The 5′ Untranslated Region of the Major Immediate Early mRNA Is Necessary for Efficient Human Cytomegalovirus Replication

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ABSTRACT The human cytomegalovirus (HCMV) immediate early 1 (IE1) and IE2 proteins are critical regulators of virus replication. Both proteins are needed to efficiently establish lytic infection, and nascent expression of IE1 and IE2 is critical for reactivation from latency. The regulation of IE1 and IE2 protein expression is thus a central event in the outcome of HCMV infection. Transcription of the primary transcript encoding both IE1 and IE2 is well studied, but relatively little is known about the posttranscriptional mechanisms that control IE1 and IE2 protein synthesis. The mRNA 5′ untranslated region (5′ UTR) plays an important role in regulating mRNA translation. Therefore, to better understand the control of IE1 and IE2 mRNA translation, we examined the role of the shared 5′ UTR of the IE1 and IE2 mRNAs (MIE 5′ UTR) in regulating translation. In a cell-free system, the MIE 5′ UTR repressed translation, as predicted based on its length and sequence composition. However, in transfected cells we found that the MIE 5′ UTR increased the expression of a reporter gene and enhanced its association with polysomes, demonstrating that the MIE 5′ UTR has a positive role in translation control. We also found that the MIE 5′ UTR was necessary for efficient IE1 and IE2 translation during infection. Replacing the MIE 5′ UTR with an unstructured sequence of the same length decreased IE1 and IE2 protein expression despite similar levels of IE1 and IE2 mRNA and reduced the association of the IE1 and IE2 mRNAs with polysomes. The wild-type MIE 5′-UTR sequence was also necessary for efficient HCMV replication. Together these data identify the shared 5′ UTR of the IE1 and IE2 mRNAs as an important regulator of HCMV lytic replication.

IMPORTANCE The HCMV IE1 and IE2 proteins are critical regulators of HCMV replication, both during primary infection and during reactivation from viral latency. Thus, defining factors that regulate IE1 and IE2 expression is important for understanding the molecular events controlling the HCMV replicative cycle. Here we identify a positive role for the MIE 5′ UTR in mediating the efficient translation of the IE1 and IE2 mRNAs. This result is an important advance for several reasons. To date, most studies of IE1 and IE2 regulation have focused on defining events that regulate IE1 and IE2 transcription. Our work reveals that in addition to the regulation of transcription, IE1 and IE2 are also regulated at the level of translation. Therefore, this study is important in that it identifies an additional layer of regulation controlling IE1 and IE2 expression and thus HCMV pathogenesis. These translational regulatory events could potentially be targeted by novel antiviral therapeutics that limit IE1 and IE2 mRNA translation and thus inhibit lytic replication or prevent HCMV reactivation.

KEYWORDS human herpesvirus, human cytomegalovirus, HCMV, mRNA translation, protein synthesis, major immediate early gene, IE1, IE2, lytic replication, human herpesviruses

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Human cytomegalovirus (HCMV) is a betaherpesvirus that establishes mainly asymptomatic lifelong infection in healthy individuals. However, HCMV is a significant cause of disease in newborns and immunocompromised adults. Primary infection of immune-naive newborns causes over 5,000 cases of long-term birth defects each year in the United States alone, including deafness, microcephaly, mental defect, and in some cases, death. Reactivation of latent HCMV infection in immunocompromised adults, typically after transplant, can lead to severe complications, including organ failure, neutropenia, and death. Thus, HCMV is a significant human pathogen that causes disease in multiple patient populations.

Two critical regulators of the HCMV replication cycle are the major immediate early proteins IE1 and IE2 (1). Both IE1 and IE2 are encoded by immediate early genes and expressed to high levels within hours after virus entry. In addition, their reexpression is thought to be critical for HCMV reactivation. IE1 helps establish lytic infection by preventing the deposition of suppressive chromatin marks on viral genomes and also inhibits the type I interferon response by binding to the STAT1 transcription factor (2–4). IE2 is a broadly acting transcriptional activator, which stimulates the expression of host genes (5–9) and HCMV early genes needed for viral DNA replication (10–17). Viruses lacking IE1 are viable but replicate poorly after low-multiplicity infections (18, 19), while deletion of IE2 completely blocks virus replication (20, 21). The regulation of IE1 and IE2 expression is thus a central event in the HCMV life cycle.

Given the important role of IE1 and IE2 in HCMV replication, much work has been done to understand the factors controlling their expression. The mature IE1 and IE2 transcripts arise from a common precursor RNA by alternative splicing (22). Mature mRNAs containing exons 1 to 4 encode the IE1 protein. An exon-skipping event gives rise to the mature IE2 mRNA, composed of exons 1 to 3 and exon 5 (10, 23–26). Early in infection, the HCMV major immediate early promoter (MIEP) drives robust expression of the IE1/2 precursor RNA. Multiple factors regulate MIEP activity, including the cellular transcription factors AP1, SP1, NF-κB, and MEF2D (27–33). In addition, viral proteins, including pTRS1, pUL83, pp71, and the IE1 and IE2 proteins themselves, modulate MIEP activity (34–39). Later in infection, IE2 binds a *cis*-repression sequence (crs) in the MIEP and represses its activity (40–44), and alternative MIE promoters become active (45), allowing for continuous, but dynamic, IE1 and IE2 expression throughout infection.

While less studied, posttranscriptional regulatory events also control IE1 and IE2 expression. For example, cellular factors regulate IE1/IE2 alternative splicing (23–26). In addition, the rate of synthesis of the IE1 and IE2 proteins changes throughout infection (46), potentially due to the effects of cellular microRNAs (47). Consistent with this, the amount of mature IE1 and IE2 mRNAs associating with polyribosomes, or polysomes, changes as infection progresses (48, 49). However, little is known of the factors controlling IE1 and IE2 mRNA translation and how these mechanisms might contribute to HCMV replication.

An important factor regulating mRNA translation is the 5′ untranslated region (5′ UTR) preceding the mRNA coding region (50). Though sometimes viewed exclusively as translational enhancers, 5′ UTRs can either enhance or repress mRNA translation. RNA binding proteins (RBPs) can recognize specific sequences in the 5′ UTR and facilitate ribosome recruitment (51) to enhance translation. Alternatively, RNA structures in the 5′ UTR can impede ribosomal subunit scanning or translation start site recognition (52–56) or serve as binding sites for RBPs that suppress translation (50, 57–59). Experimentally determining the impact of a 5′ UTR on mRNA translation is therefore critical for understanding the regulation of protein expression.

To better understand the posttranscriptional regulation of IE1 and IE2 protein expression, we investigated the role of the shared 5′ UTR of the IE1 and IE2 mRNAs (MIE 5′ UTR) in the control of mRNA translation and virus replication. Using a combination of biochemical approaches and recombinant viruses, we found that the sequence of the MIE 5′ UTR is an important determinant of IE1 and IE2 mRNA translation efficiency (TE). In a cell-free system, the MIE 5′ UTR repressed translation. In contrast, in transfected cells the MIE 5′ UTR enhanced the expression of a reporter gene and increased

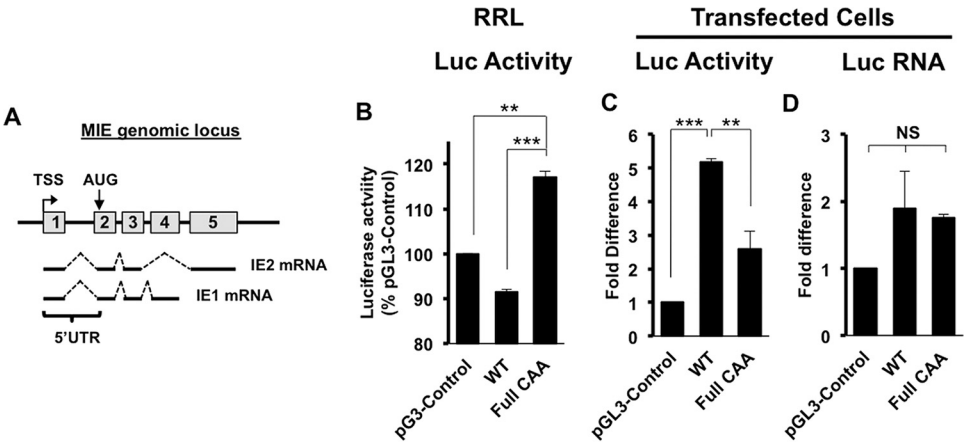

FIG 1 The MIE 5′ UTR increases reporter gene expression outside the context of infection. (A) Cartoon showing the arrangement of the IE1 and IE2 coding region, the mature IE1 and IE2 mRNAs, and the shared MIE 5′ UTR. (B) *In vitro*-transcribed, capped, and polyadenylated luciferase reporter mRNAs were translated in rabbit reticulocyte lysates, and luciferase activity was measured. Data show the relative luciferase activity compared to the pGL3-Control reporter, which was set to 100%. (C and D) The luciferase reporter plasmids were transfected into HeLa cells, and luciferase activity (C) and RNA levels (D) were measured 24 h after transfection. Changes in luciferase activity and RNA abundance are relative to pGL3-Control, which was set to 1. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$; NS, not significant.

its association with polysomes. During HCMV infection, replacement of the majority of the MIE 5′ UTR with an unstructured sequence of equal length led to a defect in IE1 and IE2 mRNA association with polysomes, decreased IE1 and IE2 protein expression, and diminished HCMV replication. These studies show that the MIE 5′ UTR is necessary for efficient IE1 and IE2 protein expression and identify the MIE 5′ UTR as a critical determinant of HCMV replication.

