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Human Cytomegalovirus US3 Gene Expression Is Regulated by a Complex Network of Positive and Negative Regulators

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One immediate early gene of human cytomegalovirus, the US3 gene, causes retention of major histocompatibility locus class I heavy chain proteins in the endoplasmic reticulum and is postulated to have a role in viral pathogenicity. Expression of the US3 gene is regulated by a number of *cis*-acting elements. In addition, numerous viral proteins are involved in regulating US3 gene expression. US3 transcription was activated modestly by a virion protein, ppUL82. The immediate early proteins encoded by UL122–123 (IE1 and IE2) further activate US3 expression, with the activation enhanced by expression of pTRS1. Other proteins, the immediate early protein encoded by UL37ex1/UL38 and the early protein, pUL84, inhibited IE1 and IE2 activation of US3 expression. US3 transcription is regulated both positively and negatively by a complex network of viral proteins, the interaction of which contributes to precise regulation of US3 gene expression. The ability of pUL37ex1/UL38 to repress expression of the immediate early US3 gene while activating early gene expression suggests that pUL37ex1/UL38 may function to switch viral gene expression from immediate early to early genes. © 1999 Academic Press

INTRODUCTION

Human cytomegalovirus (HCMV) is an important opportunistic pathogen and causes a wide variety of clinical syndromes in immuno-suppressed individuals including pneumonitis in bone marrow transplant recipients (see Britt and Alford, 1996 for a review). Replication of the virus requires the temporally regulated expression of immediate early (IE), early, and late genes (see Mocarski, 1996 for a review). The large genome, the time period required for viral replication (~5 days), and the complex patterns of transcription suggest that multiple mechanisms are utilized to precisely regulate gene expression.

The IE genes (UL122–123, IRS1/TRS1, US3, and UL36–38) are the first group of genes to be expressed following viral infection and are transcribed in the absence of *de novo* protein synthesis. IE gene expression is activated by proteins located in the tegument of the virus that are released into the cell upon infection. The HCMV genes UL82, UL69, and TRS1/IRS1 encode tegument proteins with known regulatory functions. ppUL82 (the phosphorylated protein encoded by UL82, also known as pp71; Chee *et al.,* 1990) activates expression of UL122–123 [the major IE (mIE) gene] through consensus ATF or Ap-1 binding sites (Liu and Stinski, 1992). IRS1/TRS1 are genes partially located in repeated regions of the viral genome and encode proteins (pTRS1/pIRS1) that have common amino termini but different carboxyl termini (Chee *et al.,* 1990). Proteins encoded by the UL69 gene, pUL69, activate a variety of promoters and work synergistically with pIRS1/TRS1 to activate the mIE and the IRS1/TRS1 promoters (Winkler *et al.,* 1994; Winkler and Stamminger, 1996; Romanowski *et al.,* 1997).

Proteins encoded by the IE genes have regulatory roles and influence both cellular and viral gene expression. Expression of the mIE gene is subject to complex regulation and results in the synthesis of two predominant proteins, IE1 and IE2, that are involved in autoregulation and regulation of the expression of other viral genes (see Mocarski, 1996 for a review; Stenberg and Stinski, 1985; Hermiston *et al.,* 1987, 1990; Cherrington and Mocarski, 1989).

TRS1/IRS1 are transcribed at IE times of infection and continue to be transcribed throughout the replication cycle. Thus not only are pTRS1/pIRS1 located in the virion, these proteins are also newly synthesized at IE times of infection (Romanowski and Shenk, 1997). The protein products of the TRS1 and IRS1 genes augment activation of viral gene expression by IE1 and IE2 (Stasiak and Mocarski, 1992; Romanowski and Shenk, 1997) as well as cooperating with pUL69 in activating the mIE and IRS1/TRS1 promoters (Romanowski *et al.,* 1997). An additional IE transcriptional unit contained within IRS1, $IRS1^{263}$, encodes a protein that shares amino acids with the carboxyl terminal end of pIRS1 (Romanowski and Shenk, 1997). $pIRS^{263}$ represses IE1- and IE2-mediated transcriptional activation of an early and a late gene promoter.

UL36–38 is a complex gene cluster that includes open reading frames UL36, UL37, and UL38. Transcription of UL36–38 results in the synthesis of a number of alterna-

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tively spliced RNAs (Tenney and Colberg-Poley, 1991). Proteins encoded by UL36–38 work synergistically with US3 proteins in regulating *hsp70* transcription and are required for lytic (*ori*Lyt dependent) replication of the virus and activation of early viral gene expression (Iskendrian *et al.,* 1996; see Colberg-Poley, 1996 for a review). In particular, UL37 plays a role in contributing to activation of early viral gene expression (Colberg-Poley *et al.,* 1998).

Early genes are dependent on IE proteins for expression and generally encode proteins involved in DNA replication. Two early genes with regulatory roles are UL84 and the UL112–113 gene cluster, both of which are required for *ori*Lyt-dependent replication of the viral genome (Pari and Anders, 1993; Iskenderian *et al.,* 1996; Kerry *et al.,* 1996; Sarisky and Hayward, 1996). UL112–113 are needed for maximal expression of several early promoters (Pari and Anders, 1993). The protein encoded by UL84 associates with IE2 and blocks activation of an early gene promoter by IE2 as well as augmenting IE2-mediated transcriptional repression of the mIE promoter (Spector and Tevethia, 1994; Gebert *et al.,* 1997).

