The Human Cytomegalovirus Gene Products Essential for Late Viral Gene Expression Assemble into Prereplication Complexes before Viral DNA Replication[▽]

Hiroki Isomura,^{1*} Mark F. Stinski,³ Takayuki Murata,¹ Yoriko Yamashita,² Teru Kanda,¹ Shinya Toyokuni,² and Tatsuya Tsurumi¹

Division of Virology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan¹; Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan²; and Department of Microbiology, Carver College of Medicine, University of Iowa, 51 Newton Road, Iowa City, Iowa 52242³

Received 24 February 2011/Accepted 8 April 2011

The regulation of human cytomegalovirus (HCMV) late gene expression by viral proteins is poorly understood, and these viral proteins could be targets for novel antivirals. HCMV open reading frames (ORFs) UL79, -87, and -95 encode proteins with homology to late gene transcription factors of murine gammaherpesvirus 68 ORFs 18, 24, and 34, respectively. To determine whether these HCMV proteins are also essential for late gene transcription of a betaherpesvirus, we mutated HCMV ORFs UL79, -87, and -95. Cells were infected with the recombinant viruses at high and low multiplicities of infection (MOIs). While viral DNA was detected with the recombinant viruses, infectious virus was not detected unless the wild-type viral proteins were expressed in *trans*. At a high MOI, mutation of ORF UL79, -87, or -95 had no effect on the level of major immediate-early (MIE) gene expression or viral DNA replication, but late viral gene expression from the UL44, -75, and -99 ORFs was not detected. At a low MOI, preexpression of UL79 or -87, but not UL95, in human fibroblast cells negatively affected the level of MIE viral gene expression and viral DNA replication. The products of ORFs UL79, -87, and -95 were expressed as early viral proteins and recruited to prereplication complexes (pre-RCs), along with UL44, before the initiation of viral DNA replication. All three HCMV ORFs are indispensable for late viral gene expression and viral growth. The roles of UL79, -87, and -95 in pre-RCs for late viral gene expression are discussed.

Human cytomegalovirus (HCMV) is a member of the beta-herpesvirus family. The viral genome is 240,000 bp long and has at least 150 predicted open reading frames (ORFs) (9, 57). Although infection by HCMV occurs in most individuals, it is usually asymptomatic. The virus is reactivated under immunosuppressive conditions and causes pneumonitis, hepatitis, retinitis, and gastrointestinal diseases. The virus replicates productively in terminally differentiated cells such as fibroblasts, epithelial and endothelial cells, and monocyte-derived macrophages (11, 12, 18, 28, 41, 42, 48).

During productive infection, HCMV genes are expressed in a temporal cascade, with temporal phases designated immediate-early (IE), early, and late. The major IE genes (MIE) UL123 and UL122 (IE1/IE2) play a critical role in subsequent viral gene expression and the efficiency of viral replication (19, 20, 24, 33–35). The early viral genes encode proteins necessary for viral DNA replication (36). Following viral DNA replication, delayed early and late viral genes are expressed which encode structural proteins for the virion. Expression of true late (gamma 2 class) viral genes is prevented by inhibition of viral DNA synthesis. UL75 (gH), UL99 (pp28), and the middle transcription start site of UL44 are examples of HCMV genes

that are true late transcripts (26, 29, 32, 53). While the regulation of HCMV immediate-early and early gene expression has been studied extensively, little is known about the specific mechanisms of regulating late gene expression.

With HCMV, the IE2 protein can be found in microfoci early after infection and is associated with parental viral genomes by direct DNA contact. Some of the microfoci enlarge to form replication compartments (RCs) at late times after infection (43). However, the HCMV IE genes alone are not sufficient for activation of the true late viral genes. Which HCMV early genes are required for late gene expression is not known.

ORFs 18, 24, 30, and 34 of murine gammaherpesvirus 68 (MHV-68) are essential for late gene transcription (3, 54, 55) and have homology to HCMV ORFs UL79, -87, and -95. The MHV-68 gene products function specifically to stimulate the promoters of late genes, since their absence has little effect on early viral gene expression or DNA replication. Therefore, late gene expression depends not only on viral DNA replication but also on these virally encoded *trans*-acting factors.

HCMV viral DNA replication proteins, including DNA polymerase processivity factor (UL44) and single-stranded DNA binding protein (SSB) (UL57), are localized in large nuclear structures that resemble herpes simplex virus type 1 (HSV-1) RCs (37, 39). In HSV-infected cells, small viral prereplication sites or foci containing the virus-encoded SSB (ICP8) protein were detected when viral DNA synthesis was blocked by the inhibitor phosphonoacetic acid (PAA) (6, 30,

* Corresponding author. Mailing address: Division of Virology, Aichi Cancer Center Research Institute, 1-1, Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan. Phone: 81-52-762-6111, ext. 7032. Fax: 81-52-763-5233. E-mail: hisomura@aichi-cc.jp.

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38). With HCMV, the SSB (UL57) protein did not overlap with viral DNA until after the initiation of viral DNA synthesis (37). In the absence of PAA, bromodeoxyuridine (BrdU) was incorporated into viral DNA and the DNA was found associated with progeny virus (7, 58).

In this report, we show for the first time that HCMV ORFs UL79, -87, and -95 are essential for late viral gene expression of UL44, -75, and -99 and for infectious virus production but not for viral DNA replication. The UL79, UL87, and UL95 proteins assemble with the viral genome before viral DNA replication into microfoci containing the early viral gene UL44.

MATERIALS AND METHODS

Cells and viruses. Primary human foreskin fibroblast (HFF) cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in 5% CO₂ as described previously (44). To inhibit viral DNA replication, PAA (Sigma, St. Louis, MO) (200 µg/ml) or 9-[(1,3-dihydroxy-2-propoxy)methyl] guanine (ganciclovir [GCV]; Calbiochem, Darmstadt, Germany) (450 µM) was added to the medium at the time of infection and maintained throughout infection.

To generate HFF cells expressing the HCMV UL79, -87, or -95 ORF, a retroviral system was used following a protocol from the lab of G. Nolan (http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html). The UL79 and -95 ORFs were amplified by PCR from bacterial artificial chromosome (BAC) DNA of HCMV Towne, using primer pairs XhoIUL79ORFF-HindIIIUL79ORFR and XhoIUL95ORFF-HindIIIUL95ORFR, respectively. The sequences for the PCR primers are shown in Table 1. The PCR products were digested by the restriction endonucleases XhoI and HindIII and cloned into pLBC (2) (kindly provided by H. P. Kiem, Fred Hutchinson Cancer Research Center, with permission from G. Nolan, Stanford University) containing the mcherry coding sequence (kindly provided by M. Matsuda, Kyoto University Graduate School of Medicine, Kyoto, Japan) fused to the C terminus of the ORF. pLBC is a shuttle vector containing the EBNA1 ORF and the OriP sequence of the Epstein-Barr virus (EBV) genome for construction of recombinant retroviruses. The UL87 DNA sequence contains a restriction endonuclease HindIII site 815 nucleotides (nt) from the ATG start codon. The HindIII site is required for cloning of the ORF into pLBC. The region from nt 1 to 814 of UL87 was first amplified by PCR using the primer pair XhoIUL87ORFF-UL87HindIII(815)R and then cloned into pLBC at the corresponding restriction endonuclease site, and the DNA was sequenced (Aichi Cancer Center Research Institute Central Facility). The region from nt 815 to 2826 was then amplified by PCR using the primer pair UL87(815)HindIIIF-HindIIIUL87ORFR and cloned into pLBC containing the region from nt 1 to 814 of UL87 at the HindIII site, and the DNA was sequenced. A retrovirus stock was prepared by transfecting the shuttle vector containing each ORF into the packaging cell line Phoenix-GALV (17) (kindly provided by H. P. Kiem, with permission from G. Nolan). HFFs were infected with the recombinant retrovirus to generate a population of pUL79-, -87-, or -95-mcherry-expressing cells under puromycin selection.

