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Human Cytomegalovirus pUL93 Is Required for Viral Genome Cleavage and Packaging

Bernadette M. DeRussy, Ritesh Tandon

Department of Microbiology and Immunology, University of Mississippi Medical Center, Jackson, Mississippi, USA

Human cytomegalovirus (HCMV) pUL93 is essential for virus growth, but its precise function in the virus life cycle is unknown. Here, we characterize a UL93 stop mutant virus (UL93st-TB40/E-BAC) to demonstrate that the absence of this protein does not restrict viral gene expression; however, cleavage of viral DNA into unit-length genomes as well as genome packaging is abolished. Thus, pUL93 is required for viral genome cleavage and packaging.

Human cytomegalovirus (HCMV) protein pUL93, a putative tegument protein, is required for the growth of HCMV (1, 2), but the exact function of this protein is unknown. There is no functional study done to date on HCMV pUL93; however, pUL17, the positional homolog of pUL93 in herpes simplex virus 1 (HSV-1), has been shown to be required for the localization of capsids to the DNA replication compartments in the infected cell nucleus, where viral genome cleavage and packaging take place (3, 4). pUL17 has also been found to interact with pUL25, a homolog of HCMV pUL77, to form the capsid vertex-specific component (CVSC), which is found at the 12 vertices of the icosahedral capsid (4–10). The exact function of the CVSC is currently unknown, but it is thought to aid in capsid stability and nuclear egress (10–13). pUL25-null mutants package viral DNA only transiently and produce mostly empty capsids, while pUL17-null mutants completely abolish DNA packaging (3, 14). It is important to note that pUL17 and pUL93 are positional homologs that share only 1.5% sequence identity, 1.9% sequence similarity, and no distinct conserved domains (based on amino acid ClustalW alignment). Moreover, several HCMV proteins have been found to have very different functions compared to their homologs in other herpesviruses, highlighting the importance of studying HCMV proteins independently. For example, HCMV pUL50 and pUL53 were presumed to recruit host protein kinase C (PKC) for disruption of the nuclear lamina based on data from other herpesviruses but were instead found to recruit viral protein kinase pUL97 for this purpose (15). Targeting of essential structural viral proteins holds great promise for the development of antivirals that would be highly specific and effective and also less susceptible to the development of resistance be-

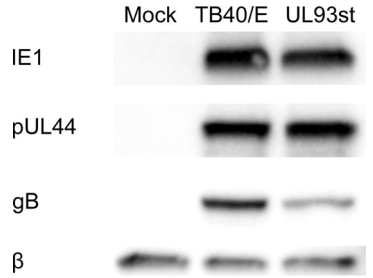
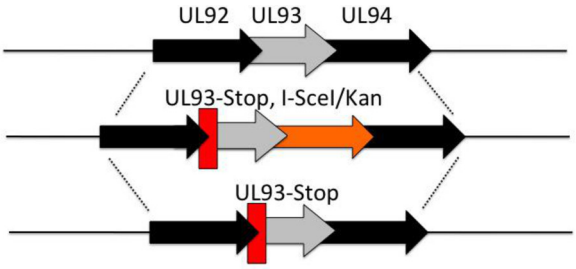


FIG 2 pUL93 is not required for viral gene expression. HF were infected with WT-TB40/E or UL93st-TB40/E-BAC virus (UL93st) at an MOI of 3.0 or mock infected, and cells were harvested at 72 h postinfection for immunoblot assays probing for early (IE1), delayed early (pUL44), and late (gB) viral proteins.

A.



B.

Virus	Titers (pfu/ml)
WT-TB40/E in HF	7.8×10^7
UL93st in FLAG-UL93 HF	2.24×10^8
UL93st in HF	N.D.

FIG 1 Construction and growth of a UL93 stop mutant virus. (A) Schematic of the UL92-to-UL94 region in TB40/E-BAC (top line) with other bacmid constructs shown below. A UL93 stop mutant virus (UL93st-TB40/E-BAC) was constructed using two-step BAC recombineering (17). In the first step, a kanamycin (Kan) cassette fused to the UL93 stop mutation and an I-SceI site was inserted in the BAC genome using homologous recombination. The Kan cassette was then spliced out utilizing the I-SceI site, leaving only the UL93 stop mutation in the genome. (B) Final virus yields were determined at day 10 postinfection for wild-type TB40/E (WT-TB40/E in HF), UL93st-TB40/E-BAC in complementing cells (UL93st in FLAG-UL93 HF), and UL93st-TB40/E-BAC in noncomplementing cells (UL93st in HF). N.D., not detected, as the number of PFU in this preparation was below the threshold of detection in the assay.

