**Abstract**

The human cytomegalovirus (HCMV) UL26 gene encodes a virion protein that is important for high titer viral replication. To identify specific domains within the UL26 protein that contribute to viral infection, we created a panel of site-directed UL26 mutant viruses and assessed their impact on phenotypes attributed to UL26. We find that the C-terminal 38 amino acids of the UL26 protein are absolutely necessary for UL26 function. A stop-insertion mutant that produced a truncated UL26 protein lacking this region behaved identically to UL26-null viruses. This included reduced accumulation of IE1 protein at early time points, smaller plaque size, reduced virion stability, and growth with similarly attenuated kinetics. This C-terminal truncation decreased the amount of UL26 packaged into the virion resulting in reduced delivery of UL26 to newly infected cells. Further, this C-terminal truncated UL26 exhibited substantially reduced nuclear localization compared to wildtype UL26. Translation of UL26 mRNA is initiated from two separate in frame methionines that give rise to a long and a short isoform of UL26. We find that the N-terminal 34 amino acids, which are unique to the long isoform of UL26, are also important for the function of the UL26 protein. A viral mutant that produces only the short isoform of UL26 and lacks these N-terminal 34 amino acids exhibits delayed IE1 accumulation, and demonstrates intermediate defects in viral plaque size, virion stability and viral growth kinetics. Ablation of the short UL26 isoform in the presence of the long UL26 isoform did not impact any of the *in vitro* phenotypes tested. These experiments highlight important domains within the UL26 protein that contribute to HCMV infection.

**Introduction**

Human cytomegalovirus (HCMV), a betaherpesvirus, is a widespread opportunistic pathogen. HCMV causes severe disease in various immunosuppressed populations including the elderly, cancer patients receiving immunosuppressive chemotherapy, transplant recipients, and AIDS patients [1,2]. HCMV infection is also a substantial cause of rejection in allograft recipients (kidney, liver, heart and bone marrow) [3–6]. Further, congenital HCMV infection is a major cause of birth defects resulting in permanent disabilities in approximately one in a thousand live births [7–9]. Congenital HCMV infection can result in multiple organ system abnormalities, although central nervous system damage is the most prevalent sequela, which occurs in the majority of symptomatic newborns [2,10].

HCMV is a relatively large virus, with a ~240-kb DNA genome that encodes >200 open reading frames. The viral particle is enveloped and its genome is encased within a protein capsid. Packaged in between the capsid and the viral envelope is a protein layer called the tegument, a structural feature unique to herpes viruses [11–13]. Tegument proteins perform diverse functions during viral infection. Some tegument proteins are important for structure and assembly of virions such as those encoded by UL32 and UL99 [14–16]. Tegument proteins are delivered to the cellular cytoplasm upon viral membrane fusion and many function at the earliest steps of infection. Such examples include pp71, which serves as a transcriptional activator of viral genes, and also suppresses the Rb tumor suppressor [17–19], and pp65 which antagonizes innate immunity and the interferon response [20,21]. Tegument proteins are therefore critical at multiple steps during HCMV infection; at early times, they initiate a cellular environment conducive to viral replication, and later, they help assemble viral particles.

While many HCMV tegument proteins are known to be important for HCMV replication, the mechanisms through which many of these proteins contribute to the infectious cycle are unclear. One such tegument protein is encoded by the UL26 gene, which has been found to be critical for high-titer viral replication [22–24]. The UL26 protein is expressed with early kinetics, and synthesis of the protein initiates at one of two start codons resulting in 21- or 27-kDa products [25]. HCMV strains containing a UL26 deletion grow to lower final titers, with slower growth kinetics, and exhibit a small plaque phenotype [23,24]. UL26 has been implicated in transcriptional activation of the immediate early promoter [23,25]. Deletion of UL26 also impacts the structural characteristics of virions. These mutants are less stable than...
wildtype virions and contain hypophosphorylated tegument constituents [23,24]. Consistent with a nuclear function early during infection, and a role in viral assembly at late time points, the U126 protein localizes to the nucleus at early times post infection, and to viral assembly compartments at late time points [23].

