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Human Cytomegalovirus US3 and UL36-38 Immediate-Early Proteins Regulate Gene Expression

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We have established the ability of the human cytomegalovirus (HCMV) UL36-38 and US3 immediate-early (IE) gene products to alter gene expression in human cells by using transient transfection assays. The cellular heat shock protein 70 (*hsp*70) promoter was transactivated following cotransfection with the HCMV IE regions in nonpermissive HeLa cells by UL36-38, US3, or IE1 and in permissive human diploid fibroblasts (HFF) by IE1 or IE2. Moreover, *hsp*70 expression was synergistically increased in HeLa cells cotransfected with US3 and UL36, with US3 and UL37, or with US3 and UL37x1. The synergistic transactivation of *hsp*70 expression by US3 and UL36-38 was not observed in HFF cells. Synergy was also not observed in HeLa cells between US3 and UL38, an early gene product encoded by the UL36-38 IE locus. Synergistic transactivation of *hsp*70 expression in HeLa cells required the syntheses of UL36-38 and US3 IE proteins, since nonsense mutants were not functional. *hsp*70 expression increased with increasing amounts of transfected US3 and UL37 DNA and occurred at the level of stable *hsp*70-promoted RNA. In contrast to the broad *hsp*70 response, promoters from the HCMV UL112 early gene and another cellular gene, brain creatine kinase, both responded strongly only to singly transfected IE2 in HeLa cells. Nevertheless, IE2 transactivation of the UL112 promoter was further stimulated by cotransfection of IE1 or of UL36-38 in both HeLa and HFF cells. Thus, different patterns of promoter transactivation and interactions between HCMV IE gene products in transactivation were found in HFF cells and in HeLa cells. These results establish the ability of the HCMV US3 and UL36-38 proteins to alter cellular and viral gene expression and are consistent with involvement of cellular transcription factors in HCMV IE regulation of gene expression.

Human cytomegalovirus (HCMV), a human herpesvirus of considerable medical importance, is a major cause of birth defects and of life-threatening infections in immunosuppressed patients. Nevertheless, the regulatory mechanisms used by this pathogen during its replication are just being deciphered.

To initiate infection, HCMV relies heavily on the host cell machinery to transcribe and translate its gene products. In permissive human cells, expression of the virus genome is temporally regulated (17, 39, 61). The first viral proteins to appear, known as immediate-early (IE) proteins, are regulatory in nature in that they alter the expression of other temporal classes of HCMV genes (7, 18, 47, 50, 52) as well as cellular genes (19, 21, 33, 43) and heterologous viral genes (16, 21, 27, 43, 48, 60). The large (230-kbp) HCMV genome encodes IE gene products from at least three distinct regions (17, 61), the major IE (MIE) (1, 34, 54, 56), the UL36-38 (35, 57, 58, 63), and the US3 (62) loci. IE1 and IE2 are encoded by the MIE locus and result from differential use of promoters and alternative splicing such that various proteins are produced at different times of infection (1, 51, 54, 55). IE1 and IE2 can act synergistically in the activation of HCMV early promoters (7, 18, 47, 50) and have also been shown to regulate the expression of their own and heterologous gene promoters (9, 10, 16, 21, 26, 27, 36, 41–43, 48, 52, 53, 60). Activities of the UL36-38 and US3 IE gene products have not yet been demonstrated.

HCMV infection of permissive human cells stimulates expression of cellular growth genes (3, 4, 12, 19, 45). Transient stimulation of the cellular proto-oncogenes, c-*myc*, c-*fos*, and c-*jun*, occurs immediately upon infection and appears to involve attachment of virion particles to the cell rather than de novo IE protein action (3, 4). However, stimulation of RNA synthesis from another cellular gene, heat shock protein 70 (*hsp*70), occurs within a few hours of infection, requires de novo protein synthesis, and is consistent with transactivation by HCMV IE gene products (12, 45).

We sought to measure the ability of the US3 and UL36-38 IE gene products to alter expression of cellular and HCMV early gene promoters. Because expression of the cellular *hsp*70 and brain creatine kinase (*ckb*) genes (12, 45) as well as viral genes is increased following infection of permissive cells, we selected for our analyses these cellular promoters and a representative, characterized HCMV early gene promoter (UL112), which is known to be transactivated by the HCMV IE1 and IE2 gene products (50). Besides measuring the effects of individually transfected UL36-38 and US3 genes on expression from *hsp*70, *ckb*, and UL112 promoters, we also analyzed interactions between the UL36-38, US3, and MIE gene products by cotransfecting the promoter-indicator genes with pairwise combinations of the IE genes. Differences in contributions made by the host cell for transactivation of gene expression were examined by comparing transfections of permissive human diploid fibroblast (HFF) cells and nonpermissive HeLa cells. Our results indicate that the HCMV IE gene products, including those of the UL36-38 and US3 IE gene products, can act alone or in combination to regulate expression of viral and cellular promoters in a manner that is dependent upon the promoter, the host cell, and the IE gene products present.

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TABLE 1. Transfected DNAs

HCMV gene	Plasmid name	Reference(s) or source
Wild type		
IE1	pRR59	13
IE2	pBK1	13
US3	p339	13
UL36-38	p302	13
UL36 cDNA	p326	13, 58
UL37x1/38 cDNA	p327	13, 58
UL37/36 cDNA	p334	13, 58
UL37 cDNA	p414	This work
UL38	p406	13
gB	p370	13
Mutant		
US3	p424	This work
UL36	p425	This work
UL37x1	p426	This work
UL37	p428	This work
Target		
*hsp*70-CAT	pHBCAT	64
*hsp*70-*neo*	p423	This work
ckb-CAT	pckbΔNde	29
UL112-CAT	p358-CAT	50
SV40-*lacZ*	pCH110	Pharmacia

MATERIALS AND METHODS

Cells and transfections. HFF cells (Viromed Laboratories, Inc.; used before passage 16) and HeLa cells were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (DMEM/10).

DNA expression constructions. The constructions used in these studies are listed in Table 1. All of the constructions containing HCMV sequences are from strain AD169, except gB (Towne strain). pRR47 contains the 6.7-kbp *Eco*RI-*Sal*I fragment (nucleotides [nt] 168817 to 175524) that includes the upstream sequences from the MIE region and the open reading frames of IE1 and IE2, cloned into pUC18. pRR49, the cloned MIE promoter enhancer, contains the *Bal*I-*Hpa*II fragment (nt 173678 to 174402) cloned as a *Hin*dIII-*Hpa*II fragment into pUC18 and then subcloned into the pBSII SK+ vector (Stratagene) generating p325. pRR59 contains the 3.67-kbp *Cla*I fragment (nt 170811 to 174479) that includes the MIE upstream sequences and the IE1 open reading frame, cloned into pUC18. pBK1, encoding IE2 gene products, was generated by cleavage of pRR47 with *Acc*I and religation, thereby removing the exon 4 coding sequences of IE1 (nt 170952 to 172366). The US3 region was cloned as a 1.69-kbp *Sma*I fragment (nt 193421 to 195108) into pGem4, producing p339. The UL36-38 recombinant, p302, was generated by *Eco*RI-*Kpn*I (nt 47634 to 53104) cleavage of HCMV *Eco*RI Q and insertion into pBSII SK+ vector. The cDNAs (57, 58) for UL36 (nt 48095 to 49809), UL37x1/38 (nt 51001 to 52709), and UL37/UL36 (nt 48095 to 52709) and a DNA encoding UL38 (nt 51001 to 52249) were placed under the IE1 promoter/enhancer in p325 to produce p326, p327, p334, and p406. p414, which produces UL37, was generated from the UL37/UL36 cDNA (p334) by cleavage with *Sal*I (nt 48314 to 49832) and religation, removing thereby the UL36 coding sequences. The gB coding sequences (nt 80524 to 83640) from an adenovirus-gB construction (38) were placed downstream of the HCMV MIE promoter contained in p325. An *Xba*I fragment containing the simian virus 40 (SV40) polyadenylation signal (nt 2533 to 2770) (6) was placed downstream of the gB coding sequences resulting in the gB expression clone p370.

