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Four of Eleven Loci Required for Transient Complementation of Human Cytomegalovirus DNA Replication Cooperate To Activate Expression of Replication Genes

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As previously shown, 11 loci are required to complement human cytomegalovirus (HCMV) DNA replication in a transient-transfection assay (G. S. Pari and D. G. Anders, J. Virol. 67:6979–6988, 1993). Six of these loci encode known or candidate replication fork proteins, as judged by sequence and biochemical similarities to herpes simplex virus homologs of known function; three encode known immediate early regulatory proteins (UL36-38, IRS1/TRS1, and the major immediate early region spanning UL122-123); and two encode early, nucleus-localized proteins of unknown functions (UL84 and UL112-113). We speculated that proteins of the latter five loci might cooperate to promote and regulate expression of the six replication fork proteins. To test this hypothesis we made luciferase reporter plasmids for each of the replication fork gene promoters and measured their activation by the candidate effectors, expressed under the control of their respective native promoters, using a transient-cooperativity assay in which the candidate effectors were subtracted individually from a transfection mixture containing all five loci. The combination of UL36-38, UL112-113, IRS1, or TRS1 and the major immediate early region produced as much as 100-fold-higher expression than the major immediate early region alone; omitting any one of these four loci from complementing mixtures produced a significant reduction in expression. In contrast, omitting UL84 had insignificant (less than twofold), promoter-dependent effects on reporter activity, and these data do not implicate UL84 in regulating HCMV early-gene expression. Most of the effector interactions showed significant positive cooperativity, producing synergistic enhancement of expression. Similar responses to these effectors were observed for the each of the promoters controlling expression of replication fork proteins. However, subtracting UL112-113 had little if any effect on expression by the UL112-113 promoter or by the simian virus 40 promoter-enhancer under the same conditions. Several lines of evidence argue that the cooperative interactions observed in our transient-transfection assays are important to viral replication in permissive cells. Therefore, the data suggest a model in which coordinate expression of multiple essential replication proteins during permissive infection is vitally dependent upon the cooperative regulatory interactions of proteins encoded by multiple loci and thus have broad implications for our understanding of HCMV biology.

Human cytomegalovirus (HCMV) is a common opportunistic pathogen associated with significant morbidity in immunocompromised individuals and neonates (42). Currently available antiviral drugs target the lytic-phase replication machinery, but in spite of their biomedical importance and fundamental interest, the mechanisms of HCMV DNA synthesis and its regulation are not completely understood. It is known that viral DNA synthesis utilizes both a *cis*-acting replicator, *ori*Lyt (2, 4, 20, 41), and a set of *trans*-acting viral proteins. Transient complementation of HCMV *ori*Lyt-mediated DNA replication requires proteins encoded within 11 distinct loci (44, 45). In HCMV-infected cells, blocking expression of individual proteins expressed by members of this set of loci also blocks viral DNA replication (49, 54), implying that the transient-transfection assay accurately reflects requirements during infection. Six of the defined loci encode herpesvirus group-common proteins whose roles in DNA synthesis are predicted by their homology to essential herpes simplex virus type 1 (HSV-1) replication functions (6, 8, 68) and by basic biochemical studies (1, 3, 15, 18, 24, 29, 69). The six proteins encoded include the DNA polymerase (pUL54) and a candidate polymerase accessary protein (pUL44), the single-stranded DNA-binding protein (pUL57), and three proteins (pUL70, pUL102, and pUL105) homologous to HSV-1 helicase-primase subunits. These six proteins probably act coordinately at the replication fork to carry out the reactions necessary for DNA replication.

The finding that five additional loci were needed to complement HCMV DNA replication was surprising because homologous proteins were not required to complement HSV-1 or Epstein-Barr virus DNA synthesis (16, 17, 43). Moreover, none of these five showed evidence of similarity to the seventh essential HSV-1 replication component, the origin-binding protein UL9 (8, 13, 14). Instead, these loci, spanning UL36-38, UL84, UL112-113, the major immediate early region (MIE) UL122-123, and IRS1/TRS1, encode proteins that are without obvious homologs in alpha- and gammaherpesviruses, and their roles in complementing DNA replication are not known. UL36-38, MIE, and IRS1/TRS1 proteins are expressed under immediate early conditions (60, 63) and have been shown to modulate expression of cellular and/or viral gene targets (10–12, 48, 60, 62). In contrast, the UL84 and UL112-113 loci both

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TABLE 1. Plasmids used in this study

Plasmid	Site/vector	Coordinates	Locus	Reference
Reporter plasmids				
pAI1	*Bgl*II/pGL2-Basic	56511–56702	UL44 promoter	This paper
pAI3	*Bgl*II/pGL2-Basic	103374–103831	UL70 promoter	This paper
pAI5	*Bgl*II/pGL2-Basic	146385–146522	UL102 promoter	This paper
pAI7	*Bgl*II/pGL2-Basic	151329–151649	UL105 promoter	This paper
pAI13	*Bgl*II/pGL2-Basic	80996–81441	UL54 promoter	This paper
pLHB2	*Nhe*I-*Bgl*II/pGL2-Basic	90280–90509	UL57 promoter	This paper
pAI15	*Bgl*II/pGL2-Basic	160154–160568	UL112-113 promoter	This paper
Effector plasmids				
pZP8	*Kpn*I/pGEM7Zf(−)	42045–53104	UL36-38	45
pAI12	Same as for pZP8	pZP8Δ49168–53104	ΔUL36-38	This paper
pZP13	*Aat*II-*Fsp*I/pGEM7Zf(−)	120502–123363	UL84	44
pZP13FS	Same as for pZP13	Frameshift at 122665	UL84	44
pZP18	*Sph*I-*Sac*I/pGEM7Zf(−)	146036–151125	UL102	44
pZP3	*Sma*I/pGEM7Zf(−)	189500–193108	IRS1	45
pZP24	*Sph*I-*Sac*I/pGEM7Zf(−)	Δ*Msc*I 163028–163950	UL112-113	44
pZP24EcoRIFS	Same as for pZP24	Frameshift at 162053	UL112-113	This paper
pZP24SalIFS	Same as for pZP24	Frameshift at 161831	UL112-113	This paper
pZP24PstIFS	Same as for pZP24	Frameshift at 160860	UL112-113	This paper
pSVH			UL122-123	62
pON2334	*Xho*I-*Eco*RI/Bluescript SK(−)		TRS1	60
pON2336	Same as for pON2334	Frameshift at *Not*I	TRS1	60
pXEXX-6.1	*Sal*I/pT7-1	222722–228794	TRS1	28

express early-temporal-class, nucleus-localized proteins with unknown functions (22, 67). pUL84 recently was shown to complex with the IE2 86-kDa protein (50, 57), but the significance of this association remains unknown. Studies of the UL112-113 locus have characterized the transcripts, promoter, and encoded proteins and their expression but have not elucidated its function (27, 58, 59, 66, 67). We previously speculated that proteins encoded by each of these five loci may regulate expression of replication proteins (44). In the experiments described here, we have directly tested their abilities to regulate promoters for each of the replication fork protein genes. Results indicate that UL36-38, UL112-113, IRS1/TRS1, and the MIE proteins cooperatively promote expression of the replication genes. In contrast, UL84 did not appear to participate in regulating HCMV early-gene expression under these conditions.