RESULTS

The MIE 5′ UTR enhances mRNA translation *in vitro*. 5′ UTRs can either enhance or inhibit translation depending on their length, sequence composition, the presence of RNA structure, and the cellular milieu. To understand how the MIE 5′ UTR affects mRNA translation, we measured the effect of the MIE 5′ UTR on the expression of a luciferase reporter gene. Comparing the MIE 5′-UTR sequences from multiple clinical and laboratory isolates (AD169, Towne, Toledo, Merlin, HAN20, and TB40/E) revealed that the MIE 5′ UTR was 100% conserved across multiple strains, suggesting a conserved role in regulating IE1 and IE2 mRNA translation. We cloned the 136 nucleotides of the MIE 5′ UTR upstream of a luciferase reporter gene (Fig. 1A), *in vitro* transcribed the 5′UTR-luciferase fusion, and measured luciferase activity in rabbit reticulocyte lysates (RRLs). We compared the results to those obtained with the empty vector, where the 5′ UTR consists of 32 nucleotides derived from the multicloning site. To control for the effect of the additional length of the MIE 5′ UTR on luciferase expression, we also compared our results to those of a reporter in which the 5′ UTR consisted of 136 nucleotides composed of CAA trinucleotide repeats. Translation efficiency is inversely correlated with 5′-UTR length and RNA secondary structure. CAA repeat sequences contain no RNA secondary structure; therefore, comparing the effects of the MIE 5′ UTR to a CAA repeat sequence of the same length allows us to control for the effects of 5′-UTR length on translation and suggests that any observed phenotypes result from changes to either RNA structure and/or the removal of specific RBP binding sites (60). Compared to the CAA control, the MIE 5′ UTR repressed reporter expression in RRLs (Fig. 1B). We performed similar experiments in cells transfected with the above-described luciferase reporters and observed different results (Fig. 1B). In transfected cells, the MIE 5′-UTR-luciferase construct was the most active, while the empty vector control was the least active. Luciferase RNA levels were approximately the same for the

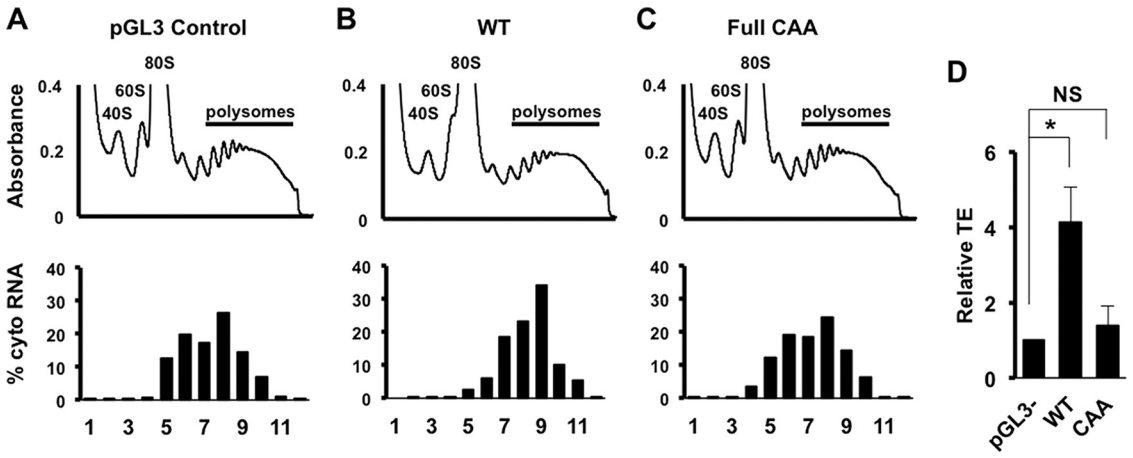

FIG 2 The MIE 5′ UTR increases the translation of a luciferase reporter gene. (A to C) The indicated luciferase reporter plasmids were transfected into HeLa cells, and cytoplasmic lysates were resolved through 10 to 50% linear sucrose gradients to separate ribosomal subunits, individual ribosomes (80S), and polysomes (top panels). Representative OD_{254} absorbance profiles for cytoplasmic lysates are shown (bottom panels). Quantitative reverse transcriptase PCR (qRT-PCR) analysis was performed to determine the percentage of the cytoplasmic RNA in each gradient fraction. The results of one representative experiment of a total of three are shown. (D) The translation efficiency (TE) for each reporter was determined by dividing the amount of the RNA in polysomes (fractions 9 to 12) by the total amount of RNA in the gradient. The graph shows the means and standard errors of the means for three independent experiments. *, $P \leq 0.05$; NS, not significant.

different constructs (Fig. 1C), indicating that the change in luciferase activity is due to a change in luciferase protein expression rather than increased luciferase transcription.

To more directly measure the effect of the MIE 5′ UTR on translation, we determined the translation efficiency of each reporter. The translation efficiency is the efficiency with which an mRNA is translated into protein, which can be determined by measuring the amount of an mRNA associated with polysomes. Importantly, the amount of mRNA associated with polysomes has previously been shown to correlate with more-efficient translation (61–66). Cytoplasmic lysates were resolved through linear sucrose gradients to separate ribonucleoprotein complexes, ribosomal subunits, monosomes, and polysomes. Importantly, this assay compares the distribution of RNA throughout the gradient and thus measures mRNA translation efficiency independent of RNA abundance.

Transfection of the reporters did not affect the overall levels of translation, as judged by the similar abundance of polysomes in each sample (Fig. 2, top panels) and the similar distribution of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) RNA across the gradient (data not shown). The empty vector (Fig. 2A) and CAA (Fig. 2C) 5′-UTR RNAs showed a similar distribution across the gradients, with the majority of the mRNA concentrated in fractions containing light polysomes (<3 ribosomes). In contrast, the MIE 5′-UTR mRNA was more abundant in fractions containing heavier polysomes (Fig. 2B), consistent with more-efficient translation compared to the two control transcripts. We quantified the translation efficiency of each reporter by calculating the ratio of mRNA in fractions containing polysomes to the total amount of mRNA in the entire gradient. The translation efficiency of the mRNA containing the MIE 5′ UTR was increased 3.95-fold relative to the empty vector control (Fig. 2D), while the translation efficiency of the CAA 5′-UTR reporter was not statistically different from the control. Thus, while the MIE 5′ UTR repressed translation compared to the CAA reporter in a cell-free system, it enhanced translation in intact cells. We conclude that the MIE 5′ UTR enhances translation outside the context of infection.

The MIE 5′ UTR is a critical determinant of HCMV replication. We next determined the role of the MIE 5′ UTR on IE1 and IE2 protein expression during HCMV infection. To do so, we constructed and analyzed two recombinant viruses with mutations in the MIE 5′ UTR (Fig. 3A). In the first recombinant (Full CAA), we replaced nucleotides +1 to +110 of the MIE 5′ UTR with CAA trinucleotide repeats, as in our

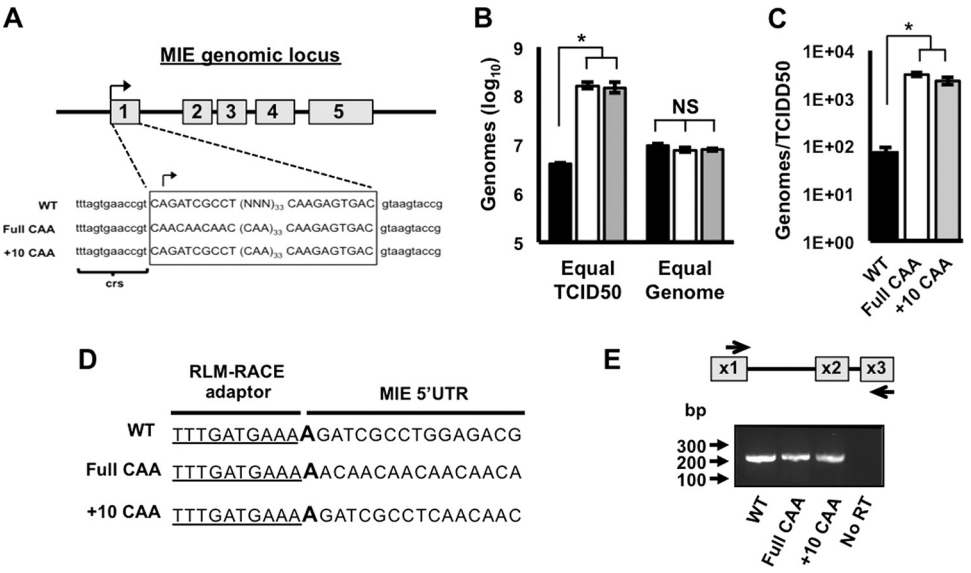

FIG 3 Mutations in the MIE 5′ UTR decrease HCMV infectivity. (A) Cartoon showing the mutations in the IE 5′ UTR for the Full CAA and +10 CAA viruses. (B) Primary fibroblasts were infected with either an equal number of $TCID_{50}$ units (MOI, 3) or an equivalent number of viral genomes. The number of intracellular HCMV genomes was quantified by real-time PCR at 6 h after infection. (C) The number of HCMV genomes in virus stocks was measured by real-time PCR and divided by the number of $TCID_{50}$ units to obtain the genome/$TCID_{50}$ ratio. The results are the means from two independent experiments. *, $P \leq 0.05$; NS, not significant. (D) The transcription start site of the MIE transcript was identified for wild-type virus and each recombinant using RLM-5′ RACE. Fibroblasts were infected with an equal number of genomes of each virus, and 5′RACE was performed on total RNA using gene-specific primers recognizing MIE exon 2. Shown is the portion of the sequence of the resulting PCR product that was used to identify the transcription start site. (E) Cells were infected and harvested as described for panel D, and RT-PCR was performed on total RNA using primers specific for exon 1 and exon 3 of the MIE transcript. An agarose gel showing the resulting PCR product is shown. A PCR product of the expected size for the spliced cDNA (220 bp) was observed with each virus. A control sample in which reverse transcriptase was omitted (no RT) is shown for RNA isolated from cells infected with wild-type virus.

