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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 SmInf92, in which pM92 expression was disrupted by an insertional/framesshift mutation. During infection, SmInf92 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. pM92 and pM79 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-terminal 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 FLAG sequence was PCR amplified from pYD-C746 and recombined in 10.1-FLAG-tagged pM92 (MRC5-FLAG-pM92) and pM92 (MRC5-UL92flag), MRC5 cells were transduced with lentivirus reconstituted from pYD-C780 or C678, respectively, and allowed to recover for 48 h. To create cells containing the vector control (MRC5-vector), MRC5 cells were transduced 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. SMiri2 was reconstituted in 10.1-flagM92 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 SMiri2 or ADinUL92 with other recombinant viruses, titers for all of the viruses were determined in 10.1-flagM92 or MRC5-UL92flag cells, respectively.

BAC recombination. Recombinant BAC clones in the present study were created by using a linear recombination-based BAC recombinering 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 pSM36r 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 locus, which has been known to be dispensable for MCMV infection in vivo and in vitro (33–35). The clone pSMiri2 carried a frameshift mutation in the MCMV gene M92 (Fig. 1A). To construct pSMiri2, the FRT-bracketed GalK/kanamycin 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 insert within M92 to create a frameshift mutation. The clone pSMflag2 contained an N-terminally 3×FLAG-tagged M92 coding sequence (Fig. 1A). To construct pSMflag2, 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 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/Cre 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). pADgfp was similar to pADgfp except that the 5’ terminus of UL79 coding sequence was tagged with 3×FLAG. The clone pADm92L2 was created in a similar manner to that of pSMm92, 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 from infected cultures. Cell-free and cell-associated viruses were collected at indicated times and titers were determined by TCID₅₀ assay in 10.1-M92 cells. The detection limit of the TCID₅₀ is indicated by a dashed line.

Viral DNA and RNA analysis. Intracellular DNA was determined by reverse transcription-coupled qPCR (RT-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 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 transcript 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 fluoride 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 followed by HRP-coupled secondary antibodies, and visualized by 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 recombining (13, 26), producing mutant BAC pMSmM92 (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 pSMgp BAC in MEF10.1 cells produced virus (termed SMgp), 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 pMSmM92 failed to show any sign of CPE even at 2 weeks posttransfection (Fig. 1B). However, pM92-deficient virus (termed SmM92) was rapidly reconstituted to wild-type levels from pMSmM92 upon transfection into MEF 10.1 cell that stably expressed N-terminally 1×FLAG-tagged pM92 (10.1-FLAGM92). Thus, the defect of SmM92 is the direct result of pM92 ablation.

To more precisely define the growth defect of SmM92, we performed growth curve analyses to quantify the defect of the recombinant virus and validate the essentiality of pM92 (Fig. 1C). SmM92 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 pH92 to proteins with known function. To acquire basic information and gain insights into the role of pH92, 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, pSMflagM92, in which the M92 coding sequence was tagged with a FLAG tag at the N terminus (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 (pflagM92) migrated at the expected size of 25 kDa, became detectable at 24 hpi, and accumulated at more abundant levels.

