

# Transcription of True Late ( $\gamma_2$ ) Cytomegalovirus Genes Requires UL92 Function That Is Conserved among Beta- and Gammaherpesviruses

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**Human cytomegalovirus-encoded UL92 plays an essential role in viral replication that has not been resolved. We show here that this gene controls the accumulation of true late ( $\gamma_2$ ) viral transcripts, a property shared with several other recently evaluated genes (UL79, UL87, UL91, and UL95) conserved among beta- and gammaherpesviruses. When the UL92 mutant virus was evaluated, function was fully complemented by either the natural protein or the homologous Rh127 protein from rhesus cytomegalovirus. N-terminal epitope-tagged UL92 protein is functional, follows complex early-late expression kinetics, and localizes in the nucleus within viral replication compartments. UL92 severely impacts the late (72-h postinfection) expression of nine genes encoding virion proteins (UL32, UL55, UL73, UL75, UL80, UL86, UL99, and UL115), as well as UL91 and itself, but does not influence the levels of UL44, UL82, or UL83 accumulation. Although viral DNA is made at normal levels, viral capsid accumulation in the nucleus is severely compromised in UL92 mutant virus-infected cells, and mature virions are not observed in the cytoplasm. Taken together, UL92 is a key regulator of late viral gene expression, apparently functioning with four other beta- or gammaherpesvirus gene products in a pattern that appears reminiscent of gene regulation in T4 DNA bacteriophage.**

Human cytomegalovirus (HCMV) causes significant disease in the developing fetus, as well as in immunocompromised individuals (1), exhibiting a broad tropism for differentiated myeloid, epithelial, endothelial, mesothelial, fibroblast, and neuronal cell types. In all of these settings, viral genes are expressed in a coordinated cascade that is characteristic of all herpesviruses. HCMV replication, although tightly regulated, is prolonged, such that viral late gene expression and progeny accumulate only after 2 to 3 days of infection. Like other herpesviruses, such as the alphaherpesvirus herpes simplex virus 1 (HSV-1), transcription is carried out by host RNA polymerase (Pol) II modified by viral proteins. Like HSV-1, HCMV relies on a virion tegument protein to transactivate immediate-early (IE; also called  $\alpha$ ) genes (2–4), relying on UL82-encoded pp71 to act on cellular Daxx/ATRAX (5, 6). Once expressed, the IE gene products, IE2-p86 (7) and IE1-p72 (8, 9), coordinate (10, 11) the recruitment of host RNA Pol II to activate delayed early (DE; also called  $\beta$ ) genes (1). HCMV mutants provided key evidence for a direct role of these IE genes in regulation of gene expression, while unveiling their role in modulating cytokine activation, programmed cell death, and chromatin remodeling (12–16). Once activated, viral UL112–113 gene products associate with specialized nuclear sites (17, 18), guided by IE2-p86, and this leads to recruitment of ppUL84 and ppUL44, the viral DNA Pol processivity (proc) factor into a complex that recognizes the origin of viral DNA replication, oriLyt, to initiate DNA synthesis (19–22), as well as the enhanced production of late ( $\gamma$ ) viral gene products that are required for the assembly, maturation, and release of progeny (23).

Like other herpesviruses, HCMV encodes distinct categories of late genes, commonly referred to as leaky late (or  $\gamma_1$ ) and true late (or  $\gamma_2$ ); the former are expressed independent of viral DNA synthesis, and the latter are not expressed at all when viral DNA synthesis is blocked by specific inhibitors (1, 24). Genes in the true late category include UL99 (encoding pp28) (25, 26), UL94 (27), UL75 and UL115 (encoding gH/gL) (28–30), UL32 (encoding pp150)

(31, 32), the middle transcription start site of UL44 (33–35), and a start site located within UL122 (IE2-p86) encoding IE2-p40 (also called L40) (10, 36). Many other viral transcripts are detected at late times (37). True late gene regulation in HCMV (35, 38, 39), like HSV-1 (40) is controlled by a small, TATA box-proximal region. An unusual, TATT sequence predominates in HCMV  $\gamma_2$  genes (27, 29, 33, 37, 39, 41).

Gammaherpesviruses, such as the mouse  $\gamma$ MHV-68 and human Epstein-Barr virus (EBV), have provided direct insights into the function of a distinct set of genes that are conserved in beta-herpesviruses but not in alphaherpesviruses, the so-called betagamma genes (42, 43). Open reading frames (ORFs) 18, 24, 30, 31, and 34 are homologs of HCMV UL79, UL87, UL91, UL92, and UL95, respectively, and all are involved in regulating late gene expression in  $\gamma$ MHV-68 (44–47). EBV BcRF1, the homolog of  $\gamma$ MHV-68 ORF24 and HCMV UL87, interacts with RNA Pol II and functions as a TATA-binding protein (TBP) with specificity for TATT (48, 49). All five HCMV betagamma genes are essential for replication (50, 51), suggesting that control of late transcription via RNA Pol II in this subfamily also relies on a late transcription complex (52) that may include UL79 (32), TBP-like UL87 (48), and UL95 and UL91 (53). In HCMV evidence suggests that this complex associates with the ppUL44 polymerase processivity sliding clamp independent of DNA synthesis (52). Reliance on a simple promoter, the presence of an unusual promoter element (TATT), and the requirement for specific viral genes to modify the core transcription machinery are reminiscent of bacteriophage

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T4, where the transcription machinery associates with the DNA Pol proc sliding clamp to regulate late genes (54).

Here, we evaluate HCMV UL92 gene function, completing the set of genes influencing late gene activation in  $\gamma$ MHV-68 (44–47). Like the other betagamma genes, UL92 is conserved in HCMV strains, as well as in chimpanzee (42, 43), rhesus macaque (55, 56), and rodent (57, 58) cytomegaloviruses (CMVs), in addition to all characterized gammaherpesviruses. We show that full-length UL92 is necessary for the transcriptional activation of true late genes within the nucleus of infected cells. This characterization implicates conserved betagamma gene function controlling transcription of true late genes, findings that are consistent with a modification in host RNA Pol II machinery. (In an accompanying paper, D. Yu et al. [59] demonstrate the role of MCMV protein pM92 in gene expression and suggest that the function of UL92 is a potential target for therapeutic intervention in cases of CMV infection.)

## MATERIALS AND METHODS

**Plasmid construction.** To construct a plasmid expressing UL92 with three tandem FLAG epitope (3FLAG) tag at the N terminus, the full-length UL92 ORF was amplified by PCR from the HCMV Towne-BAC genome (GenBank accession no. AY315197) (60) and cloned in-frame into the HindIII-XbaI sites of p3FLAG-CMV10 vector (Sigma, St. Louis, MO) to produce p3FLAG-UL92. N-terminally 3FLAG-tagged rhesus macaque CMV (RhCMV) rh127 expression plasmid was constructed by substituting full-length rh127 ORF in p3FLAG-UL92. RhCMV strain 68-1 DNA (GenBank accession no. NC\_006150) (61, 62) was generously provided by Peter Barry, University of California Davis. An influenza virus hemagglutinin (HA) epitope-tagged UL93 protein expression plasmid was constructed by inserting a PCR fragment containing the sequence of the UL93 ORF, along with a C-terminal HA epitope tag, into the HindIII-XbaI sites of pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA). For the construction of 3FLAG-UL92 expressing lentiviral vector, the 3FLAG-UL92 ORF was inserted into BamHI-NheI sites of pLV-EF1 $\alpha$ -MCS-IRES-Puro lentiviral vector (Biosettia, San Diego, CA) to produce pLV-3FLAG-UL92.

