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# Transactivation of the Cytomegalovirus ICP36 Gene Promoter Requires the α Gene Product TRS1 in Addition to IE1 and IE2

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**Very little is known about the human cytomegalovirus functions that activate γ (late) gene expression. We have investigated the regulation of the human cytomegalovirus γ gene encoding the ICP36 major late DNA-binding protein family (UL44). Transactivation of the ICP36 gene promoter was found to be absolutely dependent on the *trs*1 gene product when expressed in cells in conjunction with *ie*1 and *ie*2 gene products. Transactivation occurred poorly or not at all when any one of these three transactivators was omitted. TRS1 is a member of the US22 family of proteins and is encoded by a region near the L-S junction of the viral genome within the *c* repeat and adjacent $U_S$ sequences. TRS1 is highly homologous to IRS1, which is encoded from the other copy of the *c* repeat, and plasmid constructs carrying the *irs*1 gene were also able to mediate transactivation of the ICP36 promoter. RNA blot analysis of steady-rate RNA throughout infection showed that the *trs*1 transcript was expressed with the kinetics of an α gene but its accumulation was delayed relative to that of *ie*1 and *ie*2 transcripts. On the basis of these experiments, TRS1 and IRS1 are proposed to be important intermediaries in the cascade of cytomegalovirus gene expression.**

Human cytomegalovirus (CMV) is a medically important betaherpesvirus that typically causes disease in immunocompromised persons such as AIDS patients and those undergoing transplant surgery. It is also a common infectious cause of congenital birth defects (2). The virus has a 230-kbp double-stranded DNA genome in which over 200 open reading frames (ORFs) have been predicted (6). The large genome size and protein-coding capacity as well as the slow replication cycle of this virus have impeded characterization of the vast majority of these ORFs. In common with other herpesviruses, however, gene expression falls into at least three kinetic classes, α (immediate early), β (delayed early), and γ (late). The α genes are the first to be expressed following entry of the virus into the cell, and expression of these genes does not require prior protein synthesis.

The major α gene locus located within the $U_L$ region of the genome (between 169 and 175 kbp) consists of an extremely strong promoter-enhancer driving expression of 5′-coterminal transcripts encoding *ie*1 and *ie*2 gene products via differential splicing (1, 3, 48, 50–52, 54, 57). IE1, a 72-kDa (72 K) (491-amino-acid) protein, has been shown by transient transfection analyses to autoregulate the *ie*1 promoter-enhancer via the host cell factor NF-κB, which interacts with sites in the 18-bp repeats (8, 43). The *ie*2 gene encodes a number of proteins expressed immediately after infection and also encodes a predicted 40K protein as a γ gene product (48). The prominent 86K (579-amino-acid) IE2 has been shown by several laboratories to be a transactivator of a variety of CMV and non-CMV promoters, particularly when expressed together with IE1 (11, 13, 21, 31, 41, 47, 56). By transient assay analysis, IE1 and IE2 have been implicated in the regulation of a number of β genes, including those encoding gp48 (5, 31), DNA polymerase (49), the UL112-UL113 family of phosphoproteins (47), and the strongly expressed 2.7-kb transcript (19, 23, 33). In addition to its role as a transactivator, IE2 also appears to mediate autorepression of the *ie*1/*ie*2 transcription unit (20, 41, 49) through a *cis*-repression signal located just upstream of the transcriptional start site (7, 30, 40).

The γ (late) genes may be classified as either $\gamma_1$ (leaky late) or $\gamma_2$ (true late). $\gamma_1$ gene transcription and translation start prior to viral DNA replication, but maximum expression occurs after replication. $\gamma_2$ gene expression is strictly dependent on viral DNA replication. The α proteins encoded by IE1 and IE2 appear sufficient for the activation of at least one late gene, encoding pp65 (11), a gene showing transcriptional patterns characteristic of either a β or $\gamma_1$ gene (17). The regulation of other γ genes of CMV remains uncharacterized. The cascade model of gene regulation predicts that their expression would be dependent on the expression of viral transactivators earlier in infection.

Previous work from this laboratory has investigated promoter elements controlling the expression of ICP36 (major late DNA-binding protein family or UL44) gene in order to gain some insights into γ gene regulation. ICP36 has been suggested to be the functional homolog of herpes simplex virus type 1 UL42, one of the seven viral proteins shown to be essential for viral DNA replication (61). UL42 has been shown to stimulate viral DNA polymerase processivity in vitro (16, 22), and this activity has been shown to be preserved by ICP36 acting on the CMV DNA polymerase (12).

The promoter of the ICP36 gene was previously shown to use three transcriptional start sites that were expressed differentially throughout infection (29) (Fig. 1B). Transcription initiation from start sites 1 and 3 was shown to take place from early times postinfection (8 h postinfection [hpi]) to late times (more than 36 hpi), showing kinetics of β or $\gamma_1$ promoters. Start site 2 was shown to have characteristics of a $\gamma_2$ gene and was active only after the initiation of viral DNA replication. ICP36 expression is initiated from a single start codon located within all three of these transcripts, and although translation starts at early times, maximal synthesis occurs at late times of infection and may be influenced by posttranscriptional events (17, 36).

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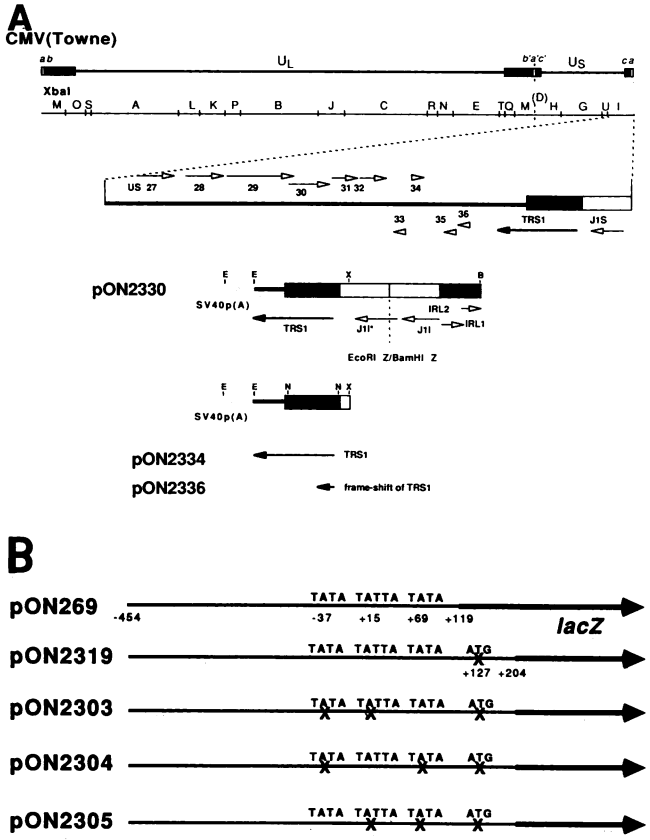