MATERIALS AND METHODS

Cells and virus. All experiments were done with low-passage-number (<15) human foreskin fibroblast cells and HCMV strain AD169 (ATCC VR-538). Cell and virus culture were as previously described (44). Nucleotide sequence coordinates are taken from the published data for the HCMV strain AD169 genome (8), accession number X17403.

Plasmids. Plasmids used in this study that are not commercially available are detailed in Table 1. pGL2-Basic and pGL2-Control vectors were purchased from Promega Corp. (Madison, Wis.). Plasmids expressing the candidate effector proteins encoded within UL36-38, UL84, UL112-113, IRS1, TRS1, and MIE, or corresponding mutated versions, under the control of their respective native promoters were as described elsewhere (44, 45, 60, 62). Luciferase reporter plasmids were made by isolating the known or predicted promoter regions of the UL44, UL54, UL57, UL70, UL102, UL105, and UL112-113 loci by PCR and ligating them into the *Bgl*II site of the vector pGL2-Basic. Synthetic oligonucleotides used for PCR primers had *Bam*HI or *Bgl*II sites within a 10-nucleotide extension at their 5′ ends to facilitate cloning (Table 2); correct-sized PCR products were treated with *Bam*HI plus *Bgl*II and gel purified prior to ligations. pLHB2 was an exception in that the PCR fragment was cleaved to produce *Nhe*I and *Bgl*II ends and inserted into similarly treated pGL2-Basic. The DNA sequences of all cloned promoter fragments were verified.

Transfections. Most experiments were done by using the Chen and Okayama calcium phosphate transfection protocol to introduce plasmid DNAs into human fibroblast (HF) cells (9), just as was done for transient complementation of DNA replication (44, 45) but substituting luciferase reporter plasmids in place of an *ori*Lyt-containing plasmid. For the calcium phosphate transfections, 10^5 HF cells were plated on 6-cm-diameter dishes 24 h prior to DNA addition, and subsequent steps were as described elsewhere (45). Transfection mixtures contained a total of 5 μg of DNA per dish (2.5 μg of the indicated luciferase reporter plasmid plus 0.5 μg of each test effector plasmid), as specified in the figure legends. Many later transfections, noted in the figure legends, were done with the lipofectamine reagent (catalog no. 18324-012; Gibco BRL, Gaithersburg, Md.) by following the vendor's recommended protocol (21). For those experiments 3×10^5 HF cells per well were plated on six-well plates. Each well received a total of 1 μg of DNA (0.5 μg of the luciferase reporter plasmid plus 0.1 μg of each test effector plasmid), as detailed in the figure legends. For both calcium phosphate and lipofectamine transfection experiments, the transfected cells were harvested for the luciferase assay at 96 h after transfection.

Luciferase assay. Luciferase activity was measured by using the Promega luciferase assay system (Promega Corp.) per the vendor's recommendations and a liquid scintillation counter (Wallac model 1409) with coincidence counting turned off. A standard curve was constructed by using purified firefly luciferase (Boehringer-Mannheim, Indianapolis, Ind.) to ensure that output was measured in the linear range.

Statistical analysis. The effects of incorporating or omitting individual loci, and the magnitude of cooperativity, were assessed by using classical analysis-of-variance techniques (53). Outliers were identified by a robust regression method (46) and eliminated prior to the analysis. In the analysis of variance, separate parameters are estimated for the magnitude of each effector and combined effector responses. Predictions for each experimental outcome are then available as sums of the appropriate parameter estimates. Because the activity of the luciferase reporter was log-normally distributed, all analyses were performed on the log scale. Predictions were therefore obtained by taking antilogs.

RESULTS

UL36-38, UL112-113, IRS1, and MIE proteins cooperate to activate expression of UL44. To determine whether all or several of the loci spanning the UL36-38, UL84, UL112-113, IRS1/TRS1, and MIE open reading frames (ORFs) encode proteins that cooperate to activate expression of replication genes, we cotransfected a set of five plasmids expressing the candidate effector proteins under the control of their respective native promoters, together with a replication gene promoter plasmid. We first tested the ability of these candidate effectors to cooperate in activating expression from the UL44 promoter-luciferase plasmid (pAI1) because this promoter has been characterized previously (36, 60) and because a segment almost identical to that previously characterized is sufficient for

TABLE 2. Synthetic oligonucleotides for PCR

Oligonucleotide	Sequence	Coordinates
UL44Pro1	5′-ATCGAGATCTATCCCGGACAGCGTGCAAGTC-3′	56511–56531
UL44Pro2	5′-ATCGGGATCCGGTACCACTGGCGCTTTAAGG-3′	56702–56682
UL70Pro1	5′-ATCGAGATCTCGTCGCGCCGGCACGATGCA-3′	103374–103393
UL70Pro2	5′-ATCGGGATCCGAGGCCTGATCCGTGGAGAA-3′	103831–103812
UL102Pro1	5′-ATCGGGATCCGGCGCGCCGATGACGACAGG-3′	146383–146402
UL102Pro2	5′-ATCGAGATCTCCTCGGACAGAAGAGAGTCG-3′	146521–146502
UL105Pro1	5′-ATCGAGATCTGCAAACGGGCGTGCTGGGCG-3′	151649–151630
UL105Pro2	5′-ATCGGGATCCAGCCGCTCCGAGCGCACCTTC-3′	151329–151349
UL112Pro1	5′-CACAGGATCCGCTGTCCGCACAGAGGTAACAAC-3′	160155–160177
UL112Pro2	5′-CACAAGATCTAGCGCAACGCTCGTCGGAG-3′	160567–160549
UL54Pro1	5′-GTCAAGATCTATGCATCAGACGACGGTGGTC-3′	80996–81016
UL54Pro2	5′-TGACGGATCCGAATTCAACTCGTACAAGCA-3′	81440–81421
UL57lili54	5′-AGTCAGATCTATGGTGGATGAACGG-3′	90280–90294
UL57lili55	5′-ATTAGGATCCGCTAGCGGTCTGGCTG-3′	90509–90494

