trans-Activation and Autoregulation of Gene Expression by the Immediate-Early Region 2 Gene Products of Human Cytomegalovirus

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The major immediate-early (IE) gene region mapping at coordinates 0.71 to 0.74 in the genome of human cytomegalovirus (HCMV) gives rise to a series of overlapping spliced IE mRNAs that are all under the transcriptional control of the complex IE68 promoter-enhancer region. We show here that one of the phosphorylated nuclear proteins encoded by this region behaves as a powerful but nonspecific *trans*-activator of gene expression. In transient chloramphenicol acetyltransferase (CAT) assay experiments with Vero cells all relatively weak heterologous target promoters tested, including those of herpes simplex virus IE175 and delayed-early genes, adenovirus E3, the enhancerless simian virus 40 early gene, and the human beta interferon gene, were stimulated between 30- and 800-fold by cotransfection with the *Hind*III C fragment of HCMV (Towne) DNA. In contrast, expression of the homologous HCMV IE68-CAT gene but not SV2-CAT was specifically repressed. Inactivation mapping studies of the effector DNA, together with dose-response comparisons with subclones from the region, revealed that an intact 7.1-kilobase sequence encompassing both the IE1 and IE2 coding regions (exons 1 to 5) in the major IE transcription complex was required for both the nonspecific *trans*-activation and autoregulatory responses. The IE1 coding region alone (exons 1 to 4) was inactive, but both functions were restored by insertion of the IE2 coding region (exon 5) in the correct orientation downstream from the IE1 coding region. Internal deletions or inserted terminator codons in IE1 (exon 4) still gave efficient *trans*-activation and autoregulation, whereas the insertion of terminator codons in IE2 (exon 5) abolished both activities. Finally, IE2 (exon 5) sequences only (under the direct transcriptional control of the strong simian CMV IE94 promoter) were still able to specifically down regulate IE68-CAT expression but failed to exhibit *trans*-activation properties. Therefore, the IE2 gene product(s) of HCMV appear likely to be key control proteins involved in gene regulation during HCMV infection.

All of the major classes of DNA viruses that replicate in mammalian cell nuclei encode immediate-early (IE) gene products that behave as *trans*-activators of subsequent viral gene expression and are themselves under the control of complex upstream promoters and regulatory or enhancer regions. The large T antigens of papovaviruses, the E1A gene products of adenoviruses, the IE175 (ICP4) and IE110 (ICP0) proteins of herpes simplex virus (HSV), the E2 proteins of papillomaviruses, and the *rep* gene products of parvoviruses all appear to fit into this classification. They also all share the characteristic of being hydrophilic nuclear phosphoproteins whose mRNAs are synthesized immediately after infection and in the absence of de novo protein synthesis. Most of these proteins are needed within their own systems for efficient synthesis of subsequent viral genes (usually acting at the transcriptional level), but they may also show various degrees of relaxed specificity or cross-reactivity with heterologous viral or cellular promoters. Several of these gene products also demonstrate negative autoregulatory features when acting upon their own *cis*-acting promoter regions (4, 26, 27, 29, 39), and E1A also inhibits heterologous enhancer elements (2, 40).

Among the cytomegaloviruses (CMVs), IE-class genes and gene products have been described in some detail for human CMV (HCMV), simian CMV (SCMV), and murine

CMV systems. In all instances, a major IE nuclear phosphoprotein referred to as IE68, IE94, or IE98, respectively, is produced in abundance after reversal from a cycloheximide block in infected permissive cells (11, 12, 14, 16, 20, 36). Additional minor IE proteins have also been described for HCMV (34) and murine CMV (16). The three major IE gene products have limited amino acid homology (1, 33; K.-T. Jeang, Ph.D. thesis, Johns Hopkins University, Baltimore, Md., 1984) and are under the control of complex and partially homologous 5'-upstream promoter-regulatory regions that characteristically display strong constitutive promoter activity with some enhancerlike properties (3, 5, 13, 15, 23, 28, 38; Jeang, Ph.D. thesis). The major IE68 and IE94 gene products of HCMV and SCMV are believed to be encoded by spliced IE mRNAs consisting of three small upstream exons (the first being a noncoding leader sequence) plus a large fourth exon (1, 13, 33). The subsequent discovery of additional minor IE mRNA species in HCMV that splice these same upstream exons onto a fifth large exon downstream of exon 4 has necessitated the introduction of the terminology IE1 to describe the major IE gene product of exons 1, 2, 3, and 4 and IE2 to describe the family of minor IE gene products produced from exons 1, 2, 3, and 5 (34). The same situation occurs within the SCMV (Colburn) major IE gene region (Y.-N. Chang, K.-T. Jeang, and G. S. Hayward, unpublished data).

Superinfection studies with SCMV indicated that this virus encodes or induces powerful *trans*-activators of gene expression that act on both the HSV delayed-early (DE)

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38K-CAT and IE175-CAT targets in transient assays (24). Similarly, HCMV infection was shown to stimulate expression of both specific HCMV DE promoter targets and a heterologous rabbit β -globin target (7, 30, 35), and Tevethia and Spector (37) reported that coinfection with HCMV could provide factors that complement E1A-negative adenovirus deletion mutants. Subsequently, Everett (6), Koszinowski et al. (18), and Spector and Tevethia (31) showed more directly that cotransfection with plasmid DNA encompassing the major IE gene regions of HCMV or murine CMV leads to *trans*-activation of gene expression from hybrid target genes or complementation of E1A mutants in transient assays. Some investigators have also presented preliminary evidence that HCMV may introduce a virion factor that specifically *trans*-activates the HCMV major IE promoter both in transient assays (30) and in permanent cell lines (35).

Overproduction of the HCMV IE68 or SCMV IE94 mRNA occurs in the absence of de novo viral protein synthesis (12, 36) or after infection of nonpermissive rodent fibroblast cells (12, 20). Furthermore, a reduction in the level of the HCMV major IE68 protein expressed at later stages of infection in permissive cells has also been observed, and the gene product has been implicated as a negative regulator of its own expression or stability in COS cells (32). These results all hint at either a specific shutoff mechanism or possibly autoregulation acting on the HCMV major IE promoter, perhaps by a mechanism similar to that demonstrated for the HSV IE175 gene (4, 8, 26, 27).

In the present study we set out to (i) examine the target specificity for *trans*-activation of heterologous promoter constructions by the HCMV IE gene product(s) in transient cotransfection assays; (ii) attempt to identify the minimal HCMV gene product(s) necessary for *trans*-activation; and (iii) determine whether the HCMV IE gene products might exhibit autoregulatory effects upon the IE68 promoter region. Although they are nonpermissive for HCMV (Towne) infection, we chose to use Vero cell cultures for our experiments. Both the isolated IE68 and IE94 promoters and gene products were already known to be expressed very efficiently in Vero cells after either transient or permanent DNA transfection procedures (15, 23, 28; J. D. Mosca, L. Sha, P. O'Hare, M. C. Pizzorno, and G. S. Hayward, unpublished data). In addition, this was the system that had been used successfully for most of our previous *trans*-activation studies with herpesvirus IE genes (21, 24–28), and thus we could validly compare the properties and specificities of the different herpesvirus *trans*-activators.

