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The Human Cytomegalovirus Gene UL79 Is Required for the Accumulation of Late Viral Transcripts▽

Yi-Chieh Perng, Zhikang Qian,† Anthony R. Fehr,† Baoqin Xuan,† and Dong Yu*

Department of Molecular Microbiology, Washington University School of Medicine, Saint Louis, Missouri 63110

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In this study, we adopted a conditional protein genetic approach to characterize the role of the human cytomegalovirus (HCMV) gene UL79 during virus infection. We constructed AD*dd*UL79, a recombinant HCMV in which the annotated UL79 open reading frame (ORF) was tagged with the destabilization domain of a highly unstable variant of the human FKBP12 protein (*dd*FKBP). The *dd*FKBP domain targets the tagged protein for rapid proteasomal degradation, but the synthetic ligand Shield-1 can stabilize *dd*FKBP, allowing accumulation of the tagged protein. AD*dd*UL79 failed to replicate without Shield-1, but it grew at wild-type levels with Shield-1 or in human foreskin fibroblasts overexpressing hemagglutinin (HA)-tagged UL79 (HF-UL79HA cells), indicating an essential role of UL79 and the effectiveness of this approach. Without Shield-1, representative immediate-early and early viral proteins as well as viral DNA accumulated normally, but late transcripts and proteins were markedly reduced. UL79 was transcribed with early-late kinetics, which was also regulated via a positive-feedback loop. Using HF-UL79HA cells, we found that the UL79 protein localized to viral replication compartments during HCMV infection. Finally, we created a second UL79 mutant virus (AD*in*UL79$_{stop}$) in which the UL79 ORF was disrupted by a stop codon mutation and found that AD*in*UL79$_{stop}$ phenocopied AD*dd*UL79 under the destabilizing condition. Taking these results together, we conclude that UL79 acts after viral DNA replication to promote the accumulation of late viral transcripts. Importantly, the comparative analysis of AD*dd*UL79 and AD*in*UL79$_{stop}$ viruses provide additional proof for the power of the protein stability-based conditional approach to dissect the role of viral factors in HCMV biology.

Human cytomegalovirus (HCMV) is the prototypical betaherpesvirus and a ubiquitous opportunistic pathogen that infects the majority of the world's population. Upon primary infection, HCMV establishes a lifelong persistent and latent/recurrent infection in a host (10). Even though HCMV infection is usually asymptomatic in healthy individuals, it is a significant source of severe disease in immunocompromised adults, such as AIDS patients and transplant recipients. Importantly, HCMV is the leading infectious cause of birth defects in newborns (7). Additionally, there is evidence that HCMV is a possible risk factor in the development of vascular disease, such as atherosclerosis, transplant vascular sclerosis, and coronary restenosis after angioplasty surgery (21, 30, 37, 41, 49, 51, 66). To acquire a comprehensive understanding of HCMV biology and facilitate the effort to develop effective therapeutics to combat disease caused by HCMV, it is imperative to dissect the roles of previously uncharacterized viral genes in both acute and latent infections of this virus.

During lytic infection, HCMV genes are expressed in a highly ordered temporal cascade. Viral transcripts accumulate in three different kinetic classes, namely, immediate early (IE), early, and late. The HCMV major IE (MIE) genes UL123 (IE1) and UL122 (IE2) play a critical role in predisposing the cellular environment to infection, and they act as transactivators to induce transcription of early genes. Many early genes encode proteins involved in viral DNA replication or predisposing the cellular environment to infection (24, 25, 35, 36). The transcript accumulation of early genes is independent of viral DNA replication; however, the continued accumulation of a subset of genes (i.e., early-late) is enhanced by the onset of viral DNA replication (50). Following viral DNA replication, late viral genes that mainly encode structural proteins start to transcribe, ultimately leading to the assembly and release of infectious particles. Although late gene transcription is tightly coupled to viral DNA replication, the underlying mechanism is poorly understood.

HCMV contains a 240-kb double-stranded DNA genome that contains at least 166 putative open reading frames (ORFs) and several microRNAs (9, 11, 13, 15, 22, 42, 43). Previously we and others have used genome-wide mutagenic approaches to classify the entire set of HCMV genes that encode annotated ORFs into three functional categories (14, 63), and we found that about 40 genes are essential for the HCMV laboratory strain AD169 to replicate in human fibroblasts. Many of the HCMV genes have not been experimentally characterized and lack homologues with known functions in other herpesviruses (39), and their functions therefore remain elusive. With the advent of the infectious bacterial artificial chromosome clone-based system for HCMV (BAC-HCMV) (4, 64), the functions of many HCMV genes have started to be elucidated. However, defining the role of essential genes remains challenging because of a paucity of a reliable system to propagate null mutant viruses. The conventional complementation approach, namely, the use of cells expressing a viral gene in *trans* to support the growth of the null mutant, has been reported for only a few essential HCMV genes (39). To overcome this technical hurdle, we and others have recently adopted a con-

* Corresponding author. Mailing address: Department of Molecular Microbiology, Washington University School of Medicine, Saint Louis, MO 63110. Phone: (314) 362-7367. Fax: (314) 362-1232. E-mail: dongyu@borcim.wustl.edu.

† These authors contributed equally to this work.

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ditional approach (3) to facilitate the analysis of proteins critical to viral growth and pathogenesis (20, 45). This approach takes advantage of a mutant variant of the human FKBP12 protein that is highly unstable and rapidly degraded when expressed in mammalian cells (3). Using this method, a recombinant virus in which a viral ORF of interest is tagged with the destabilization domain of this FKBP variant (*dd*FKBP) that confers rapid instability can be constructed. The addition of the cell-permeable, synthetic ligand Shield-1 stabilizes the fusion protein, allowing propagation of the recombinant virus. Upon withdrawal of Shield-1, *dd*FKBP targets the fusion protein for rapid degradation, thus allowing the study of the virus infection in the absence of the protein of interest. *dd*FKBP tagging offers a robust genetic tool to study the role of viral genes, particularly the ones that are essential for HCMV infection.

In the present study, we characterized the role of viral gene UL79 in HCMV replication. UL79 was previously classified as being essential for HCMV replication in fibroblasts by two large-scale mutagenic analyses (14, 63). To define its function during virus replication, we engineered a recombinant HCMV virus (AD*dd*UL79) in which the annotated UL79 ORF was tagged with *dd*FKBP. Using the *dd*FKBP approach, we found that UL79 was critical for the accumulation of late viral transcripts but not viral DNA synthesis. This phenotype was further validated by our analysis of a second UL79 mutant virus in which the UL79 ORF was disrupted by a stop codon mutation. Furthermore, UL79 was expressed with early-late kinetics, was regulated by a positive-feedback loop, and primarily localized to viral nuclear replication compartments during infection.

MATERIALS AND METHODS

Plasmids, antibodies, and chemicals. pYD-C169 is a retroviral vector derived from pRetro-EBNA (29). It was created by inserting a PCR fragment containing the sequence of the UL79 ORF along with a C-terminal hemagglutinin (HA) tag into the multiple cloning site of pRetro-EBNA. pYD-C630 was derived from pGalK (56) and carried the 110-amino-acid destabilization domain of the human FKBP12 protein variant that contained the F36V and L106P destabilizing mutations (*dd*FKBP) (3). In this vector, *dd*FKBP was followed by a GalK/kanamycin dual expression cassette that was flanked by the Flp recognition target (FRT) sequence.

The primary antibodies used in this study included anti-β-actin (clone AC15; Abcam), anti-pUL44 (clone 10D8; Virusys), anti-IE2 (MAB8140; Chemicon), anti-HA (H6908; Sigma), anti-PCNA (clone F-2; Santa Cruz), and anti-FKBP12 (8/FKBP12; BD Biosciences). Other primary antibodies used in this study were anti-IE1 (52), anti-pp28 (48), anti-UL38 (52), anti-UL69 (31), anti-pp71 (6), and anti-pp150 (all generous gifts from Thomas Shenk, Princeton University). Secondary antibodies used for immunoblotting were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG (Jackson Laboratory). The secondary antibodies used for immunofluorescence were Alexa Fluor 568-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen-Molecular Probes).

The synthetic chemical ligand Shield-1 (Shld1) that was used to regulate the stability of *dd*FKBP-tagged proteins was purchased from Cheminpharma (Farmington, CT).