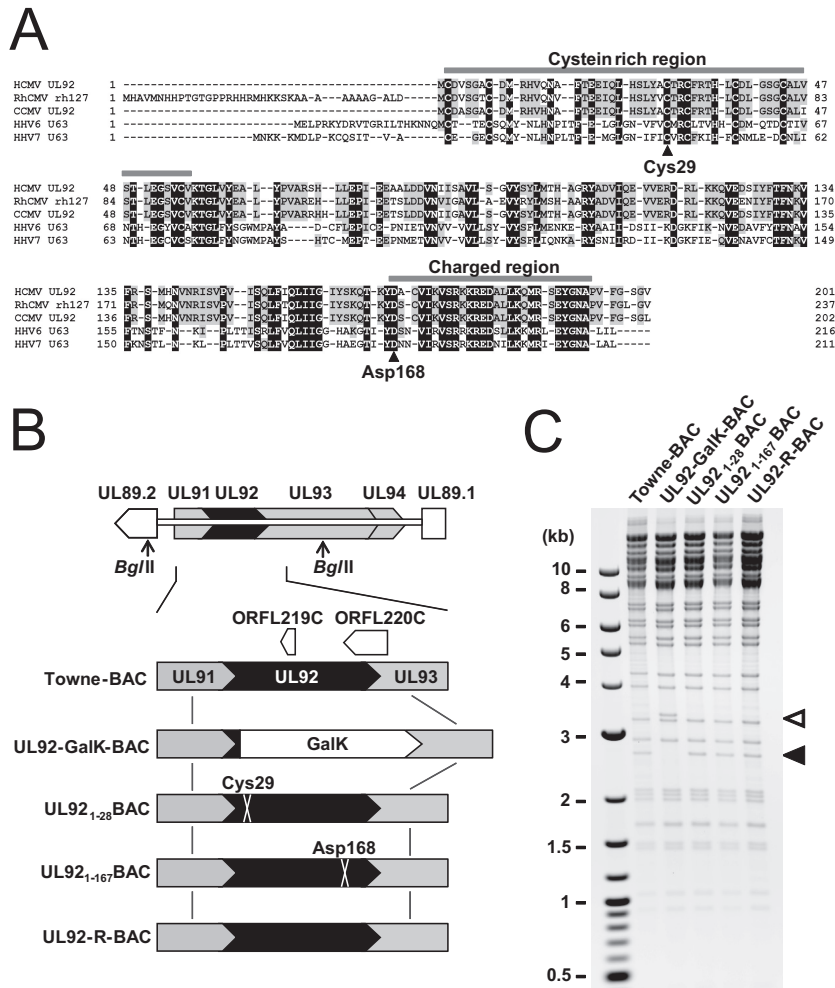
**Cells.** Primary human foreskin-derived fibroblasts (HFs) and 293T cells were cultured as described previously (53). HFs between passages 8 and 15 were used in all experiments. To generate HFs expressing 3FLAG-UL92, a lentivirus stock was prepared by transfecting pLV-3FLAG-UL92, psPAX2 (63), and VSV-G expressing plasmid into 293T cells. Low-passage HFs were transduced with lentiviral vector by spinoculation (1,000  $\times$  g for 1 h) and selected using 0.5  $\mu$ g/ml of puromycin (Invitrogen, Carlsbad, CA).

**BAC mutagenesis and recombinant viruses.** Bacterial artificial chromosomes (BACs) recombining was carried out using GalK positive/negative selection protocols (64), as described for UL91 (53). To create UL92-GalK-BAC, double-stranded linear DNA carrying overhang of 50 bp of homology flanking both sides of the desired deletion region from Towne-BAC, in addition to the GalK cassette, was amplified by PCR using the pGalK plasmid (64) as a template with the primers 5'-GGCACGTCCAGAACGCGTTTACCGAGGAGATCCAGTTACTCTGCTCTACTctgttgacaattaatcatcgga-3' and 5'-GGTCGAGCACCAGATGTAGAGGCAATTGCTCATCGTCAGCGAACCGCGCtcagcactgtctgctcctt-3'. In the PCR primer sequences, nucleotides homologous to Towne-BAC DNA are indicated in uppercase letters, and nucleotides in the GalK cassette are indicated in lowercase letters. The double-stranded linear DNA was introduced into the SW102 *Escherichia coli* (64) carrying Towne-BAC by electroporation, and recombinants containing the GalK cassette were selected in galactose and chloramphenicol. UL92<sub>1-28</sub>BAC was generated by replacing the GalK cassette using a linear fragment containing a single nucleotide insertion to introduce a translational stop plus frameshift at the Cys29 codon in UL92. The linear fragment was obtained

by PCR from Towne-BAC DNA with the primer 5'-GGCACGTCCAGAACGCGTTTACCGAGGAGATCCAGTTACTCTGCTCTAGCGTAGCAGCGCTGCTTTTCGCACG-3', where the boldface "T" and the underlining indicate the insertion of a frameshift and the stop codon, respectively, and the primer 5'-GGTCGAGCACCAGATGTAGAGGCAATTGCTCATCGTCAGCGAACCGCGC-3'. The PCR-amplified DNA was transformed into SW102 *E. coli* containing UL92-GalK-BAC and selected on 2-deoxy-galactose minimal agar plates. UL92<sub>1-167</sub>BAC was also generated in this manner by a single nucleotide insertion to introduce a translational stop plus frameshift at the Asp168 in UL92. To create a marker rescue virus (UL92-R-BAC), the UL92<sub>1-28</sub>BAC was first replaced by a GalK cassette and then subjected to a second round of recombination by replacing GalK cassette with wild-type UL92. To make 3FLAG-UL92-BAC, the double-stranded linear fragment was amplified by PCR using the p3FLAG-UL92 plasmid as a template and the primers 5'-CCTGTCGTCCTGCCCTTCCTGTTCCTCCGCCCCCAAACTGTGAGCTGACGCTAGCagccaccatggactacaagaccatgacgg-3' and 5'-GGTCGAGCACCAGATGTAGAGGCAATTGCTCATCGTCAGCGAACCGCGCCTCAAACGCCAGATCCG AATACAGG-3'. In these PCR primer sequences, the boldface "T" and the underlining indicate the insertion of a frameshift and the stop codon at Asp78 in UL91 ORF, respectively, and nucleotides homologous to those in the Towne-BAC are shown in uppercase letters, and the nucleotides in the three tandem FLAG epitope tag are shown in lowercase letters. DNA was transformed into SW102 *E. coli* containing UL92-GalK-BAC and selected on 2-deoxy-galactose minimal agar plates. All generated BACs were assessed by fragment length polymorphism (RFLP) with restriction enzymes, PCR analysis, and DNA sequencing of the insert region. To generate recombinant viruses, BAC DNA and pp71 expression plasmid (53) were transfected into HFs or 3FLAG-UL92 expressing HFs. Infectious titers were determined by standard plaque assay. For the secondary spread assay, HFs were transfected with UL92-GalK-BAC, the pp71 expression plasmid, and one of the p3FLAG constructs in the presence or absence of UL93-HA and then analyzed as described previously (53). For live cell imaging and the quantification of fluorescence intensities, cell cultures were analyzed with a Live Cell Imaging System InCuCyte ZOOM (Essen Bioscience, Ann Arbor, MI) (53, 65).

