Contributions of the Human Cytomegalovirus U_L30-Associated Open Reading Frames to Infection

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ABSTRACT Transposon-based insertional mutagenesis screens have assessed how disruption of numerous human cytomegalovirus (HCMV) open reading frames (ORFs) impacts *in vitro* viral replication. Insertional mutagenesis of the HCMV U_L30 gene was previously found to substantially inhibit production of viral progeny. However, there are a number of putative U_L30-associated ORFs, and it is unclear how they impact viral replication. Here, we report on the contributions of the eight U_L30-associated ORFs to infection. We find that deletion of the canonically annotated U_L30 ORF substantially reduces production of infectious virus at both high and low multiplicities of infection (MOI). This deletion likely has complex effects on viral replication, as we find that it reduces the expression of neighboring non-U_L30-associated ORFs. Mutation of the initiating methionine of the canonical U_L30 ORF indicated that it is dispensable for high- and low-MOI infection in the highly passaged AD169 strain, although it is important for low-MOI infection in the less-passaged TB40/E strain. Comutation of eight methionines in the U_L30 region results in a low-MOI viral replication defect, as does mutation of the TATA box responsible for the most abundant U_L30 transcript, which is found to be necessary for the accumulation of multiple U_L30-associated protein isoforms during infection. In total, our data indicate the importance of the U_L30-associated ORFs during low-MOI HCMV infection and further highlight the difficulty associated with the functional interrogation of broadly disruptive mutations: e.g., large deletions or transposon insertions.

IMPORTANCE Viral genes and their products are the critical determinants of viral infection. Human cytomegalovirus (HCMV) encodes many gene products whose roles during viral infection have not been assessed. Elucidation of the contributions that various HCMV gene products make to infection provides insight into the infectious program, which could potentially be used to limit HCMV-associated morbidity, a major issue during congenital infection and in immunosuppressed populations. Here, we explored the role of HCMV's U_L30-associated gene products and found that they are important for HCMV replication. Future work elucidating the mechanisms through which they contribute to viral infection could highlight novel avenues for therapeutic intervention.

KEYWORDS BAC-mediated recombineering, human cytomegalovirus, U_L30, viral gene expression

Human cytomegalovirus (HCMV) is a widespread betaherpesvirus that infects more than 50% of adults over the age of 40 in industrialized countries and a far higher percentage of adults and children in developing countries (1, 2). While infection of immunocompetent individuals is frequently asymptomatic, the virus is an opportunistic pathogen that can cause severe morbidity and mortality in immune-immature and immunocompromised individuals, such as infants, transplant recipients, and cancer and AIDS patients (3–6). Notably, congenital HCMV infection occurs in ~30,000 infants born each year in the United States and causes virally induced birth defects, including

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deafness, intellectual disabilities, and other central nervous system damage, in up to 20% of these children (7). Current treatments for HCMV infection are often toxic (8), have poor bioavailability (9), and result in the emergence of drug-resistant strains (10, 11). Given the prevalence and severity of HCMV infection among immunosuppressed populations, the lack of a suitable treatment or vaccine remains a significant issue.

HCMV has one of the largest genomes of any known human virus, with a double-stranded, 230-kb genome that is translated in both the sense and antisense directions (12). Ribosome profiling data suggest that the genome contains over 750 open reading frames (ORFs) (Merlin strain) (13). For the vast majority of these ORFs, their contribution to HCMV infection is unknown. This lack of knowledge represents a significant barrier toward therapeutic development as, to date, most clinically successful antiviral pharmaceuticals target the specific activities of viral gene products.

Previous studies have begun to globally address this issue. Functional maps of the AD169 and Towne laboratory strains of HCMV have been made via insertional mutagenesis or deletion of predicted viral ORFs. These ORFs were then subsequently classified based on their observed contributions to successful *in vitro* infection of fibroblasts (14, 15). For the AD169 study, approximately 150 ORFs were insertionally disrupted, with ~43 ORFs being found to be essential for HCMV infection in fibroblasts and ~30 ORFs exhibiting a ≥ 10 -fold reduction in viral progeny produced relative to wild-type (WT) infection of fibroblasts, and these results were largely consistent with the study in the Towne strain (14, 15). In both studies, a small ORF, U_L30, was found to be important for HCMV infection, with insertional mutagenesis of U_L30 in AD169 resulting in a ≥ 50 -fold defect in production of viral progeny (15).

The canonical U_L30 ORF was computationally predicted to be a 366-bp ORF that, if translated, would code for a 121-amino-acid (~14-kDa) protein (16, 17). While little is known about the protein product, proteomic experiments have identified several U_L30 peptide sequences that accumulate to a greater extent during the later times of infection (18). A study analyzing the subcellular localization of HCMV ORFs found that a C-terminally FLAG-tagged U_L30 exhibits subnuclear localization when exogenously expressed outside of the context of infection and induces the loss of Cajal bodies, subnuclear organelles where nuclear ribonucleoproteins are assembled (19). A microarray study showed the presence of U_L30 RNA 5 and 8 days postinfection (dpi) in CD34⁺ progenitor cells infected at a multiplicity of infection (MOI) of 10 with the AD169 strain of HCMV (20). Additionally, a study that examined the abundance of transcripts in the U_L30 to U_L32 region of the HCMV genome identified several transcripts of various lengths (0.6 to 6.0 kb) that accumulate at late times of infection (96 h postinfection [hpi]) (21). According to this study, the most abundant transcript in this region was a 0.6-kb transcript that starts upstream and ends in the middle of the canonical U_L30 ORF. Consistent with these results, ribosome profiling in this region shows a high abundance of ribosomes in the U_L30 region (13). Beyond these studies, little is known about the expression or function of U_L30 during HCMV infection.