MATERIALS AND METHODS

Plasmid DNA. All of the target hybrid promoter-CAT DNA plasmids, including pSV2-CAT, pA10-CAT, pTK-CAT, p38K-CAT, pIE175-CAT, pIE175(5' Δ -380)-CAT, pIE175(5' Δ -108)-CAT, p β IFN-CAT, pSAA-CAT, pE3-CAT, and pNRP-CAT, have been described elsewhere (21, 24–27). The pCATwt760 target plasmid containing the HCMV(Towne) IE68 promoter regulatory sequences from -760 to +10 was described by Stinski and Roehr (35). Plasmid pTJ148 containing the SCMV(Colburn) *Hind*III H DNA fragment, which encompasses the entire major IE transcription unit, was described by Jeang et al. (13). The HCMV(Towne) major IE effector plasmids were derived directly from our standard sets of *Bam*HI or *Hind*III fragments cloned in pBR322. Plasmid pRL103 contains the 20.8-kilobase (kb) *Hind*III C fragment and includes the complete HCMV major IE transcription unit (map coordi-

nates 0.71 to 0.74). Plasmids pRL105 and pRL107 contain the *Hind*III E (17.2 kb) and *Hind*III G (13.4 kb) fragments of HCMV (Towne) DNA, which encompass both the L- and S-segment inverted repeats (19). Plasmid pRL42 represents the right-hand 6.0-kb *Bam*HI-*Hind*III subfragment of pRL103, and pRL20 represents the adjacent internal 5.3-kb *Bam*HI T subfragment.

The structures of the related inserts in pRL43a, pRL43b, pRL44, pRL45, pRL48a, pRL49, pRL51, pRL53, pRL55, pRL56, pMP10, pMP11, pMP12, pMP14, pMP15, pMP16, pMP17, pMP18, and pMP19 are shown in either Fig. 4b or 7. Plasmids pRL43a and pRL43b were constructed by inserting the *Bam*HI T fragment (exons 5 and 6) from pRL20 in the forward (sense) and backward (antisense) orientations at the single *Bam*HI site in pRL42 downstream of exon 4. Plasmid pRL44 represents a 1.35-kb *Eco*RI deletion from the upstream 5' end of pRL43a. Similarly, plasmid pRL45 represents a 3.4-kb *Sal*I deletion from the downstream 3' end of pRL44, which removes most of the putative exon 6 sequences and terminates approximately 400 base pairs (bp) 3' to the IE2 poly(A) signal. Plasmids pRL48a and pRL48b consist of the same HCMV insert sequences as does pRL42 but with an 800-bp *Bam*HI-to-*Bgl*II fragment derived from pSV2-NEO inserted in either orientation at the *Bam*HI site directly downstream from the exon 4 coding sequences. Deletion of a 1.35-kb *Eco*RI fragment from the 5'-upstream side of the pRL48a insert produced plasmid pRL49. Plasmid pRL51 was formed by first cloning the 5.2-kb *Sac*I-*Bam*HI fragment of pRL20 into a derivative of the pUC19 vector and then moving the same insert as a *Sac*I-to-*Hind*III fragment into pTJ278, thus placing HCMV exons 5 and 6 directly under the control of the SCMV IE94 promoter-enhancer region (15). This construction lacks 167 bp at the 5' end of exon 5, and therefore the first in-phase initiator ATG codon in the leader sequence represents amino acid 85 encoded by exon 5. Plasmid pRL53 contains the 5.0-kb *Xho*I fragment from pRL44 inserted at the *Sal*I site in pGH59, a derivative of the 1.9-kb pKP54 vector (15). This insert contains exons 1 to 4 intact plus the first 614 bp of exon 5. Plasmid pRL55 was derived from pRL45 by the internal deletion and rejoining of the *Bam*HI and *Bgl*II sites, thus removing 474 bp at the 3' end of exon 4, including the predicted TAA terminator codon. Plasmid pRL56 was derived from pRL53 by deleting sequences between the *Sma*I site in the polylinker at the 3' end of the insert and the *Sma*I site in exon 5, thus removing 457 of the 494 amino acids encoded by exon 5. Plasmids pMP10, pMP11, pMP12, and pMP14 were also derived from pRL45: the first two were derived by insertion of a 14-bp oligonucleotide, containing an *Xba*I site plus terminator codons in all three reading frames, at either the first or second *Eco*RV site in exon 4, and the other two were derived by insertion of the same oligonucleotide at the *Sma*I or *Stu*I site in exon 5. Similarly, plasmids pMP15 and pMP16 were prepared by inserting the same oligonucleotide terminator at either the *Xho*I or *Stu*I site within exon 5 in pRL51. Plasmid pMP17 contains the intact IE1 coding regions plus transcriptional control signals within a 4.0-kb *Clal* fragment produced from pRL45 DNA and inserted into pKP54. Plasmid pMP18 was produced from pRL45 DNA by partial cleavage with *Acc*I and rejoining to delete only the 4.1-kb *Acc*I fragment encompassing exon 4. Finally, plasmid pMP19, which encodes truncated forms of both IE1 and IE2, was constructed from pMP11 by deleting sequences between the *Nru*I site in the vector and the *Sma*I site in exon 5.

Short-term DNA transfection assays. Transient expression assays involved transfection of one or more plasmid DNAs

by the calcium phosphate procedure into subconfluent Vero cell cultures, followed by a glycerol boost at 4 h and harvesting at 40 to 48 h (24). No more than 5.0- μ g quantities of total plasmid DNA without any other carrier DNA was used for 5×10^5 cells in each 35-mm-diameter well of six-well culture dishes. All extracts were assayed either directly or after appropriate dilution by incubation with acetyl coenzyme A and [14 C]chloramphenicol for 45 min. Acetylated products were fractionated by thin-layer chromatography, visualized by autoradiography, and excised from the plate for radioactivity determinations in a scintillation counter. Chloramphenicol acetyltransferase (CAT) activity is expressed as the percentage of input [14 C]chloramphenicol converted to the 1'- and 3'-monoacetylated forms. Unless otherwise stated, the total input DNAs in samples in all dose-response experiments were made up to equal quantities by the addition of pBR322 DNA.

RESULTS

Cotransfection of hybrid CAT target genes with HCMV effector DNA. The major IE gene complex of HCMV appears to encode a transcriptional *trans*-activator that has been shown to increase the level of steady-state globin mRNA synthesized under the transcriptional control of the HSV type 1 glycoprotein D promoter in short-term DNA transfection assays (6). To examine other promoter targets in transient CAT assays, plasmids containing HCMV *Hind*III-C (pRL103) or SCMV *Hind*III-H (pTJ148), which include the intact major IE gene coding regions (IE1 plus IE2) of both viruses, were used in cotransfection experiments in Vero cells with target plasmid DNAs containing hybrid CAT genes driven by HSV IE or DE promoters (Fig. 1a). Measurements of CAT enzyme activity in cell extracts revealed up to 50- and 800-fold stimulation effects with added pRL103 DNA on expression from HSV IE175-CAT and 38K-CAT, respectively, and up to 8- and 80-fold effects, respectively, with pTJ148 DNA. Another plasmid containing the complete major IE gene complex of HCMV(Towne) DNA within the *Xba*I E fragment (pMSDT-E) was also positive, but two other plasmids, pRL105 and pRL107, with large *Hind*III inserts encompassing the entire internal L-plus-S inverted repeat segments of HCMV were inactive (data not shown).