Cells and viruses. Primary human newborn foreskin fibroblasts (HFFs) and embryonic lung fibroblasts (MRC5) were propagated in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, nonessential amino acids, sodium pyruvate, and penicillin-streptomycin. To create HFFs expressing the HA-tagged UL79 (HF-UL79HA cells) or empty vector (HF-vector cells), retrovirus stocks were prepared from Phoenix-Ampho cells (29) that were transfected with the retroviral vector pYD-C169 or pRetro-EBNA, respectively. HFFs were transduced with retrovirus three times and then allowed to recover for 72 h to generate a pool of cells expressing the protein of interest.

Various BAC-HCMV clones were constructed to reconstitute recombinant HCMV viruses. Two BAC-HCMV clones, pAD/Cre and pAD-GFP, were used as the parental clones to produce wild-type viruses AD*wt*.2 and AD*wt*, respectively. pAD/Cre carries the full-length genome of HCMV strain AD169, and pAD-GFP is identical to pAD/Cre except that it contains a simian virus 40 (SV40) early promoter-driven green fluorescent protein (GFP) gene in place of the viral US4-US6 region (52, 64). All other recombinant BAC clones were constructed by using a linear recombination protocol in the bacterial strain SW105 that contained an arabinose-inducible Flp gene for the transient expression of Flp recombinase (56). To create a BAC clone in which the UL79 ORF was tagged with the *dd*FKBP sequence at its N terminus, a cassette that contained the *dd*FKBP sequence followed by the GalK/kanamycin dual marker was amplified by PCR from pYD-C630 with a pair of 70-bp primers. The 5′-terminal 50-bp sequences of these primers were homologous to the viral sequence immediately upstream or downstream of the N terminus of the UL79 ORF (5′-TCG TCCATCGTCATTGTCGTCACCGTCGCTACCCGCTCACCGAGCGAACG ATGGGAGTGCAGGTGGAAACCATC-3′ and 5′-TTGCCCGTGCGGACCC GCGGGACGGCGGGGTTCTCTTCGTCGCGGGCCATGCTGGAGCTCC ACCGCGGGAAGTTC-3′). The cassette was recombined into pAD-GFP at the N terminus of the UL79 ORF by linear recombination, and the resulting transformants were selected on kanamycin-containing LB plates to identify clones carrying the marker cassette. As the GalK/kanamycin marker was also flanked by FRT sites, it was then removed from the BAC by adding arabinose to a fresh culture to induce Flp recombinase expression and plating for isolation of single colonies lacking kanamycin resistance. Therefore, the final clone (AD*dd*UL79) contained the *dd*FKBP sequence along with a small FRT site fused in frame at the N terminus of UL79 (see Fig. 1A). To create a BAC clone in which the UL79 ORF was prematurely terminated by a stop codon, a cassette that contained a stop codon followed by the FRT-bracketed GalK/kanamycin dual marker was amplified by PCR from pYD-C649 with a pair of 70-bp primers. The 5′-terminal 50-bp sequences of these primers were homologous to the viral sequence immediately upstream or downstream of the 5th amino acid of the UL79 ORF (5′-GTCGTCACCGTCGCTACCCGCTCACCGAGCGAACGATGATGGCC CGCGACTGATCACTATAGGGCGAATTGGGTA-3′ and 5′-GTAAAGGA GAATTTGCCCGTGCGGACCCGCGGGACGGCGGGGTTCTCTTCAGG GAACAAAAGCTGGAGCTCC-3′). The cassette was recombined into the UL79 ORF of pAD-GFP, and the GalK/kanamycin marker was then removed by Flp/FRT recombination as described above (see Fig. 7A). The final clone (AD*in*UL79$_{stop}$) contained a stop codon along with a small FRT site inserted in frame at the 6th amino acid codon of the UL79 ORF. All recombinant BACs were verified by restriction digestion, PCR, and direct sequencing analysis.

To reconstitute virus, 2 μg of the BAC-HCMV DNA and 1 μg of the pp71 expression plasmid were transfected into HFFs or HF-UL79HA cells by electroporation (64), and the culture medium was changed 24 h later. For reconstitution of *dd*FKBP-tagged virus, the synthetic chemical ligand Shield-1 was added every 48 h to maintain the concentration at 1 μM. The recombinant virus was harvested by collecting cell-free culture supernatant when the entire monolayer of infected cells was lysed. Virus was amplified by collecting cell-free culture supernatant from HFFs infected at a multiplicity of infection (MOI) of 0.01 in the presence of Shield-1. To remove any residual Shield-1, virus-containing culture supernatants were then purified by ultracentrifugation through a 20% D-sorbitol cushion at an average relative centrifugal force of 53,000 × *g* for 1 h, resuspended in DMEM with 10% fetal calf serum, and saved as viral stocks. HCMV titers were determined by 50% tissue culture infectious dose ($TCID_{50}$) assay. For *dd*FKBP-tagged virus, the titer was determined in duplicate in HFFs in the presence of 1 μM Shield-1 (unless indicated otherwise). For AD*in*UL79$_{stop}$ virus, the titer was determined in duplicate in HF-UL79HA cells.

Analysis of viral growth kinetics. HFFs, HF-vector cells, or HF-UL79HA cells were seeded in 12-well plates overnight to produce a subconfluent monolayer. Cells were then inoculated with recombinant HCMV viruses for 1 h at an MOI of 0.1 for multistep growth analysis or an MOI of 3 for single-step growth analysis. The inoculum was removed, the infected monolayers were rinsed with phosphate-buffered saline, and infected cells were cultured in medium in the presence or absence of Shield-1 (1 μM). To infect cells with virus in the presence of ligand Shield-1, the ligand was added every 48 h to maintain its concentration. At various times postinfection, cell-free virus was collected by harvesting the medium from infected cultures, and the titers of the virus were determined by $TCID_{50}$ assay.

DNA and RNA analysis. Intracellular viral DNA was measured by quantitative PCR (qPCR) as previously described (46). HCMV-infected cells were collected at various times postinfection, resuspended in lysis buffer (200 mM NaCl, 20 mM Tris [pH 8.0], 50 mM EDTA, 0.2 mg/ml proteinase K, 1% sodium dodecyl sulfate [SDS]), and incubated at 55°C overnight. DNA was extracted with phenol-chloroform, treated with RNase A (100 μg/ml), extracted again with phenol-

TABLE 1. Primers and probes used for RT-qPCR

Transcript	qPCR reaction	Primer sequence	6FAM-TAMRA TaqMan probe sequence[a]
IE1	TaqMan	5′-CAAGTGACCGAGGATTGCAA-3′ 5′-CACCATGTCCACTCGAACCTT-3′	5′-TCCTGGCAGAACTCGTCAAACAGA-3′
UL32	SYBR green	5′-GGTTTCTGGCTCGTGGATGTCG-3′ 5′-CACACAACACCGTCGTCCGATTAC-3′	NA
UL79	SYBR green	5′-CCGCACGGGCAAATTCTCCT-3′ 5′-TGGTCCGAGACACCCAGGTTGTT-3′	NA
UL82	SYBR green	5′-TGCTGATGTCTGCCGCGGTAC-3′ 5′-CGGGCACTGATCCTGACCGG-3′	NA
UL93-99	SYBR green	5′-GTGTCCCATTCCCGACTCG-3′ 5′-TTCACAACGTCCACCCACC-3′	NA
*dd*FKBP-UL79	SYBR green	5′-TGGAAACCATCTCCCCAGGAGA-3′ 5′-TCACCTCCTGCTTGCCTAGCAT-3′	NA
GAPDH	SYBR green	5′-CTGTTGCTGTAGCCAAATTCGT-3′ 5′-ACCCACTCCTCCACCTTTGAC-3′	NA

[a] 6FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; NA, not applicable.

chloroform, precipitated with ethanol, and resuspended in nuclease-free water (Ambion). Viral DNA was quantified by qPCR as previously described using a TaqMan probe (Applied Biosystems) and primers specific for the HCMV UL54 gene (46). Cellular DNA was quantified with SYBR PCR master mix (Clontech) and a primer pair specific for the human β-actin gene as previously described (46). The accumulation of viral DNA was normalized by dividing the number of UL54 gene equivalents by the number of β-actin gene equivalents. The accumulation of wild-type viral DNA at 4 h postinfection (hpi) was arbitrarily set at 1.