The titers of wild-type (wt) HCMV Towne, RHAUL95UL87myc, RUL95HAUL87myc, RHAUL95UL87mycflagUL79, RUL95HAUL87mycflagUL79, RdlUL79, RdlUL87, and RdlUL95 were determined by standard plaque assays on either HFF cells (for the parental and epitope-tagged viruses) or HFF cells expressing pUL79-, -87-, or -95-mcherry (for the deletion mutants), as described previously (35). The titers of the viruses were also determined by detection of green fluorescent protein (GFP) fluorescent foci in monolayers of HFF cells infected with serial dilutions of virus. At various times after infection, cells and supernatant were collected and subjected to three freeze-thaw cycles. Virus titers were determined by the 50% tissue culture infective dose (TCID₅₀) assay on HFF cells (for the parental and epitope-tagged viruses) or HFF cells expressing pUL79-, -87-, or -95-mcherry (for the deletion mutants). We used the Reed-Muench method to calculate TCID₅₀. The wt and recombinant viruses contained the GFP gene substituted for the dispensable, 10-kb unique short 1 (US1) to US12 region.

Viral DNA replication. After infection at a multiplicity of infection (MOI) of 3 or 0.01, cells were collected at 6 h and 2, 3, and 4 days postinfection (dpi). Cells in 35-mm plates in triplicate were suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, and 20 µg/ml RNase A) containing 50 µg/ml proteinase K. The input and replicated viral DNAs were detected by real-time PCR using HCMV gB primers and probe as described previously (20, 24). Real-time PCR with 18S rRNA gene primers and probe purchased from Applied

Biosystems (Foster City, CA) was also performed to serve as an internal control for input DNA. Data are averages for three independent experiments.

Mutagenesis of HCMV BAC DNA. A rapid homologous recombination system with *Escherichia coli* expressing bacteriophage lambda recombination proteins *exo*, *beta*, and *gam* (kindly provided by D. L. Court, NIH) was employed as described previously (10). BAC DNA of HCMV Towne was kindly provided by F. Liu (University of California) (9). When a recombinant HCMV BAC DNA with deletion of an ORF was constructed, the FRT sequence was inserted into the middle of the ORF to reduce the effect of the 34-bp sequence on the neighboring genes to a minimum. Moreover, nucleotides were not deleted to rule out the possibility that the regulatory region of the neighboring genes was removed. To generate the recombinant HCMV BAC DNAs of BACdlUL79Kan+FRT, BACdlUL87Kan+FRT, and BACdlUL95Kan+FRT (Fig. 1a), the double-stranded DNAs for recombination were amplified by PCRs using the plasmid pACYC177 (NEB) as a template and the primer pairs BACdlUL79FRTFKanF-BACdlUL79FRTKkanR, BACdlUL87FRTFKanF-BACdlUL87FRTKkanR, and BACdlUL95FRTFKanF-BACdlUL95FRTKkanR, respectively. The primer sequences are shown in Table 1.

To generate BACUL95N neo+St and BACUL95C neo+St, the double-stranded DNAs were amplified by PCRs using the plasmid pRpsL-neo (Gene Bridges, Dresden, Germany) as a template and the primer pairs BACUL95Nneo+StF-BACUL95Nneo+StR and BACUL95Cneo+StF-BACUL95Cneo+StR, respectively. The primer sequences are shown in Table 1. Plasmids pACYC177 and pRpsL-neo (Gene Bridges) contained a kanamycin resistance (Kan^r) gene and both Kan^r and streptomycin sensitivity genes, respectively. The amplified double-stranded DNAs for recombination contained a Kan^r gene flanked by the 34-bp minimal FRT site (5'-GAAGTTCTATTTCTAGAAAGTATAGGAAGCTT C-3') (15) or the RpsL-neo gene cassette (Gene Bridges) and 70 bp of homologous viral DNA sequence. After digestion with DpnI at 37°C for 1.5 h, the PCR product was gel purified and transformed into DY-380 containing the parental HCMV BAC DNA. After homologous recombination, the mutated BAC DNA containing the Kan^r-plus-FRT sequence or the RpsL-neo gene cassette was resistant to kanamycin (Fig. 1).

To excise the Kan^r sequence from the mutated HCMV BAC DNA with the FRT sequence, FRT-mediated recombination was employed as described previously (24). Plasmid pCP20 (kindly provided by G. Hahn, Max von Pettenkofer Institute, Munich, Germany) was transformed into DH10B containing the recombinant HCMV BAC DNA. HCMV BAC DNA without kanamycin was selected on LB plates containing ampicillin and chloramphenicol. To screen recombinant BAC DNA with deletion of the UL79, -87, or -95 ORF, PCR analysis was performed using the primer pair UL79detectF-UL79detectR, UL87BACdetectF-UL87BACdetectR, or UL95BACdetectF-UL95BACdetectR, respectively. The primer sequences are shown in Table 1. The PCR products were sequenced to confirm the recombination (Aichi Cancer Center Research Institute Central Facility).

Reverse selection was performed as described previously (21, 51). Since RpsL confers streptomycin sensitivity, the mutated BAC DNA was selected on the basis of increased streptomycin resistance by use of a counterselection modification kit (Gene Bridges). To construct BACHAUL95 and BACUL95HA, the oligonucleotides BAColigoHAUL95 and BACUL95Coligo, respectively, were used for reverse selection (Fig. 1b). The oligonucleotide sequences are shown in Table 1.

To construct recombinant BACHAUL95UL87myc or BACUL95HAUL87myc (Fig. 1b), the reverse procedure was repeated after construction of BACHAUL95 or BACUL95HA. The double-stranded DNAs were amplified by PCRs using the primers BACUL87Cneo+StF and BACUL87Cneo+StR and transformed into DY-380 containing BACHAUL95 or BACUL95HA. The primer sequences are shown in Table 1. BACHAUL95neo+St and BACUL95Haneo+St were selected on LB plates containing kanamycin. After reverse selection was performed using the oligonucleotide BACUL87oligo, BACHAUL95UL87myc and BACUL95HAUL87myc were selected on the basis of increased streptomycin resistance as described above (Fig. 1).

To insert a Flag epitope into the N terminus of UL79 of BACHAUL95UL87myc or BACUL95HAUL87myc, the double-stranded DNA was amplified by PCR using the primers BACUL79Nfneo and BACUL79NRneo and transformed into DY-380 containing BACHAUL95UL87myc or BACUL95HAUL87myc, respectively. The primer sequences are shown in Table 1. BACHAUL95UL87mycneo+StUL79 and BACUL95HAUL87mycneo+StUL79 were selected on LB plates containing kanamycin. After reverse selection was performed using the oligonucleotide BAColigoflagUL79, BACHAUL95UL87mycflagUL79 and BACUL95HAUL87mycflagUL79 were selected on the basis of increased streptomycin resistance as described above (Fig. 1).