Nonsense mutations in the UL36, 37, 37x1, and US3 coding sequences. The US3, UL36, UL37, and UL37x1 open reading frames were mutated by insertion of a double-stranded stop linker that encodes translation stop codons in all three frames (forwards and backwards) at restriction sites close to the 5′ end of the coding sequences (Fig. 3). The sequence of the linker contains the rare restriction sites *Sfi*I, *Not*I, *Stu*I, and *Bgl*I, which were used to verify insertion. The orientation and, therefore, the number of encoded amino acids were determined by nucleotide sequencing with a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). p424, a US3 mutant, was generated by cleavage of p339 DNA with *Spo*I (nt 194668) and blunt-end ligation to the stop linker. The UL36 cDNA expression clone, p326, was digested with *Pml*I (nt 49665) and blunt-end ligated to the stop linker to produce p425. Mutants in UL37 (p428) and UL37x1/38 (p426) cDNAs were created by digesting p414 and p327 with *Acc*I (nt 52693), filling of the ends with the Klenow fragment of DNA polymerase, and insertion of the stop linker by blunt-end ligation.

Target promoter-reporter DNA constructions. The human *hsp*70-chloramphenicol acetyltransferase (CAT) construction contains 2.4 kbp of upstream sequences (64). An *hsp*70-neomycin (*neo*) reporter construct, p423, was prepared by isolating the *hsp*70 promoter as a *Hin*dIII-*Bam*HI fragment and inserting this fragment in front of the *neo* resistance coding sequences in pNVD at the *Hin*dIII and *Bgl*II sites (28). The rat *ckb* promoter-CAT construction (29) contains a *Hinc*II-*Xma*III fragment (189 bp) from the *ckb* gene (2) inserted into the pUC^{PL}CAT vector (5). The HCMV early gene promoter CAT construction p358-CAT (50) contains the UL112 promoter, located upstream of the UL112-113 open reading frames (8). Efficiency of DNA uptake and expression was measured by cotransfection of an independent reporter, the SV40 early enhancer/promoter-*lacZ* construction (pCH110; Pharmacia). This internal control was unresponsive to all of the IE gene products except IE2, which only stimulated its expression a maximum of twofold (14).

Transfections of human cells. Permissive HFF cells were transfected by using the DEAE-dextran method (50). Briefly, 0.1 to 10 μg of DNA in DMEM containing 0.4 mg of DEAE-dextran per ml and 50 mM Tris-HCl, pH 7.4, was added to HFF cells which had been subcultured the previous day into 10-cm plates. The transfected cells were incubated at 37°C for 2 h, washed twice with phosphate-buffered saline (PBS) and then once with DMEM/10, overlaid with fresh DMEM/10, and incubated further. Cells were harvested 48 h after transfection and assayed for CAT and β-galactosidase (β-Gal) activities as described below.

Nonpermissive HeLa cells in 10-cm plates were transfected with 0.1 to 10 μg of each DNA by using calcium phosphate precipitation (23). Four hours after transfection, the cells were treated with 20% glycerol in PBS, washed twice with DMEM, overlaid with DMEM/10, and incubated at 37°C. For RNA analysis and some CAT experiments, HeLa cells were transfected as described by Horlick et al. (30). Cells were harvested 48 h after transfection and assayed for RNA and for β-Gal and CAT activities.

Enzymatic assays. Transfected cell extracts were assayed for CAT activity (22). Briefly, a portion of the cells were harvested into 0.25 M Tris-HCl, pH 7.4. The protein extracts were frozen and thawed thrice, debris was pelleted, and the

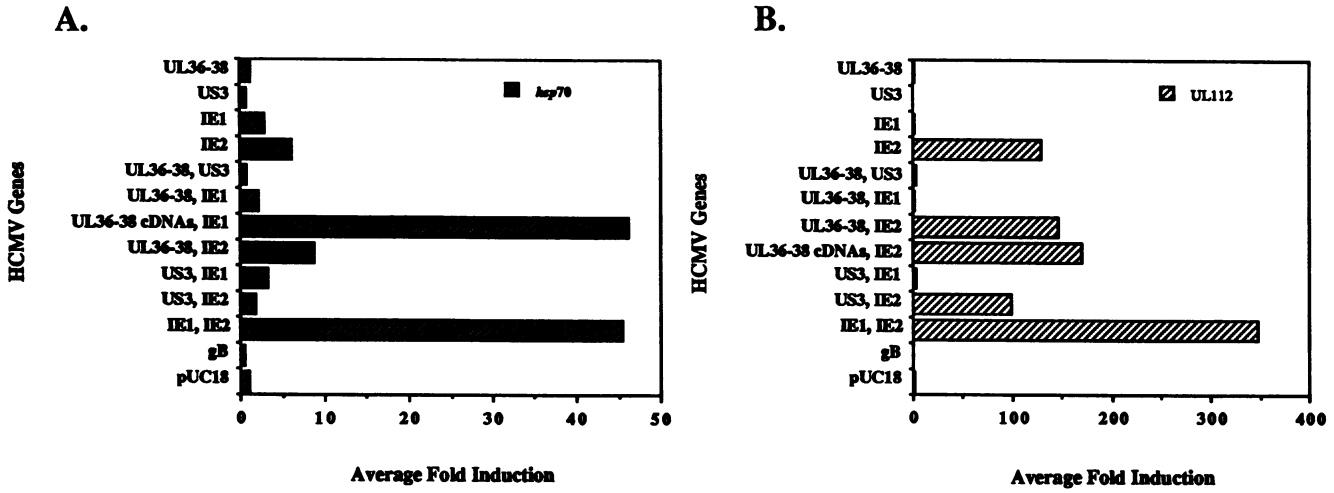


FIG. 1. (A) HCMV IE proteins transactivate the human *hsp*70 promoter in permissive human cells. The average fold induction of the *hsp*70 promoter-CAT construction by the HCMV IE gene products obtained in four separate experiments is shown. HFF cells were transfected with pHBCAT (5 to 10 μg), pCH110 (1 to 2 μg), and the indicated HCMV gene or control vector (2.5 to 5.0 μg). Protein extracts were harvested 48 h after transfection and assayed for β-Gal activity, and 20 to 100 μg was assayed for CAT activity for 1 to 6 h. (B) HCMV IE proteins transactivate the HCMV UL112 promoter in permissive human cells. The average fold induction of the UL112 construction was obtained by transfecting HFF cells in three of the experiments shown in panel A in parallel with p358-CAT (10 μg), the indicated HCMV gene or control vector (2.5 to 5.0 μg), and pCH110 (1 to 2 μg). The cells and extracts were treated as described for panel A. Following standardization to β-Gal activity, the fold induction of each group was determined by comparison with cells cotransfected with pUC18 DNA as described in Materials and Methods. An increase or decrease of twofold or greater was considered an indication of activation or repression, respectively. For combinations, the sum of each DNA individually transfected is the expected additive value; values for the combined cotransfected DNAs twofold greater than the expected additive effect were considered synergistic.