To examine a relatively weak nonherpesvirus promoter target, we carried out a dose-response experiment with increasing amounts of pRL103 effector DNA cotransfected with plasmid DNA containing the A10-CAT hybrid gene driven by an enhancer-minus version of the simian virus 40 (SV40) T-antigen promoter. Sixfold activation was detected with only 0.05 μ g of pRL103 DNA, and this increased up to 250-fold with 5.0 μ g of effector DNA (Fig. 1b). In further experiments, we found that all heterologous hybrid CAT genes tested responded well to *trans*-activation by pRL103 DNA, with the level of stimulation ranging from 10- to 500-fold (Fig. 1c). Responsive targets included the minimal SV40 early-region promoter (A10-CAT), the HSV type 1 thymidine kinase promoter (TK-CAT), the adenovirus type 5 E3 promoter (E3-CAT), the Epstein-Barr virus *Not*I repeat gene promoter (NRP-CAT), and two cellular gene promoters, a rat acute-phase serum amyloid-inducible promoter (SAA-CAT) and the human beta interferon gene promoter (IFN-CAT). In other experiments with lower basal expression, the level of E3-CAT expression was also induced more than 50-fold (data not shown).

Evidence for specific inhibition of IE68-CAT expression. In distinct contrast to the broad-spectrum activation of hetero-

FIG. 1. *trans*-activation of heterologous target promoters in transient expression assays in Vero cells. (a) Response of the HSV IE175-CAT and 38K-CAT hybrid genes (1.0 μ g of input target plasmid DNA) to cotransfection with 0.5 μ g of effector plasmid DNA containing either the HCMV (Towne) major IE gene region (pRL103) or the SCMV (Colburn) major IE gene region (pTJ148). (b) Dose-response of the SV40 minimal early promoter in A10-CAT target DNA (0.5 μ g) to increasing amounts of HCMV IE effector DNA (pRL103, 0.05 to 5.0 μ g). (c) Responses of a series of heterologous viral and cellular target promoter plasmids (1.0 μ g each) to cotransfection with HCMV IE effector DNA (pRL103, 1.5 μ g [+]); basal levels (-). Total input DNA amounts in all samples were adjusted with pBR322 to 1.5 (a), 5.0 (b), or 2.5 (c) μ g. Levels of CAT activity are given as percent conversions of [14 C]chloramphenicol (Cm) to monoacetylated forms (3-Ac and 1-Ac).

ologous targets, cotransfection with pRL103 DNA reduced expression from the constitutively strong HCMV IE68 promoter in pCATwt760. However, to obtain a convincing demonstration of this effect from a promoter with such high basal expression introduced some difficulties. In the previous experiments, the heterologous target CAT plasmid DNAs (which have an average size of 5 to 6 kb) were usually cotransfected with no more than equal microgram quantities of the 24-kb effector pRL103 plasmid DNA, i.e., at a 1:4 molar ratio of effector to target DNA. To keep basal expression from IE68-CAT at sufficiently low levels for detection of any possible activation, we initially used only 1/30 as much target DNA (0.03 μ g; Fig. 2a, left panel). Although a 10-fold inhibition was observed, the molar ratio of input effector to target DNA was as high as 10:1, thus creating the possibility of promoter or other competition effects. Nonetheless, additional experiments carried out with lower ratios of effector

to target DNA always revealed between three- and sevenfold inhibition but without giving clearly linear dose responses (Fig. 2a, center and right panels).

To determine whether another strong promoter-enhancer region might respond to the inhibitory mechanism, we undertook a series of experiments to compare the activities of IE68-CAT, SV2-CAT, and A10-CAT target genes after cotransfection with pRL103 DNA in parallel experiments over

FIG. 2. Negative effects on IE68-CAT expression of cotransfection with HCMV IE effector DNA. (a) Shutoff of basal IE68-CAT by cotransfection with pRL103 DNA at various effector/target DNA ratios (+). Amounts of input DNA in micrograms are given above the lanes. Control basal expression samples (–) were cotransfected with pBR322 DNA only. Lane B, Cotransfection with *Bam*HI-cleaved pRL103 effector DNA. (b) Dose-response comparisons of expression from IE68-CAT (●), SV2-CAT (□), and A10-CAT (○) targets in the presence of increasing amounts of cotransfected HCMV IE effector DNA (pRL103). Data from three separate paired series of experiments with different amounts (0.1, 0.2, and 0.5 μg) of input IE68-CAT and SV2-CAT target DNA are given. The two parallel control series for A10-CAT received 2.0 μg of target DNA in each sample. The levels of CAT activity (percent conversion) and amounts of input pRL103 DNA (in micrograms) are both plotted on a logarithmic scale. Basal levels for target DNA cotransfected with 5.0 μg of pBR322 DNA are plotted on the extreme left-hand side of the graph. All other samples received increasing amounts of effector DNA plus compensating smaller amounts of pBR322 DNA to total 5.0 μg.

a wide range of input DNA concentrations. Representative dose-response measurements of CAT activity in the extracts of cotransfected Vero cells over a 150-fold range of input effector DNA (0.03 to 5.0 μg) are shown in Fig. 2b. For the control A10-CAT target gene DNA a nearly linear increase in activity was observed over the whole range of effector to target DNA from molar ratios of 1:240 up to 1:2. At the highest amounts of input pRL103 DNA tested, the A10-CAT activity reached 250-fold stimulation, a level eight times greater than that of basal expression from an equimolar amount of SV2-CAT DNA in a parallel sample. On the other hand, with the SV2-CAT hybrid gene as the target, a maximum stimulation of only two- to fourfold was obtained in each of the three experiments for which results are shown in Fig. 2b. In contrast, expression from the homologous IE68-CAT target gene (in plasmid pCATwt760) was inhibited significantly in all three experiments (Fig. 2b) even at an input ratio as low as 1:25. Maximum inhibition levels of three-, eight-, five-, five-, and threefold occurred at ratios of effector pRL103 DNA to target pCATwt760 DNA below 3:1 in five separate experiments of this type carried out with different input target DNA levels. Again, although there was no clear dose dependence for inhibition, the trends were sufficiently consistent over a wide range of effector to target DNA ratios to conclude that the strong IE enhancer-containing promoter-regulatory regions of SV40 and HCMV respond differently to cotransfection with the HCMV IE gene complex. In two experiments, cleavage of the input pRL103 DNA with *Bam*HI reduced the inhibition of IE68-CAT expression from sevenfold to only twofold (e.g., Fig. 2a, center panel, lane B). Therefore, despite the relatively weak inhibitory action of the cotransfected IE68 effector plasmid on IE68-CAT basal expression, these results seemed unlikely to be caused by promoter competition or cell killing effects.

