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Human Cytomegalovirus Tegument Protein pp71 (ppUL82) Enhances the Infectivity of Viral DNA and Accelerates the Infectious Cycle

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Three tegument proteins of human cytomegalovirus (HCMV), ppUL82 (pp71), pUL69, and ppUL83 (pp65), were examined for the ability to stimulate the production of infectious virus from human diploid fibroblasts transfected with viral DNA. Although viral DNA alone had a low intrinsic infectivity of 3 to 8 plaques/μg of viral DNA, cotransfection of a plasmid expressing pp71 increased the infectivity of HCMV DNA 30- to 80-fold. The increase in infectivity produced by pp71 was reflected in an increased number of nuclei observed to express high levels of the major immediate-early proteins IE1 and IE2. Cotransfection of viral DNA with plasmids directing expression of IE1 and IE2 also resulted in extensive IE1 and IE2 expression in the transfected cells; however, the infectivity of viral DNA was only marginally increased. pp71 also facilitated late gene expression, virus transmission to adjacent cells, and plaque formation. In contrast, expression of pUL69 reduced the pp71- and IE1/IE2-mediated enhancement of HCMV DNA infectivity and also failed to produce any increase in the number of cells expressing IE1 and IE2 over that seen with viral DNA alone. Expression of pp65 did not alter the infectivity of HCMV DNA, nor did it modify the effects of pp71 or pUL69. These results imply that pp71 plays a critical role in the initiation of infection apart from its function as a transactivator of IE1 and IE2.

Human cytomegalovirus (HCMV) is a betaherpesvirus which typically causes asymptomatic infections in healthy individuals, although more serious complications can arise from congenital infection of newborns and acute or reactivated infection of immunodeficient individuals (for reviews see references 3 and 12). Gene expression during lytic HCMV replication proceeds in the three temporal classes characteristic of the herpesvirus family: immediate-early (IE), early, and late (for a review, see reference 22). Since expression of early and late HCMV genes is largely dependent on the synthesis and activity of the IE proteins, IE gene regulation is thought to be a likely control point for the initiation of lytic replication after primary infection or during reactivation from latency. Among the IE proteins, the most abundant and well studied are expressed from the major IE (MIE) locus: the 72-kDa (IE1) and the 86-kDa (IE2) products. These proteins are powerful transregulators of viral and cellular gene expression and are likely critical for promoting the onset of HCMV replication (11, 14, 17, 21, 29, 30, 43, 44). IE1 and IE2 are expressed via differential splicing of a single mRNA transcript initiated from the MIE promoter (MIEP) (reviewed in reference 22). The MIEP is adjacent to a powerful enhancer element comprised of an extensive and complex array of binding sites for cellular transcription factors. Although the MIE enhancer has been the subject of extensive study in vitro, little is known regarding its activity in the various cell lineages which HCMV infects in vivo, in particular during the earliest stages of infection.

All herpesvirus virions contain a tegument which separates the nucleocapsid from the envelope. A large number of proteins comprise the tegument, and some of these proteins may be released into infected cells following fusion of the viral envelope with the cell membrane. In this manner, tegument proteins may exert important regulatory roles immediately after entry. Two alphaherpesviruses, herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV), each contain tegument proteins which have been shown to transactivate IE promoters and enhance the production of infectious virus in cells transfected with their respective genomes. For example, the HSV-1 tegument protein VP16 (also called Vmw65 or αTIF) markedly increases the transcriptional activity of the HSV-1 IE genes by forming a complex with cellular components, including Oct-1 and HCF, which subsequently binds to regulatory elements upstream of the IE coding regions (reviewed in reference 34). When expressed constitutively in a permissive cell line, VP16 can also increase the efficiency of virus production following transfection of HSV-1 DNA (47). Two tegument proteins of VZV, encoded by open reading frame (ORF) 10 and ORF 62, have likewise been shown to activate one or more VZV IE genes (24, 26). The ORF 10 protein, which is the homolog of HSV-1 VP16, also interacts with Oct-1 and HCF (23), while the ORF 62 protein has been shown to bind to specific DNA elements upstream of IE, E, and late genes (51). Both the ORF 10 and ORF 62 tegument proteins, whether expressed constitutively or transiently, enhance the infectivity of VZV or HSV-1 viral DNA (24, 26).

The HCMV tegument is estimated to contain 20 to 25 proteins, of both viral and cellular origin (2, 7, 8, 42). Several of these tegument proteins have been identified, and a number of the corresponding genes, including the three which are the focus of this work, have been mapped (references 2 and 42 and references therein). ORF UL82 encodes a 71-kDa phosphoprotein referred to either as the upper tegument protein, ppUL82, or pp71 (27, 33, 36). A second phosphoprotein, termed the lower matrix protein, ppUL83, or pp65, is the 65-kDa product of ORF UL83 (27, 33, 36). A third tegument protein, pUL69, is encoded by the UL69 ORF. Multiple forms of this protein, ranging in size from 105 to 116 kDa, have been detected within infected cells (48), whereas only a 110-kDa form has been found in virions (50).

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Recently, pUL69 and pp71 were shown to increase expression of reporter genes driven by HCMV MIE, HCMV early, and heterologous promoters in transient transfection assays (16, 48, 49). Activation by both gene products resulted in the accumulation of reporter gene mRNA and is thought to reflect increased transcriptional initiation, although the mechanism of transactivation is unknown. In addition, synergistic activation of the MIEP was also observed when pp71 and pUL69 were coexpressed in transfected cells (49). Together, these experimental results suggest that activation of transcription from the MIEP by tegument proteins pUL69 and pp71 may augment the initiation of lytic replication following infection of the host cell. In contrast to pUL69 and pp71, pp65 has no known effect on MIEP-mediated transcription in transient assays (16). Although pp65 is nonessential for replication in tissue culture cells (39), a recent study has shown that pp65 can help protect infected cells from cytotoxic T lymphocytes by blocking the presentation of IE1-derived peptides with the major histocompatibility antigen (9). However, the observation that this tegument protein transits to the nucleus immediately after infection (6, 10, 38, 52) and is involved in the phosphorylation of viral proteins (39), including IE1 (9), indicates that pp65 could nonetheless have other early regulatory activities.