Transcript accumulation was analyzed by reverse transcription coupled to qPCR (RT-qPCR) as previously described (62). Total RNA was extracted using the Trizol reagent (Invitrogen) and treated with the TURBO DNA-free reagent (Ambion) to remove genomic DNA contaminants. cDNA was reverse transcribed with random hexamer primers using the high-capacity cDNA reverse transcription kit (Applied Biosystems). For IE1, cDNA was quantified by qPCR using Maxima probe/Rox 2× qPCR master mix (Fermentas), and a TaqMan probe (Applied Biosystems) and a primer pair specific for IE1. Alternatively, cDNA was quantified using SYBR Advantage qPCR premix (Clontech) and primer pairs specific for viral genes UL32, UL79, UL82, UL93-99, the *dd*FKBP sequence of *dd*FKBP-UL79, or the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cellular gene (16, 38, 57). All of the primer pairs and TaqMan probes are listed in Table 1. cDNA from infected cells was used to generate a standard curve for each gene examined. The standard curve was then used to calculate the relative amount of a specific transcript present in a sample. The amounts of IE1, UL32, UL79, UL82, and UL93-99 were normalized using GAPDH as an internal control.

Protein analysis. Protein accumulation and subcellular localization were determined by immunoblotting and immunofluorescence, respectively. For immunoblotting, cells were washed with phosphate-buffered saline and lysed in the sodium dodecyl sulfate (SDS)-containing sample buffer. Virion proteins were prepared by purifying cell-free virions from culture medium of infected cells by ultracentrifugation through a sorbitol cushion and resuspending the cells in SDS-containing sample buffer. Proteins were resolved by electrophoresis on an SDS-containing polyacrylamide gel, transferred to a polyvinylidene difluoride (PVDF) membrane, hybridized with primary antibodies, reacted with the HRP-conjugated secondary antibody, and visualized by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) (62).

For immunofluorescence, cells grown on glass coverslips were fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 15 min, incubated with a primary antibody, and subsequently labeled with an Alexa Fluor 488- or 568-conjugated secondary antibody (Invitrogen-Molecular Probes). Labeled cells were also counterstained with TO-PRO-3 (Invitrogen-Molecular Probes) to visualize the nuclei. Finally, labeled cells were mounted on slides with Prolong Gold antifade reagent (Invitrogen-Molecular Probes). Images were captured using Zeiss LSM Image software with a Zeiss LSM 510 META confocal laser scanning microscope.

RESULTS

Construction of a BAC-HCMV clone carrying the *dd*FKBP-tagged UL79 and propagation of recombinant virus. The HCMV UL79 ORF has been reported to be essential for HCMV replication in fibroblasts (14, 63). To study the function of UL79 in virus infection, we adopted a protein-based viral genetic approach (3, 20, 45). We constructed a recombinant BAC-HCMV clone, pAD*dd*UL79, in which the annotated UL79 coding sequence was tagged with a 110-amino-acid destabilization domain derived from a mutant variant of the human FKBP protein (*dd*FKBP) (3) at its N terminus (Fig. 1A). This recombinant clone was derived from the parental BAC clone (pAD-GFP) which was also used to reconstitute wild-type virus (AD*wt*) in this study (64). In cells infected with recombinant virus (AD*dd*UL79) that was derived from pAD*dd*UL79, the *dd*FKBP-tagged UL79 protein was anticipated to be expressed but directed for rapid degradation by the *dd*FKBP tag. However, the *dd*FKBP tag should be stabilized in the presence of the synthetic chemical ligand Shield-1, and therefore, the tagged protein should retain its activity. This *dd*FKBP tagging approach offers some unique advantages for creating mutant viruses. It abrogates protein products of essential genes during virus infection without the need for creating null mutant virus and complementing cell lines. As only one virus stock is needed for the comparative study of HCMV infection in the presence or absence of a viral protein product, it eliminates the need for creating marker rescued virus and avoids the potential complication resulting from an altered particle-to-PFU ratio in mutant virus. Finally, it provides evidence that it is the protein encoded by the tagged ORF and not other potential products derived from the UL79 locus that is important for viral replication.

Transfection of pAD*dd*UL79 into human foreskin fibroblasts (HFFs) did not result in any detectable productive viral infection when the cells were surveyed for 30 days. However, when Shield-1 was added at a concentration of 1 μM, a cyto-

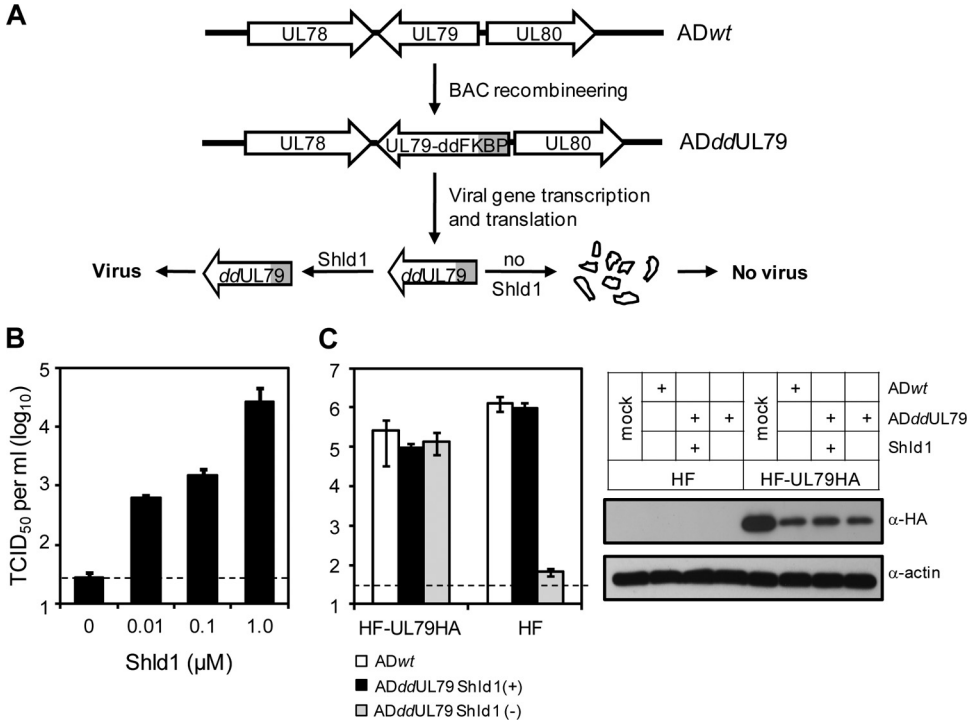

FIG. 1. (A) Schematic diagram for creating recombinant virus AD*dd*UL79 and applying a destabilization domain (*dd*FKBP)-mediated protein stability-based approach to regulate gene function. AD*dd*UL79 carried the UL79 ORF that was tagged at its N terminus with *dd*FKBP (indicated by gray box) and expressed the *dd*FKBP-UL79 fusion protein. Without the small-molecule ligand Shield-1 (Shld1), *dd*FKBP should direct the entire fusion protein for rapid degradation, thus effectively depleting UL79 proteins during infection. However, Shield-1 should bind to *dd*FKBP, protect the fusion protein from degradation, and maintain the accumulation of the UL79 proteins, thus allowing productive virus replication. BAC, bacterial artificial chromosome. (B) Dose-dependent regulation of AD*dd*UL79 replication by Shield-1. Human foreskin fibroblasts (HFFs) were infected with AD*dd*UL79 at a multiplicity of infection (MOI) of 3 in the presence of various concentrations of Shield-1 (as indicated). At 96 h postinfection (hpi), supernatants of infected cells were collected and analyzed for the presence of virus by $TCID_{50}$ assay in HFFs maintained in 1 μM Shield-1. (C) HFFs overexpressing the hemagglutinin (HA)-tagged UL79 complemented the growth of AD*dd*UL79 in the absence of Shield-1. Normal HFFs (HF) or HFFs overexpressing the HA-tagged UL79 (HF-UL79HA) were infected with the AD*wt* (wild-type virus control) or AD*dd*UL79 virus at an MOI of 3 with Shield-1 or without Shield-1. At 120 hpi, the titers of cell-free virus were determined as described above for panel B (left panel), and total cell lysates were analyzed for the expression of the tagged UL79 by immunoblotting using the anti-HA antibody (α-HA) (right panel). The antibody to actin was used as a loading control. The detection limit of the $TCID_{50}$ assay is indicated by the dashed line.

pathic effect (CPE) was observed, similar to that in transfection with wild-type pAD-GFP. Recombinant virus AD*dd*UL79 produced from pAD*dd*UL79 transfection was then amplified by infecting fresh HFFs in the presence of Shield-1, purified by ultracentrifugation, and resuspended in fresh medium to generate a virus stock. To confirm that viral growth was dependent on the presence of Shield-1, HFFs were infected with AD*dd*UL79 at an MOI of 3 with various concentrations of Shield-1. Infectious culture supernatants were collected at 96 h postinfection (hpi), and the titer of cell-free virus was determined by $TCID_{50}$ assay (Fig. 1B). The rescue of AD*dd*UL79 replication by Shield-1 was dose dependent. While AD*dd*UL79 failed to produce detectable progeny without Shield-1, its replication and CPE were indistinguishable from those of the wild-type virus with 1 μM Shield-1 (Fig. 1B and C). Our results suggest that Shield-1 efficiently regulates the replication of AD*dd*UL79 recombinant virus.