FIG. 1. (A) Structure of the human CMV (Towne) genome and plasmid constructs. The top line is a schematic representation of the genome, and the *Xba*I restriction map is shown below. The *a* sequence repeat is depicted as an open block and the *b* and *c* sequence repeats are shown as filled blocks. These repeats are present in the inverse orientation (*a'*, *b'*, and *c'*) at the L-S junction. Below the genome, the *Xba*I fragment is expanded to show the predicted ORFs in this region as open arrows (6). TRS1 is distinguished by a dark arrow. In addition, the structure of pON2330 derived from pON232 (37) is shown. pON2330 is an L-S junction fragment composed of the *Bam*HI Z L-terminal fragment and the *Eco*RI Z S-terminal fragment. The 239-bp fragment containing the SV40 early polyadenylation signal [SV40 p(A); stippled bar] is indicated. In addition to TRS1, pON2330 also contains other ORFs, IRL1 and IRL2 in the *c* sequence and J1I and J1I* in the *a* sequence repeats. J1I* is a 251-amino-acid ORF that would be predicted to lie across the *a-a* junction. pON2334 carries the 2.6-kbp *Eco*RI-*Xho*I fragment encoding TRS1 with the SV40 polyadenylation signal as in pON2330. pON2336 carries the same *Eco*RI-*Xho*I fragment but with a frameshift mutation created in amino acid 13 of TRS1 by selective digestion, fill-in, and religation at the 5' *Not*I site. Approximate restriction enzyme site positions are indicated for *Eco*RI (E), *Xho*I (X), *Not*I (N), and *Bam*HI (B). (B) ICP36 promoter target constructs. The ICP36 promoter region is shown with nucleotide positions relative to transcription start site 1. The three TATA elements of the ICP36 promoter are depicted, and an X indicates where point mutations were introduced to destroy a TATA box. The ICP36 start codon is also shown with an X when mutated from ATG to CCA. *E. coli lacZ* is indicated by a thick arrow.

The studies described in this report were performed to determine whether the ICP36 promoter was responsive to IE1 and IE2 or to other viral gene products as would be predicted by the cascade model of herpesvirus gene expression. These studies enabled the identification of a previously unrecognized transactivator protein, TRS1, an α gene product that acts together with IE1 and IE2 to increase expression from the ICP36 promoter in transient transfection assays.

(A preliminary presentation of this work was made at the Third International Cytomegalovirus Workshop and the 16th International Herpesvirus Workshop in June and July 1991.)

## MATERIALS AND METHODS

**Viruses and cell culture.** Human foreskin fibroblast (HFF) cells were grown in Dulbecco's minimum essential medium (DMEM; GIBCO, Grand Island, N.Y.) supplemented with 10% NuSerum (Collaborative Research Inc., Waltham, Mass). Infection with human CMV (Towne strain) was performed at a multiplicity of infection of 5 to 10 PFU per cell, using frozen stocks of CMV as previously described (44). Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was dissolved in medium at a concentration of 50 μg/ml, added to cells 20 min prior to the time of infection, and maintained throughout the infection period. Phosphonoformic acid (Sigma) was dissolved in a medium at a concentration of 200 μg/ml and added to cells immediately following the 1 h of adsorption.

**Plasmid constructs.** Restriction enzymes, *Escherichia coli* DNA polymerase I Klenow fragment, T4 DNA ligase, and T4 DNA polymerase (New England Biolabs Inc., Beverley, Mass.) were used by established protocols (32). Sequenase (United States Biochemical Corp., Cleveland, Ohio) was used as recommended by the manufacturer.

Point mutations within the ATG start codon of pON268 and within the three TATA elements of the ICP36 promoter in pON268 were made by using the system described by Kunkel (25). An 18-mer oligonucleotide was constructed to alter the ICP36 ATG to CCA, creating pON2319. Oligonucleotides (24-mers) were constructed to make the following changes to the TATA elements: TATA element 1, TAT ATAA to TGCATAA; TATA element 2, TATTATTA to TG CTATTA; and TATA element 3, TATAAAA to TGCAAAA (Fig. 1B). The *Sma*I-*Xba*I insert from pON268 was cloned into Bluescript (SK-) (Stratagene, La Jolla, Calif.) and transformed into competent cell preparations of the *dut ung E. coli* strain CJ236. Single-stranded DNA that contained uracil deoxynucleotides in place of thymidine could then be prepared. The oligonucleotides were annealed to this single-stranded DNA, and the double-stranded plasmid was synthesized by using excess deoxynucleotides (Pharmacia, LKB Biotechnology, Piscataway, N.J.), T4 DNA polymerase, and T4 DNA ligase. Transformation into competent *E. coli* DH5α therefore resulted in selection of the newly synthesized strand containing thymidine and the desired mutation. The insert was then subcloned back into the pON1 vector (45), containing the *lacZ* indicator gene. The mutations were confirmed by sequencing of the double-stranded DNA used in the transfections (44). pON2303 contained mutations in the ATG and TATA elements 1 and 2. pON2304 contained mutations in the ATG and TATA elements 1 and 3. pON2305 contained mutations in the ATG and TATA elements 2 and 3.

pON2330 was constructed by cloning a 239-bp *Eco*RI fragment containing the simian virus 40 (SV40) early polyadenylation signal (bp on the SV40 genome 2533 to 2770) from pON829 (59) into the unique *Eco*RI site 39 bp downstream of the TRS1 stop codon in the construct pON232 (38). pON2334 was constructed by subcloning the 2.6-kbp *Xho*I-*Eco*RI fragment from pON232 (38) into Bluescript (SK-).

