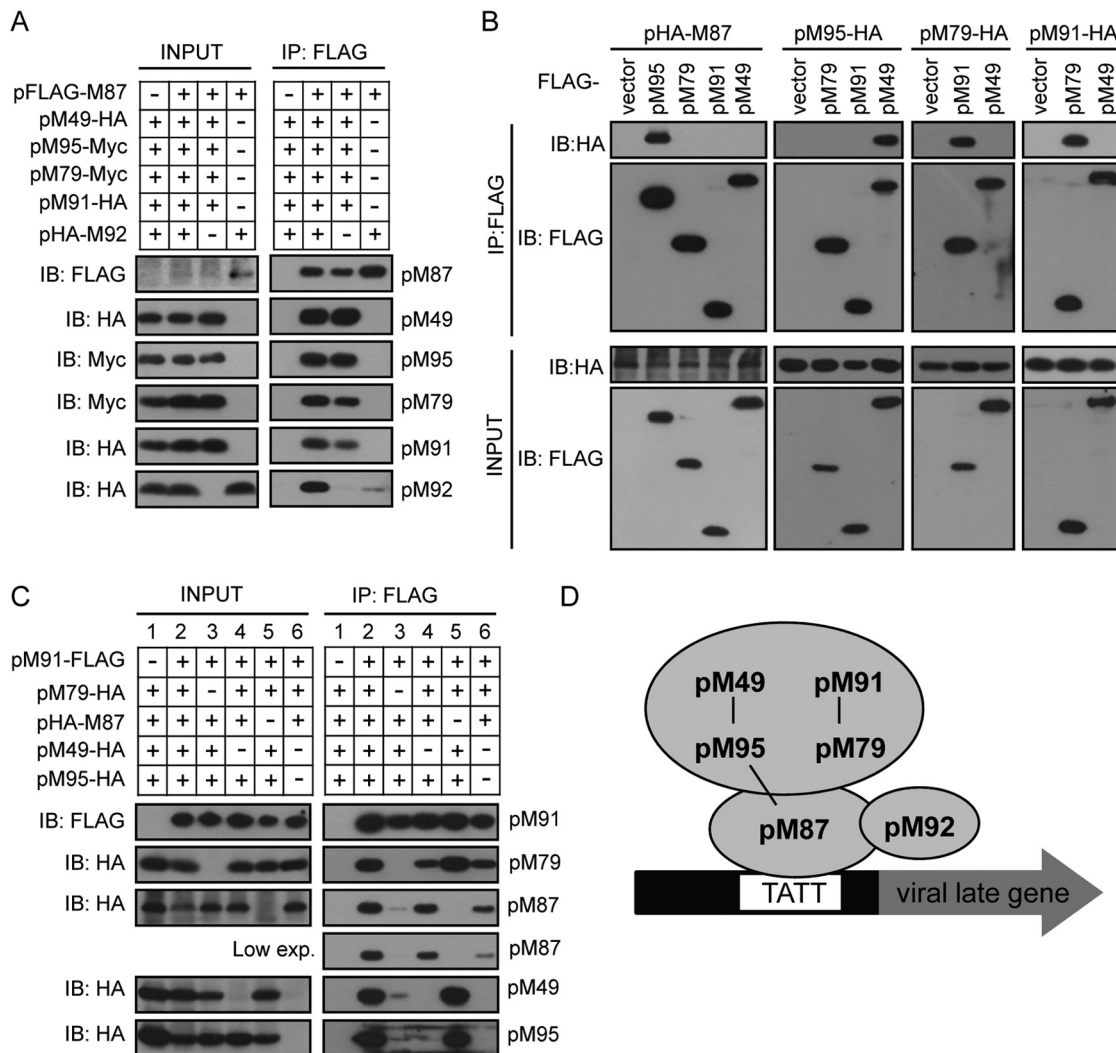


FIG 2 Organization of the vTF complex. (A) pM87 interacts with pM92. The FLAG-tagged pM87 expression plasmid or the empty vector was cotransfected with the indicated HA- or Myc-tagged vTFs into HEK293T cells. Cell lysates were immunoprecipitated by anti-FLAG M2 beads and assayed by immunoblotting. (B) Pairwise interaction screening of five vTFs via coimmunoprecipitation. The experiments were performed as described in the legend to panel A, except that the indicated plasmids were used in the samples. (C) Additional interactions were established after coexpression. The experiments were performed as described in the legend to panel A, except that various combinations of vTF subunits, as indicated, were coexpressed in the samples. exp., expression. (D) Model of the MCMV vTF complex. Three pairs of vTF subunits (pM87-pM95, pM95-pM49, and pM91-pM79) form strong interactions, as indicated by black lines. Two of them (pM49-pM95 and pM91-pM79) bind strongly with each other to form a stable subcomplex. The subcomplex binds to pM87 through multiple interfaces. pM92 weakly binds to the complex by interacting with pM87.

to an immunofluorescence assay with rabbit anti-FLAG and mouse anti-HA antibodies. Results showed that pM91 localized in the nucleus and was strongly associated with pM79 (Fig. 3D), indicating that pM91 colocalized with pM79 in replication compartments.

