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Human Cytomegalovirus ie1 Transactivates the α Promoter-Enhancer via an 18-Base-Pair Repeat Element

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The expression of α (immediate-early) genes of cytomegalovirus is regulated via a complex enhancer that consists of several different repeat elements. We describe here the autoinduction of expression from the α promoter-enhancer by the most abundant α gene product, a 491-amino-acid nuclear phosphoprotein referred to as ie1. We defined the 18-base-pair repeat element within the α enhancer as the signal through which ie1 acts to regulate gene expression. This element contains an NFκB site that may play an important role in ie1 autoregulation. Our analysis, which relied on deletions through the enhancer as well as reconstitution of responsiveness to a promoter with synthetic 18-base-pair repeats, strongly implicated ie1 in the transcriptional transactivation of the α promoter through its enhancer.

Human cytomegalovirus (CMV), one of five human herpesviruses, has a double-stranded linear DNA genome of 230 kilobase pairs (kbp) (Fig. 1A) encoding over 100 gene products which fall into three kinetic classes and are expressed in a cascade fashion (35; E. S. Mocarski, Transfusion Rev., in press). The α (immediate-early) genes are the first to be expressed after infection, require no de novo protein synthesis, and reach maximal transcription rates by 3 to 8 h postinfection. Three α gene regions have been mapped on the CMV genome (1, 18, 37, 41, 42), with one (0.728 to 0.751 map units [13, 37, 42]) much more abundantly expressed than the others (35). This region consists of four distinct genes: 1, 2A, 2B, and 3 (*ie1*, *ie2A*, *ie2B*, and *ie3*) (14), some of which are expressed via differential splicing from a strong promoter-enhancer (3, 13, 33, 34, 39). The most highly expressed of these four is the *ie1* gene, which encodes a 491-amino-acid (aa) nuclear phosphoprotein (11, 20, 33, 38) referred to as the major immediate-early protein. On the basis of its abundant expression and nuclear localization, *ie1* has been suspected to play a role in regulating other viral genes; however, as yet only one of the CMV α genes, *ie2A*, has been clearly implicated as having a regulatory function (13, 25).

The CMV α promoter-enhancer is very active in uninfected cells and contains repeat elements of 16, 18, 19, and 21 bp (Fig. 1B) that play an important role in transcriptional enhancement (3, 36). Human, murine, and simian CMVs all carry *ie1*-like genes that are abundantly expressed from a strong enhancer-promoter (3, 6, 12, 39). Deletion analyses on the human CMV enhancer have suggested that the repeats act in an additive manner and have implicated the 19-bp repeat as an essential element for enhancer-dependent expression in uninfected HeLa cells (3, 36). Sequence-specific DNA-binding proteins that interact with each of these repeat elements have been identified in uninfected HeLa cells (9, 10, 12). By analogy to other systems, these elements could be sites of action for transcription factors and may confer either positive or negative regulatory characteristics on gene expression (for a review, see reference 15). Consistent with a potential role in transcriptional regulation, the 18-bp repeat contains a sequence that is similar to both the NFκB enhancer element of immunoglobulin genes (30) and a human immunodeficiency virus enhancer element (23). The 19-bp repeat element contains a sequence that is similar to the cyclic AMP-responsive element (22). We have been interested in determining the role played by these repeat elements in the transactivation of the *ie1* promoter-enhancer, as well as identifying the proteins involved in α gene activation.

Immediately after infection of cells in culture with CMV, a virion component signals the transactivation of CMV α genes (31, 36), a process that appears to be analogous to the activation of the α genes of herpes simplex virus (HSV) (2, 4, 24, 26). In CMV, the enhancer is required for virion transactivation (W. C. Manning and E. S. Mocarski, unpublished data); however, the specific target sequences within the enhancer region have not yet been defined, nor has the virion component responsible for α gene transactivation been rigorously characterized.