I am interested in characterizing the regulatory mechanisms used to control expression of the HCMV US3 gene. The US3 gene encodes a number of proteins synthesized from alternatively spliced mRNAs (Colberg-Poley *et al.,* 1992; Tenney *et al.,* 1993). Although the US3 gene is not essential for replication in cell culture (Kollert-Jons *et al.,* 1991; Jones and Muzithras, 1992), US3-encoded proteins are involved in activating expression of *hsp70* and also cause retention of major histocompatibility complex class I heavy chains in the endoplasmic reticulum (Colberg-Poley *et al.,* 1992; Tenney *et al.,* 1993; Ahn *et al.,* 1996; Jones *et al.,* 1996). Expression of the US3 gene is activated by viral infection with US3 transcripts detectable at ~1 h after infection and increasing in abundance until 3 h p.i. (Biegalke, 1995). After the initial burst of transcription, expression of the US3 gene is repressed with the level of US3 transcripts markedly decreased by 5 h p.i. The complex pattern of US3 expression is controlled by number of *cis*-acting elements including a silencer element (Chan *et al.,* 1996; Thrower *et al.,* 1996), an enhancer element (Weston, 1988), and a transcriptional repressive element (*tre;* Biegalke, 1995, 1998; Lashmit *et al.,* 1998). Although the *cis*-acting elements controlling US3 transcription have been well characterized, little is known about the *trans*-acting factors involved in US3 regulation. Here, I have begun to examine the effect of virally encoded *trans*-acting proteins on US3 expression.

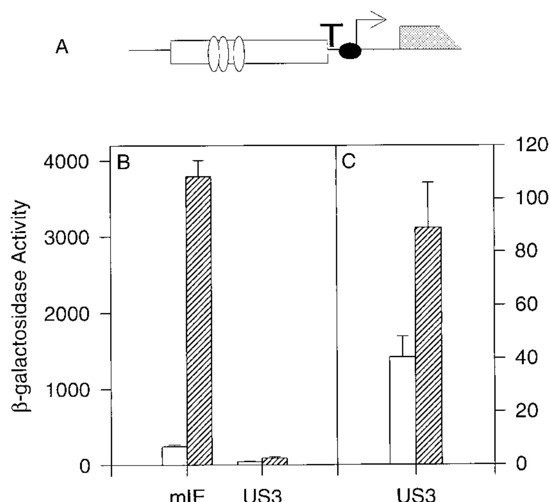

FIG. 1. Regulation of US3 expression by ppUL82. (A) Diagram of the regulatory region of the US3 gene present in the *lacZ* reporter plasmid, pBJ171. Open rectangle, enhancer element; open ovals, predicted ATF binding sites (according to DNASIS analysis); T, TATA box; black oval, *tre* (transcription repressive element); bent arrow, transcription start site; open ended rectangle, coding region. (B) Effect of ppUL82 (pp71) on expression of the mIE and US3 promoters. Human diploid fibroblasts were transfected with the US3 (pBJ171) or the mIE (pEQ176) reporter gene plasmid along with a control plasmid (pBJ201, open rectangles) or a plasmid expressing ppUL82 (pBJ203, cross-hatched rectangles) using DEAE-dextran. β-galactosidase activity was measured ~2 days after transfection by determining the fluorescence of media containing the cleavage product of methyl-umbelliferyl-β-D-galactoside (MUG). The β-galactosidase activities (MUG fluorescence units) are the means of two experiments (plus one standard deviation). Levels of enzyme activity obtained with a promoterless *lacZ* containing plasmid (pEQ3) were subtracted from the test values. (C) The data for the US3 expression plasmid (pBJ171) in the presence or absence of ppUL82 from (B) is shown enlarged here.

RESULTS

Influence of tegument proteins on US3 expression

Activation of US3 expression is dependent on infection but does not require viral protein synthesis (Biegalke, 1995). This suggests that a structural protein(s) of the virus is involved, either directly or indirectly, in the initial activation of US3 transcription. Transient expression assays were utilized to determine whether any of the known transcriptional activators found in the virion (ppUL82, pUL69, or pIRS1/TRS1) could activate expression from the US3 promoter in a manner analogous to activation by infection.

The US3 regulatory region contains three consensus ATF binding sites (Fig. 1A), suggesting that ppUL82 would function to activate expression of US3, similar to ppUL82 activation of mIE gene expression. The effect of ppUL82 on US3 transcription was examined in transient expression experiments. Using DEAE dextran, a reporter gene plasmid (pBJ171) expressing the *lacZ* gene under the control of the US3 enhancer, promoter, and *tre* (Biegalke, 1995), was transfected into human diploid fibroblasts along with a control plasmid (pBJ201; Biegalke, 1997), which contains the mIE promoter and expresses no open reading frame, or a plasmid (pBJ203), which expresses the ppUL82 open reading frame under the control of the mIE promoter. Levels of β-galactosidase

activity were determined 40–48 h after transfection by measuring the fluorescence of the cleavage products of 4-methylumbelliferyl-β-D-galactoside (Biegalke, 1995).

ppUL82 is a very efficient transactivator of the mIE promoter as previously reported by Liu and Stinski (1992) and depicted in Fig 1B. Expression of ppUL82 activated expression of the mIE promoter–reporter gene construct (pEQ176; Biegalke and Geballe, 1990) 15-fold above the basal level of promoter activity (Fig. 1B). Surprisingly, ppUL82 had only a slight effect on the US3 promoter–reporter gene construct, increasing levels of β-galactosidase activity about twofold above basal levels (Figs. 1B and 1C). These data suggested that virion proteins other than ppUL82, such as pUL69 or pTRS1/pIRS1, contribute to the activation of US3 transcription seen after infection.

ppUL69 and pTRS1/IRS1 were assayed for an effect on US3 expression, both singly and in combinations including ppUL82. pUL69 and pTRS1 alone or together were unable to activate the US3 promoter (data not shown). No additional activation of the US3 promoter by ppUL82 was seen in the presence of pUL69 or pTRS1 (data not shown). Thus the identified transcriptional activators found in the virion particle have only a small effect on US3 expression, suggesting that other virion proteins are involved in activating US3 expression.

