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Murine Cytomegalovirus Protein pM92 Is a Conserved Regulator of Viral Late Gene Expression

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In this study, we report that murine cytomegalovirus (MCMV) protein pM92 regulates viral late gene expression during virus infection. Previously, we have shown that MCMV protein pM79 and its human cytomegalovirus (HCMV) homologue pUL79 are required for late viral gene transcription. Identification of additional factors involved is critical to dissecting the mechanism of this regulation. We show here that pM92 accumulated abundantly at late times of infection in a DNA synthesis-dependent manner and localized to nuclear viral replication compartments. To investigate the role of pM92, we constructed a recombinant virus SM*in*92, in which pM92 expression was disrupted by an insertional/frameshift mutation. During infection, SM*in*92 accumulated representative viral immediate-early gene products, early gene products, and viral DNA sufficiently but had severe reduction in the accumulation of late gene products and was thus unable to produce infectious progeny. Coimmunoprecipitation and mass spectrometry analysis revealed an interaction between pM92 and pM79, as well as between their HCMV homologues pUL92 and pUL79. Importantly, we showed that the growth defect of pUL92-deficient HCMV could be rescued in *trans* by pM92. This study indicates that pM92 is an additional viral regulator of late gene expression, that these regulators (represented by pM92 and pM79) may need to complex with each other for their activity, and that pM92 and pUL92 share a conserved function in CMV infection. pM92 represents a potential new target for therapeutic intervention in CMV disease, and a gateway into studying a largely uncharted viral process that is critical to the viral life cycle.

Human cytomegalovirus (HCMV), a prototypical member of the betaherpesvirus subfamily, is a ubiquitous pathogen limited to the human host (1). After the resolution of acute infection, HCMV establishes a persistent, life-long infection characterized by alternate stages of virus production and latency (1). In immunocompetent hosts, the infection is typically asymptomatic. However, in immunocompromised hosts, lytic infection, during both primary infection and reactivation from latency, can cause significant morbidity and mortality (1, 2). HCMV is the leading viral cause of birth defects, such as deafness and mental retardation, in perinatally infected infants (3, 4). It is a major cause of retinitis and blindness in AIDS patients (2). It is a common source of infectious complications in transplant recipients and cancer patients (5). Emerging evidence also provides a possible association of HCMV infection with cardiovascular disease and proliferative diseases such as cancer (6, 7). Currently there is no vaccine to this virus, and antiviral therapies are limited by poor toxicity scores, low availability, and emergence of resistant viruses (8). Understanding the role of viral genes in lytic infection is paramount and will yield novel targets for antiviral therapies.

Murine CMV (MCMV) is the homologue of HCMV and model of choice to study CMV biology and pathogenesis. It shares conserved features with HCMV with regard to virion structure, genome organization, gene expression, tissue tropism, and clinical manifestations (9–12). Many genes of MCMV are conserved in HCMV, and its ability to infect mice provides a tractable small animal model to investigate virus infection *in vivo*. The use of MCMV to explore conserved viral genes will shed light on the roles of their counterparts in the replication and pathogenesis of HCMV.

The lytic replication cycles of herpesviruses are characterized by highly ordered cascades of gene expression, which can be sequentially divided into immediate-early (IE), early (E), and late (L) phases (1). The expression of IE genes only requires cellular factors and viral proteins associated with incoming virions. IE proteins transactivate the expression of early genes that are required for viral DNA synthesis. Many of the early proteins localize to viral nuclear replication compartments, where viral DNA synthesis, late gene transcription, and viral genome encapsidation take place (1). After viral DNA synthesis, late genes, many of which encode structural proteins, are expressed to allow virion assembly, maturation, and egress. It is also worth noting that some genes have both early and late properties; their transcriptions start prior to viral DNA synthesis, but the accumulation of their transcripts is enhanced considerably after DNA synthesis.

Although the regulation of IE and E gene expression has been studied extensively, less is known regarding the regulation of late gene expression during CMV infection. Previously, we have shown that MCMV protein pM79 is dispensable for viral DNA synthesis but is a key regulator of late gene transcription during MCMV infection (13). pM79 is conserved in both beta- and gammaherpesviruses, and its homologues in HCMV (pUL79) and MHV68 (ORF18) have been shown to play similar roles during virus infection (14–16). Identifying additional viral regulatory factors in late gene expression will be critical to understand this process. In the present study, we report that pM92 is another key regulator of MCMV late gene transcription. Like pM79, pM92 is also conserved in both beta- and gammaherpesviruses. Its homo-

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logues include pUL92 of HCMV, ORF31 of MHV68, and U63 of HHV-6. However, the role of pM92 during MCMV infection has not been defined, even though genome-wide mutagenesis analyses have previously shown that both MHV68 ORF31 and HCMV pUL92 are essential for lytic virus replication (17–19). Here, we create a pM92-deficient MCMV mutant virus and show that, in the absence of pM92, MCMV is capable of synthesizing its DNA at wild-type levels but unable to efficiently produce late gene products. The M92 gene products abundantly accumulate at 20 h postinfection (hpi) and localize to nuclear replication compartments. We also provide evidence that pM92 interacts with pM79 during virus infection and that their HCMV homologues pUL92 and pUL79 interact as well. These results support the role of pM92 as a key regulator of viral late transcription and suggest that pM92 and pM79 are part of a multicomponent regulatory complex controlling late transcription. Finally, we demonstrate the functional conservation between MCMV pM92 and HCMV pUL92 by rescuing the growth defect of pUL92-deficient HCMV virus with pM92 expression *in trans*. pM79 and pM92 offer attractive targets for novel antivirals, and MCMV provides a powerful system to dissect the regulatory mechanism of CMV late gene transcription, as well as to test antivirals targeting steps other than viral DNA synthesis. (In an accompanying paper, E. S. Mocarski and S. Omoto [20] discuss the particular role of HCMV-encoded UL92 as a key regulator of late viral gene expression.)

MATERIALS AND METHODS

Plasmids, antibodies, and chemicals. pYD-C433, pYD-C569, pYD-C245, and pYD-C618 were retroviral vectors derived from pRetro-EBNA (21). pYD-C433 and pYD-C569 contained the C-terminally hemagglutinin (HA)-tagged M38 and M79 coding sequences, respectively. pYD-C245 expressed the red fluorescent protein (DsRed) (22) from an internal ribosome entry site (IRES). pYD-C618 was derived from pYD-C245 and carried the N-terminally 1×FLAG-tagged M92 coding sequence that was expressed together with DsRed as a bicistronic transcript. pYD-C755 (a gift from Roger Everett, University of Glasgow Center for Viral Research), pYD-C678, and pYD-C780 were pLKO.1-based lentiviral expression vectors that carried a puromycin resistance marker (23, 24). Both pYD-C780 and pYD-C678 were derived from pYD-C755, and they carried the C-terminally 3×FLAG-tagged M92 and UL92 coding sequences, respectively. pYD-C191 carried a kanamycin selection cassette bracketed by two Flp recognition target (FRT) sites. pYD-C630 was derived from pGalK (25) and carried a FRT-bracketed GalK/kanamycin dual selection cassette (26). pYD-C746 was derived from pYD-C630, where a 3×FLAG sequence preceded the FRT-bracketed selection cassette.

The primary antibodies included: anti-actin (clone AC15; Abcam), anti-FLAG polyclonal rabbit antibody (F7425) and monoclonal mouse antibody (F1804) (Sigma), rat anti-HA (11867423001; Roche), anti-MCMV IE1 (CROMA101) and E1 (CROMA103) (generous gifts from Stipan Jonjic, University of Rijeka, Rijeka, Croatia), and anti-MCMV M44 (3B9.22A) and gB (2E8.21A) (generous gifts from Anthony Scalzo, University of Western Australia). The secondary antibodies used for immunoblotting were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, goat anti-rabbit IgG, and goat anti-rat IgG (Jackson Laboratory). The secondary antibodies used for immunofluorescence were Alexa Fluor 594-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen/Molecular Probes).