luciferase reporters. The 10 nucleotides 5′ of the splice donor site in exon 1 and the 17 nucleotides of the MIE 5′ UTR in exon 2 were left unchanged to limit potential effects on exon 1/exon 2 splicing and the context of the translation site. The second recombinant (+10 CAA) was identical to the Full CAA recombinant, except that the first 10 nucleotides of the wild-type 5′ UTR were left unchanged to avoid potential effects on the crs site in the MIEP (67), which is critical for repression of MIEP activity by IE2 binding (42, 44, 68). Two independent isolates of each recombinant were analyzed. Virus was recovered after transfection of either recombinant genome into fibroblasts, indicating that neither mutation resulted in a complete block to HCMV replication.

Using the 50% tissue culture infectious dose ($TCID_{50}$) method to quantify virus stocks, we consistently obtained low-titer stocks with either recombinant virus. However, fibroblasts infected with the recombinant viruses showed greater cytopathic effect than wild-type virus after high multiplicity of infection (MOI, 3) (data not shown). The increase in cytopathic effect with the recombinant viruses suggested that the $TCID_{50}$ assay did not accurately measure the levels of infectious virus present in the stocks. Consistent with this idea, 6 h after infection with an equal number of $TCID_{50}$ units, cells infected with the recombinant viruses contained more intracellular genomes than did those infected with the wild-type virus (Fig. 3B). Quantitative PCR (qPCR) analysis of virus stocks revealed that the recombinant virus stocks had approximately 50-fold more genomes per infectious unit than the wild-type virus (Fig. 3C). Thus, the two recombinant viruses had a decrease in the particle-to-infectious-unit ratio compared to that of wild-type HCMV.

To confirm that the defect in particle-to-infectious-unit ratio did not result from changes to the architecture of the MIE transcript, we determined if either mutation affected transcription start site usage or altered splicing. Using RNA ligase-mediated

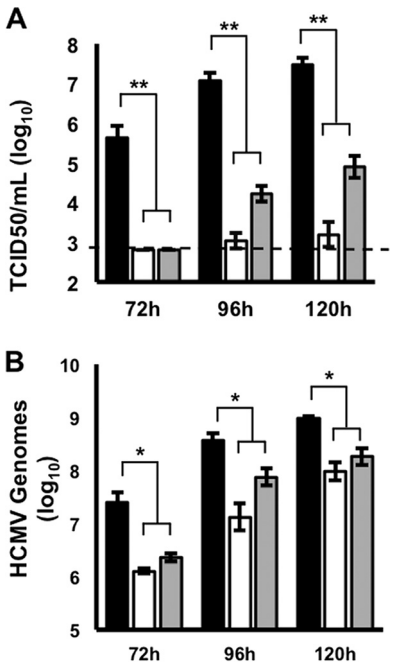

FIG 4 Mutations in the IE 5′ UTR decrease HCMV replication. Fibroblasts were infected with an equivalent number of genomes of wild-type HCMV or the indicated mutants. The number of genomes was equivalent to a multiplicity of three $TCID_{50}$ units per cell of wild-type virus. Cell-free virus in the supernatant was quantified using either the $TCID_{50}$ method (A) or real-time PCR analysis of viral genomes (B). The graphs show the averages and standard deviations from three independent experiments. The dashed line indicates the limit of detection. *, $P \leq 0.05$; **, $P \leq 0.005$.

rapid amplification of cDNA ends (RLM-RACE), we found that neither mutation affected transcription start site selection (Fig. 3D). Similarly, reverse transcription-PCR (RT-PCR) analysis using primers specific for exons 1 and 3 showed that splicing of the first three exons of the MIE transcript was not affected by either mutation (Fig. 3E). Thus, the defect in the particle-to-infectious-unit ratio observed with the Full CAA and +10 CAA viruses was not due to changes in MIE transcription start site usage or splicing.

To determine if replacement of the MIE 5′ UTR affected HCMV replication, we performed single-step growth analysis on the wild-type, Full CAA, and +10 CAA viruses. The amount of cell-free virus was measured over a time course of infection using both the $TCID_{50}$ assay and qPCR. Fewer infectious virions (Fig. 4A) and HCMV genomes (Fig. 4B) accumulated after infection with the Full CAA and +10 CAA viruses than after infection with the wild-type virus. Similar results were obtained with an independent isolate of each virus. Thus, the wild-type sequence of the MIE 5′ UTR is necessary for efficient HCMV replication.

The MIE 5′ UTR is necessary for efficient IE1 and IE2 protein expression during HCMV infection. We next determined how the replacement of the MIE 5′ UTR affected HCMV protein expression. Cells were infected with an equivalent number of genomes of each virus, and the levels of the IE1 and IE2 protein and RNA were measured at 6 h after infection. Both IE1 and IE2 protein levels were reduced after infection with either the Full CAA or the +10 CAA virus compared to levels obtained with wild-type virus (Fig. 5A), although there was no statistically significant difference in IE1 or IE2 mRNA levels (Fig. 5B). We also measured viral protein expression throughout a single round of virus replication (Fig. 5C). The two recombinants expressed less IE2 protein than did the wild-type virus at each time after infection. IE1 levels were also decreased, though the effect was more pronounced with the Full CAA virus. The defect in IE1 expression decreased over time, likely due to accumulation of the long-lived IE1 protein over the course of the lytic cycle. The early protein UL44 and the late pp28 protein were also expressed to reduced levels and with delayed kinetics, consistent with the defect in

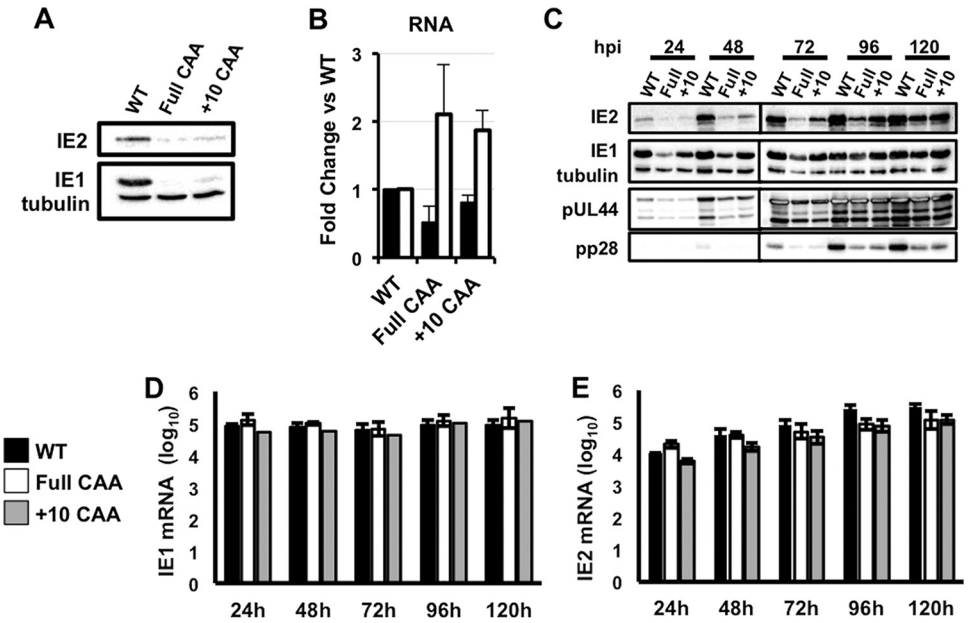

FIG 5 The MIE 5′ UTR is required for efficient IE1 and IE2 protein accumulation during infection. (A) Fibroblasts were infected with an equivalent number of genomes of wild-type HCMV (WT) or the indicated recombinants. Western blots showing IE1 and IE2 protein levels at 6 h after infection. (B) Cells were infected as described for panel A, and the abundance of the IE1 (black bars) and IE2 (white bars) RNAs was determined by qRT-PCR at 6 h after infection. (C) Cells were infected as described for panel A, and viral protein expression was measured by Western blotting over a time course of infection. hpi, hours postinfection. (D and E) Cells were infected as described for panel A, and qRT-PCR was used to quantify the abundance of the IE1 (D) or IE2 (E) mRNAs over a time course of infection. There was no statistical difference in IE1 and IE2 RNA levels between wild-type virus and the recombinants.