Collectively, we conclude that pM91 has similar expression kinetics as pM79 and that it colocalizes with pM79 during infection.

pM91 is essential for MCMV replication. MCMV pM79, HCMV pUL79, and HCMV pUL91 were shown to be essential for viral late gene expression (16, 27, 29). As a pM79 partner, pM91 is also likely to be involved in this process. To understand the role of pM91 during infection, we engineered a pM91-deficient virus. Considering that the 5'-flanking region of the *M91* coding sequence overlaps part of *M90*, we were unable to delete the entire open reading frame of pM91. Instead, we constructed a recombinant bacterial artificial chromosome (BAC), pSM_{in}91, in which pM91 translation was

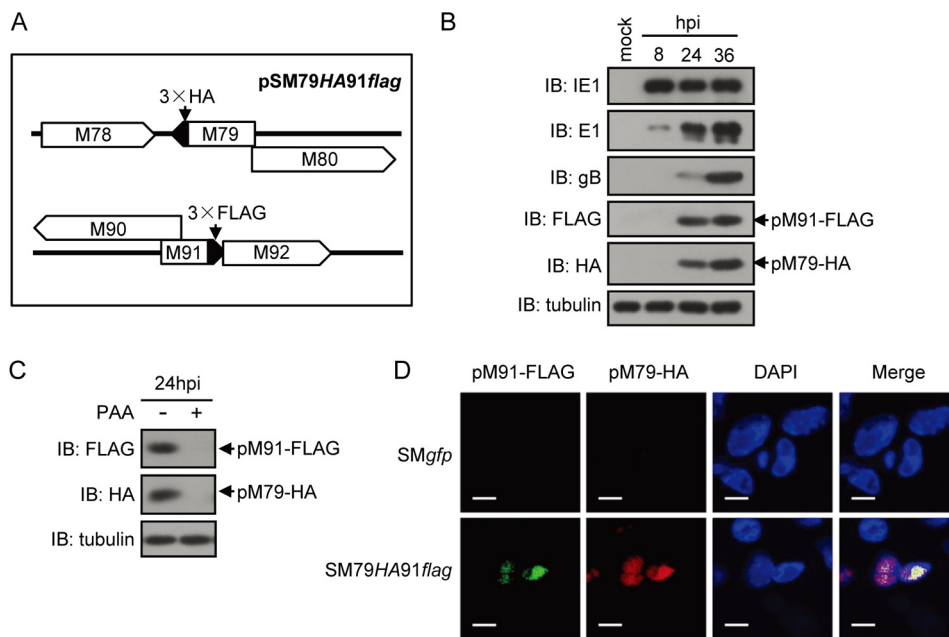


FIG 3 Expression pattern and localization of pM91 and pM79 during infection. (A) Representative diagram of the modifications in the genome of the MCMV BAC pSM79HA91flag. Fragments encoding a 3×HA tag or 3×FLAG tag were inserted in frame at the 3' end of the *M79* or *M91* coding sequence, respectively. (B) pM91 and pM79 expression patterns. MEF10.1 cells were infected with the SM79HA91flag virus at an MOI of 2. Cells were harvested at the indicated hours postinfection (hpi), lysed, and analyzed by immunoblotting. pM91 was detected with anti-FLAG mouse antibodies, and pM79 was detected with anti-HA antibodies; α -tubulin was included as a loading control. (C) pM91 and pM79 expression and viral DNA synthesis. MEF10.1 cells were infected with the SM79HA91flag virus at an MOI of 2 in the presence or absence of the viral DNA synthesis inhibitor PAA (200 μ g/ml). At 24 hpi, cells were harvested and analyzed as described in the legend to panel A. (D) pM91 colocalized with pM79. MEF10.1 cells were infected with SMgfp or SM79HA91flag virus at an MOI of 2, fixed at 24 hpi, and subjected to immunostaining with the rabbit anti-FLAG antibody and mouse anti-HA antibody to detect pM91 (green) and pM79 (red), respectively. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei (blue). Bars = 10 μ m.

disrupted by a premature stop codon by an insertional/frameshift mutation without interference with pM90 expression (Fig. 4A). We then transfected wild-type BAC pSMgfp or recombinant BAC pSM $in91$ into MEF10.1 cells. Both BACs were successfully transfected into MEF10.1 cells, with green fluorescent protein (GFP)-positive cells being present at 2 days posttransfection (Fig. 4B). However, only SMgfp spread to form plaques at 6 days posttransfection, whereas SM $in91$ produced only GFP-positive cells, indicating that this mutant virus had a significant replication deficiency (Fig. 4B, left). Unfortunately, the virus production of SM $in91$ was not rescued by pM91 expression in *trans* (Fig. 4B, right), and no mutant virus could be collected for further studies.

