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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 SMim92, in which pM92 expression was disrupted by an insertional/frameshift mutation. During infection, SMim92 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 reinitis 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-
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 entry site (IRE); 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 carrying the N-terminally 1×FLAG-tagged M92 and UL92 coding sequences, respectively. pYD-C191 carried a kanamycin selection cassette bracketed by two Flp recognition target (FRT) sites. pYD-C746 was derived from pYD-C630, where a 3×FLAG sequence was inserted in vivo in pYD-C746 and carried the C-terminally 3×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 carrying 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 (1186743001; 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 IgM, 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 (PAAC) (284720-10G; Sigma-Aldrich), l-(+)-arabinose (A325625G; 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% CO2 in a humidified atmosphere. To create cell lines stably expressing N-terminally 1×FLAG-tagged pM92 (10.1flagM92), MEF10.1 cells were transduced three times with pYD-C618-derived retrovirus and allowed to recover for 48 h. DisRed-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) pSmM92 (see below). The cell line yielding the highest titer at 5 days posttransfection was used in this study. To create cell lines expressing C-terminally 3×FLAG-tagged pM92 (MRC5-M92flag) or pUL92 (MRC5-UL92flag), MRC5 cells were transfected with lentivirus reconstituted from pYD-C780 or C678, respectively, and allowed to recover for 48 h (30). To create cell lines containing the vector control (MRC5-vector), MRC5 cells were transfected with pYD-C755-derived 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 SMgfp and SMflag2 were reconstituted in MEF10.1 cells. SMgfp was reconstituted in 10.1flagM92 cells. To reconstitute recombinant HCMV, BAC-HCMV DNA, pp71-expression plasmid, and G403-expression plasmid were cotransfected into MRC5-UL92flag 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 (TCID50) assay in the appropriate cell type. In experiments where comparative analysis was performed between SMgfp or ADinUL92 with other recombinant viruses, titers for all of the viruses were determined in 10.1flagM92 or MRC5-UL92flag 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). pSMgfp, 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 pSM92 carried a frameshift mutation in the MCMV gene M92 (Fig. 1A). To construct pSMgfp2, the FRT-bracketed GalK/kanamycin dual selection cassette was PCR amplified from pYD-C630 and recombined into pSMgfp 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-nucleotide insertion within M92 to create a frameshift mutation. The clone pSMflag92 contained an N-terminally 3×FLAG-tagged M92 coding sequence (Fig. 1A). To construct pSMflag92, a fragment containing the FRT-bracketed GalK/kanamycin dual selection cassette was PCR amplified by 3×FLAG sequence that was PCR amplified from pYD-C746 and recombined into the N terminus of the M92 coding sequence of pSMgfp. 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/Crc that carried the full-length genome of HCMV strain AD169 (31). pADgfp, used as the wild-type clone here, had a green fluorescent protein (GFP)
gene in place of the viral US4-US6 region (31, 36). pADflagUL79 was similar to pADgfp except that the 5′ terminus of UL79 coding sequence was tagged with 3×FLAG. The clone pADM1nUL92 was created in a similar manner to that of pSMind92, except that the insertion replaced nt 125 to 406 of the UL92 coding sequence carried in pADgfp. 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.

Cell-free and cell-associated viruses were collected at indicated times and titers were determined by TCID₅₀ assay in 10.1-flagM92 cells. The detection limit of the TCID₅₀ is indicated by a dashed line.

DNA and RNA analysis. Intracellular DNA was measured by quantifying intracellular DNA by using a primer pair specific for the mouse actin gene (13). Cellular DNA was quantified by using 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 pSMgfp 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 SMgfp 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-primered 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 (Clontech) 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 SMgfp 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-
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 with 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 pM92 (pM92-flag) migrated at the expected size of 25 kDa, whereas transfection of pSM92-flag failed to show any sign of CPE even at 2 weeks posttransfection (Fig. 1B). However, pM92-deficient virus (termed SM92) was rapidly reconstituted to wild-type levels from pSM92 upon transfection into MEF 10.1 cell that stably expressed N-terminally 1×FLAG-tagged pM92 (10.1-flagM92). Thus, the defect of SM92 is the direct result of pM92 ablation.

To more precisely define the growth defect of SM92, we performed growth curve analyses to quantify the defect of the recombinant virus and validate the essentiality of pM92 (Fig. 1C). SM92 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 of SM92. Both single-step and multistep growth curve analyses indicated that SM92 failed to show any sign of CPE even at 2 weeks posttransfection (Fig. 1B). However, pM92-deficient virus (termed SM92) was rapidly reconstituted to wild-type levels from pSM92 upon transfection into MEF 10.1 cell that stably expressed N-terminally 1×FLAG-tagged pM92 (10.1-flagM92). Thus, the defect of SM92 is the direct result of pM92 ablation.