Inactivation mapping of the nonspecific *trans*-activator function. A series of experiments involving specific restriction enzyme cleavage of the input HCMV IE effector pRL103 DNA were carried out with either TK-CAT or A10-CAT target DNAs (Fig. 3a and b). In these experiments the control uncut DNA sample gave 25- to 30-fold stimulation. Release of the insert HCMV DNA effector sequences from the pBR322 vector DNA with *Hind*III had little effect on *trans*-activating ability, and similarly, linearization with *Xba*I or *Eco*RI reduced the activity no more than two- to fourfold. On the other hand, cleavage with *Bam*HI, *Bgl*II, *Cla*I, *Nco*I, *Sst*I, or *Xho*I always reduced activity between 8- and 30-fold. Although several of the restriction enzymes used give multiple cleavages within the large insert of pRL103, a comparison of the inactivation data with the physical map (Fig. 4a) suggests that *trans*-activation was essentially eliminated by interruption of the effector DNA at all sites tested that lie within either the IE1 gene coding region (*Bam*HI and *Bgl*II) or the IE2 gene coding region (*Cla*I and *Xho*I) or both (*Nco*I and *Sst*I). *Hind*III, *Xba*I, and *Eco*RI were the only restriction enzymes tested that failed to abolish pRL103 *trans*-activation, and none of them cleaves within the major IE gene complex. Incubation with the *Sal*I enzyme, which cleaves 400 bp to the left of the proposed 3' poly(A) site for the IE2 transcript (i.e., outside the known coding exons for either IE1 or IE2), consistently abolished *trans*-activation in these experiments, suggesting that even the putative exon 6 region may play some role (but see below). These rather surprising results narrowed the requirements for *trans*-activation to within an intact 10.2-kb region encompassing all five known exons for genes IE1 and IE2

(and possibly also exon 6) but excluded any need for the IE3 gene, which maps further to the left within the *Hind*III C fragment (36).

Reconstruction of the intact minimal IE1-plus-IE2 transcription unit. To confirm and extend the inactivation mapping analysis, a series of smaller HCMV subclones based on the pRL103 plasmid were constructed (Fig. 4b) and examined for *trans*-activation properties. Plasmid pRL42 contains a 6.0-kb insert from the extreme right-hand side of pRL103

FIG. 3. Inactivation mapping of the HCMV IE effector plasmid. (a) Autoradiograph showing the results of a CAT activity assay with 1.0 μ g of A10-CAT target DNA after cotransfection with 1.0 μ g each of restriction-enzyme-cleaved samples of pRL103 DNA. Basal and uncut controls on the left-hand side of the autoradiograph represent cotransfection with 1.0 μ g each of pBR322 DNA or supercoiled pRL103 DNA, respectively. (b) Comparison of residual stimulatory activities on TK-CAT and A10-CAT targets after cleavage of cotransfected effector pRL103 DNA with different restriction enzymes. Where range bars are shown, results are averages from three separate experiments.

FIG. 4. Correlation of functional activity with physical maps of the HCMV IE region. (a) Localization of known restriction enzyme cleavage sites in the *Hind*III-C insert of pRL103 effector DNA for those enzymes used in the inactivation experiments (Fig. 3). The IE genes (IE1, IE2, and IE3) are indicated by open bars (or broken lines). Unmapped regions are enclosed by parentheses. The solid bar shows the location of the major IE promoter-enhancer region (ENH) for IE1 and IE2 transcripts. (b) Structures of the first series of plasmid inserts derived from the major IE region of pRL103. Open and solid bars depict the locations of the five known IE exons and the putative sixth exon and the general structures of the spliced major IE1 and minor IE2 classes of mRNA, respectively. Stippled bars indicate the positions of the IE68 promoter-enhancer region in most of the plasmids or of the added IE94 promoter-enhancer region in pRL51. Arrows in pRL43a and pRL43b show the relative orientations of the inserted *Bam*HI T fragment sequence. Triangles indicate the inserted SV40-derived early-gene poly(A) and splice signals in pRL48a, pRL48b, and pRL49. Abbreviations: Ba, *Bam*HI; E, *Eco*RI; H, *Hind*III; Sa, *Sal*I; Sc, *Sac*I; Xh, *Xho*I.

and includes the intact major IE gene promoter plus the complete coding region (exons 1, 2, 3, and 4) for IE1, although it lacks all of exon 5 plus the 3' noncoding sequences and the predicted poly(A) signal for the major

1.9-kb IE1 mRNA species. Although pRL42 failed to *trans*-activate 38K-CAT and A10-CAT targets (Fig. 5a and b), we discovered that addition of the 5.3-kb HCMV *Bam*HI T DNA fragment from pRL20 behind the IE1 coding region in pRL42 completely restored activity in one orientation (pRL43a) but not in the other (pRL43b). The DNA of pRL20 itself, which contains all of exon 5 plus additional 3' se-

quences, including the putative exon 6, had no stimulatory activity on its own (data not shown) but when inserted in the natural configuration adjacent to exon 4 of the IE1 gene in pRL43a, it reconstructed the entire complex HCMV major IE transcription unit. Since insertion of *Bam*HI-T in the backward orientation in pRL43b failed to provide any stimulatory activity, the effect cannot involve complementation between the two DNA fragments. Therefore, we conclude that rejoining of either the IE2 coding region or the IE1 poly(A) signal to the truncated IE1 transcription unit represented the key event for restoration of activity.

Additional cotransfection studies with derivatives of pRL43a revealed that a plasmid with a 5'-upstream *Eco*RI deletion (pRL44) and another with a 3'-downstream *Sal*I deletion (pRL45) were both still fully active (Fig. 5a and b). This result contrasts with that obtained in the inactivation studies described above to the extent that sequences beyond the *Sal*I site and adjacent to the 3' end of IE2 appeared to be necessary for full activity with linearized pRL103 DNA, but they could be deleted from the smaller uncleaved effector DNAs without any deleterious effect. The possibility of contributions under some circumstances from the additional putative exon 6 region 3' to IE2 exon 5 is under further investigation. Therefore, the minimal region defined as necessary and sufficient for *trans*-activating activity in transient assays in Vero cells encompasses only the known HCMV IE1 and IE2 regions and lies within a 7.1-kb sequence between the *Eco*RI site 1,200 bp upstream from the IE1 mRNA start site and the *Sal*I site 400 bp downstream from the IE2 poly(A) site.

