Cytomegalovirus UL91 Is Essential for Transcription of Viral True Late (γ2) Genes

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Human cytomegalovirus-encoded UL91 is a betagamma gene that is essential for viral replication. Here we show that the 111-amino-acid (aa) UL91 protein controls accumulation of true-late (γ2) viral transcripts. The primate betaherpesvirus conserved N-terminal region from aa 1 to 71 is sufficient to fully reconstitute function. Evaluation of viral DNA, RNA, and antigen revealed that UL91 protein is expressed with leaky-late (γ1) kinetics, localizes in the nucleus without influencing viral DNA synthesis, and must be present from 48 h postinfection to support full expression of late viral transcripts and proteins. In the absence of UL91, viral capsid assembly in the nucleus of infected cells is significantly reduced, and mature, cytoplasmic virions fail to form. Taken together, the evidence shows that UL91 regulates late viral gene expression by a mechanism that is apparently conserved in betaherpesviruses and gammaherpesviruses.
transcription by influencing RNA Pol II. Indeed, a specific late transcription complex has been described for HCMV (53) that is composed of UL79 (36), TBP-like UL87 (49), and UL95 proteins and associates with the IE2-p86/ppUL84/ppUL144/UL112-113 protein complex, which is known to control initiation of DNA synthesis (53).

Here, we have dissected the function of the HCMV UL91 gene, an essential gene (51, 52) with homologs in all characterized HCMV strains as well as in both chimpanzee CMV (54, 55) and rhesus macaque CMV (RhCMV) (56, 57). Even though sequence homologs are retained in primate CMVs, only the genome position adjacent to UL92 is retained in rodent CMV (58, 59) and gammaherpesviruses. We show that the N-terminal 71 amino acids (aa) of full-length UL91 suffice for transcriptional activation of true-late genes within the nucleus of infected cells. This characterization adds to the list of betagamma genes that may specifically modify RNA Pol II to transcribe late genes.

**MATERIALS AND METHODS**

**Plasmid construction.** For construction of a UL91-3myc-expressing plasmid, the full-length UL91 ORF was amplified by PCR from the HCMV Towne-BAC genome (GenBank accession number AY315197) (60) and cloned in frame between BglII and Xhol sites of pON2780 (61), resulting in a three tandem c-myc epitope (3myc) tag at the C terminus of UL91 to produce pLNXC-UL91-3myc. To make a UL91-3myc-expressing lentiviral vector, the UL91-3myc ORF was inserted between XbaI and BamHI sites of the pLV-EF1α-MCS-IRES-Puro lentiviral vector (Biolent, Carlsbad, CA) containing 4.5 g/ml glucose, 10% fetal bovine serum (SH1245OH; Atlanta Biologicals, Lawrenceville, GA), 1 mM sodium pyruvate, 2 mM l-glutamine, and 100 U/ml penicillin-streptomycin (Cellgro, Manassas, VA) at 37°C with 5% CO2. HFs between passages 8 and 15 were used in all experiments. For establishment of UL91-3myc-expressing HFs, a lentivirus stock was produced by cotransfection of pLV-UL91-3myc, pSAX2 (64), and plasmid expressing the G protein of vesicular stomatitis virus with 293T cells by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Low-passage HFs were transduced with lentiviral vector and selected in 0.5 μg/ml of puromycin (Invitrogen, Carlsbad, CA).

**BAC mutagenesis and recombinant viruses.** Bacterial artificial chromosomes (BACs) containing the HCMV genome were constructed in SW102 *Escherichia coli* by linear recombination in a two-step selection protocol (65). The first step in generating UL91 mutant BACs was recombination between the parental Towne-BAC DNA (60) and a linear PCR fragment containing a Galk cassette flanked by 50-500 bp UL91 homology arms. The double-stranded linear fragment was obtained by PCR using the pGalk plasmid (65) as a template and the oligonucleotide primers 5'-GGCGGCCCATAAIAAGCAAGTGTGCCTGCGGCAGGCCAGCAGGCCACGCGCCGcctgtagaattactagcatacgca-3' and 5'-AACGCGTTCTGACGTGGCACACACGCGCCATGACGCTGACG-3', with nucleotides homologous to Towne-BAC shown by uppercase letters and the nucleotides in the 3myc tag shown in lowercase letters. To make UL91-mCherry-BAC, mCherry DNA was PCR amplified from pTREG3 (Clontech, Palo Alto, CA) and used to replace the 3myc tag in pLNXC-UL91-1,71-3myc (pLNXC-UL91-1,71-mCherry). The double-stranded linear fragment for recombineering was obtained by PCR using pLNXC-UL91-1,71-mCherry as a template and the primers UL91-1,50-5-F and 5'-AACGCGTTCTGACGTGGCACACACGCGCCATGACGCTGACG-3', with nucleotides homologous to Towne-BAC shown in uppercase letters and the nucleotides in mCherry shown in lowercase letters. For construction of UL91-1,50-5-BAC, the FK506 binding protein destabilization domain (FKBP-DD) was amplified by PCR with pBMN-HA-YFP-FKBP(L106P)-IRES-HcRed-tan-mCherry shown in lowercase letters. For construction of UL91-1,50-5-DDBAC, the region from aa 1 to 71 of UL91 was amplified by PCR from pTRE3G (Clontech, Palo Alto, CA) and used to replace the 3myc tag in pLNXC-UL91-1,71-3myc (pLNXC-UL91-1,71-DD). The double-stranded linear fragment for recombineering was obtained by PCR using pLNXC-UL91-1,71-DD as a template and the primers UL91-1,50-5-F and 5'-AACGCGTTCTGACGTGGCACACACGCGCCATGACGCTGACG-3', with nucleotides homologous to Towne-BAC shown in uppercase letters and the nucleotides in the FKBP-DD shown in lowercase letters. All generated BACs were verified by restriction enzyme digestion, PCR analysis, and DNA sequencing. Recombinant viruses were generated by transfecting BAC DNA and pp71 expression plasmid (66, 67) into HFs by lipofection with calcium phosphate (61). UL91-3myc-expressing HFs were used for propagation of UL91-1,4-BAC virus. For recovery of UL91-1,50-5-BAC virus, transfected cells were cultured in the presence of 1 μM Shield-1. Infectious titers of all recombinant viruses were determined by plaque assay on HFs or UL91-3myc-expressing HFs. For purposes of imaging and determination of fluorescence intensities, live cultures were analyzed with the live cell imaging system IncuCyte Zoom (Essen Bioscience, Ann Arbor, MI) (68).