supernatant was assayed for protein concentration. Protein concentrations were quantified with the Bio-Rad Protein Determination Kit. CAT assays were performed by addition of 1 to 100 μg of total protein to [^{14}C]chloramphenicol in the presence of 1 to 4 mM acetyl coenzyme A for 10 to 60 min (HeLa cells) or in the presence of 4 mM acetyl coenzyme A for 1 to 6 h at 37°C (HFF cells). After separation of the reaction products by ascending thin-layer chromatography, the percent conversion in each sample was quantified with an AmBis radioanalytic imaging system. The fold induction for each sample was determined by comparison to the percent conversion for cells cotransfected with the same promoter-indicator gene fusion and vector (pUC18) DNA. In some experiments the fold induction was normalized for transfection efficiency by comparison of β-Gal activities.

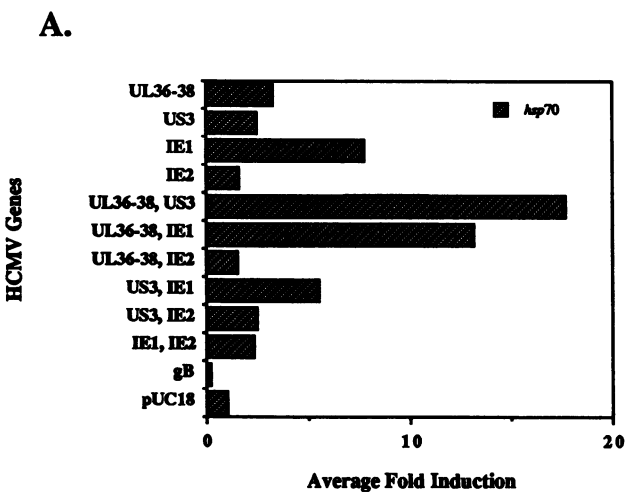
β-Gal assays were performed as previously described (20). Briefly, a portion of the transfected cells were harvested into 200 μl of lysis buffer (100 mM sodium phosphate, pH 7.2, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 2.5 mM EDTA, 0.125% Nonidet P-40) and lysed by repeated pipetting or vortexing. Cell debris was pelleted by centrifugation, and aliquots of the supernatants were assayed for protein concentration as described above and for β-Gal activity by the conversion of chlorophenol red β-galactopyranoside substrate (Boehringer-Mannheim, 4 mg/ml) to chlorophenol red with a 575-nm filter on a Molecular Devices V*max* kinetic microtiter plate reader with the ΔSoft program.

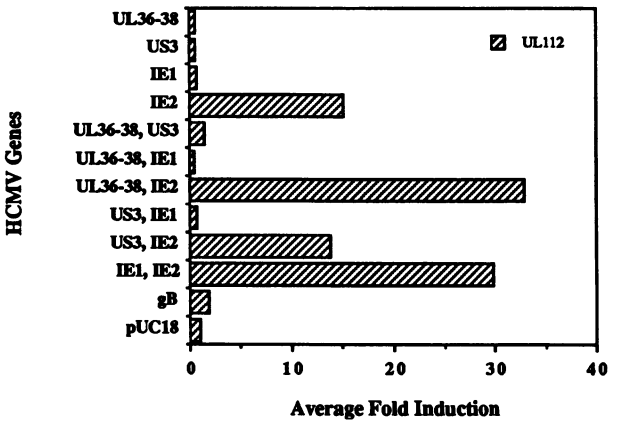
RNA studies. Transfected HeLa cells were harvested into guanidinium isothiocyanate solution, and RNA was isolated by acid phenol extraction (11) and lithium chloride precipitation (44). Total RNA was assayed by primer extension (30), except that after reverse transcription, samples were treated with NaOH to hydrolyze RNA, neutralized, and precipitated (2). Each reaction contained the oligonucleotide 5′-CGTGCAATCCATCTTG-3′ (complementary to bases +26 to +41 from the start of translation of the neomycin resistance gene). The reaction products were resolved on 7% polyacrylamide gels containing 8 M urea and quantified with an AmBis radioanalytic imaging system. The fold induction was calculated by comparison with the amount of *hsp*70-*neo* RNA obtained from cells cotransfected with the *hsp*70-*neo* construct and pUC18 DNA.

Nucleotide sequence accession number. The EMBL accession number for the complete HCMV genome sequence is X17403 (8).

RESULTS

Transactivation of the *hsp*70 promoter in HFF cells. We had previously shown that induction of cellular *hsp*70 gene expression during permissive HCMV infection of HFF cells occurs at IE times of infection and requires de novo protein synthesis (12, 45). These findings were consistent with a role of HCMV IE gene products in the transactivation of cellular *hsp*70 gene expression. To establish which HCMV IE gene product may play a role in *hsp*70 transactivation, we measured the ability of the HCMV IE gene products encoded from the UL36-38, US3, IE1, and IE2 loci, alone and in pairwise combinations, to transactivate expression from the *hsp*70 promoter-indicator construction upon cotransfection of HFF cells (Fig. 1A). In these experiments, HCMV gB expressed from the MIE enhancer/promoter was used as a negative control for transactivation and for promoter competition effects. Comparison to cells transfected with indicator constructions and vector DNA (pUC18) was used to determine the fold induction in each experimental group. An increase or decrease of twofold or greater was considered an