Since the HSV-1 and VZV tegument transactivators possess the ability to alter the infectivity of transfected viral DNA, an infectivity assay was developed for HCMV DNA to determine whether pUL69, pp65, and pp71 might have further functional homology to their HSV-1 and VZV counterparts. Human foreskin fibroblasts (HFF) permissive for HCMV replication were cotransfected with purified HCMV viral DNA and plasmids expressing the tegument proteins, both individually and in combination. Plaque formation, MIE protein synthesis, late protein synthesis, and virus spread were then measured. Our results imply that pp71 plays an important role in initiating productive infection and that its activation of MIE gene expression alone is not sufficient for this process.

MATERIALS AND METHODS

Plasmids. pCMV71 contains the HCMV Towne strain UL82 ORF driven by the HCMV MIEP (16). pCMV71R was constructed by digesting pCMV71 with *Xba*I to liberate the 2.2-kb UL82 ORF, which was then religated in the reverse orientation. pHM160 contains the UL69 ORF of HCMV Towne driven by the HCMV MIEP in the expression vector pCB6 (48). pCGN fuses a nine-amino-acid epitope of the influenza virus hemagglutinin protein (flu tag) to the amino terminus of the target protein and expresses it from the HCMV MIEP (46). pCGN65 was constructed by PCR amplification of the UL83 ORF from HCMV DNA by using primers which incorporated *Xba*I and *Bam*HI restriction endonuclease cleavage sites into the 5′ and 3′ ends, respectively, of the PCR product. Ligation of this product into the cognate sites in pCGN resulted in fusion of the entire UL83 ORF, minus the initiator methionine, to the flu tag. pCGNIE1 and pCGNIE2 express cDNA copies of the 72-kDa IE1 mRNA or the 86-kDa IE2 mRNA as flu tag fusions in pCGN (53). Immunoblot assays detecting the flu epitope established that pCGN65, pCGNIE1, and pCGNIE2 all produced polypeptides of the expected mass. pGL3-hCMV-MIEP contains the firefly luciferase gene under the control of the HCMV MIEP (35). pRL45 contains a 6.5-kb DNA fragment comprising the MIE locus subcloned from HCMV Towne, and it expresses the principal MIE gene products (30). pMP18 and pMP17 were derived by deleting the MIE exons 4 (UL123) and 5 (UL122), respectively, from pRL45 and therefore express either IE1 or IE2 from the native MIEP (30). pEGFP-N1 (Clontech) expresses the *Aequorea victoria* green fluorescent protein (GFP), optimized for cell sorter analysis, under control of the HCMV MIEP (5).

Viral DNA purification. HCMV viral DNA was isolated essentially as previously described (13). Primary HFF monolayers grown in Dulbecco's modified minimal essential medium (DMEM) containing 10% fetal calf serum (FCS) were infected with plaque-purified isolates of HCMV AD169 or Towne at a multiplicity of infection (MOI) of 0.1 to 0.01 PFU/cell. Following adsorption for 1 h, DMEM containing 5% FCS was added, and the infected cells were incubated at 37°C until cytopathic effect became extensive (13 to 17 days postinfection). The medium was then collected and cleared of cell debris by centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was layered onto a sorbitol cushion (20% D-sorbitol, 50 mM Tris [pH 7.2], 1 mM $MgCl_2$), and the virus was pelleted by centrifugation at 55,000 × *g* for 1 h at 4°C in a Beckman SW28 rotor. The virions were resuspended in 2 ml of 50 mM Tris [pH 8.0]–1 mM $MgCl_2$, an equal volume of lysis buffer (150 mM Tris [pH 8.0], 1 mM $MgCl_2$, 0.2 mM EDTA, 200 mM NaCl, 1% sodium sarkosyl, 200 μg of proteinase K per ml) was added, and the lysate was incubated at 37°C for 3 to 5 h. The liberated viral DNA was extracted four times by gently rocking with an equal volume of phenol plus chloroform (1:1, vol/vol), extracted twice more with chloroform, and then precipitated with ethanol. The precipitate was washed with 80% ethanol, air dried briefly, and resuspended in TE (10 mM Tris [pH 8.0], 1 mM EDTA) overnight. Alternatively, the extracted DNA was dialyzed extensively against TE, under vacuum, until the volume was reduced to approximately 0.5 ml.

Transfections and infectivity assays. HFF between 16 and 24 doublings (passages 8 to 12) after isolation were used for all transfections. Cells grown in monolayer to approximately 80% confluency were harvested with trypsin and washed twice with DMEM containing 10% FCS. The washed cells were suspended at 10^7 cells/ml in DMEM containing 10% FCS, and 0.4 ml of the suspension was added to an electroporation cuvette with a 4.0-mm electrode gap. Viral DNA (2 μg) and plasmid DNA (5 to 15 μg) were added to the cell suspension and mixed thoroughly with the cells by repeated passage through a wide-bore pipette. DNA was electroporated into cells with a single pulse from a Gene Pulser unit (Bio-Rad) set at 260 V and 960 μF, after which the cells were immediately diluted into DMEM containing 10% FCS and plated. After 2 to 3 days, the cell monolayers were overlaid with DMEM containing 5% FCS and 1% low-melting-point agarose (Life Technologies). When the plaques became readily apparent (approximately 12 to 14 days posttransfection), the infected monolayers were fixed with 10% formalin in phosphate-buffered saline (PBS) and stained with 0.03% methylene blue (Sigma Chemical Co.), and the plaques were counted by microscopic examination. All transfections were repeated at least twice.

To correlate plaque yield with IE gene expression, HFF were transfected as described above, but the pEGFP-N1 expression vector (1 μg) was included. Approximately 24 h after transfection, the cells were harvested with trypsin, washed twice, and resuspended in 2 ml of PBS containing 10% FCS. The total number of cells surviving each transfection (typically 25 to 50%) and the fraction expressing GFP (typically 3 to 20%) were determined by analyzing an aliquot (10%) with a Becton Dickinson Profile-II FACS (fluorescence-activated cell sorting) analyzer. The remaining cells were divided to seed one 18-mm^2 coverslip for IE staining and one or more culture dishes to score for plaque outgrowth as outlined above. In some cases, transfected cells were diluted with untreated HFF to ensure sufficient separation of plaques during the outgrowth. On day 2 following transfection, the cells seeded onto the coverslips were fixed and stained for IE expression as described below. To estimate the total number of IE-expressing cells in a transfection, all IE-positive nuclei on the entire area of the coverslip were counted, and this total was corrected for the fraction of the total transfected cell population contained on the coverslip (2×10^5 to 4×10^5 cells), to calculate the total number of IE-positive cells generated by the transfection.