**Secondary spread assay.** As described previously (61), HFs were transfected with UL91-1,4-BAC or UL91-1,50-5-BAC, the pp71 expression plasmid, and one of the pLNXC constructs. Medium including 0.1% human gamma globulin (Carimune; CSL Behring, King of Prussia, PA) was changed every other day until day 10 posttransfection, when cells were
were analyzed at a magnification of 25,000, randomly selected for each sample, and three independent image fields were embedded in epoxy resin. Thin sections were counterstained with a cacodylate buffer, postfixed with the same buffer with 1% osmium tetraoxide.

Fluorescence microscopy equipped with ZEN 2009 acquisition and analysis software (Carl Zeiss, Thornwood, NY) for transmission electron microscopy (TEM). HFs infected at an MOI of 3. Total RNA was isolated at the indicated time points with the RNeasy minikit (Qiagen, Valencia, CA) for DNA and RNA analyses. Accumulation of intracellular viral DNA was measured by quantitative PCR as previously described (71), with a slight modification. HFs were infected at an MOI of 0.3, and cells were collected at the indicated time points. The infected cells, in lysis buffer (20 mM Tris-HCl [pH 8.0], 200 mM NaCl, 50 mM EDTA, 1% SDS, 0.2 mg/ml protease K), were incubated at 55°C overnight. DNA was extracted with phenol-chloroform, followed by RNase A treatment, and resuspended in nuclease-free water. Viral DNA was quantified by qPCR directed at the IE1 exon 2 region. The accumulation of viral DNA was normalized by dividing the number of IE1 gene equivalents by the number of β-actin gene equivalents. The amount of viral DNA at 4 hpi was set at 1. Viral RNA was analyzed by reverse transcription coupled to qPCR (qRT-PCR). HFs were infected at an MOI of 3. Total RNA was isolated at the indicated time points with the RNeasy minikit (Qiagen, Valencia, CA) followed by Dnase I (Invitrogen, Carlsbad, CA) to treat to remove genomic DNA contamination. Viral RNA was reverse transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) and cDNA was quantified using qPCR and Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) in a 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA). The RNA samples that did not undergo a reverse transcription reaction were also amplified by qPCR to confirm there was no genomic DNA contamination. The following primers were used for qPCR and qRT-PCR (36, 72, 73): IE1, 5'-GGGGCAAGAGATCCCTCCA-AAA-3' and 5'-TGGTTGCAATCTCCCGG-3'; IE2, 5'-TTACACGGTGTGTCGTCG-3' and 5'-GGCCGAAAACATCGTATCAAC-3'; UL53, 5'-TCACTATGGTTGTTAAAGAGT-3' and 5'-ACCCGACTCAGGATGTCG-3'; UL75, 5'-CAGCGGCTCCACGCTCT-3' and 5'-CCGGGTTTGTACTGCTGT-3'; UL79, 5'-TACGCGGACCGCAGACGATC-3' and 5'-CGGGGAAACACCGTATCGGAGA-3'; UL80, 5'-GACGACAAACAGCGCGAAGA-3' and 5'-CTGGGAAAGACGCAAGAGT-3'; UL83, 5'-GGGACACACCCCTATCGGAGAGA-3' and 5'-CTGGAAGGAGACCAGCAGAT-3'; UL92, 5'-ACCAGCGGATCTGCGAATC-3' and 5'-CCGGCGTTTCTGGCTCGTGG-3'; UL99, 5'-GGTTCCTGCGACGCGATC-3' and 5'-CGGGCGTTTCTGGCTCGTGG-3'; UL115, 5'-AGGATGTCGTTAAGTCCGACA-3' and 5'-ACGGCCGACGTTAACGTTAAGG-3'; and 5'-CTGGCGTTGCTGCGAATC-3' and 5'-GCTGGAAAACTTCCACGCT-3'. 