This fragment contains the TRS1 ORF plus 322 bp upstream of the TRS1 start codon. The SV40 poly(A) signal was added as before at the unique *Eco*RI site 39 bp downstream of the TRS1 stop codon (Fig. 1A).

A frameshift mutation within the TRS1 ORF was created by partial digestion of pON232 with *Not*I, which cuts this construct twice, once at nucleotide 30 (disrupting amino acid 13) of TRS1 and at nucleotide 1560 (disrupting amino acid 523) of TRS1. Plasmids which were cut only once were selected, and the *Not*I site was filled in with excess deoxynucleotides (Pharmacia), using the Klenow fragment of DNA polymerase. The ends were joined with T4 DNA ligase, and after transformation of competent *E. coli* DH5α, DNA from individual colonies was tested by restriction enzyme digestion to identify the plasmid with the most 5′ *Not*I site destroyed. The desired frameshift was confirmed by sequencing of the double-stranded DNA, using Sequenase and a primer complementary to sequence 50 bp upstream of the *Not*I site (44). This construct, pON2331, was used to isolate the TRS1 ORF as for pON2334. Thus, the 2.6-kbp *Xho*I-*Eco*RI fragment of pON2331 containing the frameshifted TRS1 sequence was then cloned into Bluescript (SK-), and the SV40 polyadenylation signal within a 239-bp *Eco*RI fragment from pON829 (59) was cloned into the *Eco*RI site 39 bp downstream of the TRS1 stop codon as before to create the construct pON2336 used in transfection analyses.

Plasmids pON303G, pON308G, $pON308_{FS}$, pON303 ΔAcc, pON249, and pON232 have been described previously (7, 8, 18, 38). The pXbaI library of the Towne strain of CMV was kindly provided by M. Stinski (58), and the AD169 strain cosmid clone pCM1015 was provided by B. Fleckenstein (15). pON2401, containing approximately 2.5 kb of sequence complementary to the β2.7 transcript, was provided by G. Duke (11a).

**Transient assays and RNA analysis.** HFF cells grown in 35-mm dishes were transfected by the DEAE-dextran method as previously described (45) within 36 h of being seeded. The ratio of effector to target DNA in cotransfections of two constructs was 1:0.5 (1 μg:0.5 μg). Where two effector constructs were cotransfected with a target construct, a ratio of 1:1:0.5 was used. Alteration of this ratio did not affect β-galactosidase (β-Gal) levels appreciably. Briefly, the DNAs were added to 0.5 ml of DMEM–50 mM Tris hydrochloride (pH 7.5), and DEAE-dextran (Pharmacia Fine Chemicals) was added to 200 μg/ml. The transfection mix was added to wells containing $5 \times 10^5$ to $10 \times 10^5$ cells for 3.5 to 4 h before being washed once and replaced with 2 ml of fresh DMEM supplemented with 10% NuSerum. The cells were incubated at 37°C, and 48 h posttransfection, the medium was replaced with medium containing a fluorogenic substrate for β-Gal, 4-methylumbelliferyl-β-D-galactoside (MUG), at a concentration of 150 μg/ml. Aliquots of this medium were taken at time intervals 72 and 96 h posttransfection, and β-Gal activity was measured by a fluorometric assay as previously described (18). β-Gal levels described here represent the ratio of MUG fluorescence units obtained after cotransfection of the effector and target plasmids over levels obtained after transfection of the target plasmid alone.

In each figure, the results of one representative experiment are shown and numbers are the averages of two or three separate cotransfections. The cotransfection experiments were repeated on at least three separate occasions. Levels of β-Gal activity that were at least threefold over levels observed after transfection of a target construct alone were considered significant. Within any experiment, the relative levels of β-Gal activity for each cotransfection remained constant. Each transfection experiment included control cotransfections of the effector construct pON303G (IE1+IE2) (8), with the target pON249 (18) containing the *ie*1 promoter-enhancer driving the *E. coli lacZ* gene. Autostimulation of the enhancer by IE1 and IE2 as described previously (8) was consistently observed, and β-Gal levels measured 72 or 96 h posttransfection were at least sixfold or greater over levels measured after transfection of the target alone. In addition, cotransfection of pON249 with the effector plasmid pXbaE, which contains the major immediate-early transcription unit expressing IE1 and IE2, was also used as a positive control in the majority of experiments (data not shown).

Total cell RNA from mock-infected or CMV (Towne)-infected HFF cells was prepared by the guanidinium isothiocyanate method as previously described (9, 33). For RNA blot analysis, 5 μg of RNA was separated on a 1% agarose–1.2 M formaldehyde gel, blotted onto nitrocellulose, and probed with DNA labelled by the random priming method (14) with [α-$^{32}$P]CTP (Amersham Corp., Arlington Heights, Ill.). A *trs*1-specific probe was created by isolation of a 0.6-kbp *Hin*cII-*Eco*RI DNA fragment from pON2333. A probe complementary to IE1 sequence was prepared by isolation of a 3.8-kbp *Xba*I-*Hin*dIII fragment from pON308G (8). A probe complementary to sequence of the early $\beta_{2.7}$ transcript (18) was prepared by isolation of a 2.0-kbp *Nsi*I fragment from pON2401 (11a).

## RESULTS

**Identification of a factor that functions together with IE1 and IE2 to transactivate the ICP36 promoter.** Earlier studies had shown that the target construct pON269 containing 572 bp of the ICP36 promoter linked to the *E. coli lacZ* gene transfected into HFF cells could be transactivated by CMV infection (29). We initially tested whether IE1 and IE2 were capable of transactivating the ICP36 promoter in transient assays. The effector construct pON303G, containing the genomic region encoding IE1 and IE2, was transfected into HFF cells together with the target construct pON269. Expression of β-Gal from pON269 was measured by a MUG cleavage assay at 72 h posttransfection (see Materials and Methods) (18). The results of such experiments indicated that IE1 and IE2 were unable to transactivate the ICP36 promoter (Fig. 2).