In order to better understand the *cis*- and *trans*-acting factors involved in the regulation of CMV α gene expression, we adopted a strategy, similar to that used to localize the HSV-1 α *trans*-inducing factor (αTIF; [4, 24]), which would enable us to identify regions of the CMV genome that might encode an α *trans*-inducing factor. A series of CMV (Towne) *Xba*I fragment clones representing different regions of the CMV genome were cotransfected (with DEAE-dextran) into human fibroblast cells (31) along with a target indicator plasmid, pON260 (Fig. 1B), which carries 658 bp of the α promoter-enhancer sequence (−14 to −672 relative to the transcription start site) fused to the *lacZ* indicator gene. Expression of β-galactosidase (β-gal) from pON260 was measured by a 4-methylumbelliferyl-β-D-galactoside cleavage assay (8). Another construct, pON249 (Fig. 1B), containing sequences extending further upstream than those in pON260 (to −1144 relative to the transcription start site), was also used as a target in some of the cotransfection experiments. pON260 and pON249 both carried a complete enhancer with all repeat elements, including three 16-bp repeats, four 18-bp repeats, five 19-bp repeats, and two 21-bp repeats. In addition, pON260 and pON249 contained two and four high-affinity nuclear factor 1-binding sites, respectively, upstream of the enhancer (12, 14).

Among the CMV *Xba*I fragment clones used in cotransfection with the α promoter-enhancer target (pON260), only the *Xba*I E fragment, which carried the *ie1*, *ie2A*, *ie2B*, and

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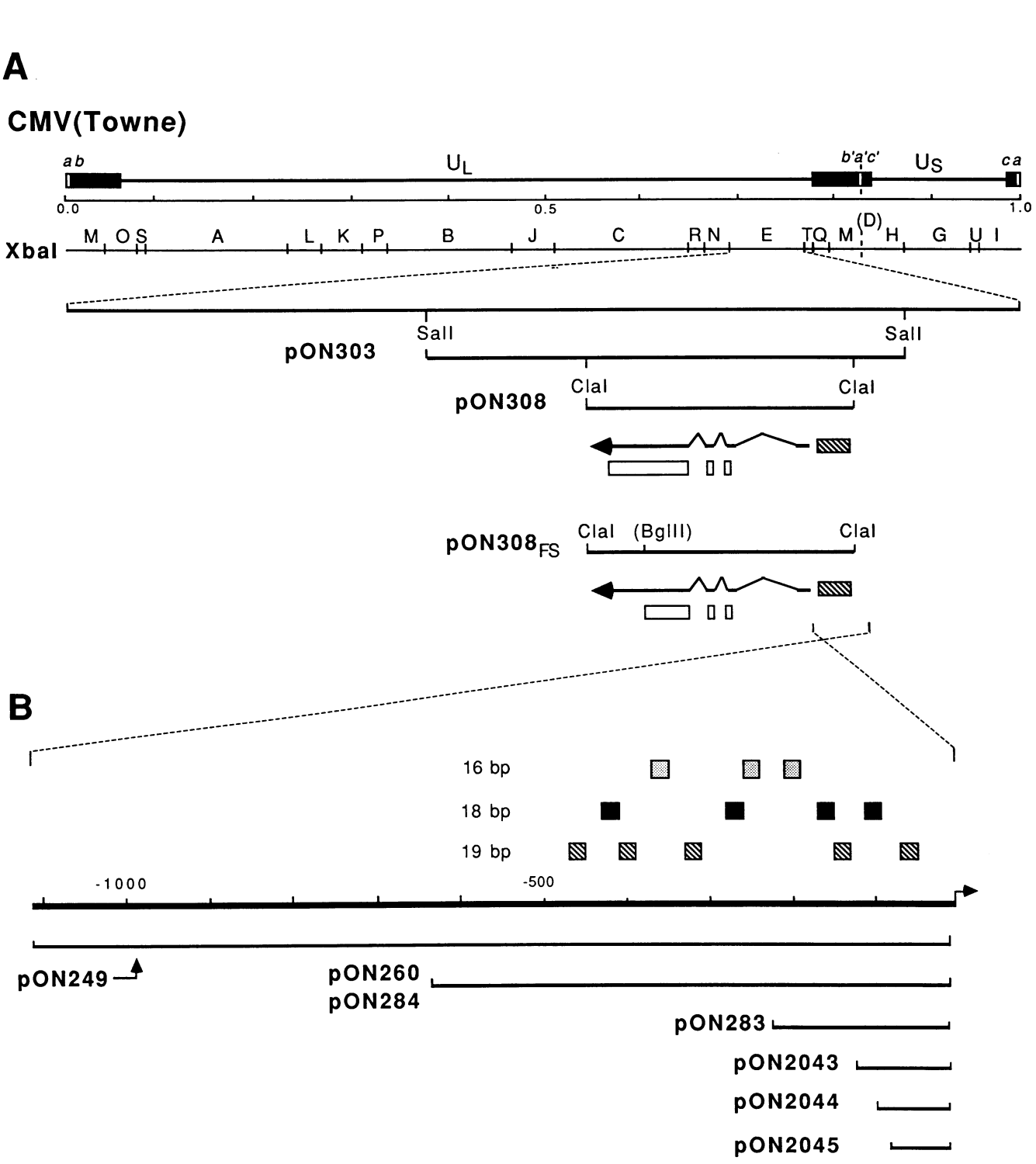