Late gene expression and virus spread. For experiments examining late gene expression, HFF were first transfected with plasmids and HCMV DNA as described above except that immediately after transfection, each sample was distributed into four wells of a six-well culture dish, each containing one 18-mm^2 coverslip. As a control, untransfected HFF seeded previously onto coverslips were infected at the same time with HCMV Towne at an MOI of 0.01 PFU/cell. On each of the next 4 days, one coverslip from each transfection or the infection control was fixed, blocked, and stored at 4°C until collection of all time points had been completed. When all samples had been harvested, they were double stained in unison for IE2 and the late gene product pp28. The fraction of those cells expressing IE2 which also expressed pp28 was then assessed by fluorescence microscopy. To document the emergence of nascent plaques early after transfection (virus spread), samples taken over the first 4 days were instead stained for the MIE proteins and total nuclear DNA.

Immunofluorescent staining. Cells growing on 18-mm^2 coverslips were prepared for staining by fixation in 4% paraformaldehyde in PBS for 10 min at room temperature, followed by permeabilization with Triton X-100 and blocking with 1% bovine serum albumin in PBS for 30 min at room temperature. All subsequent antibody dilutions and washes were done in PBS containing 0.1% bovine serum albumin. For infectivity and virus spread assays, total IE1 and IE2 were detected by staining sequentially with a mouse monoclonal antibody (MAb) reactive with an epitope shared by IE1 and IE2 (MAb 810; Chemicon), followed by a Texas Red-coupled, goat anti-mouse secondary antibody. For experiments which examined late gene expression, IE2 and pp28 were detected simultaneously by staining first with a cocktail composed of a rabbit antiserum (1:100 dilution) reactive with an epitope unique to IE2 (gift of M. Pizzorno and G. S. Hayward) plus a mouse monoclonal hybridoma supernatant (1:5 dilution) reactive with pp28 (gift of A. J. Levine [28]). This was followed with a second cocktail of goat anti-rabbit fluorescein isothiocyanate (FITC) plus goat anti-mouse Texas red.

To document early spread of virus, coverslips containing fixed and blocked cells were stained for IE1 plus IE2 with MAb 810, followed by a Texas red-conjugated secondary antibody. Prior to viewing, the coverslips were treated with 100 μg of RNase A per ml for 30 min at room temperature, and nuclear DNA was then counterstained with YOYO-1 (Molecular Probes), a nucleic acid binding oxacyanine dye with spectral characteristics similar to those of FITC. Individual fields were examined by confocal microscopy for IE expression (Texas

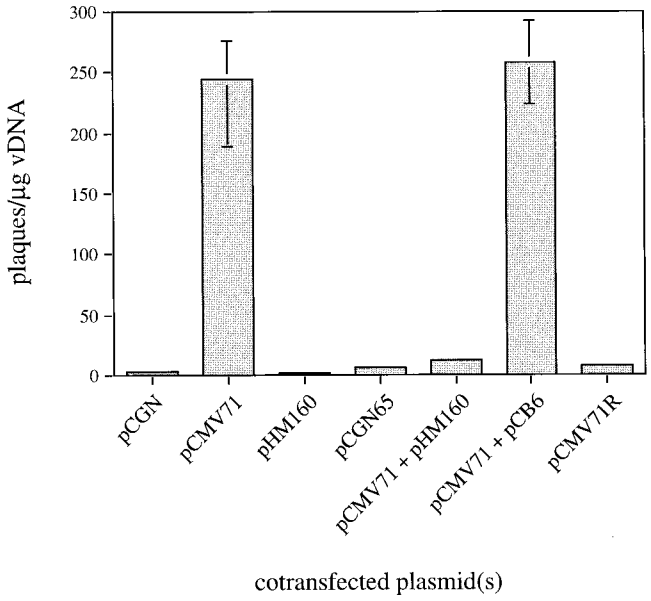

FIG. 1. Effect of tegument proteins pp71, pUL69, and pp65 on the infectivity of HCMV DNA. HFF were electroporated with 2 μg of purified HCMV Towne viral DNA (vDNA) together with 5 μg of either a control expression vector (pCGN) containing the HCMV MIEP or 5 μg of a vector expressing the tegument protein pp71 (pCMV71), pUL69 (pHM160), or pp65 (pCGN65). The total plasmid mass was adjusted to 10 μg with pCGN. Following transfection, cells were plated, and the resulting plaques were quantified by methylene blue staining 12 to 14 days after transfection. pCMV71R is a derivative of pCMV71 containing the UL82 ORF in the opposite orientation with respect to the MIEP. pCB6 is the parent vector of pHM160. The mean infectivity and range from at least two independent experiments are shown.

red) compared with the total number of nuclei present (YOYO-1). Stained coverslips were mounted in phosphate-buffered glycerol and viewed on a Nikon Optiphot II microscope by epifluorescence or confocal microscopy, using a Bio-Rad MRC600 confocal laser scanning microscope.

Luciferase assays. HFF were transfected with 0.1 μg of pGL3-hCMV-MIEP plus 2 μg of effector plasmid pCMV71, pHM160, pCGNIE1, or pCGNIE2 and plated in a 10-cm-diameter dish. After 48 h, the cells were washed once with PBS and lysed for 10 min in 0.5 ml of a solution containing 0.1 M potassium phosphate (pH 7.8) and 0.2% Triton X-100. Luciferase enzymatic activity in the cell lysates was analyzed in a Monolight 2010 luminometer (Analytical Luminescence Laboratory) according to the manufacturer's protocol. Relative light unit (RLU) readings were normalized as RLU/milligram of protein, using total protein concentrations determined for each lysate by the Bradford assay (Bio-Rad). Assays were performed in triplicate, and the average with standard deviation is reported.