In the current study, we explore U_L30's contributions to infection. Our results indicate that deletion of the U_L30 ORF significantly attenuates productive infection at high or low MOI. However, more-targeted and less-disruptive mutations that ablate expression of the canonical U_L30 ORF do not substantially impact high-MOI infection, suggesting that wholesale deletion of the U_L30 ORF induces additional deficits independent of U_L30 protein expression. These observations were largely consistent for U_L30 deletion mutants in the laboratory-adapted AD169 strain, as well as the clinical TB40/E strain of HCMV. In contrast, at low MOI, we find that translation of the U_L30-associated ORFs is important for viral replication and that the canonical U_L30 start codon is important for low-MOI infection in a TB40/E background.

RESULTS

Deletion of the HCMV U_L30 gene inhibits HCMV infection. Transposon insertion into the U_L30 ORF has been reported to result in a ≥ 50 -fold defect in viral titer (15). To

FIG 1 Deletion of the U_L30 ORF attenuates viral infection. MRC5 fibroblast cells were infected with (A) WT, ΔU_L30, or U_L30stmut (AD169 strain, MOI of 3.0, *n*=4), (B) WT and ΔU_L30 repair viruses (AD169 strain, MOI of 3.0, *n*=2), and (C) WT, ΔU_L30, or U_L30stmut (AD169 strain, MOI of 0.01; 11 and 13 dpi, *n*=4; 1, 7, and 15 dpi, *n*=2). At the indicated times postinfection, virus was harvested for analysis of viral progeny by TCID₅₀. (D) MRC5 fibroblast cells were infected with WT, ΔU_L30, and U_L30stmut viruses (AD169 strain, MOI of 3.0). At the indicated times postinfection, viral DNA was harvested for analysis by qPCR (*n*=3). The relative abundance of viral DNA was quantified using an IE1 probe and normalized to cellular DNA via a GAPDH probe. (E) MRC5 fibroblast cells were infected with 100 PFU of WT, ΔU_L30, and U_L30stmut viruses in the AD169 strain. At 11 dpi, plaque sizes were quantified by ImageJ (WT, *n*=54; ΔU_L30, *n*=64; ΔU_L30 repair, *n*=32; U_L30stmut, *n*=44). Error bars represent the standard error of the mean (SEM). •, *P* < 0.05, ••, *P* < 0.01, and ns, not significant, by Van Waerden test.

corroborate these findings and to explore the contributions of the canonical U_L30 ORF to infection, we employed bacterial artificial chromosome (BAC)-mediated recombining to target U_L30 in two ways. First, we created a virus in which the U_L30 ORF was completely deleted (ΔU_L30), and second, we created a virus in which the start codon (ATG) was mutated to an ATC (U_L30stmut) to prevent translation of a potential U_L30 protein, a strategy we have previously employed to prevent translational initiation during HCMV infection (22). During high-MOI infection, deletion of the U_L30 ORF resulted in a greater than 20-fold defect in viral replication (Fig. 1A). These results are consistent with previous studies that inserted green fluorescent protein (GFP) into the U_L30 ORF in AD169 (15) or deleted U_L30 in the Towne strain (14). Both of these studies observed a greater growth defect (≥50-fold) than we observed here, but these differences are likely due to differences in the type of mutation and strain. In contrast, mutation of the U_L30 start codon had no detectable effect on viral replication at a high MOI (Fig. 1A). These results suggest that deletion of the U_L30 ORF may inhibit HCMV replication independently of the expression of the U_L30 protein. Subsequent repair of the U_L30 deletion rescued the replication of ΔU_L30, suggesting that second-site mutations are not responsible for the ΔU_L30 mutant's decreased replication (Fig. 1B). During low-MOI infection, the ΔU_L30 virus exhibited a substantially greater defect in viral replication, accumulating to titers 60- to 100-fold less than WT at 11 and 13 dpi, respectively (Fig. 1C). The start codon mutation resulted in a much smaller, 2- to 3-fold growth defect, a difference that failed to be statistically significant (Fig. 1C). These results suggest that expression of the canonical U_L30 ORF is dispensable for peak viral titers during infection of fibroblasts.

At an MOI of 3.0, deletion of U_L30 had a minor, statistically insignificant effect on viral DNA accumulation, and the U_L30 start codon mutation did not impact viral DNA accumulation (Fig. 1D), suggesting that the U_L30 region of the genome is not important for viral

FIG 2 Deletion of the U_L30 ORF results in reduced expression of viral proteins. MRC5 fibroblast cells were infected with WT, ΔU_L30 , and $U_L30stmut$ viruses (AD169 strain, MOI of 3.0). (A) At the indicated times postinfection, protein was harvested, and expression levels of representative IE, E, and L gene products were examined by Western blotting. (B) At 4 hpi, nuclear and cytoplasmic fractions were separated, and protein and DNA were extracted from each fraction ($n=3$). Total and nuclear genomes were quantified by qPCR, and protein was quantified by Western blotting. Error bars represent SEM.

DNA replication. In contrast, analysis of viral plaque size revealed that plaques generated by the ΔU_L30 virus are about 55% smaller than those formed by WT virus, while the repair virus displayed WT-like plaque sizes (Fig. 1E). Plaques generated by the $U_L30stmut$ virus displayed WT-like plaque sizes (Fig. 1E). These results suggest that the U_L30 region is necessary for optimal cell-to-cell spread in a way that is independent of translation of the canonical U_L30 ORF.