**Chemicals and antibodies.** Protein synthesis inhibitor cycloheximide (CHX) and HCMV DNA synthesis inhibitor phosphonoformate (PFA) were purchased from Sigma (St. Louis, MO). Proteasome inhibitor MG132 was obtained from Calbiochem (San Diego, CA). Mouse anti-FLAG (clone M2) monoclonal antibody was purchased from Sigma (St. Louis, MO). Mouse monoclonal antibodies against pp150 (clone 36-14) (31) and MCP (clone 28-4) (66) were kindly provided by William Britt, University of Alabama, Birmingham, AL. Other antibodies used in the present study were described previously (53).

**Immunoblotting.** 293T cells were transfected with the 3FLAG-UL92 or 3FLAG-rh127 constructs in Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and lysed in 2% sodium dodecyl sulfate (SDS)-containing sample buffer with  $\beta$ -mercaptoethanol at 48 h posttransfection. HFs infected with various viruses at the indicated multiplicity of infection (MOI) and times postinfection and processed as described previously (53). Extracellular virus particles were purified as described previously (67). Briefly, HFs were infected with Towne-BAC or 3FLAG-UL92-BAC virus (MOI of 3) and harvested at 4 dpi. Cellular debris was removed by low-speed centrifugation, and extracellular virus particles were collected on a 30% sucrose cushion (SW 41 rotor, Beckman L-80 ultracentrifuge, 24,000 rpm, 1 h) and resuspended and solubilized in 2% SDS-containing sample buffer in the presence of  $\beta$ -mercaptoethanol. Proteins were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Billerica, MA), probed with primary antibodies, and reacted with horseradish peroxidase-conjugated secondary antibody. A chemiluminescent signal was detected with an ECL Western blotting detection reagent (GE Healthcare, Buckinghamshire, United Kingdom).



**FIG 1** Construction of UL92 mutant BAC clones. (A) Amino acid sequence alignment of UL92 homologs from HCMV (AY315197), RhCMV (NC\_006150), CCMV (NC\_003521), HHV6 (NC\_001664), and HHV7 (NC\_001716). Identical amino acids (black) and CMV conserved amino acids (gray) are highlighted. The positions of Cys29 and Asp168 where individual translational stop-frameshift mutations were introduced are indicated by black arrowheads. The N-terminal cysteine-rich region and a highly charged C terminus are indicated by gray bars. (B) Schematic diagrams of the UL89.2-to-UL89.1 region in Towne-BAC with other UL92 mutant BACs shown below. (C) Electrophoretic separation of BglII-digested Towne-BAC and UL92 mutant BACs on 0.8% agarose as a diagnostic for integrity of the genomes. Insertion of GalK cassette into the UL92 locus results in an ~0.7-kb increase in the size of the BglII fragment (white arrowhead) compared to the 2.6-kb band (black arrowhead) in Towne-BAC and UL92 mutant BACs.

**Microscopy.** Immunofluorescence analysis (IFA) and transmission electron microscopy (TEM) were performed as described previously (53).

**DNA and RNA analyses.** Accumulation of intracellular viral DNA or RNA was measured by quantitative PCR (qPCR) or reverse transcription coupled to qPCR (qRT-PCR) (53). Primers (5'-AGTACGACGCGTGTGTCATC-3' and 5'-ATCCGAATACAGGTGCGTTT-3') were used for detection of the UL92 transcript. Other primers used in the present study were described previously (53).

**RESULTS**

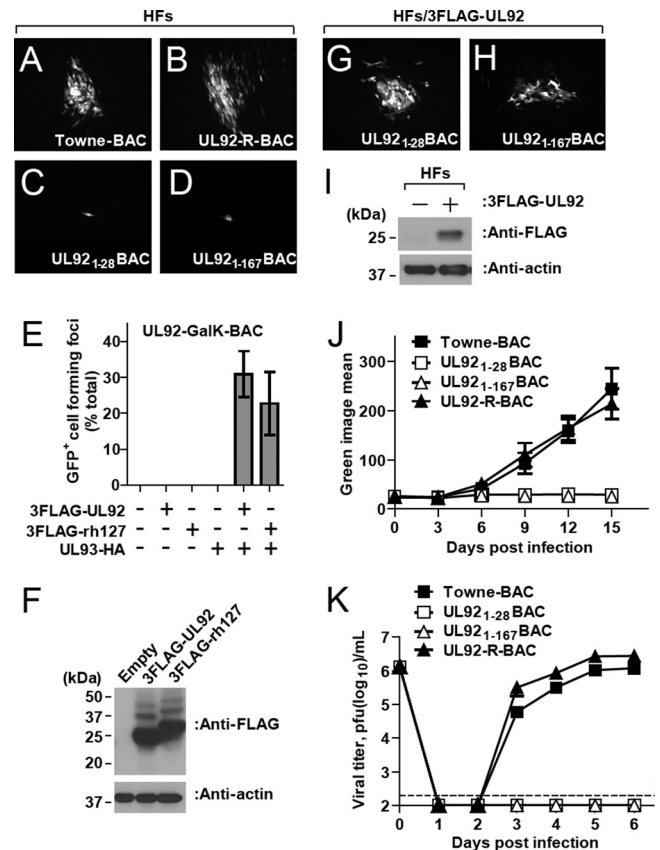
**Construction of UL92 mutant BAC clones.** In order to study function of UL92 in HCMV replication, UL92 mutant viruses were constructed from Towne-BAC (60) in a manner that did not interfere with expression or functions of adjacent genes. Insertion and deletion mutagenesis has indicated that the highly conserved UL92 gene is essential for replication in both AD169 and Towne strains of HCMV (50, 51), but that overlap with the adjacent UL91 (53), as well as a UL93, makes it difficult to be confident in the

independent importance of UL92. The overlapping C terminus of UL91 is dispensable (53), but the overlapping UL93 region is of unknown function. Alignment analysis revealed that UL92 homologous amino acid sequences from primate betaherpesviruses are highly conserved across the protein, and the N-terminal region of UL92 contains an N-terminal cysteine-rich region and a highly charged C terminus (Fig. 1A). Based on this information, Towne-BAC UL92 was substituted with a GalK cassette to generate UL92-GalK-BAC as an intermediate to introduce individual translational stop-frameshift mutations at Cys29 and Asp168 (UL92<sub>1-28</sub>-BAC and UL92<sub>1-167</sub>-BAC, respectively; Fig. 1A and B). In addition, a marker rescue virus (UL92-R-BAC) was generated from UL92<sub>1-28</sub>-BAC, as described in Materials and Methods. These mutant BACs were designed to disrupt UL92 within the cysteine-rich N terminus (UL92<sub>1-28</sub>-BAC), as well as to remove the highly charged C terminus (UL92<sub>1-167</sub>-BAC) without altering UL93. Two short ORFs (ORFL219C and ORFL220C) of

unknown function are translated at low levels from the cDNA strand in this region (68). The 21-amino-acid (aa) ORFL219C remained intact in both mutant viruses, and the 50-aa ORFL220C was intact in UL92<sub>1-28</sub>BAC but was mutated (C-terminal 6-aa YLLCFE to VLGLLV) in UL92<sub>1-167</sub>BAC. The genomic integrity of all mutant and control BAC clones was verified by RFLP with restriction enzymes (Fig. 1C and data not shown), and the entire UL92 locus was sequenced to ensure that only the intended changes were introduced.