RESULTS

Infectivity of HCMV DNA is enhanced by pp71. Three observations led us to examine whether the HCMV tegument proteins pp71, pUL69, and pp65 might influence the infectivity of transfected HCMV DNA. First, the HCMV proteins pUL69 and pp71 transactivate a plasmid-based HCMV MIEP in transfection assays. Second, pp65, an abundant component of the tegument, rapidly migrates to the nucleus upon infection (6, 10, 38, 52) and may participate in the phosphorylation of IE1 (9). Finally, tegument proteins of HSV-1 and VZV, which regulate IE gene expression in these viruses, have been found to alter the infectivity of transfected viral DNA (24, 26, 47). Therefore, several expression plasmids encoding HCMV tegument proteins were cotransfected singly or in combination into HFF along with purified HCMV Towne DNA, and the infectivity of the viral DNA was determined by plaque assay (Fig. 1). When Towne DNA was transfected, either by itself or with a control vector, it produced approximately 1 to 4 plaques/μg of viral DNA. However, the infectivity increased to an average of 245 plaques/μg when a vector expressing pp71 (pCMV71) was included in the transfection. Cotransfection of Towne DNA with plasmid pCMV71R, a derivative of pCMV71 in which the UL82 ORF had been positioned in the opposite translational polarity with respect to the MIEP, did not significantly affect the infectivity of Towne viral DNA. Vectors expressing either pUL69 (pHM160) or pp65 (pCGN65) also did not substantially alter the infectivity of cotransfected Towne DNA. The lack of an effect on infectivity by pp65 may be related to the observation that the pp65 ORF can be deleted from the viral genome without affecting the replication of the virus in cell culture (39).

To search for potential cooperation between pp71 and the other tegument proteins, pCMV71 was cotransfected with pCGN65 or pHM160. The pCGN65 vector had no effect on the stimulation of infectivity produced by pCMV71 and was therefore not further studied (data not shown). Surprisingly, cotransfection of pHM160 with pCMV71 consistently reduced the increased infectivity mediated by pCMV71 4- to 20-fold (Fig. 1 and data not shown). Control transfections with pCB6, the parental expression vector of pHM160, failed to produce any such reduction, indicating that the UL69 sequence is required to negate the effect of pCMV71.

A dose-response assay further demonstrated that expression of pp71 was necessary for the enhancement of infectivity (Fig. 2A). On average, a 30-fold increase in the infectivity of Towne DNA was observed with as little as 50 ng of cotransfected pCMV71 (1:1 molar ratio of plasmid to viral DNA), and the response saturated between 0.8 and 3.2 μg of plasmid DNA. Moreover, it was possible to demonstrate that the effect of pCMV71 was not strain specific by cotransfecting pCMV71 with the HCMV AD169 DNA. This resulted in a stimulation of the infectivity of AD169 DNA similar to that found with Towne DNA, indicating that the effect of pp71 was not unique to HCMV Towne (Fig. 2B). As was observed for Towne DNA, plasmid pCGN also failed to increase the infectivity of AD169 DNA. The experimental results in Fig. 1 and 2 indicate that the stimulation of infectivity produced by pCMV71 cannot be attributed to titration of negative regulatory factors (due to additional copies of the MIEP) or to a nonspecific effect of the DNA encoding pp71 but, rather, that stimulation is sensitive to the level of pp71 expression and appears to act on multiple strains of HCMV.

Increased infectivity correlates with enhanced expression of IE1 and IE2. Since pp71 has been shown to transactivate a plasmid-based reporter controlled by the HCMV MIEP (16), additional experiments were undertaken to determine whether the increase in infectivity mediated by pp71 might correlate with a change in MIE gene expression from the transfected viral genome. Transfections were done as described for the experiments in Fig. 1 but in addition included pEGFP-N1, a plasmid which expresses a FACS-optimized variant of GFP. Approximately 24 h after electroporation, the surviving cells were removed from the dishes with trypsin, and an aliquot (10%) of each transfection was analyzed by FACS for GFP expression to determine the total number of transfected cells in each sample; this number was used to normalize the subsequent assays for transfection efficiency. Additional portions of the cells were used for two further measurements. One fraction was plated onto coverslips and examined by immunofluorescence the next day (2 days after transfection) for expression of IE1 and IE2. The remaining portion was further incubated at 37°C to score for plaque outgrowth. In addition to dramatically increasing the infectivity of transfected viral DNA, cotransfection with pCGN71 (in comparison to pCGN) also resulted in a substantial increase in the number of cells expressing the MIE

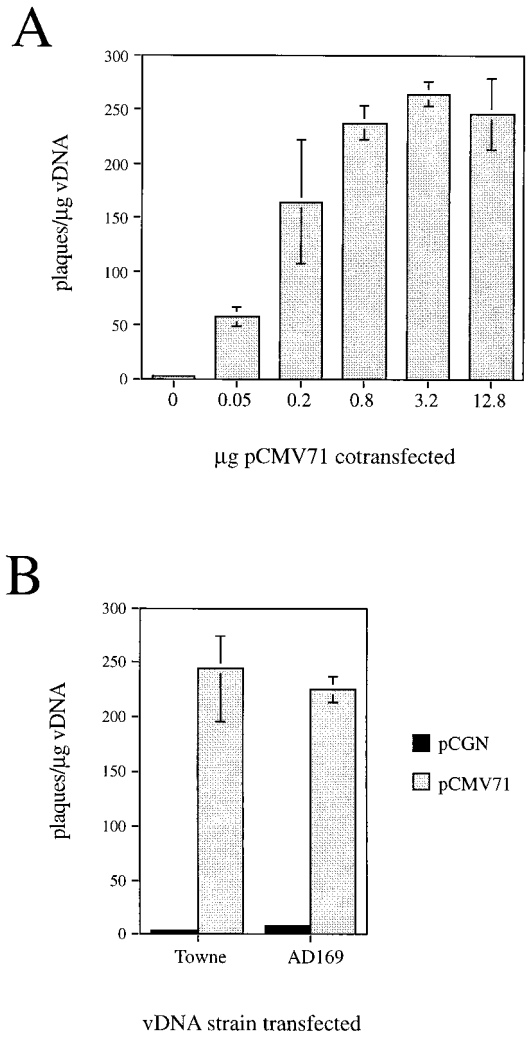

FIG. 2. Dose response and strain specificity of pp71 activity. (A) Titration of the pCMV71 expression vector. Transfections were performed as described in the legend to Fig. 1 with the indicated amounts of pCMV71. The total plasmid mass was adjusted to 15 μg with pCGN. vDNA, viral DNA. (B) Response of HCMV AD169 to pCMV71. Purified viral DNA from the Towne or AD169 strain of HCMV (2 μg) was transfected into HFF along with 5 μg of either pCMV71 or the pCGN control vector. After 12 to 14 days, the resulting plaques were stained with methylene blue and counted. The mean infectivity and range from at least two independent experiments are shown.

proteins (Table 1). Cotransfection with pHM160 again did not increase viral DNA infectivity and surprisingly also failed to stimulate MIE protein synthesis.