HCMV replication. To better understand the defect in IE1 and IE2 expression, we measured IE1 and IE2 mRNA levels. IE1 and IE2 mRNA levels were similar following infection with wild-type virus and either recombinant (Fig. 5D and E). We conclude that the MIE 5′ UTR is necessary for efficient IE1 and IE2 protein expression and for the subsequent expression of early and late proteins needed for HCMV replication.

The discrepancy between IE1 and IE2 protein and mRNA levels suggested that the MIE 5′ UTR regulates IE1 and IE2 mRNA translation during infection. We therefore measured the translation efficiency of the IE1 and IE2 mRNAs at 24 h after infection with the wild-type or recombinant viruses using sucrose density gradient centrifugation. The overall levels of polysomes were similar in cells infected with each virus, indicating that all viruses had the same effect on overall levels of translation (Fig. 6, left panels). In cells infected with wild-type virus, more IE1 and IE2 mRNA was present in fractions containing polysomes than in those containing monosomes, consistent with efficient IE1 and IE2 translation early in infection (Fig. 6A). Both IE1 and IE2 were less efficiently translated after infection with the +10 CAA virus, as evidenced by the decrease in RNA abundance in fractions containing heavy polysomes (Fig. 6C). A more-pronounced decrease in IE1 and IE2 translation efficiency was seen during infection with the Full CAA virus; compared to wild-type virus, less IE1 and IE2 RNA was found in the fractions containing polysomes (fractions 9 to 12), and more of each RNA was present in fractions containing ribosomal subunits and monosomes (fractions 3 to 7) (Fig. 6B). In contrast, the cellular GAPDH RNA had the same distribution throughout the gradient after infection with each virus, demonstrating that the MIE 5′-UTR mutations specifically impact translation of the IE1 and IE2 RNAs.

To quantify the differences in IE1 and IE2 RNA translation, we calculated the translation efficiency (TE) for IE1 and IE2 after infection with each virus (Fig. 7). We found that the IE1 RNA is translated almost twice as efficiently as the IE2 RNA at 24 h after infection (TE, 2.28 and 1.20, respectively), which closely agrees with previous measurements of IE1 and IE2 translation efficiency at this time point (69). After infection

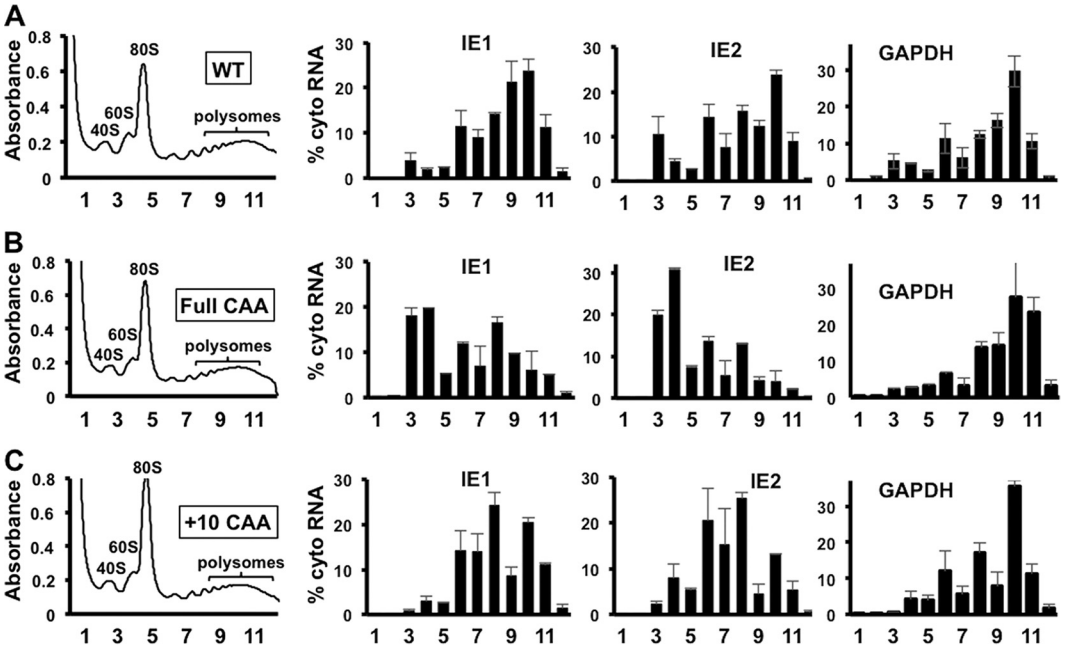

FIG 6 The MIE 5′ UTR is necessary for efficient association of the IE1 and IE2 mRNAs with polysomes. Fibroblasts were infected with an equal number of genomes of wild-type (WT) HCMV (A), the Full CAA mutant (B), or the +10 CAA mutant (C). The number of genomes used was equivalent to a multiplicity of three $TCID_{50}$ units per cell with wild-type virus. At 24 h after infection, cytoplasmic lysates were resolved through 10 to 50% linear sucrose gradients, and the abundance of polysomes in each sample was determined by continuous OD_{254} absorbance monitoring during fractionation of the gradient. Data for a representative experiment of a total of three are shownon the left. The percentages of the IE1, IE2, or GAPDH RNAs in each fraction of the gradient were determined by qRT-PCR. The graphs show the means and standard deviations from three independent experiments.

with the +10 CAA virus, the TE of the IE1 RNA was decreased 2.15-fold. IE2 mRNA translation was reduced to a similar extent (2.8-fold). The reduction in TE was even greater after infection with the Full CAA virus and affected IE1 and IE2 to a similar degree (7.6- and 10-fold, respectively). We conclude that the defect in IE1 and IE2 protein expression with the Full CAA and +10 CAA viruses results from decreased IE1 and IE2 mRNA translation. Together with the data above, these results identify the 5′ UTR of the IE1/2 mRNA as a critical regulatory element controlling IE1 and IE2 protein expression and HCMV replication.

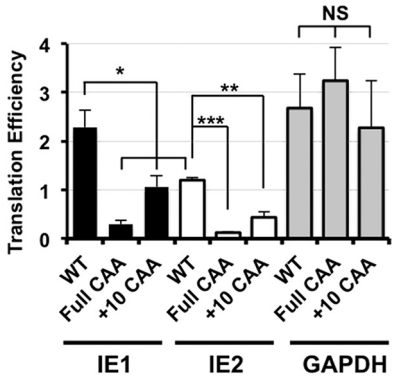

FIG 7 The MIE 5′ UTR is necessary for efficient IE1 and IE2 translation during HCMV infection. The translation efficiency of the IE1 (black bars), IE2 (white bars), and GAPDH (gray bars) mRNAs in cells infected with wild-type (WT), Full CAA, or +10 CAA viruses at 24 h after infection was calculated using the data in Fig. 6. The translation efficiency is the amount of RNA in polysomes (fractions 9 to 12) divided by the amount of RNA in monosomes (fractions 3 to 7). NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

DISCUSSION

5′ UTRs play important roles in regulating mRNA translation and can either enhance or repress protein synthesis. Our results show that in the context of HCMV infection, the MIE 5′ UTR serves to enhance translation and thus demonstrate the importance of posttranscriptional regulatory mechanisms in regulating IE1 and IE2 protein expression. We found that the MIE 5′ UTR enhances translation outside the context of infection and is necessary for efficient IE1 and IE2 mRNA translation in HCMV-infected cells. Replacement of the MIE 5′ UTR with a synthetic sequence of the same length decreased the particle-to-infectious-unit ratio but did not affect HCMV entry. Rather, replacement of the MIE 5′ UTR resulted in decreased IE1 and IE2 protein expression, delayed and reduced early and late protein expression, and decreased virus replication. Together these data identify the MIE 5′ UTR as a critical determinant of HCMV replication.

Our results also show that the MIE 5′ UTR is a critical determinant of HCMV replication. The replication defect of viruses lacking the MIE 5′ UTR likely arises from decreased IE1 and IE2 protein levels, which result in a cascading defect in early protein levels, DNA accumulation, and late protein expression. While mutation of the MIE 5′ UTR could affect aspects of RNA biology not directly measured by our assays, the decrease in the association of the IE1 and IE2 mRNAs with polysomes when the MIE 5′ UTR is mutated suggests that the defect in IE1 and IE2 protein expression is due to a defect in IE1 and IE2 mRNA translation. As cytoplasmic lysates were used for our polysome analysis, this result demonstrates a defect in mRNA translation independent of potential effects on RNA export. The fact that more IE1 and IE2 RNA is found in gradient fractions containing ribosomal subunits and monosomes after infection with the Full CAA and +10 CAA viruses suggests that the defect lies in translation initiation. This suggests that the MIE 5′ UTR helps recruit factors that facilitate ribosome recruitment.

RNA binding proteins (RBPs) serve as bridges between specific 5′-UTR sequences and 43S preinitiation complexes (50). Perhaps specific RBPs recognize sequences and/or RNA secondary structures in the MIE 5′ UTR during HCMV infection to facilitate 43S recruitment. At least some of these factors are likely cellular, as the MIE 5′ UTR also enhanced translation of a reporter gene in transfected cells. These factors may be specific for human cells, as our experiments in RRLs and transfected and infected human cells gave differing results. Perhaps human cells contain an RBP that is absent in RRLs and promotes the translation of mRNAs containing the MIE 5′ UTR. In addition, HCMV encodes multiple RBPs that could also bind the MIE 5′ UTR to influence IE1 and IE2 mRNA translation (70). Experiments to define the specific MIE 5′-UTR sequences needed to enhance translation and the associated host and/or viral factors will likely provide further insight into translational control of IE1 and IE2 protein expression.

