Identification of the Interaction Domain of the Small Terminase Subunit pUL89 with the Large Subunit pUL56 of Human Cytomegalovirus†

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ABSTRACT: The small terminase subunit pUL89 of human cytomegalovirus (HCMV) is thought to be required for cleavage of viral DNA into unit-length genomes in the cleavage/packaging process. Immunoprecipitations with a UL89-specific antibody demonstrated that pUL89 occurs predominantly as a monomer of approximate M_r 75.000 together with a dimer of approximate 150.000. This was confirmed by gel permeation chromatography. In view of its putative function, pUL89 needs to be transported into the nucleus. By use of laser scanning confocal microscopy, pUL89 was found to be predominantly localized throughout the nucleus and in particular in viral replication centers of infected cells. By immunofluorescence, we demonstrated that both terminase subunits co-localized in viral replication centers. Furthermore, analysis with pUL89 GST-fusion protein mutants showed that amino acids 580−600 may represent the interaction domain with pUL56. To verify this result, a recombinant HCMV genome was constructed in which the UL89 open reading frame was disrupted. By transfection of the deletion BACmid alone, we showed that it has a lethal phenotype. Cotransfection assays demonstrated that, in contrast to pUL89 wild-type, a plasmid construct encoding a pUL89 variant without aa 580−590 as well as one encoding a variant without aa 590−600 could not complement the HCMV-pUL89 null genome, thus, suggesting that the 20 aa sequence GRDKALAVEQFISRFNSGYIK is sufficient for the interaction with pUL56 and in conclusion required for DNA packaging.

Human cytomegalovirus (HCMV)[1], one of eight human herpesviruses, can cause serious, life threatening diseases. Maturational events of HCMV DNA replication and capsid assembly require efficient translocation of viral gene products into the nucleus of infected cells. Several gene products of HCMV have been identified that appear to be involved in the process of DNA replication, cleavage, and packaging (*1*). DNA replication leads to the formation of head-to-tail linked concatemeric DNA which must be resolved into unit-length genomes during packaging. Proteins involved in viral DNA cleavage and packaging are so-called terminases. Terminases were first described as specific enzymatic proteins for dsDNA bacteriophages with multiple activities required for the whole packaging process (*2*−*4*). Terminases are heteromultimers composed of a small subunit that binds the DNA and a large subunit that has, besides an endonuclease activity, an ATPase activity mediating the translocation of DNA into the preformed capsid (*5*−*13*).

Our previous results together with the observations from Underwood et al. (*14*) and Krosky et al. (*15*) indicate that the terminase of human cytomegalovirus consists of the highly conserved proteins pUL56 and pUL89 (*16*−*21*). While it is supposed that the large terminase subunit pUL56 (i) mediates the specific binding to packaging elements, so-called *pac* motifs, on the concatamers; (ii) provides energy for translocation of the DNA to the procapsids; and (iii) associates itself with the capsid for enabling the entry of the DNA, the small subunit pUL89 seems to be mainly required for cleavage of concatemeric DNA into unit-length genomes (*20*). Both proteins are highly conserved throughout the herpesviruses. Recently, we demonstrated by structural analysis of single particles that pUL56 forms dimers of two open rings, a characteristic of DNA metabolizing proteins (*22*).

Adelman et al. (*23*) demonstrated that the HSV-1 homologue of pUL56, pUL28, plays a role in DNA packaging as a sequence-specific DNA-binding protein. Despite the importance of these proteins and the intensive research into biochemical and functional aspects of terminases, there is still a lack of information regarding their intracellular localization. In this study, we characterized the small terminase subunit pUL89 of HCMV. We could demonstrate that (i) the protein is expressed as a 75 kDa protein and has a tendency to dimerize, (ii) it is translocated in infected cells

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[1] Abbreviations: HCMV, human cytomegalovirus; BAC, bacterial artificial chromosome; GST, glutathione-*S*-transferase; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; IPTG, isopropyl β-D-1-thiogalactopyranoside; MOI, multiplicity of infection; PAGE, polyacrylamide gel electrophoresis; PAA, phosphonoacetic acid; PMSF, phenylmethanesulfonyl fluoride; ORF, open reading frame.

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Table 1: Oligonucleotide Primers Used in PCR

Construct	Sense primer (5' - 3')	Antisense primer (5' - 3')
GST-UL89-1	CGGGATCCATGTTGCGCGGAGACTCGGCC	CCGCTCGAGGATGGAGGTCTGCGAGAGGAAGTCG
GST-UL89-2	CGCGGATCCCGCGTGCTGCAGTACCTCATCCAC	CCGCTCGAGCACTTGCGAGTTGAGGCTGATGAAGG
GST-UL89-3	CGGATCCCCCACCTTCATCAGCCTCAACTCGC	CCGCTCGAGTTTGATGTAGCCCGAGTTGAAACGC
GST-UL89-4	CGGATCCGACATCTTTAAGCAGAAAGCCACCGTGTTC	CCGCTCGAGCTAGCTGACCCTGAAACGGATGGC
GST-UL89-5	CGCGGATCCCGCGACAAGGCGCTGGCCGTGGAACAG	CCGCTCGAGGATGTAGCCCGAGTTGAAACGCGAGAT
	TTCATCTCGCGTTTCAACTCGGGCTACATC	GAACTGTTCCACGGCCAGCGCCTTGTCGCG

into viral replication centers, (iii) aa 580−600 are required for the interaction with the large terminase subunit pUL56, and (iv) a UL89 construct without this region was unable to complement a UL89-null genome.

MATERIAL AND METHODS

Cells and Viruses. Human foreskin fibroblasts, HFF-cells, at passage 10−20 were grown in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin (5 U/mL), and streptomycin (50 μg/mL). Preparation of HCMV AD169 and infection of HFF at an MOI of 3 was carried out as described before (*24*). Inhibition of the viral polymerase was achieved by addition of PAA (200 μg/mL) during the entire infection.

Insect cells 5B1-4 (High five) were grown in TC-100 medium supplemented with glutamine, gentamicin (60 μg/mL), and 10% fetal calf serum. Recombinant baculovirus expressing rpUL89 containing an N-terminal Anti-Xpress antibody epitope was generated by site-specific transposition of the expression cassette into the bacmid bMON14272 as described by Life Technology.