Other chemicals used in the present study included phosphonoacetic acid (PAA) (284270-10G; Sigma-Aldrich), L-(+)-arabinose (A3256-25G; Sigma-Aldrich), TO-PRO3 iodide (T3605; Invitrogen), Dynabeads (Novex, Life Technologies), benzonase (Novagen, Fisher Scientific), and 4 to 12% gradient polyacrylamide NuPAGE gels (Novex, Life Technologies).

Cells and viruses. Mouse embryonic fibroblast 10.1 cells (MEF10.1) (27), human embryonic lung fibroblasts (MRC5) (28, 29), and human foreskin fibroblasts (HFF) were propagated in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, nonessential amino acids, 1 mM sodium pyruvate, and 100 U of penicillin-streptomycin/ml. Cells were maintained at 37°C and 5% CO_2 in a humidified atmosphere. To create cell lines stably expressing N-terminally 1×FLAG-tagged pM92 (10.1-*flag*M92), MEF10.1 cells were transduced three times with pYD-C618-derived retrovirus and allowed to recover for 48 h. DsRed-positive cells were cloned by limiting dilution. Clonal cell lines were tested for their ability to produce virus upon transfection with the mutant MCMV bacterial artificial chromosome (BAC) pSM*in*92 (see below). The cell line yielding the highest titer at 5 days posttransfection was used in this study. To create cells expressing C-terminally 3×FLAG-tagged pM92 (MRC5-M92*flag*) or pUL92 (MRC5-UL92*flag*), MRC5 cells were transduced with lentivirus reconstituted from pYD-C780 or C678, respectively, and allowed to recover for 48 h (30). To create cells containing the vector control (MRC5-vector), MRC5 cells were transduced with pYD-C755-derived vector lentivirus. Transduced cells were then selected with 1 μg of puromycin/ml and maintained with 0.5 μg of puromycin/ml.

To reconstitute recombinant MCMV or HCMV viruses, confluent MEF10.1 or MRC5 cells were electroporated with corresponding MCMV or HCMV BAC DNA (see below), respectively. Recombinant MCMV SM*gfp* and SM*flag*92 were reconstituted in MEF10.1 cells. SM*in*92 was reconstituted in 10.1-*flag*M92 cells. To reconstitute recombinant HCMV, BAC-HCMV DNA, pp71-expression plasmid, and G403-expression plasmid were cotransfected into MRC5-UL92*flag* cells by electroporation (31). Cells were plated on a 10-cm plate, the medium was changed at 24 h posttransfection, and virus was harvested by collecting cell-free culture medium after the entire monolayer of cells was lysed. Alternatively, virus stocks were produced by collecting cell-free supernatant from infected culture at a multiplicity of infection (MOI) of 0.001. Virus titers were determined in duplicate by a 50% tissue culture infectious dose ($TCID_{50}$) assay in the appropriate cell type. In experiments where comparative analysis was performed between SM*in*92 or AD*in*UL92 with other recombinant viruses, titers for all of the viruses were determined in 10.1-*flag*M92 or MRC5-UL92*flag* cells, respectively.

BAC recombineering. Recombinant BAC clones in the present study were created by using a linear recombination-based BAC recombineering protocol that we have previously established (26). Recombination was carried out in *E. coli* strain SW105 that harbored either the MCMV or HCMV BAC clone and expressed an arabinose-inducible *Flippase* gene for transient expression of Flp recombinase (25).

Recombinant MCMV BAC clones were derived from the parental clone pSM3fr that carried a full-length genome of the MCMV Smith strain (32). pSM*gfp*, used as the wild-type clone in this study, contained the green fluorescent protein (GFP) expression cassette at the C terminus of the IE2 loci, which has been shown to be dispensable for MCMV infection *in vivo* and *in vitro* (33–35). The clone pSM*in*92 carried a frameshift mutation in the MCMV gene M92 (Fig. 1A). To construct pSM*in*92, the FRT-bracketed GalK/kanamycin cassette was PCR amplified from pYD-C630 and recombined into pSM*gfp* at nucleotide (nt) 358 of the M92 coding sequence. The selection cassette was then removed by arabinose induction of Flp recombinase and subsequent Flp-FRT recombination (26), leaving an 88-nt insert within M92 to create a frameshift mutation. The clone pSM*flag*92 contained an N-terminally 3×FLAG-tagged M92 coding sequence (Fig. 1A). To construct pSM*flag*92, a fragment containing the FRT-bracketed GalK/kanamycin selection cassette preceded by a 3×FLAG sequence was PCR amplified from pYD-C746 and recombined into the N terminus of the M92 coding sequence of pSM*gfp*. The selection cassette was subsequently removed by Flp-FRT recombination, resulting in the 3×FLAG fused in frame with the M92 coding sequence. Recombinant HCMV BAC clones were derived from the parental clone pAD/Cre that carried the full-length genome of HCMV strain AD169 (31). pAD*gfp*, used as the wild-type clone here, had a green fluorescent protein (GFP)