Dose-response patterns obtained with different IE1-plus-IE2 effector plasmids. The use of single-dose samples only in cotransfection experiments can occasionally lead to misleading results. For example, we have consistently observed a biphasic dose-response curve for *trans*-activation of 38K-CAT target DNA by the HSV type 1 IE175 effector gene in p*Xho*I-C DNA (P. O'Hare and G. S. Hayward, unpublished data). In that situation a strong stimulatory effect of low input doses of effector DNA is often totally abolished at higher input doses. The phenomenon does not appear to be caused by cell killing or other nonspecific effects because parallel cotransfections with similar doses of effector DNA do not inhibit SV2-CAT expression (D. Ciufo and G. S. Hayward, unpublished data). In several experiments at single doses with pRL43a DNA we came across a similar phenomenon. Cotransfection of 2.0 μ g of A10-CAT target DNA with 2.0 μ g of pRL103, pRL44, or pRL45 DNA gave between 10- and 20-fold stimulation, whereas pRL42, pRL43a, and pRL43b DNA all appeared not to *trans*-activate (Fig. 5a). Yet other experiments with the same A10-CAT target DNA but carried out at lower input doses of pRL43a gave over 40-fold stimulation (data not shown). This was not the case for pRL42 or pRL43b.

An extensive dose-response comparison of the *trans*-activation effects on 38K-CAT target DNA with the same series of deletion plasmids is shown in Fig. 5b. In these experiments the effector plasmids pRL43a and pRL45 (and also pRL44, data not shown) both gave initial 20-fold positive *trans*-activation effects with only 0.01 μ g of input DNA, which represents a 5- to 10-fold lower amount than was needed with the parent pRL103 plasmid. In contrast, pRL42 and pRL43b failed to induce 38K-CAT activity to any detectable extent even at 300-fold higher levels of input DNA. At medium and high input DNA levels plasmids pRL43a and pRL45 produced divergent trends: whereas stimulation continued unabated up to 165-fold at 3.0 μ g with

FIG. 5. Comparison of *trans*-activation patterns obtained with different subfragments from the major IE locus. (a) Autoradiograph showing the results of a single-dose cotransfection experiment with 2.0 μ g of A10-CAT target DNA and 2.0 μ g of various effector DNAs. (b) Dose-response experiment showing the effects on 38K-CAT target DNA (2.0 μ g) of cotransfection with a 1,000-fold range of input effector DNA samples (0.003 to 3.0 μ g) in a standard transient assay in Vero cells. Parallel experiments were carried out with effector plasmids encoding both IE1 and IE2 intact (pRL103 [□], pRL43a [●], and pRL45 [▲]), plasmids containing only the IE1 coding region intact plus complete or partial deletions of exon 5 (pRL42 [△], pRL43b [○], pRL49 [▽], and pRL53 [◇]), or a plasmid containing the exon 5 portion of IE2 only (pRL51 [□]). Data from two separate experiments are shown for pRL43a to indicate the sometimes abrupt decrease in activity observed at the highest input doses with this plasmid. *, Basal expression level with 2 μ g of SV2-CAT. The structures of the inserts in each effector plasmid used are shown in Fig. 4b. Cm, [¹⁴C]chloramphenicol; Ac, monoacetylated forms of chloramphenicol.

pRL45 DNA, the activity with pRL43a first plateaued at 40-fold in both experiments for which results are shown and then fell dramatically to only 3- to 4-fold at 3.0 μ g in one experiment. The plateauing and shutoff effects on 38K-CAT (or A10-CAT) expression at high doses of pRL43a DNA were observed consistently over a number of experiments with this plasmid and occasionally also with pRL103 DNA but never with the smaller deleted derivatives pRL44 and pRL45. We have no explanation for this effect at present, although it could represent either more effective autoregulation of IE gene expression by the larger effector DNAs or the activation of additional perhaps lethal viral gene product(s) encoded within the flanking DNA sequences in pRL43a and pRL103. These results demonstrate that additional undefined interactions involving regions adjacent to the IE1 and IE2 coding sequences appeared to produce complex and subtle differences in the characteristics of the different effector plasmids and emphasize the need for careful dose-response analysis in such assays.

In an attempt to reconstruct a functionally complete IE1 transcription unit that should ensure efficient expression of the IE68 gene product (without any IE2 sequences present), we inserted an 850-bp *Bam*HI-to-*Bgl*II fragment derived from SV40 DNA in both orientations at the *Bam*HI site downstream from exon 4 in pRL42. This fragment contains the same poly(A) and small-t-antigen splice signals that were used in the pSV2-NEO and pSV2-CAT constructions. However, neither the plasmid with an "early" (pRL48a) or "late" (pRL48b) orientation of the added "SV2" cassette nor another similar construction with a 5' *Eco*RI deletion (pRL49) had any positive effect on expression from A10-CAT or 38K-CAT target DNA, and in fact they sometimes reduced the basal activity of the heterologous target DNAs severalfold (e.g., pRL49 in Fig. 5a).

Inhibition of IE68-CAT expression represents autoregulation by the HCMV IE gene products. We showed above that in contrast to all other target genes tested, including a control heterologous target gene giving similar strong basal expression (SV2-CAT), the levels of IE68-CAT activity were reduced 3- to 10-fold by cotransfection with the pRL103 effector plasmid DNA. However, these experiments did not rule out the possibility of either competition for limiting cellular factors that might be specific for the HCMV IE promoter or secondary effects related to stimulation of expression of other genes encoded within the 20-kb insert in pRL103 DNA. The subsequent availability of smaller subcloned plasmids that also contained the IE promoter-enhancer regions but varied in their *trans*-activation properties provided a means to control for these effects. Therefore, additional dose-response experiments in which the IE68-CAT target DNA was cotransfected with plasmid pRL43a, pRL43b, pRL44, pRL45, or pRL49 were carried out. On this occasion, we did not compensate for different amounts of cotransfected effector DNA with pBR322 vector DNA but instead added 3.0 μ g of pBR322 DNA as a nonspecific control for the highest doses of effector DNA in a separate sample. We particularly wish to emphasize the results obtained with pRL43a and pRL43b DNA (Fig. 6a). As described above, these two plasmids contain identical DNA sequences except that the *Bam*HI-T (IE2) region was inserted in the sense orientation relative to both IE1 and the IE68 promoter in pRL43a and in the antisense orientation in pRL43b. The maximal inhibition observed with 3.0 μ g of pRL43b DNA was nearly fivefold relative to that observed with pBR322 carrier DNA sequences and occurred at a 10:1 molar ratio of effector to target DNA. This difference pre-

FIG. 6. Evidence that IE68 inhibition is an autoregulatory function that requires the HCMV IE gene products. (a) Demonstration of inhibition of IE68-CAT expression by cotransfection with pRL43a DNA but not with pRL43b DNA. Input amounts of target pCATwt760 effector DNA and pBR322 carrier DNA (in micrograms) are shown above the lanes, and the resulting percent conversions of [14 C]chloramphenicol (Cm) to monoacetylated forms (Ac) are given below the lanes. (b) Demonstration that inhibition requires the IE2 gene region. The results of dose-response cotransfection experiments are presented showing the inhibition of IE68-CAT expression by three plasmids containing the intact IE1-plus-IE2 transcription unit (pRL43a, pRL44, and pRL45) but not by plasmids containing only the IE1 coding region (pRL43b and pRL49).

sumably represents the true extent of competition effects between the excess of IE68 promoter sequences in the effector plasmid over those in the target DNA. In contrast, pRL43a DNA gave a maximum inhibition of 50-fold relative to the level obtained with an equal amount of cotransfected pBR322 DNA at the same 10:1 ratio. Thus, an additional 10-fold inhibition was obtained with the plasmid in which the intact IE1-plus-IE2 transcription unit was regenerated. Similar results were obtained with pRL44 and pRL45 (Fig. 6b), whereas pRL49 DNA (and also pRL42, data not shown) did not cause inhibition.