BAC Mutagenesis. For generation of HCMV-GFP−BAC-ΔUL89, exon 2 of ORF UL89 (nt 133449−134499) was deleted in the parental BAC HCMV-GFP by the ET mutagenesis procedure using linear PCR fragments (*25*). A PCR fragment was generated using the following oligonucleotides: UL89.for, 5'-ACCCTGAACGGATGGCGTGTATATCGTCACACAGGTAGGTGGCCATGATGCCCCGAAAAGTGCCACCTGCAGAT-3'; UL89.rev, 5'-TCTGTCTCCTCTCTACACAGAGCATCCGAGGACAAAACTTCCACTTGCTGGTGACACAGGAACACTTAACGGCTGA-3'containing approximately 20 nt homologous to the kanamycin resistance gene of pori6K-F5 (*26*) and 50 nt homologous to the regions upstream and downstream of UL89 exon 2. The resulting fragment was introduced into *Escherichia coli* containing the parental HCMV-GFP-BAC for homologous recombination as previously described (*27*). Bacterial clones were selected on agar plates containing 25 μg/mL kanamycin plus 17 μg/mL chloramphenicol and checked for the presence of mutant BACs by restriction analysis and agarose gel electrophoresis. The kanamycin resistance marker was subsequently excised by site-specific recombination in *E. coli* using *Flp* recombinase (*28*−*29*).

BAC Isolation. BAC DNA was obtained from 500-mL overnight cultures using Nucleobond PC500 columns (Macherey Nagel, Düren, Germany) according to the instructions of the manufacturer.

Immunofluorescence and Immunoprecipitation. For immunofluorescence, HFF cells were grown on cover slips. At the appropriate time point, cells were fixed with 3% paraformaldehyde as described previously (*30*).

Detection of HCMV pUL89 was carried out with pAb-UL89 (*19*) for 45 min at room temperature prior to further incubation for 45 min with Cy3-labeled goat anti-human $F(ab')_2$ fragments. For double staining, HCMV pUL89-specific human pAbUL89 and IE1-specific monoclonal antibody mAb pp63−27 (provided by T. Stamminger, 31) was used in the first incubation. In the second incubation, Cy3-labeled anti-human and Cy2-labeled anti-mouse $F(ab')_2$ fragments were used.

For immunoprecipitation, infected or mock-infected cells (T-75 cm^2 flasks; 2×10^6 cells) were radiolabeled 60 h post-infection with 50 μCi/mL [^{35}S]methionine for 12 h. Total cell extracts were prepared from labeled cultures by solubilization in immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% NP-40, 5 mM EDTA, 25 mM iodacetamide, 0.4% sodiumdeoxycholate, 1 mM PMSF, and 100 U/mL Trasylol) and ultrasonic treatment. Insoluble material was sedimented for 30 min at 100 000*g*. Comparable amounts of extracts and pUL89-specific antibody (1:20) were used for precipitation as described previously (*16*).

Plasmid Construction and Oligonucleotides. Plasmid GST-UL89 was generated by digestion of pcDNA-UL89 (*20*) and pGEX-5X-1 (Amersham Pharmacia Biotech, Freiburg, Germany) as described previously (*19*). The 2.025 bp fragment encoding the HCMV pUL89 was ligated in-frame into pGEX-5X-1, yielding pGEX-UL89.

Oligonucleotide-directed mutagenesis of GST-UL89 was performed. Constructs GST-UL89-1, GST-UL89-2, GST-UL89-3, GST-UL89-4, and GST-UL89-5 were generated using the QuickChangeTM site-directed mutagenesis kit (Stratagene) as recommended by the supplier and pairs of synthetic oligonucleotides as described in Table 1 (construct GST-UL89 served as template). The construct GST-UL89-5 with overlapping regions between GST-UL89-3 and GST-UL89-4 was chosen based on computer analysis that suggests aa residues 580−600 are exposed at the surface of the protein. All mutations were verified by DNA sequencing.

For construction of plasmids pUC-UL89P, pUC-UL89PΔ10aa1, pUC-UL89PΔ10aa2, and pUC-UL89PΔ9aa, respective fragments were obtained using cosmid pCM1106 as a template for PCR and ligation into pUC-18. The pairs of synthetic oligonucleotides are described in Table 2.

Table 2: Oligonucleotide Primers Used in PCR

Construct	Sense primer (5' - 3')	Antisense primer (5' - 3')
pUC-UL89P	CCCAAGCTTGCACCTCGGCCGATTCGCGCGGAATTACG	CGCGGATCCTTGGAGGCGAGCGTGGTGGCGGCGGCGGTAG
pUC-UL89PΔ10aa1 (1740-1770 bp)	CGGGATCCATGTTGCGCGGAGACTCGGCC	CCGGAATTCCTAGCTGACCCTGAAACGGATGG
pUC-UL89PΔ10aa2 (1770-1800 bp)	CCGCTCGAGGCCTCGCAAGAGCTCGTCTCC	CCGCTCGAGGAACTGTTCCACGGCCAGCGCC
pUC-UL89PΔ9aa (300-327 bp)	CGTTTCGCCCGCAGAAGCTGGACG	CGTCCAGCTTCTGCGGGCGAAACG

Table 3: pUL89 Mutants Used in this Study

construct	DNA sequence w/o GST	amino acid sequence w/o GST	Predicted M_r with GST
GST-UL89	1−2025 bp	1−675	101.000
GST-UL89-1	1−600 bp	1−200	48.000
GST-UL89-2	541−1200 bp	180−400	50.200
GST-UL89-3	1171−1800 bp	390−600	49.100
GST-UL89-4	601−2025 bp	200−675	78.250
GST-UL89-5	1741−1800 bp	580−600	28.200

Protein Purification. A fresh overnight culture of *E. coli* BL21 carrying the plasmid pGEX-UL89, pGEX-UL89-1, pGEX-UL89-2, pGEX-UL89-3, pGEX-UL89-4, pGEX-UL89-5, or pGEX-5X-1 was diluted 1:100 in LB medium (1 L) containing 50 μg/mL ampicillin and incubated at 37 °C. After the cells reached an OD_{600} of 0.5, the GST-protein expression was induced by addition of IPTG to a final concentration of 0.1 mM and incubated for 2 h at 37 °C. Sedimented cells were lysed in 40 mL binding buffer (20 mM sodium phosphate, 0.15 M NaCl, pH 7.4) containing 250 μL 1 M $MgCl_2$, 25 μL 1 M MnCl, 40 μL of DNase I (10 mg/mL), and 400 μL of lysozyme (10 mg/mL), incubated on ice for 30 min, and sonicated on ice. Undissolved material was sedimented for 40 min at 5 000*g* at 4 °C and passed through a 0.45 μm filter. The purification was performed with a 1 mL GSTrap column by using an Äkta Prime machine (Amersham Pharmacia Biotech) according to the instruction of the manufacturer. The column was washed with 3 bed volumes of binding buffer prior to loading the proteins. Elution was performed with 10 bed volumes of elution buffer (50 mM Tris-HCl, 10 mM glutathione, pH 8.0), and 10 fractions were collected. The fractions were analyzed by SDS−PAGE. Fractions containing the GST-UL89 proteins (Table 3) were stored at −80 °C.