FIG. 1. Organization of the human CMV genome and plasmid constructs used in these experiments. (A) The 230-kbp human CMV genome is depicted on the top line, with the large boxes indicating inverted repeats flanking the unique (U) regions of the L and S components (35). An *Xba*I restriction map is shown below. A *Sal*I fragment cloned in pON303 (31) carrying both the *ie1* and *ie2A* genes and a *Cla*I fragment cloned in pON308 (31) carrying the intact *ie1* gene are depicted in the expanded region. pON303G (see text) is the same *Sal*I fragment and pON308G is the same *Cla*I fragment cloned into pGEM-2 (Promega Biotec). The splicing pattern of the *ie1* gene (four exons and three introns) is depicted along with the protein coding sequences (□) (33). ▨, *ie1* gene enhancer (−118 to −524). ie1 is a 491-aa protein beginning at an initiation codon within the second exon. A frameshift introduced into the unique *Bgl*II site in the fourth exon of *ie1* resulted in the construct pON308$_{FS}$, which is predicted to express a truncated 367-aa ie1. (B) Human CMV α promoter-enhancer regions fused to the *lacZ* indicator gene and used as target constructs in cotransfection experiments. Endpoints of deletions are indicated below and the positions of 16- (▩), 18- (■), and 19- (▧) bp repeat elements are indicated above. pON260 was constructed by deleting the CMV promoter-enhancer sequences between −762 (a *Bal*I site) and −1144 (a polylinker *Sal*I site 6 bp upstream of −1144) in pON249 (8). pON284 carries a CMV DNA fragment identical to that in pON260 but lacks an SV40 origin-enhancer region. pON284 was constructed by cloning a 4.0-kbp *Eco*RI fragment (containing the entire promoter-enhancer and most of the *lacZ* sequences) from pON260 into a 3.0-kbp *Eco*RI fragment from pON3 (containing the carboxyl-terminal sequences of *lacZ*, SV40 polyadenylation signal, and pBR322-based replicon [W. C. Manning and E. S. Mocarski, Virology, in press]). pON283 was created by digesting pON249 with *Bal*1 (sites at −672 and −763 relative to the transcription start site) and subsequent treatment with BAL 31 nuclease (19). pON283 was then linearized at −225 (*Xho*I linker site) and digested with BAL 31 to create pON2043, pON2044, and pON2045. Sizes of deletions were determined by polyacrylamide gel electrophoresis. The precise deletion endpoints, determined by dideoxynucleotide sequence analysis (28), were as follows: pON260 and pON284, −672; pON283, −219; pON2043, −131; pON2044, −103; pON2045, −88 (all relative to the transcription start site).