expression of UL44 in transient complementation of DNA replication (45). The tested UL44 promoter spanned nucleotides 56511 to 56702 of the published sequence. Salient results are presented in Fig. 1A and can be summarized as follows. When all of the five candidate effector plasmids were cotransfected with pAI1, luciferase expression was amplified as much as 100-fold in comparison with expression with no effectors (Fig. 1A). In later experiments that used lipofectamine instead of calcium phosphate transfection procedures, the amplification relative to expression with no effectors was even greater than 100-fold. To determine which of these loci encode proteins that participate in activating the UL44 promoter, the candidate effectors were individually subtracted from the transfection mixture. Omitting either IRS1-, UL36-38-, UL112-113-, or MIE-expressing plasmids from these cotransfections reproducibly reduced UL44 promoter expression between 2- and 10-fold in comparison with the transfections using all effectors (MIE reduced expression most dramatically, and IRS1 reduced it the least). In contrast, omitting UL84 from the transfection mixture always resulted in slightly increased luciferase expression. Although there was considerable variability in absolute levels of luciferase expression between experiments, the noted trends, when expressed as the percent of activation with all effectors, were clear and reproducible (Fig. 1A). These results argue that proteins encoded by MIE, UL36-38, IRS1, and UL112-113 each participate in activating expression from the UL44 promoter.

Results consistent with the findings above were obtained when individual effector plasmids were cointroduced into HF cells with pAI1 or with only MIE (Fig. 1B). In these experiments IRS1-, UL36-38-, and MIE-expressing plasmids, transfected alone in the amount used in the subtraction experiments, activated the UL44 promoter slightly (two- to fivefold), whereas UL84- and UL112-113-expressing plasmids transfected alone had minimal effects or even appeared to inhibit expression slightly. As previously shown by Stasiak and Mocarski for TRS1 (60), IRS1 cooperated with MIE to synergistically activate expression of the UL44 promoter. The combination of UL36-38 and MIE also cooperatively activated the UL44 promoter. In contrast, UL112-113 failed to significantly augment MIE induction, suggesting that UL112-113 induction of the UL44 promoter requires cooperation with an additional activator(s); alternatively, adequate expression of the delayed-early UL112-113 locus, which is known to be activated by MIE (58), may require additional activating proteins under these conditions (see Fig. 3F). Not surprisingly, given the result shown in Fig. 1A, the UL84-expressing plasmid also failed to cooperate with MIE to further activate expression of the UL44 promoter.

As an additional test, we repeated these experiments, substituting frameshifted or mutated versions of each respective effector plasmid in place of the wild-type (Fig. 1C). Because in our DNA replication experiments we used a TRS1 frameshift instead of an IRS1 mutant, we tested two different plasmids expressing wild-type TRS1 for direct comparison of TRS1- and IRS1-containing transfection cocktails. We also made frameshift mutations in UL84, UL112, and UL113. The UL36-38 locus was supplied in these experiments by pZP8, which spans ORFs UL33 to UL38. To confirm the role of the UL36-38 locus we used a pZP8-derived plasmid with a deletion which eliminated most of the UL36-38 region coding capacity and the predicted promoter regions but left intact the UL33-UL35 ORFs and associated promoters and polyadenylation signals (pAI12) (Table 1). Our results show that, in every case, the frameshifted or mutated effectors failed to activate expression; substituting the individual mutant plasmids for the wild-type plasmid in transfection mixtures produced levels of luciferase expression comparable to those obtained by omitting the corresponding wild-type plasmid (Fig. 1C, bars 5 to 14). These results indicate that for TRS1, as already shown (60), and for UL112-113, the observed regulatory interactions are not due simply to promoter competition or other irrelevant plasmid effects and that encoded proteins are needed for cooperative activation of the UL44 promoter. Likewise, we concluded that the UL36-38 region contributes a protein(s) essential to cooperative activation.

Although either IRS1 or its terminal repeat counterpart TRS1 functioned in the nonquantitative transient-replication assay (44), both the strain Towne (TRS1-M; pON2334) and strain AD169 (TRS1-J; pXEXX-6.1) TRS1-expressing plasmids produced severalfold-higher levels of luciferase expression when substituted for IRS1 (Fig. 1C, compare bar 2 with bars 3 and 4). Thus, either omitting TRS1 or substituting with the frameshifted version reduced overall levels of expression 5- to 10-fold in comparison with mixtures containing all effectors including TRS1. This percent reduction relative to the all-effector mixture is comparable to that produced by omitting, or substituting mutated versions of, UL36-38 or UL112-113 plasmids. Despite this greater potency of TRS1, replacing the IRS1 plasmid with TRS1 plasmid in the subtraction experiment did not affect the overall pattern of interaction between the effectors, except that the reduction relative to all effectors was greater upon omitting TRS1 than upon omitting IRS1 (compare Fig. 1A and 2A). Replacing IRS1 with TRS1 also pro-