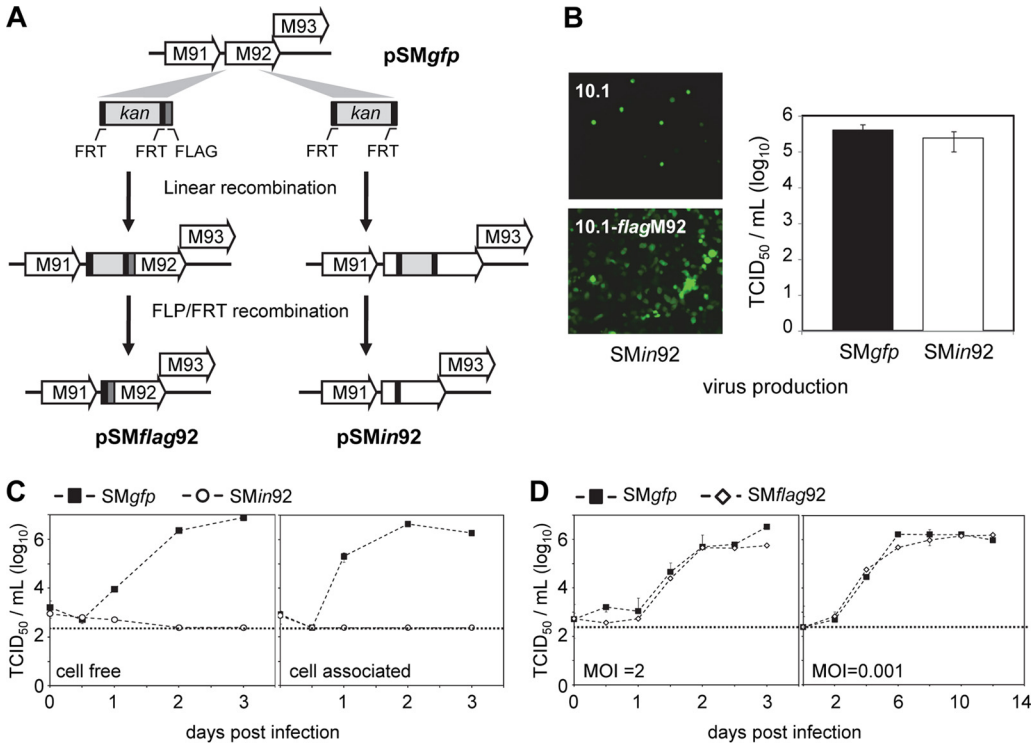


FIG 1 pM92 is essential for MCMV replication in fibroblasts. (A) Diagram depicting the construction of MCMV BACs used in the present study by BAC recombineering. To create pSM*in*92, an 88-nt insert was introduced (indicated by the black bar) at nt 358 of the M92 coding sequence, resulting in a frameshift mutation. pSM*flag*92 was created by fusing a 3×FLAG tag in frame at the N terminus of the M92 coding sequence (indicated by the shaded bar). (B) Growth of SM*in*92 virus in MEF 10.1 cells expressing FLAG-tagged pM92 (10.1-*flag*M92). Left panels are fluorescent images of virus-driven GFP expression in MEF 10.1 cells or 10.1-*flag*M92 cells, both of which were transfected with pSM*in*92. Images were taken at 7 days posttransfection. Right panels are titers of cell free virus at 72 hpi from 10.1-*flag*M92 cells infected with either SM*gfp* or SM*in*92 at an MOI of 2. (C and D) Growth kinetic analysis of MCMV recombinant viruses used in the present study. MEF 10.1 cells were infected with indicated viruses at an MOI of 2 (for single-step growth analysis) or 0.001 (for multistep growth analysis). Cell-free and cell-associated viruses were collected at indicated times and titers were determined by $TCID_{50}$ assay in 10.1-*flag*M92 cells. The detection limit of the $TCID_{50}$ is indicated by a dashed line.

gene in place of the viral US4-US6 region (31, 36). pAD*flag*UL79 was similar to pAD*gfp* except that the 5′ terminus of UL79 coding sequence was tagged with 3×FLAG. The clone pAD*in*UL92 was created in a similar manner to that of pSM*in*92, except that the insertion replaced nt 125 to 406 of the UL92 coding sequence carried in pAD*gfp*. All of the BAC clones were validated by restriction digestion, PCR analysis, and direct sequencing, as previously described (37).

Viral growth analysis. MEF10.1 or MRC5 cells were seeded in 12-well plates overnight to produce a confluent monolayer. Cells were inoculated with recombinant viruses for 1 h at an MOI of 2 for single step or 0.001 for multistep growth analysis. The inoculum was removed, the infected monolayer was rinsed with phosphate-buffered saline (PBS), and fresh medium was replenished. At various times postinfection, cell-free virus was collected in duplicate by harvesting medium from infected cultures. Cell-associated virus was collected by rinsing infected cells once with PBS and scraping cells into fresh medium. Cells were lysed by one freeze-thaw cycle, followed by sonication. Lysates were cleared of cell debris by low-speed centrifugation, and supernatants were saved as cell-associated virus. Virus titers were determined by $TCID_{50}$ assay.

DNA and RNA analysis. Intracellular DNA was measured by quantitative PCR (qPCR) as previously described (13). Briefly, cells were collected in a lysis buffer (200 mM NaCl, 20 mM Tris [pH 8.0], 20 mM EDTA, 0.2 mg of proteinase K/ml, 0.4% sodium dodecyl sulfate [SDS]), and lysed by incubation at 55°C overnight. DNA was extracted with phenol-chloroform and treated with RNase A (100 μg/ml) at 37°C for 1 h. Samples were extracted again with phenol-chloroform, precipitated with ethanol, and resuspended in nuclease-free water (Ambion). Viral DNA was quantified by qPCR using SYBR Advantage qPCR Premix (Clontech) and a primer pair specific for the MCMV IE1 gene (13). Cellular DNA was quantified by using a primer pair specific for the mouse actin gene (13). A standard curve was generated using serially diluted pSM*gfp* BAC DNA or cellular DNA and was used to calculate relative amounts of viral or cellular DNA in a sample. The amount of viral DNA was normalized by dividing IE1 equivalents over actin gene equivalents. The normalized amount of viral DNA in SM*gfp* infected cells at 10 hpi was set at 1.

Intracellular RNA was determined by reverse transcription-coupled qPCR (RT-qPCR) as previously described (13). Total RNA was extracted by TRIzol reagent (Invitrogen) and treated with Turbo DNA-free reagents (Ambion) to remove contaminating DNA. First-strand cDNA synthesis was performed with a high-capacity cDNA reverse transcription kit using random hexamer-primed total RNA (Applied Biosystems). Each sample also included a control without the addition of reverse transcriptase to determine the level of residual contaminating DNA. cDNA was quantified by using SYBR Advantage qPCR Premix (Clonetech) and primer pairs specific for viral genes or cellular β-actin (Table 1). A standard curve was generated for each gene using serially diluted cDNA from infected cells and was used to calculate the relative amount of a transcript in each sample. The amounts of viral transcript were normalized by dividing viral transcript equivalents over actin equivalents. The normalized amount of transcript during SM*gfp* infection at 10 hpi in the absence of PAA was set to 1.

Protein analysis. Protein accumulation was analyzed by immunoblotting. Cells were washed, and lysates were collected in sodium dodecyl sulfate (SDS)-containing sample buffer. Proteins were resolved by SDS-

TABLE 1 Primers used in PCR analysis

Primer	Sequence (5′–3′)
MCMV IE1 forward	CAGGGTGGATCATGAAGCCT
MCMV IE1 reverse	AGCGCATCGAAAGACAACG
MCMV M34 forward	TACTCTGATCGCGAACAGCA
MCMV M34 reverse	GCGTTGGTGGTTCGTGTCTG
MCMV M37 forward	ATGACGGCGGTCCTCTCCAT
MCMV M37 reverse	ACGTGTACGTCTTCCGTGCC
MCMV M45 forward	GGAACTCCTTGGTCATGCGA
MCMV M45 reverse	TTCCCCAAGTTCCCTAAGAG
MCMV M46 forward	CTGAACCTATAGCGCAGGAC
MCMV M46 reverse	ACTATAATAGCGATCGGCAG
MCMV M55 (gB) forward	GCGATGTCCGAGTGTGTCAAG
MCMV M55 (gB) reverse	CGACCAGCGGTCTCGAATAAC
MCMV M74 forward	AGGAGGCTGTGACTTTGAAA
MCMV M74 reverse	CTCATCAGCCGTTACTCGAG
MCMV M85 forward	TTTCATGAGGAGCATGTTGC
MCMV M85 reverse	CTGCATCTCGCTCTCCATGA
MCMV M92 forward	AAACCCACGGAGAATGCGAT
MCMV M92 reverse	ACGAACAGCTGACCTATGACGC
MCMV M96 forward	TCGAGGCGTTCGGTCCTGAT
MCMV M96 reverse	CGCATTCTCGGATACTCGCT
MCMV M102 forward	AGACCAGTACGGCGATCCAG
MCMV M102 reverse	AGCTTCCTGTAGGCGTGCGT
MCMV M112/113 (E1) forward	GAATCCGAGGAGGAAGACGAT
MCMV M112/113 (E1) reverse	GGTGAACGTTTGCTCGATCTC
MCMV M116 forward	TCCTTGGTGGTGATGGCGGT
MCMV M116 reverse	GCATCCCGTACCTGACCACA
Mouse actin forward	GCTGTATTCCCCTCCATCGTG
Mouse actin reverse	CACGGTTGGCCTTAGGGTTCA
M92 forward[a]	ATGTTCTCACACGGCCGAGA
M92 reverse[a]	CTAGCGGCCTGTTCGAAACG
UL92 forward[a]	ATGTGCGACGCCTCGGGCGCCTG
UL92 reverse[a]	AACGCCAGATCCGAATACAGGTG

[a] Primer used in PCR analysis of cellular DNA from pM92- or pUL92-expressing MRC5 cells.

containing polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride membrane. Proteins of interest were detected by hybridizing the membrane with specific primary antibodies, followed by HRP-coupled secondary antibodies, and visualized by using SuperSignal West Pico enhanced chemiluminescent (ECL) substrate (Thermo Scientific).