Collectively, these data expand our understanding of the regulatory mechanisms controlling IE1 and IE2 protein expression by showing that proper regulation of IE1 and IE2 mRNA translation is important for efficient HCMV replication. Interestingly, these data also suggest that transcriptional and translational control of IE1 and IE2 expression could be linked. We recently found that later in infection multiple transcription start sites generate a series of mRNAs encoding full-length IE1 and IE2 proteins (45). These mRNAs differ only in their 5′-UTR sequence and thus would be predicted to translate with different efficiencies. This could explain previous studies showing temporal regulation of the association of the IE1 and IE2 mRNAs with polysomes throughout the HCMV lytic cycle (46, 49). While our data show an enhancing role for the MIE 5′ UTR during lytic infection in fibroblasts, it is also possible that the MIE 5′ UTR has different effects in different settings and cell types. For example, regulated IE1 and IE2 mRNA translation could potentially serve to dampen the effects of spurious IE1 and IE2 transcription during latency. In this regard, it is important to note that these experiments were performed using HCMV strain AD169, and thus additional viral factors in the ULb′ locus of clinical strains (71–73) could directly or indirectly affect MIE 5′-UTR regulation due to changes in the cellular environment. The results presented here thus

TABLE 1 Sequences of primers and oligonucleotides used in this study

Primer name	Sequence (5′→3′)
qRT-PCR GAPDH F	CTGTTGCTGTAGCCAAATTCGT
qRT-PCR GAPDH R	ACCCACTCCTCCACCTTTGAC
qRT-PCR IE1 F	CAAGTGACCGAGGATTGCAA
qRT-PCR IE1 R	CACCATGTCCACTCGAACCTT
qRT-PCR IE2 F	TGACCGAGGATTGCAACGA
qRT-PCR IE2 R	CGGCATGATTGACAGCCTG
UL99 F	GTGTCCCATTCCCGACTCG
UL99 R	TTCACAACGTCCACCCACC
qRT-PCR Fluc F	ACAAAGGCTATCAGGTGGCT
qRT-PCR Fluc R	CGTGCTCCAAAACAACAACG
MIE UTR-pGL3 F	GAGGCCTAGGCTTTTGCAAAACAGATCGCCTGGAGACGCCATCCAC
MIE UTR-pGL3 R	TATGTTTTTGGCGTCTTCCATCGTGTCAAGGACGGTGAGTCACTCT
5′ RACE OUT	GCTGATGGCGATGAATGAACACTG
5′ RACE IN	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG
IEX2R OUT	TCAGGGTCCATCTTTCTCTTGGCA
IEX2R IN	ATCGTGTCAAGGACGGTG
IEX1F	CCAAGAGTGACT
IEX3R	TTCTTCGGCCAACTCTGGAAACAG
Full CAA Ultramer-pGL3	GAGGCCTAGGCTTTTGCAAAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACA ACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACACAAGAGTGACTCACCGTCCTTGACACGATGGAAGACGCC AAAAACATA
IE UTR del KS F	AGGCGTGTACGGTGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGGAATTCGAGCTCGGTACCCGG
IE UTR del KS R	AGCCAAGGGGGTGGGCCTATAGACTCTATAGGCGGTACTTACGTCACTCTTGATCCCGGGAAAAGTGCCACC
T7 Primer WT UTR F	CATCAAGCTTTAATACGACTCACTATATCAGATCGCCTGGAGACGCCATC
T7 Primer Full CAA F	GCTTTAATACGACTCACTATAGCAACAACAACAACAACAAC
T7 pGL3 F	GCTTTAATACGACTCACTATAGGGCATTCCGGTACTGTTG
luc pA R	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTACACGGCGATCTTTCCGCCCTTC
Full CAA oligo F	AGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGCAACAACAACAACAACAACAACAACAACAACAACAA CAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACCAAGA GTGACG
Full CAA oligo R	CCAAGGGGGTGGGCCTATAGACTCTATAGGCGGTACTTACGTCACTCTTGGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG TTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGCGGTTCA CTAAAC
+10 CAA oligo F	AGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCCAACAACAACAACAACAACAACAACA ACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACA ACAACAACAACCAAGAGTGACG
+10 CAA oligo R	CCAAGGGGGTGGGCCTATAGACTCTATAGGCGGTACTTACGTCACTCTTGGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG TTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGGGCGATCTGAC
IE UTR CAA F	AGGCGTGTACGGTGGGAGGTCTAT
IE UTR CAA R	CCAAGGGGGTGGGCCTATAGAC

provide the rationale for future work to define the role of the MIE 5′ UTR in other infection settings.

MATERIALS AND METHODS

Cells and viruses. HeLa cells and MRC5 primary fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma) containing 10% fetal bovine serum (Gibco) and penicillin and streptomycin (Sigma). Virus stocks were grown on MRC5 fibroblasts, and their titers were determined using the 50% tissue culture infectious dose ($TCID_{50}$) assay, unless otherwise noted. In all experiments, AD*in*GFP (74) was used as the wild-type HCMV strain.

Recombinant viruses were made using a two-step recombination process as described previously (37, 45, 75, 76). Briefly, SW105 *Escherichia coli* isolates containing the AD*in*GFP bacterial artificial chromosome (BAC) were induced to be recombination competent by incubation at 42°C for 15 min. The bacteria were then electroporated with a kanamycin-levansucrase (KanSacB) expression cassette containing 50 nucleotides of sequence flanking the desired insertion site to guide recombination into the HCMV genome. The KanSacB recombination template was generated by PCR using the primers IE UTR del KS F and IE UTR del KS R, listed in Table 1. After recombination, bacteria were selected for growth on kanamycin, and the lack of gross recombination was confirmed by restriction digestion. Correct insertion of the KanSacB cassette was determined by PCR amplification of the region containing the KanSacB cassette followed by sequencing of the amplicon. Correct insertants were subjected to a second round of recombination to replace the Kan/SacB cassette with the intended mutation. To generate the Full CAA recombinant, the oligonucleotides "Full CAA oligo F" and "Full CAA oligo R" were annealed and then amplified using primers IE UTR CAA F and IE UTR CAA R. The PCR product was then electroporated into bacteria containing the Kan/SacB insertion, and recombinants were selected for by growth on LB agar containing 6% sucrose, which is toxic in the presence of the SacB gene. Replica plating was used to confirm the

absence of the kanamycin cassette prior to restriction digestion to monitor genomic integrity. To generate the +10 CAA recombinant, the oligonucleotides +10 CAA F and +10 CAA R were annealed and amplified with primers IE UTR CAA F and IE UTR CAA R prior to recombination and selection as described above. The region from +500 to −500 nucleotides surrounding the recombination site was PCR amplified and sequenced to confirm the absence of unintended mutations. For recombinants with the correct genotype, BAC DNA was purified (Nucleobond) and electroporated into MRC5 fibroblasts to generate infectious virus. Two independent isolates of each recombinant virus were generated from the wild-type BAC; both isolates were characterized. Primer and oligonucleotide sequences are listed in Table 1.

Plasmids. The luciferase reporter vectors were made by cloning the indicated 5′-UTR sequence into the HindIII and NcoI sites of pGL3-Control (Promega) using Gibson assembly (NEB). To generate the wild-type MIE 5′-UTR reporter, the MIE 5′ UTR was PCR amplified using primers MIE UTR-pGL3 F and MIE UTR-pGL3 R using cDNA from HCMV-infected cells as a template. To generate the Full CAA reporter, the Full CAA ultramer was PCR amplified using primers MIE UTR-pGL3 F and MIE UTR-pGL3 R. The PCR products were cloned into pGL3 control using Gibson assembly. Primer and ultramer sequences are listed in Table 1.

Luciferase assays. Luciferase assays were performed essentially as described previously (77). HeLa cells were transfected with 0.5 μg of each plasmid using polyethylenimine (PEI; Sigma) as the transfection reagent. Twenty-four hours after transfection, the cells were lysed in 1× passive lysis buffer (Promega) for 10 min at room temperature. Eight microliters of lysate was mixed with 40 μl luciferase reagent (Promega), and luciferase activity was measured using a luminometer (Molecular Devices LSII Max). The amount of luciferase activity was normalized to the protein content of each sample as determined by the Bradford assay. All transfection data are the means from at least three independent experiments performed on multiple days. For *in vitro* translation reactions, the indicated 5′-UTR-luciferase reporter fusion was PCR amplified with a 5′ primer containing a T7 promoter and a reverse primer that added a poly(A) tail to the end of the luciferase open reading frame (ORF). The sequences of the primers used for PCR are listed in Table 1. The PCR product was then used as the template in an *in vitro* transcription reaction mixture (mMessageMachine T7 in vitro transcription kit; Ambion) containing 7-methylguanosine, resulting in capped, polyadenylated reporter mRNA. Rabbit reticulocyte lysates (RRLs; Promega) were programmed with 1 μg mRNA, and the *in vitro* translation reaction was allowed to proceed for 90 min at 30°C. Luciferase activity was determined using a luminometer as described above. Each *in vitro* translation assay was performed a minimum of three times.