To screen the recombinant BAC DNAs containing HA-UL95, UL95-HA, Flag-UL79, and UL87-myc, PCR analysis was performed using the primer pairs UL95NdetectF-UL95NdetectR, UL95CdetectF-UL95CdetectR, UL87CdetectF-UL87CdetectR, and UL79NdetectF-UL79NdetectR, respectively. The primer sequences are shown in Table 1. The PCR products were

TABLE 1. PCR primers and oligonucleotides for construction of plasmids and HCMV BAC DNAs for this study

Primer or oligonucleotide	Sequence (5'-3')
XhoIUL79ORFF	CCGCTCGAGATGATGGCCCCGCGACGAAGAGAACCC
HindIIIUL79ORFR	CCCAAGCTTTCACGTCGTTAGCCAGCGTCGGCATAT
XhoIUL95ORFF	CCGCTCGAGATGATGGCGGCGGCGGTGGTGCAGCGGAG
HindIIIUL95ORFR	CCCAAGCTTTTAGATTCAACGTGATGAGACCCGCGTCGTTACAGC
XhoIUL87ORFF	CCGCTCGAGATGGCCGCGCTGCGCCGCGCCGCTCGGCTGCGAC
UL87(815)HindIIIR	CAACGCCCAGCCAAAGCTTTTGCAGCTCCAGC
UL87(815)HindIIIF	GCTGGAGCTGCAAAAGCTTTGGCTGGGCGTTG
HindIIIUL87ORFR	CCCAAGCTTTTCGTGATGCAAACCGCGCTCGCGGCGACGTG
BACdIUL79FRTFKanF	GCGGCCGTGTTTCGATGAAACGCGCGCCGCGCTCTCAGCCAGCGCCTGTGTCAACCCGCG CTTGAGCGGCGGAAGTTCCTATTCTCTAGAAAGTATAGGAACCTCAACTCAGCAAAAG TTCGATTTATTCAAC
BACdIUL79FRTRKanR	TTGCGCGCCAGGCTTCGCGGAAAACGACCGGCAGGCCGGTGTGGCGCTGCACAAAGC GCGTCAGCAGTCGAAGTTCCTATACTTTCTAGAGAATAGGAACCTCTAATGCTCTGCCA GTGTTACAACCA
BACdIUL87FRTFKanF	GTCAGTCCGCGCGACCCGCGCCGAGATCGCGTTCGACGTTACGTATGCATTATCTCGCG GAAGTTCCTATTCTCTAGAAAGTATAGGAACCTCAACTCAGCAAAAGTTCGATTTATT CAAC
BACdIUL87FRTRKanR	GTCTCCGACCGCCGTTGACAGTGTTCACGCCATCTCTCCCCGTACCGAGCGTACATGAGA GAAGTTCCTATACTTTCTAGAGAATAGGAACCTCTAATGCTCTGCCAGTGTTCACAACCA
BACdIUL95FRTFKanF	ACGACGCCGCCACGCGCTCTTTCTACGTCGACACGACGTGCTGGAGCGTTTCGCGGCCG GAAGTTCCTATTCTCTAGAAAGTATAGGAACCTCAACTCAGCAAAAGTTCGATTTATT CAAC
BACdIUL95FRTRKanR	CACGGTCAGCATTGCGTAACGCATAATCACGCACACAAAGCGACGGCAAAGGCTCAGCC GGAAGTTCCTATACTTTCTAGAGAATAGGAACCTCTAATGCTCTGCCAGTGTTCACA ACCA
BACUL95Nneo+StF	CTTCTGCAGCTCCGCGTAGCGCTCCTGGATCTTGGCGGCCGAGTCTCCGCGCAACGGCCT GGTGATGATGGCGGGATC
BACUL95Nneo+StR	TTTCTCTCTCTCGCCGCTGCCGCTAACCTCCGCTCGCACCAACCGCCGCCGCTCAGAA GAACTCGTCAAGAAGG
BACUL95Cneo+StF	CGGAAAGTTGCTGGACGCCCTCTCGCTGAACGACGCGGGTCTCATCAGTTGAATGGCC TGGTGATGATGGCGGGATC
BACUL95Cneo+StR	TCAAGGTCGACGCGCATCAGTCCTTTAAGAGCTGTTTGTGACCGACGTCATAGTCAGA AGAACTCGTCAAGAAGG
BAColigoHAUL95	TTGCGCTTCTGCAGCTCCGCGTAGCGCTCCTGGATCTTGGCGGCCGAGTCTCCGCGCAAC ATGTACCCATACGATGTTCCAGATTACGCTGCGGCGGCGGTGGTGCGAGCGGAGGTTA GGCGGCAGCGGCGAGAGGAGAGGAAAAAGATG
BACUL95Coligo	CCACGTCGGAAGTTGTCTGGACGCCCTCTCGCTGAACGACGCGGGTCTCATCAGTTGA ATTACCCATACGATGTTCCAGATTACGCTCTATGACGTCGGTCAACAAACAGCTCTTAA AGGACGTGATGCGCGTCGACCTTGAGCGACA
BACUL87Cneo+StF	CAGCGTTGACGGCAGTTCTGAACCCACGTCGCCGCGAGCGCGGTTTGCATCACGAGGCC TGGTGATGATGGCGGGATC
BACUL87Cneo+StR	GTCGGACGCTCCTCCGACGAAACGCCGCGGCGGCAGCGGCCGCGGCTTCCATCATCAG AAGAACTCGTCAAGAAGG
BACUL87oligo	TCCGTACGCGTTGACGGCAGTTCTGAACCCACGTCGCCGCGAGCGCGGTTTGCATCACG AGAACAGAACTGATCTCTGAAGAAGACCTGTGATGGAAGCCGCGGCCGCTGCCGCC GCGGCGTTTCGTCCGAGGAGCGTCCGACGCCG
BACUL79NNeo	CACAGCCTGCGGCTGCTGCTCGTCCATCGTCATTGTCGTCACCGTCGCTACCCGCTCACC GAGCGAACGGGCTGGTGATGATGGCGGGATC
BACUL79NNeo	GATTGGCGCAAGTAAAGGAGAATTTGCCCTGTGCGGACCCGCGGACGCGGGGTTCTCT TCGTCGCGGGCTCAGAAGAAGTCTCAAGAAGG
BAColigoflagUL79	GCGGCTGCTGCTCGTCCATCGTCATTGTCGTCACCGTCGCTACCCGCTCACCAGCGAAC GATGGACTACAAAGACGATGACGACAAGGCCCGCGACGAAGAGAACCCCGCCGCTCCC GCGGGTCGCACaGGCAAATTCCTTTACTTG
UL79detectF	CGCCAGGCCTTCCCGGGGCTGG
UL79detectR	CCGTAGAGCGTGCCTAGGGAGAAGAGG
UL87BACdetectF	CGATCCGTAAGCCGCGGTTACGG
UL87BACdetectR	CGTAAAGTCAAATGGCGGCGTGCG
UL95BACdetectF	GGGAAAAGAACGCGGCTGGGTCTCG
UL95BACdetectR	GCTGGAGAGTGACAGCTCCACGTGAGC
UL95NdetectF	AGTTCGGGGTGATCATCT
UL95NdetectR	ACGACACCACTAGGGACGAC
UL95CdetectF	AAGTTGCTGGACGCCCTCTC
UL95CdetectR	ACACGCATCACCGTCAAAG
UL87CdetectF	CCCATTGTGTGTCTCTCAT
UL87CdetectR	CCGTACCGTCGTCCATTAAC
UL79NdetectN	GGTCGTAACGGGCGAGAAAGCCG
UL79NdetectR	CTGTCCGCGGACATCTTCTCGC