Intracellular localization of proteins of interest was analyzed by using an immunofluorescence assay. Cells were seeded onto coverslips 24 h prior to infection. At 24 hpi, the cells were washed with PBS, fixed, permeabilized with methanol (−20°C) for 10 min, and blocked with 5% fetal bovine serum in PBS at room temperature for 1 h. Cells were incubated with primary antibodies for 30 min at room temperature and subsequently labeled with secondary antibodies coupled to Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen-Molecular Probes). Cells were counterstained with TO-PRO3 and mounted on slides with Prolong Gold antifade reagent (Invitrogen-Molecular Probes). Confocal microscopic images were captured by a Zeiss LSM510 Meta confocal laser scanning microscope.

Protein interactions were analyzed by coimmunoprecipitation. For MCMV, MEF 10.1 cells transiently expressing C-terminally HA tagged M38 or M79 were infected with SM*flag*92 at an MOI of 2. Cells were collected at 48 hpi and lysed by incubation with extraction buffer (50 mM Tris [pH 8.0], 300 mM NaCl, 0.5% NP-40) for 15 min. In the meantime, 1 μg of FLAG-antibody was conjugated to 25 μl of Dynabeads (Novex, Life Technologies) by incubation in conjugation buffer (0.02% Tween 20 in PBS [pH 7.2]) for 20 min at room temperature. Lysates were cleared by centrifugation, the pellet of cellular debris was saved, and the supernatant was incubated with FLAG antibody-conjugated Dynabeads in the presence of endonuclease Benzonase (800 U/ml) (that digested DNA and prevented DNA-mediated, nonspecific interactions among DNA-binding proteins). After an overnight incubation, beads were washed four times with extraction buffer, and supernatant was saved as flowthrough. Washed beads were mixed with NuPAGE LDS sample buffer (Invitrogen) and boiled to elute FLAG-associated proteins. Pellets, flowthrough, and eluted samples were analyzed by an SDS-PAGE gel, followed by immunoblotting. For HCMV, HFF cells were infected with AD*gfp* or AD*flag*UL79 (16) at an MOI of 3. Infected cells were collected and lysed at 72 hpi, and immunoprecipitation was performed as described above. After elution, the samples were resolved on an SDS-PAGE gel, and protein bands were visualized by silver staining using a ProteoSilver Plus silver stain kit (Sigma).

RESULTS

pM92 is essential for MCMV replication in fibroblasts. The M92 open reading frame (ORF) is predicted to encode a gene product of 231 amino acids (aa) and is a sequence homologue to the HCMV ORF UL92 (202 aa). UL92 is essential for virus replication during HCMV infection (19), but the importance of M92 in MCMV infection in fibroblasts has not yet been established. To investigate this, a frameshift mutation was introduced at nt 358 of the predicted M92 ORF by BAC recombineering (13, 26), producing mutant BAC pSM*in*92 (Fig. 1A). This insertion is not expected to interfere with the expression of neighboring genes, particularly the 5′-terminally overlapping M93. Transfection of wild-type pSM*gfp* BAC in MEF10.1 cells produced virus (termed SM*gfp*), resulting in a complete cytopathic effect (CPE) of the monolayer and full spread of the virus-driven GFP expression at 5 days posttransfection, whereas transfection of pSM*in*92 failed to show any sign of CPE even at 2 weeks posttransfection (Fig. 1B). However, pM92-deficient virus (termed SM*in*92) was rapidly reconstituted to wild-type levels from pSM*in*92 upon transfection into MEF 10.1 cell that stably expressed N-terminally 1×FLAG-tagged pM92 (10.1-*flag*M92). Thus, the defect of SM*in*92 is the direct result of pM92 ablation.

To more precisely define the growth defect of SM*in*92, we performed growth curve analyses to quantify the defect of the recombinant virus and validate the essentiality of pM92 (Fig. 1C). SM*in*92 failed to produce detectable levels of cell-free or cell-associated progeny through the entire course of analysis, indicating that pM92 is essential for MCMV replication at steps prior to virus release.

pM92 is a 25-kDa protein that accumulates to high levels at late times of infection. A thorough search of available nucleotide and amino acid sequence databases failed to identify any significant homology of pM92 to proteins with known function. To acquire basic information and gain insights into the role of pM92, we first characterized potential protein and transcript products from this gene. Since no antibody was available for detecting the M92 protein product, we created a recombinant MCMV BAC, pSM*flag*92, in which the M92 coding sequence was tagged with 3×FLAG at the N terminus (Fig. 1A). Transfection of pSM*flag*92 BAC in MEF 10.1 cells rapidly reconstituted recombinant virus, termed SM*flag*92. Both single-step and multistep growth curve analyses indicated that SM*flag*92 grew similarly to SM*gfp* (Fig. 1D), suggesting that the 3×FLAG tag did not interfere with the function of M92 or neighboring gene M91 (Fig. 1A). 3×FLAG-tagged pM92 (p*flag*M92) migrated at the expected size of 25 kDa, became detectable at 24 hpi, and accumulated at more abundant