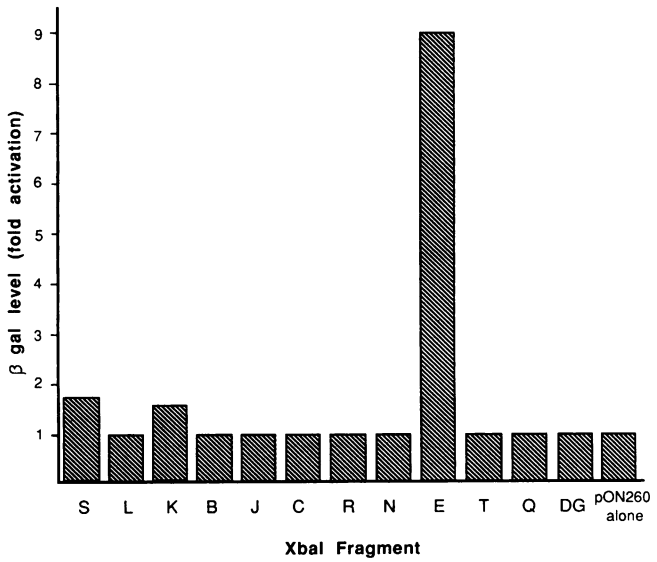

FIG. 2. Survey of human CMV DNA fragments for transactivation of the α promoter-enhancer. The target construct pON260 (10 μg) was cotransfected with individual plasmid clones of CMV (Towne) *Xba*I fragments (20 μg) (40) into human fibroblast cells by using DEAE-dextran (31). Transfected cells were overlaid with Dulbecco minimum essential medium (GIBCO Diagnostics) supplemented with 10% NuSerum (Collaborative Research) containing 150 μg of 4-methylumbelliferyl-β-D-galactoside per ml as described previously (8). 4-Methylumbelliferyl-β-D-galactoside cleavage (β-gal activity) was measured at 48 and 72 h postcotransfection on duplicate dishes. β-gal levels are depicted as fold activation measured after cotransfection with an *Xba*I fragment compared with transfection of pON260 alone, and the values represent an average of β-gal activities measured from 12 separate transfection experiments. The β-gal activities ranged no more than 10 percent above or below the indicated values. Doubling the amount of pON260 DNA (to 20 μg) in the transfection did not detectably increase β-gal levels.

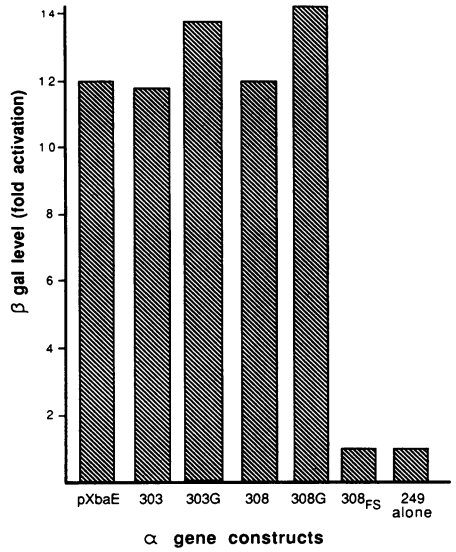

FIG. 3. Transactivation of the α promoter-enhancer required the expression of *ie1*. The target construct pON249 (10 μg) was cotransfected with either constructs carrying α genes or an α gene mutant (20 μg) (Fig. 1) and assayed as described for Fig. 2.

ie3 genes (13, 37), reproducibly transactivated expression of β-gal (Fig. 2). In order to determine which of the α genes within *Xba*I E was responsible for transactivation, we used two previously described (31) plasmids carrying different α gene combinations (pON303 [carrying *ie1* and *ie2A*; Fig. 1A] and pON308 [carrying *ie1*] [Fig. 1A]) in cotransfection experiments. pON303, pON308, and pXbaE transactivated β-gal expression from pON249 to similar levels, suggesting that the function resided in ie1 itself (Fig. 3). As described below, ie1 was able to transactivate the murine CMV α promoter-enhancer (pON405; see Fig. 5); however, target constructs carrying other promoters, including the human CMV $\beta_{2.7}$ promoter (pON241) and the HSV-1 α4 promoter (pON105), were unresponsive to ie1 transactivation (Fig. 4).