To overcome this problem, we created another recombinant BAC, pSM $dd91$, with an alternative approach using our previously reported intein-mediated modulation of protein stability (imPS) system (38). The intein domain is divided into N- and C-terminal fragments (Int^N and Int^C, respectively), each of which is fused to an external protein sequence (extein). Int^N and Int^C could associate and refold into an active intein, mediating protein *trans*-splicing and thus ligating the two exteins. In BAC pSM $dd91$, a fragment encoding a protein-destabilizing domain, SopE, and Int^N were inserted in frame at the 3' end of the *M91* coding sequence, just upstream of the stop codon (Fig. 4A). Theoretically, in the cells with empty vectors (MEF10.1-Ctrl cells), pM91 should be degraded with SopE. On the contrary, in the presence of Int^C, protein splicing should remove SopE from pM91 and stabilize the protein (Fig. 4C). As expected, we successfully harvested the mutant virus with HA-mCherry-Int^C-FLAG-expressing MEF10.1 cells (MEF10.1-Int^C cells).

To determine the replication capacity of the SM $dd91$ recombinant viruses, we measured multiple-step growth curves. MEF10.1-Ctrl cells or MEF10.1-Int^C cells were

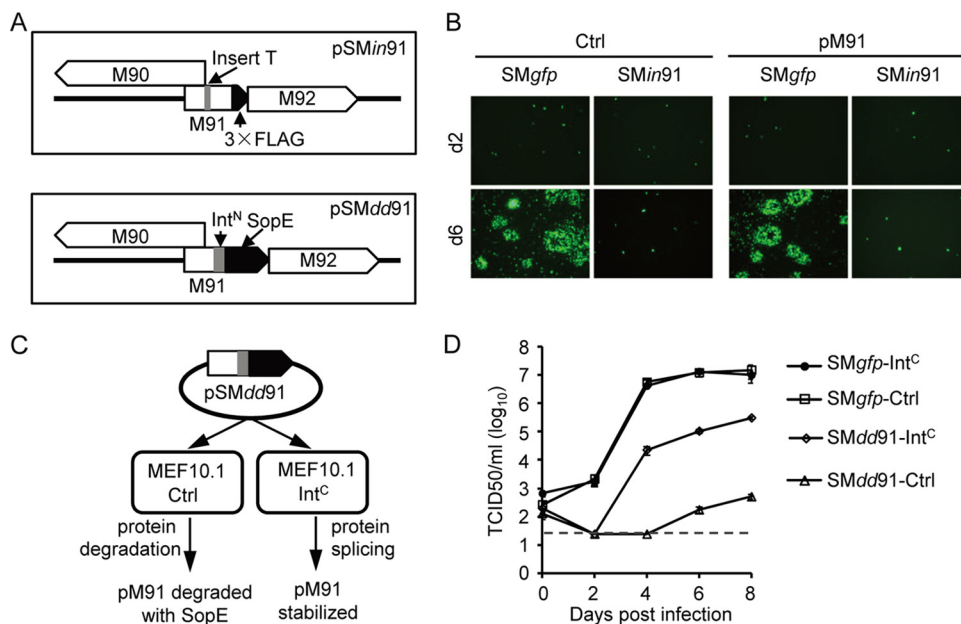


FIG 4 *M91* is essential for MCMV replication in fibroblasts. (A) Representative diagram of the pM91-deficient recombinant BACs pSMin91 and pSMdd91. pSMin91 has a single nucleotide insertion (T before nt 210) (indicated by the gray box) within the *M91* coding sequence, resulting in a frameshift mutation. pSMdd91 contains the SopE coding sequence (indicated by the black box) and part of the gp41-1 split intein (Int^N; indicated by the gray box) at the 3' end of the *M91* coding sequence. (B) Control (Ctrl) or pM91-expressing (pM91) MEF10.1 cells were transfected with wild-type BAC pSMgfp or mutant BAC pSMin91, and GFP signals were observed using fluorescence microscopy. (C) Working model of the pM91-deficient virus using the intein-mediated modulation of the protein stability system. Without Int^C, SopE induces rapid degradation of the entire fusion protein, effectively depleting pM91 during infection. However, after intein splicing, SopE is released from pM91 and pM91 expression is rescued, supporting viral growth. (D) Control (Ctrl) or HA-mCherry-Int^C-FLAG (short for Int^C)-expressing MEF10.1 cells were infected with SMgfp or SMdd91 at an MOI of 0.01. At the indicated time points, the supernatants of infected cells were harvested and analyzed by the 50% tissue culture infective dose (TCID₅₀) assay using MEF10.1-Int^C cells. The detection limit of the TCID₅₀ assay is indicated by the dashed line.