FIG. 1. Structure of recombinant HCMV BAC DNAs. (a) Diagram of recombinant BAC DNAs of wt, RdlUL79+F, RdlUL87+F, and RdlUL95+F viruses. When the recombinant HCMV BAC DNA with mutation of the ORF was constructed, the FRT sequence was inserted into the middle of the ORF, and then the Kan^r gene was excised by FLP-mediated recombination, leaving only 34 bp of the FRT sequence. (b) Diagram of recombinant BAC DNAs with an epitope fused to the UL95 and UL87 ORFs. When an HA epitope fused to the UL95 ORF was inserted, reverse selection was performed as described in Materials and Methods to remove the drug resistance cassette. To construct BACUL95N neo+St or BACUL95C neo+St, a marker cassette containing the RpsL gene, conferring increased sensitivity to streptomycin, and the neomycin resistance marker, providing kanamycin resistance, was inserted into the N or C terminus of the UL95 ORF, respectively. Intermediate BAC clones were isolated based on resistance to kanamycin. In a second round of homologous recombination, the entire marker cassette was replaced with the sequence containing an HA epitope by counterselection using an oligonucleotide, as described in Materials and Methods. To construct a recombinant BAC with a myc epitope fused to the C terminus of the UL87 ORF, the reverse selection was repeated after construction of BACHAUL95 or BACUL95HA as described in Materials and Methods. To construct a recombinant BAC with a Flag epitope fused to the N terminus of the UL79 ORF, the reverse selection was repeated after construction of BACHAUL95UL87myc or BACUL95HAUL87myc as described in Materials and Methods. (c to e) Growth curves of parental HCMV, RUL95HAUL87myc, RHAUL95UL87myc, RUL95HAUL87mycflagUL79, and RHAUL95UL87mycflagUL79 at an MOI of 3 (c and d) or 0.01 (e). Virus titers were determined by TCID₅₀ assay as described in Materials and Methods.

FIG. 1—Continued.

sequenced to confirm the recombination and excision (Aichi Cancer Center Research Institute Central Facility).

Antibodies. To detect fusion proteins tagged with hemagglutinin (HA) or Flag, a rat (3F10; Roche) or rabbit (R4-TP1511; Reagent, Taipei, Taiwan) polyclonal antibody, respectively, was used. To detect fusion proteins with the myc epitope, mouse monoclonal antibody 9B11 or rabbit polyclonal antibody 7D10 (Cell Signaling, Boston, MA) was used. Antibodies to detect IE1 and IE2 proteins, IE2, UL44, UL57, TATA-binding protein (TBP), pp28, and cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were NEA-9221 (Perkin Elmer, Boston, MA), CA006 (EastCoast Bio, North Berwick, ME), CH167 (Santa Cruz, Santa Cruz, CA), CA004 (EastCoast Bio), and MAB374 (Chemicon, Temucula, CA), respectively.

In situ extraction of proteins prior to confocal microscopy. Confluent HFFs in 24-well plates were infected with the recombinant viruses at an MOI of 3. At various times after infection, cells grown on glass coverslips were fixed with 50 μ g/ml of dithiobis succinimidylpropionate (DSP) (Thermo, Rockford, IL), which is a homobifunctional *N*-hydroxysuccinimide (NHS) ester cross-linking agent, for 10 min at 4°C as described previously (56). To remove non-matrix-bound proteins from the nucleus or the cytoplasm, cells were treated with TNE buffer (10 mM Tris-HCl, pH 7.8, 1% NP-40, and 0.15 M NaCl) and fixed with 2% paraformaldehyde for 60 min at 4°C.

Microscopy. Cells were washed three times with high-salt TPBS (0.01 M sodium phosphate [pH 7.3], 0.5 M NaCl, 0.1% Tween 20, and 0.1% Triton X-100) and incubated with the indicated primary antibody at 4°C overnight. After three washes with TPBS at room temperature, the cells were incubated at 37°C for 30 min with secondary anti-mouse IgG conjugated with Alexa Fluor 594 or 680 (Molecular Probes), anti-rabbit IgG conjugated with Alexa Fluor 594 or 680 (Molecular Probes), or rat IgG antibody conjugated with Alexa Fluor 594 (Molecular Probes). When an HA or myc fusion protein was detected in the infected cells, Can Get Signal immunostain (Toyobo, Osaka, Japan) was used to enhance the intensity of the specific signal. The slides were mounted in ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Image acquisition was performed using a Carl Zeiss Meta confocal laser scanning microscope (Carl Zeiss MicroImaging, Inc.) equipped with a PlanApo $\times 63$ or $\times 100$ 1.4-numerical-aperture (NA) oil-immersion objective lens. The

LSM 510 Meta confocal microscope is equipped with a HeNe laser to excite Alexa Fluor 594 and 680 fluorophores. We first examined localization of the protein when cells were stained for either antigen and then confirmed that no immunofluorescence was observed with alternate filters under the same conditions.

FISH assay. HCMV BAC DNA was labeled with SpectrumOrange by use of a nick translation kit (Abbott Molecular, Abbott Park, IL) according to the manufacturer's recommendations. Fluorescence *in situ* hybridization (FISH) analysis was performed essentially as previously described (25), with minor modifications. Cells grown on glass coverslips were fixed with 2% paraformaldehyde at 4°C for 60 min after *in situ* extraction as described above and permeabilized with blocking buffer (2.5% bovine serum albumin [BSA], 0.2 M glycine, and 0.1% Triton X-100) containing RNase (100 μ g/ml) for 1 h before hybridization. Each coverslip was placed conversely on the glass slide with 5 μ l of hybridization buffer (2 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 10% dextran sulfate, and 50% formamide) and a DNA probe. After being sealed in a hybridization bag, the slides were immersed in a water bath at 85°C for 5 min, followed by incubation at 37°C overnight. Anti-UL44 antibody was used to detect UL44 antigen after FISH analysis. The coverslip was washed three times in 2 $\times$ SSC containing 50% formamide at 50°C for 3 min each, three times in 2 $\times$ SSC at 50°C for 3 min each, and once in 0.1 $\times$ SSC at 60°C for 10 min. The SpectrumOrange fluorophore has an absorbance maximum of 560 nm and a fluorescence emission maximum of 588 nm and was excited with a HeNe laser.

Western blot analysis. Cells were harvested at the indicated times postinfection, washed with phosphate-buffered saline (PBS), and treated with lysis buffer as described previously (19, 20). Twenty-microgram aliquots of proteins were loaded into each lane for sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. Enhanced chemiluminescence detection reagents (GE Healthcare UK Ltd., Buckinghamshire, United Kingdom) and secondary horseradish peroxidase-labeled anti-mouse, -rabbit, or -rat IgG antibody (Zymed, San Francisco, CA) were used according to the manufacturers' instructions.

Northern blot analysis. Cytoplasmic RNAs were isolated 1, 2, and 3 days after infection as described previously (5, 16). Twenty micrograms of cytoplasmic RNA was subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and transferred to a maximum-strength Hybond N+ membrane

(GE Healthcare UK Ltd.). Northern blot analysis with the IE1, UL75, or UL99 DNA probe was performed as described previously (20, 22, 24). A radioactive probe was generated by labeling with [³²P]dCTP as described previously (24).

RNAse protection assay. A ³²P-labeled antisense UL44 RNA probe was constructed as described previously (24). Twenty micrograms of RNA was hybridized to the ³²P-labeled antisense UL44 promoter probe at 37°C overnight before digestion with RNase T1 (100 U) as described previously (19, 27). The protected RNA fragments were subjected to electrophoresis in denaturing 6% polyacrylamide gels followed by autoradiography on Hyperfilm MP (GE Healthcare UK Ltd.).

