Cytomegalovirus UL91 Is Essential for Transcription of Viral True Late (γ_2) 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 (γ_2) 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 (γ_1) 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 beta-herpesviruses and gammaherpesviruses.

Human cytomegalovirus (HCMV) is responsible for significant disease in the developing fetus as well as in the immunocompromised host (1). This virus replicates in a wide variety of fully differentiated cell types, including myeloid, epithelial, endothelial, mesothelial, fibroblast, and neuronal cells, where viral genes are expressed in a coordinated cascade. The viral replication cycle is tightly regulated, although it is very much prolonged compared to better-studied herpesviruses, such as the alphaherpesvirus herpes simplex virus 1 (HSV-1). Herpesvirus transcription is carried out by host RNA polymerase II (Pol II) modified by viral proteins. In fibroblasts, virion protein pp71 activates expression of the HCMV immediate-early (IE, also called α) genes (2–4), acting primarily through modulation of nuclear Daxx/ATRAX (5, 6). Next, IE2-p86 (7) together with IE1-p72 (8, 9) function in a tightly coordinated manner (10, 11) to recruit host RNA Pol II to delayed-early (DE, also called β) genes (1), processes that have been experimentally dissected using laboratory-propagated strains, such as Towne, but that are preserved in clinical strains as well (1). Experience has attested to the value of viral mutants in dissecting the direct contribution of gene products to regulation of gene expression from an indirect role in deflecting cytokine activation, programmed cell death, and chromatin remodeling (12–16).

Once activated, DE gene products carry out viral DNA synthesis and support production of late (γ) viral gene products, leading to the assembly, maturation, and release of progeny virus (17). IE2-p86 recruits DE proteins encoded by UL112–113 to specialized nuclear sites (18, 19), which in turn recruits ppUL84 and ppUL44, the viral DNA Pol processivity (Proc) factor, forming a complex that recognizes the origin of viral DNA replication, ori-Lyt, to initiate DNA synthesis (20–23). The IE2-p86/ppUL84/ppUL44/UL112–113 protein complex recruits single-stranded DNA binding protein, DNA Pol, and helicase-primase (22, 23), ensuring timely production of viral DNA starting about 24 h postinfection (hpi) and peaking at 48 hpi. Viral DNA is packaged into preformed capsids starting at 48 hpi and peaking at 72 to 96 hpi (1). Nucleocapsid maturation and release is completed over an approximately 24-h time period (17, 24). All herpesviruses build a similar DNA replication complex, such that ORF50/RTA plays an analogous organizing role in gammaherpesviruses (25, 26). HCMV UL112–113 proteins can replace RTA to activate lytic rep-

lication of Kaposi's sarcoma herpesvirus (KSHV, also called HHV-8) (27), suggesting functional conservation between a beta-herpesvirus and a gammaherpesvirus.

Like all herpesviruses, HCMV encodes at least two distinct categories of late genes: one (leaky-late, or γ_1) is expressed in the presence of viral DNA synthesis inhibitors, and expression of the other (true-late, or γ_2) is dependent on active viral DNA synthesis (1, 28). Seven characterized viral genes fall into the true-late category: UL99 (encoding pp28) (29, 30), UL94 (31), UL75 and UL115 (encoding gH/gL) (32–34), UL32 (encoding pp150) (35, 36), the middle transcription start site of UL44 (37–39), and a transcription start site located within the C-terminal portion of IE2-p86 encoding IE2-p40 (also called L40) (10, 40). The promoters of true-late genes UL99 and UL44 contain a small, TATA box-proximal region sufficient to confer proper regulation (39, 41), similar to the properties of true-late gene regulation in HSV-1 (42). When assayed in the context of the viral genome, UL99 promoter transcription is regulated authentically when sequences upstream of the TATA box (–40) are deleted (41), and transcription also does not require sequences downstream of +6 (43). Besides the similarity to HSV-1 (42), this simple promoter requirement is reminiscent of bacteriophage T4, where small virus-encoded proteins link late gene transcription to the replication machinery by bridging the host RNA Pol to the DNA Pol Proc factor (44).