Polysome analysis. Polysomes were resolved using linear sucrose gradients as described previously (45). Briefly, cells were treated with 100 μg/ml cycloheximide for 10 min at 37°C prior to harvest. Cell pellets were resuspended in polysome buffer (20 mM Tris-HCl [pH 7.4], 140 mM KCl, 5 mM $MgCl_2$) containing 0.1% Triton X-100 and 10 mM dithiothreitol (DTT) and disrupted by 5 passes through a 27-gauge needle. Nuclei were removed by centrifugation for 5 min at 2,500 × *g*, followed by centrifugation for 10 min at 13,000 × *g* to remove insoluble debris. The clarified cytoplasmic lysate was layered onto linear 10 to 50% sucrose gradients prepared in polysome buffer and spun in an ultracentrifuge (Becton-Dickinson) for 2 h at 32,500 rpm in an SW41 swinging bucket rotor without brake. The gradient was fractionated using a gradient fractionation system (Brandel) with continuous absorbance monitoring at an optical density at 254 nm (OD_{254}). Gradient fractions were extracted with TRIzol and treated with Turbo DNase (Applied Biosystems), and the RNA was converted to cDNA as described below.

5′RACE. The transcription start site of the MIE mRNA was determined using the RLM-RACE kit (Ambion) as described before (45) according to the manufacturer's directions. Briefly, total RNA was isolated at 6 h after infection and treated with calf intestinal phosphatase (CIP) to dephosphorylate uncapped and degraded RNAs. The m^7G mRNA cap was then removed with tobacco acid pyrophosphatase (TAP), and an RNA oligonucleotide was ligated to the 5′ end of the RNA. The RNA was reverse transcribed using random hexamers as primers. The resulting cDNA was amplified in a nested PCR, using primers 5′ RACE OUT and IEX2R OUT in the first round and primers 5′ RACE IN and IEX2R IN in the second round. Primer sequences are listed in Table 1. The resulting PCR product was cloned into the pCR-Blunt vector (ThermoFisher), and the insert was sequenced by Sanger sequencing.

Analysis of nucleic acid abundance. Analysis of RNA abundance was performed essentially as described previously (45, 61). Briefly, cells or frozen cell pellets were resuspended in 1 ml of TRIzol. Two hundred microliters of chloroform was added, and the samples were vortexed and spun for 10 min at 15,000 × *g*. An equal volume of isopropanol was added to the aqueous layer, and the RNA was pelleted by centrifugation at 15,000 × *g* for 30 min. The RNA was resuspended in 1× DNase buffer containing 20 U DNase (Turbo DNase free kit; Ambion) and incubated for 30 min at 37°C. The DNase was inactivated per the manufacturer's protocol, and the RNA was quantified using a spectrophotometer (NanoDrop). An equal mass of RNA from each sample, typically 0.5 μg, was added to a 1× reverse transcription reaction mixture (High Capacity cDNA reverse transcription kit; ThermoFisher) together with random hexamer primers. The reaction mixture was incubated at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. For real-time PCRs, 2 μl of the reverse transcription reaction mixture was mixed with 1× SYBR green master mix and 0.5 μM gene-specific primers (Table 1) and subjected to PCR under the following conditions: 95°C for 5 min, 40 cycles of 95°C for 10 s and 55°C for 30 s, and 72°C for 7 min. The absolute quantification method was used to determine transcript abundance by comparing the abundance of each PCR product to a standard curve generated from 10-fold serial dilutions of a DNA standard specific for each primer pair.

HCMV DNA was quantified essentially as described previously (45, 61, 76, 78). Briefly, samples were incubated in DNA extraction buffer (400 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA) containing

proteinase K (10 mg/ml) overnight at 37°C. The samples were extracted with phenol-chloroform, digested with RNase A (10 mg/ml) for 1 h at 37°C, and extracted again with phenol-chloroform, and the DNA was precipitated with isopropanol and resuspended in 10 mM Tris-HCl (pH 8.0). qPCRs using primers specific to the UL99 gene (UL99F and UL99R [Table 1]) were used to determine the number of HCMV genomes by comparing the threshold values to a series of HCMV BAC DNA standards containing from 10^8 to 10^1 HCMV genomes.

Analysis of MIE exon 1 and 2 splicing. cDNA was generated from total RNA as described above. The cDNA was amplified with a primer specific to the end of MIE exon 1 (primer IEX1F) and a primer recognizing MIE exon 3 (primer IEX3R). Primer sequences are listed in Table 1. The PCR product was visualized on a 2% agarose gel and run according to the predicted size of 220 bp. A control reaction in which reverse transcriptase was omitted was performed to confirm that the PCR product was derived from cDNA rather than contaminating genomic DNA.

Analysis of protein expression. Western blot analysis was performed as described before (79). Briefly, cells were scraped and stored as dry frozen pellets at −80°C until analysis. Cell pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate) containing 1× cOmplete protease inhibitor cocktail (Roche), and the protein concentration was determined by the Bradford assay. Equal amounts of protein were resolved on SDS-PAGE gels and then transferred to nitrocellulose membranes (Amersham). The membranes were blocked in TBS-T (20 mM Tris-HCl [pH 7.6], 140 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk for at least 1 h prior to incubation with primary antibody. Mouse monoclonal antibodies were diluted in 1% bovine serum albumin (BSA) in TBS-T and incubated with the membrane for 1 h at room temperature. Rabbit polyclonal antibodies were diluted in 5% BSA in TBS-T and incubated overnight at 4°C. Membranes were washed with TBS-T, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody, and then visualized by chemiluminescence using a digital imager (Bio-Rad). Antibodies specific for the following proteins were used: IE1 (1:100 [80]), UL44 (1:1,000; Virusys), pp28 (1:100 [81]), tubulin (1:10,000; Sigma). All experiments were performed a minimum of three times, and representative results from a single experiment are shown in the figures.

Viral growth analysis. Cells were infected with HCMV at the indicated multiplicity of infection in a minimum volume of complete growth medium. Virus was incubated with cells for 1 h at 37°C with rocking every 15 min, after which time the inoculum was removed and replaced with fresh medium. The amount of virus in cell-free supernatants was quantified using either the $TCID_{50}$ method to measure infectious units or qPCR as described above to enumerate HCMV genomes as previously described (37, 82). For single-step growth analysis, cells were infected at a multiplicity of 3, or the equivalent number of viral genomes, as described in the text. Supernatants were harvested at 24-h intervals and stored at −80°C until use.

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REFERENCES

1. Stinski MF, Thomsen DR, Stenberg RM, Goldstein LC. 1983. Organization and expression of the immediate early genes of human cytomegalovirus. J Virol 46:1–14.
2. Nevels M, Paulus C, Shenk T. 2004. Human cytomegalovirus immediate-early 1 protein facilitates viral replication by antagonizing histone deacetylation. Proc Natl Acad Sci U S A 101:17234–17239. https://doi.org/10.1073/pnas.0407933101.
3. Zalckvar E, Paulus C, Tillo D, Asbach-Nitzsche A, Lubling Y, Winterling C, Strieder N, Mucke K, Goodrum F, Segal E, Nevels M. 2013. Nucleosome maps of the human cytomegalovirus genome reveal a temporal switch in chromatin organization linked to a major IE protein. Proc Natl Acad Sci U S A 110:13126–13131. https://doi.org/10.1073/pnas.1305548110.
4. Saffert RT, Penkert RR, Kalejta RF. 2010. Cellular and viral control over the initial events of human cytomegalovirus experimental latency in CD34+ cells. J Virol 84:5594–5604. https://doi.org/10.1128/JVI.00348-10.
5. Caswell R, Bryant L, Sinclair J. 1996. Human cytomegalovirus immediate-early 2 (IE2) protein can transactivate the human hsp70 promoter by alleviation of Dr1-mediated repression. J Virol 70:4028–4037.
6. Caswell R, Hagemeier C, Chiou CJ, Hayward G, Kouzarides T, Sinclair J. 1993. The human cytomegalovirus 86K immediate early (IE) 2 protein requires the basic region of the TATA-box binding protein (TBP) for binding, and interacts with TBP and transcription factor TFIIB via regions of IE2 required for transcriptional regulation. J Gen Virol 74(Part 12): 2691–2698. https://doi.org/10.1099/0022-1317-74-12-2691.
7. Yurochko AD, Huong SM, Huang ES. 1999. Identification of human cytomegalovirus target sequences in the human immunodeficiency virus long terminal repeat. Potential role of IE2-86 binding to sequences between −120 and −20 in promoter transactivation. J Hum Virol 2:81–90.
8. Lukac DM, Harel NY, Tanese N, Alwine JC. 1997. TAF-like functions of human cytomegalovirus immediate-early proteins. J Virol 71:7227–7239.
9. Petrik DT, Schmitt KP, Stinski MF. 2007. The autoregulatory and transactivating functions of the human cytomegalovirus IE86 protein use