Real-time RT-PCR analysis. For detection of RNA, whole-cell RNA was purified and then converted to cDNA with reverse transcriptase (RT) (Roche) as described previously (24). The no-RT control failed to detect any input viral or plasmid DNA and was similar to the mock control. Amplifications were achieved in a final volume of 25 µl containing Platinum Quantitative PCR Supermix-UDG cocktail (Invitrogen). Each reaction mixture was described previously (23). HCMV MIE gene primers and reporter probes were described previously (19, 21, 23). Thermal cycling conditions and standard curve analysis were described previously (23). Real-time PCR with glucose-6-phosphate dehydrogenase (G6PD) primers and probes (50) was also performed to serve as an internal control for input RNA. Real-time RT-PCR assays were performed in triplicate. An arbitrary RNA in the isolated RNAs was set to 1.0, and a standard curve was constructed using serial dilutions of cDNA from the RNA set to 1.0. A constant amount of the RNAs was quantitated based on the standard curve.

RESULTS

Construction of recombinant viruses with mutated or epitope-tagged UL79, -87, and -95 ORFs. To determine the effects of HCMV UL79, -87, and -95 on viral DNA replication and late mRNAs, we constructed recombinant viruses with mutations to interrupt the ORFs of UL79, -87, and -95. HFF cells stably expressing UL79, -87, or -95 protein fused in frame at the C terminus with the fluorescent protein mcherry were isolated by using a recombinant retrovirus as described in Materials and Methods. These cells were used to grow the recombinant HCMVs. Since a drug resistance gene is necessary to select the recombinant BAC DNA from the parental BAC DNA, we first constructed a recombinant BAC DNA with a Kan^r gene flanked by a 34-bp minimal FRT site and then excised the Kan^r gene by FRT-FLP recombination as described in Materials and Methods and as diagrammed in Fig. 1a. The FRT sequence was inserted into the middle of the ORF to minimize the effect of the 34 bp on the neighboring genes.

We also constructed recombinant viruses with a Flag, HA, or myc epitope fused to the N or C terminus of the UL79, UL87, or UL95 ORF (Fig. 1a and b). Since the UL89 gene is essential (9, 57) and the UL95 ORF overlaps with UL89, insertion of an HA epitope into the N terminus of UL95 may impair the function of the UL89 protein. Therefore, we constructed recombinant viruses with an HA epitope fused to the N or C terminus of the UL95 ORF (Fig. 1a and b). Since UL86 is transcribed in a direction opposite from that for UL87, we inserted a myc epitope into the C terminus of the UL87 ORF. Since the TGA stop codon of the UL87 ORF and the ATG start codon of the UL88 ORF overlap, a myc epitope was inserted in front of the TGA stop codon of the UL87 ORF. To insert an epitope into the N or C terminus of the ORF, a reverse selection procedure was performed as described in Materials and Methods. As shown in Fig. 1b, we inserted the RpsL-neo gene cassette (Gene Bridges), conferring kanamycin resistance and increased sensitivity to streptomycin. Intermediate BAC clones were isolated based on resistance to kanamycin. The integrity of these clones was checked by digestion with HindIII, and the insertion of the marker cassette in the

correct location was confirmed by PCR as described in Materials and Methods. In a second round of homologous recombination, the RpsL-neo cassette was replaced with the native sequence plus an epitope by counterselection as described in Materials and Methods. Recombinant constructs were isolated based on increased resistance to streptomycin as described previously (51), and the recombinant BAC with insertion of an epitope was screened by PCR. The diagrams in Fig. 1b indicate the relative binding sites of the primers. When an epitope was inserted into the appropriate site in the HCMV BAC, the molecular weights of the PCR products should be increased due to the insertion of an epitope. The proper insertion of an epitope in the recombinant BAC DNAs was checked by PCR using the primer pairs shown in Fig. 1b (data not shown). DNA sequencing confirmed the recombination (data not shown), and the integrity of the mutant BACs was checked by digestion with HindIII (data not shown). We compared the growth of these recombinant viruses with that of the parental virus (wt) at a high or low MOI (3 or 0.01). At the high MOI for RHAUL95UL87myc and at high and low MOIs for RHAUL95UL87mycflagUL79, replication was seen at approximately the same titers as those for the wt (Fig. 1c, d, and e). RUL95HAUL87myc and RUL95HAUL87mycflagUL79 exhibited a slight growth defect at 4 dpi. Since we cannot rule out the possibility that an HA epitope with the UL95 C terminus impairs the function of the UL89 protein, we used RUL95HAUL87mycflagUL79 in subsequent experiments, which rendered results similar to those for RHAUL95UL87myc and RHAUL95UL87mycflagUL79.

UL79, -87, and -95 are required for HCMV growth but have little effect on viral DNA replication. Complementary cells were used to determine the titers of the recombinant viruses, as described in Materials and Methods. The wt virus grew to similar titers in both HFF and complementary cells (Fig. 2a). To determine whether HCMV UL79, -87, and -95 proteins are required for viral growth or DNA replication, HFF cells expressing or not expressing the complementary viral proteins were infected with the recombinant viruses at an MOI of 3. Virus titers were assayed at 1, 4, 5, 7, and 9 dpi, using the complementary HFF cells, and DNAs from equal numbers of infected cells were collected at 2, 3, and 4 dpi as described in Materials and Methods. Recombinant viruses with the UL79, -87, or -95 ORF mutated did not produce infectious virus in HFF cells not expressing the complementary viral protein (Fig. 2b, c, and d, respectively). The y axes in the graphs show relative amounts of viral DNA on a logarithmic scale. The relative amounts of viral DNA were similar in both HFF cell types at the high MOI (Fig. 3a to c).

At the low MOI, viral DNA of RdlUL79 or RdlUL87 was approximately 5-fold lower in the complementary cells than that at 3 dpi or 2 and 4 dpi, respectively, in the HFF cells (Fig. 3d and e). There was no significant difference in the relative amounts of viral DNA of RdlUL95 in both cell types (Fig. 3f). The differences detected with RdlUL79 and RdlUL87 at the low MOI may have been due to the preexpression of UL79 and UL87 in the complementary HFF cells.

Effects of UL79, UL87, and UL95 proteins on MIE gene transcription at a low MOI. To determine why viral DNA replication was lower with RdlUL79 and RdlUL87 at a low MOI, HFF cells in the presence or absence of the complemen-

FIG. 2. UL79, -87, and -95 are required for viral growth. HFF cells in the presence or absence of the complementary proteins were infected at an MOI of 3. Virus titers were determined by TCID₅₀ assay as described in Materials and Methods. (a) Parental virus; (b) RdlUL79; (c) RdlUL87; (d) RdlUL95.

tary viral proteins were infected with the deletion mutants at an MOI of 0.01 and harvested at 1, 2, and 3 dpi. The quantity of MIE RNAs was analyzed by real-time RT-PCR. HCMV MIE gene primers and reporter probes were described previously (19). As shown in Fig. 4a and b, the steady-state levels of the MIE RNAs from RdlUL79- or RdlUL87-infected complementary cells were approximately 5- to 10-fold lower than those in the wild-type HFF cells. There was no significant difference detected with RdlUL95-infected HFF cells in the presence or absence of the complementary viral proteins (Fig. 4c). The lower level of viral DNA replication detected at a low MOI with RdlUL79 and RdlUL87 (Fig. 3d and e) was due to preexpression of the UL79 and UL87 proteins and to the lower level of MIE expression. Preexpression of UL79 and UL87 had no effect on wt virus replication at a high MOI (Fig. 2a). Since there was no difference in viral DNA replication at an MOI of 3, all subsequent experiments were done at a high MOI.

UL79, -87, and -95 proteins are required for expression of true late (gamma 2) viral mRNAs. To determine whether the UL79, -87, and -95 proteins are required for expression of true late (gamma 2) mRNAs, HFF cells in the presence or absence of the complementary viral proteins and in the presence or absence of PAA were infected with the recombinant viruses at an MOI of 3. Cytoplasmic RNA was harvested at 1, 2, or 3 dpi and analyzed by Northern blot analyses, using either the IE1, UL75 (gH), or UL99 (pp28) DNA probe as described in Materials and Methods. The steady-state levels of IE1 RNA were similar at 1 dpi in both cell types infected with the recombinant viruses, as expected (Fig. 5a to c, left panels).