infected with the SMgfp or SMdd91 virus at an MOI of 0.01, and the supernatants were collected at the various time points indicated below. The viral titer, determined by a 50% tissue culture infective dose (TCID₅₀) assay, was measured using MEF10.1-Int^C cells. As shown in Fig. 4D, the growth of the wild-type virus (SMgfp) in MEF10.1-Int^C cells was similar to that in MEF10.1-Ctrl cells, indicating that HA-mCherry-Int^C-FLAG expression does not affect SMgfp virus growth. In contrast, the recombinant virus SMdd91 almost failed to grow in the MEF10.1-Ctrl cells, as the levels of the viruses collected at 8 days postinfection (dpi) were almost undetectable (5×10^2 TCID₅₀ units/ml). Nevertheless the levels of the viruses collected from SMdd91-infected MEF10.1-Int^C cells reached 3×10^5 TCID₅₀ units/ml at 8 dpi. Although the level of growth of the recombinant virus SMdd91 in complementary cells (MEF10.1-Int^C) was not rescued to that of the wild-type virus SMgfp, the imPS system indeed successfully controlled the stability of pM91 during virus infection and provided a unique approach to study the function of this essential gene.

Taken together, our findings suggest that pM91 is a factor essential for MCMV replication.

pM91 is required for late viral gene expression but dispensable for viral DNA replication. Using the pM91-deficient virus (SMdd91), we investigated the specific role of pM91 during infection. First, we examined the viral gene expression profile with reverse transcription (RT)-coupled quantitative PCR (qPCR) analysis. MEF10.1 cells were infected with pM91-deficient virus (SMdd91) or wild-type virus (SMgfp) at an MOI of 2. Total RNA was extracted to assess the RNA levels of the genes indicated below. At the early time point (8 hpi), depletion of pM91 had a minimal impact on immediate early gene *m123* (IE1) or early gene *M112/113* (E1) transcripts (Fig. 5A and B). However, at the

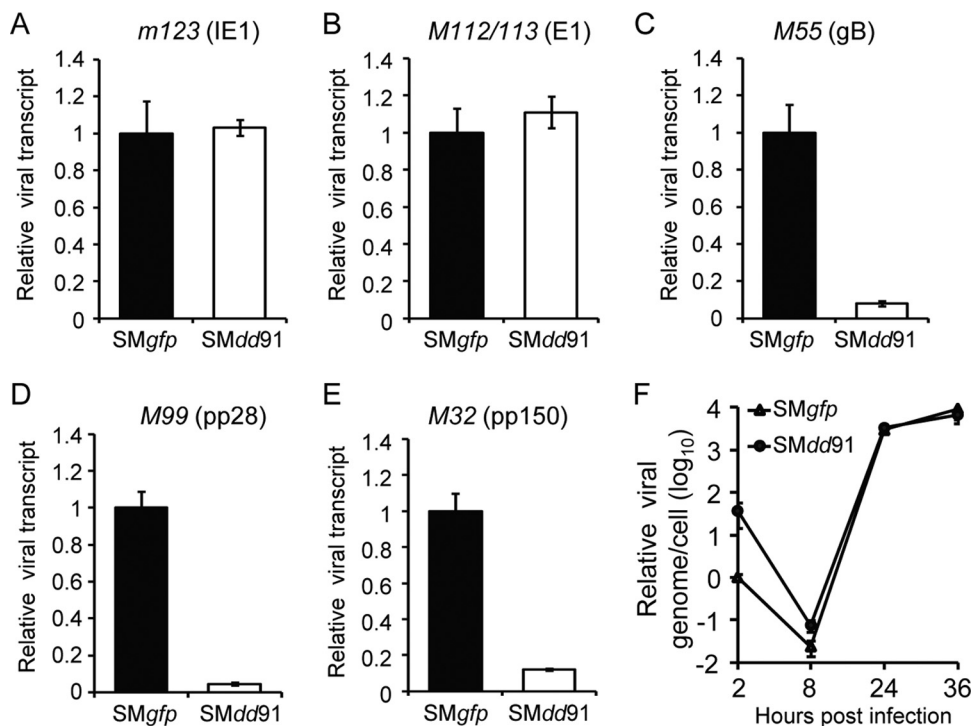


FIG 5 pM91 is required for late gene expression but dispensable for viral lytic DNA replication. MEF10.1 cells were infected with wild-type virus (SMgfp) or pM91-deficient virus (SMdd91) at an MOI of 2. At 8 hpi (A, B) or 36 hpi (C, D, E), total RNA was extracted from the harvested cells and subjected to RT-qPCR. (A to E) The mRNA expression levels of the viral genes *m123* (immediate early gene; IE1), *M112/113* (early gene; E1), and *M55* (gB), *M99* (pp28), and *M32* (pp150) (late genes) were normalized to the mouse *Gapdh* gene expression level, and the normalized amount of transcript during SMgfp infection was set to 1. (F) Depletion of pM91 had a minimal effect on viral DNA synthesis. MEF10.1 cells were infected as described in the legend to panel A, and intracellular DNA was isolated from infected cells at the indicated times. DNA levels were analyzed by qPCR, and the values were normalized to those for the actin gene. The quantity of DNA from SMgfp virus-infected cells at 2 hpi was set to 1.

late time spot (36 hpi), the levels of three important late gene transcripts, *M55* (gB), *M99* (pp28), and *M32* (pp150), were 12.4-, 21.9-, and 8.3-fold lower, respectively, when pM91 was absent (Fig. 5C to E). The results suggest that pM91 is required for viral late gene transcription.