In addition to increasing the absolute number of IE-expressing cells, pCMV71 also increased the intensity of the observed IE fluorescence to levels typical of infected cells. In the presence of transfected pCMV71, over 80% of the IE-positive nuclei were essentially indistinguishable from infected nuclei, showing a characteristically strong and uniform nuclear IE fluorescence (data not shown). Conversely, in the absence of pCMV71, only about 15% of all IE-positive nuclei stained with an intensity comparable to that of infected cell nuclei; the majority displayed a weaker, sparse, punctate signal (data not shown). Two further points of interest are evident from the observations related in Table 1. First, even in the presence of transfected pCMV71, only a minority of transfected cells ever expressed detectable levels of the IE proteins. Second, among those cells which do express IE proteins, the majority never achieve productive infection (Table 1). As it is unlikely that the cells expressing GFP had never received viral DNA, we feel that the simplest interpretation is that there exist strong constraints on MIEP activity and HCMV replication, even within permissive cells.

The preceding results are, however, still consistent with the hypothesis that activation of MIE gene expression by pp71 is sufficient to promote productive replication of transfected HCMV DNA. The critical function of pp71 may then be to augment the development of a threshold level of MIE proteins required for viral replication. If this is the case, then cotransfection of vectors which generate levels of IE1 and IE2 similar to those seen with pCMV71 should also increase the infectivity of viral DNA to the same extent as pCMV71. To test this hypothesis, two sets of expression vectors for IE1 and IE2, containing either cDNA copies of IE1 and IE2 or subcloned HCMV genomic DNA, were substituted for pCMV71 in the infectivity assays (Fig. 3). pRL45, pMP17, and pMP18 were cloned from the genomic Towne MIE locus and express all of the MIE products, IE1 only, and IE2 only, respectively. Alternatively, pCGNIE1 and pCGNIE2 express cDNA copies of IE1 and the 86-kDa IE2 (see Materials and Methods). Expression of IE1 or IE2 individually had little effect on HCMV DNA infectivity. However, coexpression of the MIE proteins, using either pRL45 or pCGNIE1 plus pCGNIE2, resulted in an average infectivity of about 20 plaques/μg, considerably less than the level observed with coexpression of pp71 (Fig. 3A). We cannot rule out the possibility that the levels of MIE protein expression from IE1- and IE2-containing plasmids are lower than those induced from the viral genome by pp71; subtle differences in the level of MIE expression could influence infectivity to different extents. However, we favor the view that pp71 does not enhance infectivity simply by increasing the level of MIE expression because the intensity of IE1- and IE2-specific immunofluorescence resulting from transfection with pCGNIE1 plus pCGNIE2 was at least equal to that observed following pCMV71 transfection (data not shown).

Since expression of IE1 and IE2 did, however, show a small but reproducible effect on infectivity, the potential for synergism of IE1 and IE2 with pp71 on infectivity was investigated in further transfections (Fig. 3B). HFF transfected with pRL45, pCMV71, and Towne DNA showed a slight increase in infectivity compared to pCMV71 alone. Similar results were obtained when pRL45 was replaced with pCGNIE1 and pCGNIE2. Consistent with the results shown in Fig. 3A, cotransfection of either pCGNIE1 or pCGNIE2 along with pCMV71 had little effect on the enhancement of infectivity mediated by pCMV71 alone. It is notable that pHM160, which antagonized

TABLE 1. Effects of pp71 and pUL69 on IE1 and IE2 expression and plaque formation

Cotransfected plasmid	IE-positive cells/plaques[a]		
	Expt 1	Expt 2	Expt 3
pCGN	189/0	211/2	348/4
pHM160	125/0	270/2	929/4
pCMV71	1,170/296	4,158/608	6,156/634

[a] For each experiment shown, the total number of cells which expressed MIE proteins (IE1 plus IE2) 2 days after the indicated transfection is presented together with the total number of plaques which resulted from the same transfection. Within each experiment, IE and plaque values were normalized to reflect equal numbers of transfected cells, using GFP expression (see Materials and Methods). Typical transfections yielded between 5×10^4 and 1.5×10^5 GFP-positive cells, which was taken as the number of cells receiving viral DNA.