RESULTS

Generation of UL91 mutant BACs. In order to assess the role of UL91 in HCMV replication, we constructed a series of UL91 mutants in Towne-BAC without impacting the expression or function of adjacent genes. Insertion and deletion mutagenesis has indicated that the highly conserved UL91 gene is essential for replication in both the AD169 and Towne strains of HCMV (51, 52). Alignment of amino acid sequences of UL91 homologs from pri-
respectively) (Fig. 1A and C), as described in Materials and Methods. These mutant BACs were designed to disrupt UL91 near the predicted N terminus (UL91-6BAC), as well as to truncate the poorly conserved C-terminal region (UL91-71BAC) and eliminate only the last 14 aa, including the region of overlap with UL92 (UL91-97BAC). Furthermore, UL91-6BAC is specific for UL91 and did not disrupt any of the predicted spliced gene products arising from the super splice acceptor (74), whereas both UL91-71BAC and UL91-97BAC disrupted predicted spliced gene products. ORFL218C, a recently identified translational product from the opposite DNA strand in the UL91 region (75), remained intact in all of these viruses. Restriction enzyme digestion was employed to ensure that the rescued and mutant BACs had the expected changes and did not exhibit any gross DNA rearrangements (Fig. 1D). In addition, DNA sequence analysis revealed that unwarranted mutations had not been incorporated into UL91 or the region flanking this gene.

UL91 is essential, but its C-terminal region is dispensable for HCMV replication. We transfected each of the UL91 mutant BACs into HFs to try to rescue virus without complementation. Fluorescence microscopy was used to identify transfected cells by GFP expression from a cassette in the parental Towne-BAC viral genome (60). As shown in Fig. 2A to F, Towne-BAC,

**FIG 1** Generation of UL91 mutant BACs. (A) Amino acid sequence alignment of UL91 (GenBank accession number AY315197) and homologs from RhCMV (NC_006150), CCMV (NC_003521), HHV6 (NC_001664), and HHV7 (NC_001716). Identical amino acids (in black) and CMV conserved amino acids (in gray) are highlighted. The black arrowheads indicate the positions of the amino acids where individual translational stop-frame shift mutations were introduced. (B) DNA sequence surrounding the UL92 ATG with the predicted amino acid sequence of the overlapping UL91 region shown above and UL92 shown below. (C) Schematic representation of the UL89.2 to UL89.1 region of Towne-BAC where UL91 mutants were introduced (expanded region). (D) Restriction enzyme digestion analysis for diagnosis of indicated UL91 mutant BACs. The BAC DNAs were digested with the restriction enzyme BglII and separated by 0.8% agarose gel electrophoresis. Insertion of the GalK cassette resulted in an approximately 0.9-kb increase in the size of the BglII fragment (white arrowhead) compared to 2.6-kb band (black arrowhead) in Towne-BAC and UL91 mutant BACs.
UL91-l71BAC, UL91-97BAC, and UL91-R-BAC all replicated, with obvious virus spread evident by day 10 posttransfection, whereas UL91-GalK-BAC and UL91-l73BAC produced only single GFP-positive cells, indicating a complete replication defect. Replication was reconstituted when these constructs were complemented by cotransfection with a c-myc epitope-tagged UL91 (UL91-3myc) expression plasmid (Fig. 2G and H). RhCMV rh126 also complemented the defect (Fig. 2I), demonstrating functional conservation of this gene in another primate CMV. These results reinforce earlier studies (51,52) and further demonstrate that the UL91 region with highest sequence similarity among related viruses, aa 1 to 71, was absolutely essential for replication. Furthermore, the phenotype of UL91-3myc demonstrated a crucial role for the conserved amino terminus of UL91. When taken together with the UL91-l71BAC and UL91-97BAC abilities to fully replicate, UL91 function appeared independent of fusion proteins that may result from the RNA super splice acceptor, located between aa 37 and 38. Finally, the novel ORFL218C did not contribute to replication properties assigned to UL91.

Next, we established UL91-transduced HFs by using a lentivirus expressing UL91-3myc from the EF-1α promoter in order to have complementing cells to propagate UL91-6BAC virus. Expression of UL91-3myc in the transduced HFs was confirmed by immunoblotting with anti-myc antibody (data not shown). Consistent with the transient complementation, UL91-3myc-expressing HFs supported the replication of UL91-6BAC (data not shown), producing titers of mutant virus nearly identical to parental Towne-BAC. These cells supported the production of mutant virus stocks to facilitate the investigation of UL91 function during infection.

To characterize the growth defect of UL91-6BAC virus further and compare it to replication-competent UL91 mutant BACs (UL91-l71BAC and UL91-97BAC), HFs were infected with these mutant viruses or controls (Towne-BAC and UL91-R-BAC) at an MOI of 0.01, and virus replication was quantified by means of GFP signal intensity determined via IncuCyte live cell imaging. (K) Single-step growth curves of Towne-BAC and UL91 mutant BAC-derived viruses. HFs were infected with the indicated viruses at an MOI of 3, and viral titers were determined for culture supernatants by plaque assay at the indicated time points. The data point at 0 dpi indicates the input virus dose. The detection limit of the plaque assay is indicated by a dashed line.

