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The IE2 Gene Products of Human Cytomegalovirus Specifically Down-Regulate Expression from the Major Immediate-Early Promoter through a Target Sequence Located near the Cap Site

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The 82-kDa IE2 protein of human cytomegalovirus (HCMV) acts as both a powerful nonspecific *trans* activator of heterologous promoters and a negative autoregulator of HCMV immediate-early gene expression in transient assays. We show here that the highly specific down-regulation effect occurs in permissive diploid human fibroblast cells as well as in nonpermissive Vero cells and that the target sequences are conserved within the major immediate-early promoters of both HCMV and simian cytomegalovirus. The response sequences were localized between −67 and +30 in the simian cytomegalovirus IE94 promoter and upstream of position +9 in the HCMV IE68 promoter. Deletion of sequences downstream of −14 in a target IE68-CAT gene abolished the negative phenotype and resulted in a reporter gene that was stimulated instead of inhibited by cotransfection with IE2 effector DNA. Insertion of an oligonucleotide containing sequences from between −17 and +9 into the IE68-CAT deletion construction restored autoregulation in either orientation. Furthermore, this same oligonucleotide transferred the full down-regulation phenotype when inserted at +10 into the nonresponsive IE175 promoter from herpes simplex virus. Therefore, a specific response signal that acts at the DNA level must lie within these boundaries. Additional analysis with inserted oligonucleotides containing deletions or point mutations revealed that essential components of the signal lie between positions −12 and +5. Therefore, negative autoregulation by HCMV IE2 in DNA cotransfection systems resembles that for simian virus 40 large T antigen and herpes simplex virus IE175 by acting through a signal located near the cap site, but the target sequence itself bears no resemblance to those utilized in these other viral systems.

Cytomegaloviruses (CMV) exhibit a very limited host cell range for permissive lytic-cycle infection in cell culture. Although the viruses adsorb to and enter many cell types, viral gene expression is often never initiated or (in the case of rodent fibroblasts) is limited to the immediate-early (IE) proteins (12, 16). For human cytomegalovirus (HCMV) in particular, stable IE mRNA fails to accumulate after infection of many types of transformed human and mammalian cell lines (17, 18). Nevertheless, the isolated major IE promoter region from HCMV, as well as those from simian cytomegalovirus (SCMV) and murine cytomegalovirus, behaves as a powerful constitutive enhancer element in these same cell types, both in transient assays and in permanent cell lines (1, 7, 13, 15, 24, 27, 29). Even in permissive diploid human fibroblasts (HF), HCMV IE mRNA is overexpressed in the presence of cycloheximide (14, 40) and is also down-regulated at late times during the replication cycle (35, 37). These observations suggest that viral as well as cell type-specific factors may be influencing the levels of IE gene expression in different host cells.

In previous studies, both the IE1 and IE2 gene products (5, 30, 36) have been suggested to functionally contribute to this autoregulation. However, in our analysis in Vero cells, down-regulation by IE1 appeared to be a nonspecific effect, whereas that by IE2 was specific for the HCMV IE68 promoter (30). The IE2 gene product is also a powerful nonspecific *trans* activator of both HCMV delayed early promoters (6, 34) and a variety of heterologous promoters in transient assays (10, 21, 23, 30) and can complement the defect in E1A-minus mutants of adenovirus (33).

The IE1 and IE2 genes themselves are encoded by complex spliced mRNAs that are under the transcriptional control of a single major IE promoter region (37). The two HCMV proteins contain a small common NH_2-terminal domain of 87 amino acids (encoded by exons 2 and 3) and have large unique COOH-terminal domains encoded by exon 4 (IE1) or exon 5 (IE2). The COOH-terminal domain of IE2 participates in both *trans* activation and autoregulation, but the common NH_2-terminal domain is required only for *trans* activation (30).