independent mechanisms for promoter binding. J Virol 81:5807–5818. https://doi.org/10.1128/JVI.02437-06.
10. Hermiston TW, Malone CL, Witte PR, Stinski MF. 1987. Identification and characterization of the human cytomegalovirus immediate-early region 2 gene that stimulates gene expression from an inducible promoter. J Virol 61:3214–3221.
11. Iwamoto GK, Monick MM, Clark BD, Auron PE, Stinski MF, Hunninghake GW. 1990. Modulation of interleukin 1 beta gene expression by the immediate early genes of human cytomegalovirus. J Clin Invest 85:1853–1857. https://doi.org/10.1172/JCI114645.
12. Geist LJ, Monick MM, Stinski MF, Hunninghake GW. 1991. The immediate early genes of human cytomegalovirus upregulate expression of the interleukin-2 and interleukin-2 receptor genes. Am J Respir Cell Mol Biol 5:292–296. https://doi.org/10.1165/ajrcmb/5.3.292.
13. Crump JW, Geist LJ, Auron PE, Webb AC, Stinski MF, Hunninghake GW. 1992. The immediate early genes of human cytomegalovirus require only proximal promoter elements to upregulate expression of interleukin-1 beta. Am J Respir Cell Mol Biol 6:674–677. https://doi.org/10.1165/ajrcmb/6.6.674.
14. Geist LJ, Monick MM, Stinski MF, Hunninghake GW. 1992. Cytomegalovirus immediate early genes prevent the inhibitory effect of cyclosporin A on interleukin 2 gene transcription. J Clin Invest 90:2136–2140. https://doi.org/10.1172/JCI116099.
15. Monick MM, Geist LJ, Stinski MF, Hunninghake GW. 1992. The immediate early genes of human cytomegalovirus upregulate expression of the cellular genes myc and fos. Am J Respir Cell Mol Biol 7:251–256. https://doi.org/10.1165/ajrcmb/7.3.251.
16. Geist LJ, Monick MM, Stinski MF, Hunninghake GW. 1994. The immediate early genes of human cytomegalovirus upregulate tumor necrosis factor-alpha gene expression. J Clin Invest 93:474–478. https://doi.org/10.1172/JCI116995.
17. Kline JN, Geist LJ, Monick MM, Stinski MF, Hunninghake GW. 1994. Regulation of expression of the IL-1 receptor antagonist (IL-1ra) gene by products of the human cytomegalovirus immediate early genes. J Immunol 152:2351–2357.
18. Greaves RF, Mocarski ES. 1998. Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus IE1 mutant. J Virol 72:366–379.
19. Mocarski ES, Kemble GW, Lyle JM, Greaves RF. 1996. A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to a failure in autoregulation. Proc Natl Acad Sci U S A 93:11321–11326. https://doi.org/10.1073/pnas.93.21.11321.
20. Marchini A, Liu H, Zhu H. 2001. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. J Virol 75:1870–1878. https://doi.org/10.1128/JVI.75.4.1870-1878.2001.
21. Yu D, Silva MC, Shenk T. 2003. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. Proc Natl Acad Sci U S A 100:12396–12401. https://doi.org/10.1073/pnas.1635160100.
22. Stenberg RM, Witte PR, Stinski MF. 1985. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. J Virol 56:665–675.
23. Awasthi S, Isler JA, Alwine JC. 2004. Analysis of splice variants of the immediate-early 1 region of human cytomegalovirus. J Virol 78:8191–8200. https://doi.org/10.1128/JVI.78.15.8191-8200.2004.
24. Du G, Dutta N, Lashmit P, Stinski MF. 2011. Alternative splicing of the human cytomegalovirus major immediate-early genes affects infectious-virus replication and control of cellular cyclin-dependent kinase. J Virol 85:804–817. https://doi.org/10.1128/JVI.01173-10.
25. Hou W, Torres L, Cruz-Cosme R, Arroyo F, Irizarry L, Luciano D, Marquez A, Rivera LL, Sala AL, Luo MH, Tang Q. 2016. Two polypyrimidine tracts in intron 4 of the major immediate early gene are critical for gene expression switching from IE1 to IE2 and for replication of human cytomegalovirus. J Virol 90:7339–7349. https://doi.org/10.1128/JVI.00837-16.
26. Martinez FP, Cruz R, Lu F, Plasschaert R, Deng Z, Rivera-Molina YA, Bartolomei MS, Lieberman PM, Tang Q. 2014. CTCF binding to the first intron of the major immediate early (MIE) gene of human cytomegalovirus (HCMV) negatively regulates MIE gene expression and HCMV replication. J Virol 88:7389–7401. https://doi.org/10.1128/JVI.00845-14.
27. Meier JL, Keller MJ, McCoy JJ. 2002. Requirement of multiple cis-acting elements in the human cytomegalovirus major immediate-early distal enhancer for viral gene expression and replication. J Virol 76:313–326. https://doi.org/10.1128/JVI.76.1.313-326.2002.
28. Hunninghake GW, Monick MM, Liu B, Stinski MF. 1989. The promoter-regulatory region of the major immediate-early gene of human cytomegalovirus responds to T-lymphocyte stimulation and contains functional cyclic AMP-response elements. J Virol 63:3026–3033.
29. Isomura H, Tsurumi T, Stinski MF. 2004. Role of the proximal enhancer of the major immediate-early promoter in human cytomegalovirus replication. J Virol 78:12788–12799. https://doi.org/10.1128/JVI.78.23.12788-12799.2004.
30. Lashmit P, Wang S, Li H, Isomura H, Stinski MF. 2009. The CREB site in the proximal enhancer is critical for cooperative interaction with the other transcription factor binding sites to enhance transcription of the major intermediate-early genes in human cytomegalovirus-infected cells. J Virol 83:8893–8904. https://doi.org/10.1128/JVI.02239-08.
31. Lashmit PE, Lundquist CA, Meier JL, Stinski MF. 2004. Cellular repressor inhibits human cytomegalovirus transcription from the UL127 promoter. J Virol 78:5113–5123. https://doi.org/10.1128/JVI.78.10.5113-5123.2004.
32. Macias MP, Huang L, Lashmit PE, Stinski MF. 1996. Cellular or viral protein binding to a cytomegalovirus promoter transcription initiation site: effects on transcription. J Virol 70:3628–3635.
33. Hennighausen L, Fleckenstein B. 1986. Nuclear factor 1 interacts with five DNA elements in the promoter region of the human cytomegalovirus major immediate early gene. EMBO J 5:1367–1371.
34. Bresnahan WA, Shenk TE. 2000. UL82 virion protein activates expression of immediate early viral genes in human cytomegalovirus-infected cells. Proc Natl Acad Sci U S A 97:14506–14511. https://doi.org/10.1073/pnas.97.26.14506.
35. Cantrell SR, Bresnahan WA. 2005. Interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early gene expression and viral replication. J Virol 79:7792–7802. https://doi.org/10.1128/JVI.79.12.7792-7802.2005.
36. Cantrell SR, Bresnahan WA. 2006. Human cytomegalovirus (HCMV) UL82 gene product (pp71) relieves hDaxx-mediated repression of HCMV replication. J Virol 80:6188–6191. https://doi.org/10.1128/JVI.02676-05.
37. Cristea IM, Moorman NJ, Terhune SS, Cuevas CD, O'Keefe ES, Rout MP, Chait BT, Shenk T. 2010. Human cytomegalovirus pUL83 stimulates activity of the viral immediate-early promoter through its interaction with the cellular IFI16 protein. J Virol 84:7803–7814. https://doi.org/10.1128/JVI.00139-10.
38. Romanowski MJ, Garrido-Guerrero E, Shenk T. 1997. pIRS1 and pTRS1 are present in human cytomegalovirus virions. J Virol 71:5703–5705.
39. Romanowski MJ, Shenk T. 1997. Characterization of the human cytomegalovirus irs1 and trs1 genes: a second immediate-early transcription unit within irs1 whose product antagonizes transcriptional activation. J Virol 71:1485–1496.
40. Liu B, Hermiston TW, Stinski MF. 1991. A cis-acting element in the major immediate-early (IE) promoter of human cytomegalovirus is required for negative regulation by IE2. J Virol 65:897–903.
41. Pizzorno MC, Hayward GS. 1990. The IE2 gene products of human cytomegalovirus specifically down-regulate expression from the major immediate-early promoter through a target sequence located near the cap site. J Virol 64:6154–6165.
42. Cherrington JM, Khoury EL, Mocarski ES. 1991. Human cytomegalovirus ie2 negatively regulates alpha gene expression via a short target sequence near the transcription start site. J Virol 65:887–896.
43. Macias MP, Stinski MF. 1993. An in vitro system for human cytomegalovirus immediate early 2 protein (IE2)-mediated site-dependent repression of transcription and direct binding of IE2 to the major immediate early promoter. Proc Natl Acad Sci U S A 90:707–711. https://doi.org/10.1073/pnas.90.2.707.
44. Reeves M, Murphy J, Greaves R, Fairley J, Brehm A, Sinclair J. 2006. Autorepression of the human cytomegalovirus major immediate-early promoter/enhancer at late times of infection is mediated by the recruitment of chromatin remodeling enzymes by IE86. J Virol 80:9998–10009. https://doi.org/10.1128/JVI.01297-06.
45. Arend KC, Ziehr B, Vincent HA, Moorman NJ. 2016. Multiple transcripts encode full-length human cytomegalovirus IE1 and IE2 proteins during lytic infection. J Virol 90:8855–8865. https://doi.org/10.1128/JVI.00741-16.
46. Stenberg RM, Depto AS, Fortney J, Nelson JA. 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. J Virol 63:2699–2708.
47. Murphy E, Vanicek J, Robins H, Shenk T, Levine AJ. 2008. Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: implications for latency. Proc Natl Acad Sci U S A 105:5453–5458. https://doi.org/10.1073/pnas.0711910105.
48. Stenberg RM, Thomsen DR, Stinski MF. 1984. Structural analysis of the