**FIG 2** Replication properties of UL91 mutant BAC-derived viruses. (A to H) Typical fluorescent images of GFP-positive cells at 10 days posttransfection of HFs with Towne-BAC (A), UL91-GalK-BAC (B), UL91-l73BAC (C), UL91-97BAC (D), UL91-3myc (E), and UL91-R-BAC (F). The images from the secondary spread assay by cotransfection of UL91-GalK-BAC (G) or UL91-3myc (H) with the UL91-3myc expression plasmid are also shown. Original magnification, ×100. (I) Complementation of UL91-GalK-BAC by UL91-3myc or rh126-3myc in the secondary spread assay with HFs (upper panel). 293T cell lysates at 48 h posttransfection with the indicated plasmids were subjected to immunoblotting with anti-myc antibody (lower panel). The arrow and asterisk indicate myc-tagged proteins and nonspecific bands (for loading control), respectively. (J) Multistep growth curves of Towne-BAC and UL91 mutant BAC-derived viruses. HFs were infected with the indicated viruses at an MOI of 0.01, and virus replication was quantified for 15 days by means of GFP signal intensity using the IncuCyte live cell imaging system. All Towne-BAC derivatives constitutively expressed the same fluorescent marker cassette, facilitating direct comparisons. UL91-6BAC virus replication was not detected, whereas UL91-l71BAC, UL91-97BAC, and UL91-R-BAC viruses exhibited viral replication kinetics that paralleled parental Towne-BAC (Fig. 2J). As expected, only single GFP-positive cells formed following infection with UL91-6BAC, and there was no subsequent spread even when examined at 15 days postinfection (dpi), indicating this mutant virus was incapable of replication. Next, UL91-6BAC virus was evaluated under synchronous infection conditions (Fig. 2K). Following infection at an MOI of 3, mutant virus did not produce any detectable progeny under conditions where control viruses replicated well, producing progeny by 3 dpi and exceeding 10^6 PFU/ml in the culture medium by 6 dpi. These results demonstrated that uniform, high-MOI infection does not overcome the replication defect, and they reinforced the requirement for the N-terminal region from aa 1 to 71 in viral replication.

**UL91 is expressed with leaky-late kinetics.** Although UL91 is the most abundantly expressed of the five betagamma homologs that have been implicated in γMHV-68 late gene expression (75), UL91 is translated at very low levels during infection compared to other late proteins. In order to facilitate studies on UL91 protein...
expression and localization, we constructed a recombinant virus with c-myc-tagged UL91 protein, based on the ability of this protein to fully complement mutant viruses when transduced into HFs (Fig. 2F).

The C-terminal tag used to produce full-length UL91-3myc-complementing cells was not useful in the context of the viral genome, because this modification interrupts the N terminus of UL92 (Fig. 1B), which is also essential (51, 52). An N-terminal myc-tagged UL91 was prepared in a retroviral expression vector, but it did not produce detectable protein in transduced HFs (data not shown). To solve this problem, we took advantage of observations showing that the region of UL91 downstream of aa 72 was completely dispensable for viral replication and generated UL91-3myc-BAC by replacing aa 72 to 101 of UL91 with three tandem c-myc epitopes (Fig. 3A). This virus replicated as well as Towne-BAC in HFs followed over 15 days (Fig. 3B), similar to UL91-3myc-BAC described above. Thus, UL91-3myc-BAC exhibited viral replication kinetics indistinguishable from parental Towne-BAC. Next, we subjected UL91-3myc-BAC to an immunoblot analysis to assess the time course of UL91 accumulation. When HFs were infected with UL91-3myc-BAC virus at an MOI of 3, UL91-myc protein was detected as early as 24 hpi and reached a steady state by 48 hpi at an apparent size (~13 kDa) predicted from the sequence. UL91-myc expression was sustained at least until 120 hpi (data not shown), indicating that expression starts early and continues through the late phase of infection. No other species of myc-tagged viral proteins was observed, suggesting the translation products from the RNA splice acceptor do not accumulate to substantive levels. UL91-myc levels were markedly decreased when the viral DNA synthesis inhibitor PFA was included at 300 μg/ml through 72 hpi (Fig. 3C), an expression pattern consistent with leaky-late kinetics.

In order to determine whether UL91 contributes to viral DNA synthesis, HFs were infected with UL911-6 BAC or Towne-BAC at an MOI of 0.3, and total DNA was purified at the indicated times postinfection and subjected to qPCR analysis (Fig. 3D). The accumulation of viral DNA in UL911-6 BAC-infected cells was indistinguishable from parental Towne-BAC. Treatment with 300 μg/ml of PFA resulted in the expected reduction of the viral DNA levels. These data demonstrate that viral DNA synthesis proceeds completely independent of UL91.

UL91 localizes in the nucleus in infected cells. To investigate the localization of UL91 during infection, HFs were infected with UL91-3myc-BAC virus at an MOI of 0.3 and analyzed at 72 hpi by IFA with anti-myc antibody. When either 3.7% or 2% formaldehyde was used as a fixative, the signal from myc-positive cells was difficult to detect (data not shown). When the general GFP signal was eliminated by fixing cells with methanol-acetone (1:1), myc epitope staining was detected in kidney-shaped nuclei of all UL91-3myc-BAC-infected cells (Fig. 4A to D), and occasional cells also showed cytoplasmic staining (Fig. 4C). Given the known low levels to which natural UL91 is expressed in infected cells (75), a complementary approach with UL91-mCherry-BAC was undertaken (Fig. 4E). UL91-mCherry was functional and supported viral replication (Fig. 4F), and it revealed a uniform UL91 nuclear staining pattern (Fig. 4G to L) without any sign of cytoplasmic staining. Both UL91-3myc (Fig. 4M) and mCherry-tagged UL91 (data not shown) were produced at the expected size when separated on denaturing gels and evaluated by immunoblotting. We confirmed the IFA analysis by fractionating UL91-3myc-BAC-infected cells. UL91-3myc protein was detected as a dual band in the cytoplasm, peaking at 48 hpi, whereas nuclear UL91-3myc corresponded to the slower migrating of the two species and continued to increase at late times (48 and 72 hpi). The nuclear localization pattern was only observed in virus-infected cells; UL91-3myc showed a diffuse distribution in uninfected lentiviral vectortransduced HFs (data not shown). Taken together, these data demonstrate that the fully functional N-terminal portion between
aa 1 and 71 of UL91 is recruited from the cytoplasm starting between 24 and 48 hpi and associates with a replication compartment-like nuclear location at late times of infection.