Here, we analyzed specific domains of U126 that contribute to U126-dependent phenotypes through the creation of a panel of mutant U126 viruses. Site-directed mutagenesis was employed to target both of U126’s initiation methionines and to introduce stop codons throughout the U126 ORF. Analysis of these mutant viruses indicates that the U126 short isofrom is dispensable in vitro when in the presence of the U126 long isofrom. In contrast, the extra N-terminal 34 amino acids of the long U126 isofrom was found to be important for U126-dependent phenotypes, exhibiting intermediate defects in plaque size and virion stability in comparison to wildtype and U126-null viruses. Lastly, the carboxy terminal 38 amino acids were found to be critical for wildtype localization and tegumentation of the resulting U126 protein. These studies indicate that distinct domains of U126 contribute to different U126-dependent phenotypes and shed light on how these domains contribute to HCMV replication.

Materials and Methods

Cell Culture and Viruses

MRC5 fibroblasts (passages 23–29) were cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum. The wild type HCMV strain used in this study was BAdwt, a bacterial artificial chromosome (BAC) clone of Ad169 [26,27]. Cells were grown to 3.2×10^5 cells per cm². Prior to infection, cells were starved for 24 hours. In all infections, viral inoculums were added to cells for a 2 hr adsorption period and then aspirated. For experiments involving measurement of viral titers via plaque assay, unbound virus was inactivated through a sodium citrate wash (40 mM sodium citrate, 10 mM KCl, and 135 mM NaCl, pH 3.0) followed by a DMEM wash immediately following viral adsorption.

BAC Mutagenesis

All U126 mutants were derived from the BAdwt clone of Ad169 (Genebank accession number: FJ527563) [26,27]. The U126 mutants constructed are: BAdUL26 double methionine deletion (referred to as DBmet in the text); BAdUL26 1st Methionine deletion (referred to as 1stmet in the text); BAdUL26 2nd Methionine deletion (referred to as 2ndmet in the text); BAdUL26 double methionine deletion rescue (referred to as DBR rescue in the text); BAdUL26 #68 stop codon mutant (referred to as #68stop in the text); BAdUL26 #107 stop codon mutant (referred to as #107stop in the text); BAdUL26 #146 stop codon mutant (referred to as #146stop in the text); BAdUL26 #185 stop codon mutant (referred to as #185stop in the text). Wild type BAdwt is referred as WT in the text and BAdUL26 transposon insertion virus [22] is referred to as UL26TI in the text. Red recombineering was used to construct the viral mutants in either a one-step or two-step PCR recombination process as previously described [28]. Briefly, for the two step PCR, a PCR amplified Kan/Isce I cassette from the pEPkan-S vector containing U126 flanking sequences was recombined into BAdwt through electroporation into E. coli (strain SW105) containing BAdwt. Recombination was screened by growth in kanamycin. The Kan/Isce I cassette containing BAC was then electroporated into GS1783 cells, which contain an arabinose-inducible I-Scel restriction site used for negative selection [29]. In the second step of Red recombineering, a double-stranded DNA oligo containing the mutant sequence of interest was transformed into GS1783 competent cells containing the Kan/Isce I cassette to allow recombination and insertion of the mutant sequence into the Kan/Isce I cassette site. Recombinants were negatively selected on arabinose, positively selected on chloramphenicol and screened for loss of kanamycin resistance. Restriction enzyme analysis of all BAC clones was performed to rule out large-scale aberrant recombination events. Further, all recombinant BAC clones were sequenced to confirm the presence of the inserted mutation and confirm the lack of any additional mutations in the U126 gene. The UL26 DBmetA mutant was created by deletion of the 2nd Methionine from the UL26 1stmetA and second reaction to insert the point mutation) were as follows (3′ to 5′): UL26 1stmetA insertion: F-GCGGCGCTCTGCGTCCTGACGGCAGCGCAAATCATTAG AGGGTTTACGCGGAGAGGG; R- GCAGGCGGCTCTGCGTCCTGACGGCAGCGCAAATCATTAG AGGGTTTACGCGGAGAGGG.

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was quantified with specific primer pairs targeting UL83 (pp65), Biosystems) according to manufacturer's instructions. Viral DNA accumulation was performed using Fast SYBR green master mix, a model 7500 acid was quantified and checked for purity through 260:280 proteinase K, and 40

900 mM Tris-HCl, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml serum albumin (BSA), 5% goat serum, 5% human serum, and 0.3% Triton X-100. Cells were incubated with anti-U126 sera [7H19] that had been diluted 1:2 in PBS containing 0.05% Tween-20 for 1 hr. Slides were subsequently washed with PBS containing 0.01% Tween-20 three times, incubated with fluorescein isothiocyanate conjugated-anti-mouse secondary antibody for 1 hr, and washed three times in the same buffer lacking antibody. Coverslips were washed in 90% alcohol and mounted in FluoreMount-G2 antifade reagent (Molecular Probes) and DAPI (4', 6'-diamidino-2-phenylindole). Confocal images were captured with FV1000 Olympus laser scanning confocal microscope. All images were captured under identical confocal settings.