To determine whether other viral gene products might be needed to transactivate the ICP36 promoter, effector plasmids containing individual *Xba*I fragments encompassing the majority of the CMV genome (Towne strain) (58) were cotransfected with the target pON269. A cosmid clone pCM1015 from the AD169 strain of CMV composed of *Hin*dIII fragments O, Y, N, and J was tested as an alternative to the Towne strain *Xba*I A fragment (15). Again, no significant transactivation of the ICP36 promoter was observed (data not shown).

To test the hypothesis that IE1 and IE2 may be necessary for activation of ICP36 in conjunction with other viral gene products, pON303G (IE1 + IE2) and each pXba plasmid (or pCM1015) were cotransfected together with pON269. The results of a representative experiment are shown in Fig. 2. Significant transactivation (sixfold) of pON269 was observed with pXbaI and to a lesser extent with pXbaGD (almost fivefold) and pXbaD (almost fourfold). These levels were comparable to levels observed after CMV infection of cells transfected with the pON269 plasmid (see Fig. 4A). The

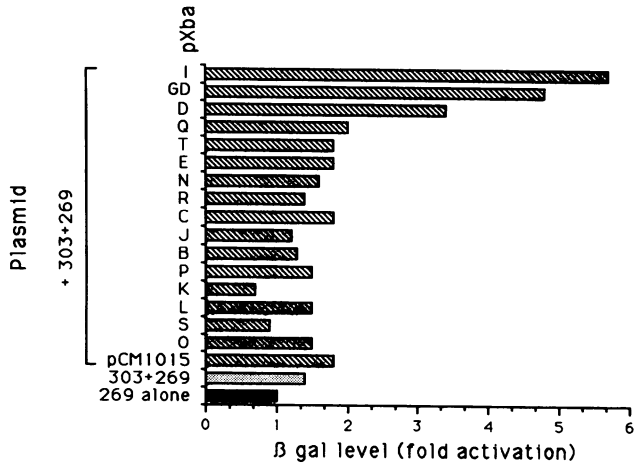


FIG. 2. Activation of the ICP36 promoter by IE1, IE2, and a factor within the S-terminal region of CMV. Target plasmid pON269 (0.5 μg, containing 572 bp of the ICP36 promoter linked to the *E. coli lac*Z gene) was transfected alone (filled bar), with 1 μg of effector plasmid, pON303G (IE1+IE2) (stippled bar), or with 1 μg of pON303G and 1 μg of individual plasmid clones of the CMV (Towne) genome as *Xba*I fragments (hatched bars). One cosmid clone, pCM1015, containing *Hin*dIII fragments O, Y, N, and J of the AD169 strain, was used in place of the *Xba*I A fragment of the Towne strain. β-Gal activity was measured 72 h after cotransfection, and the values shown here are the averages of cotransfections performed in duplicate or triplicate in a single experiment. These data are representative of those from experiments performed on at least three separate occasions. β-Gal levels are expressed as fold activation measured after cotransfection with effector and target clones over transfection of the target plasmid (pON269) alone.

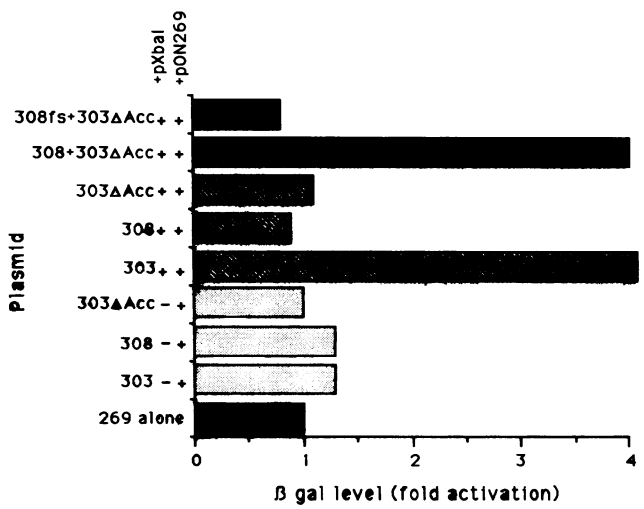


FIG. 3. Cotransfection of pXbaI and plasmids expressing IE1 and IE2 individually with target construct pON269. pON269 (0.5 μg) was transfected either alone (filled bar) or together with 1 μg of plasmid expressing either IE1 and IE2 (pON303G), IE1 alone (pON308G), or IE2 alone (pON303ΔAcc) (stippled bars). The hatched bars represent the additional cotransfection of 1 μg of pXbaI to the plasmids described above. The vertically striped bars show the results of transfection of four constructs, the target pON269 together with effectors pON303ΔAcc (IE2) and pXbaI and either pON308G (IE1) or pON308$_{FS}$ (a frameshift within IE1). The β-Gal levels shown were measured 72 h posttransfection as described in the legend to Fig. 2.

remaining plasmids were unable to transactivate pON269 significantly. The three pXbaI plasmids giving the strongest transactivation of pON269 have the *c* and *a* sequence repeat regions of the genome in common.

**Transactivation by the *Xba*I-I plasmid requires both IE1 and IE2.** The effector plasmid pON303G expressed both IE1 and IE2. To test whether pXbaI could transactivate the ICP36 promoter with either IE1 or IE2 individually, plasmids expressing either IE1 (pON308G) or IE2 (pON303 ΔAcc) (7, 8) were cotransfected with pXbaI and the target pON269. The results of a representative experiment are shown in Fig. 3. In the presence of pXbaI, pON269 could not be activated by cotransfection of either pON308G (IE1) or pON303ΔAcc (IE2) alone. This was in contrast to the fourfold activation observed with pXbaI and pON303G (IE1+IE2). As expected, without pXbaI, IE1 and IE2 together or individually were unable to activate the ICP36 promoter. The requirement for both IE1 and IE2 was confirmed by cotransfecting four plasmid constructs into HFF cells: three effector plasmids, pON308G (IE1), pON303 ΔAcc (IE2), and pXbaI, together with the target pON269. Transactivation of pON269 was restored to levels comparable to those obtained when IE1 and IE2 were present on the same plasmid (approximately fourfold).