**UL91 function is required late during infection.** In order to determine the point in viral replication where UL91 function is needed, we constructed a conditional virus mutant in which aa 72 to 101 of UL91 was substituted with FKBP-DD, and this mutant was designated UL91-DD-BAC (Fig. 5A). Based on available information (76), the UL91-DD fusion protein should rapidly degrade and produce a null condition in the absence of the stabilizing ligand, Shield-1, but continue to be expressed and functional in the presence of this ligand (70, 77). Thus, UL91-DD-BAC facilitated conditional expression of UL91 during infection.

On one hand, transfection of the UL91-DD-BAC into HFs in the absence of Shield-1 resulted in single GFP-positive cells at 10 days posttransfection (Fig. 5B), consistent with a full replication defect, similar to UL91 null virus. On the other hand, the growth defect of UL91-DD-BAC virus was restored in the presence of Shield-1 (1 μM) to levels that paralleled parental Towne-BAC (Fig. 5C). These data showed that DD-regulated UL91 functions in a Shield-1-dependent manner.

**FIG 4** The UL91 protein localizes in the nucleus during infection. (A to D) HFs were infected with Towne-BAC (A and B) or UL91-3myc-BAC (C and D) viruses at an MOI of 0.3. The cells were fixed with methanol-acetone at 72 hpi and examined by IFA. UL91-3myc protein was visualized with mouse anti-myc 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). Original magnification, ×630. Bars, 20 μm. (E) Schematic representation of the genome structure of UL91-mCherry-BAC, constructed from Towne-BAC. (F) Growth curves of Towne-BAC and UL91-mCherry-BAC viruses. HFs were infected with the indicated viruses at an MOI of 0.3, virus supernatants were collected at the indicated time points, and viral titers were determined by plaque assay. The data point at 0 dpi indicates the input virus dose. The detection limit of the plaque assay is indicated by a dashed line. (G to L) HFs were infected with Towne-BAC (G to I) or UL91-mCherry-BAC (J to L) viruses at an MOI of 0.3 and fixed with 3.7% formaldehyde at 72 hpi. The fluorescent signals from mCherry (G and J) and nuclear staining with DAPI (H and K) are shown. The merged images (I and L) include virally encoded GFP fluorescence, which identified the infected cells (green). Original magnification, ×1,000. Bars, 20 μm. (M) Immunoblot of cytoplasmic (Cyt) and nuclear (Nuc) fractions from HFs infected with UL91-3myc-BAC virus immunoblotted with anti-myc antibody for the detection of UL91-3myc protein at the indicated times. The arrow and asterisks indicate UL91-3myc species and nonspecific bands, respectively. Antibodies against β-actin and histone H1 were used to demonstrate the purity of cytoplasmic and nuclear fractions, respectively.
1-dependent manner, establishing conditional expression of this essential late gene product.

Next, UL91-DD-BAC virus was propagated in HFs in the presence of Shield-1 to compare growth properties with control virus. Supernatant virus was collected by centrifugation and then resuspended in fresh medium to minimize the impact of free Shield-1 in the inoculum. To assess the Shield-1 dependency of UL91-DD-BAC replication, virus-infected HFs (MOI, 0.01) were carried in the presence or absence of stabilizing ligand, and viral replication was monitored over 15 days by means of GFP signal intensity by IncuCyte imaging (Fig. 5D). UL91-DD-BAC replication in the presence of Shield-1 was similar to parental Towne-BAC, whereas, viral replication was not detected at all in the absence of Shield-1. Infection with UL91-DD-BAC virus in the absence of Shield-1 resulted in single GFP-positive cells by fluorescence microscopy at 15 dpi (data not shown). Similar results were obtained using an MOI of 3 by assessing virus titers in cell culture supernatants at daily intervals through 6 dpi (Fig. 5E). Remarkably, given previous experience with similar conditional expression of HCMV genes (36, 70, 77), it was surprising to observe such a complete replication block in the absence of Shield-1 that was sustained even at a high MOI. Furthermore, replication of UL91-DD-BAC in the presence of Shield-1 was indistinguishable from that of parental Towne-BAC (compare Fig. 5E to 2J). In contrast to the complete block to accumulation of progeny virus, UL91-DD-BAC DNA synthesis continued unimpeded in the presence or absence of Shield-1 (Fig. 5F). These data reinforce the very late defect imposed in UL91 mutants and further indicate that UL91 function is a highly suitable antiviral target.