**Virus Purification**

To produce partially purified virions, the analysis of their constituent proteins, WT and #185stop virus stocks were first clarified by low speed centrifugation and then centrifuged through a sorbitol cushion at 26K rpm for 1 hr. The virion pellet was then resuspended in T.N. buffer containing 20 mM Tris-HCl pH 7.4, 100 mM NaCl, and 1.5% BSA and purified by centrifugation through a glycerol tris-tratrate gradient as previously described [23]. Bands containing virions were collected and diluted 4-fold with T.N. buffer. Virions were pelleted by centrifugation at 21 K rpm for 1 hr and resuspended in T.N. buffer. For western analysis of viral preps, disruption buffer was added to a final concentration of 50 mM Tris [pH 7.0], 2% SDS, 5% 2-mercaptoethanol, and 2.75% sucrose. A rabbit UL26 N-terminal-specific antibody was generated by Biomatik (http://www.biomatik.com/) using the following underlined sequence: SRRAPDGGLNLDD. The methionine preceding this sequence is the 2

**Analysis of Viral Plaque Formation and Viral Stability**

Replicate cultures of MRC5 fibroblasts were infected with 25 PFU of the indicated recombinant virus. Representative plaques at day 15 post infection for each virus are shown. Areas of representative plaques for each virus were quantified by Image J and normalized to the WT plaque size. To investigate the stability of virion infectivity, an equivalent number of plaque forming units from freshly thawed viral stocks were incubated at 37°C for 0, 4, 8, or 20 hours. After the indicated incubation period, confluent MRC5 fibroblasts were infected. The percentage of plaques remaining relative to the 0 h control was plotted.

To investigate virion stability after trypsin exposure, infected MRC5 cells [MOI = 3.0] were harvested when the CPE reached 80%. The media containing infectious virus was reserved, and cells were scraped in a small volume of media and sonicated. The sonicated cells and reserved culture media were combined and centrifuged at 6,000 rpm for 30 min. The supernatants were then sedimented at 38,000×g for 60 min. The pellets containing virus were resuspended in serum-free minimal essential medium. Two hundred μl of either 2.5% trypsin (Invitrogen) or media was mixed with 1.8 ml of the resuspended virus, and incubated at 37°C for 30 or 60 minutes for the trypsin treated, or 0 minutes for the media control. To inactivate the trypsin, at the end of the prescribed intervals, calf serum was added to a final concentration of 10%.
The suspension was then tittered by plaque assay. The percentage of plaques remaining relative to the media control was plotted.

Statistical Analysis

Statistical significance was assessed by a non-paired two tailed homoscedastic student’s t-test unless otherwise indicated. A probability of value (p) <0.05 was considered statistically significant. For comparison of the viral growth between wildtype and the #185stop mutant from 48–120 hpi a homoscedastic paired two-tailed test of viral titers at each time point was performed. Averages are plotted with either standard deviation (SD), or standard error of the mean (SE) as indicated.

Results

Construction of U26 Mutant Viruses

Viruses containing deletions in the U26 gene have been shown to be growth attenuated [23,24]. These viruses grow to reduced titers, have decreased virion stability and exhibit decreased plaque size [23,24]. The U26 protein is expressed from a spliced mRNA transcript that is also responsible for the expression of the U29, U185, U27, and U29/28 open reading frames [25,30] (Fig. 1A). Previously characterized U26-null viruses contain large deletions within the U26 open reading frame which could impact the expression of the other open reading frames that are expressed from this mRNA transcript. Further, it is unclear how the two separate isoforms of the U26 protein contribute to HCMV infection. To address these issues, and to map the domains of U26 that impact viral replication, we employed BAC-mediated recombinering to create a panel of viruses containing site-directed U26 mutations. This panel included viruses containing a mutation ablating one or both initiating methionines as well as viruses containing stop codon insertions throughout the U26 open frame.