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Address correspondence to Ritesh Tandon, rtandon@umc.edu.

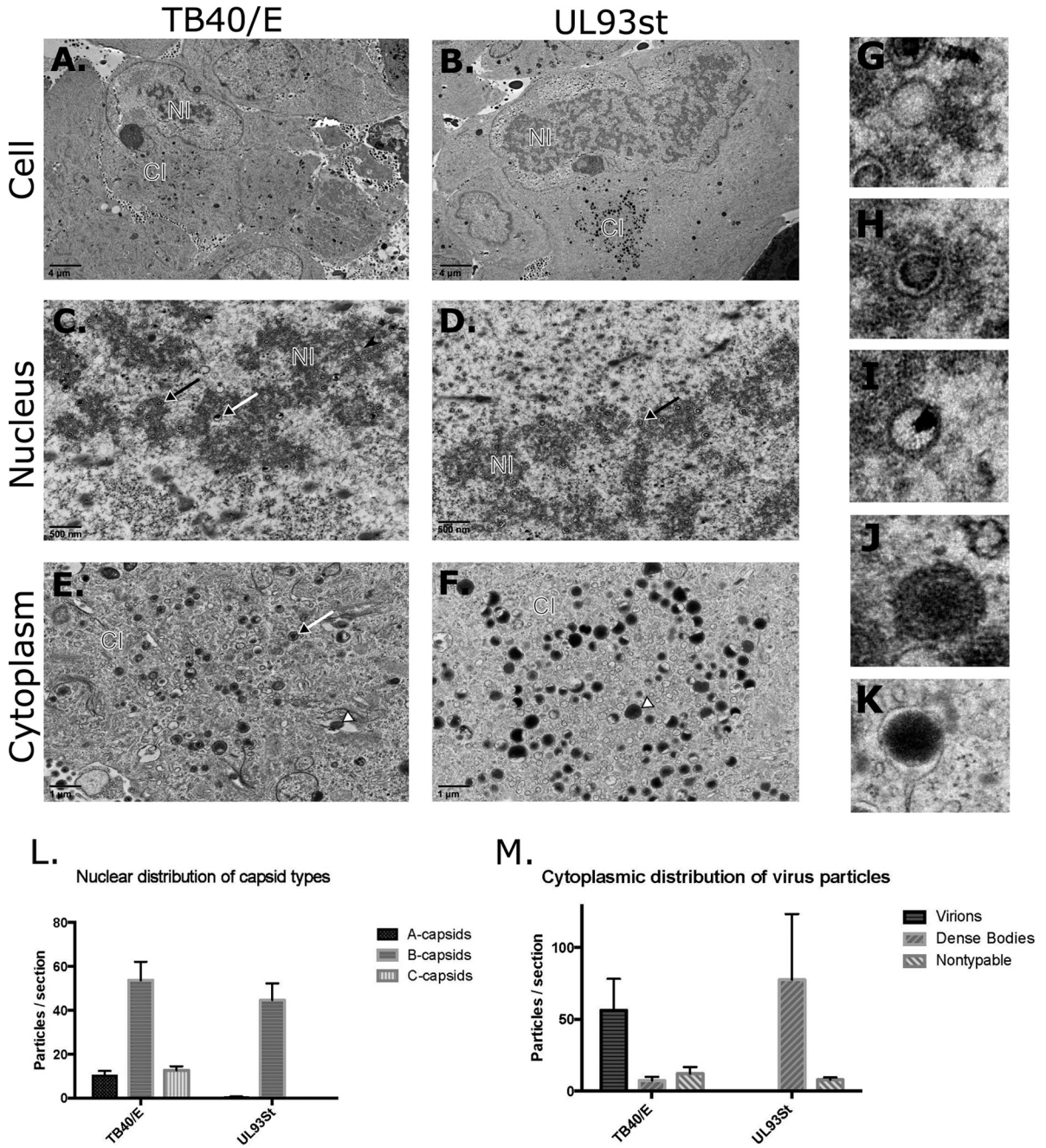

FIG 3 pUL93 is required for nucleocapsid maturation. (A to F) Transmission electron micrographs (TEM) of HF infected with WT-TB40/E (A, C, and E) or UL93st-TB40/E-BAC (UL93st) (B, D, and F) viruses at an MOI of 3.0 and fixed for processing at 4 days postinfection. (A and B) A single infected cell showing nuclear inclusion (NI) as well as cytoplasmic inclusion (CI). (C and D) Nuclear inclusion illustrating A- (black arrowhead), B- (black-tailed arrow), and C- (white-tailed arrow) capsids. (E and F) Cytoplasmic inclusion illustrating mature virions (white-tailed arrow) and dense bodies (white arrowhead). Bars, 4 μm (A and B), 500 nm (C and D), and 1 μm (E and F). (G to K) Magnified images (not to scale) of A-capsids (G), B-capsids (H), C-capsids (I), virions (J), and dense bodies (K) are shown. (L and M) A-, B-, and C-capsids in the nucleus (L) and virions, dense bodies, and nontypeable particles in the cytoplasm (M) were enumerated in 4 individual infected cells, and the mean values are displayed.

cause the mutation of a structural protein would likely compromise capsid assembly and maturation (16). Characterization of pUL93 will aid in the development of these promising antivirals in addition to advancing our understanding of HCMV maturation events.

Here, we have characterized a UL93 stop mutant virus. We report that although pUL93 is not required for expression of different kinetic classes of viral genes, DNA-containing C-capsids and virions cannot be detected in the nucleus and the cytoplasm, respectively, of infected cells in the absence of pUL93. Upon further investigation, pUL93 was found to be required for the cleavage of viral genomic DNA into unit-length genomes.

pUL93 is dispensable for viral gene expression. To study the role of pUL93 in the HCMV life cycle, we engineered a UL93 stop mutant (UL93st-TB40/E-BAC) by replacing the initiating codon (ATG) in UL93 with a stop codon (TAG) using two-step bacterial artificial chromosome (BAC) recombineering (Fig. 1A) (17–19). BAC constructs were validated by restriction fragment length polymorphism (RFLP) and PCR sequencing of the region of the BAC genome containing this change (data not shown). Upon transfection in human foreskin fibroblasts (HF), this mutant BAC did not yield any detectable infectious virus particles in cell culture medium. To enable the growth of this virus, we constructed pUL93-complementing cells by transducing HF with a lentiviral vector (pLV-EF1α-MCS-IRES-Puro; cDNA-pLV01 [20]) that expressed FLAG-tagged pUL93. These complementing cells were then transfected with UL93st-TB40/E-BAC to grow