In this report, we set out to further evaluate the role of IE2 negative autoregulation in HCMV and SCMV infections, with particular emphasis on identifying the presumed target sequences in the viral major IE promoter. The promoter plus enhancer regions of the HCMV IE68 and SCMV IE94 genes both have large and complex upstream domains that contain multiple copies of many cellular transcription factor-binding sites, including NFI, CRE, SRE, and NFkB elements (1, 2, 8, 11, 13, 15, 41). Although the specific organization of these 5′ upstream binding sites within the two enhancer regions has been greatly rearranged during their evolutionary divergence, the overall intron and exon structures of the IE1 and IE2 mRNAs and in particular the sequences around the cap site and in the large noncoding leader regions are highly conserved. Similarly, the key features and domains of the IE2 *trans*-activator proteins of HCMV and SCMV are relatively well conserved, with large stretches in the COOH half of the proteins having between 60 and 85% amino acid homology.

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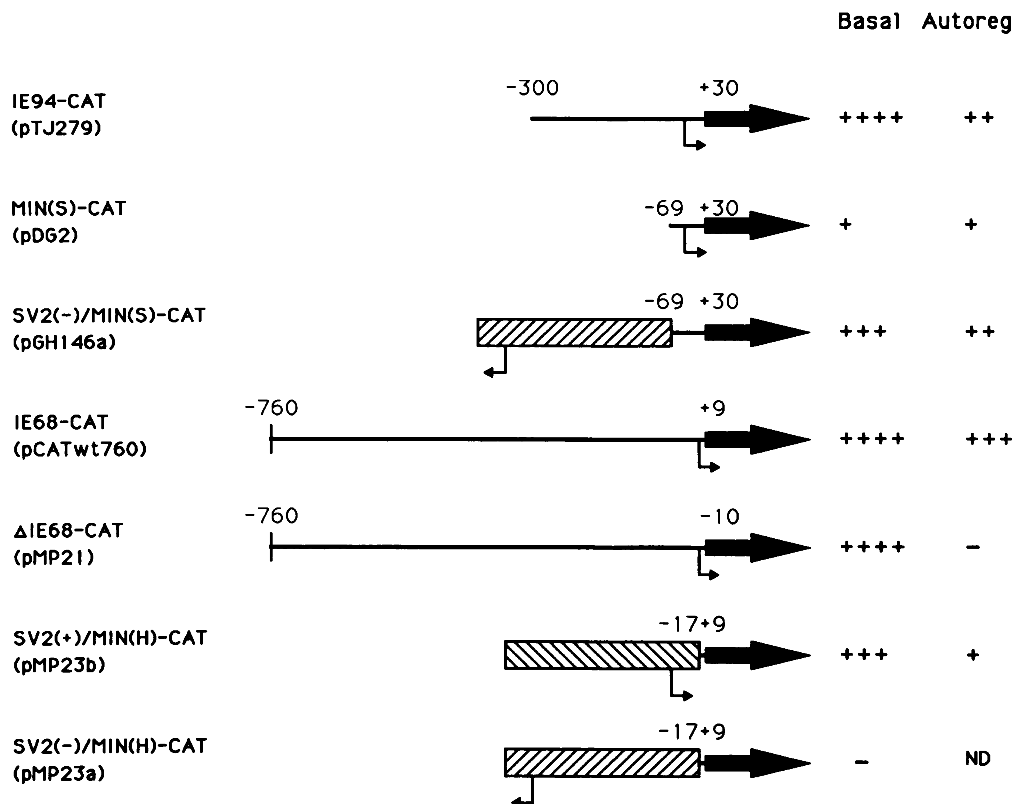