**UL92 is essential and its C-terminal charged region is crucial for HCMV replication.** In order to characterize the replication properties of UL92 mutant virus without complementation, we first transfected each of the mutant BACs into HF cells and identified transfected cells, as well as viral spread, by using green fluorescent protein (GFP) expression from a cassette within the viral genome (60). Towne-BAC and UL92-R-BAC generated similar sizes and morphologies of GFP-positive plaques, whereas UL92<sub>1-28</sub>BAC and UL92<sub>1-167</sub>BAC only produced single GFP-positive cells that remained at 10 days posttransfection (Fig. 2A to D). Thus, the replication defect observed in genome-wide mutagenesis (50, 51) was sustained. Replication of UL92<sub>1-28</sub>BAC and UL92<sub>1-167</sub>BAC was restored by transient expression of 3FLAG-UL92 (data not shown), a finding consistent with the direct role of this protein and not other potential gene products from this region. Because homologous recombination between the viral genome and 3FLAG-UL92-expressing plasmid might complicate this result, we performed additional complementation experiments with UL92-GalK-BAC using UL92 together with UL93 expression plasmids in a secondary spread assay. Replication of UL92-GalK-BAC was reconstituted when complemented by transfection with 3FLAG-UL92 in the presence but not in the absence of HA-tagged UL93 (Fig. 2E and F). These results reinforce earlier studies (50, 51) and show UL92 is necessary for viral replication. Finally, RhCMV rh127 also complemented replication (Fig. 2E and F), demonstrating functional conservation of this gene in another primate CMV.

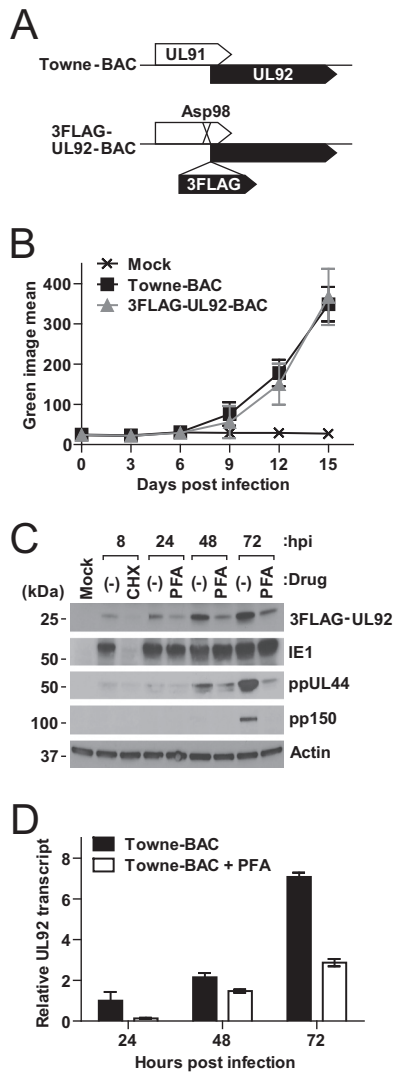
Based on the transient complementation results, we established 3FLAG-UL92 expressing HF cells using lentivirus-mediated transduction for propagation of UL92<sub>1-28</sub>BAC and UL92<sub>1-167</sub>BAC viruses. 3FLAG-UL92 expressing HF cells supported the replication of UL92<sub>1-28</sub>BAC and UL92<sub>1-167</sub>BAC (Fig. 2G and H), producing titers of mutant virus nearly identical to parental Towne-BAC. Expression of 3FLAG-UL92 in the transduced HF cells was confirmed by immunoblotting (Fig. 2I). These results demonstrate that 3FLAG-tagged UL92 is fully functional and the phenotypes of UL92<sub>1-28</sub>BAC and UL92<sub>1-167</sub>BAC viruses was a direct result of UL92 disruption. In order to further access the growth defect of UL92 mutant viruses, HF cells were infected with mutant viruses or controls (Towne-BAC and UL92-R-BAC) at an MOI of 0.01, and virus replication was quantified based on the GFP signal intensity, which is expressed from a cassette within the viral genome. UL92<sub>1-28</sub>BAC and UL92<sub>1-167</sub>BAC viruses failed to replicate, whereas UL92-R-BAC virus exhibited similar viral replication kinetics to parental Towne-BAC (Fig. 2J). When UL92 mutant viruses were evaluated under synchronous infection condition at an MOI of 3, neither mutant virus produced any detectable progeny where control viruses replicated well (Fig. 2K). Taken together, these data demonstrate that UL92 is essential for viral replication and a high MOI of UL92-null virus did not overcome a very tight replication block. This pattern is very similar to the phenotype of UL91 mu-



**FIG 2** Replication properties of UL92 mutant BAC-derived viruses. (A to D) Typical GFP-positive plaque phenotypes at 10 days posttransfection of HF cells with Towne-BAC (A), UL92-R-BAC (B), UL92<sub>1-28</sub>BAC (C), and UL92<sub>1-167</sub>BAC (D). Original magnification,  $\times 100$ . (E) Secondary spread assay evaluation of plasmids 3FLAG-UL92 or 3FLAG-rh127 to complement UL92-GalK-BAC in the presence or absence of UL93-HA. (F) 293T cell lysates at 48 h posttransfection with indicated plasmids were subjected to immunoblotting with anti-FLAG antibody. The antibody against  $\beta$ -actin was used as a loading control. (G and H) Fluorescent images of GFP-positive cells at 10 days posttransfection of 3FLAG-UL92-expressing HF cells with UL92<sub>1-28</sub>BAC (G) and UL92<sub>1-167</sub>BAC (H). Original magnification,  $\times 100$ . (I) Lysates from 3FLAG-UL92-expressing HF cells were subjected to immunoblotting with anti-FLAG antibody. The antibody against  $\beta$ -actin was used as a loading control. (J) Multistep growth curves (MOI of 0.01) of Towne-BAC and UL92 mutant viruses in HF cells. Virus replication was quantified for 15 days based on the GFP signal intensity, which is expressed from a cassette within the viral genome. (K) Single-step growth curves (MOI of 3) of Towne-BAC- and UL92 mutant BAC-derived viruses in HF cells. Virus titers were determined by plaque assays of supernatants collected at daily intervals. The data point at 0 dpi indicates the input virus dose. The detection limit of the plaque assay is indicated by a dashed line.

tant virus (53). In addition, the highly charged aa 168 to 201 C-terminal region is crucial for UL92 function.