As we observed a significant defect in HCMV replication upon deletion of U_L30 , we wanted to examine the impact on the viral life cycle. We monitored the accumulation of representative immediate early (IE), early (E), and late (L) genes during infection at a high MOI (3.0). We found that mutation of the start codon of U_L30 had no effect on IE, E, or L protein levels relative to WT HCMV (Fig. 2A). In contrast, deletion of the U_L30 ORF resulted in reduced accumulation of IE1 at 8 hpi (Fig. 2A). Densitometry analysis estimated that IE1 levels were reduced by about half in the absence of U_L30 relative to WT at 8 hpi. Additionally, the deletion of the U_L30 ORF resulted in decreased protein levels of U_L26 at 48 hpi and pp28 at 96 hpi (Fig. 2A). These results suggest that in a high-MOI infection, the complete deletion of the U_L30 ORF induces a significant defect in viral protein expression, in contrast to the U_L30 start mutation, which exhibits no defect.

Deletion of the U_L30 ORF does not impact nuclear genome delivery. The defects in immediate early protein expression observed upon U_L30 deletion could have resulted from decreased nuclear delivery of viral genomes. To determine whether the ΔU_L30 or the $U_L30stmut$ virus had a defect in nuclear genome delivery, we measured the accumulation of nuclear genomes during infection. Separation of cytoplasmic and nuclear fractions was confirmed by Western blotting, but no difference in the percentages of nuclear genomes was detected between the WT and mutant viruses (Fig. 2B). These experiments suggest that the ΔU_L30 and $U_L30stmut$ viruses are not defective for nuclear genome delivery.

Deletion of the canonical U_L30 ORF attenuates U_L31 and U_L32 RNA abundance. Previously published ribosome profiling data reported a high ribosome density on mRNA containing the U_L30 ORF at 72 hpi, as well as on other short putative ORFs

FIG 3 Ribosome profiling and RNA transcripts in the U_L30 region. (A) A MochiView (40) output of ribosome profiling and RNA-seq data from Stern-Ginossar et al. shows high ribosome density and RNA levels in the U_L30 region 72 hpi (13). (B) Transcript mapping of the U_L30 regions by Ma et al. and Mitchell et al. shows many overlapping RNA transcripts containing U_L30 sequence (21, 23). (C and D) MRC5 fibroblast cells were infected with WT virus (AD169 strain, MOI of 3.0) in the presence or absence of PAA. At 24, 48, and 72 hpi, RNA was harvested, and 5' RACE was performed as described in Materials and Methods. The sequence confirmed 5' transcript ends from the gels match transcript ends identified by Ma et al. and are denoted by the green and orange asterisks. The primers used are indicated by the red arrows in panel A.

immediately upstream of U_L30: one previously identified as U_L30A and two others which we termed U_L30B and U_L30C (Fig. 3A) (13). The high ribosome density ends in the middle of U_L30 at a location just downstream of a polyadenylation signal sequence (13). Additionally, several overlapping, U_L30-containing, transcripts have been reported, many of which terminate at the polyadenylation signal in the middle of U_L30 (13, 21, 23) (Fig. 3B). The most abundantly expressed transcript identified in this region is a 0.6-kb transcript that starts immediately upstream of U_L30A and ends at the polyadenylation signal in the middle of U_L30 (21). We performed 5' rapid amplification of cDNA ends (5' RACE) in this region with a primer for U_L30 and identified the same 0.6-kb transcript that accumulates over time (Fig. 3C). The accumulation of this 5' end is inhibited by phosphonoacetic acid (PAA) treatment, which inhibits viral DNA replication. This result suggests that this U_L30 transcript is a true late transcript (Fig. 3C). Several transcripts in this region also contain

FIG 4 Deletion of the UL30 ORF affects the RNA levels of some surrounding ORFs during HCMV infection. (A to H) MRC5 fibroblast cells were infected with WT or Δ UL30 viruses (AD169 strain, MOI of 3.0). At the indicated times postinfection, RNA was harvested and cDNA was synthesized. The relative levels of several surrounding ORFs were quantified as described in Materials and Methods, and RNA levels were normalized to WT 24 hpi (A, $n=3$; B, $n=4$; C, $n=2$; D, $n=4$; E, $n=3$; F, $n=3$; G, $n=2$; H, $n=2$). (I) MRC5 fibroblast cells were infected with WT, Δ UL30, or UL30stmut viruses (AD169 strain, MOI of 3.0). At the indicated hours postinfection, protein was harvested and expression of UL32 was examined by Western blotting. *, $P < 0.05$, **, $P < 0.01$, and ns, not significant, by Student's t test.

all or portions of the UL32 ORF (Fig. 3B), which has been shown to be essential for HCMV infection (15) and is critical for virion maturation. 5' RACE with a primer for UL32 verified a transcript that starts upstream of UL32, and accumulation of this 5' end was also inhibited by PAA treatment (Fig. 3D). Downstream of UL30, a UL29 transcript and a UL28/UL29 spliced transcript have been identified (23). The 5' end of these spliced transcripts has not been identified, although it is hypothesized that a TATA box at the end of UL30 is responsible for transcription of these RNAs (23). Lastly, an RNA antisense to UL27 to UL30 has also been identified (24) (data not shown). Collectively, these studies highlight the transcriptional complexity of this region and suggest that the deletion of the UL30 ORF could impact the expression of these transcripts.

To address the possibility that the deletion of the UL30 ORF was impacting the expression of local viral RNA, we measured the accumulation of a number of viral transcripts in this region. Primers amplifying the UL31 and UL32 ORFs were used, although UL31, UL32, and several uncharacterized ORFs may be on overlapping transcripts that may be detected by both primer sets. The abundance of UL31 and/or UL32 RNA was significantly decreased during high-MOI Δ UL30 infection (Fig. 4A and B). Disruption of the UL31 ORF has been previously shown to have no effect on viral growth (15), although it has recently been shown to be important for nucleolar reorganization (25) and to inhibit cGAS during infection, thereby attenuating DNA sensing and subsequent antiviral signaling (26). Deletion of UL31 has been reported to induce the accumulation of specific antiviral genes, including *IFNB1*, *RANTES*, and *IL6* (26). During Δ UL30 infection, we did not observe a statistically significant increase in the accumulation of these

antiviral RNAs (Fig. 4C), suggesting that any observed decrease in U_L31 RNA abundance does not substantially impact U_L31's predicted inhibition of cGAS.