Protein Purification of Recombinant, Baculovirus-Expressed rpUL89. High-five cells (4×10^8) expressing the recombinant pUL89 (*20*) were harvested 48 h post-infecton. Sedimented cells were lysed in 50 mL of cation exchange buffer (20 mM MES, pH 6.5, 150 mM NaCl, and protease inhibitors) and sonicated on ice. Cell lysates were sedimented and passed through a 0.2 μm filter prior to loading onto an equilibrated anion exchange column (HiTrap Q HP, 1 mL bed volume; Amersham Bioscience). The purification was performed at 4 °C by using an ÄKTA Prime (Amersham Bioscience) as described before (*20*). Fractions containing the protein were subjected to a second chromatographic step using a molecular size exclusion column (HiLoad 16/60 Superdex 200 prep grade) and an Äkta FPLC (Amersham Bioscience) as described (*20*). Molecular mass markers, thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) were analyzed by gel permeation chromatography as well.

In Vitro Translation. Plasmid pcDNA-UL56 (0.5 μg) and plasmid pHM123 (0.5 μg), encoding HCMV IE1, were incubated with [^{35}S]methionine (10 mCi/mL) and 20 μL of TNTT7 Quick Master Mix (Promega, Madison, WI) in a final volume of 30 μL for 1 h at 30 °C. Translation products were analyzed by SDS−PAGE.

SDS−PAGE and Autoradiography. The proteins of the supernatants or precipitated proteins were separated on 10% polyacrylamide gels, transferred to nitrocellulose sheets, and subjected to Western blot analysis or stained with coomassie brilliant blue. The antibody pAbUL89 (1:20) specific for pUL89 was used as the primary antibody prior to incubation with horseradish peroxidase-conjugated anti-human $F(ab')_2$ fragments (1:500). After immunostaining, radioactive blots or gels were subjected to autoradiography.

In Vitro Binding Assay. For in vitro binding analysis, equal amounts of GST fusion proteins (see above) loaded on glutathione-sepharose 4B (Amersham Biosciene) were incubated with in vitro translated pUL89 or IE1 for 2 h at 4 °C in 500 μL of binding buffer (0.05% Nonidet P-40, 50 mM Hepes, 10% glycerol, 0.1% BSA, and 300 mM NaCl, pH 7,3). Samples were washed with binding buffer and subsequently subjected to SDS−PAGE, consecutive fixation, and autoradiography. For quantification of radioactive signals, a BioImager (Raytest, Germany) was used.

PreScission Protease Treatment. To cleave the GST-tag from GST-UL89, after binding to the GSTrap column, the protein was incubated with 80 U PreScission protease (Amersham Bioscience) for 12 h at 4 °C under rotation. After removal of the GST-tag from GST-UL89 during incubation with the protease, the resulting proteins were eluted in 10 fractions (1 mL) of PreScission buffer (50 mM Tris, pH 7.0, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT). The fractions containing the protein (0.97 mg/mL) were stored at −80 °C.

Protein/Protein Interaction by Chemical Cross-Linking. pUL89 (300 ng) was incubated for 5 min at room temperature

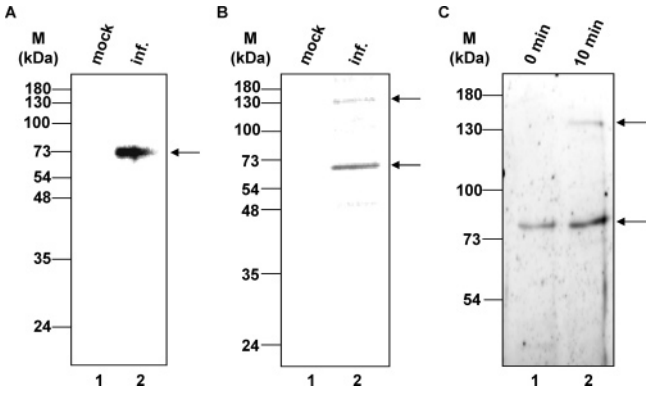

FIGURE 1: Identification of HCMV pUL89 in infected HFF cells. (A) Lysates of mock-infected (lane 1) or HCMV-infected HFF cells (lane 2) at 72 h post-infection were subjected to 8% SDS−PAGE prior to transfer onto a nitrocellulose membrane. Immunoblot analysis was performed using the pUL89-specific antibody pAbUL89. (B) HCMV infected (lane 2) or mock-infected cells (lane 1) were radiolabeled 60 h post-infection with 50 μCi/mL [^{35}S]-methionine for 12 h. At 72 h post-infection, cells were immunoprecipitated with pAbUL89 prior to 8% SDS−PAGE and autoradiography. (C) Analysis of the association of pUL89 with itself by protein cross-linking using a bacterial fusion protein. The purified bacterial protein pUL89 was either untreated or reacted with glutaraldehyde for 10 min, followed by SDS−PAGE and Western blot analysis.