Thus, the failure of the subcloned plasmid DNA preparations that retained the complete IE68 promoter-regulatory sequences but failed to act as *trans*-activators to significantly inhibit IE68-CAT expression makes it difficult to explain the inhibition observed with pRL43a, pRL44, and pRL45 DNA solely by promoter-specific competition effects. We conclude that there must be a negative response element within the major IE promoter-enhancer region of IE68-CAT that is

specifically recognized by an autoregulatory *trans*-acting gene product encoded within the IE1-plus-IE2 transcription unit.

Examination of the requirements for IE1 versus IE2 gene products. The results presented above show that proteins encoded within the intact IE1-plus-IE2 gene complex are required for both *trans*-activation and autoregulation, and they also imply that the IE1 product alone may be insufficient. However, they do not discriminate between the possibilities that IE2 alone may be sufficient for activity or that both the IE1 and IE2 gene products may be essential. Furthermore, although the tested plasmids that contained the intact IE1 coding region alone without IE2 sequences (pRL42 and pRL43b) were inactive, our immunofluorescence studies reported elsewhere (R. L. LaFemina, M. C. Pizzorno, J. D. Mosca, and G. S. Hayward, submitted for publication) revealed that transient expression of the IE68 nuclear antigen from these two plasmids in DNA-transfected Vero cells is weak and aberrant compared with that from pRL103, pRL43a, pRL44, and pRL45. Furthermore, even the addition of the SV40 3' poly(A) and splicing signals (pRL48a, pRL48b, and pRL49) resulted in a new cytoplasmic localization pattern for the IE68 protein rather than the strongly expressed and exclusively nuclear localization observed with the IE1-plus-IE2 plasmids. Therefore, the low levels of expression of IE1 from these particular plasmids may be insufficient for a valid assay of the *trans*-activating properties of IE1.

To resolve these questions, we constructed a second set of plasmids in which portions of either the IE1 or IE2 coding region were deleted in such a way that the other gene product(s) could still be expressed. The structures of two new prototype plasmid inserts in this series (pRL51 and pRL53) are shown in Fig. 4b for comparison with the first set of plasmids, and a detailed analysis of the residual open reading frames in pRL45, pRL53, pRL51, and 11 additional constructions derived from them is shown in Fig. 7. In plasmids pRL53, pRL56, and pMP17, all exon 5 sequences beyond codons 205 (*Xho*I), 50 (*Sma*I), and 13 (*Cla*I), respectively, have been deleted, leaving the entire IE1 transcription unit with its own poly(A) site and 3' nontranslated regions intact. Even so, in cotransfection assays none of these IE1 plasmids proved to be able to *trans*-activate (e.g., Fig. 5b, pRL53, and Fig. 8, pMP17), although in contrast to pRL42, pRL43b, and pRL49, etc., all three plasmids expressed the normal nuclear form of the IE68 antigen at high efficiency (LaFemina et al., submitted).

Similarly, two internal deletions were made in pRL45 to create plasmids lacking part or all of exon 4. Plasmid pRL55 was derived by joining the *Bam*HI and *Bgl*II sites, thus removing 147 codons from the COOH-terminal acidic domain of exon 4 but leaving intact the IE1 poly(A) signal and all of the IE2 coding regions and transcriptional control signals (exons 1, 2, 3, and 5). In plasmid pMP18, the entire exon 4 coding region from the *Acc*I site before the splice acceptor to the *Acc*I site just after the *Bam*HI site was deleted, leaving intact all of IE2 together with the IE1 poly(A) site. These two constructions both proved to exhibit normal *trans*-activation (Fig. 8), although as expected, expression of the IE68 antigen itself was absent or aberrant (LaFemina et al., submitted).

In assays for autoregulation, pRL53 and pMP17 each gave weak (twofold) inhibition of both the IE68-CAT and SV2-CAT targets (Fig. 9), whereas pRL55 and pMP18 showed 5- to 15-fold inhibition of IE68-CAT at doses at which SV2-CAT was stimulated 4- to 8-fold. Therefore, the IE2 gene

products encoded by exons 1, 2, 3, and 5 can function independently to give both *trans*-activation and specific autoregulation, whereas the IE1 gene products alone exhibit only weak nonspecific inhibitory effects.

Insertion of terminator codons and separation of autoregulation from *trans*-activation. To unambiguously assign the key functional activity to the exon 5 domain and not to exon 4, we also created variant plasmids with terminator codons inserted into these two coding regions in pRL45 DNA. Plasmids pMP10 and pMP11 contain a 14-bp synthetic oligonucleotide inserted at internal *Eco*RV sites to truncate the IE1 gene product after either amino acid 46 or 189 in exon 4. Similarly, plasmids pMP12 and pMP14 contain the same 14-bp oligonucleotide inserted at the *Sma*I or *Stu*I site to truncate the IE2 gene product after amino acid 50 or 456 of exon 5. Only pMP12 and pMP14 proved to produce the typical bright nuclear immunofluorescence with anti-IE68 monoclonal antibody (LaFemina et al., submitted), yet in cotransfection assays pMP10 (and pMP11, data not shown), but not pMP12, stimulated 38K-CAT activity (Fig. 8). Even in dose-response experiments pMP12 failed to activate 38K-CAT expression and in common with several other IE1-expressing plasmids, actually produced negative effects at the highest doses (data not shown). Cotransfection experiments with mixtures of pMP10 and pMP12 at various ratios provided some weak support for a synergistic effect between the IE1 and IE2 gene products, especially at relatively low IE1 (pMP12) and high IE2 (pMP10) input ratios, but with no more than a threefold additional stimulation overall (data not shown).

Again, in single-dose assays for autoregulation (Fig. 9), pMP10 specifically inhibited IE68-CAT expression eightfold and activated SV2-CAT expression sevenfold, but pMP12 gave three- to fourfold nonspecific inhibitory effects on both IE68-CAT and SV2-CAT targets. In contrast, pMP19, a derivative of pMP11 that in addition lacks most of the exon 5 sequences (i.e., is truncated in both IE1 and IE2), had no significant positive or negative effects on either IE68-CAT or SV2-CAT expression. Similarly, in dose-response assays with IE68-CAT target DNA, pMP10 DNA alone gave almost as strong an inhibitory effect as did pRL45 DNA, whereas pMP12 was relatively inactive (Fig. 10a). Note that in these experiments pRL45 DNA produced 20- to 50-fold inhibition of IE68-CAT expression, a much greater effect than was ever observed with pRL103 DNA.