To provide additional proof that Shield-1 regulated AD*dd*UL79 replication by controlling the stability of the *dd*FKBP-tagged UL79 protein, we wanted to examine the expression of the tagged protein in fibroblasts infected with AD*dd*UL79. Surprisingly, we could not detect the tagged protein during infection regardless of Shield-1 treatment using an anti-FKBP12 antibody. This was not due to the inability of the antibody to recognize the *dd*FKBP domain, because we have used the same antibody to detect other *dd*FKBP-tagged viral proteins (data not shown). Moreover, we were able to readily detect the *dd*UL79 protein from cells transfected with a plasmid overexpressing *dd*UL79 in the presence of Shield-1 using the anti-FKBP12 antibody (data not shown). Therefore, our inability to detect the tagged protein is likely the result of the low-level accumulation of the UL79 protein during HCMV infection. As an alternative approach to confirm that the UL79 ORF encoded a protein essential for HCMV growth, we tested the growth of AD*dd*UL79 in UL79-overexpressing HFFs (HF-UL79HA cells) that were made by transduction of retrovirus carrying the C-terminally HA-tagged UL79 ORF. HF-UL79HA cells expressed the tagged protein at the predicted size (35 kDa) (Fig. 1C). In normal HFFs, AD*dd*UL79 grew like wild-type virus did in the presence of Shield-1 but could not replicate without it. In contrast, AD*dd*UL79 virus could replicate in HF-UL79HA cells in the absence of Shield-1, producing virus titers similar to those of AD*wt*. Therefore, the growth

defect of AD*dd*UL79 in the absence of Shield-1 is the direct result of the abrogation of UL79.

UL79 is essential for HCMV replication. To characterize the role of UL79 in HCMV replication, we first analyzed the growth kinetics of the AD*dd*UL79 virus in HFFs. For progeny virus collected at each time point, we determined the titer of virus in the presence and absence of Shield-1 to determine whether there were additional escape mutations acquired during the course of infection. Under the multistep growth condition, AD*dd*UL79 replicated indistinguishably from AD*wt* in the presence of Shield-1. However, without Shield-1, the production of AD*dd*UL79 was almost undetectable by 12 days postinfection (dpi), whereas AD*wt* growth peaked and the titers reached 1×10^7 $TCID_{50}$ units/ml (Fig. 2A). It was noted that at 15 dpi in the absence of Shield-1, AD*dd*UL79 started to produce very low levels of progeny that could be determined even in the absence of Shield-1. We interpreted these results to mean that AD*dd*UL79 might acquire spontaneous escape mutations after a prolonged period under nonpermissive conditions.

Next, we examined the single-step growth kinetics of AD*dd*UL79 to determine whether the defect was dependent on the MOI. At an MOI of 3 and in the absence of Shield-1, AD*dd*UL79 produced almost no virus by 4 dpi and very low levels of progeny ($<1 \times 10^3$ $TCID_{50}$ units/ml) by 6 dpi, while AD*wt* reached peak titers of 1×10^7 $TCID_{50}$ units/ml (Fig. 2A). In contrast, Shield-1 treatment restored the growth of AD*dd*UL79 to wild-type levels. The very low levels of AD*dd*UL79 produced after 5 dpi in the absence of Shield-1 might be due to the slight leakiness of Shield-1 regulation of *dd*FKBP degradation (3). It was unlikely that this low level of virus growth represented escape mutants because they could not establish a second round of infection without Shield-1, their titers did not increase over time, and their titers could not be measured without Shield-1.

Taken together, these data indicate that UL79 is essential for HCMV replication in cultured fibroblasts and that *dd*FKBP-mediated Shield-1 regulation allows for tight control of UL79 function during the virus infection cycle.

UL79 is required for viral replication at late stages of infection. To determine when UL79 is required during the viral replication cycle, we stabilized UL79 protein accumulation by Shield-1 at various time points and for different lengths of time in infected cells and then determined the impact of such temporal accumulation of UL79 proteins on virus production. To determine whether UL79 was required at early times, Shield-1 was added at the onset of virus infection but was removed from the medium at 24 or 48 hpi. To determine whether UL79 was required at late times during infection, Shield-1 was added at 24 or 48 hpi. In both experiments, the titers of virus in the supernatants of infected cells were determined at 120 hpi (Fig. 2B). When we added Shield-1 as late as 48 hpi, AD*dd*UL79 replication was indistinguishable from virus grown in the continuous presence of Shield-1. In contrast, when Shield-1 was present for only the first 48 h during infection, almost no viral progeny were produced, similar to AD*dd*UL79 infection in the complete absence of Shield-1. Taken together, these data indicate that UL79 is required for viral replication during the late stages of viral infection.

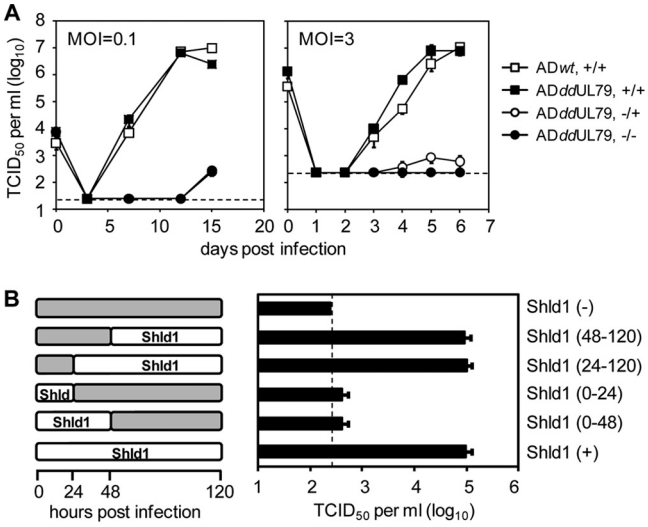

FIG. 2. UL79 is essential for HCMV replication at late stages of infection. (A) Growth kinetic analysis of AD*dd*UL79 in the presence or absence of Shield-1. HFFs were infected with AD*wt* or AD*dd*UL79 at an MOI of 3 for single-step growth analysis or at an MOI of 0.1 for multistep growth analysis. Infected cells were cultivated in the presence (+) or absence (−) of 1 μM Shield-1, and supernatants were collected at the indicated times and analyzed for the presence of virus by $TCID_{50}$ assay in HFFs maintained in 1 μM Shield-1 (+/+ or −/+). To test the potential occurrence of spontaneous escape mutants after prolonged growth of recombinant virus, supernatants from cells infected with AD*dd*UL79 in the absence of Shield-1 were also analyzed by $TCID_{50}$ assay in the absence of Shield-1 (−/−). (B) UL79 is required at late stages of HCMV infection. HFFs were infected with AD*dd*UL79 at an MOI of 0.1, Shield-1 (Shld1) was added at the indicated times postinfection, and infected cells were cultivated with Shield-1 for various time periods (indicated by the white boxes in the schematic drawing in the left panel). Supernatants were collected at 120 hpi and analyzed for the presence of virus by the $TCID_{50}$ assay in HFFs maintained in 1 μM Shield-1 (right panel). The number of hours of Shield-1 treatment are shown in parentheses to the right of the graph. The detection limits of the $TCID_{50}$ assay are indicated by dashed lines in the graphs.

UL79 is required for late viral gene expression. To determine which step of the viral replication cycle was compromised in the absence of UL79, we first examined the viral protein accumulation profile in the absence of Shield-1. The accumulation of viral immediate-early proteins (IE1-72 and IE2-86) or early/early-late proteins (UL38, UL44, UL69, and pp65) in cells infected with AD*dd*UL79 was not affected (Fig. 3 and data not shown). In contrast, the true late proteins, pp28, pp71, and pp150, accumulated at markedly reduced levels.