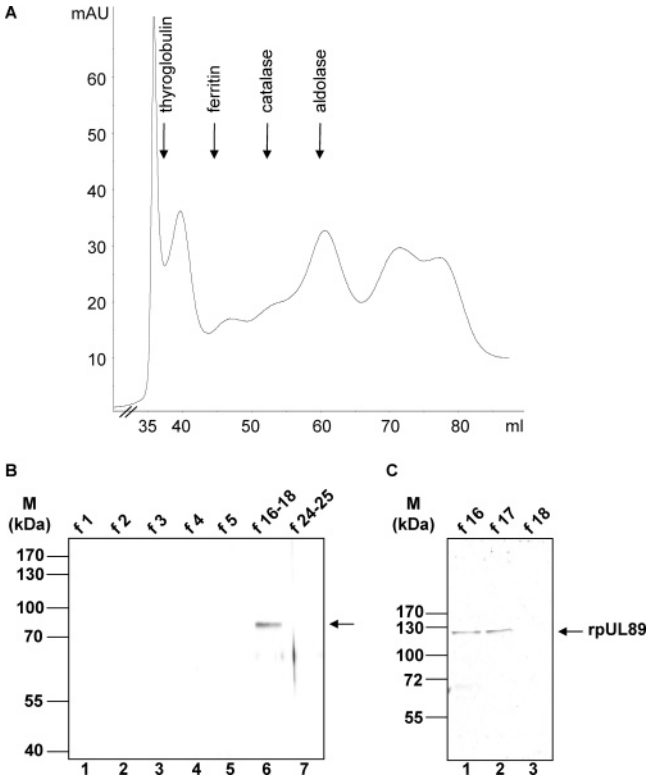

FIGURE 2: Purification of pUL89. (A) Gel permeation chromatography of rpUL89. The protein was subjected to chromatography through a HiLoad 16/60 Superdex 200 prep column using an ÄKTA Explorer. The flow-through was recorded at OD_{280}. The column was calibrated with the molecular weight standards aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) as indicated by arrows. (B) Analysis of the peak fractions by urea gel electrophoresis. The fractions f1−f5 (corresponding to 35−42.5 mL), f16/17/18 (corresponding to 58−62.5 mL), and f24/25 (corresponding to 70−73 mL) were separated on the gel prior to immunoblot analysis with monoclonal antibody against the express tag of rpUL89. (C) Analysis of fractions 16, 17, and 18 by 8% SDS−PAGE and Western blot analysis using the anti-Xpress antibody. The molecular weight markers (M) are indicated on the left.

in PBS in a final volume of 20 μL. The cross-linking reaction was started by addition of 0.1% (v/v) glutaraldehyde to a final concentration of 0.01%. The reaction was stopped by addition of 10 μL of SDS-loading buffer and denaturation of the samples for 2 min at 95 °C. The proteins were separated by 8% SDS−PAGE and analyzed by Western blot with the specific antibody pAbUL89.

Cotransfection Assays. For transient complementation of the HCMV pUL89-null genome, 1 μg of HCMV-GFP-BAC-ΔUL89, together with 2 μg off either pUL89 expression plasmid pUC-UL89P, pUC-UL89PΔ10aa1 (Δ aa580−590), pUC-UL89PΔ10aa2 (Δ aa590−600), or pUC-UL89PΔ9aa (Δ aa100−109), was transfected into hTERT-RPE1 cells (Clontech) using the Transfectin reagent (Bio-Rad) according to the manufacturer's instructions. Ten days after transfection, the cells were analyzed for plaque formation using fluorescence microscopy. The parental HCMV-GFP-BAC and HCMV-GFP−BAC-ΔUL89 transfected together with HCMV cosmid clone pCM1106 (*32*) comprising the UL89 ORF plus flanking homologous regions were used as positive controls.

RESULTS

Characterization of pUL89 in Infected Cells. To determine the expression of pUL89 in infected cells, immunoblots with mock and HCMV-infected cells were performed. The monospecific antibody pAbUL89 (*19*), which was purified from hightiter human serum by column affinity chromatography (Affigel 10/15-pUL89), recognized a 75 kDa protein in HCMV-infected cells (Figure 1A, lane 2), that corresponds with the predicted size of the amino acid sequence. In the control, no protein was detected (Figure 1A, lane 1). To further investigate the presence of pUL89 in infected cells, immunoprecipitation with pAbUL89 was performed prior to SDS−PAGE and autoradiography. Interestingly, the 75 kDa protein and an additional band of approximately 150 kDa were detected in infected cells (Figure 1B, lane 2). It is reasonable to speculate that the latter protein may represent a dimer of pUL89 and that pUL89 also forms a dimer as the large terminase subunit pUL56. Therefore, the interaction of pUL89 with itself was examined by chemical protein cross-linking using a bacterial fusion protein. The GST-UL89 fusion protein was purified including cleavage of the GST-tag and subjected to treatment with glutaraldehyde for 0 and 10 min before the proteins were separated by SDS−PAGE and analyzed by immunoblotting. Again, a new protein band appeared at 150 kDa, indicative of the dimer (Figure 1C, lane 2). To further analyze the molecular size of pUL89, the protein was expressed as a recombinant protein utilizing a baculovirus system and subjected to molecular size-exclusion chromatography together with marker proteins (Figure 2A). The peak fractions f1−f5 (corresponding to 35−42.5 mL), f16/17/18 (corresponding to 58−62.5 mL), and f24/25 (corresponding to 70−73 mL) were analyzed by urea gel electrophoresis followed by Western blot (Figure 2B). Interestingly, the only observed rpUL89 was in fraction 16−18, thus, indicating that pUL89 neither forms high molecular weight complexes (fraction 1−5) nor monomers under native conditions (fraction 24/25). Analysis of fractions 16 and 17 by 8% SDS−PAGE and Western blot analysis demonstrated