The absolute requirement for IE1 was confirmed by transfection of pON308$_{FS}$ in place of pON308G. pON308$_{FS}$ contains a frameshift mutation at amino acid position 367 within the IE1-coding region previously shown to abolish IE1 function (8). Transactivation levels were reduced to the basal levels observed after transfection of the target alone (Fig. 3).

**Transactivation of target plasmids containing point mutations within the TATA elements of the ICP36 promoter.** The ICP36 promoter region in pON269 contains three TATA elements and three transcription start sites that are activated differentially during infection such that start sites 1 and 3 are functional at both early (8 hpi) and late (36 hpi) times of infection whereas start site 2 is functional only at late times of infection and is nonfunctional when viral DNA replication is inhibited. Previous transfection-infection studies using promoter-deletion constructs had shown that start sites 1 and 3 and their associated TATA elements were able to function as separable independently regulated promoters, whereas start site 2 was inactive in such assays (29). To determine individual start site use within the context of the entire promoter region, a series of point mutations was made within each of the three TATA elements in the target plasmid pON268 (Fig. 1B). pON268 differs from pON269 by an additional 85-bp region from +119 to +204 relative to transcriptional start site 1 which includes the ICP36 start codon (29). In pON2319 and each construct carrying TATA mutations, a point mutation was made in the ICP36 start codon (ATG to CCA) to ensure efficient translation of *lac*Z. Point mutations were made such that only one TATA element remained wild type each construct; pON2303 contained only wild-type TATA 3, pON2304 contained only wild-type TATA 2, and pON2305 contained only wild-type TATA 1.

Initially, these target constructs were tested for their ability to be transactivated by viral infection. The results in Fig. 4A show β-Gal levels measured 72 h after infection of HFF cells transfected with the different target plasmids. The control target construct pON2319, which was wild-type for all three TATA elements, was transactivated approximately

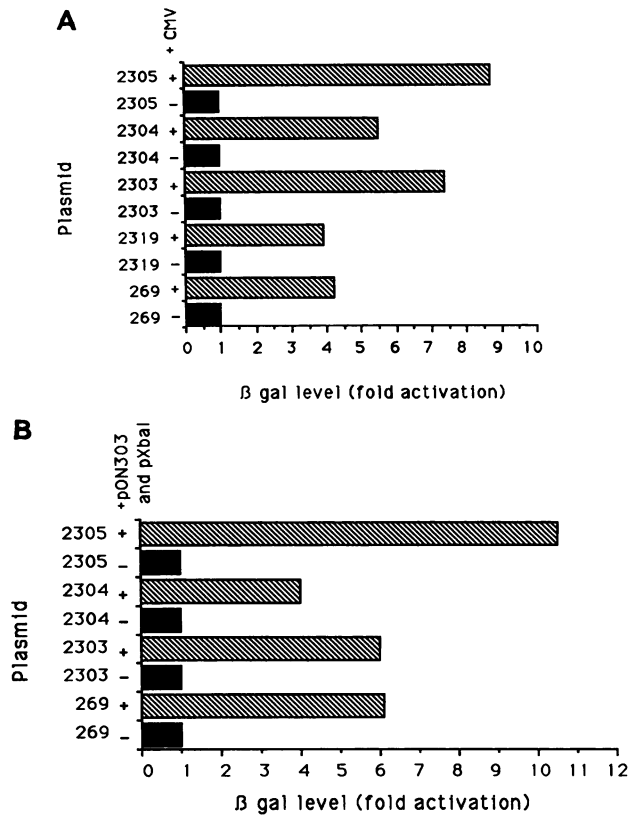


FIG. 4. (A) Transactivation of ICP36 promoter-*lac*Z target constructs by viral infection. Target plasmids (1 μg) pON269, pON2319, pON2303, pON2304, and pON2305 were transfected into HFF cells and either mock infected (filled bars) or infected with 5 PFU of CMV (Towne) per cell (hatched bars). The β-Gal levels shown were measured 72 h postinfection as described in the legend to Fig. 2. (B) Transactivation of ICP36 promoter-*lac*Z target constructs by IE1, IE2, and *Xba*I-I. Target plasmids (0.5 μg) pON269, pON2303, pON2304, and pON2305 were transfected either alone (filled bars) or together with 1 μg each of effector plasmids pON303G (IE1+IE2) and pXbaI (hatched bars). β-Gal levels were measured 96 h post-transfection as described in the legend to Fig. 2.

fourfold over the level obtained in cells transfected with the target plasmid but not infected with CMV. This level was comparable to those observed with the target pON269. Target plasmids containing only one functional TATA element within the context of the entire promoter region were individually transactivated by viral infection. However, contrary to our expectations, expression was increased by the removal of TATA elements. Thus, pON2305 containing TATA 1 alone, pON2304 containing TATA 2 alone, and pON2303 containing TATA 3 alone were each transactivated approximately six- to ninefold (Fig. 4A).

A similar pattern was observed when these target constructs were transactivated by using plasmids carrying viral transactivators (pON303G and pXbaI; Fig. 4B). This finding suggests that the factors involved in activation of the ICP36 promoter could be supplied or induced by the transfected viral genes. Again, all three TATA elements were individually responsive when in the context of the entire promoter region. However, the highest level of activity (11-fold) was obtained with the target pON2305 with only TATA 1 functional. pON2304 (TATA 2 alone) and pON2303 (TATA 3 alone) were transactivated to lower levels of four- and sixfold, respectively, over levels obtained with the target alone. Consequently, in remaining experiments pON2305 was used as the target construct in order to exploit its responsiveness to IE1, IE2, and proteins expressed by the *Xba*I I fragment.