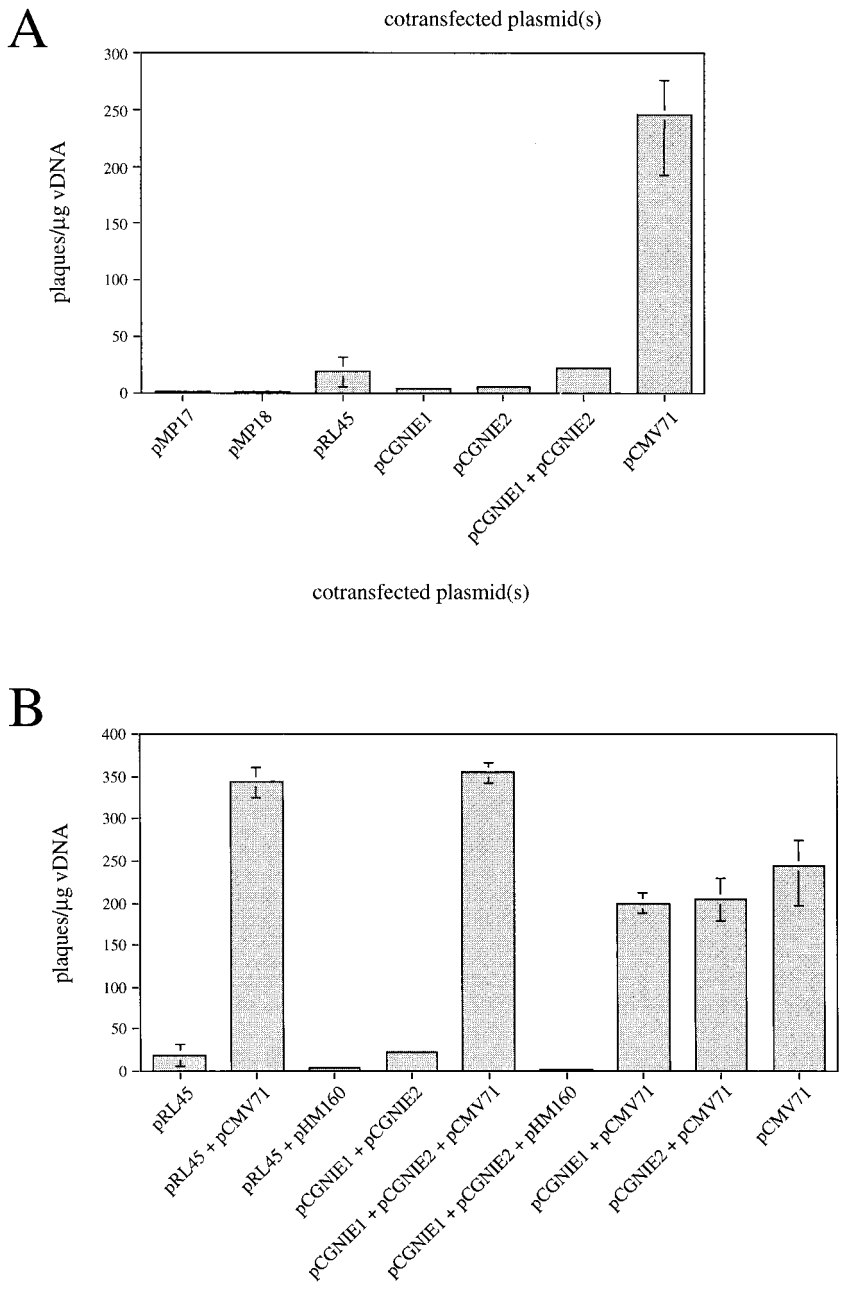

FIG. 3. Influence of the MIE proteins on the infectivity of HCMV DNA. Transfections of Towne viral DNA (vDNA) and the indicated plasmids (5 μg of each) were performed as indicated in the legend to Fig. 1. Total plasmid mass was equalized, as required, with pCGN. (A) Comparison of pCMV71 with MIE protein expression vectors. A vector expressing the entire genomic MIE locus (pRL45), deletion derivatives expressing IE1 (pMP17) or IE2 (pMP18), or vectors expressing cDNA copies of IE1 (pCGNIE1) or the 86-kDa form of IE2 (pCGNIE2) were cotransfected with Towne viral DNA. After 12 to 14 days, the resulting plaques were stained with methylene blue and counted. (B) Cotransfection of the tegument and MIE expression vectors. Transfections were performed with Towne viral DNA plus combinations of MIE protein expression vectors, as in panel A, and pCMV71 or pHM160. The mean infectivity and range from at least two independent experiments are shown.

the effect of pCMV71, again negated the modest enhancement produced by IE1 and IE2 expression.

Tegument and IE protein expression vectors transactivate the MIEP. Since pUL69 has been reported to transactivate a plasmid-based MIEP (49), it was somewhat surprising that pUL69 antagonized the effects of pp71 (Fig. 1) and the MIE proteins (Fig. 3B) in the infectivity assays and was unable to stimulate MIE gene expression from transfected viral genomic DNA (Table 1). To confirm that the preparations of the tegument and IE protein expression vectors used in our infectivity studies were able to transactivate the MIEP, HFF were electroporated with the expression vectors and pGL3-hCMV-

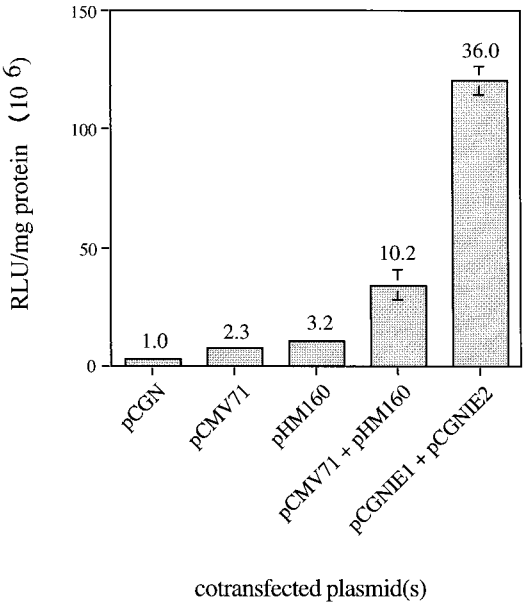

FIG. 4. MIEP transactivation by tegument and MIE proteins. HFF were transfected with 0.1 μg of a reporter plasmid expressing firefly luciferase from the MIEP, pGL3-hCMV-MIEP, and 2 μg each of the tegument or MIE protein expression vectors as described in the text. Extracts were prepared 48 h posttransfection and analyzed for luciferase enzyme activity, which is reported as RLU/milligram of total protein. Each transfection was repeated at least three times. The mean fold activation relative to the pCGN vector control is indicated by the number over each bar, and the standard deviation of the mean is shown by the error bars.

MIEP, a reporter construct containing the firefly luciferase gene under the control of the HCMV MIEP. Consistent with the results of transactivation studies reported previously (16, 49), cotransfection of pGL3-hCMV-MIEP with pCMV71 or pHM160 resulted in elevated luciferase activity relative to the pCGN vector (Fig. 4). As also noted previously (49), a synergistic activation of the MIEP was seen when pCMV71 and pHM160 were cotransfected. Notably, the combined activation of the MIEP by pCMV71 and pHM160 (10-fold) was substantially less than that resulting from cotransfection of pCGNIE1 and pCGNIE2 (36-fold). Thus, although displaying different effects on the infectivity of HCMV DNA, pp71, pUL69, and the MIE proteins all activated the plasmid-based MIEP under the conditions used for the infectivity assays. These results, together with the results of Fig. 3, are consistent with our conclusion that activation of the MIE locus is not of itself sufficient to permit HCMV to undergo a lytic replication cycle, though such activation may still be necessary.

pp71 promotes late gene expression, facilitates virus spread, and accelerates plaque development. Since cotransfection of the pp71 expression vector with viral DNA increased the number of cells expressing the MIE proteins (Table 1), we asked if this resulted in activation of the gene expression cascade and ultimately promoted expression of a late viral protein. To examine this, transfected cells were fixed and reacted with antibodies specific for IE2 and the true late gene product pp28, and the fraction of cells expressing IE2 which also expressed pp28 was determined by immunofluorescence over the first few days following transfection (Table 2). When pCMV71 was included, pp28 expression from the viral genome was observed in up to 40% of IE2-expressing cells after 2 days and in as many as 90% of the IE2-positive cells by day 4 after transfection. In fact, the efficiency of pp28 expression following cotransfection with the viral genome and pCMV71 was similar to that observed in parallel infections with virus (Table 2). Within this time period, pp28 was rarely detected in the IE2-expressing cells observed in control transfections with viral DNA and the pCGN vector.