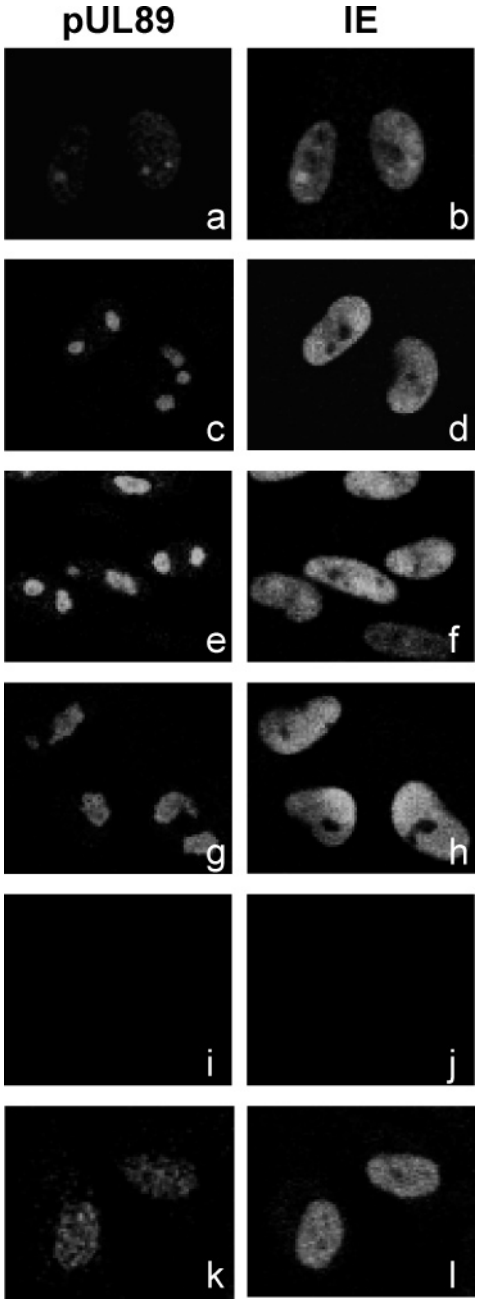

FIGURE 3: Confocal microscopy analysis of the intranuclear distribution of HCMV pUL89. HFF cells were mock-infected (i and j) or infected with HCMV for 12 (a and b), 24 (c and d), 48 (e and f), and 72 h (g and h) and subjected to immunofluorescence with antibodies directed against pUL89 (pAbUL89; panels a, c, e, g, and i) and against the IE1 protein (mAb63−27; panels b, d, f, h, and j). HFF cells infected with HCMV in the presence of PAA were stained with antibodies against pUL89 (k) and against IE1 (l) at 72 h post-infection, respectively.

that the detected protein migrated with an M_r of 150.000, the molecular mass of a dimer (Figure 2C). These results demonstrated that pUL89 is expressed in infected cells as a monomer of 75 kDa with a tendency to dimerize.

Intracellular Distribution of pUL89. To examine the precise intracellular distribution of pUL89, immunofluorescence was performed. Coverslip cultures were mock-infected or infected with HCMV (MOI of 1) for 12, 24, 48, and 72 h prior to immunofluorescence and analysis by confocal microscopy. In infected cells, pAbUL89 detected antigen predominantly in the nucleus (Figure 3). Distribution and intensity of fluorescence changed during the course of infection. At 12−24 h post-infection, nuclear staining in pre-replication centers was observed, that increased over time until late time points after infection when in addition to homogeneous nuclear staining bright intranuclear patches were detected (Figure 3a,c,e,g). As a control for nuclear distribution, double staining with the antibody mAb pp63−27 against IE1 was performed. The IE1 migrates into the nucleus after translation, where it serves as a regulatory protein of the synthesis of early and late HCMV mRNAs. In infected cells, a homogeneous staining of IE1 throughout the nucleus was observed (Figure 3b,d,f,h). To determine whether the translocation of pUL89 is an early event, immunofluorescence was performed in the presence of phosphono acetic acid (PAA; 200 μg/mL), an inhibitor of viral DNA replication. At 72 h post-infection, double staining with antibodies directed against the HCMV pAbUL89 and IE1 proteins, respectively, was undertaken. The immediate early proteins were localized in the nucleus, as expected, as well as pUL89 (Figure 3k). The pattern of the pUL89 immunofluorescence equals that of untreated cells at early times of infection (Figure 3a). These experiments showed that pUL89 is translocated into the nucleus early after infection and that localization in intranuclear patches is dependent on viral DNA replication.

Co-Localization of pUL89 with the Terminase Subunit pUL56. To analyze the interaction of pUL89 with the terminase subunit pUL56, laser scanning confocal microscopy was performed. At early times after infection, pUL56 and pUL89 were observed in nuclear spots, representing pre-replication centers (Figure 4a−f). At 48 h after infection, both proteins were found to localize to replication centers (Figure 4g−i). The fluorescence pattern changed after 72 h post-infection to brighter staining of the replication centers (Figure 4j−l). Mock-infected HFF revealed no specific immunofluorescence signals (Figure 4m,n). We concluded from these results that both proteins are localized in the viral replication centers as required for DNA-packaging proteins.

Identification of pUL89 Domains Required for Interaction with pUL56. To verify a physical interaction of the HCMV terminase subunits, in vitro binding assays were carried out using GST-fusions of the wild-type or of mutant pUL89 proteins. To this end, GST-UL89, GST-UL89-1, GST-UL89-2, GST-UL89-3, GST-UL89-4, GST-UL89-5 (Figure 5A), or GST itself was immobilized on glutathione-sepharose beads and incubated either with in vitro translated [^{35}S]-methionine-labeled pUL56 (Figure 5B, upper panel) or [^{35}S]-methionine-labeled IE1 (Figure 5B, lower panel). The amount of bound material was analyzed by SDS−PAGE and autoradiography. As shown in Figure 5B, pUL56 interacted specifically with GST-UL89-3, GST-UL89-4, and GST-UL89-5 but not with GST alone, GST-UL89-1, and only marginally with GST-UL89-2. In vitro translated IE1, used as a negative control, did not interact with the GST fusion proteins. To estimate the intensity of the pUL56−pUL89 interaction, quantification was performed using bioimaging analysis of fluorographs from three different experiments (Figure 5C). Interestingly, the binding of GST-UL89-5 was stronger than GST-UL89-3, but slightly weaker than GST-UL89-4. These experiments led to the suggestion that at least one region necessary for the interaction of pUL89 with pUL56 is located between aa 580 and 600 of pUL89.