As shown previously (22), for the UL75 probe, three mRNA

size classes were detected that corresponded to the UL75, -76, -77, and -78 mRNAs (Fig. 5a to c, middle panels). These viral mRNAs have different start sites, as reported previously (49). The direction of transcription of UL75 is opposite from that of UL76, -77, and -78 (32). The early-late UL76 to -78 transcripts were greatly reduced in the presence of PAA (Fig. 5b and c). The UL75 transcript is a late transcript and was not detected in HFF cells in the presence of PAA or without expressing the complementary protein (Fig. 5a to c, middle panels).

The UL99 ORF is located in a complex region of the viral genome, within a series of 3'-coterminal transcripts (52). All of the transcripts utilize a common polyadenylation site downstream of the UL99 ORF (52). The pp28 tegument protein is translated from a 1.6-kb mRNA (1). Northern blot analysis using the UL99 probe showed that in the presence of PAA, the 1.6-kb mRNA from the UL99 promoter was not detected, and thus UL99 is a true late gene (Fig. 5b and c, right panels), as reported previously (1). Consistent with a previous report (52), analysis of PAA-treated RNA showed that mRNAs initiating upstream of each of the potential ORFs in this region contained delayed early transcripts (Fig. 5b and c, right panels). The 1.6-kb mRNA was not detected in HFF cells in the presence of PAA or without expressing the complementary protein (Fig. 5a to c, right panels). We concluded that HCMV UL79, -87, and -95 are essential for expression of true late viral mRNAs.

The HCMV UL44 transcription unit initiates at three distinct sites separated by approximately 50 nucleotides and differentially regulated during productive infection. Two of these start sites, the distal and the proximal sites, are active at early times, whereas the middle start site is inactive until late times

FIG. 3. UL79, -87, and -95 are not required for viral DNA replication. HFF cells in the presence or absence of the complementary protein were infected at an MOI of 3 (a to c) or 0.01 (d to f). Viral DNA was quantified by real-time PCR with gB primers and probe as described in Materials and Methods. Real-time PCR with 18S primers and probe were also performed to serve as an internal control. Data are averages for three independent experiments. (a and d) RdlUL79; (b and e) RdlUL87; (c and f) RdlUL95.

(29). Expression from the late start site is dependent upon viral DNA replication and has an effect on late viral gene expression (22). We also determined the effect of UL79, UL87, or UL95 protein on UL44 transcription. To detect all transcripts derived from the different start sites, an RNase protection assay was performed as described in Materials and Methods. Con-

sistent with previous reports (21, 22, 29), when the complementary cells were treated with PAA, the middle transcript was not detected (Fig. 6b, lane 8, and c, lane 6), as shown previously (21, 22). Thus, the middle transcript was a true late viral RNA. Three major transcripts initiating at the spatially distinct start sites were detected in the recombinant virus-infected

FIG. 4. MIE RNA levels are similar in HFF cells at a low MOI but not in HFF cells preexpressing UL79 and UL87. A real-time RT-PCR assay to detect MIE transcripts was performed after infection with the recombinant viruses at a low MOI in HFF cells in the presence or absence of the complementary viral proteins. Total RNA was harvested 1, 2, and 3 days after infection with RdlUL79 (a), RdlUL87 (b), or RdlUL95 (c) at an MOI of 0.01 and was assayed by MIE-specific primers and probe as described in Materials and Methods. The assay was performed in triplicate, and standard errors of the means were determined. HCMV RNAs were normalized to G6PD RNA. Preexpression of UL79 and UL87, but not that of UL95, affected MIE RNA levels at a low MOI.

FIG. 5. Northern blot analyses of late gene transcription after infection with recombinant viruses. HFF cells in the presence or absence of the complementary protein were infected with the mutant recombinant viruses at an MOI of 3. Cytoplasmic RNA was harvested at 1, 2, and 3 dpi in the presence or absence of PAA as described in Materials and Methods. Northern blots are shown for IE1 (left), UL75 (gH) (middle), and UL99 (pp28) (right). 28S and 18S rRNAs served as controls for equal amounts of RNA loading. Lanes in panel a: 1, 3, and 5, HFF cells; 2, 4, and 6, HFF cells expressing the UL79 fusion protein; 1 and 2, 1 dpi; 3 and 4, 2 dpi; 5 and 6, 3 dpi. Lanes in panels b and c: 1, 3, 5, and 7, HFF cells; 2, 4, 6, and 8, HFF cells expressing the UL87 (b) or UL95 (c) fusion protein; 1 and 2, 1 dpi; 3 and 4, 2 dpi; 5 and 6, 3 dpi (b) or 2 dpi in the presence of PAA (c); 7 and 8, 2 dpi in the presence of PAA (b) or 3 dpi (c). (a) RdlUL79-infected cells; (b) RdlUL87-infected cells; (c) RdlUL95-infected cells.

FIG. 6. RNase protection assay for UL44 transcripts after infection with recombinant viruses. Cytoplasmic RNAs were harvested 1, 2, and 3 days after infection with RdiUL79 (a), RdiUL87 (b), or RdiUL95 (c) at an MOI of 3. Twenty micrograms of RNA was hybridized to the 32 P-labeled antisense UL44 promoter probe at 37°C overnight before digestion with RNase T1. The protected RNA fragments were subjected to electrophoresis in denaturing 6% polyacrylamide gels. Lanes in panel a: 1, probe lacking RNase T1; 2, 4, and 6, HFF cells; 3, 5, and 7, HFF cells expressing the UL79 fusion protein; 2 and 3, 1 dpi; 4 and 5, 2 dpi; 6 and 7, 3 dpi. Arrow 1, 2, or 3 indicates the transcript initiating at start site 1, 2, or 3, respectively. Lanes in panels b and c: 1, 3, 5, 7, and 9, HFF cells; 2, 4, 6, 8, and 10, HFF cells expressing the UL87 (b) or UL95 (c) fusion protein; 1 and 2, 1 dpi; 3 and 4, 2 dpi; 5 and 6, 3 dpi (b) or 2 dpi in the presence of PAA (c); 7 and 8, 2 dpi in the presence of PAA (c) or 3 dpi (b).

complementary cells (Fig. 6a, lanes 5 and 7, b, lanes 4 and 6, and c, lanes 4 and 8). In contrast, the middle transcript (arrow 2) was not detected in HFF cells infected with RdlUL79, RdlUL87, or RdlUL95, while the two early transcripts (arrows 1 and 3) were detected (Fig. 6a, lanes 4 and 6, b, lanes 3 and 5, and c, lanes 3 and 7). The amount of proximal transcript (arrow 3) from RdlUL79 or RdlUL95 was a little larger at 2 and 3 dpi or at 2 dpi, respectively, in the HFF cells than in the complementary cells (Fig. 6a, lanes 4 to 7, and c, lanes 3 and 4). In contrast, the amount of distal transcript (arrow 1) of RdlUL79, RdlUL87, or RdlUL95 at 3 dpi in the HFF cells was a little smaller than that in the complementary cells (Fig. 6a, lanes 6 and 7, b, lanes 5 and 6, and c, lanes 7 and 8). These variations in the presence or absence of UL79, -87, and -95 are not understood and require further investigation. Taking these data together, we concluded that true late transcription from the late middle start site of UL44 requires the UL79, UL87, and UL95 viral proteins.