**FIG 5** Characterization of a conditional UL91 mutant virus. (A) Schematic representation of the genome structure of UL91-DD-BAC, constructed from Towne-BAC. The C-terminal region (aa 72 to 101) of UL91 in Towne-BAC was replaced with FKBP-DD. (B and C) Typical fluorescent images of GFP-positive cells at 10 days posttransfection of HFs with UL91-DD-BAC in the absence (B) or presence (C) of 1 μM Shield-1 in the medium. (D) Multistep growth curves of Towne-BAC and UL91-DD-BAC viruses in the presence (+) or absence (-) of 1 μM Shield-1 in the medium. HFs were infected with viruses at an MOI of 0.01, and virus replication was quantified for 15 days by means of GFP signal intensity via IncuCyte live cell imaging. (E) Single-step growth curves of Towne-BAC and UL91-DD-BAC viruses in the presence (+) or absence (-) of 1 μM Shield-1 (Shld1) in the medium. HFs were infected at an MOI of 3, and viral titers were determined for culture supernatants by plaque assay in the presence of Shield-1 at the indicated time points. The data point at 0 dpi indicates the input virus dose. The detection limit of the plaque assay is indicated by a dashed line. (F) Quantitative PCR analysis of viral DNA replication of UL91-DD-BAC virus in the presence (+) or absence (-) of 1 μM Shield-1 in the medium. HFs were infected with UL91-DD-BAC virus at an MOI of 0.3, and total DNA was isolated at the indicated times postinfection. The accumulation of viral DNA was as described for Fig. 3D. (G) HFs were infected with UL91-DD-BAC virus at an MOI of 3. Infected cells were cultivated with 1 μM Shield-1 for various time periods (indicated by the black boxes in the schematic in the left panel). The right panel shows the viral titers in the supernatants of the infected cells, determined in the presence of 1 μM Shield-1 at 120 h postinfection.
Conditional control of UL91 function with Shield-1 allowed us to evaluate the timing of UL91 function during viral replication. HFs were infected with UL91-DD-BAC virus at an MOI of 3 and treated with Shield-1 at various time points to produce functional UL91 protein. The titer of supernatant virus was determined at 120 hpi (Fig. 5G). When Shield-1 was introduced into the infected cultures at 24 or 48 hpi and treatment was continued to 120 hpi, viral titers were as high as those produced in the continuous presence of Shield-1. In contrast, when Shield-1 was present in cultures only until 24 hpi, progeny failed to accumulate at all, similar to the results when Shield-1 was left out of the cultures from the start of infection. When the infected cells were treated with Shield-1 until 48 hpi, progeny virus was produced at an intermediate level, suggesting that the presence of functional UL91 until 48 hpi allowed some viral replication to proceed. Taken together, these data demonstrate that UL91 function is necessary only during the late phase of viral replication, well after the initiation of viral DNA replication. Because removal of Shield-1 as late as 48 hpi resulted in a 100-fold reduction in titers, these data also suggest a UL91-specific antiviral agent may be useful even when treatment is initiated days after a known exposure to HCMV, such as following tissue and organ transplantation.

**UL91 function is required before capsid assembly.** In order to identify the step in viral replication that was compromised in the absence of UL91, cells were infected with UL911-6BAC or Towne-BAC viruses at an MOI of 3 and then analyzed at 5 dpi by TEM. Infection of all cells was confirmed with GFP and fluorescence microscopy. In control Towne-BAC-infected cells, a juxtanuclear cytoplasmic assembly compartment (AC) was observed and contained the expected virions, noninfectious enveloped particles (NIEPs) and dense bodies (DBs) (Fig. 6A). All three types of capsid particles (A, lacking a scaffold or dense core; B, containing a scaffold; C, containing a dense core [78]) were observed in the nucleus (Fig. 6B). In contrast, cells infected with UL911-6BAC virus did not show any evidence of an organized AC or the maturation of virus particles in the cytoplasm (Fig. 6C). Even in the nucleus, very few capsid forms were present, although an occasional example of intranuclear capsid forms (A, B, and C) was observed (Fig. 6D). Taken together with other data, mutant virus replication was blocked and not simply delayed. Nuclear capsids were then quantified by counting particles in micrographs of randomly selected nuclei. We observed, on average, 20- to 30-fold fewer capsid forms in cells infected with UL911-6BAC than in Towne-BAC (Fig. 6E). Cells infected with UL91-DD-BAC virus in the absence of Shield-1 exhibited comparable reduced levels of nuclear capsids at 4 and 5 dpi as virus grown in the presence of Shield-1 (data not shown). Taken together, these data indicate that the deletion or depletion of UL91 affects an essential replication step following viral DNA replication but before capsid assembly during infection. Furthermore, these data establish that the process of HCMV DNA synthesis proceeds independent of UL91 and accumulation of viral capsids in the nucleus.

**UL91 plays a crucial role in controlling late viral protein accumulation.** In order to further investigate the UL91 mutant phenotype, we assessed the levels of viral proteins during infection with UL911-6BAC virus. HFs were infected at an MOI of 3, and cell lysates were separated on denaturing polyacrylamide gels followed by immunoblotting to detect representative viral IE (IE1), DE (ppUL44), and late (gB, pp28, pp150, pp65, and major capsid protein [MCP]) proteins (Fig. 7A). On one hand, accumulation levels of IE1 as well as ppUL44 were similar to parental Towne-BAC. On the other hand, the accumulation levels of pp28, pp150, and MCP were markedly reduced, particularly at 72 and 96 hpi, gB was modestly decreased at these times, and levels of the tegument protein pp65 were somewhat higher. We also compared patterns of viral protein expression by UL911-DD-BAC (MOI, 3) in the presence or absence of Shield-1. These patterns (Fig. 7B) paralleled those shown in Fig. 7A. Like the parental Towne-BAC and UL911-6BAC comparison, both IE1 and ppUL44 accumulated to similar levels in UL911-DD-BAC-infected cells, independent of Shield-1. Just like UL911-6BAC, the accumulation of late antigens pp28, pp150, and MCP were strongly reduced at 72 and 96 hpi in the absence of Shield-1, while gB was modestly reduced and pp65 was somewhat higher than controls. These data reinforce the remarkably tight conditional regulation of UL91 function by Shield-1, which acts through the added FKBP-derived DD domain [76]. Thus, UL91 plays a crucial role in expression of true-late (γ) proteins (pp28 and pp150) and also influences levels of other late proteins (MCP and, possibly, gB).