These results confirm and extend previous observations with promoter deletion constructs used to define the activation of each individual TATA element (29). The activity of constructs carrying TATA 1 or TATA 3 alone were the greatest, being at the level of wild type or higher, whereas TATA 2 was inactive. In the work described here, TATA 2 is active, albeit to a slightly lesser degree than TATA 1 or 3. Clearly, TATA 2 is responsive to transactivation by viral infection. It has been shown that all three start sites are utilized in transient transfection assay by RNase protection of RNA expressed from an intact ICP36 promoter construct (28). The increased efficiency of the individual TATA elements in the target constructs used in this study may have been achieved by the greater sensitivity afforded by point mutation in which general structural and spatial relationships within the promoter have been maintained.

**Mapping transactivator function to TRS1, a member of the US22 gene family.** The next series of experiments was conducted to define the transactivating factor within the *Xba*I I fragment. Plasmid pXbaI is an S-terminal 11.7-kbp fragment of the genome (Fig. 1A). The nucleotide sequence of this region in the AD169 strain of CMV encompasses predicted ORFs US27 to US36 of the unique short region and the TRS1 and J1S ORFs in the *c* and *a* terminal repeats, respectively (Fig. 1A) (6).

Preliminary experiments were carried out by using pXbaI plasmids digested with various restriction enzymes that cut within individual ORFs, thereby destroying their expression. Cotransfection of these pXbaI variants with pON303G and pON269 revealed that disruption of the TRS1 ORF reduced transactivation significantly whereas disruption of other ORFs in the region had no effect (data not shown). The identification of TRS1 as the transactivating factor was supported by the significant transactivation of pON269 observed with plasmids pXbaGD and pXbaD, which contained the homolog of TRS1, IRS1. IRS1 is located in the *c* repeat at the junction between the unique long and unique short regions of the genome. Both TRS1 and IRS1 ORFs start within the *c* repeat and extend into the short unique region of the genome. The protein sequences are 100% identical in the amino-terminal 549 amino acids except for one amino acid change at position 190 in CMV strain AD169. The proteins diverge in the sequences that extend into the short unique region but still remain 55% identical (6, 60). Both of these proteins are members of the US22 family that also includes US22, US23, US24, and US26 in the short unique region and UL23, UL24, UL28, UL29, UL36, and UL43 in the long unique region (6, 60).

Given this information, a smaller plasmid based on pON232 (38) containing TRS1 and five other putative ORFs was used in place of pXbaI in cotransfection experiments (Fig. 1A). Weston and Barrell (60) have identified two potential polyadenylation signals for the TRS1 ORF, one located directly adjacent to the stop codon and another 898 nucleotides downstream of the stop codon. To ensure efficient polyadenylation of the *trs*1 transcript, an *Eco*RI fragment containing the SV40 polyadenylation signal was cloned into the *Eco*RI site 39 bp downstream of the TRS1 stop codon. Figure 5 shows that cotransfection of this construct, pON2330, together with pON303G (IE1+IE2) was capable

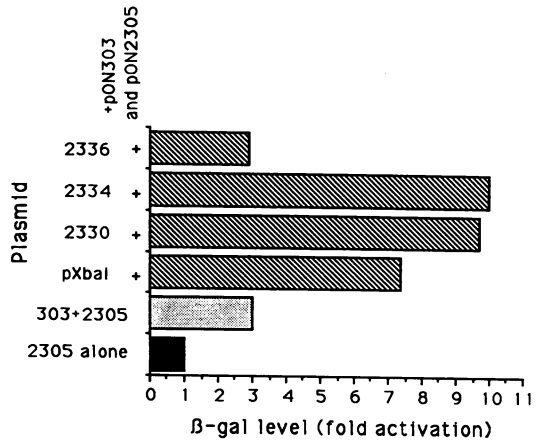


FIG. 5. Evidence that transactivation of the ICP36 promoter requires TRS1. Target plasmid (0 to 5 μg) pON2305 was transfected either alone (filled bar) or together with 1 μg of pON303G (IE1+IE2) (stippled bar). The hatched bars represent cotransfections of pON2305 with pON303G (IE1+IE2) and a series of plasmids, pXbaI, pON2330 (TRS1, J1I*, J1I, IRL1, IRL2), pON2334 (TRS1) and pON2336 (TRS1 with frameshift). β-Gal levels were measured 96 h posttransfection as described in the legend to Fig. 2.

of transactivating pON2305 to approximately 10-fold levels over transfection of the target alone, as determined by the β-Gal activity measured 96 h posttransfection. Furthermore, a construct containing only the TRS1 ORF, pON2334 (see Materials and Methods), was fully capable of transactivating pON2305 when IE1 and IE2 were also present.

Additional confirmation of the requirement for the *trs*1 gene product for transactivation was obtained by transfection of a construct pON2336 containing a frameshift mutation disrupting amino acid 13 of TRS1. This mutation shifts the ORF −1, which would produce a 57-amino-acid peptide in this frame. Figure 5 shows that cotransfection of pON303G (IE1+IE2) and pON2336 resulted in β-Gal levels comparable to those obtained when only IE1 and IE2 were present.

**Northern (RNA) analysis of the *trs*1 transcript.** To determine the kinetic class of the *trs*1 transcript, whole-cell RNA was prepared from mock-infected and CMV (Towne)-infected HFF cells at different times postinfection. Cycloheximide was also added to a parallel set of infections. RNA (5 μg per lane) was separated on 1% agarose–1.2 M formaldehyde gels and blotted onto nitrocellulose. Figure 6A shows the results of probing with a $^{32}$P-labelled 0.6-kbp DNA fragment complementary to the 3′ end of *trs*1. This probe is specific for the $U_S$ sequence unique to *trs*1 (Fig. 1A). The *trs*1 probe detected a major transcript of approximately 2.7 kb that began to be expressed at 4 hpi and increased to maximum levels by 24 hpi. A small decrease in transcript levels was observed at 48 hpi. The size of the transcript correlated well with that predicted for *trs*1, assuming an approximately 0.3-kb 3′ untranslated region based on the most 5′ predicted polyadenylation site that may be used (6, 60). After removal of the *trs*1 probe, a $^{32}$P-labelled 3.8-kbp DNA fragment from pON308G complementary to *ie*1 DNA was used as a probe in Fig. 6B. In this particular time course of infection, *ie*1 transcription (the band of approximately 2.2 kb) started at 4 hpi and was maintained throughout infection until 48 hpi. However, in analyses of RNA from other time courses of infection, transcription of *ie*l was readily detectable at 1 hpi.