Consistent with the enhanced expression of pp28 (and presumably all late viral gene products), virus spread was accelerated in the presence of pp71. By day 4 after transfection with pCMV71 and the viral genome, nascent plaques comprising 10 or more IE-positive nuclei were common (Fig. 5A), whereas in the absence of pp71, IE staining in multiple nuclei was extremely rare by day 4 (Fig. 5B). The rate of virus spread after transfection with viral DNA plus pCMV71 mirrored that observed in parallel virus infections (Fig. 5C). As predicted by the more rapid virus spread, the macroscopic development of plaques (reflected by cytopathic effect) was also accelerated in the presence of added pp71 (data not shown). When pCMV71 was included with viral DNA in transfections, plaques were two- to threefold larger when examined between days 6 and 10, after which plaques from all transfections became more similar in size. The enhanced size of nascent plaques likely results from the combination of more rapid completion of the viral growth cycle and accelerated spread of virus from the cells that initially receive the pp71 expression plasmid.

DISCUSSION

In this study, we have demonstrated an 80-fold enhancement of HCMV DNA infectivity in the presence of the HCMV tegument protein pp71, which had been expressed from a cotransfected plasmid (Fig. 1 to 3). In addition, pp71 expression facilitated progression of lytic HCMV replication following transfection of viral DNA, as was evidenced by more efficient late gene expression (Table 2) and the early spread of virus to adjacent cells (Fig. 5). This is the first example of an HCMV tegument protein having an impact on viral replication and spread. This in vitro infectivity assay may also provide a reasonable model system with which to study early events which influence lytic HCMV replication.

Liu and Stinski (16) had previously demonstrated the ability of pp71 to enhance the transcription of a reporter gene regulated by the MIEP, as well as some heterologous promoters, in transient transfection assays. Transcriptional activation by pp71 correlated with the presence of ATF or AP-1 binding sites in the target promoter sequences. We have extended those findings here by demonstrating that pp71 can activate expression of the MIE proteins IE1 and IE2 in the context of

TABLE 2. Changes in late gene expression in the presence of pp71

Cotransfected plasmid or infection	pp28$^+$ cells/IE2$^+$ cells[a]			
	2 days posttransfection		4 days posttransfection[b]	
	Expt 1	Expt 2	Expt 1	Expt 2
pCGN	0/22	ND[c]	0/19	0/3
pCMV71	40/100	10/100	90/100	60/100
Towne infection[d]	31/100	ND	95/100	ND

[a] The fraction of IE2-positive cells (up to 100 per coverslip) which also express pp28.

[b] Emerging foci bearing multiple IE2-positive nuclei were scored as 1/1. Such foci contained at least one cell expressing pp28.

[c] ND, not determined.

[d] Representative data, for comparison, from a low-multiplicity infection of HFF with the Towne strain of HCMV begun immediately following the transfection procedure.

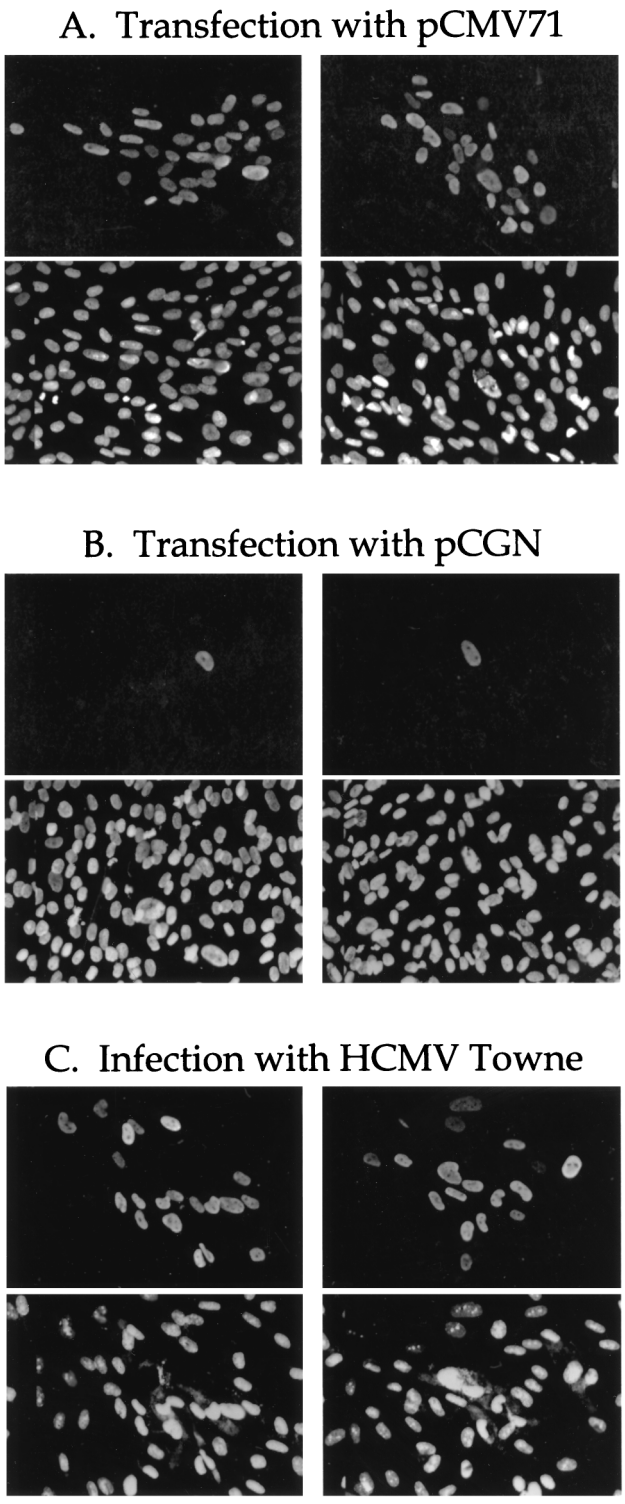

FIG. 5. Virus spread in the presence of pp71. HFF were transfected with Towne DNA plus pCMV71 (A) or the pCGN control vector (B) or were infected at low MOI (0.01 PFU/cell) with HCMV Towne (C). Following plating on coverslips and incubation at 37°C for 4 days, IE1 and IE2 proteins were colabeled with Texas red. The DNA was then nonspecifically counterstained with YOYO-1, a dye which fluoresces in the same wavelength range as FITC (see Materials and Methods). The upper images in each panel show the IE-positive cells in the field on the Texas red channel. The lower images in each panel show all nuclei in the same field on the FITC channel. Two representative fields are shown at left and right in each panel. All fields are at a magnification of ×400.