Activation and repression of US3 expression by IE proteins

The regulatory nature of IE proteins suggested that these proteins would contribute to US3 transcriptional control. Transient expression assays were utilized to examine the effects of the immediate early proteins (encoded by UL122–123, US3, UL36–38, and TRS1/IRS1) on US3 expression, either alone or in combinations as described below. IE1 and IE2 individually and synergistically activated expression from the US3 promoter as previously demonstrated (Figs. 2 and 3; Biegalke, 1997).

IRS1/TRS1 regulation of US3 expression. The effects of pTRS1, pIRS1, and pIRS1^{263} on US3 expression were assayed using transient expression experiments. DEAE-dextran was used to introduce the US3 promoter-*lacZ* reporter gene construct (pBJ171) into human diploid fibroblasts along with a control plasmid (pBJ201) or plasmids expressing pTRS1, pIRS1, and pIRS1^{263} (Romanowski and Shenk, 1997) and with or without the IE1 and IE2 expressing plasmid, pEQ276 (Biegalke and Geballe, 1991). Alone, the TRS1/IRS1 expression plasmids had no effect on US3 transcription (data not shown). However, pIRS1 and pTRS1 synergistically activated US3 expression in combination with IE1 and IE2 proteins (Fig. 2A); the synergistic effect seen with pIRS1/pTRS1 was dependent on the presence of both IE1 and IE2 (data not shown). Expression of pIRS1^{263} had only a minor effect on IE1 and IE2 activation of US3 expression (Fig. 2A). The synergistic effect of pTRS1/pIRS1 and IE1 and IE2 on US3 expression was similar to the effect seen on the IRS1^{263} and UL112 promoters (Romanowski and Shenk, 1997).

Potential autoregulation of US3 expression. The involvement of US3 proteins in activating *hsp70* expression suggested a potential autoregulatory role for the US3 proteins. The effect of US3 proteins on US3 expression was determined in transient expression assays. The US3-*lacZ* reporter plasmid, pBJ171, was introduced into cells with a control plasmid, a plasmid expressing US3 proteins under the control of the US3 promoter (pBJ174), a plasmid expressing IE1 and IE2 (pEQ276), or a combination of US3 and IE1 and IE2 expression plasmids (Fig. 2B). The expression of US3 proteins, either alone or in combination with IE1 and IE2 had no effect on pBJ171 expression (Fig. 2B).

The lack of autoregulation by US3 was confirmed in a transient expression assay, where levels of expression seen with the US3 expression plasmid, pBJ171, were compared after infection with HCMV strain Towne or HCMV strain RV670, which lacks IRS1-US11 but not US10 (Jones and Muzithras, 1992). The relative levels of expression were similar after infection with the wild-type and the mutant viruses (Fig. 2C). The similar β-galactosidase levels seen after infection with the two virus strains also confirmed the lack of a significant role for IRS1^{263} in regulating US3 expression.

Regulation of US3 expression by UL36–38. The potential involvement of UL36–38–encoded proteins in US3 transcriptional regulation was analyzed in transient expression assays similar to those described above. Plasmids expressing individual UL36–38 cDNAs (Tenney and Colberg-Poley, 1991) were transfected into cells along with the US3 promoter–reporter gene plasmid (pBJ171) and with or without pEQ276, the plasmid expressing IE1 and IE2. Levels of enzyme activity were determined as described above. Alone, the UL36–38 cDNAs had no effect on US3 transcription (data not shown). However, when analyzed in combination with IE1 and IE2, the protein encoded by the UL37ex1/UL38 cDNA (a cDNA consisting of the first exon of UL37 and the exon of UL38, Fig. 3A; Colberg-Poley *et al.,* 1992) was able to inhibit IE1 and IE2 activation of the US3 promoter (Figs. 3B and 3C). The repression was specific for the UL37ex1/UL38 open reading frame as a plasmid with a nonsense mutation in the open reading frame (p426; Colberg-Poley *et al.,* 1992) had no effect on US3-regulated expression in the presence of IE1 and IE2 (data not shown). The UL37 cDNA expression clone also had an inhibitory effect on IE1 and IE2 activation of US3 transcription (Fig. 3B), suggesting that transcriptional repression is mediated by the amino acids shared between these two cDNAs that are encoded by UL37 exon 1 (Tenney and Colberg-Poley, 1991). Neither the UL38 cDNA expression plasmid (Colberg-Poley *et al.,* 1992) nor the UL36 cDNA expression plas-