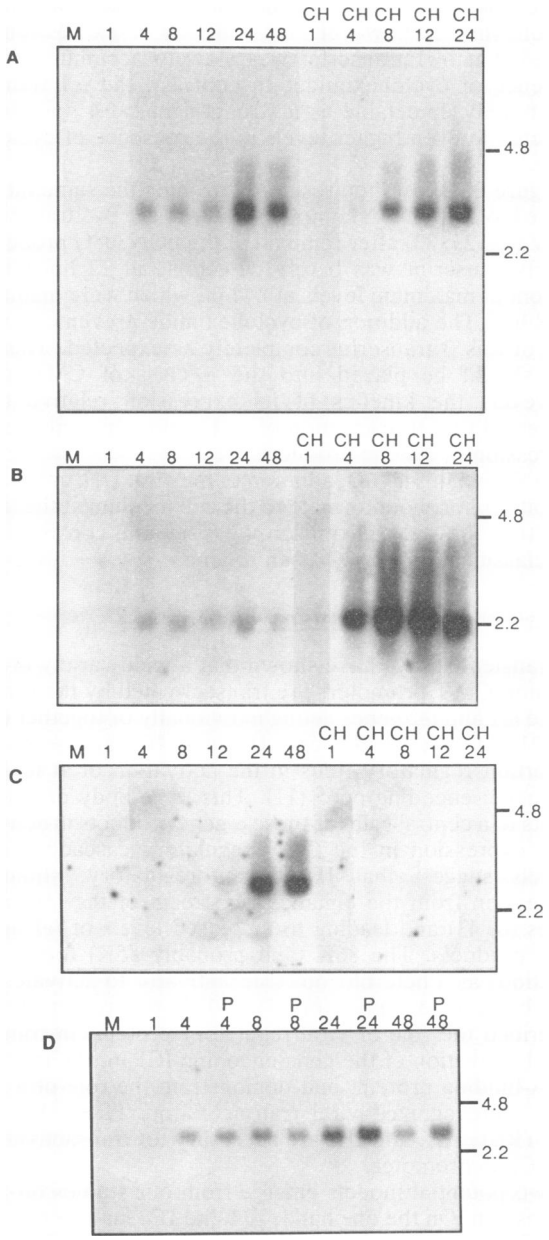


FIG. 6. Northern blot analysis of the *trs*1 transcript. Whole-cell RNA was prepared from both mock-infected(M) and CMV (Towne)-infected HFF cells at 1, 4, 8, 12, 24, and 48 hpi with or without the addition of cycloheximide (CH; 50 μg/ml) prior to infection. RNA (5 μg per lane) was electrophoresed on a 1% agarose–1.2 M formaldehyde gel and then blotted onto nitrocellulose. The RNA blot was probed with a $^{32}$P-labelled 0.6-kbp DNA probe complementary to the 3′ end of *trs*1 (A), a $^{32}$P-labelled 3.8-kbp fragment complementary to *ie*1 (B), and a 2.0-kbp fragment complementary to sequence of the $\beta_{2.7}$ transcript (18, 19, 23, 33) (C). D, whole-cell RNA was prepared from both mock-infected (M) and CMV (Towne)-infected HFF cells at 1, 4, 8, 24, and 48 hpi with or without the addition of phosphonoformate (P; 200 μg/ml) to the infection immediately following adsorption. RNA (5 μg per lane) was electrophoresed on a 1% agarose–1.2 M formaldehyde gel and blotted. The RNA blot was probed with a $^{32}$P-labelled 0.6-kbp DNA probe complementary to the 3′ end of *trs*1. The RNA marker positions (in kilobases) are shown on the right.

The 2.7-kb *trs*1 transcript was expressed when infection was carried out in the presence of the translation inhibitor cycloheximide; however, the kinetics of expression suggested that *trs*1 transcript was slow to accumulate in the presence of cycloheximide. In contrast, the *ie*1 transcript was readily detectable as a strong signal at 4 hpi and was detected in even higher levels in the presence of cycloheximide.

Figure 6C shows the results of probing the same nitrocellulose blot with DNA complementary to the $\beta_{2.7}$ transcript of CMV (19, 23, 33) after removal of the pON308G probe. This 2.7-kb transcript was barely detectable at 12 hpi but was present at maximum levels at 24 hpi, which were maintained at 48 hpi. The addition of cycloheximide prevented expression of this β transcript completely as expected. Therefore *trs*1 should be placed into the α class of CMV genes. However, the kinetics of its expression relative to *ie*1 suggested that transcription of *trs*1 is possibly enhanced by expression of α gene products.

As shown in Fig. 6D, addition of the viral DNA replication inhibitor phosphonoformate to the cell medium at the time of infection allowed *trs*1 transcript expression, consistent with the classification of *trs*1 as an α gene.

## DISCUSSION

Transient assays have shown that a wide variety of CMV and non-CMV promoters are transactivated by the products of the *ie*1 and *ie*2 genes acting individually or together (5, 11, 13, 21, 31, 41, 47, 49, 56). IE1 and IE2 apparently play important regulatory roles in the activation of at least one late gene, encoding pp65 (11). This large body of evidence points to a central role for these α gene products in activating gene expression in the CMV regulatory cascade. Current models suggest that IE1 is autoregulatory, stimulating expression from the enhancer upstream of the *ie*1 and *ie*2 genes (8, 43) and leading to increased levels of *ie*1 and *ie*2 gene products. The 86K (and probably 55K) form of IE2 functions as a heterologous transactivator to activate β and possibly γ gene expression (31, 49). In this report, we have described the role of viral regulatory proteins in transcriptional activation of the gene encoding ICP36, the major late DNA-binding protein, and demonstrate the role of a previously unrecognized viral transactivator, TRS1. We show that TRS1, IE1, and IE2 are necessary for transactivation of the ICP36 promoter.