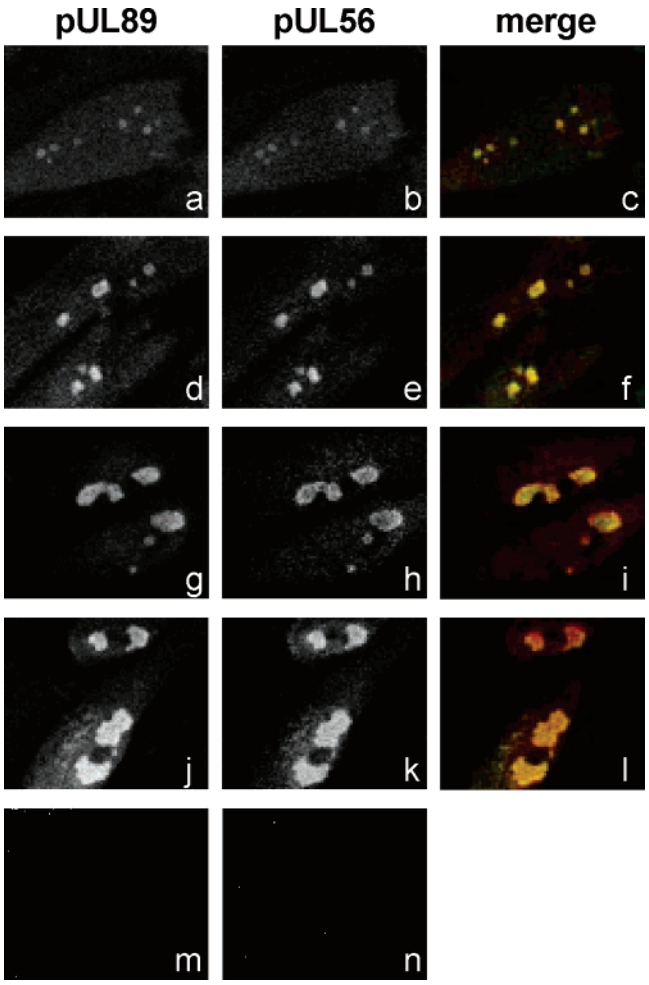

FIGURE 4: Co-localization of pUL89 with HCMV pUL56 by confocal microscopy. Mock-infected (m and n) or infected cells were double-stained with pAbUL89 and pAbUL56 directed against pUL56 at 12 (a−c), 24 (d−f), 48 (g−i), and 72 h (j−l) post-infection. The images represent the central section of the cells analyzed by laser scanning confocal microscopy. A merge of the Cy2- and Cy3-stained cells is shown to the right.

Construction of the Replication-Deficient HCMV-GFP-BAC-ΔpUL89. To verify the interaction domain of pUL89 with pUL56, an HCMV-GFP pUL89-null mutant was constructed. Since the UL89 gene consists of two exons and overlaps with other ORFs, exon 2 of UL89, which is clearly separated from other ORFs, was deleted in order to minimize any potential effect of the mutation on the neighboring genes (Figure 6A−C). For generation of the mutant BAC, the UL89 exon 2 was replaced by a kanamycin resistance marker. In the resulting HCMV-GFP-BAC-ΔUL89, the kanamycin resistance gene was removed by Flp recombinase-mediated recombination. BACs HCMV-GFP-BAC and HCMV-GFP-BAC-ΔUL89 were digested with *Hind*III and separated on an agarose gel (Figure 6D). As can be seen in the figure, a band of 7.5 kb characteristic of the parental HCMV-GFP-BAC is shifted to a lower molecular weight in HCMV-GFP-BAC-ΔUL89, giving rise to a double band at 6.5 kb.

Analysis of UL89 Deletion Constructs by Complementation Experiments with the HCMV UL89 Null Genome. Cotransfection analyses were performed to analyze the UL89 deletion constructs with the recombinant genome HCMV-GFP-BAC-ΔUL89. In contrast to HCMV-GFP-BAC, the recombinant bacmid HCMV-GFP-BAC-ΔUL89 transfected

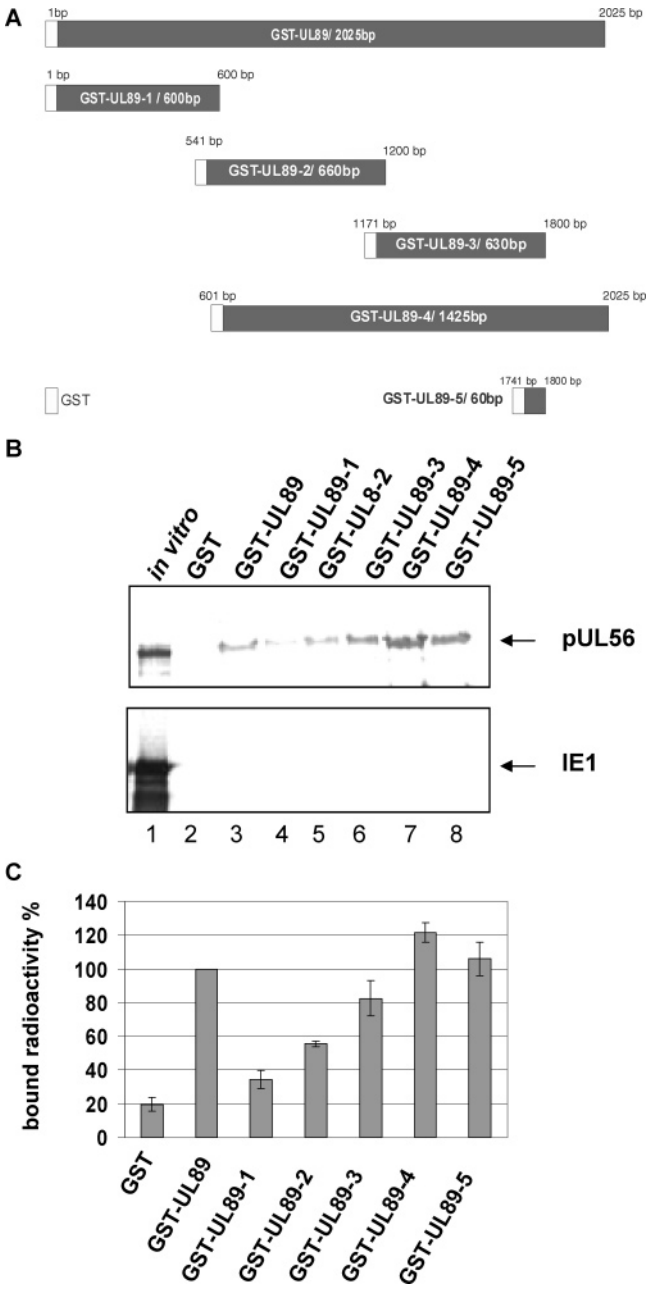

FIGURE 5: Identification of the pUL89 domains interacting with pUL56. (A) Schematic drawing of the pUL89 mutant proteins. (B) Glutathione-Sepharose 4B beads loaded with GST, GST-UL89, GST-UL89-1, GST-UL89-2, GST-UL89-3, GST-UL89-4, and GST-UL89-5 were incubated with in vitro translated UL56 or IE1. The bound material was eluted and subjected to SDS−PAGE, prior to autoradiography. The position of pUL56 and IE1 are indicated on the right. (C) Specifically the immobilized radioactivity was quantified using bioimaging analysis. The relative amounts of bound radioactivity are indicated by percent values in the diagram. Error bars on the histogram are ± standard deviation from three independent experiments.