In contrast to the substantial reductions in U_L31 and U_L32 RNA levels observed during Δ U_L30 infection, fairly minor or statistically insignificant effects were observed for the accumulation of U_L26, U_L27, and U_L29 (Fig. 4D to F), which are located close to U_L30 (Fig. 3A). We also examined the accumulation of two representative immediate early genes that are located further away, U_L37 and IE1. Slight but statistically significant reductions were observed in U_L37 RNA accumulation, whereas no statistically significant changes in IE1 RNA levels were observed during the first 72 hpi (Fig. 4G and H).

As the U_L30 deletion decreased U_L32 RNA levels, we investigated whether Δ U_L30 infection impacted U_L32 protein levels. As shown in Fig. 4I, infection with the Δ U_L30 virus exhibits reduced U_L32 protein accumulation relative to both WT HCMV and the U_L30stmut. These results are consistent with the deletion of the U_L30 ORF reducing U_L32 expression, suggesting that Δ U_L30-associated viral deficits may reflect aberrant U_L32 expression, a mutant of which displays somewhat similar phenotypes (27).

Mutation of U_L30-associated ORFs attenuates low-MOI infection. The U_L30A, U_L30B, and U_L30C ORFs were all found to possess high ribosome density (13) (Fig. 3A). Further, these ORFs appear to share a transcript with the canonical U_L30 ORF, which terminates in the middle of the U_L30 ORF (Fig. 3B) (21). These smaller U_L30 ORFs have never been functionally assessed with respect to their expression or roles during HCMV infection. We found that U_L30A and U_L30B RNAs accumulate during infection, with the highest levels of transcripts present at later times: e.g., 72 to 96 hpi (Fig. 5A). Infection with the Δ U_L30 mutant appeared to reduce the abundance of these transcripts relative to WT infection, although the changes were not statistically significant (Fig. 5A). In an attempt to address how the primary transcript that expresses U_L30, U_L30A, U_L30B, and U_L30C contributes to infection, we mutated a putative TATA box 68 bp upstream of U_L30A (Fig. 5B). This mutation was engineered to be silent with respect to U_L31, which is expressed in the opposite direction on the sense strand. After infection with this TATA box mutant (U_L30tatmut), the 0.6-kb transcript was not detectable via 5' RACE analysis, in contrast to WT infection, in which it was abundant (Fig. 5C). However, we were still able to detect the 5' end of the U_L32 transcript when the TATA box upstream of U_L30 was mutated, confirming that other RNA in this region is still detectable. These results suggest that the TATA box upstream of U_L30 is controlling transcription of the 0.6-kb transcript. The TATA box mutation reduced RNA levels of U_L30 ~2-fold at 72 hpi (Fig. 5D) and decreased U_L30A levels by 7.5-fold at the same time point (Fig. 5E). These results suggest that the TATA box upstream of U_L30 is important for accumulation of the 0.6-kb transcript and for WT levels of expression of U_L30 and U_L30A RNA. The U_L30tatmut virus exhibited reduced RNA levels of U_L30 and U_L30A (Fig. 5D and E), but it did not abolish their expression completely, which is not surprising given that additional transcripts in this region are still transcribed (Fig. 3).

To determine the extent to which the TATA box upstream of U_L30 contributes to viral replication, we examined viral growth during high- and low-MOI infections with the virus harboring the TATA box mutation. We found that TATA box mutation had little effect on viral titers of samples harvested from a high-MOI infection (Fig. 5F). By extension, this suggests that a 50% loss of U_L30 RNA has little effect during a high-MOI infection. In contrast, mutation of the TATA box upstream of U_L30 had a significant effect during a low-MOI infection, with titers at 11 and 13 dpi reduced 3.5- and 8.7-fold, respectively (Fig. 5G). These results suggest that while the TATA box upstream of U_L30 is largely dispensable for high-MOI infection, it is important for low-MOI infection.

In addition to the start codons of U_L30A and U_L30C, there are five additional in-frame methionines in this region that exhibit significant ribosome density and which could be capable of producing proteins (Fig. 5B, red asterisks). To examine how these U_L30-associated ORFs collectively contribute to HCMV infection, we generated a recombinant virus in which all 7 in-frame methionines were mutated, along with the U_L30B start codon, which is not in-frame, for a total of 8 methionine mutations (U_L30-

FIG 5 Mutation of the TATA box upstream of U_L30 results in a low-MOI growth defect. (A) MRC5 fibroblast cells were infected with WT or Δ U_L30 viruses (AD169 strain, MOI of 3.0, $n=3$). At the indicated times postinfection, RNA was harvested and cDNA was synthesized. The relative levels of U_L30A and U_L30B were quantified as described in Materials and Methods, and RNA levels were normalized to WT 24 hpi. (B) Schematic of U_L30A, U_L30B, U_L30C, and U_L30 with mass spectrometry (MS)-identified U_L30 peptides (18) and additional mutant viruses. Red asterisks denote the locations of the start codons mutated in the U_L30-8stmut virus, and a red arrow denotes the primer used for 5' RACE experiments. Bracketed bars indicate that U_L30A and U_L30C are in-frame with the first start codon of U_L30. (C) MRC5 fibroblast cells were infected with WT or U_L30tatmut viruses (AD169 strain, MOI of 3.0). At 72 hpi, RNA was harvested, and 5' RACE was performed as described in Materials and Methods. (D and E) MRC5 fibroblast cells were infected with WT or U_L30tatmut viruses (AD169 strain, MOI of 3.0, $n=2$). At the indicated times postinfection, RNA was harvested and cDNA was synthesized. The relative levels of U_L30 and U_L30A were quantified as described in Materials and Methods, and RNA levels were normalized to WT 24 hpi. (F and G) MRC5 fibroblasts were infected with WT or U_L30tatmut viruses (AD169 strain) at an MOI of 3.0 (F; $n=3$) or 0.01 (G; $n=4$). At the indicated times postinfection, virus was harvested for analysis of viral progeny by TCID₅₀. Error bars represent SEM. *, $P < 0.05$, **, $P < 0.01$, and ns, not significant, by Student's t test. •, $P < 0.05$ by Van der Waerden test.