To determine whether the effect we observed with pON308 was due to the expression of the *ie1* gene product, we introduced a frameshift mutation at the *Bgl*II site in the fourth exon of *ie1*, resulting in the plasmid construct $pON308_{FS}$ (Fig. 1). This mutation would be expected to frameshift ie1 at aa 345 and result in a premature termination of the protein, reducing its size from 491 to 367 aa. When cotransfected with pON249, $pON308_{FS}$ was inactive. Thus, an intact *ie1* open reading frame (and therefore the expression of *ie1*) was critical for transactivation. To ensure that no other sequences carried by the pON308 construct were acting in concert with *ie1*, the *Cla*I fragment from pON308, which contained the intact *ie1* gene (31, 33), was cloned into a different plasmid vector (pGEM-2 [pON308G]) and shown to be fully capable of transactivating the α promoter-enhancer in transient assays (Fig. 3). The *Sal*I fragment from pON303 containing *ie1* and *ie2A* was also cloned into pGEM-2 (pON303G) and was fully capable of transactivating pON249 (Fig. 3).

pON249 and pON260 both carry a simian virus 40 (SV40) enhancer element 3′ of the *lacZ* gene (8, 31). To determine whether this sequence element was influencing *ie1* transactivation, pON284, which carries an α promoter-regulator region identical to pON260 without the downstream SV40 enhancer, was constructed and found to be identically responsive to ie1 transactivation (Fig. 4). Thus, the presence of the SV40 enhancer did not influence the qualitative or quantitative aspects of *ie1* autoactivation.

To determine the role of CMV enhancer sequence elements responsive to ie1 transactivation, deletion mutants were constructed and used as targets. Plasmids pON249 and pON260 were both significantly transactivated by the CMV fragments carrying *ie1* (Fig. 4). In fact, pON249 was transactivated to a slightly higher level than pON260, which may be a reflection of the presence of two strong nuclear factor 1-binding sites present in pON249. First, we showed that a construct containing a deletion of sequences to −219 bp upstream of the *ie1* transcription start site (pON283) and retaining two copies each of the 18- and 19-bp repeats and one copy of the 16-bp element (but no 21-bp repeats) was transactivated, although to a reduced level (Fig. 4). Next, a series of deletion mutants was generated from pON283 by using BAL 31 nuclease, and each was tested in transient cotransfection assays. A reduction in the level of transactivation by pON308 correlated with removal of 18-bp repeats from the target constructs (Fig. 4). A deletion to −103 bp that partially disrupted the promoter-proximal 18-bp repeat (pON2044) was only slightly transactivated, and a deletion to −88 bp that completely removed this repeat (pON2045) was not stimulated at all. Thus, it appeared that the 18-bp repeat element was an important target for transactivation and that the presence of multiple copies of the element in the α promoter-enhancer was optimal for expression.