For further evaluation of the role of the IE2 gene products, we also constructed plasmid pRL51, which contains the bulk of the IE2 exon 5 coding region (starting from the *Sac*I site at codon 50) plus the putative exon 6 region, placed under the transcriptional control of the strong IE94 major IE promoter from SCMV(Colburn). Surprisingly, this plasmid proved to be unable to *trans*-activate the usual heterologous targets tested (Fig. 6b and 8) but still gave specific down regulation of IE68-CAT expression without inhibiting SV2-CAT (Fig. 9). Since the first in-phase AUG codon in pRL51 represents codon 85 of exon 5, we conclude that key sequences for *trans*-activation map either upstream within exons 2 and 3 or within codons 1 to 85 of exon 5. In contrast, codons 85 to 494 of exon 5 apparently suffice for producing the autoregulatory properties of IE2. The relative efficiency of IE68-CAT inhibition by pRL51 was further demonstrated in a dose-response cotransfection experiment in which it was compared with inhibition by pRL45 (Fig. 10b). This figure also presents the results obtained in parallel with two terminator insertion mutants of pRL51 (pMP15 and pMP16) and a third terminator insertion mutant of pRL45 (pMP14). At the

FIG. 7. Open reading frame (ORF) structures and residual functional activity of deletion and termination mutants in exons 4 and 5 of the HCMV major IE gene complex. The top portion presents a detailed map of transcriptional control features, restriction enzyme cleavage sites, and open reading frames in the 7.1-kb *EcoRI*-to-*SalI* insert in pRL45 together with the generalized structures of the IE1- and IE2-classes of spliced mRNA transcripts. The bottom portion presents the structures of the DNA and IE1 and IE2 coding regions in each of the series of variant plasmids derived from pRL45, together with their properties (+, positive; -, negative) in transient assays for *trans*-activation or autoregulation. Symbols: ■, the five known exons in IE mRNA species; ▨, the enhancer (ENH) region; □, relative sizes and locations of open reading frames predicted to encode polypeptide products; ●, known transcription start sites; ▽, potential nuclear localization signals; ◆, sites of inserted *XbaI* linker oligonucleotides containing terminator codons.

highest doses pRL45 gave 3-fold greater inhibition than pRL51 and 25-fold greater inhibition than pMP14, whereas pRL51 gave between 8- and 10-fold greater inhibition than its truncated derivatives. Therefore, we conclude that polypeptide fragments containing only 50 amino acids (pMP12, *SmaI*), 205 amino acids (pMP15, *XhoI*), or 457 amino acids (pMP14 and pMP16, *StuI*) encoded by the NH₂-terminal portion of exon 5 are all inactive in specific autoregulation, either because they lack functionally important residues or produce unstable polypeptide products. Consequently, the COOH-terminal 37 amino acids of IE2 are implicated as part of a functionally important domain for down regulation of the IE68 promoter.

DISCUSSION

We conclude that the HCMV major IE region 2 encodes a gene product that is a powerful but nonspecific *trans*-activator of gene expression in transient assays. The IE2 gene also produces a negative autoregulatory factor that acts only on an as yet undefined target site within the IE68 promoter-enhancer region. This combination of promiscuous *trans*-activation plus specific inhibition exhibited by the pRL103-series plasmids differs significantly from the properties observed previously for the individual HSV IE effector genes. For example, IE110 (ICP0) is also a nonspecific *trans*-activator of HSV DE and heterologous promoters (21,

FIG. 8. Evidence that the IE2 gene products alone are necessary and sufficient for *trans*-activation. The autoradiograph shows the results of CAT assays with extracts of Vero cells after cotransfection of 2.0 μ g of 38K-CAT (top) or A10-CAT (bottom) target DNA with 3.0 μ g each of various effector plasmid DNAs containing lesions in either exon 4 (pMP10, pMP18, and pRL55) or exon 5 (pMP12 and pMP17) or lacking exons 1, 2, 3, and 4 (pRL51). The levels of CAT activity obtained in the same experiment after cotransfection with pRL45 DNA or from basal expression of SV2-CAT are shown as controls. Cm, [14 C]chloramphenicol; Ac, monoacetylated forms of chloramphenicol.

28) but gives positive effects upon its own promoter (D. Ciufo and G. S. Hayward, unpublished data). On the other hand, IE175 (ICP4) is primarily a specific *trans*-activator of HSV DE targets, which also selectively down regulates its own promoter (4, 8, 25, 26, 28). Note that since we have not examined any HCMV DE target promoters, there could also be a third type of HCMV IE regulatory function (mapping either within or outside the major IE gene region) that acts specifically on HCMV DE-class genes. We wish to emphasize that the half-life of the CAT reporter gene mRNA (derived from pSV2-CAT) in Vero cells is greater than 8 h (J. D. Mosca, unpublished data) and is therefore usually considered sufficient to allow the use of CAT enzyme activity assays to give sensitive (but indirect) measurements of transcriptional effects. In the case of specific effects, the various hybrid target genes contain only small portions of the adjacent 5'-leader regions in addition to promoter-regulatory sequences, and the opportunity for nontranscriptional effects is very limited. However, the mechanism responsible for the nonspecific *trans*-activation effects of the HCMV IE2 gene has not been defined, and we recognize that posttranscriptional effects, mRNA stabilization, or even translational control might also have been detected.

The apparent lack of target promoter specificity displayed by the HCMV IE *trans*-activator in transient assays closely resembles the features described previously for both the HSV IE110 protein and the Epstein-Barr virus BMLF1

(MS-EA) gene products (21, 28), indicating that each major class of human herpesvirus possesses an IE-class *trans*-activator of this type. The presence of this activity presumably also complicates the situation with regard to the dose-response patterns for specific down regulation by the HCMV IE2 gene product upon its own promoter region in the IE68-CAT target DNA. One would assume that the HCMV IE68 promoter sequences are also subject to the nonspecific positive *trans*-activation function and that in addition the effector genes themselves are subject to autoregulation in these assays, especially at high input doses. Therefore, the final levels of expression must represent the summation of all of these effects plus the relatively weak nonspecific inhibitory effects of IE1. Apparently, the HCMV system resembles that for HSV IE175, in which autoregulation by IE175 both dominates over the positive effects of IE110 or VF65 *trans*-activation of IE175-CAT and also acts directly on basal expression from the minimal promoter region (26, 27). Note also that the lack of well-defined promoter target specificity for *trans*-activation by the IE2 genes of HCMV and SCMV eliminates any need to postulate a role for a cross-reacting positive virion factor to explain the 100-fold stimulation of HSV IE175-CAT expression by superinfecting

FIG. 9. Specificity of the inhibitory effects of IE2-encoding effector plasmids on expression of IE68-CAT and SV2-CAT targets. The autoradiographs show the results of parallel transient assays with 0.2 μ g of input target pCATwt760 (top) or pSV2-CAT (bottom) DNA after cotransfection with 3.0 μ g each of various deleted or truncated effector plasmids derived from pRL45, containing lesions in exon 4 (pMP18, pMP10, pRL55, and pRL51) or exon 5 (pMP17, pRL53, and pMP12) or both (pMP19 and pMP15). The structures of these plasmid inserts are shown in Fig. 7. Note that high input doses of all plasmids containing only the IE1 coding region sequences intact often gave relatively weak nonspecific shutoff effects on all target promoters tested. Cm, [14 C]chloramphenicol; Ac, monoacetylated forms of chloramphenicol.