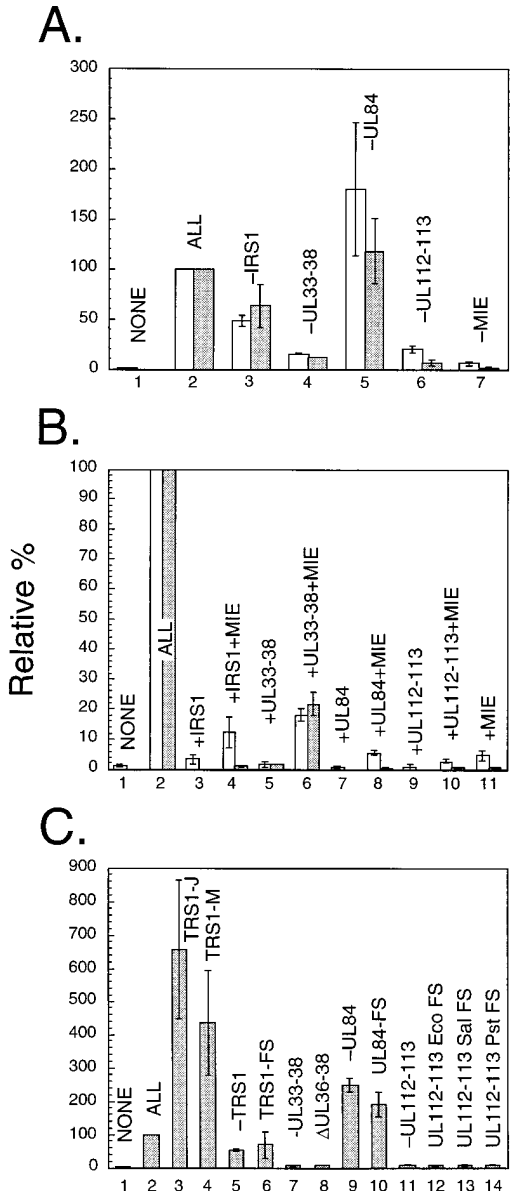

FIG. 1. At least four loci encode proteins that cooperatively activate transient expression by the UL44 promoter. Transfections were done and luciferase activity was measured as described in Materials and Methods. The shaded bars in panels A and B show results obtained with the lipofectamine reagent; all others were obtained by the calcium phosphate method. For mixtures in which a candidate effector plasmid(s) was omitted from the transfection mixture, the total DNA amount was adjusted to equivalence with pBluescript. The graphs plot measured luciferase activity relative to that obtained with all effectors, which was set to 100%. (A and B) Results of two independent experiments (open and shaded bars, respectively), each done in triplicate. Error bars plot the standard deviation for triplicate plates. (A) UL44 promoter. Transfection mixtures contained pAI1 plus either no effector plasmids (none), all five candidate effector plasmids (all), or all effectors less the indicated effector plasmid, which was replaced by an equivalent mass of pBluescript. The five candidate effector plasmids were pZP8 (UL33-38), pZP13 (UL84), pZP24 (UL112-113), pSVH (MIE), and pZP3 (IRS1). (B) Individual effectors. Transfection mixtures contained pAI1 plus the indicated effector plasmid(s). Plasmids were as listed for panel A. (C) Mutated effectors. Transfection mixtures contained pAI1 plus either no effector plasmids (none), the five candidate effector plasmids listed for panel A (all), all effectors with TRS1 in place of IRS1 (bars 3 and 4), all effectors less the indicated effector plasmid (bars 5, 7, 9, and 11), or all effectors with the indicated effector plasmid substitution (bars 6, 8, 10, and 12 to 14). The wild-type effector plasmids were those detailed for panel A plus pXEXX-6.1 (TRS1-J) and pON2334 (TRS1-M). Plasmids used to express frameshifted or deleted proteins were pON2336 (TRS1-FS), pAI12 (ΔUL36-38), pZP13FS (UL84-FS), pZP24EcoFS (UL112 113-Eco FS), pZP24SalFS (UL112-113-Sal FS), and pZP24PstFS (UL112-113-Pst FS).

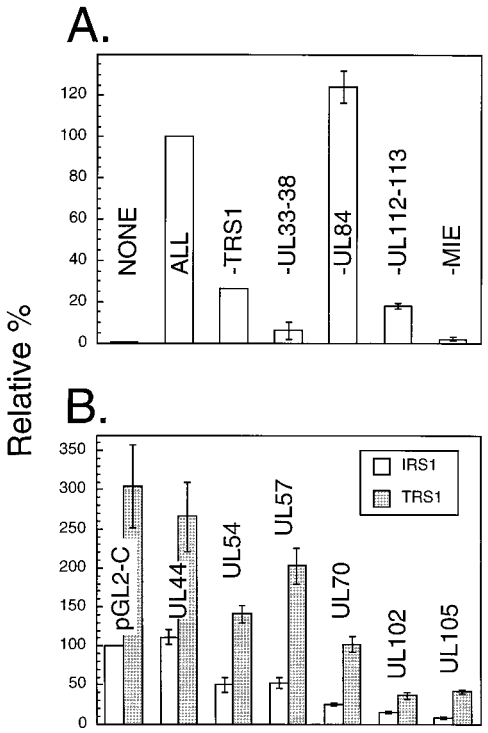

FIG. 2. Comparisons of IRS1 and TRS1 activities. Transfections were done with the lipofectamine reagent, and luciferase activity was measured as described in Materials and Methods. Results were plotted as described for Fig. 1; results of only one experiment are shown for each panel. (A) Subtraction experiment done with the UL44 reporter plasmid pAI1, as described for Fig. 1A, except that the IRS1-expressing plasmid ZP3 was replaced with pXEXX-6.1 for each transfection cocktail. (B) Comparison of relative responses to IRS1 and TRS1 by reporter constructs containing the SV40 promoter-enhancer (pGL2-C), UL44 promoter (pAI1), UL54 promoter (pAI13), UL57 promoter (pLHB2), UL70 promoter (pAI3), UL102 promoter (pAI5), and UL105 promoter (pAI7). Transfection mixtures contained the indicated reporter plasmid and pZP8, pZP13, pZP24, and pSVH, plus either pZP3 (open bars) or pXEXX-6.1 (shaded bars).

duced higher expression with other early promoter reporter plasmids (Fig. 2B), indicating that the difference is not promoter specific. We do not know whether this apparent potency of TRS1 relative to IRS1 is due to an intrinsic difference in their activating abilities or simply is due to different levels of effector expression from the plasmids used for these studies.

Together, these experiments show that the loci UL36-38, UL112-113, MIE, and IRS1 or TRS1 express proteins that can cooperate to activate expression driven by the previously characterized UL44 promoter region to much higher levels than MIE alone (more than 100-fold higher in some experiments) and that each of these contributes significantly to overall activation. Thus, although MIE is probably the single most important contributor to overall levels of UL44 promoter expression, in the absence of the other effectors its activity is severely compromised.