To determine whether reduced levels of late proteins in UL911-6BAC virus resulted from increased degradation, we evaluated the impact of the proteasome inhibitor MG132 (20 μM) on pp28 protein levels. HFs were infected with UL911-6BAC or parental Towne-BAC virus at an MOI of 3 and treated with MG132 from 72 to 96 hpi followed by immunoblot analysis. Proteasome inhibition did not restore pp28 levels in UL911-6BAC-infected cells, although there were slightly lower pp28 levels in MG132-treated Towne-BAC-infected cells (Fig. 7C). These data indicate that UL91 does not destabilize proteins, and they are consistent with a predicted contribution to increased expression levels through transcriptional or posttranscriptional mechanisms.

**UL91 is required for efficient late RNA expression.** In order to assess the impact of UL91 on late viral gene transcript levels, we performed qRT-PCR on transcripts from cells infected with UL911-6BAC or Towne-BAC virus. Total RNA was extracted from HFs infected with Towne-BAC or UL911-6BAC viruses at an MOI of 3 to assess RNA levels of 13 viral genes (Fig. 8). Consistent with the impact on protein levels, depletion of UL91 had a minimal impact on IE1 (IE or α) or UL44 (DE or B) transcripts and, consistent with the immunoblot results, a modest impact on gB (DE or B). Leaky-late (γ) transcripts encoding UL82 (pp71) and UL83 (pp65) were increased in cells infected with UL911-6BAC compared to Towne-BAC. Previously annotated true-late (γ) transcripts UL32 (pp150), UL75 (gH), UL115 (gL), and UL99 (pp28) and late (γ) transcripts UL73 (gN), UL80 (PR-AP), and UL86 (MCP) levels were markedly reduced. The level of UL91 transcript was significantly reduced at 72 hpi, suggesting autoregulation that may contribute to the tight mutant phenotype. These results demonstrate that UL91 modestly impacts leaky-late (γ) genes and is crucial for efficient expression of true-late (γ) viral genes. Furthermore, this pattern suggests that UL73, UL80, and UL86 behave more like true-late rather than leaky-late viral genes. The kinetic class of these and many other viral genes remains to be established.

**DISCUSSION**

In this study, we investigated the essential role of UL91 in HCMV replication by using two independent genetic approaches: introduction of translational stop-frame-shift mutation and addition of a conditional FKBP-DD domain [76]. When UL91 function was elimi-
nated or compromised, HCMV progeny could not be detected in the culture medium and cell-to-cell spread failed, even at high MOIs. Analyses of viral DNA, antigen, and RNA accumulation, in combination with direct imaging of infected cells by TEM, demonstrated a block in accumulation of late viral transcripts and proteins that blocked production of viral capsids. Altogether, full-length UL91 as well as the N-terminal region from aa 1 to 71 (i) is fully functional, (ii) is expressed with leaky-late kinetics, (iii) localizes predominantly in the nucleus, (iv) is dispensable for viral DNA synthesis, (v) is required from 48 hpi, and (vi) controls expression of late viral gene products. Somewhat surprisingly, there was no obvious consequence of disrupting translation of proteins that result from the super splice acceptor between aa 37 and 38 for UL91 function. Our study strongly implicates UL91 as a key regulator of late gene expression with a dramatic impact on true-late (γ2) viral genes, acting to support transcription rather than...

**FIG 6** TEM analysis of Towne-BAC or UL91_{1-6}BAC virus-infected HFs. (A to D) Cells were infected with Towne-BAC (A and B) or UL91_{1-6}BAC virus (C and D) at an MOI of 3 and fixed for TEM at 5 dpi. The nucleus (Nuc), cytoplasm (Cyt), and assembly compartment (AC) are indicated. (A) Typical viral particles are magnified and shown in the inset, with a virion indicated by an arrow, dense body (DB) by a black arrowhead, and noninfectious enveloped particle (NIEP) by a white arrowhead. (B and D) The white arrowheads point to A capsids, the black arrowheads point to B capsids, and the arrows point to C capsids. Bars, 2 μm (A and B) or 0.2 μm (panel A inset and C and D). (E) Electron micrographs of nuclei of infected cells were prepared at 5 dpi, and total intranuclear capsids were counted from micrographs. Five nuclei were randomly selected for each sample, and three independent fields were imaged from each nucleus at a magnification of ×10,000.
than reduce degradation of transcripts. UL91 and the RhCMV homolog are interchangeable, so homologs in other CMVs are likely to carry out an analogous function. UL91 functions in a manner that was first suggested in a study of γMHV-68 ORF30 (46), a positional homolog of this gene. From this and previous studies on UL79, UL87, and UL95 (53), there appears to be a common impact of HCMV betagamma genes on late gene expression, acting via a transcriptional activation mechanism.

UL91 is fully functional as a 71-aa N-terminal polypeptide. The region between aa 7 and 71 (out of 111 aa) includes the area of highest sequence similarity among primate betaherpesvirus homologs. Given this information, along with the demonstration that RhCMV rh126 complements HCMV UL91, we conclude that this small 71-aa polypeptide contains all protein-protein interaction motifs crucial to mediate transcriptional activation. Unfortunately, the amino acids that are conserved across UL91 homologs in primate betaherpesviruses are not conserved in γMHV-68 ORF30 or in other gammaherpesviruses. γMHV-68 ORF30 is itself conserved across gammaherpesviruses, such as herpesvirus saimiri and KSHV (46). A similar pattern of divergence characterizes other genes common to herpesviruses, such as HCMV ppUL44, the DNA Pol Proc factor. The identity of functionally important amino acids of HCMV UL91, as well as its interaction partners, and also whether γMHV-68 ORF30 (46) functions in a similar manner remain intriguing questions for future dissection.