Murine gammaherpesvirus 68 (γ MHV-68) open reading frames (ORFs) 18, 24, 30, 31, and 34 regulate late viral gene expression (45–48), and Epstein-Barr virus (EBV) BcRF1, a homolog of ORF24, is a TATA binding protein (TBP) homolog (49) with greater specificity for TATT and bridges to RNA Pol II (50). In HCMV, all five homologs of the γ MHV-68 genes are essential for replication (51, 52), suggesting that so-called betagamma genes (conserved in beta- and gammaherpesviruses) control late

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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/ppUL44/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 XhoI sites of pON2780 (61), resulting in a three tandem c-myc epitope (3myc) tag at the C terminus of UL91 to produce pLNCX-UL91-3myc. To make a UL91-3myc-expressing lentiviral vector, the UL91-3myc ORF was inserted between XbaI and BamHI sites of the pLV-EF1 α -MCS-IRES-Puro lentiviral vector (Biosettia, San Diego, CA) to produce pLV-UL91-3myc. A three-myc-tagged RhCMV rh126 expression plasmid was constructed by replacing the full-length rh126 ORF in pLNCX-UL91-3myc. RhCMV strain 68-1 DNA (GenBank accession number NC_006150) (62, 63) was kindly provided by Peter Barry, University of California, Davis.

Cells. Primary human foreskin-derived fibroblasts (HFs) and 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, 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, psPAX2 (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 μ g/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-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'-GCGCGCCCATAAAAACGAAAGTGTGCTGCTGCGACCCGCCA CAGCCGCCctgtgacaattaatcatcgca-3' and 5'-AACGCGTTCTGGAC GTGCTCATGTACAGGCGCCGAGACGTGCGACATtcagactgtct gctctt-3'. In the PCR primer sequences, nucleotides homologous to those in the Towne-BAC strain are shown in uppercase letters, and the nucleotides in the GalK cassette are shown in lowercase letters. Recombinants containing the GalK cassette were selected on M63 minimal medium with galactose, leucine, biotin, and chloramphenicol, followed by selection on MacConkey agar plates with galactose and chloramphenicol, as described previously (65).

For generation of UL91₁₋₇₁-BAC, a second recombination step was used

to replace the GalK cassette with a linear fragment containing a single nucleotide insertion to introduce a translational stop frameshift at the Glu7 codon in UL91. The linear fragment was obtained by PCR using Towne-BAC DNA as the template and the oligonucleotide primers 5'-GCGCGCCCATAAAA ACGAAAGTGTGCTGCTGCGACCCGCCA CAGCCGCCATGAACTC GTTGCTGGCGTGAAGTCAACCGACTGGGGG-3', where the T in boldface and underlined portion indicate insertion of the frameshift and stop codon, respectively, and 5'-AACGCGTTCTGGACGTGCCTCATG TCACAGGCGCCGAGACGTGCGACAT-3'. The linear fragments were transformed into SW102 *E. coli* containing UL91-GalK-BAC. Recombinants were selected on 2-deoxy-galactose minimal agar plates for negative selection against the GalK cassette. UL91₁₋₇₁-BAC and UL91₁₋₉₇-BAC was also generated as described above by a single nucleotide insertion to introduce a translational stop and frameshift at the Asp72 and Asp98 codons in UL91, respectively. To make a marker rescue virus (UL91-R-BAC), the UL91₁₋₆-BAC was first replaced by a GalK cassette and then subjected to a second round of recombination by replacing the GalK cassette with wild-type UL91. To create UL91-3myc-BAC, the region from aa 1 to 71 of UL91 was amplified by PCR from the Towne-BAC genome and inserted in frame between BglII and XhoI sites of pON2780, resulting in C-terminally 3myc-tagged truncated UL91 (pLNCX-UL91₁₋₇₁-3myc). The double-stranded linear fragment for recombineering was obtained by PCR using the pLNCX-UL91₁₋₇₁-3myc plasmid as a template and the primers UL91-up50-F (5'-GCGCGCCCATAAAAACG AAAGTGTGCTGCTGCTGCGACCCGCCA CAGCCGCCATGAACTCGTT GCTGGCGGAAGTCT-3') and 5'-AACGCGTTCTGGACGTGCCTCATG TCACAGGCGCCGAGACGTGCGACATgacaagctcttcagaatcagcttttg-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 pTRE3G (Clontech, Palo Alto, CA) and used to replace the 3myc tag in pLNCX-UL91₁₋₇₁-3myc (pLNCX-UL91₁₋₇₁-mCherry). The double-stranded linear fragment for recombineering was obtained by PCR using pLNCX-UL91₁₋₇₁-mCherry as a template and the primers UL91-up50-F and 5'-AACGCGTTCTGGACGTGCCTCATGTACAGGCGCCGAG ACGTGCACATgactgtacagctgctcatcgccg-3', with nucleotides homologous to Towne-BAC shown in uppercase letters and the nucleotides in mCherry shown in lowercase letters. For construction of UL91-DD-BAC, the FK506 binding protein destabilization domain (FKBP-DD) was amplified by PCR with pBMN-HA-YFP-FKBP(L106P)-IRES-HcRed-tandem (76) as a template, and the fragment was used to replace the 3myc tag in pLNCX-UL91₁₋₇₁-3myc (pLNCX-UL91₁₋₇₁-DD). The double-stranded linear fragment for recombineering was obtained by PCR using the pLNCX-UL91₁₋₇₁-DD plasmid as a template and the primers UL91-up50-F and 5'-AACGCGTTCTGGACGTGCCTCATGTACAGGCGCC CGAGACGTGCGACATgattccggttttagaagctccacatc-3', with nucleotides homologous to Towne-BAC shown in uppercase letters and the nucleotides in the FKBP-DD shown in lowercase letters. The obtained fragments were transformed into SW102 *E. coli* containing UL91-GalK-BAC and selected as described above.