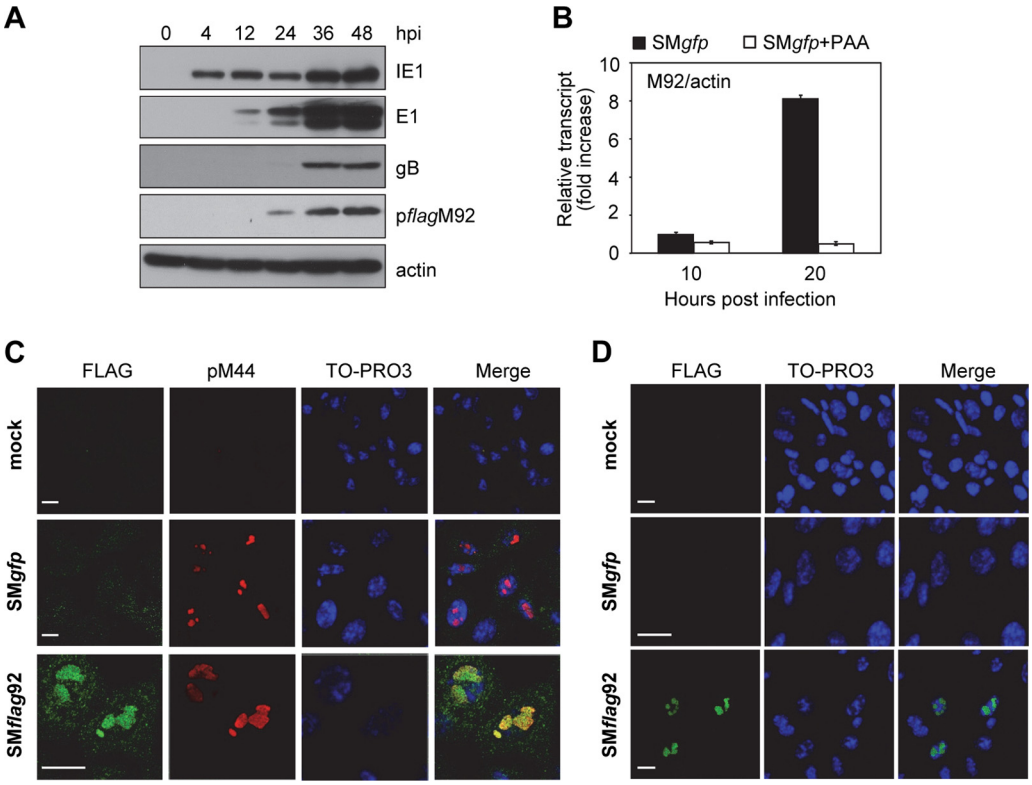


FIG 2 pM92 accumulates abundantly at late times of infection and localizes to viral nuclear replication compartments. (A) Accumulation of the M92 protein product during MCMV infection. MEF 10.1 cells were infected with SM*flag*92 virus at an MOI of 2. Total cell lysates were harvested at indicated times postinfection and analyzed by immunoblotting. The M92 protein was detected with the mouse anti-FLAG antibody, and actin was included as a loading control. (B) Accumulation of the M92 transcript during MCMV infection. MEF 10.1 cells were infected with SM*gfp* at an MOI of 2 in the presence or absence of viral DNA synthesis inhibitor PAA (200 μg/ml). Total RNA was isolated at indicated times postinfection and the M92 transcript was measured by RT-qPCR analysis with the primers listed in Table 1. The values were normalized to β-actin, and the normalized amount of M92 transcript at 10 hpi in the absence of PAA was set to the value of 1. (C and D) The M92 protein localizes to nuclear replication compartments during infection. MEF 10.1 cells were mock infected or infected with SM*gfp* or SM*flag*92 at an MOI of 2. At 24 hpi, cells were permeabilized and fixed with methanol and stained with either rabbit polyclonal (C) or mouse monoclonal (D) anti-FLAG antibody to detect the FLAG-tagged M92 protein (green). In panel C, cells were also stained with mouse antibody to pM44, which served as a marker for replication compartments. Cells were counterstained with TO-PRO3 to visualize the nuclei. Scale bars, 20 μm.

levels at 48 hpi during SM*flag*92 infection (Fig. 2A). To profile its transcription, we determined the accumulation of M92 transcript by reverse transcription-coupled quantitative PCR analysis (RT-qPCR). In agreement with its protein accumulation profile, M92 transcript levels were low at 10 hpi but increased 8-fold at 20 hpi (Fig. 2B). Importantly, M92 transcription was dramatically reduced when viral DNA synthesis was inhibited by phosphonoacetic acid (PAA) at 20 hpi (Fig. 2B). Therefore, M92 gene products accumulate abundantly in a viral DNA synthesis-dependent manner at late times of infection.

pM92 localizes to viral nuclear replication compartments during infection. To further characterize pM92, we next examined the intracellular localization of 3×FLAG-tagged pM92 during infection of SM*flag*92. Infected cells were fixed and permeabilized with methanol, which also quenched GFP fluorescence, thus allowing visualization of pM92 localization using Alexa Fluor 488-conjugated mouse anti-flag antibody by indirect immunofluorescence. pM92 localized to the nuclei of infected cells, and in particular, it localized to subnuclear structures resembling those of nuclear replication compartments (Fig. 2D). This led us to hypothesize that pM92 localized to viral replication compartments during infection. To test this, we compared intracellular localization of 3×FLAG-tagged pM92 to that of viral protein pM44 (i.e., the viral polymerase processivity factor and commonly used marker for replication compartments) during infection of SM*flag*92. Since the anti-pM44 antibody is of mouse origin, we used a rabbit anti-FLAG antibody to costain pM92 in this experiment. At 24 hpi, FLAG staining strongly colocalized with pM44 in the nuclei of SM*flag*92-infected cells (Fig. 2C), indicating that the majority of pM92 localizes within replication compartments. The rabbit anti-FLAG antibody has been previously shown to have high background staining (13). In this experiment, it also produced a diffuse, weak cytoplasmic staining, which likely represented nonspecific background since it was also present in SM*gfp*-infected control cells. Collectively, we conclude that pM92 is a nuclear protein that localizes to replication compartments during MCMV infection.

pM92 is dispensable for viral DNA synthesis but required for efficient late gene expression. To define the function of pM92, we first determined where it acted in the viral life cycle, hypothesizing that pM92 might be required for viral DNA synthesis as it localized to replication compartments (Fig. 2). However, qPCR analysis showed that viral DNA accumulation over the course of SM*in*92 infection was comparable to that during SM*gfp* infection

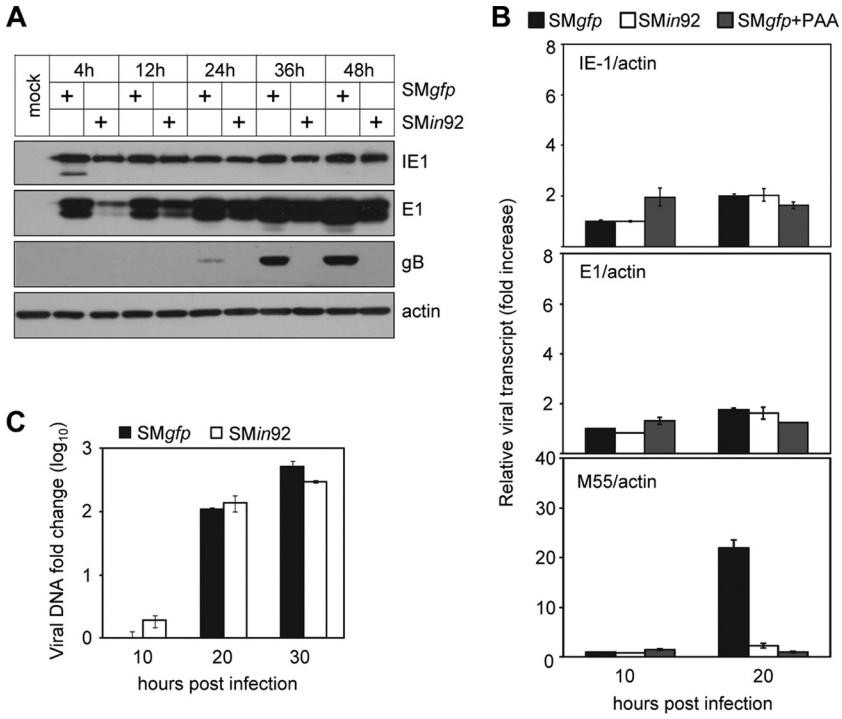


FIG 3 pM92 is dispensable for viral DNA synthesis but is required for efficient accumulation of M55 late gene products during infection. (A) Accumulation of representative viral proteins during pM92-defcient virus infection. MEF 10.1 cells were infected with SM*gfp* or SM*in*92 at an MOI of 2. Cell lysates were harvested at indicated times and analyzed by immunoblotting. (B) Accumulation of viral transcripts during pM92-deficient virus infection. MEF 10.1 cells were infected with SM*gfp* in the presence or absence of viral DNA synthesis inhibitor PAA (200 μg/ml) or with SM*in*92 at an MOI of 2. At 10 and 20 hpi, total RNA was harvested, and specific transcripts were quantified by RT-qPCR analysis using the primers listed in Table 1. The values were normalized to β-actin, and the normalized amount of transcript during SM*gfp* infection at 10 hpi in the absence of PAA was set to the value of 1. (C) Accumulation of viral DNA during pM92-deficient virus infection. MEF 10.1 cells were infected as described in panel A, total DNA was isolated from infected cells at the indicated times, and viral DNA synthesis was analyzed by qPCR. The values were normalized to β-actin, and the quantity of DNA during SM*gfp* infection at 10 hpi was set to 1.

(Fig. 3C). This result indicates that pM92 is not required for viral DNA synthesis.

To test whether pM92 is required for late gene expression, a viral event immediately downstream of viral DNA synthesis, we next examined the accumulation of immediate-early protein IE1, early protein E1 (M112/113), and late protein gB (M55) during SM*in*92 infection by immunoblot analysis. Compared to wild-type control SM*gfp*, SM*in*92 appeared to have two defects during infection. The first was a modest decrease in the accumulation of E1 protein at early times of infection. The second, and more striking defect, was that accumulation of late protein gB was reduced to undetectable levels in SM*in*92 infection (Fig. 3A). Early genes primarily function prior to viral DNA synthesis. Since no measurable defect was observed in DNA synthesis during SM*in*92 infection (Fig. 3C), the modest decrease in E1 accumulation was unlikely the main cause for the growth defect of SM*in*92. It is unclear why SM*in*92 has this minor defect in early gene expression. It may be a result of suboptimal complementation by the 10.1-*flag*M92 cells used to produce the mutant virus. It is also reminiscent of a similar observation reported for pM79-deficient virus (13). We therefore hypothesized that the inability of SM*in*92 to replicate was likely due to the failure to efficiently produce late proteins during infection.