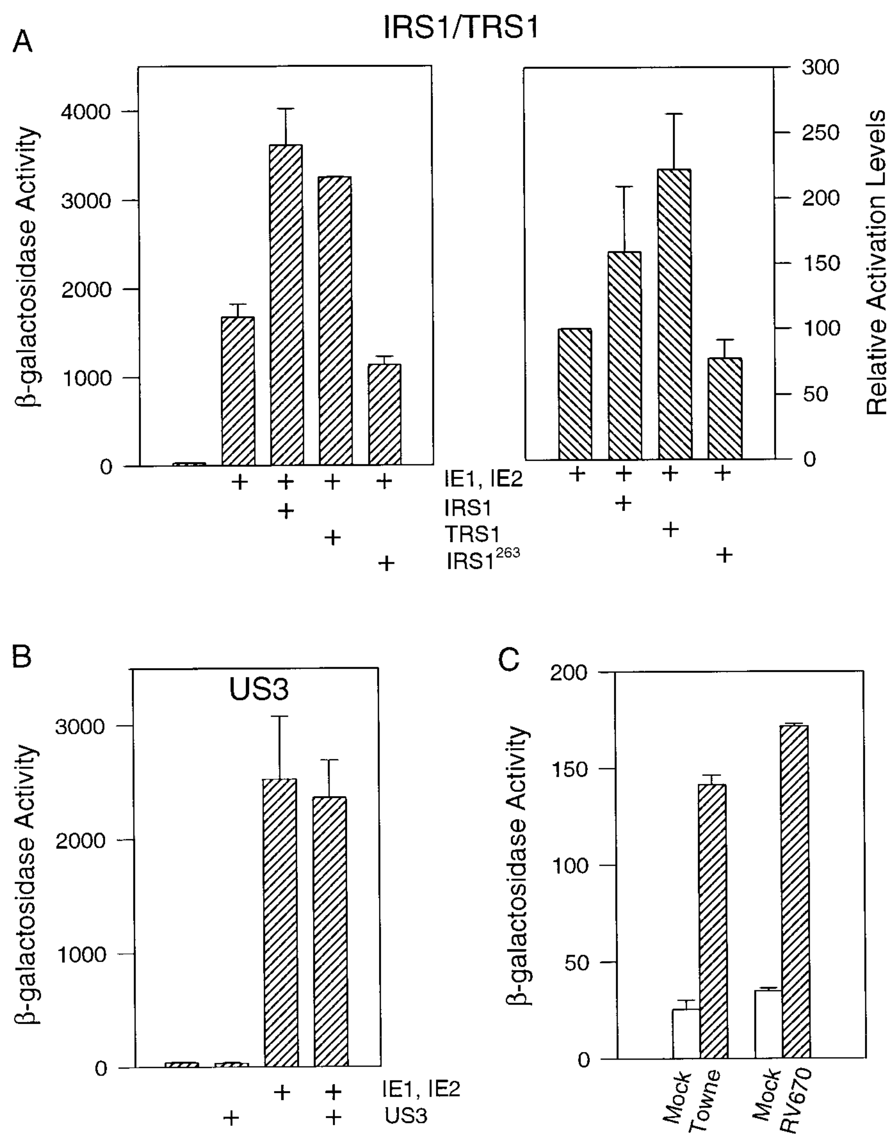

FIG. 2. Regulation of US3 expression by IRS1, TRS1, IRS1[263], and US3 proteins. (A) Analysis of the effect of IRS1, pTRS1, and pIRS1[263] on expression of the US3-reporter gene plasmid, pBJ171. pBJ171 was transfected into human diploid fibroblasts along with a control plasmid (pBJ201), a plasmid expressing the mIE proteins IE1 and IE2 (pEQ276), and plasmids expressing the IRS1/TRS1 proteins (Romanowski and Shenk, 1997). The influence of pTRS1, pIRS1 and pIRS1[263] on the US3 promoter in the presence of IE1 and IE2 is depicted as the mean of the absolute values from two experiments (plus one standard deviation) and, as the average relative activation levels (mean plus one standard deviation from eight experiments). Activation of US3 expression by IE1 and IE2 was set as a 100-fold increase in β-galactosidase activity; levels of expression obtained with the addition of plasmids expressing pTRS1, pIRS1, or pIRS1[263] were normalized to the values obtained in the presence of IE1 and IE2. (B) Analysis of the effect of US3 proteins on US3 expression. The effect of US3 proteins was assayed in the presence or absence of IE1 and IE2 proteins. The plasmid expressing US3 proteins (pBJ174) contains the US3 gene isolated from the HCMV (strain Towne) genome. (C) Comparison of the effects of HCMV strain Towne and HCMV RV670 (Jones and Muzithras, 1992) on expression of the US3 reporter gene construct, pBJ171. Cells were infected 48 h posttransfection at a m.o.i. of 10 plaque forming units per cell. One hour later, the virus was removed and cells were fed with complete DMEM. Mock, mock-infected cells. Levels of β-galactosidase activity were measured ~2 days after transfection or 16 h p.i.; enzyme levels were determined as described in the legend to Fig. 1. The β-galactosidase activities are the means of two experiments (plus one standard deviation). Levels of enzyme activity obtained with a promoterless *lacZ*-containing plasmid (pEQ3) were subtracted from the test values. The amount of DNA was kept constant in all tranfections by including control vector DNA. +, the presence of the indicated protein.

mid (p326) had an effect on IE1 and IE2 activation (Fig. 3B, data are not shown for UL36).

The effect of pUL37ex1/UL38 on IE1- and IE2-mediated activation could result from inhibition of the activity of either IE1 or IE2 or both. Transient expression assays were performed to distinguish between these possibilities. The US3 promoter–reporter gene plasmid (pBJ171) was introduced into human diploid fibroblasts along with a control plasmid or plasmids expressing IE1 and IE2 (pEQ276), IE1 alone (pEQ273; Biegalke and Geballe, 1991) or IE2 alone (pEQ326) individually or in combination with the plasmid expressing the UL37ex1/UL38