**UL92 is expressed with complex early-late kinetics.** Although these results are consistent with UL92 encoding a protein, this gene product was not detected in a recent comprehensive translational profiling experiment (68). In order to analyze UL92 protein expression and localization, Towne-BAC was engineered to tag UL92 protein with three tandem FLAG epitope at the N terminus, based on the ability of this protein to fully complement mutant viruses when transduced into HF cells (Fig. 2G and H). C-terminal tagged UL92 was not useful in the context of the viral genome because this disrupted the N terminus of UL93 (Fig. 1B), which is



**FIG 3** UL92 expression. (A) Schematic diagram of the genome structure of 3FLAG-UL92-BAC generated from Towne-BAC. Three tandem FLAG epitope tag and translational stop-frameshift mutations were introduced at N terminus of UL92 and Asp98 in UL91, respectively. (B) Multistep growth curves (MOI of 0.01) of Towne-BAC and 3FLAG-UL92-BAC viruses in HF. Virus replication was quantified for 15 days based on the fluorescence intensity from virally encoded GFP. (C) Time course of 3FLAG-UL92 expression after 3FLAG-UL92-BAC virus infection of HF at an MOI of 3 in the absence (-) or presence of CHX (50  $\mu$ g/ml) or PFA (300  $\mu$ g/ml). Shown are the accumulated levels of 3FLAG-UL92, along with IE1, ppUL44, pp150, or  $\beta$ -actin, at the indicated times as determined by immunoblot analysis. (D) UL92 transcript levels during Towne-BAC infection of HF at an MOI of 3 in the presence or absence of PFA (300  $\mu$ g/ml). Total RNA was isolated at the indicated times postinfection, and the amount of UL92 transcript was quantified by qRT-PCR using primers specific to the UL92 genes and normalized to  $\beta$ -actin. The normalized amount of UL92 transcripts in the cells infected with Towne-BAC in the absence of PFA at 24 hpi was set to 1.

also an essential gene (50, 51). Guided by this information, we took advantage of observations showing that the region of UL91 downstream of aa 72 was completely dispensable for viral replication (53) and generated 3FLAG-UL92-BAC in a virus expressing UL91<sub>1-97</sub> (Fig. 3A). We introduced a translational stop frameshift within the Asp98 codon of UL91 to avoid translation of the dispensable region. This virus exhibited similar viral replication ki-

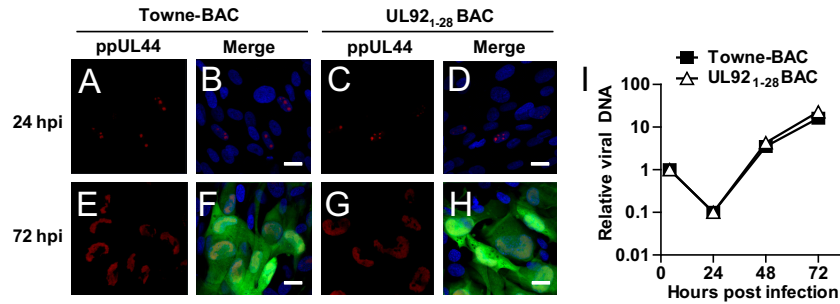
netics compared to Towne-BAC in HF over a 15-day time course under low-MOI conditions (Fig. 3B).

In order to assess UL92 protein accumulation during infection, we next subjected 3FLAG-UL92-BAC to an immunoblot analysis. When infected at an MOI of 3, 3FLAG-UL92 protein was detected in HF as early as 8 hpi and reached a steady state by 48 to 72 hpi (Fig. 3C). This tagged protein was detected in infected cells at expected size (~25 kDa), as predicted from the amino acid sequence, indicating that we had included a bona fide start codon, along with the epitope tag insertion. The immunoblot signal at 8 hpi was dependent on *de novo* translation and was not detected when cells were pretreated (1 h), infected (1 h), and maintained (8 h) in CHX (50  $\mu$ g/ml) (Fig. 3C). Signal was also absent in mock-infected cells. The accumulation of 3FLAG-UL92 protein was markedly decreased when infected cells were treated with 300  $\mu$ g/ml of PFA (Fig. 3C), an expression pattern consistent with either DE ( $\beta$ ) or leaky late ( $\gamma_1$ ) kinetics that is reminiscent of UL44 transcription where multiple promoters become activated throughout infection (33). Expression levels of representative IE (IE1), DE (UL44), and late (pp150) confirmed the desired impact of CHX and PFA treatment to the cells (Fig. 3C). To exclude the possibility that this pattern reflected the influence of the three tandem FLAG epitopes, HF were infected with Towne-BAC at an MOI of 3 and UL92 transcript levels were quantified during infection. The UL92 transcript followed a similar complex pattern, accumulating abundantly at late times of infection with levels significantly, but not completely, reduced by treatment with 300  $\mu$ g/ml of PFA (Fig. 3D). Thus, similar to the adjacent gene, UL91 (53), or to well-characterized UL44 (33), UL92 has a complex expression pattern that starts very early and continues to rise at late times in a pattern that is sensitive to inhibition of DNA synthesis.

**DNA replication compartment formation and viral DNA synthesis are independent of UL92.** In order to determine whether UL92 function is required for formation of DNA replication compartments (RCs), HF were infected with UL92<sub>1-28</sub> BAC or Towne-BAC at an MOI of 0.3. ppUL44, the virus-encoded DNA Pol proc factor, was used as a marker for RC formation. UL92<sub>1-28</sub> BAC infection resulted in formation of ppUL44-positive nuclear foci at 24 hpi that was comparable to Towne-BAC (Fig. 4A to D). The formation of RCs in UL92<sub>1-28</sub>BAC-infected cells was also indistinguishable from parental Towne-BAC at 72 hpi (Fig. 4E to H). A similar result was obtained when HF were infected with UL92<sub>1-167</sub>-BAC virus (data not shown), indicating that RCs form independently of UL92 function.

Next, in order to compare viral DNA synthesis, total DNA was purified at the indicated times postinfection from HF infected with UL92<sub>1-28</sub> BAC or Towne-BAC at an MOI of 0.3 and subjected to qPCR analysis (Fig. 4I). The accumulation of viral DNA in UL92<sub>1-28</sub>BAC-infected cells was indistinguishable from parental Towne-BAC. A similar result was obtained when HF were infected with UL92<sub>1-167</sub>BAC virus (data not shown). These results demonstrate that, similar to the impact of UL91, UL79, UL87, and UL95 (32, 53, 69), robust viral DNA synthesis does not require UL92 function.