As viral late gene transcription is activated by DNA replication, it is possible that pM91 inhibits DNA replication and further reduces late gene expression. To determine whether viral DNA replication was inhibited in the absence of pM91, we examined intracellular viral genomic copies in MEF10.1 cells infected with SMdd91 or SMgfp viruses at an MOI of 2. Total intracellular DNA was purified at the time points indicated below and subjected to qPCR analysis. The kinetics of viral genomic copies during SMdd91 infection were indistinguishable from those during SMgfp infection, suggesting that pM91 is dispensable for viral DNA replication (Fig. 5F).

The pM91-pM79 interaction is critical for virus production. Next, we decided to map the pM79-interacting region within pM91. As mentioned above, pM91 and its homologs are conserved among beta- and gammaherpesviruses. Protein sequence alignment of pM91 (MCMV) and pUL91 (HCMV) revealed that the central regions were highly conserved, while the N- and C-terminal regions were less so (Fig. 6A). Amino acids (aa) 1 to 71 of pUL91 but not the C-terminal region was previously shown to be required for HCMV replication (27). Thus, truncation mutations were first generated to define which region of pM91 was responsible for the interaction. Neither N- nor C-terminal truncation had a significant effect on the interaction, suggesting that the central region was responsible (Fig. 6B). We then performed a 10-alanine screening toward the central region. Interestingly, all the pM91 alanine mutants (A5 to A11) greatly reduced the interaction, and the A6 and A10 mutants nearly abrogated binding

contribute to the interaction between these two proteins. Therefore, we mutated four conserved negatively charged residues (E61, D62, D89, and D96) within the pM91 central region. Strikingly, the D89A/D96A or E61A/D62A double mutation greatly reduced the interaction with pM79. On the contrary, mutation of D89A and double mutation of the less conserved negatively charged residues E56A/E58A had no significant effects on the interaction between pM91 and pM79 (Fig. 6D). This result suggests that the four residues E61, D62, D89, and D96 in pM91 are crucial for the pM91-pM79 interaction.

To dissect the role of the pM91-pM79 interaction during infection, we mutated these critical residues in the context of the viral genome to cause an interaction deficiency. After transfection of the BAC mutants (pSM91^{D89A/D96A} or pSM91^{E61A/D62A}) into MEF10.1 cells, phenotypes similar to those found after pM91-deficient BAC (pSM^{ddd91}) transfection were observed (Fig. 6E). The results indicate that the pM91-pM79 interaction is critical for viral production, likely through the regulation of viral late gene expression.

DISCUSSION

Late gene expression is a critical step to complete the life cycle of CMV lytic replication. Elucidating the regulation mechanism of this process is beneficial to explore novel antiviral strategies. In this study, we demonstrated that the six MCMV vTFs (pM49, pM79, pM87, pM91, pM92, and pM95) interacted directly or indirectly with each other to form a complex, in which pM91 had a strong direct interaction with pM79 (Fig. 1 and 2). We then revealed that pM91 is expressed with kinetics similar to those of pM79 and colocalizes with pM79 in the nuclear viral replication compartments during infection (Fig. 3). Depletion of pM91 inhibited late gene transcription and virus production but had only minor effects on viral DNA replication (Fig. 4 and 5). Finally, we mapped the essential residues within pM91 for pM79 binding, identifying four conserved residues, E61, D62, D89, and D96, which were essential for the interaction between these two proteins (Fig. 6). Mutation of these residues disrupted the pM91-pM79 interaction and impaired virus production (Fig. 6E). Taken together, the pM91-pM79 interaction is critical for the formation of the viral transcription complex and plays an essential role in the regulation of viral late gene expression.

Our study provides a model to explain the organization of the MCMV vTF complex (Fig. 2D). In brief, the six viral proteins form a complex by two sets of interactions: one with a weak interaction between pM92 and pM87 (Fig. 2A) and one with strong interactions: pM87-pM95, pM95-pM49, and pM91-pM79 (Fig. 2B). In addition, two of these subunits (pM95-pM49 and pM91-pM79) strongly interact with each other (Fig. 2C) and bind to pM87 through multiple interfaces. Recently, the EBV BcRF1 protein and KSHV ORF24 protein were reported to be TBP homologs (33, 34). As they are conserved in betaherpesviruses, pM87 probably also recognizes the TATT motif within late gene promoters and recruits other proteins to initiate viral late gene expression. It is interesting that although pM91 does not directly interact with pM87, pM95, or pM49 (Fig. 2B), it could coimmunoprecipitate with pM87, pM95, and pM49 with the presence of pM79 (Fig. 2C). One possible explanation is that the formation of the strong interactions (pM87-pM95, pM95-pM49, and pM91-pM79) likely changes the conformation of these proteins and generates new binding sites. These findings indicate the complexity of the vTF complex, and further structural analysis is needed to elucidate this detailed organization.