this virus. Upon infection in noncomplementing HF, UL93st-TB40/E-BAC virus did not yield any detectable infectious virus particles in cell culture medium when monitored for up to 10 days postinfection (dpi), but in complementing HF, the UL93st-TB40/E-BAC virus grew to high titers (2.24×10^8 PFU/ml) (Fig. 1B). We used the harvested UL93st-TB40/E-BAC virus to infect noncomplementing HF at a multiplicity of infection (MOI) of 3.0. Cells were harvested at 3 dpi, and immunoblot (IB) assays probing for viral immediate early (anti-IE1/2 antibody [CH160]; P1215; Virusys Corporation, Taneytown, MD), delayed early (anti-pUL44 antibody [ICP36]; CA006-100; Virusys Corporation, Taneytown, MD), and late (anti-gB antibody; CA005-100; Virusys Corporation, Taneytown, MD) proteins in whole-cell lysates showed expression of all these classes of viral genes, indicating that pUL93 is not required for viral gene expression (Fig. 2). Nevertheless, levels of gB expression were slightly reduced in UL93st-TB40/E-BAC virus infection compared to the wild-type virus infection, indicating some impact on late gene expression.

pUL93 is required for nucleocapsid maturation. To further study the stage of the HCMV life cycle where pUL93 functions are critical, we performed transmission electron microscopy (TEM) of HF infected with either wild-type (WT) TB40/E or UL93st-TB40/E-BAC viruses. HF were infected at an MOI of 3.0 with either of the viruses and fixed for TEM at 4 dpi. Ultrastructural analysis of the nucleus and the cytoplasm of WT-TB40/E-infected cells revealed the presence of empty (A-), scaffold-containing (B-), and genome-containing (C-) capsids in the nucleus, and virions as well as dense bodies in the cytoplasm, typical of a productive infection in fibroblasts (19, 21, 22), while C-capsids and virions were not detected in cells infected with UL93st-TB40/E-BAC virus (Fig. 3A to F). We enumerated mature and immature virus particles in each infection group based on the established morphological characteristics of these particles, which are illustrated in Fig. 3G to K (23, 24). Four individual WT-TB40/E-infected cells showed an average of 10 A-capsids, 54 B-capsids, and 13 C-capsids in the nucleus, while four individual UL93st-TB40/E-BAC virus-infected cells showed an average of less than 1 A-capsid and 45 B-capsids in the nucleus (Fig. 3L). C-capsids were absent from the nuclei of UL93st-TB40/E-BAC virus-infected cells. In the cytoplasm, four WT-TB40/E-infected cells showed an average of 56 virions, 7 dense bodies, and 12 nontypeable particles, compared to no virions, 78 dense bodies, and 8 nontypeable particles in UL93st-TB40/E-BAC virus-infected cells (Fig. 3M). Thus, pUL93 is required for the production of C-capsids and mature virions.