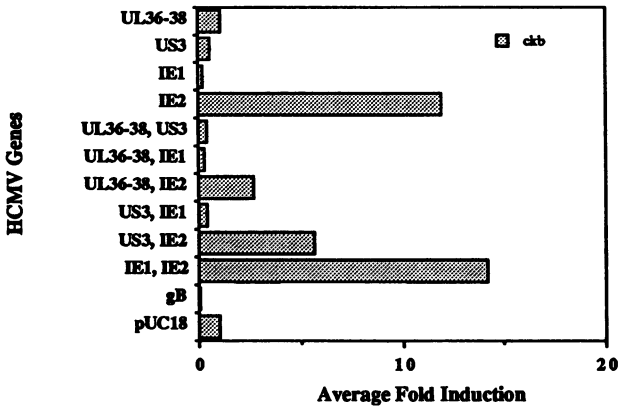


FIG. 2. (A) Transactivation of *hsp*70 promoter construction by HCMV IE genes in HeLa cells. The average fold induction of the *hsp*70 promoter-CAT construction by the HCMV IE gene products obtained in seven separate experiments is shown. HeLa cells were transfected with pHBCAT (10 μg), pCH110 (1 to 2 μg), and the indicated HCMV gene or control vector (2.5 to 10 μg). Protein extracts were harvested 48 h after transfection and were assayed for β-Gal activity and for CAT activity (10 to 100 μg) for 15 min to 1 h. (B) HCMV IE proteins transactivate the HCMV UL112 promoter in HeLa cells. The average fold induction of the UL112 construction was obtained by transfecting HeLa cells in four of the experiments shown in panel A in parallel with p358-CAT (10 μg), the indicated HCMV gene or control vector (2.5 to 5.0 μg), and pCH110 (1 to 2 μg). The cells and extracts were treated similarly to those in panel A and assayed for β-Gal activity and for CAT activity (2 to 100 μg) for 15 min to 1 h. (C) HCMV IE proteins transactivate the cellular *ckb* promoter in HeLa cells. The average fold induction of the *ckb* construction was obtained by transfecting HeLa cells in three of the experiments shown in panel A in parallel with pckbΔNde (10 μg), the indicated HCMV gene or control vector (2.5 to 5.0 μg), and pCH110 (1 to 2 μg). The cells and extracts were treated similarly to those in panel A and were assayed for β-Gal activity and for CAT activity (2 to 100 μg) for 15 min to 1 h. Following standardization to β-Gal activity, the fold induction of each group was determined by comparison with cells cotransfected with pUC18 DNA as described in Materials and Methods.

indication of activation or repression, respectively. For combinations, the sum of each DNA individually transfected is the expected additive value; values for the combined cotransfected DNAs twofold greater than the expected additive effect were considered synergistic. β-Gal activity expressed from cotransfected SV40-*lacZ* DNA was used to standardize for transfection efficiency in various groups.

Induction of *hsp*70 expression in HFF cells resulted from cotransfection with the known HCMV transactivators IE2 or IE1 (Fig. 1A). Our results for *hsp*70 transactivation by IE1 are consistent with the findings of Sinclair and his coworkers (46). Similar to the findings of others (18, 27, 37, 43, 60), there was a striking synergy between IE1 and IE2 in the transactivation of *hsp*70-CAT expression in HFF cells (to 45-fold). US3 decreased the stimulation of *hsp*70 expression by IE2, while pairwise combinations of US3 DNA with the other IE genes were unremarkable. This decrease of IE2 function was the first indication of biological activity of US3; nonetheless, we chose to study its transactivating capability on the *hsp*70 promoter in HeLa cells (see below). Although the genomic UL36-38 construction had little effect when cotransfected with IE1, synergy was observed when all three cDNA expression clones of UL36-38 were combined with IE1 (to 46-fold). Since US3 downregulation of IE2 activity was observed and the IE1 and UL36-38 cDNA synergy was comparable to that between IE1 and IE2, the UL36-38 and US3 gene products appear to regulate cellular gene expression in permissive HFF cells.

Transactivation of the HCMV UL112 early promoter in HFF cells. Because herpesvirus early genes have been found to be responsive to the viral IE gene products (21), we also examined the ability of the UL36-38 and US3 IE proteins to transactivate the HCMV UL112 early gene promoter (Fig. 1B), which has previously been shown to be transactivated by the region encoding both IE1 and IE2 gene products (50). When transfected individually, the UL36-38 and US3 gene products, as well as the IE1 gene product, appeared to be inactive in HFF cells for transactivation of UL112, whereas IE2 showed a dramatic stimulation (130-fold) of the UL112 promoter construction in HFF cells. Only cotransfection of the IE1 gene product synergistically stimulated the activity of IE2 (to 347-fold), while cotransfection of the genomic UL36-38 DNA with IE2 only enabled a modest increase (to 146-fold). Moreover, unlike the results using the *hsp*70 promoter, the UL36-38 cDNA expression clones only modestly increased further the IE2 transactivation (170-fold) of the UL112 promoter. The presence of US3 did not appre-

ciably alter the UL112 promoter transactivation by IE2. Therefore, the expression of the HCMV UL112 early gene promoter was significantly transactivated only by IE2 alone and increased linearly with 0 to 1 μg of added IE2 expression construct (14). Although IE1 and IE2 could synergistically activate the UL112 promoter, cotransfection with DNAs encoding the UL36-38 gene products and the IE2 gene product seemed to have an additive effect on UL112 expression.

Transactivation of the *hsp70* promoter in HeLa cells. Cellular transcription factors involved in HCMV-mediated transactivation may differ between the various human cell types. Differences in HeLa and HFF cells would suggest the participation of cell-type-specific factors in the transactivation of gene expression by HCMV IE gene products. We used HeLa cells to study the activity of HCMV IE gene products and to examine the involvement of cell-type-specific factors in transactivation of *hsp70*, UL112, and *ckb* promoter constructs. HeLa cells were used because the expression of transfected genes is much higher in these human cells than in permissive HFF cells. Moreover, much of our current understanding of the biochemistry of transcription comes from work with HeLa cells.

In contrast to our findings in HFF cells, the *hsp70*-CAT construction responded differently to individual HCMV IE gene products in HeLa cells (Fig. 2A). The *hsp70* promoter was negligibly transactivated by the promiscuous transactivator IE2 but moderately induced by US3 or UL36-38 and substantially induced by IE1. The enhanced expression of the *hsp70* promoter in response to IE1 is reminiscent of the activity of IE1 on the MIE promoter (10, 52) and on the interleukin 1-beta gene (33). From the UL36-38 and US3 results, we suggest that the UL36-38 and US3 gene products independently transactivate expression of the *hsp70* promoter.

We also observed activity of the UL36-38 and US3 regions upon cotransfection of pairwise combinations of the IE genes in HeLa cells (Fig. 2A). UL36-38 and US3 synergistically transactivated *hsp70* expression (to 18-fold), whereas cotransfection of UL36-38 and IE1 stimulated *hsp70* expression additively (to 13-fold). Additionally, IE2 decreased the stimulation of *hsp70* expression by either IE1 or UL36-38 in HeLa cells. The different results obtained in HFF and HeLa cells suggest a role for cell transcription factors in *hsp70* transactivation. Cellular transcription factors may affect whether or not UL36-38 proteins can act synergistically with either US3 gene products or additively with IE1 protein in stimulating *hsp70* expression.

Transactivation of the HCMV UL112 early promoter in HeLa cells. We examined UL112 promoter transactivation by the HCMV IE gene products in HeLa cells (Fig. 2B). Similar to the results in HFF cells (Fig. 1B), the HCMV early promoter construction did not respond to UL36-38, US3, or IE1 but responded positively in cells transfected with IE2 DNA (15-fold) (Fig. 2B). The magnitude of the response was much lower than that in HFF cells. Significantly, the HCMV early promoter construction was also stimulated synergistically to high levels by the combination of UL36-38 and IE2 (33-fold) or IE1 and IE2 (30-fold). This is in contrast with the results with HFF cells, in which we observed synergy only with IE1 and IE2.