**UL92 protein is recruited to the nucleus during infection and is associated with mature virus particles.** We first examined the intracellular localization of UL92 expressed alone in the absence of any other viral factors. Although no detectable FLAG staining occurred in nontransduced HF (Fig. 5A and B), 3FLAG-UL92



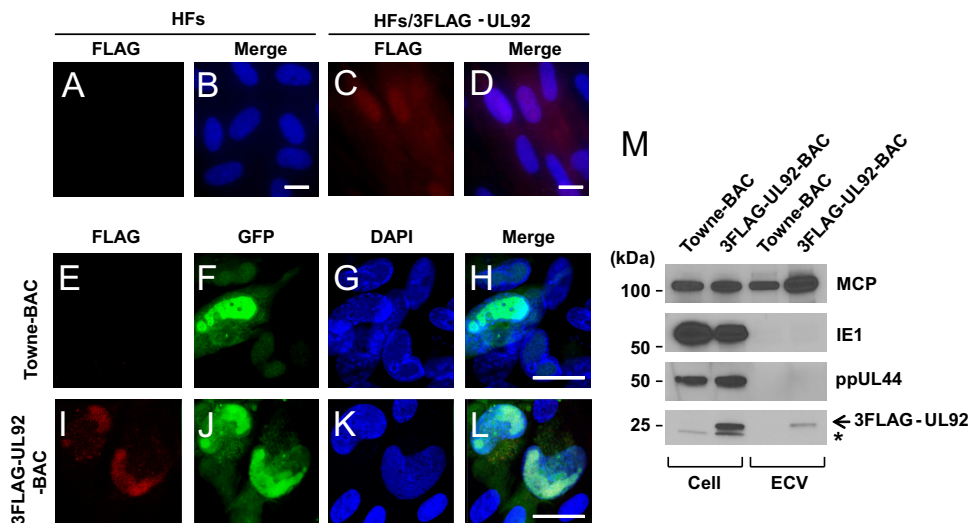
**FIG 4** UL92 function and viral DNA synthesis. The immunofluorescence of HF cells infected with Towne-BAC (A, B, E, and F) or UL92<sub>1-28</sub>-BAC (C, D, G, and H) viruses at an MOI of 0.3 was determined. Cells were fixed at 24 (A to D) or 72 (E to H) hpi for IFA. ppUL44 protein was visualized with mouse anti-ppUL44 monoclonal antibody and Alexa Fluor 568-conjugated anti-mouse IgG secondary antibody (red) (A, C, E, and G). The merged images (B, D, F, and H) include virus-encoded GFP fluorescence (green), DAPI (4',6'-diamidino-2-phenylindole) staining, which identifies nuclei (blue). Original magnification, ×630. Bars, 20 μm. (I) Viral DNA replication in HF cells infected with Towne-BAC and UL92<sub>1-28</sub>-BAC viruses at an MOI of 0.3. The accumulation of viral DNA at the indicated hpi was quantified by qPCR using primers specific for the IE1 gene and normalized to β-actin. The normalized amount of viral DNA in the cells infected with Towne-BAC at 4 hpi was set at 1.

showed a diffuse distribution in uninfected lentiviral vector-transduced HF cells (Fig. 5C and D). In order to define the cellular distribution of UL92 during infection, HF cells were infected with 3FLAG-UL92-BAC virus at an MOI of 3 and analyzed at 72 hpi by IFA with anti-FLAG antibody. Although FLAG staining was absent in HF cells infected with Towne-BAC (Fig. 5E to H), FLAG epitope staining was detected in kidney-shaped nuclei of all 3FLAG-UL92-BAC-infected cells (Fig. 5I to L) according to an intranuclear distribution pattern reminiscent of ppUL44 and RCs (see Fig. 4A and B). Therefore, UL92 is recruited to the nucleus during infection and into RCs as they form over the course of infection.

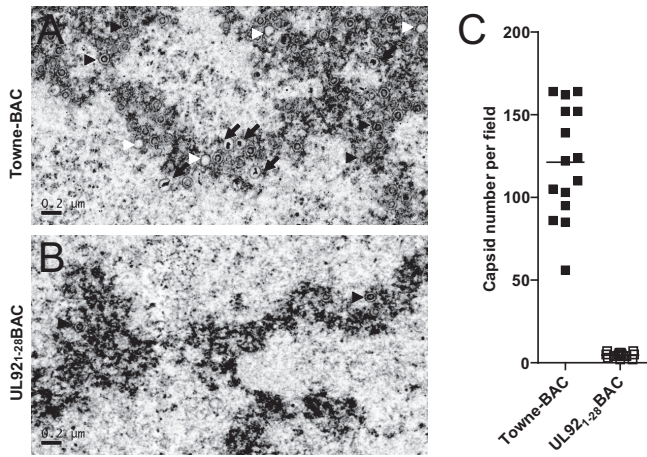
In order to determine whether UL92 associates with mature virions and dense bodies, extracellular particles were collected

from culture medium during Towne-BAC or 3FLAG-UL92-BAC infections by ultracentrifugation through a 30% sucrose cushion. These preparations were denatured, run on an SDS-polyacrylamide gel and immunoblotted to detect MCP, IE1, ppUL44, or FLAG epitope (3FLAG-UL92) (Fig. 5M). MCP, as expected, was readily detected in the sample of total cell and particle preparations. Particle preparations did not contain detectable nonstructural IE1 and ppUL44 antigens but did contain 3FLAG-UL92. Even though UL92 protein is difficult to detect immediately after infection (see Fig. 3C), it is present in extracellular virions and/or dense bodies. The levels of UL92 protein are low given the lack of detection in recent translational profiling of late-phase transcripts (68).

**UL92 has a crucial role for viral replication before capsid assembly.** In order to directly assess the step in viral replication



**FIG 5** UL92 protein is recruited to the nucleus during infection and is virion-associated. Immunofluorescence of HF cells (A and B) or 3FLAG-UL92-transduced HF cells (C and D) fixed and stained with mouse anti-FLAG monoclonal antibody and Alexa Fluor 568-conjugated anti-mouse IgG secondary antibody (red) (A and C). The merged images (B and D) include DAPI staining which identifies nuclei (blue). HF cells were infected with Towne-BAC (E to H) or 3FLAG-UL92-BAC (I to L) viruses at an MOI of 0.3. The cells were fixed at 72 hpi for IFA. 3FLAG-UL92 protein was visualized with mouse anti-FLAG monoclonal antibody and Alexa Fluor 568-conjugated anti-mouse IgG secondary antibody (red) (E and I). Shown are virally encoded GFP fluorescence (green) (F and J), DAPI staining which identifies nuclei (blue) (G and K) and the merged images (H and L). Original magnification, ×630. Bars, 20 μm. (M) UL92 protein is associated with viral particles. HF total cell lysate or culture supernatant was collected at 96 hpi from cells infected with Towne-BAC or 3FLAG-UL92-BAC (MOI of 3). Extracellular virus particles were collected by ultracentrifugation. All samples were subjected to SDS-PAGE, followed by immunoblotting with anti-MCP, anti-IE1/2, anti-ppUL44, or anti-FLAG antibodies. The arrow and asterisk indicate 3FLAG-UL92 proteins and nonspecific bands, respectively. ECV, extracellular viral particles.