To determine whether the reduced late protein accumulation in AD*dd*UL79 infection was the result of reduced viral late transcript accumulation, we analyzed the IE1 transcript (control) and late UL32 (pp150), UL82 (pp71), and coterminated UL93-99 transcripts by RT-qPCR analysis (Fig. 4) (16, 38, 57). All transcripts detected were specific and were not the result of genomic DNA contamination, as mock-infected cells and qPCR reactions done in the absence of reverse transcriptase failed to produce any products (data not shown). At 24 hpi, late transcripts were barely detectable, whereas the IE1 transcript accumulated to a high level (data not shown). Importantly, stabilization of UL79 by Shield-1 had no substantial

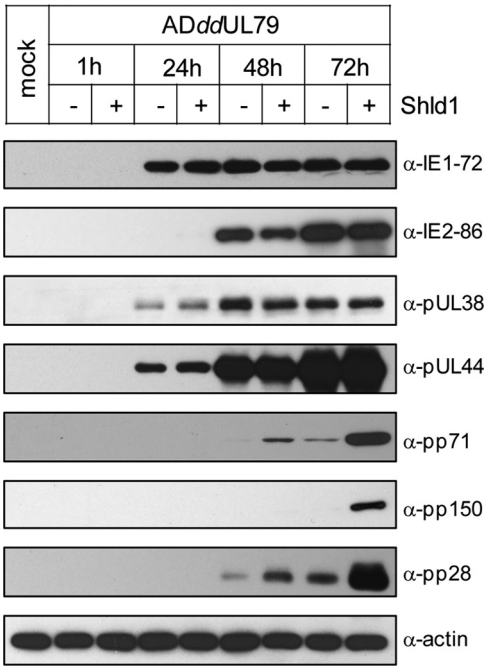

FIG. 3. UL79 is required for the efficient accumulation of late viral proteins during HCMV infection. HFFs were infected with AD*dd*UL79 at an MOI of 3 in the presence (+) or absence (−) of 1 μM Shield-1. Cells were harvested at different times postinfection. The accumulation of immediate-early proteins (IE1-72 and IE2-86), early proteins (pUL38 and pUL44), and late proteins (pp71, pp150, and pp28) were determined by immunoblot analysis.

effects on viral transcript accumulation at this time point (Fig. 4), consistent with the observation that UL79 was not required at early times of infection (Fig. 2B). At 72 hpi, even though degradation of UL79 had only a small effect on IE1 transcript

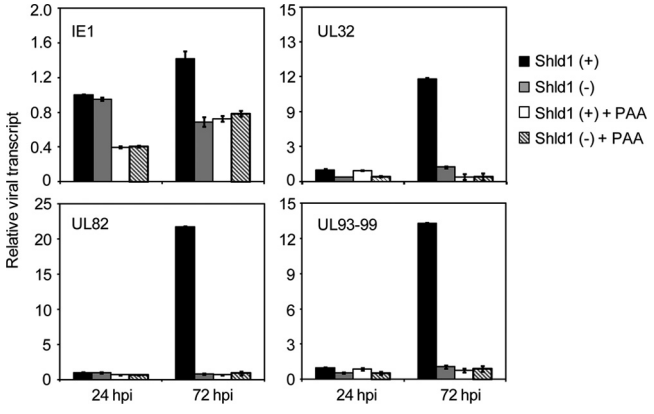

FIG. 4. UL79 is required for the efficient accumulation of late viral transcripts during HCMV infection. HFFs were infected with AD*dd*UL79 at an MOI of 3 in the presence (+) or absence (−) of Shield-1 (Shld1) (1 μM) and with or without the viral DNA synthesis inhibitor phosphonoacetic acid (PAA) (100 μg/ml). Total RNA was isolated at 24 and 72 hpi, and the amounts of selected viral transcripts were then measured by reverse transcription coupled to quantitative PCR (RT-qPCR) and normalized to that of GAPDH. The normalized amount of viral transcript at 24 hpi in the presence of Shield-1 but without PAA was set at 1.

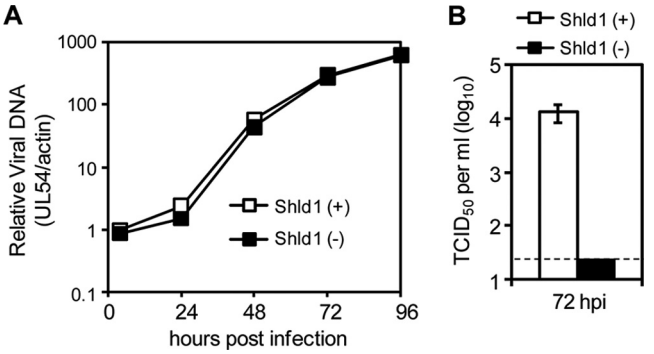

FIG. 5. Viral DNA replication is not altered in the absence of UL79 during infection. HFFs were infected with AD*dd*UL79 in the presence or absence of 1 μM Shield-1. (A) Total DNA was isolated at different times postinfection, and the accumulation of viral DNA was determined by qPCR using primers specific for the HCMV UL54 gene. (B) Supernatants of infected cells with or without Shield-1 treatment were also collected at 72 hpi and analyzed for the presence of virus by the $TCID_{50}$ assay in HFFs maintained in 1 μM Shield-1. The detection limit of the $TCID_{50}$ assay is indicated by the dashed line.

accumulation, as the viral DNA synthesis inhibitor phosphonoacetic acid (PAA) did (16), it reduced the accumulation of late transcripts almost as markedly as PAA did. The levels of late UL32, UL82, and UL93-99 transcripts when UL79 was absent were 7-, 27-, and 13-fold lower than the levels when UL79 was present, respectively (Fig. 4).

Together, our results indicate that UL79 has minimal effect on viral immediate-early and early gene expression but is critical for the accumulation of late viral transcripts and proteins.

UL79 is dispensable for viral DNA replication. As viral late gene expression is dependent on viral DNA replication, it was possible that the defect in late gene expression in the absence of UL79 was due to a defect in viral DNA replication. Thus, we examined viral DNA synthesis in the absence of Shield-1 (Fig. 5A). Surprisingly, the kinetics of viral DNA replication during AD*dd*UL79 infection in cells in the absence of Shield-1 was indistinguishable from that of infection of cells in the presence of Shield-1. To confirm that Shield-1 withdrawal was effective, supernatants from infected culture were collected at 72 hpi and measured for virus production (Fig. 5B). Infected cells in the absence of Shield-1 produced no detectable virus, indicating that UL79 function was indeed abrogated in these cells. Therefore, we conclude that UL79 acts after viral DNA replication and is specifically required for the accumulation of late viral transcripts.

The UL79 transcript is expressed with early-late kinetics and is regulated by a positive-feedback loop. To determine the kinetic class of UL79 expression, we analyzed the UL79 transcript accumulation in fibroblasts infected with AD*dd*UL79 by RT-qPCR (Fig. 6A). When the UL79 protein was stabilized, the UL79 transcript accumulated abundantly at late times of infection, and its level at 72 hpi was 5-fold higher than the level at 24 hpi. The UL79 transcript level was markedly reduced at 72 hpi when infected cells were treated with PAA, indicating that its transcript accumulation was augmented by viral DNA replication. However, in the presence of PAA, the accumulation of UL79 transcript was not completely inhibited, as the UL79 transcript levels increased almost 3-fold from 24 to 72

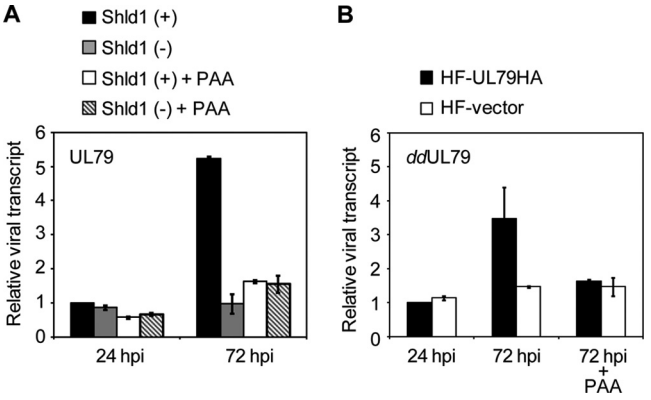

FIG. 6. UL79 is transcribed with early-late kinetics and is regulated by a positive-feedback loop. (A) HFFs were infected with AD*dd*UL79 at an MOI of 3 in the presence or absence of Shield-1 (1 μM) and with or without the viral DNA synthesis inhibitor PAA (100 μg/ml). Total RNA was isolated at 24 and 72 hpi, and the amount of UL79 transcript was measured by RT-qPCR analysis and normalized to that of GAPDH. The normalized amount of UL79 transcript at 24 hpi in the presence of Shield-1 but without PAA was set at 1. (B) HFFs expressing the HA-tagged UL79 (HF-UL79HA) or empty vector (HF-vector) were infected with AD*dd*UL79 at an MOI of 3 in the absence of Shield-1 and with or without PAA (100 μg/ml). Total RNA was isolated at 24 and 72 hpi, and the amount of *dd*FKBP-tagged UL79 transcript expressed from the viral genome was measured by RT-qPCR analysis using the primers specific to the *dd*FKBP sequence and normalized to that of GAPDH. The normalized amount of *dd*FKBP-tagged UL79 transcript at 24 hpi without PAA in infected HF-UL79HA cells was set at 1.

hpi. Therefore, as the accumulation of UL79 transcript is markedly enhanced by, but not completely dependent on viral DNA synthesis, our data suggest that UL79 is an early-late HCMV gene. However, in the present study, we cannot rule out the possibility that more than one transcript emanates from the UL79 locus and that our result reflects the combined expression profile of these transcripts rather than individual species.