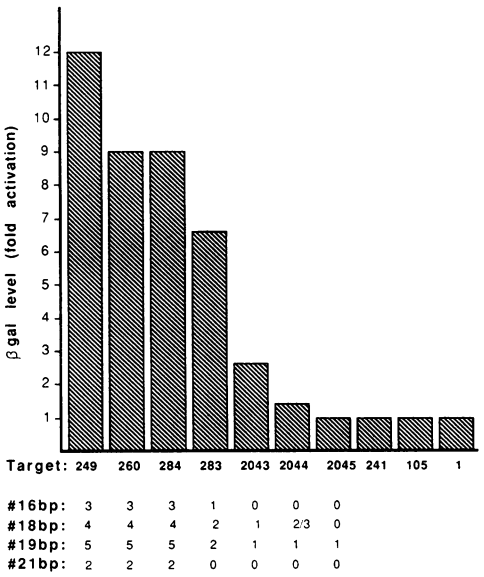

FIG. 4. Transactivation of the α promoter-enhancer by ie1 decreased with the processive removal of enhancer sequences. α promoter-enhancer constructs (10 μg) were cotransfected with pON308 (20 μg) and assayed as described for Fig. 2. Fold activity reflects the level of β-gal activity measured from cotransfection of ie1 and a specific target divided by the level measured from transfection of that specific target plasmid alone. The number of 16-, 18-, 19-, and 21-bp repeat elements carried by each target construct is indicated below the graph. α promoter-enhancer constructs are depicted in Fig. 1; pON241 carries the $\beta_{2.7}$ promoter from human CMV fused to *lacZ* (31), pON105 carries the α4 promoter from HSV-1 (D. Y. Ho and E. S. Mocarski, Virology, in press) fused to *lacZ*, and pON1 lacks a promoter altogether (31). The activities indicated ranged no more than 10 percent above or below the indicated values in replicate experiments.

In order to confirm the observation that ie1 was acting through the 18-bp repeat element to stimulate expression, we performed reconstruction experiments by placing synthetic 16-, 18-, or 19-bp repeats upstream of a promoter devoid of these elements. The promoter we chose to use was derived from the murine CMV α promoter-enhancer, which is responsive to *ie1* transactivation (Fig. 5). The intact murine CMV α promoter-enhancer (pON405) carries five full and six partial 18-bp repeats plus one full and two partial 19-bp repeats (6). Unlike the situation in the human CMV enhancer, the 18- and 19-bp repeat elements in murine CMV are well separated from the Sp1-binding site, CAAT element, and TATA element. Consistent with our studies on the human CMV α promoter-enhancer, a murine CMV α promoter fragment carried by pON407 which contained one partial 19-bp repeat (6) but lacked 18-bp elements altogether was not transactivated by human CMV ie1 (Fig. 5). When three tandem copies of a synthetic 18-bp repeat were added to pON407 (pON407.18R3; Fig. 5A), responsiveness to ie1 transactivation was reconstituted (Fig. 5B). The addition of a single copy of the 18-bp repeat to pON407 resulted in only slight ie1 transactivation (data not shown). As expected from the deletion analysis on the human CMV α promoter-enhancer, pON407-based constructs carrying three copies of either the 19- or 16-bp repeats were not responsive to ie1 transactivation (Fig. 5).

RNA blot analysis was used to determine whether ie1 acted to increase steady-state levels of RNA expressed from the α promoter-enhancer. At 48 h postcotransfection of pON284 with pON308 or pON303, whole-cell RNA was prepared (5), subjected to blot analysis after electrophoretic separation in formaldehyde-agarose gels, and hybridized with a *lacZ*-specific probe (7). As shown in Fig. 6, an increased level of steady-state RNA correlated with the stimulation of β-gal activity from target pON284 upon cotransfection with either *ie1* (lane 3) or *ie1* and *ie2A* (lane 4) compared with the RNA level measured from cells transfected with pON284 alone (lane 1). Lane 2 shows the transcriptional stimulation of pON284 upon superinfection with CMV.