FIG 6 Mutation of 8 start codons around U_L30 results in a low-MOI growth defect. (A and B) MRC5 fibroblasts were infected with WT, U_L30-8stmut, or U_L30-8stmut repair (B) viruses, or the AD169 strain at an MOI of 3.0 (A; $n = 3$) or 0.01 (B; $n = 4$). At the indicated times postinfection, virus was harvested for analysis of viral progeny by TCID₅₀. (C and D) MRC5 fibroblast cells were infected with WT, ΔU_L30, or U_L30stmut, U_L30tatmut, and U_L30-8stmut viruses (AD169 strain, MOI of 3.0). (C) MRC5 fibroblast cells were infected with 100 PFU of WT, U_L30tatmut, U_L30-8stmut, and U_L30-8stmut repair viruses (AD169 strain). At 11 dpi, plaque sizes were quantified by ImageJ (WT, $n = 54$; U_L30tatmut, $n = 38$; U_L30-8stmut, $n = 32$; U_L30-8stmut repair, $n = 30$). (D) At the indicated times postinfection, protein was harvested and U_L30 protein expression levels were examined by Western blotting. Both blots are representative of experiments completed with different stocks of viruses. Error bars represent SEM. •, $P < 0.05$, and □, $P < 0.06$, by Van Waerden test.

8stmut). These mutations were silent to overlapping or sense-strand ORFs, but mutations to in-frame methionines downstream of U_L30A, U_L30C, and U_L30 would affect the amino acid sequences of these ORFs if they are in fact translated (Fig. 5B). We examined viral replication of U_L30-8stmut in both high- and low-MOI infections and found that, similar to the U_L30tatmut, the virus with the eight start mutations grew identically to WT at high MOI, yet displayed a growth defect during a low-MOI infection (Fig. 6A and B). Repair of the 8 start mutations rescued viral replication during a low-MOI infection, suggesting that the defect is not due to mutation(s) at a secondary site (Fig. 6B). These results suggest that translation of one or more of these U_L30 ORFs is important for low-MOI infection. While the U_L30-8stmut, along with the U_L30tatmut viruses, displayed low-MOI growth defects, neither virus displayed a significant defect in plaque size (Fig. 6C). These results are consistent with the U_L30stmut virus not having an effect on plaque size (Fig. 1E).

Little is known about which U_L30-associated ORFs are expressed as proteins. Previous mass spectrometry (MS) experiments have found that peptides mapping to the canonical U_L30 ORF accumulate during infection (Fig. 5B) (18). These peptides fall within the portion of the U_L30 ORF that has a high ribosome occupancy at 72 hpi. To explore the expression of U_L30-associated proteins, we generated an antibody to one of the U_L30-associated MS-identified peptides. The canonical U_L30 ORF is 366 bp long, which would yield an expected ~14-kDa protein. Using the newly created U_L30 antibody, we observed a doublet at 25 kDa and a number of lesser, more diffuse, bands between 15 and 25 kDa (Fig. 6D) that accumulate during WT infection, all of which were absent during mock infection. These U_L30-associated bands are largely present upon infection with the U_L30stmut, which only has the canonical start codon mutated, but are completely absent during infection with the ΔU_L30 virus (Fig. 6D). Mutation of either the TATA box upstream of U_L30 (U_L30tatmut) or the eight U_L30-associated methionine codons (U_L30-8stmut) blocks the accumulation of the U_L30-associated bands

FIG 7 Deletion of the U_L30 ORF in TB40 results in a viral growth defect at a low MOI. MRC5 fibroblast cells were infected with WT, ΔU_L30 ORF deletion, and $U_L30stmut$ viruses (TB40/E strain) at an MOI of (A) 3.0 (1, 2, 3, 6, dpi, $n=4$; 4 dpi, $n=2$) or (B) 0.01 ($n=3$). At the indicated times postinfection, virus was harvested for analysis of viral progeny by TCID₅₀. (C) MRC5 fibroblast cells were infected with 100 PFU of WT, ΔU_L30 1, ΔU_L30 2, $U_L30stmut$ 1, or $U_L30stmut$ 2 (TB40/E strain). At 11 dpi, plaque sizes were quantified by ImageJ (WT, $n=62$; ΔU_L30 1, $n=30$; ΔU_L30 2, $n=64$; $U_L30stmut$ 1, $n=48$; $U_L30stmut$ 2, $n=57$). Error bars represent SEM. •, $P < 0.05$, ••, $P < 0.01$, and □, $P < 0.06$, by Van Waerden test.

between 15 and 25 kDa (Fig. 6D). These results confirm that U_L30 -associated proteins are being translated and that translation of these proteins can be initiated at a site other than the currently recognized canonical start codon. Further, the significantly larger molecular weight observed for these U_L30 proteins suggests the involvement of significant posttranslational modifications or the possibility of alternative RNA or ribosomal processing leading to increased molecular weight. In addition to the bands between 15 and 25 kDa, there is a band at about 47 kDa that is specific to infection but is not affected by any of the mutations to the U_L30 region. Given that the band is present when the U_L30 ORF is deleted, it is unclear whether or not this band is relevant to U_L30 .