Surprisingly, when UL79 proteins were destabilized, the level of UL79 transcript was drastically reduced in infected cells at 72 hpi (Fig. 6A). This suggests an a positive-feedback loop for UL79 expression which in turn amplifies the expression of late viral genes. To provide additional evidence for this autoregulation of UL79 transcription by its protein products, we tested whether we could enhance endogenous UL79 transcription during infection of UL79 recombinant virus by providing the UL79 protein in *trans*. We infected HFFs expressing the HA-tagged UL79 (HF-UL79HA cells) or empty vector (HF-vector cells) with AD*dd*UL79 in the absence of Shield-1. This experimental system allowed us to uncouple the UL79 protein accumulation (provided by HF-UL79HA cells) and endogenous UL79 transcript accumulation (from the viral genome). The endogenous UL79 transcription was analyzed by RT-qPCR using a primer pair specific to the *dd*FKBP sequence. We found that the accumulation of *dd*FKBP-UL79 transcripts in infected HF-UL79HA cells markedly increased at 72 hpi relative to that in infected HF-vector cells. This indicates that, as anticipated, the UL79 protein enhances the transcription from the UL79 gene locus (Fig. 6B).

Together, these results suggest that efficient transcription of UL79 is positively regulated by both viral DNA replication and its own protein products.

A UL79 stop codon mutant virus has the same defect as AD*dd*UL79. To provide additional proof that the defect of AD*dd*UL79 was the direct result of the loss of UL79 function, we created a second UL79 mutant virus, AD*in*UL79$_{stop}$. In this recombinant virus, we inserted a stop codon at residue Glu^6 of the UL79 ORF to abrogate the expression of UL79 protein products (Fig. 7A). The AD*in*UL79$_{stop}$ virus could be reconstituted only from transfection of the BAC clone in HF-UL79HA cells, not by transfection in normal HFFs or HF-vector cells. The titer of reconstituted mutant virus was comparable to that of wild-type virus (data not shown), and it replicated efficiently in HF-UL79HA cells but completely failed to grow in HF-vector cells (Fig. 7B). These results indicated that loss of UL79 function was solely responsible for the growth defect of AD*in*UL79$_{stop}$. Importantly, cells infected with AD*in*UL79$_{stop}$ synthesized viral DNA at wild-type levels (Fig. 7C) and accumulated the products of IE genes (IE1-72 and IE2-86) and early genes (pUL38 and pUL44) efficiently but had a marked defect in accumulation of viral late proteins (pp71 and pp28) during infection (Fig. 7D). Therefore, the defect of AD*in*UL79$_{stop}$ phenocopied that of AD*dd*UL79 under Shield-1 withdrawal, and our results provide further validation for the power of the protein stability-based conditional approach to dissect the role of viral factors in HCMV biology.

The HA-tagged UL79 protein is primarily nuclear and localizes in replication compartments during virus infection. As UL79 is required for the accumulation of late viral transcripts, it is possible that it is a nuclear protein involved in the regulation of viral late gene transcription. We first examined the intracellular distribution of UL79 expressed alone to determine its ability to localize into the nucleus in the absence of any other viral factors. We used HF-UL79HA cells because there was no antibody specific to UL79 available and because the tagged UL79 was fully functional to complement UL79-deficient virus (Fig. 1C). While no appreciable HA staining was present in normal HFFs, the staining was readily observed in HF-UL79HA cells, predominantly localizing within the nuclei, suggesting that UL79 proteins are primarily nuclear (Fig. 8A).

Next we determined whether the UL79 proteins localized to any virus-induced intracellular structures during infection, particularly replication compartments. These organized intranuclear viral structures are sites where sets of viral proteins (e.g., pUL44, pUL57, pUL117, and IE2) (1, 44, 46) and cellular proteins (e.g., p53, Nbs1, and Rad50) (18, 32) are recruited to, and viral activities, including viral DNA synthesis, late gene transcription, and DNA packaging, take place. To test our hypothesis, we examined the localization of the HA-tagged UL79 proteins expressed from HF-UL79HA cells that were infected with HCMV, as we were not able to detect tagged UL79 proteins expressed from the endogenous locus of the viral genome during infection (data not shown). The tagged UL79 proteins formed large intranuclear domains which colocalized with pUL44, the virus-encoded DNA polymerase accessory protein that has been used as a marker for replication compartments (44, 46) (Fig. 8A), indicating that a major fraction of HA-tagged UL79 proteins is localized in replication compartments. Importantly, the formation of UL79-containing large intracellular domains appeared to occur at late times