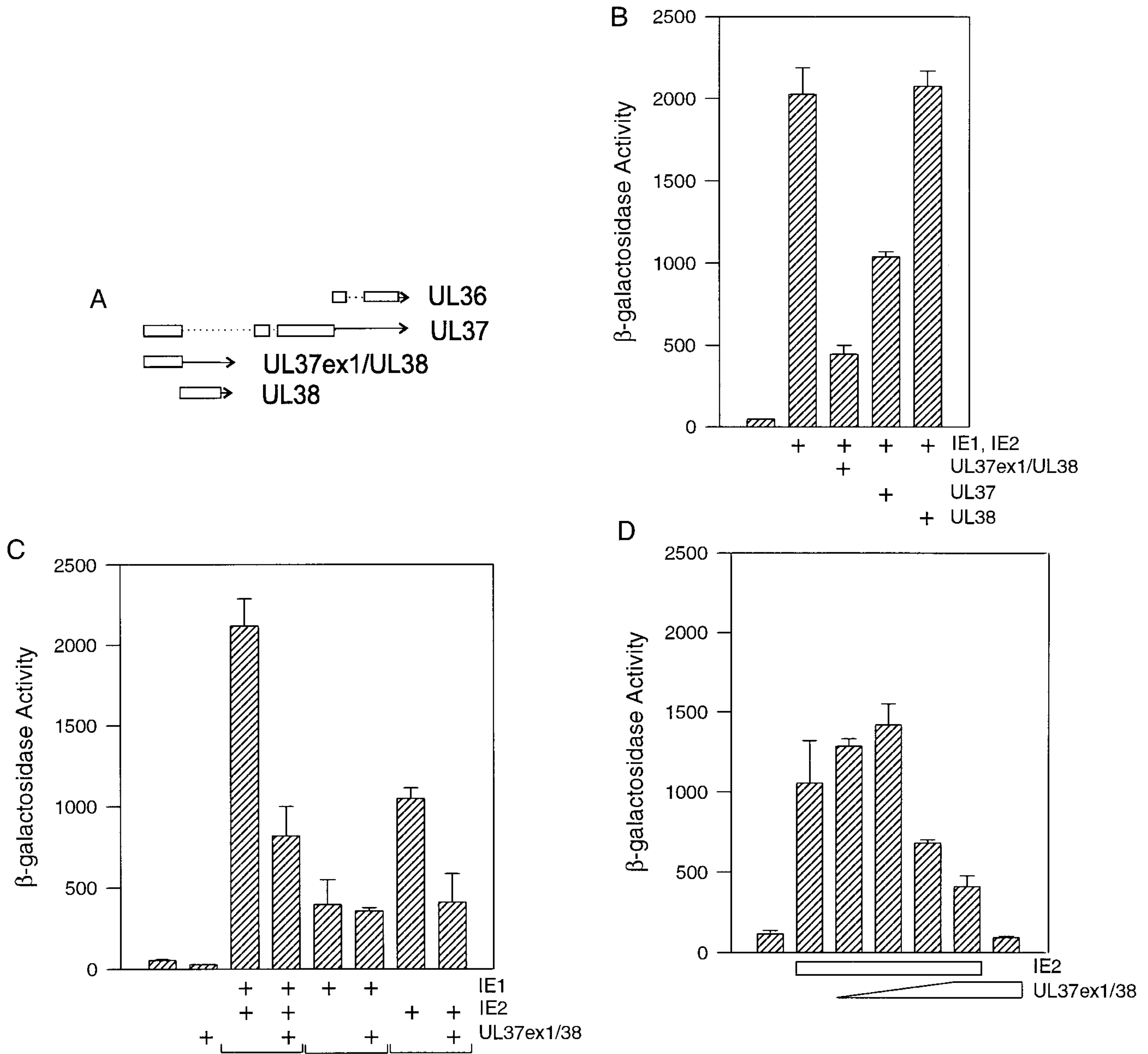

FIG. 3. Regulation of US3 expression by UL36–38. (A) Diagram of UL36–38 transcripts (orientation is reversed relative to the HCMV genome); open rectangles, predicted protein coding regions; dotted line, RNA spliced out of the mature transcript; solid line, noncoding RNA sequences (Tenney and Colberg-Poley, 1991). (B) Analysis of the effect of UL36–38–encoded proteins on US3 expression. Plasmids expressing UL36–38 cDNAs (Colberg-Poley *et al.,* 1992) were transfected into human diploid fibroblasts along with pEQ276 (the plasmid expressing IE1 and IE2) and the US3-*lacZ* reporter gene plasmid, pBJ171. (C) pUL37ex1/UL38 inhibits IE2-mediated activation of US3 transcription. The US3-lacZ reporter gene plasmid (pBJ171) was transfected into human diploid fibroblasts along with a control plasmid (pBJ201), a plasmid expressing IE1 and IE2 (pEQ276), a plasmid expressing IE1 (pEQ273), or a plasmid expressing IE2 (pEQ326) and plasmid expressing pUL37ex1/UL38 (p327; Tenney and Colberg-Poley, 1991). (D) A minimum threshold amount of pUL37ex1/UL38 is required to repress IE2 activation of US3 expression. The US3-lacZ reporter gene plasmid (pBJ171) was transfected into human diploid fibroblasts in the presence of a constant amount of IE2 (8 μg, pEQ326) and a variable amount of UL37ex1/UL38 (the amounts of the UL37ex1/UL38 expression plasmid were 0, 2, 4, 6, and 8 μg). β-galactosidase activities (MUG fluorescence units) were measured ~2 days after transfections; enzyme levels were determined as described in the legend to Fig. 1. The β-galactosidase levels are the means of two experiments (plus one standard deviation). Levels of enzyme activity obtained with a promoterless *lacZ*-containing plasmid (pEQ3) were subtracted from the test valaues. The amount of DNA was kept constant in all transfection by including control plasmid DNA. +, the presence of the indicated protein.

cDNA. Levels of β-galactosidase activity were determined as described above. As shown in Fig. 3B and 3C, pUL37ex1/UL38 repressed IE1 and IE2 activation of US3 transcription. When assayed for an effect on activation by the individual IE1 and IE2 proteins, pUL37ex1/UL38 inhibited IE2 activation of US3 transcription but had no

effect on IE1 activation (Fig. 3C). Thus the IE gene product encoded by UL37ex1/UL38 inhibits activation of the US3 promoter by IE2. A dose-response analysis demonstrated that UL37ex1/UL38 inhibited IE2 activation at a ratio of 3:4 (Fig. 3D); lower amounts of UL37ex1/UL38 had no effect on activation by IE2. This suggests that there is a requirement for a minimal amount of UL37ex1/UL38 for inhibition of the effect of IE2, with the degree of repression corresponding to an increase in the concentration of UL37ex1/UL38 above the minimum threshold level (Fig. 3D).