Transactivation of the *ckb* promoter in HeLa cells. The promoter of another cellular gene, *ckb*, whose expression is induced during HCMV infection of human cells, was examined in parallel (Fig. 2C). The induction of *ckb* expression during HCMV infection occurs later than that of *hsp70* (12), suggesting a role for different HCMV gene products in *ckb* induction. To determine if the HCMV IE gene products were capable of inducing *ckb* expression as well as *hsp70* expression, HeLa cells were cotransfected with a *ckb* promoter-indicator construction and HCMV IE genes. In contrast with the *hsp70* promoter, the UL36-38 and US3 gene products, as well as the IE1 gene product, did not appear to regulate expression from the *ckb* promoter in HeLa cells (Fig. 2C). However, as previously observed with the UL112 early promoter, the *ckb* promoter responded to IE2 (11-fold). Cotransfection of HeLa cells with IE1 and IE2 increased slightly the stimulation by IE2 to 14-fold, whereas cotransfection with either US3 or UL36-38 decreased the transactivation of the *ckb* promoter by IE2. Therefore, the UL36-38 and US3 genes downregulated the *ckb* induction by IE2. The patterns of transactivation of the UL112 and *ckb* promoters by the US3 or UL36-38 IE proteins are distinct from each other and from that of the promiscuous transactivator IE2 (16, 18, 21, 27, 42, 43, 60).

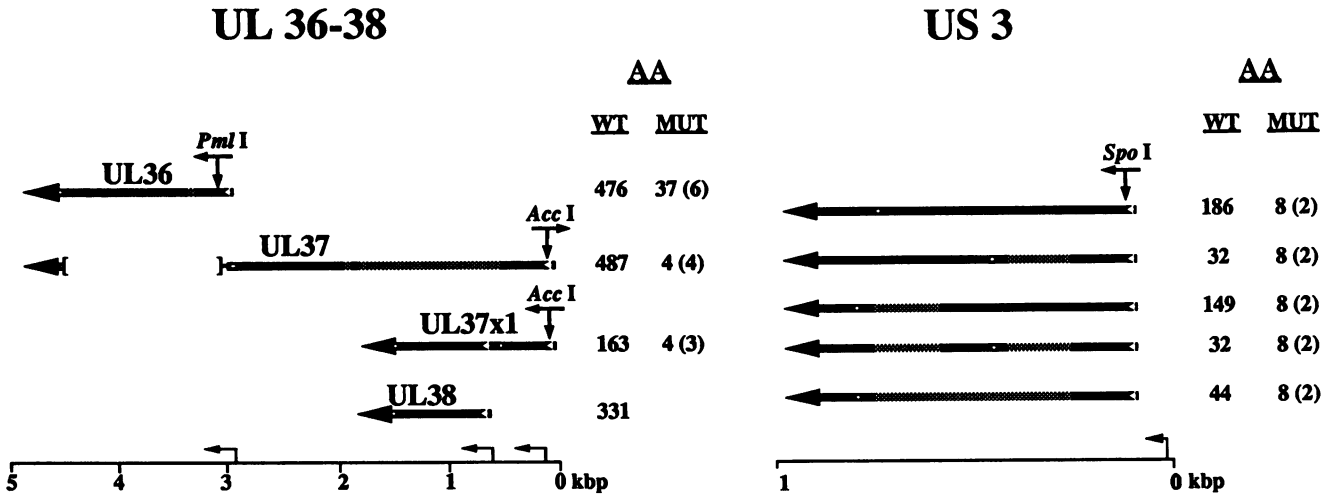
Nonsense mutations in UL36-38 and US3 abrogate transactivation of *hsp70* expression and indicate synergism between US3 and UL36-38. To allow studies examining the synergistic interactions between each of the UL36-38 gene products and US3 in HeLa cells (Fig. 2A), individual cDNA expression clones of UL36-38 or a genomic US3 clone encoding either wild-type or truncated proteins were constructed (Fig. 3). The UL36, UL37, UL37x1, and US3 open reading frames were mutated by insertion of the nonsense linker into each clone. The nonsense mutations result in predicted truncated protein products with less than 38 wild-type amino acids (aa) (less than 44 aa total). It is highly unlikely that these peptides have any activity on gene expression.

The effects of cotransfection of US3 and each of the UL36-38 cDNAs on *hsp70* transactivation were examined (Table 2). Cotransfection of HeLa cells with wild-type constructs of US3 and UL36 (10-fold), UL37 (11-fold), or UL37x1 (8-fold) cDNAs resulted in induction of *hsp70* expression above that afforded by US3 alone. No *hsp70* transactivation above that afforded by US3 alone was observed in cells cotransfected with wild-type expression clones of US3 and UL38, an early transcript encoded by the UL36-38 IE locus (58).

We demonstrated that US3 proteins are required for this synergistic transactivation of *hsp70* expression by the use of a US3 nonsense mutant (Table 2). No significant *hsp70* transactivation was seen when cotransfections were done with the mutant US3 construction.

Using nonsense mutations in UL36-38 IE genes, we could abolish synergistic transactivation of *hsp70* expression (Table 2). When combined with the wild-type US3 expression clone, nonsense mutations in UL36, UL37, and UL37x1 constructions were unable to increase *hsp70* expression above that of US3 alone. Our results demonstrate that induction of *hsp70* expression by US3 and UL36-38 requires IE protein products and does not result from DNA or RNA effects on gene expression. Moreover, our findings indicate that HCMV IE proteins encoded by US3 and each of the UL36-38 IE gene products, UL36, UL37, and UL37x1, can synergistically increase *hsp70* gene expression.

Titration of UL37 activity. Because the UL37 IE protein is predicted to be a membrane-bound glycoprotein (35), we examined *hsp70* transactivation by UL37. The dose responsiveness of the *hsp70* promoter construction to UL37 gene products was examined by transfecting different amounts of UL37 and mutant UL37 DNA. *hsp70* expression in HeLa cells was found to increase at the level of CAT enzymatic activity with increasing amounts of added UL37 DNA (Fig.



STOP LINKER

5'-CTA-GGC-CTT-AGC-GGC-CGC-TAG-3'
3'-GAT-CCG-GAA-TCG-CCG-GCG-ATC-5'

FIG. 3. UL36-38 and US3 nonsense mutants. The HCMV UL36-38 and US3 loci are shown. The promoters in the loci are represented by the bent arrows. The exons (thick lines) and introns (stippled boxes) are shown along with the start (open arrowheads) and termination of translation (open circles) in each expression clone. The UL36 (p326), UL37 (p414), UL37x1 (p327), and US3 (p339) open reading frames were mutated by insertion of the stop linker shown at the bottom of the figure. The linker has termination codons in all three reading frames and in both orientations. The insertions were placed at the indicated restriction site in each plasmid and the orientation (represented by the direction of the arrow above the corresponding restriction site) of the insert was determined by nucleotide sequencing. The predicted numbers of wild type and missense (in parentheses) amino acids encoded by each wild-type (WT) and mutant (MUT) construction are listed.