A previous study proved that KSHV ORF31-ORF34-ORF24 (pM92-pM95-pM87 in MCMV) forms a trimeric complex and ORF34 bridges the interaction between ORF31 and ORF24 (35). However, our results suggest that pM87 serves as the bridge to connect pM92 and pM95 (Fig. 2D). Another intriguing observation was that pM91 expression *in trans* could not rescue the replication of the pM91-deficient virus (Fig. 4B), while pUL91 expression could rescue the replication of the pUL91-null virus (27). These findings suggest that the conserved vTFs in beta- and gammaherpesviruses may possess different specificities and mechanisms.

Our study demonstrated that the pM91-pM79 interaction is required for formation of the vTF complex (Fig. 2C) and is essential for virus production (Fig. 6E). In addition, both pM91 (Fig. 5) and pM79 (16) are critical for viral late gene expression. It is likely that they have to interact with each other and promote the correct formation of the vTF complex during MCMV infection; otherwise, late gene transcription would be inhibited and progeny virus could not be produced. In the future, a pM91-pM79 interaction-deficient virus is needed to confirm this hypothesis.

In summary, our results shed insight into the organization of the viral late gene transcription complex during MCMV infection. Furthermore, we demonstrate the importance of pM91 and its interaction with pM79, which may provide novel strategies for the treatment of CMV-related diseases in the future.

MATERIALS AND METHODS

Plasmids and reagents. MCMV *M49*, *M79*, *M87*, *M91*, *M92*, and *M95* coding fragments were amplified from bacterial artificial chromosome (BAC) pSM*gfp* (16) and subcloned into pRetro-EBNA (39), modified to include N- or C-terminal tags (3×FLAG, 3×HA, or 3×Myc). Plasmid pEBNA-HA-mCherry-Int^C-FLAG was generated by inserting the HA-mCherry-Int^C-FLAG fragment (amplified from pLKO-dcMV-Int^C-FLAG [38]) into pRetro-EBNA. Plasmid pYD-C163 is a pRetro-EBNA-based vector containing the coding sequence of HCMV protein pUL38 (40).

Plasmid pEPKan-S2 (41), which carries a kanamycin selection cassette bracketed by two I-SceI sites, was used for BAC recombination (42). To facilitate generation of the PCR product for BAC recombination, another three plasmids were constructed. The fragment KanS was amplified from pEPKan-S2 and inserted into the pEBNA vector with an N-terminal 3×HA tag or 3×FLAG tag, resulting in plasmids pEBNA-HA-KanS and pEBNA-FLAG-KanS. To create plasmid pEBNA-Myc-KanS-Int^N-SopE, the fragment Myc-EcoRI-Int^N-SopE was amplified from pLKO-dcMV-GFP-Int^N-SopE (38) and inserted into pRetro-EBNA, and then the fragment KanS was inserted into the EcoRI site.

The primary antibodies used in this study included the following: anti- α -tubulin (66031-1-Ig; Proteintech), anti-FLAG (M20008; Abmart), anti-FLAG (F7425; Sigma-Aldrich), anti-HA (MMS-101P; Covance), anti-MCMV IE1 and E1 (generous gifts from Stipan Jonjic, University of Rijeka, Rijeka, Croatia), anti-MCMV gB (a generous gift from Anthony Scalzo, University of Western Australia), and anti-pUL38 (a generous gift from Thomas Shenk, Princeton University). Other chemicals used in this study included phosphonoacetic acid (PAA; 284270-10G; Sigma-Aldrich) and L-(+)-arabinose (Urchem).

Cells. Mouse embryonic fibroblast 10.1 (MEF10.1) cells (43), Phoenix cells (39), and HEK293T cells were propagated in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, nonessential amino acids, and 1 mM sodium pyruvate. To create MEF10.1 cells expressing pM91 (MEF10.1-pM91), the empty vector (MEF10.1-Ctrl), or HA-mCherry-Int^C-FLAG (MEF10.1-Int^C), retrovirus stocks were made by transfecting the retroviral vector pEBNA-pM91, pRetro-EBNA, or pEBNA-HA-mCherry-Int^C-FLAG, respectively, into Phoenix cells. Then, MEF10.1 cells were transduced three times with these retroviruses as described previously (44).