Interactions between IE regulators. The immediate early proteins that influence US3 expression, either positively or negatively, are synthesized at similar times during infection. Combinations of IE regulatory proteins were assayed for effects on US3 expression to determine whether pUL37ex1/UL38 or pTRS1/pIRS1 had a dominant effect on activation mediated by IE1 and IE2. Transient expression experiments were used to analyze the effects of combinations of the different regulatory-protein expressing plasmids by transfecting the plasmids expressing the regulatory proteins and the US3-lacZ reporter plasmid into human diploid fibroblasts. The β-galactosidase levels were measured as described above (Fig. 4). Expression of pUL37ex1/UL38 partially suppressed the synergistic effect of pTRS1 on IE1 and IE2 activation (Fig. 4A).

Regulation of US3 expression by early genes

In addition to the regulatory roles of virion and IE proteins, regulatory roles have also been ascribed to the early genes, UL84 and UL112–113. The potential roles of UL84 and UL112–113 in regulating US3 expression were assayed in transient expression experiments, analyzing the ability of the early proteins to regulate expression of the US3 promoter-*lacZ* reporter plasmid, pBJ171. The effects of the early proteins were measured in the presence or absence of IE proteins. Expression of UL112–113 (using the expression vector ΔPSV; Staprans and Spector, 1986) had no effect on US3 expression either alone or in the presence of the IE regulatory proteins, IE1, IE2, pTRS1/pIRS1, or pUL37ex1/UL38 (data not shown).

The effect of pUL84 on US3 expression was determined through transient expression assays as described above, assaying the effect of pUL84 alone on the US3 promoter or in combination with the IE gene products shown above to be involved in US3 regulation (Fig. 4). Expression of UL84 alone had a slight repressive effect on US3 transcription (data not shown). When expressed in combination with IE1 and IE2, pUL84 repressed activation of US3 twofold (Fig. 4A). Additionally, pUL84 also repressed the synergistic activation that occurs in the presence of IE1, IE2 and TRS1 (more than sixfold reduction in expression, Fig. 4). Thus the repressive effect of pUL84 was dominant over the transactivating proteins encoded by IE genes. The repression of US3 expression by UL84 was not dependent on a functional *tre* (data not shown). A construct expressing antisense UL84 RNA (pBJ297) had no significant effect, demonstrating that repression was specifically due to pUL84 (data not shown). A dose-response analysis demonstrated that a small amount of UL84 (ratio of UL84:TRS1:IE1,IE2 of 1:5:5) was sufficient to interfere with US3 activation by TRS1 and IE1 and IE2 (Fig. 4B).

DISCUSSION

The experiments presented above illustrate the complexity of the protein network that contributes to the regulation of US3 expression. These data are summarized in Fig. 5, where the effect of the virally encoded *trans*-acting factors on US3 expression is diagrammed. Upon infection, a number of virion proteins, including ppUL82 and pIRS1/TRS1, are postulated to enter the cell and, in the case of ppUL82, markedly activate expression of the mIE gene while activating US3 expression only modestly. The low level of US3 activation by ppUL82 suggests that an additional as yet unidentified virion protein(s) is involved either directly or indirectly in activating US3 expression.

Activation of the mIE gene (UL122–123) results in the synthesis of the abundant IE1 and IE2 proteins, which interact with pIRS1/pTRS1 to synergistically activate the US3 promoter and other IE and early promoters (Fig. 2A; Romanowski and Shenk, 1997; Romanowski *et al.,* 1997). Transcription of the TRS1/IRS1 and UL36–38 genes results in the synthesis of additional IE proteins, with pUL37ex1/UL38 inhibiting the synergistic activation of the immediate early US3 promoter mediated through IE1, IE2, and pTRS1/IRS1. The effect of pUL37ex1/UL38 on the immediate early US3 promoter is concentration dependent and contrasts to its activation of early gene promoters (Colberg-Poley *et al.,* 1998). This suggests that the increase in expression of pUL37ex1/UL38 during early times of infection (4–24 h p.i.; Tenney and Colberg-Poley, 1991) may play a critical role in switching viral gene expression from immediate early to early genes.

Expression of IE proteins activates the cascade of early and then late gene expression. Modest levels of the early gene, UL84, inhibited US3 expression in the presence of the transactivators (Fig. 4B) and suggest that during infection, pUL84 plays a dominant role in repressing US3 expression. UL84 also inhibits activation of expression of the UL112 promoter and augments the autoregulatory repression of the mIE promoter (Gebert *et al.,* 1997), suggesting that UL84 may be a key regulatory protein in the viral life cycle.

Analysis of the *cis*-acting US3 elements implicates additional regulatory proteins, as yet unidentified, in the regulation of US3 gene expression. In particular, proteins