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 using calcium phosphate (61). UL91-3myc-expressing HFs were used for propagation of UL91₁₋₆-BAC virus. For recovery of UL91-DD-BAC virus, transfected cells were cultured in the presence of 1 μ M Shield-1. Infectious titers of all recombinant viruses were determined by standard 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-GalK-BAC or UL91₁₋₆-BAC, the pp71 expression plasmid, and one of the pLNCX 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

fixed in a 3.7% formaldehyde solution for 10 min prior to evaluation by epifluorescence microscopy. Viral spread was defined as three or more adjacent green fluorescent protein (GFP)-positive cells.

Chemicals and antibodies. Cycloheximide (CHX) and phosphonoformate (PFA) were purchased from Sigma (St. Louis, MO). Shield-1 was generously provided by Tom Wandless, Stanford University. Proteasome inhibitor MG132 was purchased from Calbiochem (San Diego, CA). The following antibodies were used in immunoblot or immunofluorescence analyses: mouse anti-c-myc (clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA), anti-IE1/IE2 (clone 8B1.2; Millipore, Billerica, MA), anti-ppUL44 (clone 10D8; Virusys, Taneytown, MD), anti-pp65 (clone CH12; Virusys, Taneytown, MD), anti-pp28 (clone CH19; Virusys, Taneytown, MD), anti-gB (clone 2F12; Virusys, Taneytown, MD), anti- β -actin (clone AC-74; Sigma-Aldrich, St. Louis, MO), anti-histone H1 (clone AE-4; Santa Cruz Biotechnology, Santa Cruz, CA), horseradish peroxidase (HRP)-labeled horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), and Alexa 568-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA). Mouse monoclonal antibodies against pp150 (clone 36-14) (35) and MCP (clone 28-4) (69) were kindly provided by William Britt, University of Alabama, Birmingham.

Immunoblotting. 293T cells were transfected with the UL91-3myc or rh126-3myc plasmids in Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and lysed in 2% SDS-containing sample buffer at 48 h posttransfection. HF cells were infected with various viruses at the indicated multiplicities of infection (MOI) and times postinfection. For fractionation studies, an NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce, Rockford, IL) was used according to the manufacturer's instructions. To determine levels of protein, samples were solubilized in SDS in the presence of β -mercaptoethanol and separated on an SDS-polyacrylamide gel, followed by transfer to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore, Billerica, MA), probed with primary antibodies, reacted with the HRP-conjugated secondary antibody, and detected with ECL Western blotting detection reagent (GE Healthcare, Buckinghamshire, United Kingdom) on HyBlot CEL autoradiography film (Denville Scientific, Metuchen, NJ).