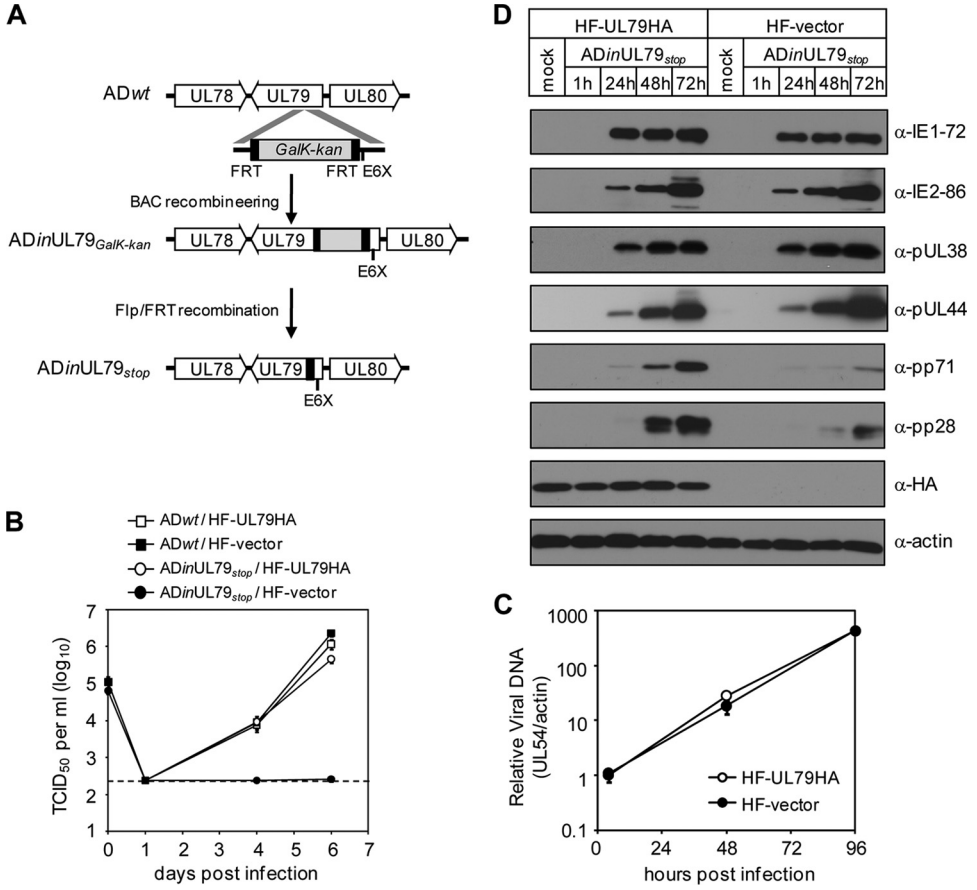

FIG. 7. A mutant virus in which the UL79 ORF has been abrogated by a stop codon mutation has the same defect as AD*dd*UL79. (A) Schematic diagram for creating recombinant virus AD*in*UL79$_{stop}$. A cassette that contained a stop codon followed by the FRT-bracketed GalK/kanamycin dual marker was amplified and recombined into the UL79 ORF of the wild-type HCMV BAC clone. The GalK/kanamycin marker was then removed by Flp/FRT recombination. The final clone, AD*in*UL79$_{stop}$, contained a stop codon along with a small FRT site inserted in frame at the 6th amino acid (E6X) of the UL79 ORF. (B) Single-step growth analysis of AD*in*UL79$_{stop}$. Complementing HF-UL79HA cells or control HF-vector cells were infected with AD*wt* or AD*in*UL79$_{stop}$ at an MOI of 3. Supernatants were collected at the indicated days postinfection and analyzed for the presence of virus by $TCID_{50}$ assay in HF-UL79HA cells. The detection limit of the $TCID_{50}$ assay is indicated by the dashed line. (C) Viral DNA replication of AD*in*UL79$_{stop}$. HF-UL79HA cells or HF-vector cells were infected with AD*in*UL79$_{stop}$ at an MOI of 3. Total DNA was isolated at different times postinfection, and the accumulation of viral DNA was determined by qPCR using primers specific for the HCMV UL54 gene. (D) Viral gene expression of AD*in*UL79$_{stop}$. HF-UL79HA cells or HF-vector cells were infected with AD*in*UL79$_{stop}$ at an MOI of 3. Cells were harvested at different times postinfection. The accumulation of immediate-early proteins (IE1-72 and IE2-86), early proteins (pUL38 and pUL44), and late proteins (pp71 and pp28) was determined by immunoblot analysis.

during infection (Fig. 8B), providing additional evidence that UL79 functions at late times to regulate gene expression.

We noted that the anti-HA antibody also appeared to generate low-level cytoplasmic staining in some normal HFFs that were infected with HCMV, even though this staining intensified in infected cells that expressed the tagged UL79 protein (Fig. 8A). This cytoplasmic staining colocalized with pp28-containing viral assembly centers (data not shown). Nonetheless, we decided not to pursue this further, as we could not rule out the possibility that the cytoplasmic HA staining represents the nonspecific recognition of assembly centers by anti-HA antibody.

The UL79 protein is not detected in HCMV virions. A proteomics study has previously shown that a single UL79-derived peptide can be detected in the HCMV virions only by the sensitive Fourier transform ion cyclotron resonance (FTICR) mass spectrometry, not by the more traditional liquid chromatography-tandem mass spectrometry (LC-MS-MS) analysis, suggesting that this peptide may represent a nonspecific contaminant (55). To determine whether the UL79 protein is a tegument protein, we infected HF-UL79HA cells or HF-vector cells with AD*dd*UL79 in the presence or absence of Shield-1. We then harvested supernatants from infected cells at 96 hpi and partially purified cell-free virions by ultracentrifugation through a sorbitol cushion. These virion samples appeared to be largely free of contamination of cellular debris, as they did not contain detectable amounts of the viral IE1 protein or the cellular PCNA protein (Fig. 9). On the other hand, the major capsid protein (MCP) was readily detected, and importantly, pUL69, a protein that is present in the tegument and dense bodies in very small amounts (55, 59), could also be detected in the virion samples (Fig. 9). Under these conditions, however, we could not detect the HA-tagged UL79 protein in the virion samples,

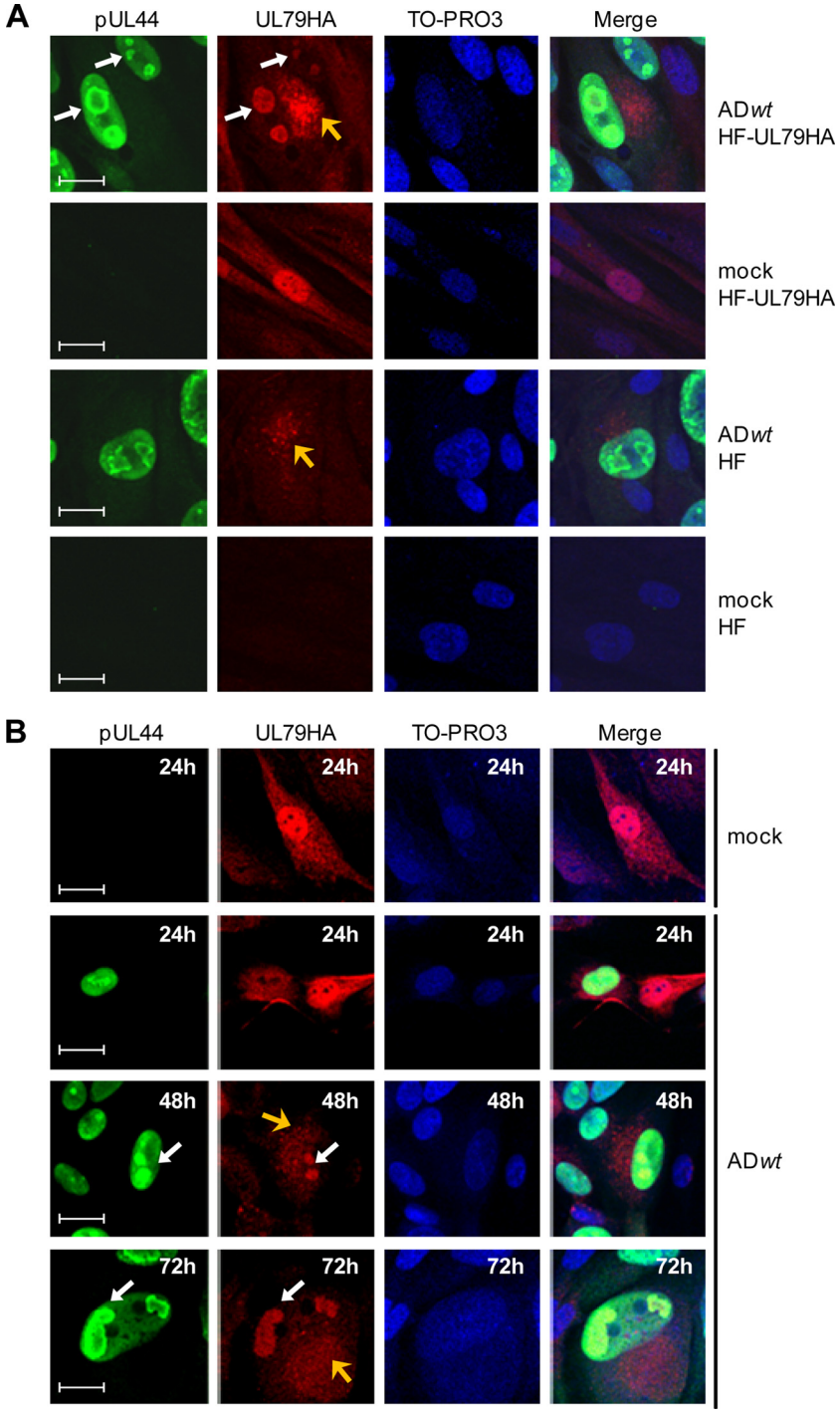

FIG. 8. UL79 proteins are primarily nuclear proteins that localize within viral replication compartments during infection. (A) Normal HFFs or HF-UL79HA cells were either mock infected or infected with the GFP-less wild-type HCMV virus AD*wt*.2 at an MOI of 0.5. At 72 hpi, the cells were harvested and examined by confocal immunofluorescence analysis for the localization of UL79 using anti-HA antibody and viral replication compartments using anti-pUL44 antibody. The cells were also counterstained with TO-PRO3 to visualize the nuclei. Mock-infected HFFs or HFFs infected with AD*wt*.2 were used as a negative control for the detection of tagged UL79 proteins by anti-HA antibody. (B) HF-UL79HA cells were either mock infected or infected with AD*wt*.2 at an MOI of 0.5. At different times postinfection, cells were examined by confocal fluorescence analysis for viral replication compartments using anti-pUL44 antibody and UL79 localization using anti-HA antibody. The cells were also counterstained with TO-PRO3 to visualize the nuclei. The white arrows indicate viral replication compartments, and the yellow arrows indicate the localization of a portion of HA staining to viral assembly center-like cytoplasmic structures. Bars, 20 μm.