A plot of manavalan hydrophobicity [31] and Chou-Fasman predicted secondary structure domains [32] of the U26 protein is illustrated in Figure 1B. The positions of specific methionine mutations or stop insertions that were engineered into BAC-Ad169 (WT) are illustrated on this plot (Fig. 1B). In an attempt to create truncated U26 reading frames that could be stably expressed, stop insertions were made at locations that approximated transitions between these predicted domains (Fig. 1B). To verify expression of these C-terminally-truncated U26 open reading frames, a rabbit polyclonal antibody was raised to a peptide in the N-terminus of U26. As shown in Figure 1C, three stop insertion viruses did not accumulate any truncated U26 protein whereas one stop insertion mutant, #185stop, did accumulate truncated U26. Mutagenesis of U26’s two initiation methionines gave the expected results; a virus containing a mutation in the first methionine expressed only the short U26 isoform, whereas a virus with a mutation of the second methionine expressed only the long U26 isoform (Fig. 1C). Further, the virus with both methionines mutated did not accumulate any U26 (DBmetΔ), nor did the previously described transposon-deleted U26 virus (UL26TI) (Fig. 1C). The repair of the DBmetΔ virus, through recombination with a DNA fragment containing the wildtype U26 N-terminus created a virus, DBrescue, which restored the expression of both U26 isoforms (Fig. 1C). The stop codon insertion mutants that did not accumulate detectable amounts of U26 behaved like U26-null viruses (data not shown) and were not analyzed in further detail. The specific mutations of the remaining recombinant viruses, and the U26 ORFs they produce, are illustrated in Fig. 1D.

N-terminal and C-terminal Domains of the U26 Protein Are Necessary for Wild-type Replication

As it has previously been found that deletion of the U26 protein impacts production of viral progeny, we wanted to elucidate how specific domains of the U26 protein contribute to viral replication. We first wanted to assess whether the double methionine deletion mutant (DBmetΔ), grew with similar kinetics as the transposon insertion mutant (UL26TI). As shown in Figure 2A and 2B, the DBmetΔ virus grew with similar kinetics at both high (3.0) and low (0.25) multiplicities of infection (MOI). Further, repair of the double methionine mutation (DBrescue) restored the viral growth kinetics to wildtype levels (Fig. 2A and 2B). These results indicate that the transposon insertion and subsequent large deletion of the U26 ORF does not substantially impact the in vitro viral growth over and above what is observed with less disruptive targeting of the UL26 initiation methionines.

To further determine how different domains of the U26 protein contribute to viral growth, we analyzed the replication of the individual methionine mutants and the #185 stop insertion mutant. At high MOI, the 2ndmet virus, which only expresses the long isoform of U26 (Fig. 1C), grew similarly to WT (Fig. 2C). The 1stmet virus, which expresses only the short isoform of U26 (Fig. 1C), displayed an intermediate growth defect. Compared to WT virus, it grew with slower kinetics and exhibited a 3-fold decrease in final titers, but grew better than a virus with the complete U26 deletion (DBmetΔ) (Fig. 2C). The #185stop mutant was more substantially attenuated, growing with reduced kinetics and to lower final titers, similar to the DBmetΔ mutant. Compared to WT virus, the stop mutant exhibited a ~10-fold reduction in final titers (Fig. 2C). Similar trends were observed during infection at a lower MOI. The 2ndmetΔ virus grew almost identically to WT, whereas the 1stmet virus displayed WT growth kinetics early during infection, but exhibited a 10-fold reduction in viral titers (Fig. 2C). Interestingly, the #185stop mutant actually grew statistically worse than the DBmetΔ mutant based on a paired two-tailed t-test of viral titers from 48–120 hpi (p<0.05) (Fig. 2D). The combined analyses of viral growth suggest that the short U26 isoform is dispensable for HCMV growth in vitro. The additional N-terminal 34 amino acids present in the long but absent in the short U26 isoform were found to be important for wildtype levels of in vitro viral growth. The short isoform still contributes to viral growth inasmuch as it grows better than the U26-null virus. In contrast, the C-terminal 38 amino acids of the U26 appear to be essential for U26 function.

The Impact of U26 Mutations on Viral Protein and Viral DNA Accumulation

It has been reported that U26 is important for wildtype levels of IE1 accumulation [23]. To further explore the impact of specific U26 domains on viral gene expression, we analyzed the accumulation of viral proteins during infection. Upon infection at an MOI = 3.0, both U26-null viruses, U26TT and DBmetΔ, accumulated less IE1 at 4 hpi, but recovered by 24 hpi (Fig. 3A). Cells infected with the #185stop mutant appeared to accumulate slightly less IE1 at 4 hpi than WT-infected cells whereas the methionine mutants accumulated WT-levels of IE1 at 4 hpi (Fig. 3A). The impact of U26 mutations on the early accumulation of IE1 was more evident during low MOI infections. At MOI = 0.25, similar to the U26-null viruses, the 1stmetΔ mutant as well as the #185stop mutant accumulated less IE1 at 4 hpi in comparison to WT infected cells (Fig. 3B). The 2ndmetΔ mutant, which expresses only the long U26 isoform, accumulated a WT-level of IE1 at 4 hpi (Fig. 3B). As with the high MOI infection, the
levels of IE1 protein recovered by 24 hpi (Fig. 3B). Our results indicate that the U126 protein is important for the early accumulation of the IE1 protein. Further, it appears that the N-terminal 34 amino acids and C-terminal 38 amino acids play a role in this phenotype in an MOI-dependent manner.