FIG. 1. Comparison of the structure and autoregulation responses of a series of reporter genes containing HCMV and SCMV major IE promoter sequences. Seven target CAT reporter gene constructions are listed under their descriptive names with the plasmid DNA nomenclature given in parentheses. The single lines represent CMV-derived promoter and leader sequences with 5′ and 3′ boundary positions indicated. Small arrows show the positions of known functional TATAAA elements in the parent SCMV IE94, HCMV IE68, and SV40 early-gene promoters. The CAT reporter gene cassette sequences (9, 20, 25) containing the CAT gene leader region, CATase-coding region, and SV40-derived splicing and poly(A) signals are designated by the heavy arrows. Hatched bars represent the added 340-bp SV40 early-gene promoter-enhancer region in the forward (+) or backward (−) orientation. The columns on the right summarize the results from experiments of the type illustrated in Fig. 2 and 3 and indicate the relative basal levels of CAT activity produced in transient assays in Vero cells together with the degree of IE2-specific down-regulation produced by cotransfection with intact HCMV IE2 effector genes. Basal: −, no detectable activity; +, ++, +++, and ++++, expression increasing in approximately fourfold steps relative to that of the minimal enhancer-minus SV40 promoter in A10-CAT. Autoregulation: −, not detectable; +, ++, and +++, increasing levels of IE2-mediated shutoff effects in approximately threefold steps observed after cotransfection with effector plasmids containing the intact HCMV IE2 gene. ND, Could not be determined.

MATERIALS AND METHODS

CMV target promoter constructions. The parent wild-type HCMV IE68(−760/+9)-CAT reporter gene in plasmid pCAT wt760 (30, 39) and the SCMV IE94(−300/+30)-CAT gene in plasmid pTJ279 (2, 15) have been described previously. Both retain portions of the major CMV IE enhancers in their natural upstream configuration. The structures of various additional forms of the minimal HCMV and African green monkey (SCMV) major IE promoters, with or without the added simian virus 40 (SV40) early-region enhancer, are summarized in the diagram shown in Fig. 1. They were constructed as follows. A 5′-deleted version of the IE94-CAT gene in plasmid pDG2 containing SCMV sequences from −69 to +30 was created by *Bal*31 deletion and the addition of a *Sal*I linker (2). A 1.7-kb *Sal*I fragment from pDG2 was then inserted into a pBR322-derived plasmid (pPOH34; 28) containing the SV40 early-region promoter-enhancer sequences from −340 to +58 with an added *Sal*I linker at the 5′ end. The resulting construction with the SV40-derived sequences in inverted orientation at the 5′ end of the SV2/MIN(S)-CAT gene is referred to as pGH146a. To prepare the SV2/MIN(H)-CAT gene, an upstream *Bam*HI-*Sac*I (−760 to −17) segment from a modified version of the pCATwt760 plasmid was removed and replaced with a *Bgl*II-*Sac*I fragment containing SV40 early-gene promoter-enhancer sequences from −340 to +58. The backward version (pMP23a), in which the enhancer was derived from plasmid pGH100a, has no interposed TATAA-box associated with the chloramphenicol acetyltransferase (CAT) gene and gave no detectable basal expression. In contrast, a forward-oriented version (pMP23b), which was derived from plasmid pGH100b, has the SV40 TATAA-box element placed upstream of the IE68(−17/+9)-CAT coding sequences and gave high basal expression.

A 3′-deleted form of the IE68 promoter, which lacks sequences between −10 and +8 (ΔIE68-CAT), was constructed by replacing sequences between the *Sac*I site in the promoter and the *Sca*I site in the CAT gene from pCATwt760 with a new 600-bp *Sac*I-*Sca*I bacterial CAT gene coding region cassette from pCATC′. This construction containing the IE68(−760/−11)-CAT gene is referred to as pMP21. A version of the IE68(−760/+9)-CAT gene with a

4-bp deletion introduced at the *Sac*I site at position −13 to −16 is referred to as pMP62.

To prepare analogous HCMV IE promoter-driven human growth hormone (HGH) reporter genes, either a 760-bp *Bgl*II fragment from pCATwt760 or a 740-bp *Bam*HI fragment from pMP21 was added at the *Bam*HI site upstream of the HGH-coding region in plasmid pϕGH (Nichols Institute, San Juan Capistrano, Calif.). The resulting IE68(−760/+9)-HGH (IE68-HGH) gene construction is contained in plasmid pMP29, and IE68(−760/−11)-HGH is contained in plasmid pMP28 (ΔIE68-HGH).