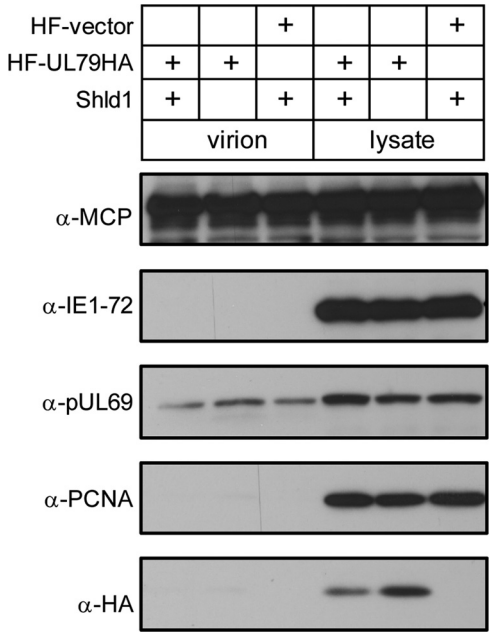

FIG. 9. The UL79 protein is not detected in HCMV virions. A total of 1×10^7 HF-UL79HA or HF-vector cells were infected with AD*dd*UL79 in the presence or absence of Shield-1 at an MOI of 5. Total cell lysates and cell-free virions were collected at 96 hpi. Cell-free virions were then partially purified by ultracentrifugation through a sorbitol cushion. Lysates of 1×10^5 cells or 20% of total virion samples harvested were loaded onto each lane of an SDS-polyacrylamide gel and analyzed by immunoblotting for MCP, IE1-72, pUL69, and cellular PCNA. For the UL79 protein, twice as many virion samples were used in the experiment, and the protein was analyzed using anti-HA antibody.

even though it was readily detectable in total lysate of infected HF-UL79HA cells. Thus, the UL79 protein is unlikely to be a major component of the HCMV virion. This was consistent with the result that UL79 appeared to be required only at late stages (i.e., after 48 hpi) (Fig. 2B) and suggested the important role of the newly synthesized UL79 protein in promoting the HCMV lytic infection cycle.

Collectively, our data indicate that UL79 plays a key role at late times after viral DNA replication to regulate the accumulation of late viral transcripts.

DISCUSSION

In this study, we applied the *dd*FKBP-mediated, Shield-1-regulated protein genetic approach (3) to investigate the role of viral gene UL79 during HCMV replication. Moreover, we also compared the effectiveness of this approach to the more traditional viral genetic approach by creating and analyzing the second UL79 mutant virus in which the UL79 ORF was disrupted by a stop codon mutation (Fig. 7). This comparative analysis provides additional proof for the power of the protein stability-based conditional approach to dissect the role of viral factors in HCMV biology. Our work represents one of the few studies that take advantage of this powerful approach to define viral gene functions in HCMV biology (20, 45). Shield-1 regulation of *dd*FKBP-tagged UL79 is robust, reversible, and precisely controlled and validates the role of UL79 as an essential HCMV gene (Fig. 1 and 2). Interestingly, we could not detect *dd*FKBP-tagged UL79 protein in cells infected with AD*dd*UL79 even with Shield-1 using an anti-FKBP12 antibody. Nonetheless, the full complementation of AD*dd*UL79 in the absence of Shield-1 by HFFs overexpressing UL79 provided additional evidence that the defect of AD*dd*UL79 was the direct result of UL79 depletion (Fig. 1C). Our inability to detect the endogenous UL79 proteins during infection is likely due to either their extremely low levels of expression or their unstable nature, similar to other HCMV proteins such as pUL21a (17). Notably, the accumulation of the HA-tagged UL79 protein overexpressed from transduced HFFs was also reduced during infection relative to that in mock-infected cells, suggesting that the turnover of UL79 proteins may be enhanced during infection (Fig. 1C). A more sensitive antibody or assay will need to be developed in order to detect and characterize endogenous UL79 proteins during HCMV infection.

Our systematic dissection of AD*dd*UL79 infection in the absence of Shield-1 allowed us to define the stage of the infection cycle where UL79 functioned. Shield-1-dependent rapid degradation/stabilization of *dd*FKBP-tagged proteins (3) gave us a tool to define the requirement of UL79 in HCMV infection in a nearly real-time manner (Fig. 2B). Treating infected cells with Shield-1 for the first 48 hpi could not rescue the growth of recombinant virus, whereas adding Shield-1 at late times, even at 48 hpi, fully restored its growth (Fig. 2), suggesting that UL79 was required at late stages of HCMV infection. Consistent with the timing of its involvement in virus infection, UL79 was expressed with early-late kinetics (Fig. 6) and localized into viral replication compartments where viral DNA replication and late gene transcription took place (Fig. 8). These spatial and temporal characteristics of UL79 support its role in regulating the accumulation of viral late gene products at transcript levels (Fig. 3, 4, and 7).

Depletion of UL79 during HCMV infection was able to uncouple viral DNA synthesis and viral late gene expression, indicating that the former is not sufficient to promote the latter (Fig. 4 and 5). For many DNA viruses, the temporal regulation of viral gene expression is a common feature of their lytic infection. For simian virus 40, the large T antigen plays an essential role in the activation of viral promoters (5, 27), and viral DNA replication attenuates in *trans* the repressor of viral late promoters (58, 67). For adenovirus, both viral *trans*-acting factors (23, 40) and viral DNA replication (53, 54) are required for its late gene expression. Viral DNA replication facilitates late gene expression directly (53) or indirectly by promoting the expression of viral *trans*-acting factors (23) or recruitment of cellular transcription factors to late promoters (54).

For herpesviruses, viral late gene expression has been studied mostly with herpes simplex virus (HSV) and murine gammaherpesvirus 68 (MHV-68). In HSV, DNA replication is required in *cis* for activity of late promoters (26, 34). HSV proteins, including ICP4, ICP8, and ICP27, are necessary for efficient expression of late genes by interacting with the general transcription machinery (19, 28, 47), and they facilitate the assembly of transcription preinitiation complexes (8, 65). In MHV-68, both viral DNA replication (12) and four viral proteins, ORF18, ORF24, ORF30, and ORF34, are required for late gene expression (2, 60, 61). HCMV UL79, UL87, and

UL95 share sequence homology with ORF18, ORF24, and ORF34, respectively, while ORF30 is conserved only in gammaherpesviruses (2, 60, 61). Both UL79 and ORF18 are dispensable for viral DNA replication but are required for the accumulation of viral late gene products (this study) (2), and UL79 also localizes to viral replication compartments (Fig. 8). However, UL79 shares only 28% amino acid identity to ORF18 (2) and is transcribed with early-late kinetics (Fig. 6), whereas ORF18 was identified as an early gene (33). This body of evidence suggests that a general regulatory principle of late viral gene expression is conserved across the herpesvirus family, but the precise mechanism may be unique to each herpesvirus.

How does UL79 regulate late viral gene expression? Three possible mechanisms are responsible for its activity. It is possible that UL79 may modulate the initiation of late viral gene transcription. It may act as a transcription activator, a function reminiscent of ORF18 (2). However, as *in silico* analysis did not reveal any significant homology of UL79 to known transcription factors or conserved DNA binding motif, UL79 may also stimulate late gene transcription by activating other transcriptional factors or dissociating repressors from late gene promoters. Alternatively, UL79 may be involved in regulation of chromatin structures of replicating viral genomes, which may in turn facilitate late viral gene expression. Finally, it is also possible that UL79 may promote the accumulation of late transcripts by maintaining their stability. In any event, the temporal expression (i.e., early-late kinetics) of UL79 may be partially responsible for its involvement in the accumulation of only the late transcripts and not the early transcripts. The precise mechanism for how UL79 selectively promotes viral late transcripts will be a key question to address in future study.

How is the expression of UL79 regulated during HCMV infection? UL79 is expressed with early-late kinetics; its transcripts accumulate even when viral DNA synthesis is inhibited, but the accumulation is markedly enhanced upon viral DNA synthesis (Fig. 6). Importantly, the efficient accumulation of UL79 transcripts also requires the accumulation of UL79 proteins themselves and is regulated by a positive-feedback loop (Fig. 6). Therefore, low-level UL79 expression appears to occur independently of viral DNA replication. Viral DNA replication may increase the copy number of DNA templates for UL79 transcription. Perhaps more importantly, any stimulatory effect of viral DNA replication on UL79 expression is further amplified via this positive-feedback loop. Therefore, it is sensible to speculate that viral DNA replication acts in *cis* and UL79 proteins function in *trans* to promote their own expression.

In conclusion, we have identified UL79 as a key viral factor bridging viral DNA replication and late gene expression during HCMV infection. We are investigating potential interactions of UL79 with cellular proteins or other viral proteins to define its mechanism during HCMV infection.

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