Transposon-mediated deletion of the U126 protein also resulted in decreased delivery of the tegument protein pp28 upon initial infection [23]. Consistent with the previous observations, cells infected with U126TI contained less pp28 protein at 4 and 24 hpi (Fig. 3A). In contrast, cells infected with the DBmetD mutant contained WT levels of pp28 at 4 and 24 hpi (Fig. 3A). This indicates the possibility that transposon-mediated deletion of U126 could have additional consequences separate from the ablation of U126 expression, e.g. a second-site mutation. However, any potential second mutation had negligible impact on infection as repair of the U126 mutation in the transposon mutant rescued viral growth [23]. The accumulation of U126 isoforms was as expected: the 1stmetD and 2ndmetD mutants accumulated only the short or long isoforms respectively whereas neither the DBmetD nor UL26TI accumulated U126 (Fig. 3A). During the early stages of infection with the #185stop mutant, there was a reduction in the amount of U126 compared to WT (Fig. 3A). However, by 48 hpi, the accumulation of #185stop-U126 was equivalent to that of WT-U126 (Fig. 3C). Analysis of the accumulation of another early protein, U144, indicated little difference in U144 levels between the panel of viruses at an MOI = 3.0 (Fig. 3A).

However, during infection at an MOI = 0.25, the 1stmetD and U126-null viruses accumulated less U144 at 24 hpi compared to WT (Fig. 3B). This difference largely disappeared by 48 hpi (Fig. 3D). In cells infected with the U126-null viruses, there also appeared to be a moderate decrease in the amount of pp28 at 48 and 72 hpi (Fig. 3C, Fig. 3D). This decrease was observed at both high and low MOI infections, and would be consistent with the delayed kinetics of infection observed with U126-null viruses. Taken together, our data indicate that at lower multiplicities of infection, the N-terminal 34 and C-terminal 38 amino acids of U126 are important for the early timing of HCMV viral gene expression, inasmuch as the deletion of these domains results in slower accumulation of IE1, and subsequently U144.

To further analyze the contribution of these U126 domains to the viral infectious cycle, we measured the accumulation of viral DNA over the course of infection with our panel of U126 mutants. At a relatively high MOI (3.0), viral DNA accumulated similarly between the WT and 2ndmetD (Fig. 4A). During infection with the 1stmetD, #185-stop and DBmetD viruses viral DNA accumulated with slower kinetics (Fig. 4A). A similar trend was observed at a lower MOI (0.25) single round of infection. Cells infected with the 1stmetD, #185-stop and DBmetD viruses accumulated DNA less rapidly than cells infected with WT or the 2ndmetD virus (Fig. 4B).

To summarize, our data indicate that the N-terminal and C-terminal domains of U126 are important for normal timing
of HCMV infection. Deletion of these domains results in a slower accumulation of viral DNA and viral proteins.

The Impact of UL26 Mutations on Viral Plaque Size and Virion Stability

We and others have observed that deletion of the UL26 open reading frame results in reduced plaque size [24]. To analyze the contribution of specific UL26 domains to this phenotype, cells were seeded with a fixed number of plaque forming units (PFU) from our panel of mutants, overlaid with agarose and incubated at 37°C for 15 days. Images of the resulting plaques were captured with subsequent analysis of the area of each plaque. Images of representative plaques are shown in Figure 5A. As shown in Figure 5, the resulting plaques were substantially smaller upon infection with the #185stop or UL26-null viruses. The plaques produced by the 1stmetΔ virus were intermediate in size between the UL26-null viruses and WT HCMV, whereas the 2ndmetΔ virus produced plaques of WT size. These results indicate that the C-terminal 38 amino acids of the UL26 protein, and to a lesser extent, the N-terminal 34 amino acids are important for WT plaque size.