CMV effector gene constructions. Both of the parental major IE region plasmids used here that contain the intact exons 1, 2, 3, 4, and 5 under the control of their own viral promoter-enhancer regions have been described previously. HCMV IE68 IE1 and IE2 are encoded by plasmid pRL45 (30), and SCMV IE94 IE1 and IE2 genes are encoded by pTJ148 (15, 30). Derivatives of pRL45 that contain inserted triple terminators to produce either a truncated IE1 gene (pMP10, ΔIE1 + IE2) or truncated IE2 genes (pMP12 and pMP14, IE1 + ΔIE2) and deletions of IE1 (pMP18, intact IE2) or IE2 (pMP17, intact IE1) were also described by Pizzorno et al. (30). Two other pRL45 derivatives containing truncated IE1 and IE2 genes (pMP20, ΔIE1 + ΔIE2) or containing both a deleted IE2 gene plus a truncated IE1 gene (pMP19, ΔIE1) were described by LaFemina et al. (19) and were used here as promoter competition controls. Another pRL45-derived plasmid with an intact IE1 gene plus a truncated IE2 gene (pMP25, IE1 + ΔIE2) was constructed by inserting an *Xba*I triple terminator oligonucleotide at the *Bcl*I site at IE2 codon 92 near the COOH end of exon 5.

An initial HCMV IE2 effector plasmid that is autoregulation positive but transactivation negative and contains exon 5 sequences only (under the transcriptional control of the SCMV IE94 promoter) was reported by Pizzorno et al. (30). Two additional plasmids (pMP24a and pMP24b) of the exon 5 type were constructed by placing a 5.2-kb *Eco*RI-*Sac*I fragment containing IE2 codons 143 to 579 from pRL51 in either orientation adjacent to the SV40 enhancer-promoter region (−340 to +58) from pGH100a and pGH100b.

Synthetic oligonucleotides and reconstructed target promoter plasmids. Synthetic oligonucleotides were synthesized by Scot Morrow (Johns Hopkins School of Hygiene, Baltimore, Md.) using either an Applied Biosystems 380A or a Milligen 7500 DNA synthesizer and purified by reverse-phase high-pressure liquid chromatography or Oligo Purification Cartridge (Glen Research Corporation, Sterling, Va.). The exact 5′-to-3′ sequences of both strands of each oligonucleotide pair were as follows: LGH153/154, GGGAGCTC GTTTAGTGAACCGTCAGATCTCG/CGAGATCTGACC GGTTCACTAAACGAGCTCCC; LGH316/317, GGGAG CTCGTTTAGTGGGTACCCAGATCTCG/CGAGATC TGGGTACCCACTAAACGAGCTCCC; LGH318/319, CC AGTGAACCGTCAGACCCG/CGGGTCTGACGGTTCAC TGG; LGH380/381, CTGGTAGTGAACCGTCA/GATCT GACGGTTCACTACCAGAGCT; LGH382/383, CGTTGCT TGAACCGTCA/GATCTGACGGTTCAAGCAACGAGCT; LGH384/385, CGTTTAGGTCACCGTCA/GATCTGACG GTGACCTAAACGAGCT; LGH386/387, CGTTTAGTGA CAAGTCA/GATCTGACTTGTCACTAAACGAGCT; LGH388/389, CGTTTAGTGAACCTGAA/GATCTTCAGG TTCACTAAACGAGCT.

The first sets of oligonucleotide pairs (LGH153/154, LGH316/317, and LGH318/319) were designed to be blunt ended and were inserted into pMP21 (ΔIE68-CAT) at the unique *Sma*I site to create pMP50a, pMP52a, and pMP51a (forward orientation) and pMP50b and pMP52b (backward orientation). Removal of a 12-bp linker fragment from between the two *Sac*I sites in pMP52a produced a variant with wild-type spacing referred to as pMP61. The second sets of oligonucleotide pairs (LGH380/381, LGH382/383, LGH384/385, LGH386/387, and LGH388/389) were designed to have a *Sac*I overhang at the 5′ end and a *Bgl*II overhang at the 3′ end. These were inserted between the *Sac*I and *Bgl*II sites at the cap site in a version of pCATwt760 that lacks the upstream *Bgl*II site (pMP64) to produce plasmids pMP66 through pMP70 (see Fig. 5). All of the promoter mutation clones were confirmed by dideoxynucleotide sequencing using a Sequenase kit from United States Biochemicals (Cleveland, Ohio).