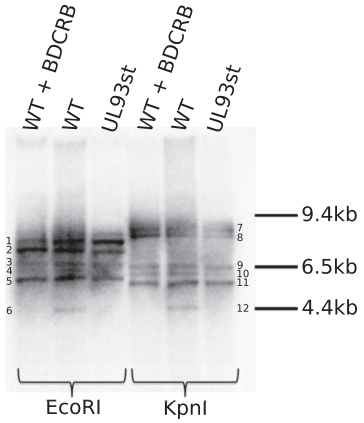

FIG 4 pUL93 is required for viral genome cleavage. DNA extracted from HF infected with WT-TB40/E in the presence of BDCRB, infected with WT-TB40/E in the absence of BDCRB, or infected with UL93st-TB40/E-BAC virus (UL93st) at an MOI of 3.0 was digested with EcoRI or KpnI, size fractionated by gel electrophoresis, and blotted. Hybridization was performed using a terminal DNA probe. Fragments labeled 6 and 12 in the autoradiographic image are terminal fragments that represent cleaved viral genomic DNA. Fragments 1, 2, 3, 4, 5, 7, 8, 9, 10, and 11 are all possible junction fragments.

pUL93 is required for viral genome cleavage. Based on the above data that show that C-capsids and virions are absent during infection with a UL93 stop mutant virus, we hypothesized that this could be due to two reasons: pUL93 is required for viral genome cleavage and packaging and it could also be necessary for maintaining capsid stability after genome cleavage and packaging. Proteolytic digestion of capsid scaffold and packaging of viral DNA are believed to occur simultaneously (25–29), and A-capsids are generated as a result of abortive attempts to package viral DNA (24, 30). Although roles of A- and B-capsids as intermediate or abortive capsid forms in the life cycle of herpesviruses have not been clearly distinguished, we hypothesized that a defect in virus genome packaging would arrest the virus capsid maturation prior to the production of C-capsids (capsids with packaged DNA). A defect after packaging would result in the instability and probable complete degradation of C-capsids, which may not be limited to the ejection of viral DNA, considering that C-capsids sustain much higher internal pressure from the packaged DNA. Thus, A-capsid numbers may not increase despite a reduction in the number of C-capsids. To explore the possibility of a DNA packaging defect, DNA was harvested from WT-TB40/E or UL93st-TB40/E-BAC virus-infected HF and digested with either EcoRI or KpnI, and Southern blot assays were performed to probe for viral terminal DNA. The use of Southern blot assays to probe for terminal and junction fragments in herpesviruses has been well established (31–35). As a control, HF were infected with WT-TB40/E and treated with 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole (BDCRB; a viral terminase inhibitor in HCMV) (36, 37) or mock treated. BDCRB selectively inhibits pUL56 and pUL89, thereby blocking viral genome cleavage without affecting viral gene expression or capsid and dense body formation (24, 38–42). The presence of the 4.4- and

4.5-kb terminal fragment in WT-TB40/E digested with EcoRI and KpnI, respectively, indicates the occurrence of genome cleavage. As expected, this band was not detected in the BD-CRB-treated control. Importantly, this band was also absent in UL93st-TB40/E-BAC virus (Fig. 4), indicating that pUL93 is required for the cleavage of viral genomic DNA. Based on the TB40/E genome sequence (GenBank accession no. EF999921.1), there can be multiple junction fragments that would bind to a terminal probe in a Southern blot assay. The number of these fragments will depend on the presence or absence of different genomic isomers in virus stock. However, all these junction fragments will be larger than the terminal fragment, as seen in the Southern blot image (Fig. 4).

Together, our data confirm an essential role of pUL93 during HCMV infection and indicate that this protein is critical for viral genome cleavage and packaging. Future studies will examine if pUL93 interacts with pUL77 to form a structure similar to the CVSC in HSV-1 and contributes to capsid stability and/or nuclear egress.

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