Previously, it has been found that the UL26 protein is important for virion stability, inasmuch as prolonged incubation at 20°C causes UL26-defective virions to lose their ability to initiate infection faster than WT virions do. [24]. To investigate how specific UL26 mutations impact virion stability, viral supernatants containing equivalent PFUs were incubated at 37°C for various times and then plated. The percentage of plaques remaining after incubation in comparison to control was plotted in Figure 6A. After 8 h of incubation, WT HCMV exhibited a less than 20% drop in infectivity (Fig. 6A). With the same incubation, the #185stop and UL26-null viruses demonstrated a >50% drop in infectivity, a statistically significant difference (Fig. 6A). After 20 h of incubation, WT HCMV lost ~40% of its infectivity (Fig. 6A). With this incubation, the #185stop and UL26-null viruses demonstrated a 70% loss of infectivity while the 1stmetΔ lost 60% of its infectivity (Fig. 6A). Different strains of HCMV have historically been found to exhibit varying sensitivities to trypsin treatment, a correlate of virion stability [33]. We therefore explored whether UL26 impacts viral trypsin sensitivity. We found that the UL26-null viruses, as well as the #185stop and 1stmetΔ viruses exhibited enhanced sensitivity to trypsin compared to wildtype HCMV (Fig. 6B). The infectious stability and trypsin
Figure 3. Accumulation of viral proteins after infection with UL26 recombinant viruses. Serum starved MRC5 fibroblasts were mock infected or infected with the indicated recombinant virus at an MOI of either 3.0 (A, C) or 0.25 (B, D). Viral proteins were harvested at 4 hr, 24 hr (A, B), 48 hr, 72 hr (C, D) post infection and processed for Western blotting using antibodies directed towards IE1, pUL44, pp28, UL26 (C-terminal specific antibody), and α-tubulin. A representative blot from two separate experiments is shown.

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Figure 4. Accumulation of viral DNA after infection with UL26 recombinant viruses. (A) Serum starved MRC5 fibroblasts were infected with the indicated recombinant virus at an MOI of either 3.0 (A) or 0.25 (B). Viral DNA was collected at 24, 48, 72 and 96 hpi. Real-time PCR was performed using HCMV-specific primers to analyze viral DNA accumulation. Values are means ± SD (n = 3).

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sensitivity results mirror each other, and suggest that the Cterminus of UL26, and to a lesser extent UL26’s N-terminal 34 amino acids, are important for infectious virion stability.

The Impact of N-terminal and C-terminal Mutations on UL26 Protein Localization

Previously, we found that the UL26 protein localized to the nucleus at early time points, and moved to the cytoplasm at later time points [23]. To determine whether specific domains of the UL26 protein were important for UL26 localization during infection, we analyzed cells infected with our panel of UL26 mutant viruses using confocal microscopy. After 5 hpi, a time at which the UL26 protein should be predominantly tegument-delivered, the UL26 protein was localized in the nucleus during WT, and 2ndmetD infection (Fig. 7A). At the same time point, and with equivalent confocal settings, there was reduced staining for nuclear UL26 protein in cells infected with the 1stmetD virus (Fig. 7A).

UL26 protein was not detectable during infection with the #185stop or the UL26-null viruses (Fig. 7A). After 72 hpi, the UL26 protein exhibits both nuclear and cytoplasmic staining during infection with the #183stop or the UL26-null viruses (Fig. 7A). At 72 hpi, the UL26 protein was not detectable during infection with the #185stop virus (Fig. 7A). After 72 h of infection with the 1 stmetΔ and 2 ndmetΔ viruses, the UL26 protein remained primarily nuclear, although there was increased cytoplasmic staining compared to the 5 hpi time point (Fig. 7B). Interestingly, after 72 h of infection with the #185stop virus, UL26 was predominantly cytoplasmic, with a reduction in nuclear staining compared to WT or the 1 st and 2 ndmetΔ viruses (Fig. 7B). Analysis of multiple fields at this time point revealed that #185stop-UL26protein was capable of accumulating in the nucleus, however it did so in a minority of cells, ~20%, in comparison to cells infected with wildtype or the methionine mutants, which exhibited nuclear UL26 in ~90% of cells (Fig. 7C). These results indicate that the #185stop mutant is defective for nuclear accumulation, suggesting that the C-terminal 38 amino acids of UL26 are important for proper nuclear localization.