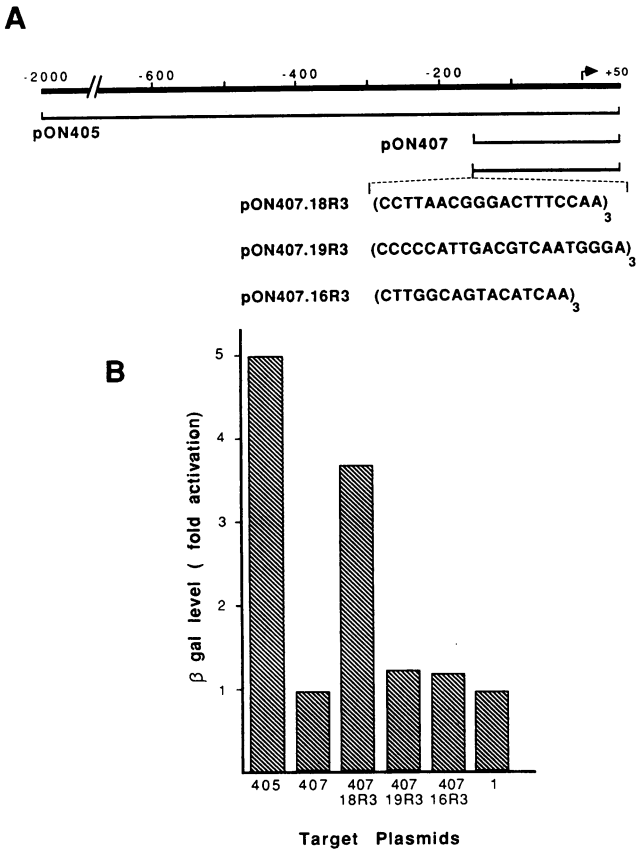

FIG. 5. (A) Murine CMV α promoter and promoter-enhancer constructs used in reconstruction experiments with synthetic human CMV α promoter-enhancer repeat elements. The top line represents the murine CMV α promoter-enhancer region from −2000 through +50 relative to the transcription start site (arrow) (6). pON407 and pON405 have been described elsewhere (Manning and Mocarski, in press). Three tandem copies of the 16-, 18-, and 19-bp repeats (the most promoter-proximal copies of the 16- and 18-bp repeats and the second upstream copy of the 19-bp repeat in the human CMV α promoter-enhancer [3, 39]) were inserted just upstream of position −146 of the murine CMV *ie1* transcription start site in pON407. To construct pON407.16R3, pON407.18R3, and pON407.18R3, pON407 was linearized at a unique *Bam*HI site adjacent to −146, filled in, and then ligated to *Hin*cII-*Sma*I fragments carrying three copies of either the 16-, 18-, or 19-bp repeats (isolated from pTZ3x16R, pTZ3x18R, or pTZ3x19R [G. W. G. Wilkinson, unpublished data], respectively). (B) Synthetic 18-bp repeat elements reconstituted transactivation by ie1. Murine CMV promoter constructs (10 μg) were cotransfected with pON308 (20 μg) and assayed as described for Fig. 2. Fold activity reflects the level of β-gal activity measured from cotransfection of *ie1* and a particular target divided by the level measured from transfection of that specific target alone.

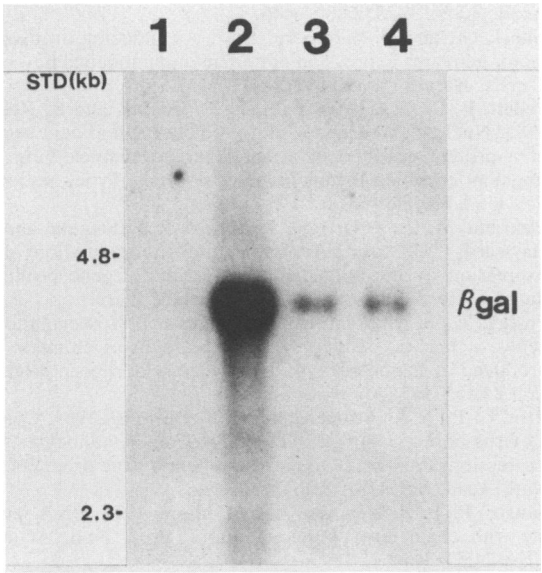

FIG. 6. RNA blot analysis to determine steady-state RNA levels in transfected cells. pON284 (10 μg) was transfected into cells alone (lane 1) or into cells that were subsequently either infected with CMV (10 PFU per cell [8]) (lane 2), cotransfected with pON303 (20 μg) (lane 3), or cotransfected with pON308 (20 μg) (lane 4). RNA was harvested (5) at 48 h postcotransfection and 24 h posttransfection. Whole-cell RNA (10 μg) from each sample was loaded onto a formaldehyde–1% agarose gel, and the RNA was transferred to nitrocellulose paper after electrophoretic separation. The blot was hybridized with a ^{32}P-labeled RNA probe complementary to the 3′-terminal 199 bp of *lacZ*, which detected a 3.6-kilobase *lacZ* transcript (indicated by β-gal to right of lanes). All procedures were as previously described (7, 8), except that a brief RNase (1 μg/ml) treatment of the blot was added before the last wash.