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levels at 48 hpi during SMflag92 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 SMflag92. 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 SMflag92. 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 SMflag92-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 SMgfp-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 SMmir92 infection was comparable to that during SMgfp infection.
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<sub>in92</sub> infection by immunoblot analysis. Compared to wild-type control SM<sub>gfp</sub>, SM<sub>in92</sub> 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<sub>in92</sub> infection (Fig. 3A). Early genes primarily function prior to viral DNA synthesis. Since no measurable defect was observed in DNA synthesis during SM<sub>in92</sub> infection (Fig. 3C), the modest decrease in 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<sub>in92</sub> infection (Fig. 3A). Early genes primarily function prior to viral DNA synthesis. Since no measurable defect was observed in DNA synthesis during SM<sub>in92</sub> infection (Fig. 3C), the modest decrease in E1 protein at early times of infection was unlikely the main cause for the growth defect of SM<sub>in92</sub>. It is unclear why SM<sub>in92</sub> has this minor defect in early gene expression. It may be a result of suboptimal complementation by the 10.1-<i>flag</i>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<sub>in92</sub> 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 transcription 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, transcription of these genes was also significantly reduced during SM<sub>in92</sub> infection. It was also noted that
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 (pM79ha) or MCMV M38 ORF as a control (pM38ha) and transfected it into MEF10.1 cells to generate expression cells. Transfected cells were subsequently infected with SMflag, 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). pM92 complexes were immunoprecipitated with pflagM92, indicated by its relative abundance in the eluted sample compared to that in the flowthrough sample. The interaction was specific, since the control pM38ha, 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 (ADflagUL79) or wild-type HCMV (ADgfp). 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 ADflagUL79-infected samples but absent in ADgfp-infected control samples were extracted. As the control, gel bands from ADgfp-infected samples with migrating positions corresponding to those of ADflagUL79-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 ADflagUL79-infected samples but not in ADgfp-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, pADinUL92, by FLP/FRT-mediated BAC recombineering (Fig. 6B). pADinUL92 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 pADinUL92 BAC into MRC5 cells failed to produce any infectious virus even after 4 weeks of incubation, whereas cells transfected with the wild-type BAC pADgfp readily developed complete CPE and a full spread of virus-driven GFP expression. This was in ac-
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). Transfection of pADinUL92 into MRC5-UL92/FLAG cells could now reconstitute infectious progeny virus, ADinUL92, with wild-type titers. Therefore, pUL92 is essential for HCMV replication, and the growth defect of the HCMV recombinant virus ADinUL92 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 cells by lentiviral transduction (MRC5-M92/FLAG). Transfection of pADinUL92 into MRC5-UL92/FLAG cells could now reconstitute infectious progeny virus, ADinUL92, with wild-type titers. Therefore, pUL92 is essential for HCMV replication, and the growth defect of the HCMV recombinant virus ADinUL92 was due to the disruption of pUL92 expression.

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
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.

### FIG 6

**A** 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, pADinUL92, by BAC recombineering. pADinUL92 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 ADinUL92 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 ADinUL92 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 TCID50 assay in MRC5-UL92 flag cells (D). The detection limit of the TCID50 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.

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<table>
<thead>
<tr>
<th>M92</th>
<th>Consensus</th>
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<tbody>
<tr>
<td>YQSMFPGGCSAELPVTEPHYDEINVVGIMTVYTLTRNADHYADTVGSIIVBGGMNLPSNALSENLQVPIVSLIG</td>
<td>C C MR N TE NLR LY C C HLCD C V BE VC TN</td>
</tr>
<tr>
<td>UL92</td>
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</tr>
<tr>
<td>YEAFLVPARSHLEETIDAEALDDIVNiSSAVGTVYIMTHAARDYDIQDQVVERDLSKQVDJISYTFKNNFKPRMNNVRIISVPIES</td>
<td>V DE N I L L H ADP V I L N E E T T V T N G</td>
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<tr>
<td>ORF31</td>
<td>Consensus</td>
</tr>
<tr>
<td>CMWMDSPGCAACLVDVPEIHYDVLASQDIHFEQIKLQADDSHEINHIKEKCCFFNF-MCMICNQAQY5LCC</td>
<td>L N T</td>
</tr>
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M92 QLFQVLIGVHVARVTDSTVIVSKRKEGGDLMMFFESYSHAPSFRTPGR 230
UL92 QLFQVLIGVHVARVTDSTVIVSKRKEGGDLMMFFESYSHAPSFRTPGR 230
ORF31 SMVHIIMJYANTTISMLFCRNTKNMTDIARKREMWY--- 200

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**References**

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(HSV-1) late gene trans factors (54, 55). Alternatively, pM92/pM79 complex 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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