alone led to the formation of single green fluorescent cells only but not to plaque formation, indicating a lethal phenotype (Figure 7A,B). We therefore examined the ability of pUL89 proteins to complement the HCMV UL89-null genome. Cotransfection with HCMV cosmid pCM1106 (*32*), plasmid pUC-UL89P carrying the authentic pUL89 ORF plus its promoter region, or plasmid pUC-UL89PΔ9aa, carrying a deletion at the N-terminus of pUL89, resulted in plaque formation and therefore phenotypic complementation (Figure

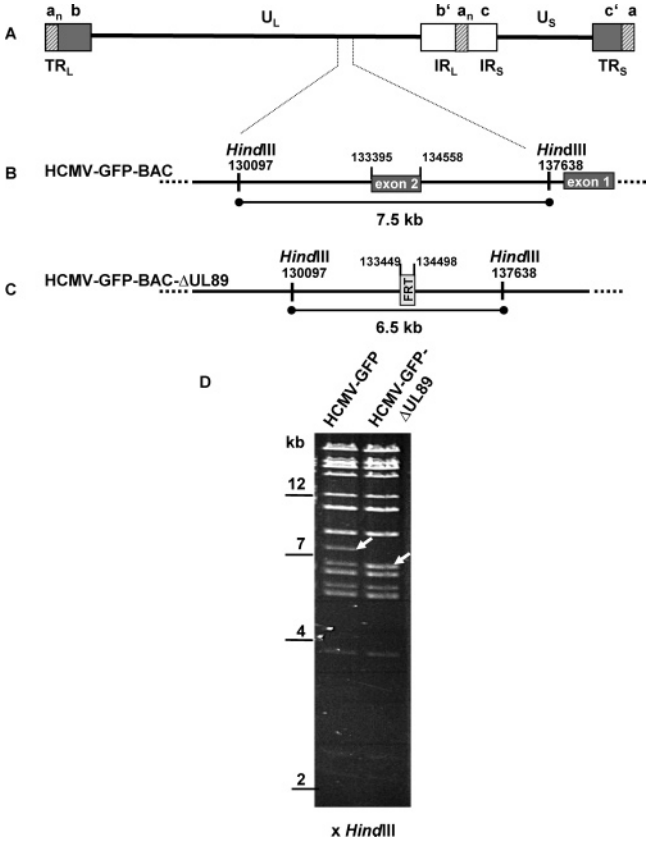

FIGURE 6: Construction of a HCMV UL89-null genome. (A) The top line represents the genome structure of the parental HCMV-GFP-BAC. (B) The region containing the ORF of UL89 (exons 1 and 2) is expanded. (C) The final BAC HCMV-GFP-BAC-ΔUL89 was constructed by replacement of UL89 exon 2 with a kanamycin resistance cassette, followed by its removal by *Flp* recombinase-mediated recombination. (D) DNA of HCMV-GFP-BAC and HCMV-GFP-ΔUL89-BAC was digested with *Hin*dIII and separated on an agarose gel. The relevant bands characterizing the BACs are indicated by arrows.

7C−E). Cotransfection with constructs containing either a deletion of aa 580−590, pUC-UL89PΔ10aa1, or a deletion of aa 590−600, pUC-UL89Δ10aa2, were not sufficient for complementation, because only single green cells were observed (Figure 7F,G). These experiments demonstrated that aa 580−600 are required for the function of pUL89 and furthermore for viral replication.

DISCUSSION

The process of herpesvirus DNA packaging requires the involvement of terminases. These enzymes (i) provide ATP for translocation of the DNA into the preformed capsids, (ii) have the ability to bind to DNA and to capsids, and (iii) have nuclease activity which releases unit-length genomes from concatemeric-replicated DNA. In the case of human cytomegalovirus (HCMV), the terminase is composed of the large subunit pUL56 and the small subunit pUL89 (*16*, *19*−*20*). Both proteins are conserved throughout the herpesviruses and have functions analogous to terminases of double-stranded DNA bacteriophages.

In the present work, we characterized the most conserved terminase subunit, pUL89 of HCMV. In infected cells, the UL89 ORF encodes proteins of approximately 75 and 150 kDa. While the prominent 75 kDa protein represents the monomer and is consistent with the predicted molecular