We have demonstrated that the 491-aa ie1 protein is capable of transactivating its own promoter in human fibroblast cells and have localized a critical *cis*-responsive signal to the 18-bp repeat elements that are part of the α enhancer. Although human CMV ie1 is the most abundantly produced α protein, it has not previously been assigned any function in human fibroblasts. Our transient assay results suggest that the function of ie1 is to augment the overall level of expression of α proteins from the α promoter-enhancer or other promoters with the 18-bp target element. Interestingly, the promoter of at least one other human CMV α gene contains the 18-bp element (41), and it will be important to determine whether ie1 transactivation contributes to the regulation of this gene as well. In addition to being active in human fibroblasts, ie1 also transactivated target constructs to similar levels in African green monkey kidney (Vero) cells (data not shown). The effects of ie1 in human fibroblasts or Vero cells are in contrast to its effects in COS-1 cells (African green monkey kidney cells expressing SV40 T antigen), in which ie1 was shown to be autorepressive (32). The reasons for these differences are not clear but may have to do with the added effects of SV40 gene products. Given that our results were obtained in human fibroblasts, which are permissive for CMV growth, we believe our data more closely resemble natural ie1 function, although it is certainly possible that ie1 could naturally exhibit repressing characteristics in some cell types and activating characteristics in others.

It is well established that the α promoter-enhancer has a high basal activity in uninfected cells and that expression is independent of ie1 autoactivation (3, 39). This characteristic of the α promoter-enhancer has led to its use in mammalian cell expression vectors. On the basis of our observations with human fibroblasts as well as with Vero cells, we would expect that expression from this promoter-enhancer could be augmented to even higher levels by the introduction of ie1 in *trans*.

It had been previously demonstrated that the human CMV α promoter-enhancer is transactivated by virus infection even when α gene expression is blocked with cycloheximide (31, 36), suggesting that a virion transactivator analogous to HSV-1 αTIF (4, 24) may be carried by CMV. The ie1 transactivation we describe here is distinct from virion transactivation, but may function in concert with this function (as well as with other transcriptional events occurring through the enhancer) during viral replication.

Besides being expressed immediately after infection in cell culture, the ie1 transcript (or polypeptide) has been detected during persistent infection of human fibroblast cells in culture (21) and during apparent latent infection of human lymphoid cells either taken from seropositive individuals or infected in culture (27, 29). Although our results do not distinguish whether ie1 autoregulation plays a more important role during the productive replication cycle or during latent infection, we speculate that it may be critical in regulating α gene expression during either maintenance of or reactivation from latency. Certainly, much more work is necessary to reveal the physiological setting in which ie1 transactivation is most crucial.

The target for human CMV ie1 transactivation, the 18-bp repeat element, has limited similarity to a core enhancer element from SV40 and is very similar to the NKκB transcription factor-binding site (30) as well as to a related sequence element in the human immunodeficiency virus long terminal repeat (23). ie1 is a nuclear phosphoprotein (11), although it does not appear to be a DNA-binding protein (32). Taken together, these characteristics imply that ie1 may interact with host cell transcription factors, possibly related to NFκB, that bind to the 18-bp element. The occurrence of related *cis*- and *trans*-acting functions in cellular and viral systems suggests they may interact in nature, as has been previously postulated for human immunodeficiency virus (23). Our observations raise the following interesting possibilities concerning the interaction between cells and viruses. (i) CMV ie1 may activate cellular genes that carry the appropriate *cis*-acting elements. (ii) Normal cellular activation of genes with NFκB-like elements may also lead to the activation of CMV gene expression. (iii) CMV ie1 may activate human immunodeficiency virus gene expression via the NFκB-like element under the appropriate circumstances.

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