4). A significant induction of *hsp*70 expression occurred with 1 μg of added wild-type UL37 DNA but not with the same amount of mutant UL37 DNA. At 10 μg, which is beyond the range used in our experiments, the transactivation by wild-type UL37 is reduced and the distinction between wild-type UL37 and mutant UL37 is obscured.

Transactivation at the level of *hsp*70-promoted RNA. The 5' end of the *hsp*70-*neo* RNA (Fig. 5A) corresponded to the correct start site for the *hsp*70 transcript as previously mapped by Hunt and Morimoto (31). Stimulation by US3 and UL37 did not alter this position, indicating transactiva-

TABLE 2. Nonsense mutations in UL36-38 and US3 genes abrogate *hsp*70 transactivation

HCMV gene	Avg induction (fold) of *hsp*70 expression	
	Wild-type US3	Mutant US3
Wild type		
UL36 (p326)	10.4	1.5
UL37x1/UL38 (p327)	7.6	1.2
UL37 (p414)	10.6	1.5
UL38 (p406)	0.8	1.0
pUC18	1.0	1.0
Mutant		
UL36 (p425)	1.0	
UL37x1 (p426)	1.2	
UL37 (p428)	2.3	

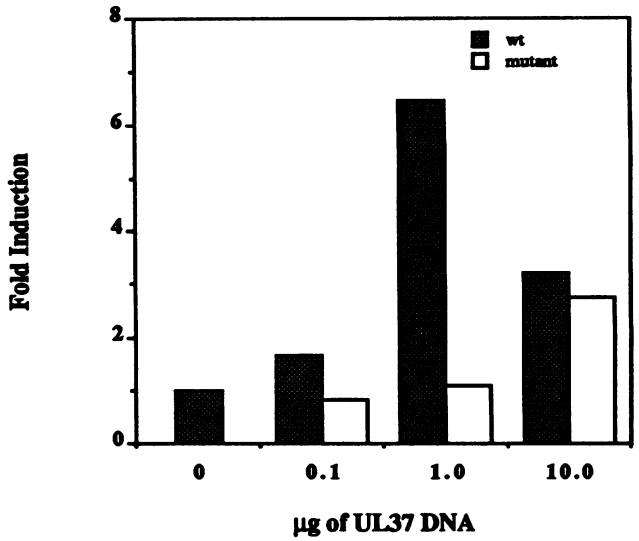


FIG. 4. Dose response of the *hsp*70-CAT construct to UL37. HeLa cells were transfected with pHBCAT (10 μg), US3 (p339; 5 μg), and different amounts (0, 0.1, 1, or 10 μg) of wild-type (wt) UL37 (p414) or mutant UL37 (p428). Protein extracts were assayed for CAT activity (1 μg for 15 min). Fold induction was determined by comparison with cells transfected with pHBCAT, US3, and pUC18.