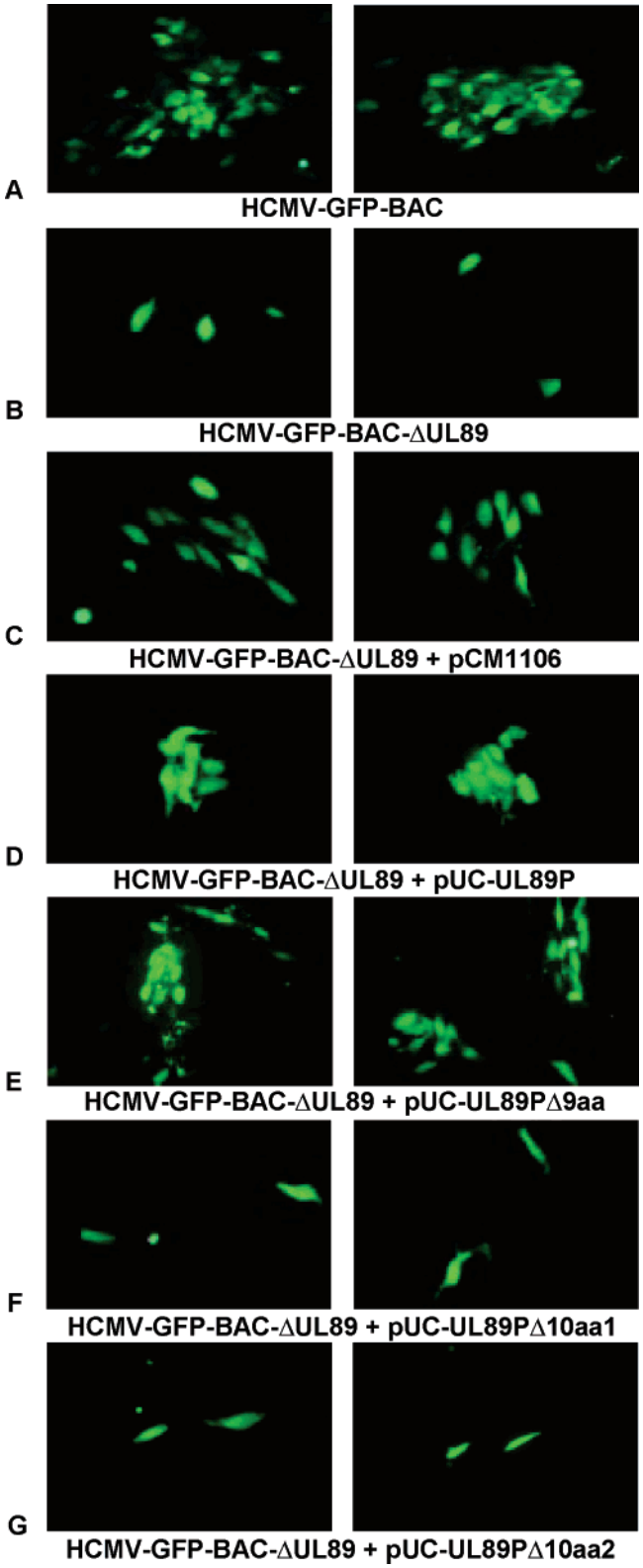

FIGURE 7: Cotransfection analysis with the recombinant genome HCMV-GFP-ΔUL89. Transfection with wild-type HCMV-GFP-BAC (A) or the recombinant BAC HCMV-GFP-BAC-ΔUL89, containing a deletion of exon 2 of UL89 (B), or cotransfection with the cosmid pCM1106 including both exons of pUL89 and flanking homologous regions (C), pUC-UL89P carrying the authentic pUL89 (D), pUC-UL89PΔ9aa with a deletion at the N-terminus (E), pUC-UL89PΔ10aa1 with a deletion of aa 580−590 in UL89 (F), or pUC-UL89PΔ10aa2 with a deletion of aa 590−600 (G) was performed. Ten days post-transfection, fluorescence microscopy was performed.

weight, the slower-migrating 150 kDa protein is observed

after immunoprecipitation with the pUL89-specific antibody. This form of pUL89 was also observed after gel permeation chromatography with marker proteins, thus, indicating that the recombinant rpUL89 forms a dimer. Interestingly, the homologous viral gene in HSV-1, UL15, encodes multiple proteins with apparent molecular weights from 80.000 to 87.000 (*33*−*34*). Only the high molecular weight pUL15 protein is a structural protein associated with DNA-containing capsids (C capsids). Some evidence was provided that suggested that the pUL15 forms are bound to the capsids by interactions with the other terminase subunit pUL28 and the portal protein pUL6 of HSV-1 (*35*). In contrast to these observations, HCMV pUL89 is not a constituent of the virion and therefore a nonstructural protein (data not shown). Our previous data suggested that the association of HCMV pUL89 with the capsid is mediated by interaction with pUL56, but pUL89 was never found together with pUL56 after DNA-cleavage (*20*). In agreement with the knowledge about the terminases of dsDNA bacteriophages (*36*), it is reasonable to speculate that, shortly after DNA cleavage, pUL89 is no longer associated with the DNA and is recycled for further cleavage/packaging processes. Furthermore, our findings indicate that pUL89 has a tendency to oligomerize, which is common for terminases of all double-stranded DNA bacteriophages analyzed so far (*36*). For instance, the small terminase subunit gp16 of bacteriophage T4 forms ring-like structures by oligomerization (*37*).

We investigated the intracellular localization of pUL89 by immunofluorescence analysis of infected cells. At early times after infection (12−24 h post-infection), staining in pre-replication centers was obtained with specific antibody pAbUL89. During infection (from 48 h post-infection onward), the fluorescence pattern changed and pUL89-specific signals were increasingly concentrated in replication centers. Experiments in the presence of phosphono acetic acid showed that this localization is dependent on viral replication. Since comparable to the large terminase subunit (*20*, *38*) also the small one is associated with viral replication centers, it is tempting to speculate that DNA packaging is closely associated with replication. Our observations are in line with those from Yu and Weller (*34*) demonstrating that the HSV-1 homologue pUL15 also localizes to replication centers.

Previously, we demonstrated only by co-immunoprecipitation that both terminase subunits interact with each other (*19*). In the study presented here, the co-localization of pUL56 and pUL89 in infected cells was examined by laser scanning confocal microscopy. We could demonstrate that both proteins were translocated into viral replication centers. These findings are consistent with the reports of nuclear translocation of the homologous HSV-1 proteins (*39*−*40*). However, Koslowski et al. (*40*) as well as Abbots et al. (*39*) demonstrated that the pUL56 homologue of HSV, pUL28, is only translocated into the nucleus in the presence of pUL15, thus, implicating that in HSV-1, pUL15 is a chaperone for pUL28. These observations are different compared to our data with HCMV. We identified previously a NLS at the carboxy terminus of pUL56, and further evidence was provided that nuclear translocation of pUL56 is mediated by the importin-dependent pathway (*41*).

Furthermore, analyses were performed to identify the interaction domain of pUL89 with pUL56. Since nothing was known so far about pUL89 domains, overlapping protein mutants were constructed and analyzed by in vitro binding assays. We were able to identify an interaction domain between aa 580−600 (GRDKALAVEQFISRFNSGYIK). This result was verified by analysis with a recombinant HCMV genome, HCMV-GFP-BAC-ΔUL89 carrying a disrupted UL89 gene. We demonstrated by transfection that the BAC HCMV-GFP-BAC-ΔUL89 has a lethal phenotype. Cotransfection assays with HCMV cosmid pcm1106, the plasmid containing the full-length UL89 ORF including its promoter, pUC-UL89P, or the plasmid with a nine amino acid deletion at the N-terminus, pUC−UL89PΔ9aa, could complement the replication-deficient genome HCMV-GFP-BAC-ΔUL89, while the mutant plasmids lacking either the region encoding aa 580−590 or the region encoding aa 590−600 were unable to complement the recombinant genome. This indicates that the interaction with the terminase subunit pUL56 is a prerequisite for the function of the HCMV terminase subunit pUL89 during viral DNA replication.

Taken together with our earlier observations (*19*−*20*) we could clearly demonstrate that both terminase subunits interact with each other and that the interaction domain is localized between aa 580 and aa 600. By complementing the HCMV UL89 null genome, we demonstrated that these amino acids were required for the function in cleavage and packaging of newly synthesized DNA.

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