UL79, UL87, and UL95 ORFs encode early viral proteins. Since HCMV ORFs 79, 87, and 95 were similar in function to MHV-68 ORFs 18, 24, and 34, we focused on their time of expression and colocalization with HCMV UL44. We constructed a recombinant virus with epitopes fused to the UL79, UL87, and UL95 ORFs as diagramed in Fig. 1b. To determine the expression kinetics of the UL87 and UL95 fusion proteins, HFF cells were infected with RUL95HAUL87myc or RHAUL95UL87myc at an MOI of 3 in the presence or absence of PAA or GCV. The infected cells were harvested at 1, 2, and 3 dpi and assayed by Western blot analysis using an antibody against the IE1, UL44, or pp28 viral protein or against the HA or myc epitope, as described in Materials and Methods. Figure 7 shows the results for the RUL95HAUL87myc-infected cells. The tagged fusion proteins were detected at 1 to 3 dpi and at 2 dpi in the presence or absence of PAA or GCV. These tagged fusion proteins were also detected in cells transfected with a plasmid containing the corresponding tagged ORF (data not shown). The expression level of the UL95 fusion protein was modestly decreased when the cells were treated with PAA or GCV, similar to that of the early viral protein of the UL44 gene (Fig. 7, compare panels a and d). The steady-state levels of these two early-late viral proteins in the virus-infected cells were affected by the inhibition of viral DNA replication. In contrast, the expression levels of the UL87 protein were similar at 1 and 2 dpi in the presence or absence of PAA or GCV (Fig. 7b). Whether posttranslational modification of these fusion proteins is essential for viral late transcripts is currently unknown. The role of the posttranslational modification in viral late gene transcription requires further investigation.

To determine the expression kinetics of the UL79 fusion protein, HFF cells were infected with RUL95HAUL87mycflagUL79 at an MOI of 3 in the presence or absence of PAA or GCV. The infected cells were harvested at 1, 2, and 3 dpi and assayed by Western blot analysis using an antibody against the Flag epitope as described in Materials and Methods. The tagged fusion protein was detected at 1 to 3 dpi and at 2 dpi in the presence or absence of PAA or GCV (Fig. 7c). The expression kinetics of the UL79 protein was similar to that of the UL87 protein (Fig. 7b and c), but there were two slower-migrating bands detected (Fig. 7c). The expression level of the highest

band was dramatically increased at 2 dpi, and when the cells were treated with PAA or GCV, the highest band almost disappeared (Fig. 7c). In contrast, the middle band was constant at 1, 2, and 3 dpi in the presence or absence of PAA or GCV (Fig. 7c). The slower-migrating UL79 protein may be modified differently after viral DNA replication, and these observations require further investigation. A faster-migrating protein in the absence of PAA or GCV at 2 and 3 dpi may be a degradation product (Fig. 7c). Figure 7d shows the expression of the MIE proteins and the UL44 and UL99 (pp28) proteins in HFF cells infected with RUL95HAUL87mycflagUL79 at an MOI of 3 in the presence or absence of PAA or GCV. Inhibitors of viral DNA replication had a slight effect on the levels of the MIE and UL44 viral proteins. pp28 protein expression was detected at 2 dpi and increased dramatically at 3 dpi. When the cells were treated with PAA or GCV, the expression of pp28 declined significantly. Taken together, these results indicate that UL79, UL87, and UL95 are expressed at early times after infection.

UL79, -87, and -95 proteins are recruited to replication compartments. The HCMV replication protein ppUL44 is localized to RCs. BrdU is incorporated into viral DNA in these compartments and is found in progeny virus (37, 39). To determine whether UL79, -87, and -95 proteins are recruited to the RCs, HFFs were infected with RUL95HAUL87myc or RHAUL95UL87myc at an MOI of 3. At 1 to 3 dpi, the cells were fixed and incubated with either a rat polyclonal antibody against the HA epitope or a mouse monoclonal antibody against the myc epitope, as described in Materials and Methods. Secondary anti-rat IgG conjugated with Alexa Fluor 594 and anti-mouse IgG conjugated with Alexa Fluor 680 were used. Figure 8a to e show the results for RUL95HAUL87myc-infected cells. There were foci staining with UL95 protein that were apparently devoid of UL87 protein at 1 dpi (Fig. 8a). Recruitment of the viral proteins into distinct foci was complete at 2 dpi (Fig. 8b). UL95 fusion protein also colocalized with UL44 protein at 2 dpi (Fig. 8d). In addition, FISH analysis demonstrated that the UL44 protein was colocalized with viral DNA (Fig. 8e). These results indicated that viral UL87 and UL95 proteins were colocalized and that UL95 was recruited into RCs.

To determine whether the UL79 protein was also recruited into the RCs, HFFs were infected with RUL95HAUL87mycflagUL79 or RHAUL95UL87mycflagUL79 at an MOI of 3. At 1 to 3 dpi, the cells were fixed and incubated with either a rabbit polyclonal antibody against the Flag epitope or a mouse monoclonal antibody against UL44 protein, as described in Materials and Methods. Secondary anti-rabbit IgG conjugated with Alexa Fluor 594 and anti-mouse IgG conjugated with Alexa Fluor 680 were used. Foci of the UL79 protein were formed at 1 dpi. While the UL79 protein was not completely colocalized with the UL44 protein at 1 dpi, it was so at 2 and 3 dpi (Fig. 8f to h). Taken together, these observations show that the UL79, -87, and -95 proteins colocalize to the viral RCs.

UL79, UL87, and UL95 proteins are recruited to micro-foci of UL44 protein before viral DNA replication. To determine whether UL79, -87, and -95 colocalized with UL44 before viral DNA replication, HFFs were infected with RUL95HAUL87myc at an MOI of 3 in the presence or absence of PAA or GCV. The cells were fixed as described

FIG. 7. UL95-HA, UL87-myc, and Flag-UL79 fusion proteins are expressed at early times after infection. Viral DNA synthesis was inhibited with PAA or GCV. Western blot analysis was performed using an antibody against immediate-early proteins pIE86 and pIE72 (UL122 and -123), UL44, the HA, myc, or Flag epitope, or GAPDH at the indicated times after infection with RUL95HAUL87myc or RUL95HAUL97mycflagUL79 in the presence or absence of PAA or GCV as described in Materials and Methods. GAPDH served as a loading control. (a) Anti-HA antibody. (b) Anti-myc antibody. (c) Anti-Flag antibody. (d) Anti-IE72/IE86, anti-UL44, anti-p28, and anti-GAPDH antibodies.

in Materials and Methods and then treated with polyclonal antibody against the HA or myc epitope and with monoclonal antibody against the UL44 protein as described in Materials and Methods. Secondary anti-rat or -rabbit IgG conjugated with Alexa Fluor 594 and anti-mouse IgG conjugated with Alexa Fluor 680 were used. After FISH analysis to detect viral DNA as described in Materials and Methods,

cells were incubated with a monoclonal antibody against the UL44 protein and then with the secondary antibody anti-mouse IgG conjugated with Alexa Fluor 680. As expected, UL44 colocalized with the viral DNA in the presence of an inhibitor of viral DNA synthesis (Fig. 9a and b). UL87 and UL95 colocalized with UL44 in the presence of PAA or GCV (Fig. 9c to f). HFF cells were also infected with

FIG. 8. Localization of UL95-HA, UL87-myc, and Flag-UL79 fusion proteins, UL44 protein, and viral DNA after infection with recombinant viruses. The cells infected with RUL95HAUL87myc (a to e) or RUL95HAUL87mycflagUL79 (f to h) were harvested at 1 dpi (a and f), 2 dpi (b, d, e, and g), or 3 dpi (c and h). Cells were fixed and treated with antibody for microscopy or for FISH analysis as described in Materials and Methods. (a to c) Colocalization of UL95-HA protein with UL87-myc protein. (d) Colocalization of UL95-HA protein with UL44 protein. (e) Colocalization of viral DNA with UL44 protein. (f to h) Colocalization of Flag-UL79 protein with UL44 protein.