Mutation of the U_L30 start codon attenuates low-MOI infection in a TB40/E background. The above experiments describe a growth defect observed when U_L30 is deleted in the laboratory-adapted AD169 strain of HCMV. To examine this result further in a more clinically relevant strain, we determined how the U_L30 deletion and U_L30 start codon mutations affected viral infection in the TB40/E background. The TB40/E strain is less passaged than AD169 and, in contrast to AD169, is able to infect several different cell types. Further, some HCMV genes behave differently in laboratory-adapted backgrounds versus those in which passage has been limited. For example, U_L84 is essential for infection in an AD169 background but is dispensable for infection with the TB40/E strain (28). After generating ΔU_L30 and $U_L30stmut$ in the TB40/E background, we found that during a high-MOI infection, the ΔU_L30 virus exhibited a slight defect in viral replication, although the difference was not quite significant for the tested number of replicates. The $U_L30stmut$ virus grew to similar titers as WT TB40/E, consistent with our results observed in the AD169 strain of HCMV (Fig. 7A). However, at low MOI, both mutant viruses display significant growth defects, with the ΔU_L30 and $U_L30stmut$ viruses growing to titers that were reduced 30- and 9-fold compared to WT, respectively (Fig. 7B). These results suggest that during a low-MOI TB40/E infection, the start codon of U_L30 makes a greater contribution to HCMV infection than in the AD169 strain of HCMV. Further, in the AD169 background, the ΔU_L30 , but not $U_L30stmut$, virus displayed a defect in plaque size (Fig. 1E). In contrast, both of these mutations resulted in a statistically significant defect in plaque size in the TB40/E strain (Fig. 7C). This defect was observed in viral stocks generated from two separate isolates of each mutant virus (ΔU_L30 1 and 2 and $U_L30stmut$ 1 and 2), suggesting that the defect is not due to a clonal second-site mutation.

DISCUSSION

Ribosome profiling data have suggested that over 750 ORFs are present in the HCMV genome (13). The function of the vast majority of these ORFs is unknown. Insertional mutagenesis strategies have created functional maps of the HCMV genome,

which highlight specific ORFs that contribute significantly to infection in cell culture (14, 15). While a very informative first step, insertional mutagenesis can impact the expression of numerous transcripts or open reading frames proximal to the insertion site, making it difficult to ascribe the resulting viral phenotypes to specific transcripts or open reading frames. To address this issue, more finely tuned and less-disruptive mutagenesis strategies are required. In this study, we performed a detailed analysis of the U_L30-associated open reading frames. We find that deletion of the U_L30 ORF results in a significant viral growth defect during high-MOI infection, consistent with earlier studies that have shown that transposon insertion in the U_L30 ORF results in a ≥ 50 -fold defect in viral replication (14, 15). However, this growth defect does not appear to result solely from the loss of the canonically predicted U_L30 protein as mutations that ablate U_L30 expression grow with WT kinetics at high MOI. Mutation of the single methionine that initiates the canonical U_L30 ORF limits the ability of HCMV to replicate at low MOI by 2- to 3-fold (Fig. 1), but this result is not statistically significant. While the exact mechanism of this Δ U_L30 defect is unclear, we speculate that decreased expression of neighboring genes is responsible. For example, we find that deletion of U_L30 reduces the RNA levels of both U_L31 and U_L32 (Fig. 4). U_L32 is an essential gene (15), and decreases in its expression have been linked to similar viral replication defects (27). These results highlight the necessity of higher-resolution mutational analysis to further interrogate the functionality of viral genes implicated by insertional mutagenesis approaches. However, given the transcriptional complexity of the HCMV genome, even small mutations targeted to proteins could have unforeseen consequences.

Many questions remain about the nature of the protein products expressed from U_L30-containing transcripts. To explore this issue, we generated a U_L30-specific antibody and found a predominant doublet at approximately 25 kDa that is lost upon infection with mutants harboring a deletion of the canonical U_L30 ORF (Δ U_L30 virus), a U_L30-associated TATA box mutation (U_L30tatmut), or the deletion of eight potential initiating methionines (U_L30-8stm) (Fig. 6). In addition, a smear of bands below 25 kDa but greater than 15 was also lost in the aforementioned mutants (Fig. 6). The apparent molecular weight of the U_L30 doublet is larger than the 13 kDa predicted from the canonical U_L30 protein. Possibilities for the increased molecular weight include posttranslational modifications (e.g., glycosylation or phosphorylation) or that U_L30 is part of a larger protein, although we were unable to detect mRNA splice variants at this locus (data not shown). In an attempt to address this issue, we tried to purify U_L30 protein products via immunoprecipitation experiments with the U_L30 antibody we generated, which would allow characterization of these products by MS. Unfortunately, these immunoprecipitations were unsuccessful. Future identification and characterization of the U_L30 protein products and their potential posttranslational modifications will enable elucidation of how they specifically contribute to HCMV infection.

Our results indicate that U_L30 contributes more to infection with TB40/E clinical isolate than the AD169 lab strain. Specifically, in the AD169 background, we see that the mutation of the canonical U_L30 start codon results in an ~ 3 -fold, insignificant growth defect during a low-MOI infection, whereas in the TB40/E background, an ~ 9 -fold defect was observed (Fig. 1 and 7). The reasons for this difference are unclear. There are a number of genetic and biological differences between laboratory-adapted and clinical isolates that could be responsible. In the U_L30 gene, there are eight base pair differences between the AD169 and TB40/E strains, resulting in amino acid changes that could potentially contribute to these strain-specific differences. Alternatively, other genetic differences outside of U_L30 could be playing a role. Laboratory and clinical isolates have several differences in a variety of important genes, including RL13, U_L36, U_L40, U_L128, U_L144, U_L146, and U_L147 (29–31). These strain differences could be responsible for the differential growth defect between AD169 and TB40/E in response to mutation of the canonical U_L30 ORF.