the viral genome (Table 1). Together with the enhancement of infectivity mediated by pp71, these results argue that pp71 can function in the context of the viral genome under conditions which can lead to productive infection. Analogous infectivity enhancement and IE gene transactivation activities are associated with the VZV ORF 10 and ORF 62 tegument proteins (24, 26) and also with the HSV-1 VP16 tegument protein (47). While these tegument proteins have no significant amino acid homology to pp71, their functional homology suggests that HCMV, like these alphaherpesviruses, has evolved to contain a virion-associated transactivator to facilitate the initial transcription of viral IE genes. While this study did not address the mechanism of infectivity enhancement by pp71, the simplest model consistent with the known features of the protein would be that pp71 transactivates IE protein synthesis at the MIE locus, and probably other IE loci also, until sufficient amounts have accumulated for autostimulation to become the prominent regulatory mode.

These experiments also have provided evidence that the MIE locus might not be the sole target of pp71 action. While the full spectrum of HCMV genes affected by pp71 is not yet known, it is clear from previous studies (22) that pp71 can activate multiple promoters. Our results suggest several reasons that activation of the MIE locus, while perhaps necessary, is unlikely to be sufficient in itself to promote lytic replication. First, cotransfection of the IE1 and IE2 expression vectors produced much greater activation of a luciferase reporter gene driven by the MIEP than did pCMV71. Second, high-level expression of IE1 and IE2 in HFF cotransfected with HCMV DNA did not increase the infectivity of the viral DNA to the degree produced by pp71. Had high-level IE1 and IE2 expression been sufficient to result in lytic replication, each of these two results would have predicted that transfection of the IE expression vectors should yield plaque outgrowth similar to that produced by the pp71 vector, yet we consistently observed the opposite. Third, coexpression of IE1, IE2, and pp71 only modestly increased DNA infectivity beyond that seen with pp71 alone. While this small increase in infectivity could have resulted from additional pp71 synthesis from pCMV71 due to activation of the MIEP by IE1 and IE2, we favor the interpretation that pp71 and the IE proteins have different roles related to early events in infection. Perhaps pp71 transactivates other IE genes which have important functions early in infection. These proteins might in turn stimulate viral DNA infectivity similarly to pp71. In this regard, VZV IE protein ORF 61 and HSV-1 IE protein ICP0, which transactivate IE, E, and late genes, also significantly enhance the infectivity of their respective viral genomes (4, 25).

Somewhat surprising was the effect of pUL69 on the infectivity of transfected HCMV DNA. This tegument protein, like pp71, transactivates the MIEP in transient transfection assays (reference 49 and Fig. 4). However, in our experiments, pUL69 did not substantially increase expression from the MIE locus in the context of the viral genome (Table 1), nor did it enhance the infectivity of transfected viral DNA (Fig. 1 and Table 1). Titration of pHM160 also indicated that the lack of an enhancement of infectivity was not the result of overexpression of pUL69 from the transfected plasmid vector (data not shown). Curiously, pUL69 expression antagonized both the pp71-mediated and the smaller IE1- and IE2-mediated increases in HCMV DNA infectivity (Fig. 1 and 3B). The pUL69 homolog in VZV, encoded by ORF 4, has also been shown to transiently transactivate VZV IE genes in transfection assays, but like pUL69, it has little effect on VZV DNA infectivity (26). Whether ORF 4 can antagonize the effects of ORF 10 or ORF 62 has not been examined. The HSV-1 homolog of pUL69,

ICP27, has been extensively studied in terms of its role as a regulator of viral gene expression. Studies with ICP27 temperature-sensitive and null mutants have demonstrated that ICP27 down-regulates IE and E genes within the HSV-1 viral genome (18, 20, 32). However, in transfection assays using reporter plasmids, ICP27 can act either as a repressor or as an activator (31, 37, 40, 45). Evidence indicates that this differential activity of ICP27 is in part due to the nature of the reporter gene transcription units; repression correlated with the presence of introns (37), while activation was influenced by certain poly(A) sites (19, 37). A similar target gene dependence may explain the pUL69-mediated activation of a nonspliced reporter gene (reference 49 and Fig. 4) in transient assays and the failure of pUL69 to enhance expression of the IE proteins (Table 1) which are derived from a genomic transcription unit that undergoes splicing. Other studies have demonstrated context-dependent promoter regulation of early and late genes of HCMV (15) and HSV-1 (1, 41).

The increased number of plaques formed following transfection of HCMV DNA together with pCMV71 also has practical implications. The functions of particular HCMV proteins can be elucidated by creating null mutations in the viral genome. The method relies on homologous recombination between transfected viral genomic DNA and a plasmid bearing a marker flanked by the target gene sequences, followed by the selection and isolation of recombinant progeny virions. Due to the low infectivity of HCMV DNA, the analysis of a large number of putative recombinant viral clones is usually not possible. We have found that expressing pp71 in transfected cells greatly increases the number of plaques obtained and thus facilitates the collection of a large pool of putative recombinant viruses. We have used this technique to make null mutations at several loci within the AD169 and Towne genomes (unpublished results).

Although the mechanism by which pp71 enhances the infectivity of viral DNA is unknown, experiments may now be designed in a comparatively simple and well-defined system to address viral or cellular activities which pp71 modulates. Of primary interest is the identification of other viral regulatory genes, particularly IE genes, which pp71 may transactivate, and also identification of other cellular and viral factors which influence the activity of HCMV IE genes. Such studies are in progress and should provide further insights into the factors which influence the initiation of the HCMV replication cycle.

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