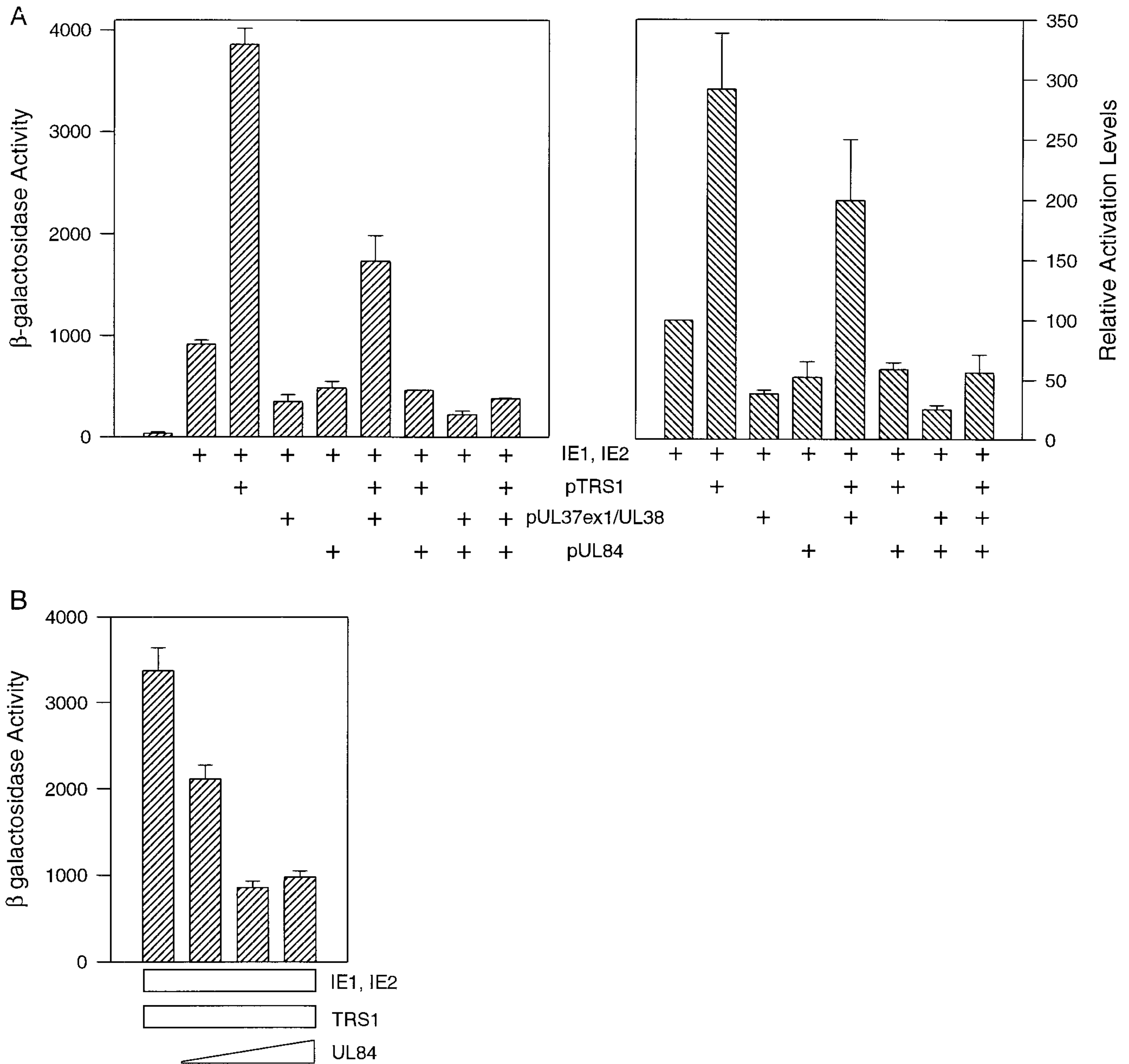

FIG. 4. Dominance of pUL84 over pTRS1 and IE1 and IE2 synergistic activation of the US3 promoter. (A) Plasmids expressing the indicated regulatory proteins were transfected into human diploid fibroblasts along with the US3-*lacZ* reporter gene plasmid (pBJ171). The data is shown as the mean of two experiments (plus one standard deviation) and, as the mean relative activation levels (mean of 5 experiments plus one standard deviation) with β-galactosidase activities normalized to the level of expression seen in the presence of IE1 and IE2 (which was set at 100). (B) A dose response analysis of the effect of UL84. One, 3, or 5 μg of the UL84 expression plasmid (pBJ296) was transfected into cells along with a constant amount of pEQ276 (5 μg, IE1 and IE2) and a constant amount of pCEP-TRS1-his (5 μg) and either pBJ171 or the control plasmid pEQ3. A constant amount of DNA was used in each transfection by varying the amount of control DNA added. Enzyme levels were measured as described in the legend to Fig. 1.

that mediate transcriptional repression via the *tre* have yet to be identified.

The regulatory network described above controls viral gene expression generally rather than US3 expression specifically. Indeed, the synergistic activation by pTRS1/pIRS1, IE1, and IE2 has been shown for a number of promoters as has the repressive effect of UL84 (Gebert *et al.,* 1997; Romanowski and Shenk, 1997; Romanowski *et al.,* 1997). The discovery of a repressive role for the IE protein, pUL37ex1/UL38, adds additional complexity to the interactions between regulatory proteins. pUL37ex1/UL38 acts as an activator of early gene expression and activates expression of an early gene promoter 30% in the presence of IE1 and IE2 (Colberg-Poley *et al.,* 1998). In contrast, pUL37ex1/UL38, in the presence of IE1 and IE2, represses US3 expression ~60% compared with

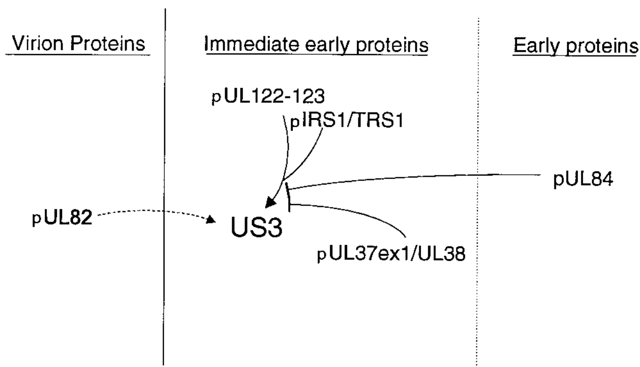

FIG. 5. Diagram of the cascade of viral proteins that modulates levels of US3 expression. The virion protein, pUL82, functions as a weak transcriptional activator of US3 and contributes to the activation of UL122–123 (Liu and Stinski, 1992). Transcription of immediate early genes leads to the synthesis of IE1, IE2 (pUL122–123), and pTRS1, which cooperate to activate US3 transcription to high levels. This effect is modulated downward by the expression of UL37ex1/UL38. UL37ex1/UL38 may play a critical role in viral infection, acting as a switch in contributing to IE gene repression and early gene activation. US3 expression is further decreased in response to expression of the early protein, pUL84. Filled arrowheads, activation; no arrowhead, repression; dashed line, weak activation.

levels of expression seen in the absence of pUL37ex1/UL38 (Figs. 3 and 4A). Thus pUL37ex1/UL38 can act as a repressor or as an activator; the differential effect of pUL37ex1/UL38 is predicted to be determined by the concentration of pUL37ex1/UL38 and influenced by the promoter region.