BAC mutagenesis and recombinant viruses. MCMV BAC pSM*gfp* (a kind gift from Dong Yu) carries the full-length genome of the MCMV Smith strain (45) with a GFP expression cassette at the C terminus of the MCMV IE2 locus (16). To modify the MCMV genome, pSM*gfp* was introduced into *Escherichia coli* strain GS1783 (a kind gift from Gregory Smith) by electroporation. All recombinant MCMV BAC clones used in this study were constructed according to a BAC recombineering protocol described previously (42). To generate BAC pSM79HA91*flag*, we first created BAC pSM79HA. pSM79HA was constructed by inserting an oligonucleotide encoding 3×HA at the 3' end of the *M79* coding sequence within pSM*gfp*. Then, the oligonucleotide encoding 3×FLAG was inserted at the 3' end of the *M91* coding sequence in pSM79HA, resulting in recombinant BAC pSM79HA91*flag*. BAC pSM*Min*91 carried a single nucleotide insertion (T inserted before nucleotide [nt] 210) within the *M91* coding sequence of pSM79HA91*flag*, resulting in a frameshift mutation. BAC pSM*Mdd*91 contained the SopE coding sequence and part of the gp41-1 split intein (Int^N) at the 3' end of the *M91* coding sequence of pSM*gfp*. BAC pSM91^{D89A/D96A} and pSM91^{E61A/D62A} carried a double mutation in the *M91* coding sequence of pSM79HA91*flag*. The primers used for mutagenesis are listed in Table 2.

The above-described BAC clones were then used to reconstitute the corresponding viruses. Two to 5 μ g of MCMV BAC DNA was electroporated into normal MEF10.1 cells or MEF10.1-Int^C cells (for reconstituted SM*Mdd*91) and plated on a 10-cm plate. The culture medium was changed after 24 h, and virus stocks were prepared by harvesting cell-free culture supernatant when 80% of the infected cells were lysed. Viruses were amplified by infecting cells at a multiplicity of infection (MOI) of 0.01 and collecting the cell-free supernatant from infected cultures. Virus titers were determined in duplicate by a 50% tissue culture infective dose (TCID₅₀) assay in MEF10.1 cells or MEF10.1-Int^C cells.

Viral growth analysis. The BAC-transfected cells were observed with a Leica fluorescence microscope. Images were captured with a Leica DFC 450 digital camera. The growth kinetics of virus SM*Mdd*91 were analyzed as previously described (44). MEF10.1-Ctrl cells or MEF10.1-Int^C cells were seeded in 12-well dishes. After 24 h, the cells were inoculated with SM*Mdd*91 virus for 1 h at an MOI of 0.01. The inoculum was removed, the cells were rinsed with fresh medium, and finally, 1 ml fresh medium was added to each well. Cell-free media from infected cultures were collected in duplicate at various times postinfection, and the virus titers in the media were determined by the TCID₅₀ assay in MEF10.1-Int^C cells.

TABLE 2 Primers used in BAC recombination

Primer name ^a	Sequence (5'-3') ^b
M91-FLAG-F	cgccgggcggaacgacgggcccacctcgcgagcgaccgcctcgaaggacGACTCAAAGACCATGACGGTG
M91-FLAG-R	cgatataggcttgcctccgcggggtctcgccgtgtgagaacatagtaCTTGTCATCGTCATCCTTGTAAATCG
M79-HA-F	acaagaaggaggaaggtgtgatcgccgacgtgctgagcgagcgctcgaATACCTTATGACGTGCCCGA
M79-HA-R	gagatatacctgtgtgggtcgtttttttttattcataaaatcaatcaAGCATAATCTGGAACATCATATGG
M91-dd-F	cgccgggcggaacgacgggcccacctcgcgagcgaccgcctcgaaggacGAGCAAAGCTGATCTCCGAGGAG
M91-dd-R	cgatataggcttgcctccgcggggtctcgccgtgtgagaacatagtaGGCGCTGCCGCGGATGTCG
M91-inT-F	acgagaccctggaagcgtgttctcgtctcgccgagtcctTcggggagcggtgacttctAGGATGACGACGATAAGTAGGG
M91-inT-R	ggattccccgggtgctgaagaagtcgacgcctccccGaatggactcgcgagacgaaaCAACCAATTAACCAATTCTGATTAG
M91-D89A/D96A-F	gtgcgacttctcaggcaacccgggaaatcccgcactcgggtcttgGCCctcgcggtgtccttattGCCcagttgcgggcggagtgtat AGGATGACGACGATAAGTAGGG
M91-D89A/D96A-R	gaagcccagcgaaccacgtctctatacactccgccgaacgtgGGCaataaggacaccgcgagCAACCAATTAACCAATTCTGATTAG
M91-E61A/D62A-F	cgcgagctcaggaagggcgatcgagcagcagaccctgGCCGCTgtgttctcgtcgcgagtcAGGATGACGACGATAAGTAGGG
M91-E61A/D62A-R	agtgcacgcctccccgtgactcggcgagacgaaacAGCGCGcagggtctcgtctCAACCAATTAACCAATTCTGATTAG

^aF, forward; R, reverse.

^bLowercase nucleotides indicate sequences homologous to parental BACs, underlined uppercase nucleotides indicate mutagenesis site, and uppercase nucleotides indicate sequences homologous to those of the plasmids used as a template.