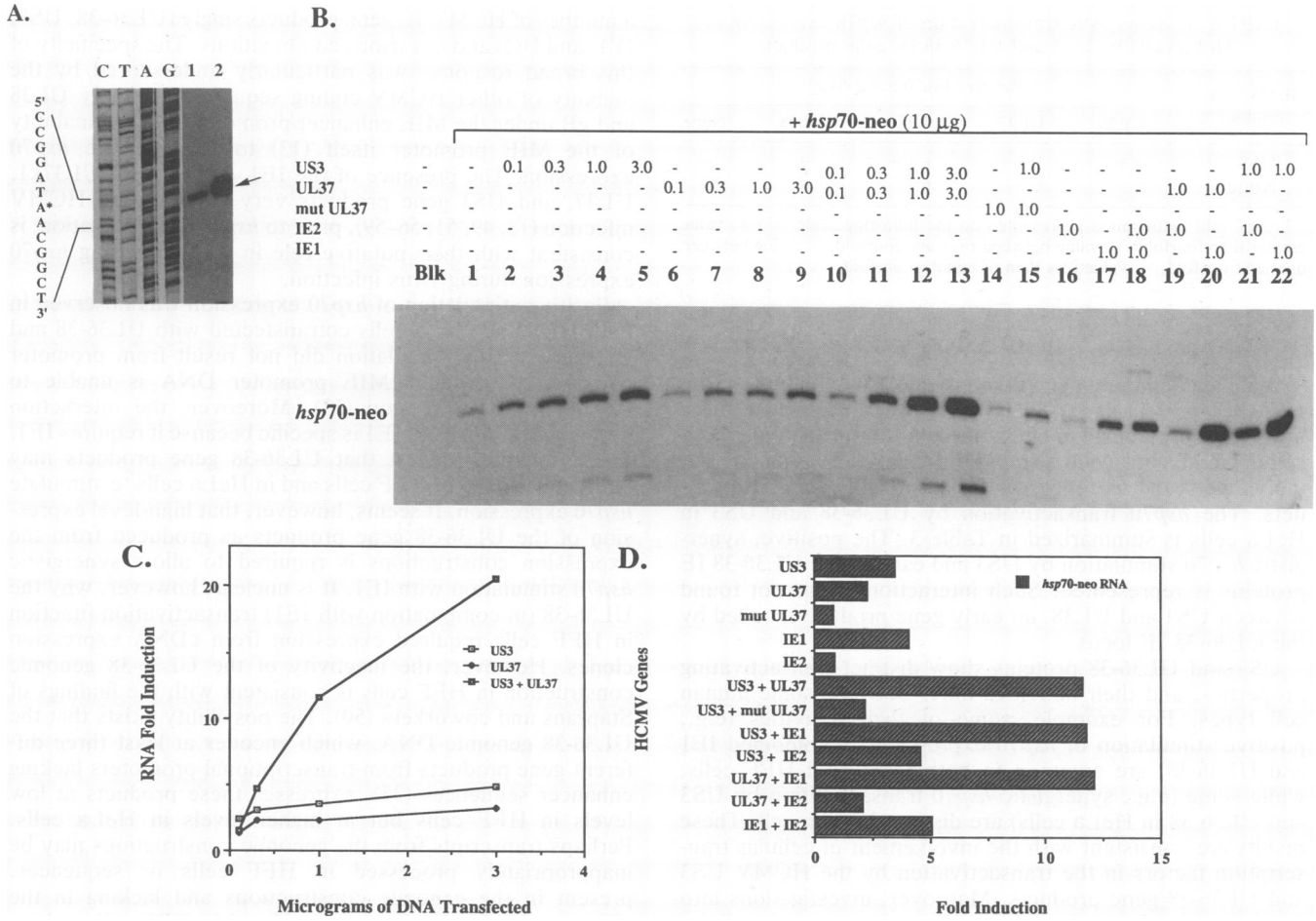


FIG. 5. *hsp*70-promoted RNA. (A) The 5′ end of the *hsp*70-*neo* transcript was mapped by primer extension. HeLa cells were cotransfected with *hsp*70-*neo* DNA (p423; 10 μg) and either pUC18 (lane 1) or with UL37 (p414; 1 μg) and US3 (p339; 1 μg) (lane 2) DNA. The arrowhead indicates the extension product corresponding to the 5′ end of the *hsp*70-*neo* transcript (marked by the asterisk) as determined by comparison to parallel sequencing reactions of *hsp*70-*neo* DNA. The sequence of the *hsp*70 RNA start site (31) is aligned vertically from 5′ to 3′. (B) The abundance of *hsp*70-*neo* transcript was monitored by primer extension as described in Materials and Methods. HeLa cells were transfected with *hsp*70-*neo* (p423; 10 μg) DNA and either pUC18 (lane 1) or with increasing amounts of US3 (p339; 0.1, 0.3, 1, and 3 μg) (lanes 2 to 5), with increasing amounts of UL37 (p414; 0.1, 0.3, 1, and 3 μg) (lanes 6 to 9), with both US3 and UL37 (0.1, 0.3, 1, and 3 μg each) DNA (lanes 10 to 13), with mutant UL37 (p428; 1 μg) (lane 14), or with mutant UL37 (p428; 1 μg) and US3 (p339; 1 μg) (lane 15). HeLa cells were also cotransfected with *hsp*70-*neo* and with IE2 (pBK1; 1 μg) (lane 16), IE1 (pRR59; 1 μg) (lane 17), both IE1 and IE2 (1 μg each) (lane 18), with UL37 (p414; 1 μg) and IE2 (pBK1; 1 μg) (lane 19), with UL37 (p414; 1 μg) and IE1 (pRR59; 1 μg) (lane 20), with US3 (p339; 1 μg) and IE2 (pBK1; 1 μg) (lane 21), or with US3 (p339) and IE1 (pRR59); 1 μg each) (lane 22). As a negative control, cells were transfected with pUC18 (lane Blk). The arrow indicates the band corresponding to the cDNA product of the *hsp*70-*neo* RNA. (C) Dose response of *hsp*70 RNA to US3, UL37, or UL37 and US3. The fold induction of the *hsp*70-*neo* RNA in each group was determined by comparison of the radioactivity in the *hsp*70-*neo* cDNA in lanes 2 to 13 of panel B with that corresponding to the cDNA products of *hsp*70-*neo* in cells transfected with *hsp*70-*neo* (p423) and pUC18 (lane 1, panel B). (D) Induction of *hsp*70-*neo* RNA abundance by HCMV IE gene products. The fold induction of *hsp*70-*neo* RNA in each group was determined by comparison of the radioactivity in the *hsp*70-*neo* cDNA in panel B from cotransfections of either US3 (lane 4), UL37 (lane 8), UL37 mutant (lane 14), IE1 (lane 17), IE2 (lane 16), IE1 and IE2 (lane 18), US3 and UL37 (lane 12), US3 and mutant UL37 (lane 15), US3 and IE1 (lane 22), US3 and IE2 (lane 21), UL37 and IE1 (lane 20), or UL37 and IE2 (lane 19) with that from cells transfected with p423 and pUC18 alone (lane 1).

tion of the authentic *hsp*70 promoter sequences. Moreover, the abundance of the *hsp*70-*neo* RNA modestly increased with increasing amounts of individually transfected US3 DNA or UL37 DNA to 3 μg (Fig. 5B and C). At 1 and 3 μg of cotransfected US3 and UL37 DNA, the abundance of *hsp*70-*neo* RNA was increased above levels present in cells transfected with either alone. Finally, the abundance of *hsp*70-*neo* RNA in cells transfected with combinations of US3 and UL37, US3 and IE1, or UL37 and IE1 was increased above the levels in cells transfected with single DNAs (Fig. 5D). Mutant UL37 singly transfected or when combined with US3 did not increase the abundance of *hsp*70-*neo* RNA above that present in cells transfected with US3 alone. The pattern of *hsp*70-promoted RNA induction for each combination of HCMV gene products correlates well with the pattern of *hsp*70-CAT fold induction previously obtained (Fig. 2A) and validates the use of CAT assays in our transactivation studies.

TABLE 3. Synergistic transactivation of *hsp*70 expression in HeLa cells by US3 and UL36-38 IE gene products

HCMV gene product	Relative *hsp*70 expression[a]				
	UL36	UL37	UL37x1	UL38	None
US3	+++	+++	+++	+	+
None	±	±	±	−	−

[a] CAT fold induction is represented on the following scale: +++, value greater than fourfold; +, value between two- and fourfold; ±, value between one- and twofold; −, value less than or equal to onefold.

DISCUSSION

We have convincingly demonstrated the ability of the HCMV UL36-38 and US3 IE gene products, whose functions were previously uncharacterized, to transactivate both cellular and viral gene expression. These proteins can act individually and in concert with the other HCMV IE products. The *hsp*70 transactivation by UL36-38 and US3 in HeLa cells is summarized in Table 3. The positive, synergistic *hsp*70 stimulation by US3 and each of the UL36-38 IE proteins is represented. Such interactions were not found between US3 and UL38, an early gene product encoded by the UL36-38 IE locus.

US3 and UL36-38 proteins show distinct transactivating properties, and their activities differ between some human cell types. For example, some of their activities (e.g., positive stimulation of *hsp*70 expression by combined IE1 and UL36-38) are common to both HeLa and HFF cells, while some (e.g., synergistic *hsp*70 transactivation by US3 and UL36-38 in HeLa cells) are distinct in each cell. These results are consistent with the involvement of cellular transcription factors in the transactivation by the HCMV US3 and UL36-38 gene products. Moreover, investigations into the breadth of activity with different viral and cellular promoters indicate that UL36-38 and US3 IE products, unlike IE2, are not promiscuous transactivators of gene expression (14).

Most significantly, the induction of *hsp*70, *ckb*, and UL112 gene expression which occurs normally during HCMV infection was reproduced by the HCMV IE induction of their promoters in transient transfections. The kinetics of *ckb* and UL112 RNA expression during HCMV infection previously suggested transactivation of these two genes by the same or simultaneously expressed HCMV gene products. These products, however, would be different from those involved in *hsp*70 stimulation, which occurs earlier. The transient assays in the current study demonstrated that HCMV IE induction of both the *ckb* and HCMV UL112 early promoters was selective and very similar. Both the *ckb* promoter and the HCMV UL112 early promoters responded to the IE2 gene products. In both HeLa and HFF cells, the UL112 promoter was dramatically activated by the combination of IE1 and IE2. Our results are in agreement with those of numerous laboratories showing synergy between IE1 and IE2 in the activation of gene expression (18, 19, 27, 37, 43, 50, 52, 60). A similar synergy was found on UL112 promoter activity in HeLa cells when UL36-38 DNA was cotransfected with IE2. These gene products stimulated UL112 activity also in HFF cells but only to an additive level. Thus, cellular transcription factors may affect the ability of UL36-38 proteins to act synergistically or additively with IE2 protein in stimulating UL112 expression.

In contrast to the limited response of both the *ckb* and UL112 promoters, the *hsp*70 promoter responded broadly to a number of HCMV IE gene products singly (UL36-38, US3, IE1, and IE2) and in various combinations. The specificity of this broad response was particularly underscored by the inability of other HCMV coding sequences, notably UL38 and gB under the MIE enhancer/promoter, and the inability of the MIE promoter itself (13) to transactivate *hsp*70 expression. The presence of the IE1, IE2, UL36, UL37x1, UL37, and US3 gene products very early during HCMV infection (12, 49, 51, 56–59), prior to *hsp*70 transactivation, is consistent with their putative role in transactivating *hsp*70 expression during virus infection.