FIG. 10. Demonstration that IE2 exon 5 sequences alone are necessary and sufficient for down regulation of IE68-CAT expression. (a) Results of dose-response CAT assays involving cotransfection of 0.5 μ g of pCATwt760 target DNA with various input amounts of effector plasmid DNAs containing terminators in either exon 4 (pMP10 [●]) or exon 5 (pMP12 [□]). Data from parallel cotransfections with the parent pRL45 plasmid DNA (▲) are shown as a positive control. (b) Results of dose-response experiments with 0.5 μ g of pCATwt760 target DNA cotransfected with plasmids encoding the IE2 exon 5 region only either before (pRL51 [●]) or after (pMP15 [○] and pMP16 [△]) insertion of terminator codons. The levels of inhibition obtained in parallel cotransfections with pRL45 (▲) and its derivative (pMP14 [□]) containing an inserted terminator at the *Sma*I site are shown for comparison.

SCMV observed previously in transient assays in Vero cells (24).

In neither the HSV nor the HCMV system have we been able to mimic the specific inhibitory effects with control plasmids containing the IE175 or IE68 promoter-regulatory regions only or with the complete effector plasmids after either cleavage within the IE gene coding regions or insertion of terminator codons. These results suggest that the inhibitory effects are mediated by the respective IE gene products themselves and are not simple effects of competition for limiting cellular factors between identical promoter regions in the target and effector plasmids. The findings that the HCMV IE2 and HSV IE175 inhibitory features do not complement or cross-react with one another and are limited to their own promoter regions (M. C. Pizzorno and G. S. Hayward, unpublished data) and that neither acts on the SV40 promoter-enhancer in SV2-CAT also indicate that we are not observing generalized enhancer repression of the type described for the adenovirus E1A gene product (2, 40). Furthermore, the relatively weak response of the complete SV40 early promoter to positive *trans*-activation by IE2 is similar to our previous observations with the HSV IE110 *trans*-activator and may simply indicate that basal expression from the enhancer-containing SV40 early promoter in SV2-CAT is already close to maximal.

The previous assumption by us and others (18, 24, 28, 31) that the HCMV major IE68 nuclear phosphoprotein was likely to be a *trans*-activator of subsequent viral gene expression appears to be incorrect (unless it acts only on specific

target promoters that we have not tested). In our initial experiments described here, cleavage of pRL103 DNA in either the coding region unique for IE1 (exon 4) or that for IE2 (exon 5) abolished stimulation of heterologous target gene expression in transient assays. However, because of the overlapping organization of the IE1 and IE2 transcription units, cleavage within the IE1 coding region (exon 4) should prevent expression of both gene products, whereas cleavage within the IE2 coding region (exon 5) should not affect expression of the IE1 (IE68) protein. The smallest contiguous plasmid that was fully functional (pRL45) contained the complete IE1-plus-IE2 transcription complex within a 7.1-kb region between *Eco*RI on the 5' side and *Sal*I on the 3' side. Because the inactive pRL42 plasmid, which encodes the complete IE1 coding region, lacks some 3' noncoding sequences plus the proposed poly(A) site for the 1.9-kb IE1 mRNA species, questions were raised about the efficiency of expression of the IE1 (IE68) gene product from this plasmid. Addition of the SV40 poly(A) site downstream of the IE1 coding region failed to restore functional activity, but again because the form of IE68 produced from these latter constructions proved to be localized in the cytoplasm rather than in the nucleus of transfected cells, the results were not definitive. Note that Everett (6) also found that the recognized HCMV IE68 coding region [even after the addition of a poly(A) site] was insufficient for the stimulation of HSV gD or β -globin promoter-driven mRNA levels that was seen with larger plasmids. Therefore, because of the complications of the overlapping and incomplete transcription units in

our early plasmid constructions, a direct and definitive demonstration of the role of IE2 gene products rather than IE1 gene products required the extensive evaluation of a new series of plasmids with deletions or internal truncations in either the exon 4 or exon 5 coding region.

With regard to the HCMV IE promoter-specific, autoregulatory activity, cleavage of pRL103 plasmid DNA inside the IE1 coding region with *Bam*HI reduced the inhibitory effect on IE68-CAT target DNA, but dose-response experiments again demonstrated a requirement for the addition of IE2-region sequences in the correct orientation in *cis* to obtain inhibitory activity. Importantly, the comparison between pRL43a and pRL43b, which possess identical but rearranged sequences, excluded the possibility that some form of specific competition effect was responsible for the inhibition. Subclones that could not express the complete IE2 gene transcription unit failed to down regulate IE68-CAT expression. Again, however, definitive mapping of this function to the IE2 and not the IE1 gene could not be obtained until the second series of internally deleted and truncated plasmids was used. Interestingly, the separation of autoregulation from *trans*-activation in plasmid pRL51 suggests that the upstream exons 1, 2, and 3, which are common on both the IE1 (IE68) protein and the IE2 gene products, are probably needed for *trans*-activation but are dispensable for autoregulation. In this regard, the suggestion by Stenberg et al. (34) that an unspliced form of IE2 mRNA (exon 5 only) may be produced from a DE-class promoter downstream of the IE1 poly(A) site is entirely consistent with our results and leads to the further prediction that this latter gene product may be involved in the shutoff of IE68 gene expression seen in HCMV (Towne) infections in permissive cells. Therefore, the report by Stenberg and Stinski (32) that IE68 is capable of negatively autoregulating expression from its own promoter in transfected COS-1 cells must now be considered suspect, although clearly, further analysis in permissive HF cells is required to resolve these issues.

The results of the functional gene mapping described here raise several interesting points about the evolution of herpesvirus genomes. First, the observation that the major IE gene transcripts of HCMV lie in the U_L portion of the physical map of the genome rather than in the inverted repeats as do their counterparts in HSV was surprising initially but is now reinforced by our evidence that the U_L region (and not the inverted repeats) encodes *trans*-activating proteins that appear to be the functional equivalents of the IE175 and IE110 gene products of HSV. Interestingly, the IE2 gene products of HCMV and SCMV show many structural features that are similar to those of E1A, large T antigen, *myc*, *fos*, etc., and also to five other identified "IE-like" nuclear regulatory proteins of herpesviruses (e.g., HSV IE110 and IE175 and Epstein-Barr virus BMLF1, BZLF1, and BRLF1). These features of the IE2 gene products include overall hydrophilic properties, a proline content of 11%, short runs of consecutive serine or acidic residues, and potential nuclear localization signals. However, none of the herpesvirus IE *trans*-activator proteins mentioned above appear to have any significant residual amino acid homology relationships to one another. Therefore, in contrast to the many closely related and conserved biochemical and structural genes of the three different herpesvirus subgroups, the analogous functional IE regulatory genes probably have totally different genetic and evolutionary origins.

After our work was submitted for publication, Hermiston et al. (10) published evidence similar to ours that the HCMV IE2 gene products can *trans*-activate the heterologous E2-

CAT target DNA in a transient assay system in permissive HF cells.

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