Promoters of other HCMV replication genes respond similarly to the UL44 promoter. We next asked whether other HCMV promoters would respond to this set of effectors and whether the expression of other replication proteins was activated by different subsets of the effectors. For these experiments, we made luciferase reporter plasmids for each of the other putative replication fork genes, UL54, UL57, UL70, UL102, and UL105. In addition, we also made a luciferase reporter plasmid containing the well-characterized UL112-113 promoter (5, 52, 58). The UL54 promoter has been studied

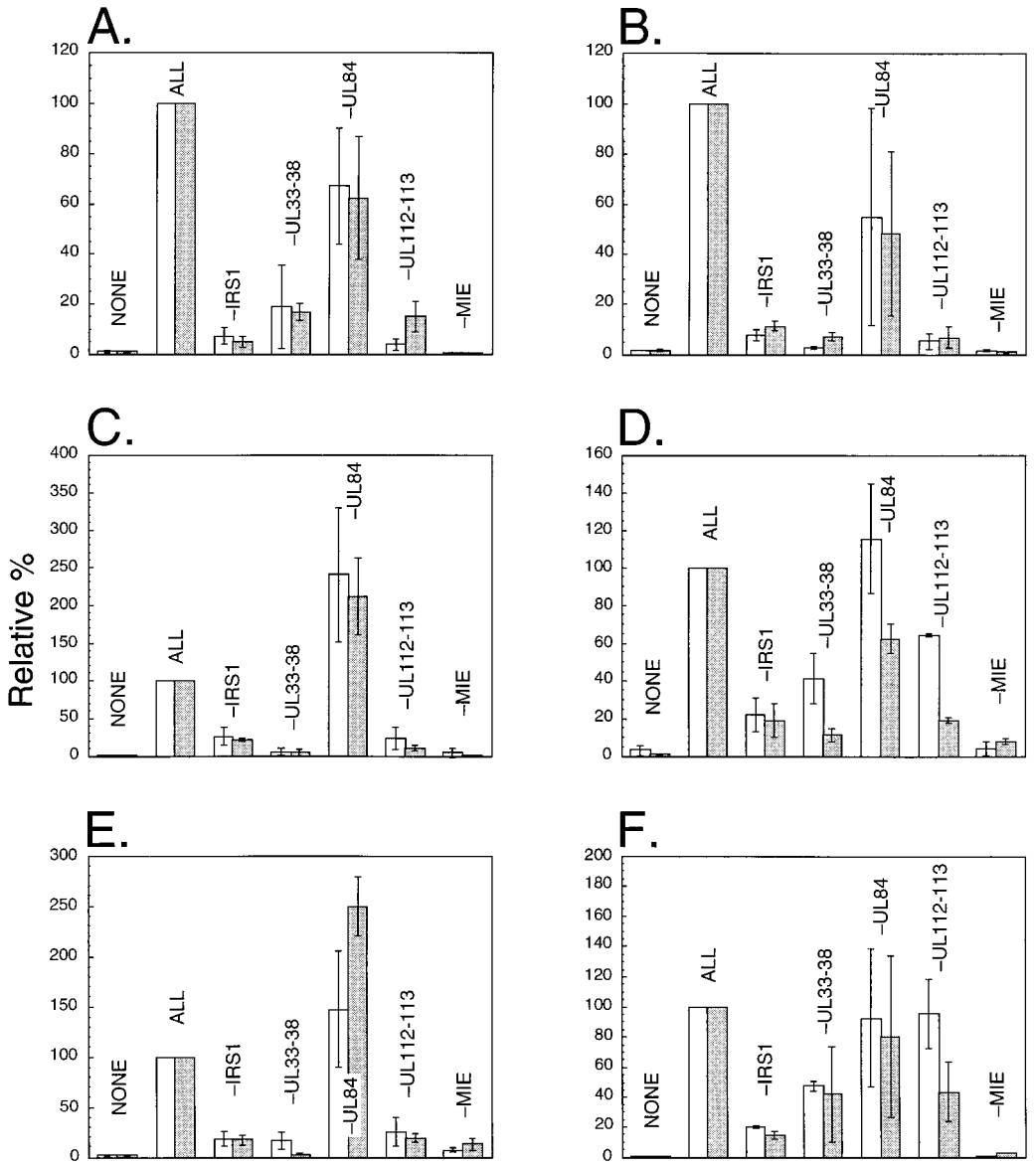

FIG. 3. Multiple early HCMV promoters are cooperatively activated by this set of loci. Transfections were done by the calcium phosphate protocol, and luciferase activity was measured as described in the text. Graphs were plotted as described for Fig. 1. Each panel presents the results of two independent experiments (open [experiment 1] and shaded [experiment 2] bars). All experiments were done as described for Fig. 1A, except that the reporter plasmid was varied as indicated for each panel. (A) UL54 promoter (pAI13); (B) UL57 promoter (pLHB2); (C) UL70 promoter (pAI3); (D) UL102 promoter (pAI5); (E) UL105 promoter (pAI7); (F) UL112-113 promoter (pAI15). Omitted plasmids were replaced with an equal mass of pBluescript, except for experiment 2 in panel C, in which case the subtracted plasmid was replaced with pZP18 (44).

(23, 31–33, 62), and the luciferase reporter plasmid that we assembled contained a region nearly identical to that previously characterized. For cases in which the promoter regions have not been described (UL57, UL70, UL102, and UL105), we assembled luciferase reporter plasmids (as detailed in Table 1) using promoter regions predicted by the DNA sequence data (8), transcript mapping (25, 55), and results of transient complementation of DNA replication (44, 45). The promoter fragments extended from well upstream of known or predicted transcription start sites downstream to the known or predicted translation start codon. Transfection experiments verified that the predicted promoter fragments were activated by virus infection (26). These reporter plasmids exhibited levels of activation comparable to UL44, with the exceptions of the predicted UL102 and UL105 promoter regions (pAI5 and pAI7, respectively), which were 5- to 10-fold less responsive than the UL44 promoter (Fig. 2B).