To determine whether the defect was at the transcriptional level, we measured the transcript accumulation of representative immediate-early (IE1), early (E1), and late (M55) genes in the presence or absence of pM92 during virus infection by RT-qPCR. The expression kinetics of these genes was validated by treatment with the DNA synthesis inhibitor PAA. As expected, both IE and E1 gene expression was resistant to PAA, whereas late gene M55 expression was markedly sensitive to PAA (Fig. 3B). Importantly, in the absence of pM92, IE1 and E1 transcripts accumulated at wild-type levels, but the accumulation of late transcript M55 was significantly reduced (Fig. 3B). Therefore, pM92 is required for efficient transcript accumulation of late gene M55 during MCMV infection.

This result led us to hypothesize that pM92 is a new member of the viral late transcription regulators, which includes recently reported pM79 (13). To test whether pM92 had a global regulatory role in viral late transcription, we examined transcript accumulation of multiple early genes (M34, M37, M45, and M102) and late genes (M46, M74, M85, M96, and M116) during SM*in*92 infection. Transcription of early genes was independent of viral DNA synthesis and consequently only modestly affected by PAA treatment (<2-fold), a finding consistent with previous reports (Fig. 4A) (13). Importantly, the effect of pM92 mutation on early gene transcription was as modest as PAA treatment, indicating that pM92 is not required for efficient early gene expression. In stark contrast, late gene transcription was dependent on viral DNA synthesis and therefore significantly sensitive to PAA (Fig. 4B). Importantly, transcription of these genes was also significantly reduced during SM*in*92 infection. It was also noted that

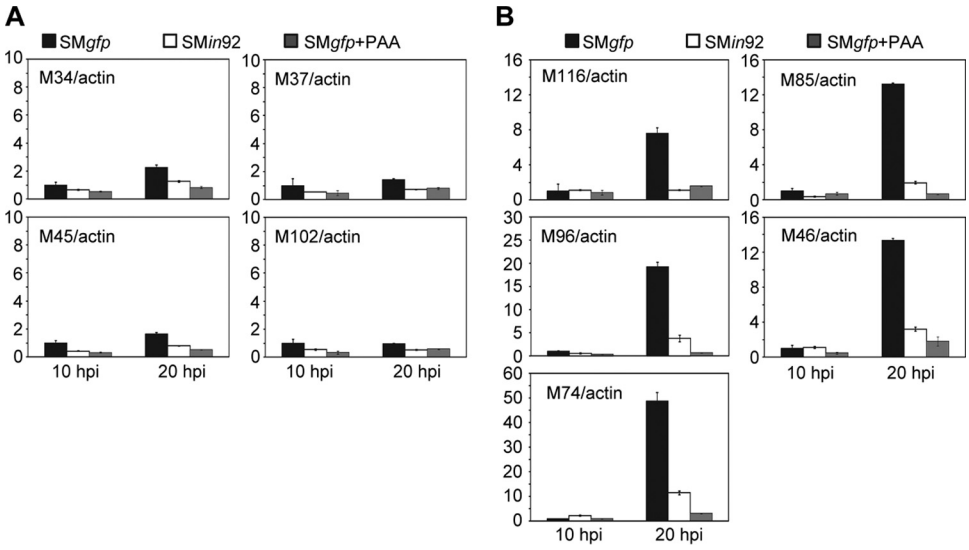


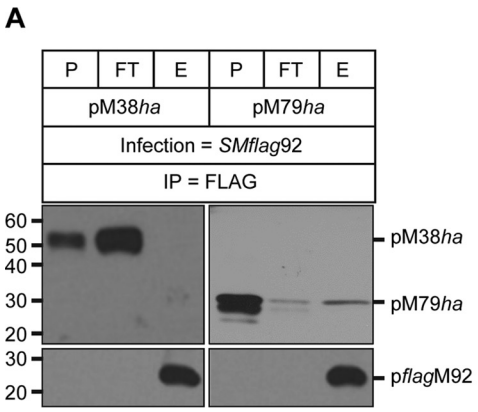
FIG 4 pM92 is required for efficient accumulation of a panel of late transcripts. MEF 10.1 cells were infected with SM*gfp* in the presence or absence of viral DNA synthesis inhibitor PAA (200 μg/ml) or with SM*in*92 at an MOI of 2. At the indicated times, total RNA was harvested and then representative early transcripts (A) and late transcripts (B) were quantified by RT-qPCR analysis using the primers listed in Table 1. The *y* axis represents relative viral transcript (fold increase). The values were normalized to β-actin, and the normalized amount of transcript during SM*gfp* infection at 10 hpi in the absence of PAA was set to 1.

transcription of individual genes showed various levels of dependency on pM92 relative to that on viral DNA synthesis. Reduction in M116 and M46 transcriptions in the absence of pM92 was comparable to that with PAA treatment, whereas the reduction in M96 and M74 transcription in the absence of pM92 was less pronounced. This is reminiscent of the previous report that different late gene transcriptions have different dependency on the viral late transcription regulator pM79 (13). Our results indicate that pM92 plays an important role in regulating late gene transcription during MCMV infection.

CMV UL92/M92 proteins interact with UL79/M79 proteins during infection. We have previously found that MCMV protein pM79 regulates viral late transcription (13). Since we showed here that pM92 also played a critical role in late gene expression, we hypothesized that these two proteins might interact and form a functional complex to exert this regulatory activity. To test this, we created a retroviral vector expressing the C-terminally HA-tagged M79 ORF (pM79*ha*) or MCMV M38 ORF as a control (pM38*ha*) and transfected it into MEF10.1 cells to generate expression cells. Transfected cells were subsequently infected with SM*flag*92, and cell lysates were collected at 48 hpi. FLAG-tagged pM92 complexes were immunoprecipitated with the mouse anti-FLAG antibody and analyzed by immunoblotting (Fig. 5A). pM79*ha* coimmunoprecipitated with p*flag*M92, indicated by its relative abundance in the eluted sample compared to that in the flowthrough sample. The interaction was specific, since the control pM38*ha*, a protein not thought to be involved in late gene regulation, was only detected in the flowthrough wash but not in the eluted sample. These results suggest that pM79 and pM92 interact during MCMV infection.