IFA. For immunofluorescence analysis (IFA), cells were fixed in methanol-acetone (1:1) or 3.7% formaldehyde followed by three washes with phosphate-buffered saline (PBS). The cells were incubated in blocking buffer containing 0.5% bovine serum albumin and 5% goat serum, diluted in PBS, for 1 h before incubation with primary antibodies. After incubation with mouse anti-c-myc (clone 9E10), cells were washed three times with PBS, and goat anti-mouse IgG conjugated to Alexa Fluor 568 (Invitrogen, Carlsbad, CA) was added. The cells were incubated with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI; Roche Diagnostics, Mannheim, Germany) for 10 min to stain nuclei. Coverslips were retrieved from the wells, mounted on glass slides with a drop of mounting medium (Gel/Mount; Biomed, Foster City, CA), and dried overnight before imaging. Images were acquired on an LSM 510 Meta confocal fluorescence microscope equipped with ZEN 2009 acquisition and analysis software (Carl Zeiss, Thornwood, NY).

TEM. For transmission electron microscopy (TEM), HF cells infected at an MOI of 3 were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h at room temperature. The cells were washed in 0.1 M cacodylate buffer, postfixed with the same buffer with 1% osmium tetroxide, washed and dehydrated through a graded series of ethanol to 100%, and embedded in epoxy resin. Thin sections were counterstained with uranyl acetate and lead citrate. The sections were examined with a JEOL JEM-1210 TEM operated at 75 kV, and the images were captured using a digital camera system, Gatan Bioscan (Pleasanton, CA) (70). The images were acquired and analyzed with Digital Micrograph software (Pleasanton, CA). For quantification of the viral capsid number, five nuclei were randomly selected for each sample, and three independent image fields were analyzed at a magnification of $\times 10,000$. The nuclear capsids were counted from the photographs.

DNA and RNA analyses. Accumulation of intracellular viral DNA was measured by quantitative PCR (qPCR) as previously described (71), with a slight modification. HF cells 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 proteinase 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). HF cells 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) treatment 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 amounts of viral RNAs were normalized using β -actin as an internal control. The following primers were used for qPCR and qRT-PCR (36, 72, 73): IE1, 5'-GGCCGAAGAATCCCTCAAAA-3' and 5'-TCGTTGCAATCCTCGG TCA-3'; UL44, 5'-TACAACAGCGTGTCTGCTCCG-3' and 5'-GGCG TAAAAACATGCGTATCAAC-3'; UL32, 5'-GGTTTCTGGCTCGTGG ATGTGC-3' and 5'-CACACAACACCGTCTGCCGATTAC-3'; UL55, 5'-TCAATCATGCGTTTGAAGAGGTA-3' and 5'-ACCGCACTGAGGA ATGTGAG-3'; UL73, 5'-CACCGAGTCCCTCTAGTCT-3' and 5'-CC GGGTTTGGTACTAGTCGT-3'; UL75, 5'-TAGCGGGCGACGCGATC A-3' and 5'-CGGGAACGGTAGTGGGGA-3'; UL80, 5'-GACACAACAA ACGCCGTAAG-3' and 5'-CGTCGTATTGACGTGACTT-3'; UL82, 5'-TGCTGATGTCTGCTGCGGTAC-3' and 5'-CGGGCACTGATCCT GACCGG-3'; UL83, 5'-GGGACACAACACCGTAAAGCCG-3' and 5'-C GTGGAAGAGGACCTGACGATGAC-3'; UL86, 5'-ACCGCCATTTC TATCACTC-3' and 5'-ATGCTTAGTGGCGTTAGGCT-3'; UL91, 5'-A CCGCCTCTTTCAACACTTT-3' and 5'-AGGATGTGCTTTGACGCAC T-3'; UL99, 5'-TTCACAACGTCCACCCACC-3' and 5'-GTGTCCCAT TCCGACTCG-3'; UL115, 5'-GAGGTGTTTCAGGGTGACAA-3' and 5'-ACGGGACGGTAACGAATAAG-3'; β -actin, 5'-CTCCATCCTGGC CTCGCTGT-3' and 5'-GCTGTCACCTTACCGTTCC-3'. The specificity of each primer pair was verified by dissociation curve analysis as well as electrophoresis followed by ethidium bromide staining.