Each of these plasmids then was tested for its activation by the set of effectors described in the legend to Fig. 1A. Results indicate that responsiveness to UL36-38, UL112-113, MIE, and IRS1 is evident for all of the promoters of replication fork genes. As with the UL44 promoter, omitting either IRS1-, UL36-38-, UL112-113-, or MIE-expressing plasmids from the cotransfection mixture reduced luciferase expression in comparison with the all-effector controls from approximately 3- to more than 10-fold (Fig. 3A to E). Removing the MIE-expressing plasmid from the cotransfection mixture almost universally produced the greatest reduction in luciferase expression. In

each case, reductions in activation following subtraction of the IRS1, UL36-38, and UL112-113 plasmids were reproducible and statistically significant. On the other hand, omission of UL84 produced variable results, dependent upon the promoter tested. Luciferase expression by UL54 and UL57 promoters (pAI13 and pLHB2, respectively) decreased by one-third to one-half when UL84 was left out of the transfection mixture (Fig. 3A and B, respectively), whereas luciferase expression by the UL70 (pAI3) and UL105 (pAI7) promoters increased by roughly the same degree (Figs. 3C and E, respectively); expression of other reporters seemed unaffected by removal of UL84 from the mixture (Fig. 3D and F). In no example was the ratio of change produced by subtracting the UL84 effector comparable to those typically produced by subtracting the IRS1, UL36-38, UL112-113, and MIE plasmids. Nevertheless, these subtle modulations were reproducible and in some cases statistically significant, and they were specific to UL84 in that substitution with other noneffector plasmids (e.g. pZP18, which expresses UL102 [Fig. 3C]) had no effect on reporter expression.

Finally, expression of the UL112-113 promoter (pAI15) was augmented by IRS1 and possibly by UL36-38 effectors (Fig. 3F), perhaps explaining why the combination of UL112-113-plus MIE-expressing plasmids was not highly cooperative in activating the UL44 promoter (Fig. 1B). Consistent with results of Colberg-Poley et al. (11), UL36-38 subtraction had a lesser effect on UL112-113 promoter expression than on most other promoters, producing only a marginally statistically significant twofold reduction (compare Fig. 3F with A through E). However, the most striking difference between pAI15 and the other promoters is that subtracting the UL112-113 effector did not produce a statistically significant loss of activity.

Some apparent variations between reporter plasmids in their relative responsiveness to specific effectors were noted. For example, luciferase expression by the UL54 and UL112-113 promoters (pAI13 and pAI15, respectively) was reduced more by omission of IRS1 than by omission of UL36-38 (Figs. 3A and F, respectively), whereas in most other examples the opposite was true (e.g., Fig. 3B and C), as it was for the UL44 promoter (Fig. 1A). As a caveat we note that, because of the degree of variation within each transfection experiment and between experiments, subtle trends indicating promoter-specific differences amongst the replication gene promoters may not be statistically significant. Also, the apparent differences may be particular to the promoter regions examined; we have not dissected any of these promoters in detail to determine whether variant upstream regions would yield similar results. In this regard, we have noted some differences in the relative responses to specific effectors across a series of UL57-promoter reporter plasmids, although each examined plasmid was responsive to all of these effectors (25). Additional experiments will be needed to address these subtleties.

Evidence that activation by UL112-113 is promoter dependent. We tested other promoters for their response to this set of effectors under the same conditions. Representative results are those obtained with the pGL2-Control plasmid, which contains the simian virus 40 (SV40) promoter and enhancer regions (Fig. 4). Subtracting either the UL36-38 or MIE plasmid resulted in roughly 10- to 20-fold-lower activity. Therefore, these two effectors are capable of upregulating expression driven by the SV40 promoter-enhancer in HF cells, just as was found with the replication gene promoters. Indeed, all promoters that we have tested in this assay to date have responded negatively to subtraction of the UL36-38 and MIE plasmids, arguing that, at least in combination with MIE proteins, UL36-38 proteins are promiscuous activators of expression in HF cells. Likewise, subtracting IRS1 or TRS1 in these experiments resulted in a significant reduction in luciferase activity. In contrast, significant changes in pGL2-Control expression were not observed upon subtraction of the UL112-113 plasmid. As noted above, expression from the UL112 promoter also did not show a significant effect of subtracting the UL112-113 plasmid (Fig. 3F), nor did expression driven by the putative promoter for an early temporal-class, small *ori*Lyt transcript (25). Thus, whereas proteins encoded by the UL36-38, IRS1/TRS1, and MIE loci appear to be comparatively broad-spectrum activators, the ability of UL112-113 proteins to upregulate expression in HF cells may be promoter specific.

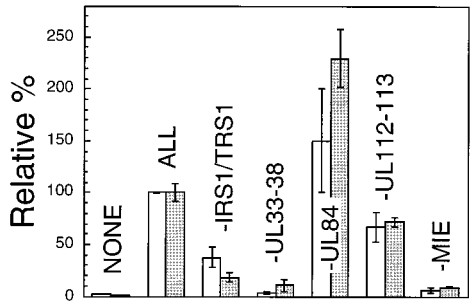

FIG. 4. Evidence that the SV40 promoter-enhancer does not respond to UL112-113. Transfections were done with the lipofectamine reagent, and luciferase activity was measured as described in Materials and Methods. Transfection mixtures contained either pGL2-Control alone (none); pGL2-Control together with pZP8, pZP13, pZP24, SVH, and either pZP3 (open bars) or pXEXX-6.1 (shaded bars) (all); or all except the indicated effector plasmid.

Some activating interactions are synergistic. The apparent differences in activation between each of the individual effectors and the collection of all five loci transfected together suggested that proteins encoded by some of the effector loci interact synergistically, as already shown for TRS1 and MIE (60). Moreover, statistical treatment of the combined data from UL44 subtraction and individual effector experiments resolved effector interactions. By this we mean that a significant difference was observed between the sum of the responses when given effectors were jointly present or absent and the sum of the responses when the same factors were individually present or absent. To examine all interactions directly, we measured expression of the UL44 promoter after cointroducing all possible combinations of the activating loci UL36-38, UL112-113, MIE, and TRS1 (Fig. 5). We omitted the UL84 plasmid from this series of transfections, because in preceding experiments it did not activate UL44 promoter expression. Data were analyzed by standard statistical approaches as described in Materials and Methods (Table 3). Basically, in this analysis each combination was considered the sum of individual contributions plus measured interaction parameters. For instance, the interaction parameter for any two effectors was derived by summing their individual activations and subtracting that sum from the measured combined activation; a statistically significant ($P < 0.05$) positive result is defined as synergy. Higher-order interactions were similarly derived by summing all individual activations plus all relevant interaction parameters. The derived interaction parameters were tested for significance. As is obvious in Fig. 5, MIE and UL33-38 plasmids were synergistic in activating the UL44 promoter. Moreover, other pairwise interactions and most higher-order combinations also showed significant positive cooperativity; only the TRS1–UL112-113 pair and other combinations involving these two were not found to interact significantly.