Since MCMV M79 and HCMV UL79 play a similar role in late gene expression during infection (13, 16), we wanted to determine whether pUL79 also interacted with pUL92, the HCMV homologue of pM92, during infection. To test this, we infected HFF cells with recombinant HCMV expressing N-terminally 3×FLAG-tagged pUL79 (AD*flag*UL79) or wild-type HCMV (AD*gfp*). Cell lysates were collected at 72 hpi and coimmunoprecipitated with anti-FLAG antibody. Immunoprecipitants were resolved by SDS-PAGE, followed by silver staining analysis. Protein bands present in AD*flag*UL79-infected samples but absent in AD*gfp*-infected control samples were extracted. As the control, gel bands from AD*gfp*-infected samples with migrating positions corresponding to those of AD*flag*UL79-specific protein bands were also extracted (Fig. 5B). The protein identities in both samples were determined by mass spectrometry analysis. Proteins that were only present in AD*flag*UL79-infected samples but not in AD*gfp*-infected samples were considered as specifically binding to pUL79. pUL92 was among the pUL79-associated viral proteins identified by this analysis (Fig. 5B). Taken together, our results suggest that two viral regulators of late gene expression, pM79 and pM92, interact during MCMV infection, and that this interaction is conserved between MCMV and HCMV.

pM92 *trans*-complements the growth of pUL92-deficient HCMV virus. MCMV pM92 and HCMV pUL92 share 50% identity and 71% similarity at the amino acid level and notably pM92 has an additional 30 aa at the N terminus (Fig. 6A). Since pM92 and pUL92 share significant sequence homology and a similar interaction partner (i.e., pM79 and pUL79, respectively), we hypothesized that pM92 and pUL92 were functional homologues. To test this, we first constructed a pUL92-deficient HCMV recombinant BAC clone, pAD*in*UL92, by FLP/FRT-mediated BAC recombineering (Fig. 6B). pAD*in*UL92 carried an 88-nt insertion at nt 124 of the UL92 ORF to replace a 282-nt segment of the coding sequence. The location of the mutation was expected not to interfere with expression of neighboring genes, namely, the overlapping 3′ terminus of UL91 or 5′ terminus of UL93. Transfection of pAD*in*UL92 BAC into MRC5 cells failed to produce any infectious virus even after 4 weeks of incubation, whereas cells transfected with the wild-type BAC pAD*gfp* readily developed complete CPE and a full spread of virus-driven GFP expression. This was in ac-



Parameters for identifying HCMV pUL92 in the pUL79 protein complex by mass spectrometry[a]

Description	Size (kDa)	Expectation[b]	Peptide count
pUL92	22.5	6.60E-24	6

FIG 5 CMV UL92/M92 proteins interact with UL79/M79 proteins during infection. (A) MEF10.1 cells expressing either HA-tagged M79 (pM79*ha*) or HA-tagged M38 (pM38*ha*) were infected with SM*flag*92 at an MOI of 2, and cell lysates were collected at 48 hpi. Cell lysates were separated into insoluble cell debris (pellet, "P") and supernatant by centrifugation. Supernatant was then immunoprecipitated with the mouse anti-FLAG antibody, washed, and eluted. Cell debris ("P"), flowthrough fraction collected by wash ("FT"), and eluted fraction ("E") were analyzed by immunoblotting with anti-FLAG and anti-HA antibodies. (B) HFF cells were infected with wild-type (AD*gfp*) or FLAG-tagged UL79 recombinant HCMV virus (AD*flag*UL79) at an MOI of 3. Cell lysates were prepared at 72 hpi and immunoprecipitated with an anti-FLAG antibody. In the top panel, immunoprecipitants were separated on a gradient polyacrylamide gel, and protein bands were visualized by silver stain. Indicated is the protein band that contained pUL92 as identified by mass spectrometry. The bottom panel shows the parameters of mass spectrometry analysis for identifying HCMV pUL92. Gel bands with the same migrating positions were extracted from both lanes of the top panel and comparatively analyzed by mass spectrometry. pUL92 was identified only in the AD*flag*UL79 samples but not in the AD*gfp* sample. Superscript letters: a, identities of remaining pUL79-binding proteins are included in a separate study; b, expectation value for peptide match. A lower value indicates a higher likelihood of the interaction. Molecular mass markers (in kilodaltons) are also indicated.

cordance with previous reports that UL92 is essential for HCMV viral replication in fibroblasts (19). To reconstitute pUL92-deficient virus, we constructed a lentivirus carrying the C-terminally 3×FLAG-tagged UL92 ORF and subsequently generated pUL92 expressing cells by lentiviral transduction (MRC5-UL92*flag*). Transfection of pAD*in*UL92 into MRC5-UL92*flag* cells could now reconstitute infectious progeny virus, AD*in*UL92, with wild-type titers. Therefore, pUL92 is essential for HCMV replication, and the growth defect of the HCMV recombinant virus AD*in*UL92 was due to the disruption of pUL92 expression.

To determine whether pM92 is the functional homologue of pUL92, we tested whether pM92 expression could *trans*-complement the growth of pUL92-deficient virus. We created a lentivirus containing the C-terminally 3×FLAG-tagged M92 ORF and subsequently generated pM92-expressing MRC5 cells by lentiviral transduction (MRC5-M92*flag*). We then infected MRC5 cells expressing pUL92 (MRC5-UL92*flag*), pM92 (MRC5-M92*flag*), or empty vector (MRC5-vector) with AD*in*UL92 at an MOI of 0.001 and determined the titer of the cell free virus produced at 14 dpi. Both infected MRC5-UL92*flag* and MRC5-M92*flag* showed spread of the pUL92-deficient virus at 14 dpi, whereas MRC5-vector showed little sign of CPE (Fig. 6C). Analysis of the final titers of infected culture supernatants indicated that pM92 could complement AD*in*UL92 to titers similar to that by pUL92 (Fig. 6D). PCR analysis of genomic DNA from MRC5-UL92*flag* and pM92 MRC5-M92*flag* confirmed that there was no cross-contamination of these two cell types (Fig. 6E). Therefore, pM92 and pUL92 have a conserved function during CMV infection.

DISCUSSION

The expression of late genes is an essential step for CMV to complete its lytic infection cycle. Key viral factors required in this process could be attractive targets for antiviral strategies to prevent CMV infection and disease. In the present study, we determined the role of MCMV protein pM92 during virus infection. pM92 accumulated at late times and localized to nuclear replication compartments during infection (Fig. 2). When pM92 was abolished, the accumulation of early gene products and viral DNA was minimally affected, but the accumulation of late gene products was markedly reduced (Fig. 3 and 4). As a result, the mutant virus failed to complete the infection cycle to produce progeny virus (Fig. 1). Therefore, pM92 is a novel regulator of viral late gene expression and thus plays an essential role in the MCMV lytic infection cycle.

Our study provides additional evidence that the regulatory mechanism of viral late gene expression is conserved between MCMV and HCMV. We have previously shown that MCMV protein pM79 and its HCMV homologue pUL79 regulate viral late gene expression (13, 16). In the present study, we demonstrated that pM92 interacted with pM79 during MCMV infection; likewise, pUL92 could interact with pUL79 during HCMV infection (Fig. 5). This suggests that during betaherpesvirus infection, a complex containing similar components of virus-encoded factors forms to promote late gene expression. How this complex functions and what additional protein components are in this complex remain important questions. Furthermore, we demonstrated that viral protein pUL92, the HCMV homologue of pM92, was also essential for virus infection (Fig. 6). Importantly, pM92 could *trans*-complement the growth of pUL92-deficient HCMV recombinant virus (Fig. 6). These experiments do not specify whether the compensation occurs at the transcriptional or translational level during HCMV infection, and further work is required to define the exact mechanism at play. Regardless, these findings suggest a conserved function for pM92 homologues among betaherpesviruses.