### TABLE 1 Primers used in PCR analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCMV IE1 forward</td>
<td>CAGGGTGATCATGAAGCTT</td>
</tr>
<tr>
<td>MCMV IE1 reverse</td>
<td>AGGGCTAGGAAAGACAGC</td>
</tr>
<tr>
<td>MCMV M34 forward</td>
<td>TACTCTGATGCCAAAGGAC</td>
</tr>
<tr>
<td>MCMV M34 reverse</td>
<td>CGGTGTGTTGCTTGTCGTC</td>
</tr>
<tr>
<td>MCMV M37 forward</td>
<td>ATGGAGGGGTTGGCATCAG</td>
</tr>
<tr>
<td>MCMV M37 reverse</td>
<td>AGGCTGAGGTCTCGCTGCT</td>
</tr>
<tr>
<td>MCMV M45 forward</td>
<td>GGAACATCTGATCCAAGGA</td>
</tr>
<tr>
<td>MCMV M45 reverse</td>
<td>GGAAGCTTCCTTGATGAGC</td>
</tr>
<tr>
<td>MCMV M55 (gB) forward</td>
<td>CGGTATGTCGAGTGTGTC</td>
</tr>
<tr>
<td>MCMV M55 (gB) reverse</td>
<td>CAGGCGAGGCTCTCTGCC</td>
</tr>
<tr>
<td>MCMV M74 forward</td>
<td>CGGATGTTGCTGCAGAAG</td>
</tr>
<tr>
<td>MCMV M74 reverse</td>
<td>CTTCATGCAGGATCAGGAG</td>
</tr>
<tr>
<td>MCMV M85 forward</td>
<td>CGGCTGAGGCTGTGGGAG</td>
</tr>
<tr>
<td>MCMV M85 reverse</td>
<td>CTCCGCACTGAGCAAGGT</td>
</tr>
<tr>
<td>MCMV M92 forward</td>
<td>AAACCCAGGAGAATAGT</td>
</tr>
<tr>
<td>MCMV M92 reverse</td>
<td>AGAACAGGAGTACATGGAG</td>
</tr>
<tr>
<td>MCMV M96 forward</td>
<td>TCGAGGCTGTGGTCGTC</td>
</tr>
<tr>
<td>MCMV M96 reverse</td>
<td>GCATTCTGGATATCATCT</td>
</tr>
<tr>
<td>MCMV M102 forward</td>
<td>AGACAGTGAGGCAGCATAC</td>
</tr>
<tr>
<td>MCMV M102 reverse</td>
<td>AGCTGCAGTTGCTGGT</td>
</tr>
<tr>
<td>MCMV M112/13 (E1) forward</td>
<td>GAATCCGAGGAGGAAGAGAT</td>
</tr>
<tr>
<td>MCMV M112/13 (E1) reverse</td>
<td>GTTGGAGGTTGGCTCAGT</td>
</tr>
<tr>
<td>MCMV M116 forward</td>
<td>TCTCTGGAAGCCCTGAG</td>
</tr>
<tr>
<td>MCMV M116 reverse</td>
<td>GCATCAGTACCTGACACA</td>
</tr>
<tr>
<td>Mouse actin forward</td>
<td>CCTGATTTTCCCCCTATCTG</td>
</tr>
<tr>
<td>Mouse actin reverse</td>
<td>CAGGGTGGTGGCTAGT</td>
</tr>
<tr>
<td>M92 forward*</td>
<td>ATGTTCCTCACAGGCGGAGA</td>
</tr>
<tr>
<td>M92 reverse*</td>
<td>CACGGCGCCTGTCGAGAAG</td>
</tr>
<tr>
<td>UL92 forward*</td>
<td>ATGTCGAGGCGCTGCGGCG</td>
</tr>
<tr>
<td>UL92 reverse*</td>
<td>AACGCGACAGTCGACAGCG</td>
</tr>
</tbody>
</table>

*Primer used in PCR analysis of cellular DNA from pM92- or pUL92-expressing MRC5 cells.
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 re-

duced when viral DNA synthesis was inhibited by phosphono-

acetic acid (PAA) at 20 hpi (Fig. 2B). Therefore, M92 gene prod-

ucts 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 exam-

ined the intracellular localization of 3×FLAG-tagged pM92 dur-

ing infection of SMflag92. Infected cells were fixed and permea-

bilized with methanol, which also quenched GFP fluorescence, thus allowing visualization of pM92 localization using Alexa Fluor 488-

conjugated mouse anti-flag antibody by indirect immunofluores-

cence. pM92 localized to the nuclei of infected cells, and in partic-

ular, it localized to subnuclear structures resembling those of nuclear replication compartments (Fig. 2D). This led us to hy-

pothesize that pM92 localized to viral replication compartments during infection. To test this, we compared intracellular localiza-
tion 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 exper-
iment. 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 pro-
duced a diffuse, weak cytoplasmic staining, which likely repre-
sented 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 local-
ized to replication compartments (Fig. 2). However, qPCR analy-
sis 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\textsubscript{in}92 infection by immunoblot analysis. Compared to wild-type control SM\textsubscript{gfp}, SM\textsubscript{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\textsubscript{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\textsubscript{in}92 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\textsubscript{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\textsubscript{in}92 infection (Fig. 3C), the modest decrease in E1 protein at early times of 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, transcription of these genes was also significantly reduced during SM\textsubscript{in}92 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 transcription regulators 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 considered as specifically binding to pM79. pM92 was also required for efficient accumulation of a panel of late transcripts. MEF 10.1 cells were infected with SM flag in the presence or absence of viral DNA synthesis inhibitor PAA (200 μg/ml) or with SMint2 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 SMflag infection at 10 hpi in the absence of PAA was set to 1.

**FIG 4** pM92 is required for efficient accumulation of a panel of late transcripts. MEF 10.1 cells were infected with SMflag in the presence or absence of viral DNA synthesis inhibitor PAA (200 μg/ml) or with SMint2 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 SMflag infection at 10 hpi in the absence of PAA was set to 1.

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-
corded 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-UL92flag). Transfection of pADinUL92 into MRC5-UL92flag 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 MRC5 cells by lentiviral transduction (MRC5-M92flag). When we infected MRC5 cells expressing pM92, 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, pM92 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 beta-herpesviruses.

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...
genes are transcribed by the cellular RNA polymerase II (RNAPII) (56–59), a 12-subunit multicomponent enzyme that requires the 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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