As UL26 is a tegument protein, and its de novo expression depends on the expression of immediate early genes [34], the majority of UL26 protein present during the first 5 h of infection is part of the virion that was delivered to the cells upon infection [25]. At 5 h post infection with #185stop, we observed substantially decreased levels of truncated UL26, both by western and by immunofluorescence (Fig. 3A & Fig. 7A). These decreased levels of UL26 protein could result from defective incorporation into the viral tegument. To explore this possibility, we gradient purified wildtype and #185stop virions. As shown in Figure 7D, #185stop virions contained reduced levels of UL26 protein compared to WT virions. There was a greater than 50% reduction in the short #185stop isoform in comparison to wildtype HCMV and a greater than 80% reduction in the long #185stop isoform (Fig. 7E). These results suggest that this C-terminal deletion decreases the tegumentation efficiency of UL26 and is likely in part responsible for the reduced abundance of UL26 protein at early time points of infection. These results coupled with the defective UL26 nuclear localization associated with this mutant suggest that...
deletion of the C-terminal 38 amino acids largely ablates the nuclear activities associated with UL26 at early times post infection.

Discussion

Herpesvirus tegument proteins play important and divergent roles during the viral life cycle. These proteins are among the first to interact with the host cell upon infection, as they are delivered to the cytoplasm after membrane fusion. At this time, they serve to institute an environment conducive to viral replication, performing a myriad of activities such as suppressing innate immunity, activating cell signaling pathways and inducing viral gene expression [17,20,21]. In addition to these early functions, tegument proteins act at the very end of infection, playing roles in the assembly, envelopment, and egress of viral particles [14,15]. We have found that the UL26 tegument protein is critical for high titer HCMV replication [23]. However, it was unclear how the long and short UL26 isoforms contribute to in vitro HCMV replication. The UL26 protein has been implicated in functioning at both early time points of infection, impacting immediate early gene accumulation [23,25], as well as at late time points during virion assembly, as inasmuch as virions lacking UL26 exhibit reduced stability [24]. However, how the different UL26 isoforms, or how specific UL26 domains contribute to these phenotypes is unclear. To address these questions we created a panel of recombinant HCMV UL26 mutants and assessed their contribution to HCMV infection. We found that the short UL26 isoform is largely dispensable for in vitro replication, whereas the N-terminal 34 amino acids of the long isofrom are required for wildtype HCMV replication. Further, we find that the C-terminal 38-amino acids of the UL26 protein are important for wildtype HCMV replication, as well as for proper nuclear localization and normal tegumentation of the UL26 protein.

The C-terminal 38 amino acids of UL26 are critical for UL26-protein function, as a truncation mutant lacking these amino acids was indistinguishable from UL26-null viruses with respect to IE1 accumulation, viral growth, plaque size, and virion stability. This C-terminal-truncated UL26 protein was less abundant at earlier times post infection, but accumulated to wildtype levels at later times post infection. This decrease in UL26 protein at early times reflects the decreased tegumentation observed in #185stop virions. This C-terminal truncated UL26 protein also displayed substantially reduced nuclear localization compared to wildtype UL26. Employing an algorithm for identification of nuclear localization sequences [35], we find that the UL26 protein contains a predicted weak nuclear localization signal close to the C-terminus (Fig. 8). The #185stop mutation falls within this sequence, and its deletion is therefore potentially responsible for the defective nuclear localization of this mutant allele (Fig. 8). Given that the #185stop mutant behaves similarly to UL26-null virus, and exhibits apparent early and late defects, it makes it difficult to definitively separate early functions from late functions with respect to their contribution to viral replication. Additional site-specific mutational analysis of this region may enable separation of the residues that are important for nuclear localization versus those important for efficient tegumentation. Given its importance for UL26 function, mutants of this C-terminal domain will be a powerful tool for further genetic and mechanistic studies into UL26’s contribution to HCMV replication. For example, in screening potentially important UL26 interacting partners, viral or host-cell factors whose binding is dependent on this C-terminal domain should be given preference.

Interestingly, the C-terminal 38 amino acids of the UL26 protein appear to be less well conserved between human, the other primate CMVs, and non-primate CMVs in comparison to other areas of the protein (Fig. 8). Two macaque CMVs, RHCMV and CyCMV, for example, contain an extra ~30 C-terminal amino acids. This increased divergence between the different UL26 proteins may indicate that this domain is important for species specific differences between the CMV strains.