Small proteins like UL91 (~13 kDa) are typically able to pass

FIG 7 UL91 plays a crucial role in controlling late viral protein accumulation. (A) HFs were infected with UL911-6BAC as well as parental Towne-BAC viruses at an MOI of 3. (B) HFs were infected with UL91-DD-BAC virus at an MOI of 3 in the presence (+) or absence (-) of 1 μM Shield-1. Cell lysates were prepared at the indicated times (hours postinfection) and analyzed by immunoblotting using anti-IE1/IE2, anti-ppUL44, anti-gB, anti-pp65, anti-pp28, anti-pp150, and anti-MCP antibodies. (C) HFs were infected as described above and treated with 20 μM MG132 from 72 to 96 hpi. Cells were harvested at 96 hpi and subjected to immunoblotting using anti-pp28 antibody, with antibody against β-actin as a loading control. WT, Towne-BAC; Mut, UL911-6BAC.

FIG 8 RNA levels during UL911-6BAC or Towne-BAC virus infection. HFs were infected at an MOI of 3. Total RNA was isolated at the indicated times postinfection, and viral transcripts were quantified by qRT-PCR using primers specific to the indicated genes and normalized to β-actin. The normalized amount of viral transcripts in the cells infected with Towne-BAC at 24 hpi was set at 1.
through the nuclear pore complex by passive diffusion, but they only accumulate in this compartment when associated with larger, nucleus-targeted proteins. The fact that UL91 is diffuse in uninfected cells yet specifically recruited to the nucleus despite the lack of a typical nuclear localization signal suggests that it partners with other viral proteins (potentially UL79, UL87, or UL95) or HCMV-induced host proteins. Even when fused to mCherry, this small aa 1 to 71 UL91 polypeptide retains localization and function, seeming to be structurally resilient. UL91 associates with the replication compartment-like region in the nucleus, the same pattern as known late gene regulators UL79, UL87, and UL95 (36, 53). Despite this localization pattern, UL91, UL79, UL87, and UL95 do not contribute to viral DNA synthesis. The gene products that are regulated by the late transcription machinery are needed for capsid formation. UL91 as well as UL79 mutant phenotypes reveal the absolute independence of viral DNA synthesis and capsid assembly, which were not previously recognized for any herpesvirus. The importance of specific late transactivators came to light because a set of five betagamma genes (ORFs 18, 24, 30, 31, and 34) in γHV-68 all were found to contribute to accumulation of late proteins (46) and transcripts (45, 47, 48). In γHV-68, the binding of RNA Pol II to late gene promoters depends on presence of ORF30 and ORF34 (48), homologs of HCMV UL91 and UL95, respectively. In EBV, the ORF24 homolog BcRF1 functions as a late gene TBP (50), indicating that betagamma gene products stimulate the assembly of the RNA Pol II transcriptional complex. Identification of interaction partners of UL91, such as UL95, as well as the dissection of UL87, a likely TBP (49), will be important for a more complete understanding of HCMV late transcription regulation.

The revelation that EBV BcRF1, the homolog of HCMV UL87 and γHV-68 ORF24, functions as a TATA binding protein with specificity for TATT, controlling late gene expression during infection (49, 50), provides key insights into how core RNA Pol II activity may be modified to promote efficient late gene transcription. Recent incisive studies of HCMV UL79, UL87, and UL95 revealed that these essential genes control transcription of late genes UL99 (pp28) and UL75 (gH) and activate the true-late element in the UL44 promoter (53). This work also provided evidence that the complex acts via ppUL44, the viral DNA Pol Proc factor. In addition, UL79 was independently shown to influence accumulation of UL82 (pp71) and UL32 (pp150) transcripts (36).

Our demonstration that HCMV UL91 protein controls viral late transcription adds to this evidence and broadens the relevance of this activity in betaherpesviruses. Our results demonstrate that UL91 controls expression of true-late genes UL32 (pp150), UL73 (gN), UL75 (gH), UL80 (PR-AP), UL86 (MCP), UL115 (gL), and UL99 (pp28), as well as leaky-late expression of UL55 (gB) and of UL91 itself. UL91 is dispensable for accumulation of UL82 (pp71), and UL83 (pp65) transcripts may even allow increased expression of pp65. This pattern suggests an impact distinct from UL79, possibly later in the cascade of late gene expression. Given the building evidence for a specific complex that modulates host RNA Pol II in both betaherpesviruses and gammaherpesviruses, it is worth noting the precision with which bacteriophage T4 recognizes very simple late transcriptional promoters in E. coli, which involves small proteins that bridge the RNA Pol to the DNA Pol Proc factor as a “sliding clamp,” providing access to promoters that is tied to viral DNA synthesis (44). Whether all five betagamma genes function in a single complex to coordinate the activity of RNA Pol II during HCMV replication remains to be determined.

In conclusion, the present study establishes that UL91, the key component in viral replication, is expressed with leaky-late kinetics and acts at late times of infection by controlling viral late gene transcription in the nucleus. A number of important issues remain to be resolved, including the following: (i) the functional mapping of UL91 protein, (ii) the identification of UL91 interaction partners, (iii) the role of UL91 in the cascade of late gene transcription, and (iv) the relationship with other betagamma gene products that influence late gene expression.

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REFERENCES