Viral infection establishes a progressively changing environment inside the cell where transcriptional activators and repressors create a complex regulatory network that influences viral gene expression. In this report, I describe these effects on US3 expression. During infection, the relative levels of activators and repressors are predicted to determine whether activation or repression of US3 or other viral genes occurs. The relative effects of the virion, IE, and early gene regulatory proteins may also vary with the cell type and influence the course of viral infection as a result of interactions with cellular proteins as well as other viral proteins. For example, repressors of gene expression may play a role in the establishment of latency, whereas activators may play a role in productive viral replication in a manner similar to lambda phage, where relative levels of regulatory proteins determine the outcome of infection, lysogeny or lytic replication. Analysis of the levels of specific viral gene expression in additional cell types, in particular monocytes, which are a source of latent virus (Söderberg-Nauclér *et al.,* 1997), may clarify these issues.

MATERIALS AND METHODS

Virus and cells

Primary human fibroblast cultures were established from skin tissue samples obtained from O'Bleness Memorial Hospital, Athens, OH. Cells were propagated in Dulbecco's minimal essential medium (DMEM) supplemented with penicillin, streptomycin, glutamine, and 10% NuSerum (complete DMEM; Collaborative Research Products, Bedford, MA). HCMV (strain Towne) and RV670 (obtained from T. Jones, Wyeth-Ayerst) were propagated in primary fibroblasts.

Plasmids

Plasmid DNA was prepared by alkaline lysis and purified by double banding on cesium chloride gradients (Maniatis *et al.,* 1982). pEQ plasmids were provided by A. Geballe (Biegalke and Geballe, 1991); pEQ3 is a control plasmid that contains the *lacZ* gene and no eukaryotic regulatory elements; pEQ276 contains the mIE gene and expresses IE1 and IE2; pEQ273 contains the mIE promoter and IE1; and pEQ326 contains the mIE promoter and the IE2 region. pCEP4-IRS1-His, pCEP4-IRS1^{263-}HIS, and pCEP4-TRS1/HIS were provided by T. Shenk and express the IRS1, IRS1^{263} or TRS1 open reading frames under the control of the mIE promoter (Romanowski and Shenk, 1997). The UL36–38 expression plasmids were provided by A. Colberg-Poley and express UL36 (p326), UL37ex1/UL38 (p327), UL37 (p414), and UL38 (p406) under the control of the mIE promoter (Colberg-Poley *et al.,* 1992). p426 is similar to p327 but contains the US3 open reading frame disrupted by stop codons in all open reading frames and was provided by A. Colberg-Poley. The UL112–113 expression plasmid, ΔPSV, was provided by D. Spector and expresses the genomic region of UL112–113 under the control of the mIE promoter (Wright and Spector, 1989).

pBJ171 expresses the *lacZ* gene under the control of the US3 enhancer, promoter, and sequences to +27 relative to the transcription start site (Biegalke, 1995). pBJ201 contains the mIE enhancer/promoter (Biegalke, 1997).

The UL82 expression plasmid, pBJ203, was constructed by combining a *Fsp*I/*Hin*dIII fragment and a *Hin*dIII/*Xho*I fragment (isolated from pCM3; Fleckenstein *et al.,* 1982) to generate the open reading frame of UL82; the resulting DNA fragment was inserted into pBJ201 so that the UL82 open reading frame is expressed under the control of the mIE promoter. The UL69 expression plasmid, pBJ313, was constructed by amplifying the UL69 open reading frame with *Pfu*I DNA polymerase (Stratagene) using oligonucleotides 43 (5′ CACAAAGCTTCGAATTCACAACACCCTTCACTCTC 3′)and 60 (5′ TATTAAGCTTCCCATGGAGCTGACTCAC 3′) and HCMV (strain AD169) DNA as a template; the resulting DNA fragment was digested with *Hin*dIII and inserted into the *Hin*dIII site of pBJ201. The plasmid expressing the US3 proteins, pBJ174, was constructed by inserting a *Eco*RV/*Nco*I fragment of the Towne genome (isolated from pRL105, provided by G. Hayward) into pUC19; the *Eco*RV/*Nco*I frag-

ment contains sequences from 5′ of the US3 enhancer to 3′ of the polyadenylation site (Weston, 1988). The UL84 expression plasmid, pBJ296, and the plasmid expressing antisense UL84, pBJ297, were constructed by amplifying the UL84 open reading frame with oligonucleotides 101 (5′ CCATGTCTAGAGCAGACACCAAGCATGCCAC 3′) and 102 (5′ CCTACTCTAGACGCCTAGTGTCCGTTTCC 3′), using Vent DNA polymerase (New England Biolabs) and HCMV (strain Towne) DNA as the template. The resulting DNA fragment was digested with *Xba*I and inserted into the *Xba*I restriction enzyme site in pBJ201.

Transfections

Human fibroblasts were transfected with plasmid DNA by using DEAE-dextran as described previously (Biegalke, 1995). Cells were washed with DMEM containing 25 mM Tris, pH 7, and then a mixture of DMEM with Tris, plasmid DNA, and DEAE dextran (400 μg/ml) was added to the cells. Four hours later, the DNA-containing mixture was removed and the cells were washed with DMEM containing Tris and media changed to complete DMEM. Forty to 48 h after transfection, DMEM containing 0.44 mM 4-methylumbelliferyl-β-D-galactoside (MUG, Sigma Chemical Co.) was added to the cells; the fluorescence of the cell culture media was measured 1–4 h later using a Dynatech fluorometer. Transfections were repeated multiple times; the relative values obtained from the transfections were consistent although there were quantitative differences in fluorescent readings obtained from the different transfections.

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