The UL26 message contains two initiating methionines which result in two in-frame UL26 protein isoforms that differ by only 34 N-terminal amino acids. It is unclear how these different isoforms contribute to HCMV infection. Our results indicate that the
**Figure 7. Localization of the UL26 protein in HCMV infected cells.** MRC5 fibroblasts were infected at an MOI of 3.0 and were fixed at either 5 hpi (A) or 72 hpi (B) and processed for immunofluorescence using an antibody specific for the C-terminus of UL26. (C) Quantification of the cells containing nuclear localized UL26 protein at 72 hpi. The percentage of total cells containing nuclear localized UL26 was plotted. UL26 localization in over one-hundred cells in separate fields was determined, **p<0.01** compared to WT virus. All counted cells contained detectable UL26 expression. (D) UL26 tegument protein composition of WT and #185stop virus. Viral particles from the supernatants of MRC5 fibroblasts infected with either WT or #185stop virus were separated from cell debris via low-speed centrifugation. Thereafter, the particles were purified by differential sedimentation in a glycerol-tartrate gradient. Resulting virion fractions were processed for Western blotting using UL26 and pp65-specific monoclonal antibodies. (E) Quantification of tegument UL26 protein in glycerol-tartrate purified virions. The amount of UL26 protein present in virions (D) was quantified after normalization to the amount of virion pp65. Quantification of UL26 and pp65 specific bands was performed using BioRad ImageLab software.

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**Figure 8. Comparison of UL26 protein sequences of various species-specific cytomegaloviruses.** The UL26 open reading frames of the indicated CMV species were aligned using the NCBI BLAST alignment tool. The black box highlights a putative NLS as predicted by the NLS mapper. The green asterisk indicates the 2nd initiation methionine and the red asterisk indicates the #185-stop insertion site.

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shorter isoform is dispensable for many of the in vitro phenotypes we assessed, including replication, plaque size, and viral stability. Despite its apparent lack of importance in vitro, the second in frame methionine is well conserved in primate CMV species (Figure 8). This suggests that the smaller isoform may be important in other settings, e.g., during in vivo infection or infection of alternate cell types. The shorter U26 isoform has been found to be preferentially packaged relative to the longer isoform during tegumentation (Fig 7C and [23]), however the mechanism governing this difference is not clear. Further, while a putative NLS was predicted in the C-terminus, and should therefore be present in both isoforms, it is not currently clear whether the different isoforms co-localize over the course of viral infection.

While the shorter U26 protein isoform is dispensable for in vitro growth, the longer U26 isoform, which contains an additional 34 N-terminal amino acids, was found to contribute to a number of in vitro phenotypes. The 1stmetΔ virus exhibited intermediate phenotypes between wildtype and U26-null viruses, including viral growth, plaque size, and viral stability. Further, the 1stmetΔ mutant displayed an MOI-dependent decrease in IE1 accumulation at 4 h. These results indicate that the 34 N-terminal amino acids are important for viral growth and argue that the long and short isoforms are not functionally redundant in vitro.

A number of questions remain about U26 and the mechanisms through which it contributes to HCMV replication. Prominently, how does the U26 protein contribute to virion stability? As a tegument protein, physical interactions between the U26 protein and other viral proteins in the virion could be important for maintaining virion stability. Alternatively, U26’s contribution to stability may occur earlier, for example in the proper assembly of virion particles. Previously we found that U26-null viruses produce virions with hypophosphorylated tegument proteins [23]. It is unclear whether this hypophosphorylation could contribute to unstable virions, or alternatively, whether the hypophosphorylation is a consequence of virion destabilization. Analysis of purified virion proteins by silver-stained gel indicates there is no dramatic difference in the proteins present in wildtype versus U26-null virus (data not shown). This suggests that large differences in virion protein constituents are likely not responsible for the decreased stability of mutant U26 viruses. Another major question is the function of U26 in the nucleus at early times. It seems likely that this nuclear U26 is responsible for impacting IE1 gene expression at early times, although the potential mechanism involved still needs to be elucidated. Our identification of the important U26 N-terminal and C-terminal domains will facilitate addressing these questions. The C-terminal 38-amino acids of U26 are important for proper U26 tegumentation, nuclear localization, and viral replication. Our studies highlight the importance of these C-terminal 38-amino acids for future study. Further functional analysis will distinguish how the specific residues within this domain contribute to U26 nuclear localization and proper tegumentation, and subsequently to HCMV replication. Given its importance to HCMV infection, elucidating the mechanisms through which U26 domains contribute to higher replication may shed light on possibilities for therapeutic intervention.

Author Contributions
Conceived and designed the experiments: CM CMS JM. Performed the experiments: CM CMS. Analyzed the data: CM CMS JM. Contributed reagents/materials/analysis tools: CM CMS JM. Wrote the paper: CM CMS JM.

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