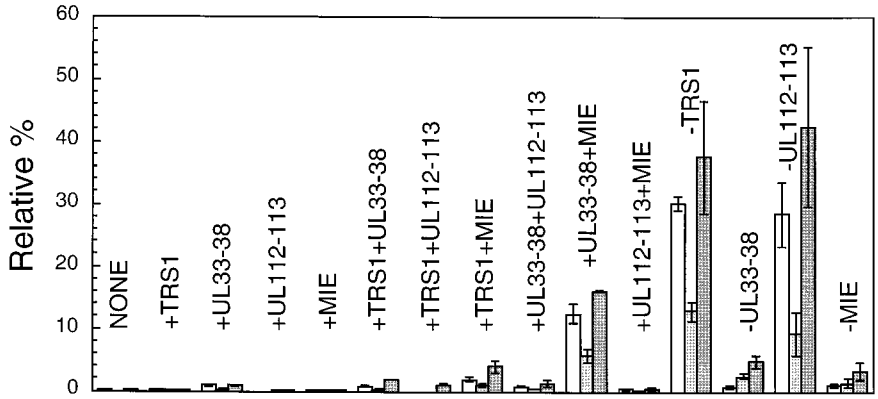

FIG. 5. Regulatory interactions among the four loci expressing proteins that activate expression of the UL44 promoter. Transfections were done with the lipofectamine reagent, and luciferase activity was measured as described in Materials and Methods. The means and standard deviations for three independent experiments, each done in triplicate, are plotted relative to the value for the mixture of all four effectors, which was set to 100% (not shown). Effectors were pXEXX-6.1 (TRS1), pZP8 (UL33-38), pZP24 (UL112-113), and pSVH (MIE). Transfection mixtures contained the indicated combinations of one, two, or three effector plasmids; mixtures denoted by "-" contained all effectors except the noted plasmid.

DISCUSSION

We have extended our previous transient-complementation studies to search for cooperative regulatory interactions between proteins encoded by several loci with unknown roles in complementing *ori*Lyt-mediated DNA replication. For these experiments, roughly equimolar mixtures of candidate effector loci were cointroduced into permissive HFs and encoded proteins were expressed under the control of their respective native promoters. This approach has the distinct advantage of preserving, to the greatest extent possible in a transient system, the regulatory relationships of proteins expressed by individual effector loci. Two major conclusions emerge. First, results show that the UL36-38, UL112-113, and IRS1 proteins cooperate with each other, and with MIE, to significantly upregulate transient expression of some HCMV early genes in HF cells. The observed interactions are not likely to be due to promoter competition artifacts because frameshift mutations within the coding regions of effector plasmids resulted in loss of function, and therefore they must reflect cooperative activation. Within the context of our assay, this cooperation resulted in expression levels much higher than those obtained with MIE activation alone (more than 100-fold higher in many experiments). In contrast, these experiments failed to find clear evidence that UL84 modulates expression of early viral genes. Second, multiple early promoters respond similarly to cooperative activation by these four effector loci. Indeed, each of the promoters driving expression of candidate replication fork proteins was responsive to each of these four activating plasmids. Thus, coordinate regulation of HCMV early gene expression in permissive HFs is a complex, multifactor process. These experiments do not reveal the mechanisms whereby these proteins cooperate. Such interactions could be due to cross-regulation between one or more of the effector loci, such that limiting activator proteins are more efficiently expressed, or to combinatorial activation of the early target loci, or both. Moreover, either or both of these could occur at any step in the series of events controlling protein accumulation. In this regard, we note that UL112-113-mediated activation of the UL54 promoter depends upon activation by MIE proteins mediated through the IR1 element, whether or not the UL112-113 proteins are expressed constitutively (30).

TABLE 3. Statistical tests for effector interactions

Effector treatment	Estimated relative %[a]	*P* value[b]
None	0.04	0.0000
TRS1	0.06	0.0000
pZP8	0.52	0.0000
pZP24	0.04	0.0000
MIE	0.06	0.0000
TRS1 + pZP8	0.70	0.0001
TRS1 + pZP24	0.02	0.3760 (NS)
TRS1 + MIE	1.78	0.0000
pZP8 + pZP24	0.73	0.0001
pZP8 + MIE	9.81	0.0000
pZP24 + MIE	0.26	0.0000
pZP8 + pZP24 + MIE	22.34	0.0001
TRS1 + pZP24 + MIE	3.30	0.2007 (NS)
TRS1 + pZP8 + MIE	23.40	0.0000
TRS1 + pZP8 + pZP24	1.72	0.0000
TRS1 + pZP8 + pZP24 + MIE	100.00	0.2767 (NS)

[a] Estimated relative percents are antilogs of the predictions from the statistical analysis described in Materials and Methods.

[b] *P* values give the probability that the interaction effect for each effector treatment is zero. NS, not significant.

The MIE locus retains a central role in activating early expression. Individually it was generally the most potent activator, and within the context of our assay its subtraction produced the greatest drop in overall expression. It is not surprising that the combination of immediate early loci spanning UL36-38, MIE, and IRS1/TRS1 can cooperatively promote expression of viral early promoters, because these have been shown individually to activate expression of viral early (10, 40, 48, 60) and cellular (11) genes; moreover, both UL36-38 and IRS1/TRS1 have been shown to cooperate with MIE proteins in activating selected targets (11, 60). The MIE region expresses several proteins via differential splicing, the most abundant and best understood of which are IE1-72kDa and IE2-86kDa (61). These two proteins synergistically activate expression of many different targets, by both direct and indirect pathways (5, 7, 19, 39, 52, 56, 62), and the IE2-86kDa protein also regulates MIE transcription (35, 37, 47, 62). In our experiments, both IE1 and IE2 proteins were expressed from pSVH (62); the relative contributions of individual IE1 or IE2 proteins to activation in the context of all effectors were not assessed.