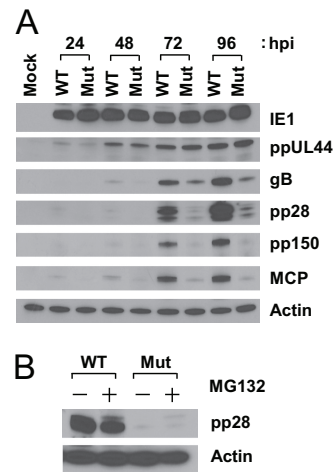


**FIG 6** TEM of Towne-BAC or UL92<sub>1-28</sub>BAC virus-infected cells. HF cells were infected with Towne-BAC (A) or UL92<sub>1-28</sub>BAC virus (B) at an MOI of 3 and fixed for TEM at 5 dpi. The white arrowheads point to A capsids, the black arrowheads point to B capsids, and the arrows point to C capsids. Scale bars are indicated. (C) Quantification of intranuclear capsids from electron micrographs. Five nuclei were randomly selected for each sample and three independent fields of image were counted from each nucleus at a magnification of  $\times 10,000$ .

where UL92 acts, we used TEM analysis of cells infected with UL92<sub>1-28</sub>BAC or Towne-BAC viruses. HF cells were infected with viruses at an MOI of 3, and successful infection was confirmed by GFP expression under fluorescence microscopy. Whereas abundant virus particles and dense bodies associated with a juxtanuclear cytoplasmic assembly compartment (AC) was observed in Towne-BAC-infected cells, there were no obvious cytoplasmic particles or organized AC in UL92<sub>1-28</sub>BAC virus-infected cells at 5 dpi (data not shown). In nuclei, all three types of capsid particles (A, lacking a scaffold or dense core; B, containing a scaffold; C, containing a dense core) (70) were detected in Towne-BAC-infected cells (Fig. 6A), but intranuclear capsid forms were difficult to find in UL92<sub>1-28</sub>BAC-infected cells. A few examples of capsid forms (A, B, and C) were identified after careful scanning of several nuclei (Fig. 6B and data not shown). Thus, UL92 is necessary for efficient capsid assembly in the nucleus.

Next, we quantified capsid forms in randomly selected nuclei. The mean numbers of intranuclear capsids per nuclear field in UL92<sub>1-28</sub>BAC-infected cells were much lower than in Towne-BAC-infected cells at 5 dpi (Fig. 6C). Cells infected with UL92<sub>1-167</sub>BAC virus showed the same reduced levels of nuclear capsids as UL92<sub>1-28</sub>BAC (data not shown). Together, these results indicate that the elimination of UL92 severely impacts an essential replication step after viral DNA replication but before capsid assembly during the viral replication cycle. Because HCMV DNA accumulated at normal levels here, as well as with other betagamma gene mutants (32, 53, 69), the accumulation and turnover of viral DNA appears to be completely independent of both capsid accumulation and the process of encapsidation of viral DNA. This surprising observation is supported by the UL91 mutant virus phenotype as well (53).

**UL92 is required for accumulation of late viral proteins.** To better identify the steps during infection where UL92 functions, we used immunoblot analysis to assess the accumulation of representative viral proteins: IE (IE1), DE (ppUL44), and late

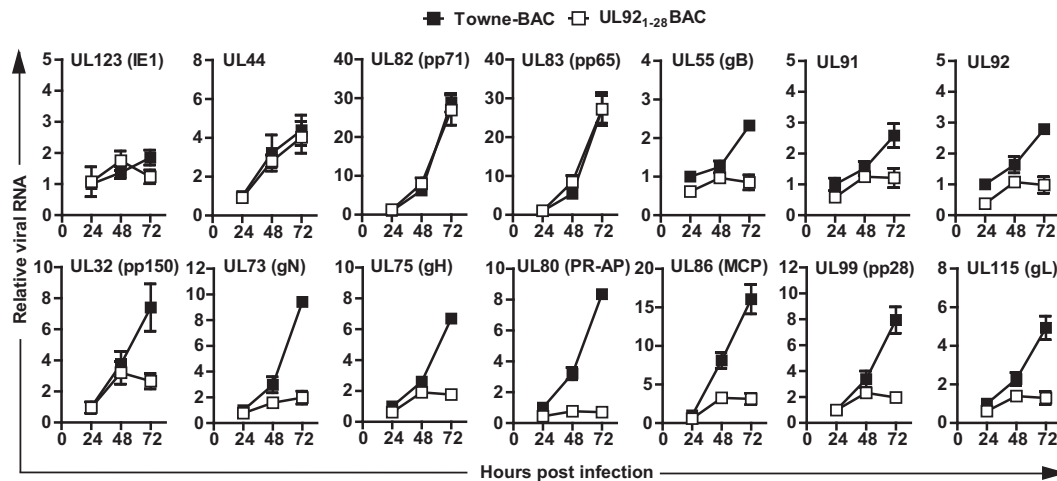


**FIG 7** Immunoblot analysis of viral proteins from HF cells infected with Towne-BAC or UL92<sub>1-28</sub>BAC virus. (A) HF cells were infected at an MOI of 3 and harvested at the indicated hpi. Shown are the accumulated levels of IE1, ppUL44, gB, pp28, pp150, MCP, or  $\beta$ -actin as determined by immunoblotting. (B) HF cells were infected as described above and maintained in the absence (-) or presence (+) of 20  $\mu$ M MG132 from 72 to 96 hpi. Cell lysates were prepared at 96 hpi and analyzed by immunoblotting with anti-pp28 antibody or with antibody against  $\beta$ -actin as a loading control. WT, Towne-BAC; Mut, UL92<sub>1-28</sub>BAC.

(gB, pp28, pp150, and MCP). When HF cells were infected with UL92<sub>1-28</sub>BAC at an MOI of 3, the accumulation of pp28, pp150, and MCP were markedly reduced at 72 and 96 hpi compared to cell infected control Towne-BAC, whereas both IE1 and ppUL44 levels were comparable, and gB was modestly decreased in the cells infected with the mutant virus (Fig. 7A). Thus, UL92 is required for the accumulation of true late ( $\gamma_2$ ) proteins (pp28 and pp150) and also influences the levels of other late proteins (MCP and, possibly, gB) in a pattern reminiscent of UL91 mutants, as well as other betagamma gene mutants (32, 53, 69).

Next, to test the possibility that UL92 has a role in reducing the degradation of viral late proteins, we assessed the impact of proteasome inhibitor on accumulation of pp28 protein, as a representative of late protein, in UL92<sub>1-28</sub>BAC-infected cells. When infected cells are treated with 20  $\mu$ M MG132 from 72 to 96 hpi, the accumulation of pp28 was not restored (Fig. 7B), suggesting that UL92 acts on the accumulation of late proteins independent of the proteasome.