Protein analysis. Protein interactions were analyzed by coimmunoprecipitation assay as previously described (30). HEK293T cells were transfected with the plasmids indicated above and collected after 48 h. Collected cells were lysed in 2 ml lysis buffer (40 mM HEPES [pH 7.4], 1 mM EDTA, 300 mM NaCl, 0.5% NP-40) supplemented with 250 units of Benzonase nuclease (which digested the DNA and prevented DNA-mediated interactions; Millipore), phenylmethylsulfonyl fluoride, and protease inhibitors (PIC; Roche), incubated at 4°C for 1 h, and centrifuged at 13,200 × *g* at 4°C for 15 min. Fifty microliters of the supernatant was saved as the input control and boiled in sodium dodecyl sulfate (SDS)-containing sample buffer. The remainder was incubated with FLAG M2 (mouse monoclonal) antibody-conjugated magnetic beads (Sigma-Aldrich) at 4°C for 4 h. Then, the beads were washed four times with 2 ml lysis buffer. The immunoprecipitants were eluted by 150 ng/μl FLAG peptide (Sigma-Aldrich). The input and elution were analyzed by immunoblotting with the antibodies indicated above.

Protein accumulation was analyzed by immunoblotting as described previously (40). Briefly, cells were collected and lysed in SDS sample buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane, hybridized with primary antibodies, reacted with horseradish peroxidase-coupled secondary antibodies, and visualized by the use of Clarity Western enhanced chemiluminescence substrate (Bio-Rad).

The intracellular localization of the proteins of interest was analyzed by immunofluorescence assay as previously described (46). Cells were seeded onto coverslips and infected with SMgfp or SM79HA91flag at an MOI of 2. At 24 h postinfection (hpi), cells were washed, fixed, permeabilized, blocked, incubated with primary antibodies, and subsequently labeled with Alexa Fluor 488 (anti-mouse)- or Alexa Fluor 568 (anti-rabbit)-conjugated secondary antibodies (Invitrogen-Molecular Probes). Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime) and mounted on slides with Prolong Gold antifade reagent (Invitrogen-Molecular Probes). Confocal microscopic images were captured by an Olympus FV1200 confocal laser scanning microscope.

RNA and DNA analysis. Relative mRNA levels were determined by reverse transcription (RT)-quantitative PCR (qPCR) as previously described (38). Total RNA was extracted with the TRIzol reagent (Invitrogen), and cDNA was reverse transcribed using a PrimeScript RT reagent kit (TaKaRa). cDNA was then quantified using SYBR Premix Ex Taq (TaKaRa) with primer pairs specific for viral genes or the mouse *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) gene (Table 3) (16). All reactions were performed

TABLE 3 Primers used in qPCR analysis

Primer ^a	Sequence (5'-3')
MCMV <i>m123</i> (IE1)-F	CAGGGTGGATCATGAAGCCT
MCMV <i>m123</i> (IE1)-R	AGCGCATCGAAAGACAACG
MCMV <i>M112/113</i> (E1)-F	GAATCCGAGGAGGAAGACGAT
MCMV <i>M112/113</i> (E1)-R	GGTGAACGTTTGCTCGATCTC
MCMV <i>M55</i> (gB)-F	GCGATGTCCGAGTGTGTCAAG
MCMV <i>M55</i> (gB)-R	CGACCAGCGGTCTCGAATAAC
MCMV <i>M99</i> (pp28)-F	GTCTGACGACGAAGACCAGG
MCMV <i>M99</i> (pp28)-R	CTGTATCGGAGTGACCACGG
MCMV <i>M32</i> (pp150)-F	GAGACGATGAGGATTGCGGT
MCMV <i>M32</i> (pp150)-R	GGCCTCGTTGTCCACCATTA
Mouse <i>Actin</i> -F	GCTGTATCCCCTCCATCGTG
Mouse <i>Actin</i> -R	CACGTTGGCCTTAGGGTTCA
Mouse <i>Gapdh</i> -F	TGGAGAAACCTGCCAAGTATGA
Mouse <i>Gapdh</i> -R	CTGTTGAAGTCGACGAGACAA

^aF, forward; R, reverse.

in two biological and two technical replicates. The amounts of viral transcript were normalized to the amount of *Gapdh*.

Intracellular DNA was measured by qPCR as previously described (16). MEF10.1 cells were infected with the *SMgfp* virus or *SMdd91* virus at an MOI of 2 and collected at the times postinfection indicated above. Viral or cellular DNA was quantified by qPCR using SYBR Premix *Ex Taq* (TaKaRa) with a primer pair specific for MCMV *m123* (IE1) or the mouse actin gene, respectively (Table 3) (16). The accumulation of viral DNA was normalized by dividing the number of *m123* gene equivalents by the number of actin gene equivalents. The normalized amount of viral DNA in *SMgfp*-infected cells at 2 hpi was set at 1.

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