Two potential models emerge from our studies on ICP36 expression. On the one hand, IE1 and IE2 may be necessary to activate *trs*1 gene expression so that a sufficient amount of TRS1 is expressed and this protein alone influences ICP36 expression. On the other hand, there may be an interplay between the products of these three regulatory genes in bringing about activation of the ICP36 promoter. These models are similar to the models that are used to explain the cooperation of IE1 and IE2 in activation of β genes such as those encoding polymerase and gp48 (5, 31, 49). The accumulation of *trs*1 transcript in the presence of cycloheximide suggests that TRS1 expression is independent of IE1 or IE2; however, the delayed accumulation of *trs*1 transcript in the absence of drug is consistent with a quantitative influence of IE1 and IE2 on *trs*1 expression. Of course, it should be noted that the behavior and apparent cooperation of these viral gene products in transient assays may differ in subtle ways when they are expressed from the viral genome during replication. Mutations need to be introduced into each of these genes in the context of the viral genome in order to accurately understand their interplay in the regulatory cascade.

The TRS1 expression plasmid, pON2334, carried 322 bp upstream of the TRS1 start codon, including the presumed *trs*1 promoter. Our results suggest that any important *cis*-acting signals controlling expression of *trs*1 or responsiveness to IE1 and/or IE2 are contained within this upstream sequence. Computer analysis of this sequence revealed the presence of an enhancer core sequence (CTTTCC) 215 bp upstream of the TRS1 start codon and 91 bp upstream of the presumed TATA element controlling expression. This site was originally described in the SV40 enhancer and shown to be important for activity (26). It remains to be seen whether this site is important for expression or activation of the *trs*1 promoter. The predicted size of the *trs*1 transcript (2.4 kb) agrees well with that detected by RNA blot analysis (2.7 kb), allowing for polyadenylation and assuming that no splicing occurs.

Here and in previous work, transcriptional control of the ICP36 promoter was shown to be independently mediated via three TATA boxes and start sites used differentially during viral replication (29). The viral proteins IE1, IE2, and TRS1 appear to be sufficient for activation of any of these start sites and are likely to act through host cell factors in a manner similar to the proposed function of IE1 and IE2 (37, 43, 46, 49, 53). The differential responsiveness of the target constructs containing point mutations in two of the three TATA elements within the ICP36 promoter has yielded further insights into the activity of this promoter. The activity of target constructs in which either TATA 1 alone or TATA 3 alone was functional was generally found to be as good as or better than that of wild-type promoter with all three TATA elements intact. This suggests that interference or competition between the different TATA elements may limit their activity in the natural configuration. Consequently, the potential rate of transcription initiation from start site 1 under the control of TATA 1, for example, may be decreased because of the requirement for factors able to bind at TATA 3, which is also transcriptionally active at the same time in the viral life cycle. A rationale for the complex promoter structure of ICP36 may be suggested if one considers its putative function as a stimulator of the viral DNA polymerase as some synthesis of ICP36 protein would be required at early times of infection before viral DNA replication (16, 22).

The studies presented in this report suggest that TATA 2 functions by itself although it appears less active in transient assays than TATA 1 or 3 alone. The previous inability to demonstrate the function of TATA 2 and start site 2 on an isolated DNA fragment (29) can now be explained as likely to be due to the removal of some critical sequence component. The particular sequences flanking TATA and start site 2 that are important for expression will need additional investigation. It is interesting to note that the promoter construct containing TATA 2 alone can function better than the wild type after viral infection (Fig. 4A), whereas it functions poorly when cotransfected with pON303G and pXbaI (Fig. 4B). This result is consistent with the existence of additional virus-encoded or -stimulated host factors that increase the activity of this TATA element. This result is also similar to the situation with herpes simplex virus type 1 $\gamma_2$ promoters, which appear to be regulated as β promoters in transient assays (42). All target constructs contained the entire ICP36 promoter region, including approximately 400 bp upstream of the TATA element 1. Preliminary experiments using a target construct in which only the region from

−59 to +204 relative to start site 1, containing all three TATA elements, have suggested that this smaller region is sufficient for transactivation by IE1, IE2, and TRS1 (data not shown). This is in agreement with the results obtained with use of similar constructs in infected cells (29).

In addition to the possible role of TRS1 in the transcriptional activation of ICP36 gene, a potential role at posttranscriptional level cannot be ruled out. Posttranscriptional controls have been suggested to play a role in the regulation of ICP36 gene expression (17). IE1 and IE2 may therefore be involved in activation of ICP36, and TRS1 may be required in order to stabilize the transcripts or to promote nuclear to cytoplasmic transport of the transcripts. Computer-assisted analysis of the predicted 802-amino-acid TRS1 ORF have shown that it does not exhibit features common to some transcription factors, such as a zinc finger domain or a leucine zipper (27, 35). However, the carboxy-terminal third of the protein is relatively charged (25%, compared with 13% in the remainder of the protein). The charged domain follows a relatively hydrophobic domain of 97 amino acids. It is interesting that although TRS1 and IRS1 diverge after amino acid 549, the proteins remain 55% identical at the protein level and the charged character at the C terminus is maintained in IRS1. A charged region may be important for protein-protein or protein-DNA interactions that might enable TRS1 (and IRS1) to act as a transactivating protein. Studies are in progress to determine which part(s) of the protein is necessary for its transactivation function. Also of interest is the relationship of TRS1 and IRS1 to other members of the US22 family. Weston and Barrell (60) have noted that TRS1 and IRS1, although highly homologous to themselves, are less homologous to some of the other members of the US22 family, US22, US23, US24, and US26. The product of the US22 gene is a soluble early protein of unknown function (39), and nothing is known about the other US22 family gene products in the unique short region. However, UL36, another member of the US22 family, is also an α gene (24, 55) that has been shown to encode a transcriptional activator of both viral and cellular genes (10). The murine CMV IE2 protein, which is unrelated to the human CMV IE2 but is homologous to US22 family members (34), is a transactivator (4). It will be of interest to determine the functions of the remaining members of the US22 family. Additional studies of TRS1 and IRS1 function on other CMV promoters will indicate the degree of specificity that each shows in its role as a transactivator of CMV gene expression.

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