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 functions 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 primate betaherpesviruses revealed strong sequence conservation across the first 70 aa, together with a highly divergent C-terminal region (aa 71 to 111) (Fig. 1A) that overlaps the N terminus of UL92 by 31 nucleotides (Fig. 1B). The N-terminal region of UL91 also contains a super-RNA splice acceptor between aa 37 and 38 that generates 3'-coterminal spliced late transcripts (74). Guided by this information, Towne-BAC UL91 was replaced with a GalK cassette to produce UL91-GalK-BAC, which was used as an intermediate to produce a rescue virus (UL91-R-BAC) as well as to introduce individual translational stop-frameshift mutations at Glu7, Asp72, and Asp98 (UL91₁₋₆BAC, UL91₁₋₇₁BAC, and UL91₁₋₉₇BAC,

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-frameshift 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.

respectively) (Fig. 1A and C), as described in Materials and Methods. These mutant BACs were designed to disrupt UL91 near the predicted N terminus (UL91₁₋₆BAC), as well as to truncate the poorly conserved C-terminal region (UL91₁₋₇₁BAC) and eliminate only the last 14 aa, including the region of overlap with UL92 (UL91₁₋₉₇BAC). Furthermore, UL91₁₋₆BAC is specific for UL91 and did not disrupt any of the predicted spliced gene products arising from the super splice acceptor (74), whereas both UL91₁₋₇₁BAC and UL91₁₋₉₇BAC 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 HF cells 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 2 Replication properties of UL91 mutant BAC-derived viruses. (A to H) Typical fluorescent images of GFP-positive cells at 10 days posttransfection of HF cells with Towne-BAC (A), UL91-GalK-BAC (B), UL91₁₋₆BAC (C), UL91₁₋₇₁BAC (D), UL91₁₋₉₇BAC (E), and UL91-R-BAC (F). The images from the secondary spread assay by cotransfection of UL91-GalK-BAC (G) or UL91₁₋₆BAC (H) with the UL91-3myc expression plasmid are also shown. Original magnification, $\times 100$. (I) Complementation of UL91-GalK-BAC by UL91-3myc or rh126-3myc in the secondary spread assay with HF cells (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. HF cells 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 determined via IncuCyte live cell imaging. (K) Single-step growth curves of Towne-BAC and UL91 mutant BAC-derived viruses. HF cells 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.

UL91₁₋₇₁BAC, UL91₁₋₉₇BAC, and UL91-R-BAC all replicated, with obvious virus spread evident by day 10 posttransfection, whereas UL91-GalK-BAC and UL91₁₋₆BAC 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 replication defects of UL91-GalK-BAC and UL91₁₋₆BAC result from disruption of UL91. The UL91 region with highest sequence similarity among related viruses, aa 1 to 71, was absolutely essential for replication. Furthermore, the phenotype of UL91₁₋₆BAC demonstrated a crucial role for the conserved amino terminus of UL91. When taken together with the UL91₁₋₇₁BAC and UL91₁₋₉₇BAC 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 HF cells by using a lentivirus expressing UL91-3myc from the EF-1 α promoter in order to have complementing cells to propagate UL91₁₋₆BAC virus. Expression of UL91-3myc in the transduced HF cells was confirmed by immunoblotting with anti-myc antibody (data not shown). Consistent with the transient complementation, UL91-3myc-expressing HF cells supported the replication of UL91₁₋₆BAC (data not shown), producing titers of mutant virus nearly identical to parental Towne-BAC. These cells supported the production of mu-

tant virus stocks to facilitate the investigation of UL91 function during infection.

To characterize the growth defect of UL91₁₋₆BAC virus further and compare it to replication-competent UL91 mutant BACs (UL91₁₋₇₁BAC and UL91₁₋₉₇BAC), HF cells 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 using the IncuCyte live cell imaging system. All Towne-BAC derivatives constitutively expressed the same fluorescent marker cassette, facilitating direct comparisons. UL91₁₋₆BAC virus replication was not detected, whereas UL91₁₋₇₁BAC, UL91₁₋₉₇BAC, 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 UL91₁₋₆BAC, and there was no subsequent spread even when examined at 15 days postinfection (dpi), indicating this mutant virus was incapable of replication. Next, UL91₁₋₆BAC 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 γ MHV-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 HFJs (Fig. 2I).