RUL95HAUL87mycflagUL79 and assayed for colocalization of UL79 with UL44. As shown in Fig. 9g and h, UL79 also colocalized with UL44 in the presence of PAA or GCV. We could not costain UL79, UL87, and UL95 with viral DNA after FISH analysis because the protein expression levels were significantly lower than those of UL44 and be-

cause FISH analysis required heat denaturation, which affects the viral proteins. However, to demonstrate the specificity of the colocalization with UL44, we tested the localization of UL57 and UL44. For HCMV, it was reported that the UL44 protein was not associated with small punctate foci containing the UL57 protein prior to viral DNA

FIG. 9. Localization of UL95-HA, UL87-myc, or Flag-UL79 fusion protein after infection with recombinant viruses in the presence of PAA or GCV. Cells infected with RUL95HAUL87myc (a to f and i) or

synthesis (37). Cells were fixed and treated with antibodies against the myc epitope fused to UL87 and with antibodies against the UL57 protein as described in Materials and Methods. Secondary anti-rabbit IgG conjugated with Alexa Fluor 594 and anti-mouse IgG conjugated with Alexa Fluor 680 were used. In the presence of PAA, the UL87 fusion protein did not colocalize with the UL57 protein (Fig. 9i). Taken together, the results show that HCMV UL79, -87, and -95 proteins colocalize with UL44 before viral DNA replication.

DISCUSSION

MHV-68 ORFs 18, 24, and 34 encode viral transacting factors for late transcription. These MHV-68 ORFs are expressed early but are not required for viral DNA replication. Since these MHV-68 proteins activate late viral promoters driving expression of an indicator gene, they are considered late viral transactivators (3, 54, 55). How late viral transactivators specifically activate a late viral promoter remains unclear. An understanding of how late viral promoters are activated would contribute to new approaches for unique antivirals. Since HCMV is a serious pathogen and current antivirals have limited efficacy and adverse effects, an analysis of HCMV late transcription factors is important for the development of new antivirals. Therefore, we determined whether the HCMV gene products homologous to the MHV-68 late viral transactivators affect the expression of true late (gamma 2) HCMV genes.

In HCMV, the promoters are simple in that they contain a core element without any requirement for upstream or downstream sequences (8, 26, 32). Transcription of UL44 initiates at three distinct start sites, which are differentially regulated during productive infection. Two of these start sites, the distal and proximal sites, are active at early times, and the middle start site is active only at late times after infection (29). The UL44 early viral promoters have a canonical TATA sequence. In contrast, the UL44 late viral promoter has a noncanonical TATA sequence (Fig. 6a). We reported that the noncanonical TATA sequence is important for late viral gene expression (21). Serio et al. (40) also reported that temporal expression from the BcLF1 late promoter of EBV is solely dependent upon a variant TATA element. Weak binding affinity of TBP for a noncanonical TATA sequence may explain in part a lack of transcription at early times after infection. It is possible that the binding affinity of TBP to a late promoter becomes stronger after the recruitment of late gene-specific transactivators and the opening of the double helix during viral DNA replication. Why the IE2 transactivators alone are not sufficient for late gene transcription is not understood. One explanation is

RUL95HAUL87mycflagUL79 (g and h) in the presence of PAA (a, c, e, g, and i) or GCV (b, d, f, and h) were treated at 2 dpi as described in Materials and Methods. (a and b) Colocalization of HCMV DNA and UL44 protein. (c and d) Colocalization of UL95-HA and UL44 proteins. (e and f) Colocalization of UL87-myc and UL44 proteins. (g and h) Colocalization of UL79-Flag and UL44 proteins. (i) Localization of UL87-myc and UL57 proteins.

that the late transactivators stabilize the binding of IE2, TBP, and RNA polymerase II to the late promoter.

At a low MOI, preexpression of UL79 or -87 negatively affected the level of MIE viral gene expression and viral DNA replication. It is also possible that this suppressive effect on the MIE promoter is required for efficient late gene transcription.

With HCMV, there are several isomers of the IE2 gene that affect early/late viral gene expression. The viral IE2-p86 protein is essential for early viral gene expression, while the IE2 isomers IE2-p60 and IE2-p40 have significant effects on late viral gene expression (50, 51). Complexes between viral DNA and the viral IE2 transactivators were detected when viral DNA started to replicate, giving rise to the development of RCs (43). Although we found HCMV UL79, UL87, and UL95 colocalized with UL44 protein in viral RCs, there is currently no evidence that the two viral proteins interact or that the IE2 protein interacts with UL79, UL87, and UL95.

Our data indicate that HCMV UL79, -87, and -95 are required for transcription of the UL44, UL75 (gH), and UL99 (pp28) late mRNAs. The UL79, -87, and -95 viral proteins are expressed before viral DNA replication and assemble at the viral RCs. RCs serve as foci for viral gene expression, in part by concentrating the viral DNA templates. The UL44 protein assembles at the sites of IE2 accumulation before viral DNA replication. Since IE2 protein is not sufficient for late gene transcription, it is very likely that recruitment of the UL44 protein along with UL79, -87, and -95 into the IE2 foci provides an environment appropriate for late gene transcription. There is no evidence that the UL44 protein interacts directly with the UL79, -87, or -95 protein. The UL44 protein is structurally homologous to the eukaryotic proliferating cell nuclear antigen (PCNA), which interacts with multiple proteins (31). Recent proteomic analyses demonstrated that the UL44 protein interacts with a variety of proteins, including transcription factors other than viral replication-related proteins (13, 45–47). Therefore, it is conceivable that pre-RCs including UL44 protein act to optimize the timing of late gene transcription. We transfected the expression plasmid(s) for UL79, UL87, and/or UL95 with IE2 and/or UL44 and a reporter driven by the UL75 or UL99 late promoter (22) in the absence of the HCMV ori and the viral proteins necessary for viral DNA replication into HFF cells and examined whether the reporter gene was activated. While the IE2 protein efficiently activated the early UL54 (HCMV DNA polymerase) promoter in the reporter assay with HFF cells, the UL75 and UL99 late promoters were not activated (data not shown). These results suggest that for HCMV, pre-RCs and RCs are required for the activation of late viral gene promoters. The complete array of viral proteins necessary for activation of a late viral promoter requires further investigation.

The HCMV RCs also contain five viral proteins homologous to herpesviral replication proteins (i.e., HSV ICP8, UL9, and the heterotrimeric helicase-primase complex UL5-UL8-UL52) that are initially assembled into viral prereplication microfoci, and the polymerase and polymerase accessory protein are subsequently recruited to these foci (4). However, when PAA was added to the medium, the HCMV UL87 protein was not colocalized with the SSB UL57 protein. With HCMV, microfoci of the HCMV UL57 protein do not seem to act as a scaffold to organize late gene transcription but may play an important role

after the initiation of viral DNA replication. Further investigation is necessary to understand when late transcription is initiated relative to the initiation of HCMV DNA replication.

In summary, we showed that UL79, -87, and -95 proteins are required for late viral gene expression and, consequently, for viral growth. We also showed that UL79, -87, and -95 proteins assemble into RCs with UL44 before viral DNA replication. HCMV, like MHV-68, controls the expression of viral genes via several viral transacting proteins that facilitate late viral gene expression after viral DNA replication. How these viral transactivators of late gene expression function will be the subject of further investigation.

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