Our data also indicate that the mutation of a TATA box upstream of U_L30, U_L30A, U_L30B, and U_L30C ablates the production of the most abundant U_L30-associated

TABLE 1 Sequences of the primers for KanR insertion into U_L30 ORFs

Primer	Sequence (5'→3') ^a
KanR insertion into U _L 30 of AD169	
Forward	GTTGAAAACGCGCATGATCTCGCGAAGCCATCTACGCGCCTGTCAGGGCG AGGATGACGACGATAAGTAGGG
Reverse	CCACACGCTCAGCCGCGACTGAACGCCGGGGCGCGCCGCTACTTGGGTTT CAACCAATTAACCAATTCTGATTAG
KanR insertion into U _L 30 of TB40/E	
Forward	GTTGAAAACGCGCATGATCTCGCGGAGCCATCTACGCGCCTGTCAGGGCG AGGATGACGACGATAAGTAGGG
Reverse	CCACACGCTCAGCCGCGACTGAGCGCCGGGGCGCGCCGCTACTTGGGTTT CAACCAATTAACCAATTCTGATTAG
KanR insertion from U _L 30A through U _L 30 of AD169	
Forward	GGCCCGAGCGCGCCGGGGAGAAGAACCCTTCCCGGGCCCCGCGTTCAAG AGGATGACGACGATAAGTAGGG
Reverse	CCACACGCTCAGCCGCGACTGAACGCCGGGGCGCGCCGCTACTTGGGTTT CAACCAATTAACCAATTCTGATTAG

^aThe KanR sequence is in boldface.

transcript (Fig. 5) and prevents accumulation of U_L30-associated proteins (Fig. 6D). These results raise interesting questions. Given that this transcript is important for U_L30 production, (i) how is U_L30 translated in the presence of upstream initiating methionines, and (ii) do these upstream ORFs regulate translation? It has been previously found that the expression of the U_L4 gene is regulated by an upstream ORF (32, 33), so modulation of U_L30 translation by upstream ORFs remains a possibility.

HCMV infection is widespread and devastating to immunocompromised populations due to the lack of safe and effective treatments. While genomic- and proteomic-scale studies provide important information on ORFs that contribute to infection, a more detailed analysis of individual ORFs is required to elucidate the function of viral genes and proteins. We have shown that the U_L30-associated start codons and the upstream TATA box are important for low-MOI infection. However, more work is required to identify the mechanisms through which U_L30 contributes to infection.

MATERIALS AND METHODS

Cell culture, viruses, and viral infection. MRC5 fibroblasts (ATCC CCL-171) and telomerase-immortalized MRC5 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (Atlanta Biologicals, Seradigm), 4.5 g/liter glucose, and 1% penicillin-streptomycin (Pen-Strep; Life Technologies) at 37°C in a 5% (vol/vol) CO₂ atmosphere. Before HCMV infection, MRC5 cells were grown to confluence at $\sim 3.1 \times 10^5$ cells per cm² in a 6-well dish. Once confluent, medium was removed, and serum-free medium was added. Cells were maintained in serum-free medium for 24 h before infection, at which point they were mock infected or infected at an MOI of 3.0 or 0.01 PFU/cell. After a 1.5- to 2-h adsorption period, the inoculum was aspirated, and fresh serum-free medium was added. Unless indicated otherwise, the strain utilized for viral infections was BADwt, derived from a bacterial artificial chromosome (BAC) clone of the HCMV AD169 laboratory strain (GenBank accession no. [FJ527563](#)) (34). The Δ U_L30, Δ U_L30 repair, U_L30stmut, U_L30tatmut, U_L30-8stmut, and U_L30-8stmut repair recombinant viruses were generated using BAC recombineering as previously described (22, 35), using the primers in Table 1 for KanR insertion and gBlocks (IDT) containing the desired mutations. Viral stocks were propagated in MRC5 fibroblasts or telomerase-immortalized MRC5 fibroblasts. Viral stock titers were determined by plaque assay, and for the assessment of viral replication, viral titer was determined via 50% tissue culture infective dose (TCID₅₀) analysis.

Analysis of DNA and transcripts during infection. Viral DNA accumulation was assessed by real-time quantitative PCR (qPCR) as previously described (36). Briefly, MRC5 fibroblasts were infected with WT or Δ U_L30 virus at an MOI of 3.0 and then, at various time points, scraped in media, washed with phosphate-buffered saline (PBS), and resuspended in lysis buffer (100 mM NaCl, 100 mM Tris [pH 8.0], 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K, 40 μ g/ml RNase A). Cells were lysed at 55°C for 3 h, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), extracted again with chloroform-isoamyl alcohol (24:1), ethanol precipitated, and resuspended in water. The DNA abundance was assessed via qPCR using Fast SYBR green master mix (Applied Biosystems), a model 7500 Fast real-time PCR system (Applied Biosystems), and Fast 7500 software (Applied Biosystems) according to the manufacturer's instructions. Viral DNA abundance was determined with a primer pair targeting the viral IE1 gene: 5'-CCATGTCCACTCGAACCTTAAT-3' (forward) and 5'-TGAACAAGTGACCGAGGATTG-3' (reverse). Host cell DNA was assessed using the following primers targeting GAPDH (glyceraldehyde-3-phosphate dehydrogenase): 5'-CATGTTCTGTCATGGGTGAACCA-3' (forward) and 5'-ATGGCATGGACTGTGGTCATGAGT-3' (reverse). IE1 and GAPDH gene equivalent values were determined using the threshold cycle ($2^{-\Delta\Delta CT}$) method, and normalized viral DNA accumulation values were achieved by dividing the abundance of IE1 gene equivalents by the abundance of GAPDH gene equivalents. To measure viral and host gene transcription during infection, RNA was isolated from infected cells (MOI of 3.0) via TRIzol (Invitrogen) extraction