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 HFJs (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 HFJs followed over 15 days (Fig. 3B), similar to UL91₁₋₇₁BAC 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 HFJs 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, HFJs 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 UL91₁₋₆BAC-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, HFJs 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 also 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

FIG 3 Expression kinetics analysis of UL91. (A) Schematic representation of the genome structure of UL91-3myc-BAC constructed from Towne-BAC. The C-terminal region (aa 72 to 101) of UL91 in Towne-BAC was replaced with a three tandem myc epitope tag. (B) Multistep growth curves of Towne-BAC and UL91-3myc-BAC-derived viruses. HFJs 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 via IncuCyte live cell imaging. (C) Time course of UL91-3myc expression following UL91-3myc-BAC virus infection of HFJs at an MOI of 3 in the absence (-) or presence of CHX or PFA. Total protein was prepared at the indicated times and analyzed by immunoblotting using anti-myc antibody. The antibody against β -actin was used as a loading control. (D) Quantitative PCR analysis of viral DNA replication of Towne-BAC and UL91₁₋₆BAC viruses. HFJs were infected at an MOI of 0.3, and total DNA was isolated at the indicated times postinfection. Comparison to PFA-treated Towne-BAC was included as a control. The accumulation of viral DNA was normalized by dividing the number of IEI gene equivalents by the number of β -actin gene equivalents. The amount of viral DNA at 4 hpi was set at 1.

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 vector-transduced HFJs (data not shown). Taken together, these data demonstrate that the fully functional N-terminal portion between

FIG 4 The UL91 protein localizes in the nucleus during infection. (A to D) HF 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, $\times 630$. 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. HF 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) HF 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, $\times 1,000$. Bars, 20 μm . (M) Immunoblot of cytoplasmic (Cyt) and nuclear (Nuc) fractions from HF 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.

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 stabiliz-

ing ligand, Shield-1, but continue to be expressed and be 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 HF 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-

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 HF cells with UL91-DD-BAC in the absence (B) or presence (C) of 1 μ M Shield-1 in the medium. (D) Multistep growth curves of Towne-BAC and UL91-DD-BAC viruses in the presence (+) or absence (-) of 1 μ M Shield-1 in the medium. HF cells 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 μ M Shield-1 (Shld1) in the medium. HF cells 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 μ M Shield-1 in the medium. HF cells 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) HF cells were infected with UL91-DD-BAC virus at an MOI of 3. Infected cells were cultivated with 1 μ M 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 μ M Shield-1 at 120 h postinfection.

1-dependent manner, establishing conditional expression of this essential late gene product.

Next, UL91-DD-BAC virus was propagated in HF cells 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 HF cells (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.

Conditional control of UL91 function with Shield-1 allowed us to evaluate the timing of UL91 function during viral replication. HF cells 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. HF cells 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 UL91-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 UL91-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_2) 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 μ M) on pp28 protein levels. HF cells 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 HF cells 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 β) transcripts and, consistent with the immunoblot results, a modest impact on gB (DE or β). Leaky-late (γ_1) transcripts encoding UL82 (pp71) and UL83 (pp65) were increased in cells infected with UL91₁₋₆BAC compared to Towne-BAC. Previously annotated true-late (γ_2) 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_1) genes and is crucial for efficient expression of true-late (γ_2) 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-frameshift mutation and addition of a conditional FKBP-DD (76). When UL91 function was elimi-

FIG 6 TEM analysis of Towne-BAC or UL91₁₋₆-BAC virus-infected HF cells. (A to D) Cells were infected with Towne-BAC (A and B) or UL91₁₋₆-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 μ m (A and B) or 0.2 μ m (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 $\times 10,000$.

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) lo-

calizes 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 (74) for UL91 function. Our study strongly implicates UL91 as a key regulator of late gene expression with a dramatic impact on true-late ($\gamma 2$) viral genes, acting to support transcription rather

FIG 7 UL91 plays a crucial role in controlling late viral protein accumulation. (A) HF cells were infected with UL91₁₋₆BAC as well as parental Towne-BAC viruses at an MOI of 3. (B) HF cells were infected with UL91-DD-BAC virus at an MOI of 3 in the presence (+) or absence (-) of 1 μ M 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) HF cells were infected as described above and treated with 20 μ M 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.

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 γ MHV-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 γ MHV-68 ORF30 or in other gammaherpesviruses. γ MHV-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 γ MHV-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 8 RNA levels during UL91₁₋₆BAC or Towne-BAC virus infection. HF cells 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 γ MHV-68 all were found to contribute to accumulation of late proteins (46) and transcripts (45, 47, 48). In γ MHV-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 γ MHV-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 func-

tion in a single complex to coordinately regulate 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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