Positive stimulation of *hsp*70 expression was observed in both HFF and HeLa cells cotransfected with UL36-38 and IE1 genes. This stimulation did not result from promoter competition, because MIE promoter DNA is unable to induce *hsp*70 expression (13). Moreover, the interaction between UL36-38 and IE1 is specific because it requires IE1. These findings suggest that UL36-38 gene products may interact with IE1 in HFF cells and in HeLa cells to stimulate *hsp*70 expression. It seems, however, that high-level expression of the UL36-38 gene products as produced from the expression constructions is required to allow synergistic *hsp*70 stimulation with IE1. It is unclear, however, why the UL36-38 (in combination with IE1) transactivation function in HFF cells required expression from cDNA expression clones. However, the inactivity of the UL36-38 genomic construction in HFF cells is consistent with the findings of Staprans and coworkers (50). The possibility exists that the UL36-38 genomic DNA, which encodes at least three different gene products from transcriptional promoters lacking enhancer sequences (35), expresses these products at low levels in HFF cells but at higher levels in HeLa cells. Perhaps transcripts from the genomic constructions may be inappropriately processed in HFF cells or sequences, present in the genomic constructions and lacking in the cDNAs, could be negatively regulated in HFF cells. Alternatively, *hsp*70 transactivation may be observed in HeLa cells, in spite of low levels of UL36-38 products, because of differences of cellular transcription factors present in these cells. Whatever the case, we were able to overcome the block by expressing the UL36-38 IE cDNAs (13, 57, 58) from the strong HCMV MIE enhancer/promoter.

The synergistic stimulation of *hsp*70 expression by US3 and UL36-38 IE gene products in HeLa cells is similar to the synergism between IE1 and IE2 in gene regulation (18, 27, 37, 43, 48, 50, 52, 60). We found the interactions were specific for the IE products as the UL38 early gene product did not show activity. *hsp*70 transactivation by UL36-38 and US3 occurred at the level of stable RNA, and the response was dose dependent. The dose-dependent *hsp*70 response to wild-type UL37 and the inactivity of UL36, UL37, and UL37x1 nonsense mutants solidifies the contention that IE protein products are required for the transactivations observed. We therefore found a clear requirement for these IE protein products in *hsp*70 transactivation.

Synergy was demonstrated by use of US3 mutant and UL36-38 mutants; *hsp*70 transactivation was absent in HeLa cells cotransfected with mutants from either IE locus compared with that in cells cotransfected with wild-type constructions. Taken together, our results establish convincingly the ability of the UL36-38 and US3 IE proteins to act as regulators of gene expression. They also suggest that these IE proteins stimulate gene expression through pathways which are independent but that can interplay positively.

The mechanisms responsible for transactivation of cellular and viral promoters by the HCMV IE genes have yet to be

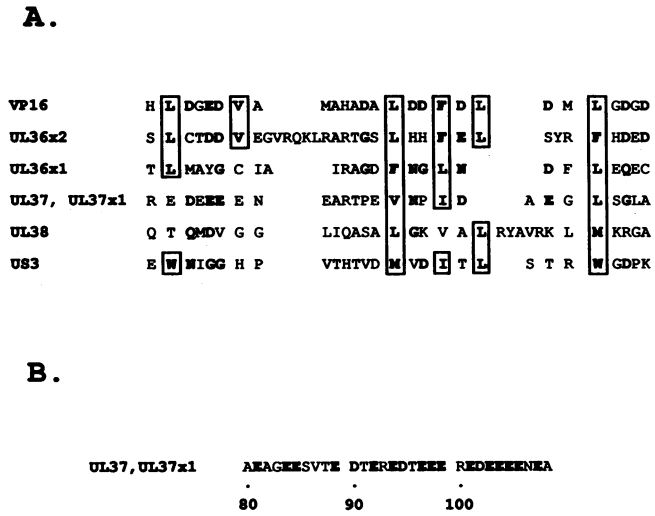


FIG. 6. (A) Alignment of the amino acid sequences of the activator domain of VP16 (15) and the predicted amino acid sequences of UL36-38 and US3 (35, 62). Shown are VP16 (aa 425 to 451), UL36x2 (aa 389 to 423), UL36x1 (aa 7 to 32), UL37 and UL37x1 (aa 100 to 126), UL38 (aa 22 to 58) and US3 (aa 97 to 124). The amino acids are shown in the single-letter code. The conserved bulky hydrophobic residues are boxed, and conserved, critical acidic residues are in boldface. Comparisons were aided by the gap sequence comparison program. (B) Acidic stretch of aa 81 to 108 of UL37 and UL37x1 (35). The acidic residues (D and E) are shown in boldface letters.

defined. IE1 has been shown to act through the 18-bp repeat motif of the MIE enhancer (10). This finding suggests that IE1 acts by regulating transcription. Previous studies with other promoters transactivated by IE2 are consistent with either transcriptional or posttranscriptional regulation. Although the proteins encoded by the MIE region have been extensively studied, only some proteins have been ascribed regulatory activity. To address the mechanism of IE2 transactivation, we have analyzed mutations of the *ckb* promoter (24, 25).

The HCMV UL36-38 locus encodes three (UL36, UL37x1, and UL38) products which are predicted to be soluble proteins (35). Of these, UL36 contains two regions with sequence homology to the bulky hydrophobic residues (Fig. 6A) located in the activation domain of VP16 (aa 425 to 451) and the three recognized classes of transcriptional activators (15, 40). The most conserved is located in UL36 exon 2 (aa 389 to 423) and contains five identical (of the six) bulky hydrophobic residues and a conservative substitution in the other position. Several of the critical acidic residues for VP16 activation (15) are conserved (6 of 10 aa) in UL36x2. The second region (aa 7 to 32), encoded by UL36 exon 1, shows conservation of the bulky hydrophobic residues (two identical and two conservative substitutions) and critical acidic residues (one identical and six conservative substitutions). The similarities are less striking between the activator domain of VP16 and the sequences of UL37, UL37x1, UL38, and US3. Nevertheless, UL37 and UL37x1 contain a highly acidic stretch of amino acids (aa 81 through 108 include 15 glutamic acid and 3 aspartic acid residues [Fig. 6B]), another feature common to some transcriptional activators (40). In contrast to UL37x1, however, the UL37 protein is predicted to be a membrane protein. Its sequences encode a hydrophobic signal sequence, a potential transmembrane domain, and multiple N-linked glycosylation sites (35). The largest unspliced US3 transcript is also predicted to encode a transmembrane glycoprotein (62). We have shown that these proteins are regulators of cellular and viral gene expression. There are precedents for membrane-bound regulators of nuclear gene expression in numerous cellular and viral proteins, which serve as protein kinases, receptors coupled to G proteins, or ion channels, and can alter the expression of nuclear genes (32). We are undertaking experiments to understand the mechanism by which the HCMV UL36-38 IE gene products alter gene expression.

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