according to the manufacturer's instructions and used to synthesize cDNA with random hexamer primers and the Superscript II reverse transcriptase system (Invitrogen). Relative quantities of gene expression were measured and normalized to GAPDH levels via the $2^{-\Delta\Delta CT}$ method using the following primers: viral U_L32, 5'-CACACAACACCGTCGTCGATTAC-3' (forward) and 5'-GGTTTCTGGCTCGTGATGTCG-3' (reverse); viral U_L31, 5'-GCTCTGCTCCACAGCGAG-3' (forward) and 5'-CGGCGTCGTGAAAGGCAC-3'; human *IFNB1*, 5'-TTGTTGAGAACCTCTGGCT-3' (forward) and 5'-TGACTATGGTCCAGGCACAG-3' (reverse); human *RANTES*, 5'-GGCAGCCCTCGCTGTCATCC-3' (forward) and 5'-GCAGCAGGGTGTGGTGTCCG-3' (reverse); human *IL6*, 5'-TTCTCCACAAGCGCTTCGGTC-3' (forward) and 5'-TCTGTGTGGGCGGCTACATCT-3' (reverse); viral U_L29, 5'-GCTGGTGGGCGCAGGATAAGTTG-3' (forward) and 5'-CCGATGCTCTCGTAGCGAAAGTC-3' (reverse); viral U_L37, 5'-CCGAGTTCTCACCGTCAATTA-3' (forward) and 5'-CTCTCCCGCTTGGTTAAG-3' (reverse); viral U_L27, 5'-CGTGCAACGTCGAGAACTGTCG-3' (forward) and 5'-GCAGGCACAGCAGCTCG-3' (reverse); and viral U_L26, 5'-AACATCGCTCGGTGATTCTTTCG-3' (forward) and 5'-ACAGCTACTTTGAAGACGTGGAGC-3' (reverse).

Analysis of viral plaque formation. Replicate cultures of MRC5 fibroblasts were infected with 100 PFU of the indicated recombinant virus. After an overnight inoculation, the inoculum was aspirated and replaced with a gel overlay (1% Low Melting NuSieve agarose [Lonza] in DMEM with 10% fetal bovine serum [FBS], 4.5 g/liter glucose, 1% penicillin-streptomycin). Areas of representative plaques at 11 dpi for each virus were quantified by ImageJ and normalized to the average WT plaque size.

Analysis of protein accumulation. Protein accumulation was assayed by Western blotting. Protein from cell lysates was solubilized in disruption buffer (50 mM Tris [pH 7.0], 2% SDS, 5% 2-mercaptoethanol, 2.75% sucrose), separated by either 10% or 8 to 20% gradient SDS-PAGE (GenScript), and transferred to nitrocellulose in either Tris-glycine or Tris-bicine transfer buffer. Blots were then stained with Ponceau S to visualize protein bands and ensure equal protein loading. The membranes were blocked in 5% milk in Tris-buffered saline-Tween 20 (TBST), followed by incubation in primary antibody. After subsequent washes, blots were treated with secondary antibody, and protein bands were visualized using the enhanced chemiluminescence (ECL) system (Pierce) and a Molecular Imager Gel Doc XR+ system (Bio-Rad). Densitometry analysis was performed using Image Lab. The primary antibodies used were antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology), α -tubulin (Cell Signaling Technology), lamin A/C (Santa Cruz Biotechnology), IE1 (37), U_L26 (7H19) (38), pp28 (39), U_L32 (pp150) (a generous gift from W. Britt), and U_L30 (generated by Biomatik). The U_L30 antibody is a polyclonal rabbit antibody against the peptide sequence QHVETLRRFLRGDSC. Secondary antibodies were rabbit polyclonal (Santa Cruz Biotechnology) and mouse monoclonal (Abcam) anti-IgG antibodies.

Analysis of cytoplasmic and nuclear viral genomes. MRC5 fibroblasts were infected with WT, Δ U_L30, and U_L30stmut viruses (MOI of 3.0). Four hours postinfection, cytoplasmic and nuclear fractions were obtained via a nuclear and cytoplasmic extraction kit (G-Biosciences). A portion of both the cytoplasmic and nuclear fractions was combined with disruption buffer and analyzed by Western blotting. Viral DNA was extracted from the remaining nuclear and cytoplasmic fractions as described above.

Analysis of 5' RNA ends. 5' RACE was performed according to the 5' RACE system for rapid amplification of cDNA ends version 2.0 and the S.N.A.P. gel purification kit from Invitrogen Life Technologies using RNA samples harvested from WT and U_L30stmut infections at 72 hpi. The following primers were used: gene-specific primer 1 (GSP1), 5'-GTTTATTCTCCCCCTC-3', and GSP2, 5'-GCCGAGAGCCGTCGTGATAGTC-3'. The resulting PCR products were run on a 1.0% agarose gel with ethidium bromide and visualized using the Molecular Imager Gel Doc XR+ system (Bio-Rad).

Analysis of statistical significance. Growth curve and plaque size data were found to be nonparametric, and their statistical significance was determined using the Van der Waerden test. For all other results, statistical significance was determined by a nonpaired one-tailed Student's *t* test. A probability value of <0.05 was considered statistically significant.

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