major immediate early gene of human cytomegalovirus. J Virol 49: 190–199.
49. Stamminger T, Puchtler E, Fleckenstein B. 1991. Discordant expression of the immediate-early 1 and 2 gene regions of human cytomegalovirus at early times after infection involves posttranscriptional processing events. J Virol 65:2273–2282.
50. Hinnebusch AG, Ivanov IP, Sonenberg N. 2016. Translational control by 5′-untranslated regions of eukaryotic mRNAs. Science 352:1413–1416. https://doi.org/10.1126/science.aad9868.
51. Hinnebusch AG. 2014. The scanning mechanism of eukaryotic translation initiation. Annu Rev Biochem 83:779–812. https://doi.org/10.1146/annurev-biochem-060713-035802.
52. Gray NK, Hentze MW. 1994. Regulation of protein synthesis by mRNA structure. Mol Biol Rep 19:195–200. https://doi.org/10.1007/BF00986961.
53. Arribere JA, Gilbert WV. 2013. Roles for transcript leaders in translation and mRNA decay revealed by transcript leader sequencing. Genome Res 23:977–987. https://doi.org/10.1101/gr.150342.112.
54. Pickering BM, Willis AE. 2005. The implications of structured 5′ untranslated regions on translation and disease. Semin Cell Dev Biol 16:39–47. https://doi.org/10.1016/j.semcdb.2004.11.006.
55. Boeras I, Seufzer B, Brady S, Rendahl A, Heng X, Boris-Lawrie K. 2017. The basal translation rate of authentic HIV-1 RNA is regulated by 5′UTR nt-pairings at junction of R and U5. Sci Rep 7:6902. https://doi.org/10.1038/s41598-017-06883-9.
56. Sobczak K, Krzyzosiak WJ. 2002. Structural determinants of BRCA1 translational regulation. J Biol Chem 277:17349–17358. https://doi.org/10.1074/jbc.M109162200.
57. Romeo DS, Park K, Roberts AB, Sporn MB, Kim SJ. 1993. An element of the transforming growth factor-beta 1 5′-untranslated region represses translation and specifically binds a cytosolic factor. Mol Endocrinol 7:759–766.
58. Zhang DL, Hughes RM, Ollivierre-Wilson H, Ghosh MC, Rouault TA. 2009. A ferroportin transcript that lacks an iron-responsive element enables duodenal and erythroid precursor cells to evade translational repression. Cell Metab 9:461–473. https://doi.org/10.1016/j.cmet.2009.03.006.
59. Iakova P, Wang GL, Timchenko L, Michalak M, Pereira-Smith OM, Smith JR, Timchenko NA. 2004. Competition of CUGBP1 and calreticulin for the regulation of p21 translation determines cell fate. EMBO J 23:406–417. https://doi.org/10.1038/sj.emboj.7600052.
60. Babendure JR, Babendure JL, Ding JH, Tsien RY. 2006. Control of mammalian translation by mRNA structure near caps. RNA 12:851–861. https://doi.org/10.1261/rna.2309906.
61. Lenarcic EM, Ziehr B, De Leon G, Mitchell D, Moorman NJ. 2014. Differential role for host translation factors in host and viral protein synthesis during human cytomegalovirus infection. J Virol 88:1473–1483. https://doi.org/10.1128/JVI.02321-13.
62. Wang X, Hou J, Quedenau C, Chen W. 2016. Pervasive isoform-specific translational regulation via alternative transcription start sites in mammals. Mol Syst Biol 12:875. https://doi.org/10.15252/msb.20166941.
63. Johannes G, Carter MS, Eisen MB, Brown PO, Sarnow P. 1999. Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. Proc Natl Acad Sci U S A 96:13118–13123. https://doi.org/10.1073/pnas.96.23.13118.
64. Floor SN, Doudna JA. 2016. Tunable protein synthesis by transcript isoforms in human cells. Elife 5:e10921. https://doi.org/10.7554/eLife.10921.
65. Chasse H, Boulben S, Costache V, Cormier P, Morales J. 2017. Analysis of translation using polysome profiling. Nucleic Acids Res 45(3):e15. https://doi.org/10.1093/nar/gkw907.
66. Arava Y, Wang Y, Storey JD, Liu CL, Brown PO, Herschlag D. 2003. Genome-wide analysis of mRNA translation profiles in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 100:3889–3894. https://doi.org/10.1073/pnas.0635171100.
67. Lashmit PE, Stinski MF, Murphy EA, Bullock GC. 1998. A cis repression sequence adjacent to the transcription start site of the human cytomegalovirus US3 gene is required to down regulate gene expression at early and late times after infection. J Virol 72:9575–9584.
68. Lang D, Stamminger T. 1993. The 86-kilodalton IE-2 protein of human cytomegalovirus is a sequence-specific DNA-binding protein that interacts directly with the negative autoregulatory response element located near the cap site of the IE-1/2 enhancer-promoter. J Virol 67:323–331.
69. Tirosh O, Cohen Y, Shitrit A, Shani O, Le-Trilling VT, Trilling M, Friedlander G, Tanenbaum M, Stern-Ginossar N. 2015. The transcription and translation landscapes during human cytomegalovirus infection reveal novel host-pathogen interactions. PLoS Pathog 11:e1005288. https://doi.org/10.1371/journal.ppat.1005288.
70. Lenarcic EM, Ziehr BJ, Moorman NJ. 2015. An unbiased proteomics approach to identify human cytomegalovirus RNA-associated proteins. Virology 481:13–23. https://doi.org/10.1016/j.virol.2015.02.008.
71. Umashankar M, Petrucelli A, Cicchini L, Caposio P, Kreklywich CN, Rak M, Bughio F, Goldman DC, Hamlin KL, Nelson JA, Fleming WH, Streblow DN, Goodrum F. 2011. A novel human cytomegalovirus locus modulates cell type-specific outcomes of infection. PLoS Pathog 7:e1002444. https://doi.org/10.1371/journal.ppat.1002444.
72. Dolan A, Cunningham C, Hector RD, Hassan-Walker AF, Lee L, Addison C, Dargan DJ, McGeoch DJ, Gatherer D, Emery VC, Griffiths PD, Sinzger C, McSharry BP, Wilkinson GW, Davison AJ. 2004. Genetic content of wild-type human cytomegalovirus. J Gen Virol 85:1301–1312. https://doi.org/10.1099/vir.0.79888-0.
73. Cha TA, Tom E, Kemble GW, Duke GM, Mocarski ES, Spaete RR. 1996. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. J Virol 70:78–83.
74. Wang D, Bresnahan W, Shenk T. 2004. Human cytomegalovirus encodes a highly specific RANTES decoy receptor. Proc Natl Acad Sci U S A 101:16642–16647. https://doi.org/10.1073/pnas.0407233101.
75. Moorman NJ, Sharon-Friling R, Shenk T, Cristea IM. 2010. A targeted spatial-temporal proteomics approach implicates multiple cellular trafficking pathways in human cytomegalovirus virion maturation. Mol Cell Proteomics 9:851–860. https://doi.org/10.1074/mcp.M900485-MCP200.
76. Ziehr B, Vincent HA, Moorman NJ. 2016. Human cytomegalovirus pTRS1 and pIRS1 antagonize PKR to facilitate virus replication. J Virol https://doi.org/10.1128/JVI.02714-15.
77. Ziehr B, Lenarcic E, Vincent HA, Cecil C, Garcia B, Shenk T, Moorman NJ. 2015. Human cytomegalovirus TRS1 protein associates with the 7-methylguanosine mRNA cap and facilitates translation. Proteomics 15:1983–1994. https://doi.org/10.1002/pmic.201400616.
78. Ziehr B, Lenarcic E, Cecil C, Moorman NJ. 2016. The eIF4AIII RNA helicase is a critical determinant of human cytomegalovirus replication. Virology 489:194–201. https://doi.org/10.1016/j.virol.2015.12.009.
79. Vincent HA, Ziehr B, Moorman NJ. 2016. Human cytomegalovirus strategies to maintain and promote mRNA translation. Viruses 8:97. https://doi.org/10.3390/v8040097.
80. Zhu H, Shen Y, Shenk T. 1995. Human cytomegalovirus IE1 and IE2 proteins block apoptosis. J Virol 69:7960–7970.
81. Silva MC, Yu QC, Enquist L, Shenk T. 2003. Human cytomegalovirus UL99-encoded pp28 is required for the cytoplasmic envelopment of tegument-associated capsids. J Virol 77:10594–10605. https://doi.org/10.1128/JVI.77.19.10594-10605.2003.
82. Arend KC, Lenarcic EM, Vincent HA, Rashid N, Lazear E, McDonald IM, Gilbert TS, East MP, Herring LE, Johnson GL, Graves L, Moorman NJ. 2017. Kinome profiling identifies druggable targets for novel human cytomegalovirus (HCMV) antivirals. Mol Cell Proteomics 16(4 Suppl 1):S263–S276. https://doi.org/10.1074/mcp.M116.065375.