Our study also provides additional evidence that viral DNA synthesis is necessary but not sufficient to drive late viral gene expression during herpesvirus infection. Inhibition of the viral

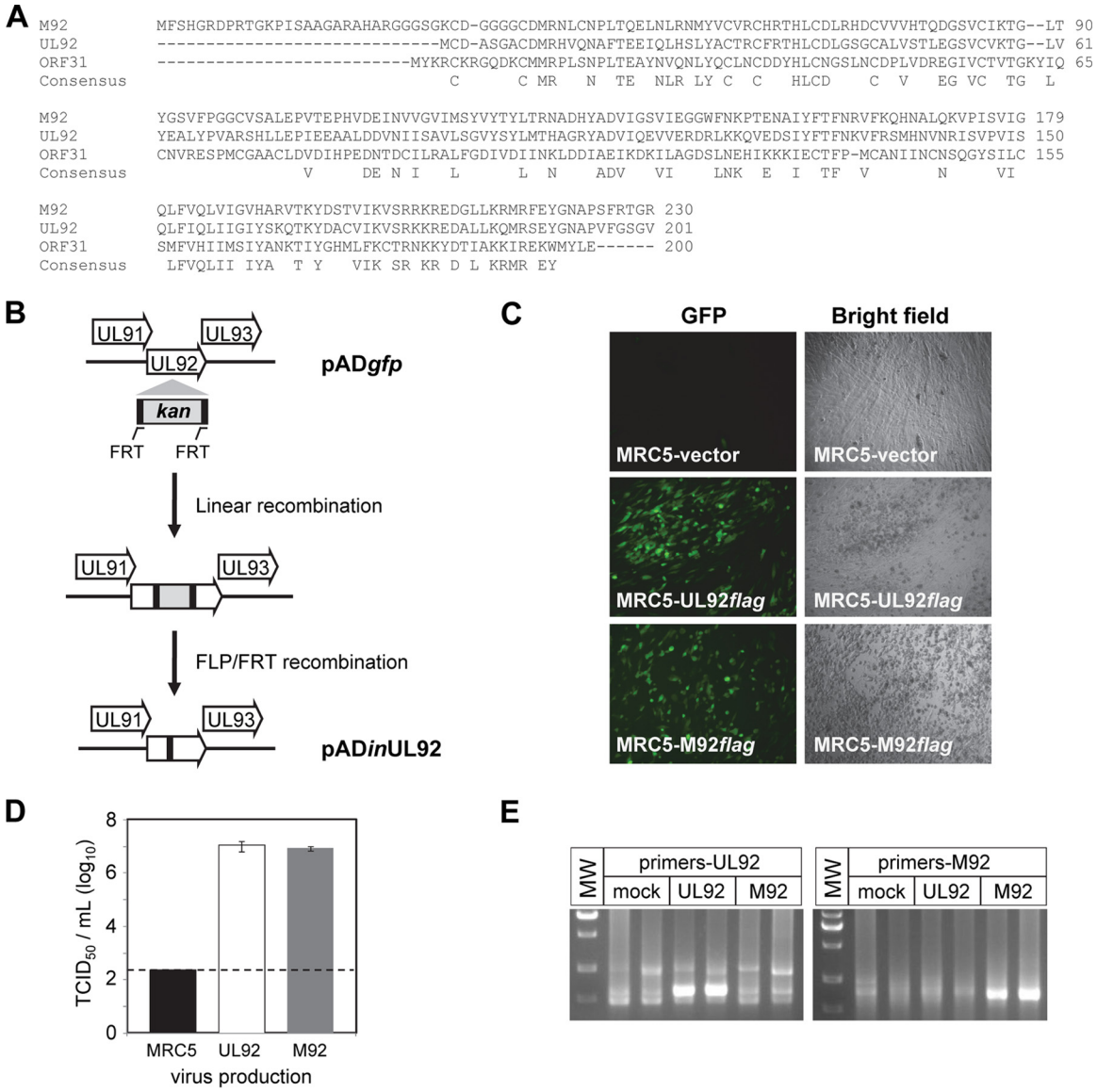


FIG 6 pM92 *trans*-complements the growth of pUL92-deficient HCMV virus. (A) Coding sequence alignment of MCMV M92 with its homologues HCMV UL92 and MHV68 ORF31. (B) Diagram depicting the construction of pUL92-deficient recombinant HCMV BAC, pAD*in*UL92, by BAC recombineering. pAD*in*UL92 carried an 88-nt insertion (indicated by black bar) at nt 124 of the UL92 ORF to replace a 282-nt segment of the coding sequence. (C and D) Growth of AD*in*UL92 virus in MRC5 cells expressing C-terminally 3×FLAG-tagged pUL92 or pM92. MRC5 cells expressing tagged pUL92 (MRC5-UL92*flag*), tagged pM92 (MRC5-M92*flag*), or the empty vector (MRC5-vector) were infected with AD*in*UL92 at an MOI of 0.001. At 14 dpi, cells were examined under a fluorescence microscope for virus-driven GFP expression (C), and titers of cell free virus were determined by $TCID_{50}$ assay in MRC5-UL92*flag* cells (D). The detection limit of the $TCID_{50}$ is indicated by a dashed line. (E) Cellular DNA of MRC5-UL92*flag* and MRC5-M92*flag* cells was isolated, and the presence of UL92 and M92 DNA in these cells was determined by PCR analysis using the primers listed in Table 1.

polymerase by PAA abolishes the accumulation of late transcripts (38–41). This dependence on viral DNA synthesis has been linked to the origin of lytic replication (oriLyt), since the oriLyt sequence is required in *cis* for proper expression of late transcripts in many herpesviruses (42–46). However, our previous data and the data presented here demonstrate that viral gene expression at late times of infection depends not only on viral DNA in *cis* but also on viral factors such as pM92 and pM79 in *trans* (13). In the absence of these viral factors, DNA synthesis kinetics were indistinguishable from those of wild-type virus despite a defect in late transcript accumulation. Therefore, pM92 does not function as a viral DNA synthesis protein; rather, it specifically acts on gene expression at late times of infection.

What is the mechanism of pM92 activity? It has been established that herpesviral genomes associate with histones during infection and require epigenetic regulation for gene expression (47–53). One possible mechanism is that the pM92/pM79 complex may activate late gene transcription by remodeling the chromatin structure of the viral genome. This could be accomplished by recruiting chromatin-remodeling complexes to the late gene loci, and rendering their promoters accessible for transcription. Such activity has been observed for herpes simplex virus 1

(HSV-1) late gene *trans* factors (54, 55). Alternatively, pM92/pM79 could play a more direct role in transcription. Herpesvirus genes are transcribed by the cellular RNA polymerase II (RNAPII) (56–59), a 12-subunit multicomponent enzyme that requires a host of accessory scaffold and regulatory proteins for its activity. The pM92/pM79 complex could play an essential role in the recruitment or assembly of these components on viral late gene promoters. Such a mechanism has been suggested for regulation of late gene expression in both HSV and gammaherpesvirus (50, 60). Finally, the activity of pM92/pM79 complex could be required at the stage of posttranscriptional modification. It is clear that accumulation of late transcripts was defective during mutant virus infection, but it remains to be determined if this defect results from a failure in transcription or an alteration in mRNA stability. HSV-1 endoribonuclease VHS-RNase is tightly regulated by at least four other viral proteins in order to prevent it from degrading viral mRNAs (61–64). Precedent for viral regulation of viral mRNA accumulation also exists in HCMV, since viral protein IE2 can inhibit its own transcription by binding to the MIEP promoter (18, 65). Thus, we cannot rule out the possibility that during pM92-deficient mutant virus infection, a defect in RNA trafficking, stability, or processing results in higher RNA turnover rates.

Efforts are under way to gain a better mechanistic understanding of the role of pM92 in late gene regulation. Identification of cellular and viral factors that interact with pM92 is anticipated to provide important insights into its function. Although pM79 is one interaction partner of pM92, it is almost certain that many additional partners exist. Furthermore, genetic and protein analysis to identify functional domains and structural elements of pM92 will be invaluable to understand the mechanistic basis for its activity and to determine additional functions that pM92 may have. Finally, it is tempting to speculate that late gene expression regulators such as pM92 and pM79 could play a role in the establishment of latency. Since both proteins are essential for the lytic viral life cycle, regulation of their activity, and/or expression may be a deciding factor for viral latency and reactivation.

In summary, we have identified pM92 as a novel late gene regulator in MCMV lytic infection, shown its interaction with another late gene regulator pM79, and demonstrated its conserved function with its HCMV homolog, pUL92. pM92 represents a potential new target for therapeutic intervention in CMV disease, and a gateway into studying a largely uncharted viral process that is critical to the viral life cycle.

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