The next most potent activator in our experiments was the pZP8 plasmid, which spans the UL36-38 region. Activation by this region has been shown to be both promoter and cell type dependent (11). Our results are consistent with previous findings and additionally provide new evidence that this locus contributes significantly to regulation of viral early-gene transcription. Like MIE, the UL36-38 region is complex in that it expresses at least four different proteins from overlapping transcripts produced by differential splicing and promoter usage (63, 64). Three of those, UL36, UL37, and UL37x1, were shown by Colberg-Poley et al. (11) to cooperate individually with US3 to activate expression of the *hsp*70 promoter in HeLa cells. It remains to be determined which of these contributed to the cooperative activation of viral early promoters observed in our experiments. Deletion of the UL36-38 region from pZP8 completely abrogated its activation of the UL44 promoter in the complementation experiments; however, replacing pZP8 with a plasmid that contains only UL36-38 produced only about one-third as much UL44 expression as pZP8 (26). This difference may be due to less efficient expression of UL36-38 proteins. However, preliminary experiments suggest this difference can be supplied in *trans* by a plasmid containing UL34 and UL35, although that plasmid had no effect in the absence of UL36-38 (26). We note that the UL34-UL35 region was not required for complementing DNA replication (45). These observations are further suggestive of the overall complexity in regulation of HCMV early-gene expression. Additional experiments will be needed to assess the possible contributions of the UL34 and UL35 ORFs.

Finally, IRS1 and TRS1 proteins are predicted to have identical sequences over their amino-terminal 550 amino acids, which are encoded within the short repeats, but diverge in their carboxyl-terminal approximately 300 residues. TRS1 was shown by Stasiak and Mocarski to cooperate with MIE to activate the UL44 promoter (60). Here we have demonstrated that IRS1 has similar capabilities and have also extended their observations to show activation of other HCMV early promoters.

Perhaps more surprising than the observed cooperative interactions among proteins encoded by the immediate early loci is the evidence that the early-temporal-class UL112-113 locus somehow participates in regulating viral early-gene expression. Like the MIE and UL36-38 immediate early loci, the UL112-113 region is expressed via differentially spliced transcripts to produce four proteins of 84, 50, 43, and 34 kDa, having a common amino-terminal segment (59, 67). Although UL112-113 transcripts are not detectable in the presence of cycloheximide (59), these proteins are seen by immunofluorescence as early as 2 h after infection and appear to localize to the characteristic intranuclear inclusions (27). Again, it remains to be determined which of these proteins contribute to cooperative activation. However, the finding that the *Sal*I and *Eco*RI frameshift mutations eliminate cooperative activation may implicate the 84-kDa protein. In most of our experiments, the UL112-113 plasmid, alone or combined with MIE, failed to show a significant activation of early reporter constructs. Thus, we might have overlooked the apparent role of this region in regulating early gene expression if we had used simpler experimental designs; these results underscore the power of transient complementation in dissecting complex processes. However, UL112-113 did activate the UL54 promoter in combination with MIE when transfected under slightly different conditions (30).

With respect to their role in transient complementation of HCMV DNA replication, our results are consistent with a requirement for UL36-38, UL112-113, MIE, and IRS1/TRS1 to adequately express essential replication fork proteins. When transfection protocols similar to those used for transient complementation of DNA synthesis were used, these loci expressed proteins that contributed to luciferase expression driven by each of the replication fork gene promoters. Clearly, a significant reduction in expression of multiple early loci might produce too little accumulation of essential replication proteins to carry out DNA synthesis, especially if one or more are limiting. Nevertheless, these studies do not rule out direct involvement in initiating or performing DNA synthesis for any of these gene products. If transcription or cognate transcriptional regulatory elements are critical components or regulators of DNA synthesis, as is the case in many other systems, including Epstein-Barr virus (17, 51), then each of these proteins becomes a candidate for involvement. Moreover, these results provide no obvious explanation of the requirement for UL84 to complement HCMV DNA replication. The UL84 protein associates with the IE2-86kDa protein (50, 57), and we anticipated that it might participate in IE2-mediated transcriptional regulation via this interaction. Although we observed reproducible promoter-dependent modulations upon subtraction of UL84, the differences were small. Therefore, we cannot yet conclude that UL84 is a significant contributor to viral early-gene expression. Instead, UL84 may well be responsible for mediating interactions with other proteins, with other types of promoters, or with *ori*Lyt.

Several lines of evidence argue that the cooperation between these effectors observed in our transient-transfection assays reflects interactions important to viral replication. First, although some aspects of viral gene expression are not accurately reproduced by transient-transfection assays, basic findings of transient-transfection analyses have been validated by using recombinant viruses (30, 32), and it is likely that results of transient-transfection experiments reflect at least a subset of the regulatory events that occur in the natural context of the viral genome. Therefore, similar interactions are predicted to occur during viral infection of permissive cells. Second, these genes were individually found to be essential for complementation of *ori*Lyt-mediated DNA replication in a transient-transfection assay in which expression of the DNA polymerase and other replication fork genes was driven by their native promoters (44, 45). Results presented here are consistent with a model in which expression of those genes in permissive human fibroblasts is vitally dependent upon this set of effectors. Finally, evidence for an essential role in viral replication in permissive cells has been obtained for one of these loci by using a complementary method. Selectively inhibiting expression of UL36-37 proteins by treating infected cells with an antisense oligonucleotide efficiently blocks viral replication in cultured cells (54). Clearly, an important challenge now is to assess the relative contributions of proteins encoded by these loci in regulating expression within the context of the HCMV genome during infection of permissive cells.

In conclusion, these data have broad implications for our understanding of HCMV biology and raise many new questions. Our results suggest that a complex interplay between several viral regulatory loci encoding at least a dozen proteins is needed to create an intracellular environment suitable for viral gene expression and replication. In this model, disregulation of or by any one of these effectors could affect expression of many early genes and might greatly inhibit or block viral replication; this could explain the failure of HCMV to replicate in some cell types in which MIE expression is not blocked (34). We have not examined the responsiveness of immediate-early or late viral promoters to proteins encoded by this set of loci nor that of cellular targets. It will be of interest to revisit

previous findings with those targets in the light of these data. For example, UL36-38 proteins have been found to cooperate with MIE to activate expression of certain cellular genes (11). Do UL112-113 and IRS1/TRS1 proteins participate in those regulatory activities as well? Likewise, we have not examined the abilities of these effectors to activate expression of these early targets in other cell types, such as endothelial cells and monocytes/macrophages, which are thought to be important sites of infection in the host. Finally, it is clear that these loci are not alone in encoding viral proteins that regulate expression during infection. For example, US3 (11), UL69 (65), and UL82 (38) all have been shown to activate expression of selected promoters. In addition, UL36 and IRS1/TRS1 are members of the US22 family of HCMV genes, which includes 13 recognized members (8). The transient-complementation method offers a useful tool to study interactions of all of these candidate regulatory loci in a variety of cell types and conditions.

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