**UL92 is required for the efficient transcription of viral late genes.** In order to further define UL92 impacts on viral late gene transcript levels, we analyzed the accumulation of viral RNA for 14 characterized genes. Total RNA was extracted from HF cells infected with Towne-BAC or UL92<sub>1-28</sub>BAC viruses at an MOI of 3 and assessed by qRT-PCR at 24, 48, and 72 hpi (Fig. 8). Whereas IE1 (IE or  $\alpha$ ) or UL44 (DE or  $\beta$ ) transcripts accumulated at comparable levels during mutant and control virus infection, gB (DE or  $\beta$ ) levels were modestly decreased in UL92-null virus, a finding consistent with the impact on protein levels. The levels of leaky-late ( $\gamma_1$ ) transcripts, UL82 (pp71) and UL83 (pp65), were unchanged; however, UL91 and UL92 transcripts were reduced at 72 hpi when UL92 was abrogated, indicating that the transcription of both UL92 and the adjacent UL91 genes are upregulated by UL92 function. The accumulations of the true-late ( $\gamma_2$ ) transcripts UL32 (pp150), UL75 (gH), UL115 (gL) and UL99 (pp28) and the



**FIG 8** UL92 is required for the accumulation of viral late transcripts. HFs were infected with both UL92<sub>1-28</sub>-BAC and parental Towne-BAC viruses at an MOI of 3, total RNA was isolated at the indicated times postinfection, and viral RNA levels were quantified by qRT-PCR using primers specific to the indicated viral genes and normalized to  $\beta$ -actin. The normalized amount of viral transcripts in the cells infected with Towne-BAC at 24 hpi was set at 1.

late ( $\gamma$ ) transcripts UL73 (gN), UL80 (PR-AP), and UL86 (MCP) were all substantially reduced in the absence of UL92. Taken together, our results demonstrate that UL92 modestly impacts leaky late ( $\gamma_1$ ) genes but is absolutely crucial for efficient expression of true-late ( $\gamma_2$ ) viral genes, following a common pattern that seems to define the set of five betagamma genes (32, 53, 69).

## DISCUSSION

In this study, we have dissected the essential functions of UL92 and shown that the 201-aa nuclear UL92 protein provides crucial late gene regulation during HCMV infection. UL92 is expressed with complex kinetics and is recruited into RCs at late times of infection. Without UL92, viral replication is blocked by the failure to accumulate late viral transcripts even though the formation of RCs and viral DNA synthesis proceed normally. UL92 is absolutely crucial for the accumulation of the true late ( $\gamma_2$ ) class of viral genes by supporting the efficient transcription rather than blocking protein degradation. As a consequence of low levels of late structural proteins, the production of viral capsids fails in UL92 mutant virus-infected cells. Given the replication defect of UL92<sub>1-167</sub>-BAC mutant virus, the highly charged aa 168 to 201 C-terminal region is crucial for function. Expression of the RhCMV homolog restored the replication defect of UL92-null virus, suggesting that other CMV homologs are likely to have similar functions. These observations place UL92 with other HCMV betagamma genes that have been characterized (32, 53, 69) and in line with hallmark work in gammaherpesviruses (44–49). The phenotype of HCMV UL92 mutants is reminiscent of the  $\gamma$ MHV-68 ORF31 mutant (45), a sequence homolog of this gene. From this and previous studies on UL79, UL87, UL91, and UL95 (32, 53, 69), there appears to be a common theme across the betagamma genes in activation of true late ( $\gamma_2$ ) gene transcription.

UL92 protein lacks an obvious nuclear localization signal and shows a diffuse distribution in uninfected HFs. This protein is specifically localized to the RC-like pattern in the nucleus at a late time of infection, suggesting that UL92 interacts with other RC gene products, potentially UL79 (32) or UL44 (69). The localization we observed for UL92 with the RC-like region in infected cells

is similar to other betagamma late gene regulators, UL79, UL87, UL91, and UL95 (32, 53, 69), suggesting that all of these may assemble and function as a complex. Despite this pattern, viral DNA synthesis proceeds normally in the absence of UL92 or the other late gene regulators (32, 53, 69). Given that sufficient accumulation of viral late proteins is required for capsid formation, viral DNA synthesis and capsid assembly are completely uncoupled, reinforcing the observation made with UL91-null virus (53). On one hand, the initiation of viral late gene expression by viral and/or host factors remains largely unexplored. A reporter plasmid driven by the previously characterized (38) UL99 late promoter (positions  $-40$  to  $+106$ ) was not activated by transient expression of UL79, UL87, UL91, UL92, and/or UL95 (data not shown), suggesting that viral late gene expression requires additional viral factors or processes that accompany infection, such as oriLyt-dependent DNA synthesis. It is possible that HCMV UL79, UL87, and UL95 control of late transcription in a complex guided via the viral DNA Pol proc factor, ppUL44 (52), although there is currently no direct evidence that any of the betagamma gene products interact with ppUL44. In gammaherpesviruses, EBV BcRF1, the homolog of HCMV UL87 and  $\gamma$ MHV-68 ORF24, acts as a TATA-binding protein, interacting preferentially with TATT to regulate late gene expression during infection (48, 49). In addition,  $\gamma$ MHV-68 ORF30 and ORF34, homologs of HCMV UL91 and UL95, regulate the binding of RNA Pol II to the late gene promoters (47). This evidence all points to a direct contribution of betagamma late gene regulators in the assembly of a late gene RNA Pol II transcriptional complex. Further studies on the interaction partners of UL92 and the regulation of UL87 since a TBP will be important to begin to dissect the mechanism of HCMV late transcriptional regulation.

The body of evidence that both HCMV UL91 (53) and UL92 control viral late transcription broadens the relevance of this process in betaherpesviruses and, separately, raises the question of how the alphaherpesviruses regulate late gene expression can be effectively achieved without homologs of these proteins. Both UL91 and UL92 are essential for the expression of the same set of true late ( $\gamma_2$ ) genes UL32 (pp150), UL73 (gN), UL75 (gH), UL80



(PR-AP), UL86 (MCP) UL99 (pp28), and UL115 (gL), as well as leaky late ( $\gamma_1$ ) expression of UL55 (gB), in addition to UL91 and UL92 themselves. It seems likely all of these act via UL87, given its relationship to gammaherpesvirus late TBP with specificity for TATT (48, 49), the sequence that also characterizes HCMV true late promoters (27, 29, 33, 37, 39, 41). In HSV-1, the late gene expression is also controlled by a small, TATA box-proximal region: however, regulation does not involve novel TATT sequences, at least based on the characterized  $\gamma_2$  gC promoter (40). Even though there are some common features, alphaherpesviruses manage without a set of regulatory genes that are conserved in beta- and gammaherpesviruses. These viruses appear to have evolved a common late gene expression strategy. The accumulating evidence for a specific complex that coordinates host RNA Pol II in beta- and gammaherpesviruses brings to mind the precision with which bacteriophage T4 recognizes simple late transcriptional promoters with small proteins that link RNA Pol to the DNA Pol proc factor as a sliding clamp to provide access to promoters as viral DNA synthesis proceeds (54). It seems tempting to speculate that betagamma genes function in a related way, as a single complex or as alternative complexes to modulate RNA Pol II recognition of HCMV late promoters to assure appropriate levels of late transcripts.

This study advances the understanding of mechanism of viral late gene transcription, completing the set of genes influencing late gene activation in beta- and gammaherpesviruses. HCMV UL92 is required for viral late gene transcription at the RC-like region in the nucleus and thus, consequently, essential for viral replication, like the other betagamma genes, UL79, UL87, UL91, and UL95. How these viral late gene transactivators interact with viral and/or host factors and its relationship to core components of RNA Pol II transcriptional machinery, as well as viral DNA synthesis, will be the subject of future investigation.

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