Heterologous target and effector genes. The herpes simplex virus type 1 (HSV-1) control CAT reporter gene 38K-CAT in plasmid pPOH1 (25), IE175(−360/−17)-CAT in plasmid pPOH40 (28), and the SV40 early promoter-enhancer region reporter gene SV2(−340/+58)-CAT in plasmid pSV2-CAT (20) have all been reported on elsewhere. An additional target IE175 reporter gene containing the putative HCMV autoregulation signal [IE175(Iwt−17/+9)-CAT, pMP41] was created by inserting the 30-mer LGH153/154 oligonucleotide pair at −17 in the IE175-CAT gene of pPOH40 by blunt-end ligation at the *Bam*HI site after filling in with Klenow DNA polymerase.

DNA transfection and transient CAT and HGH expression assays. All DNA-transfection procedures were done in monolayer cultures using calcium phosphate precipitation. Some experiments (see Fig. 2, 3, and 6) were performed using HEPES-buffered saline with air bubbling and a glycerol boost at 4 h after transfection (25). More recent experiments (see Fig. 4 and 7; Table 1) utilized the *N*,*N*-bis-(2-hydroxyethyl)-2-amino-ethanesulfonic acid (BES)-buffered saline procedure as modified from Chen and Okayama (3). Cells were plated at 5×10^5 per 35-mm well into 6-well cluster dishes the day before transfection. The medium was aspirated and replaced with 2.5 ml of fresh Dulbecco modified Eagle medium plus 10% fetal calf serum 4 h before transfection. Total amounts of DNA per well did not exceed 5 μg. Plasmid DNA and sterile H_2O were mixed to a volume of 113 μl. Then 12 μl of 2.5 M $CaCl_2$ was added and mixed. Next, 125 μl of 2× BES-buffered saline (50 mM BES [pH 6.95], 280 mM NaCl, 1.5 mM Na_2HPO_4) was added and gently mixed. The cocktail was kept at room temperature for 20 min and then added dropwise to each well and swirled. The cells were incubated at 35°C in 2.5% CO_2 for 20 h. The medium was aspirated, replaced with 2 ml of fresh Dulbecco modified Eagle medium plus 10% fetal calf serum, and incubated at 37°C in 5% CO_2. Cells were harvested 45 h after transfection and assayed for CAT activity by the method of Gorman et al. (9). Growth hormone assays were carried out in medium collected from the same cell cultures at the time of harvest. Samples of the culture medium (250 μl each) were stored at −20°C until assayed by radioimmunoassay procedure according to the instructions in the Allegro HGH kit (Nichols Institute).

RESULTS

Both the HCMV IE68 and the SCMV IE94 promoters contain *cis*-acting IE2 down-regulation signals. We showed previously that the homologous HCMV IE68 promoter, but neither the intact SV40 early promoter nor the HSV IE175 promoter, responded to IE2 down-regulation when cotransfected with the HCMV IE2 gene (30). In fact, expression

from IE175-CAT and numerous other CAT reporter genes containing weak heterologous promoters was strongly stimulated by effector plasmids encoding either HCMV IE2 alone or the IE1 plus IE2 genes. However, there was no information available about whether the SCMV major IE genes also encoded a negative autoregulatory function or whether the SCMV IE94 promoter was subject to down-regulation in a similar manner. The structures of a set of target CAT reporter genes designed to examine these questions are illustrated in Fig. 1.

In an initial experiment, target plasmid DNA samples containing the HCMV or SCMV major IE promoter-enhancer regions were cotransfected into Vero cells with control vector DNA sequences (pUC18) or with equivalent amounts of effector DNA containing the intact IE1 plus IE2 genes from either HCMV (pRL45) or SCMV (pTJ148). The results of these transient expression assays revealed that the high basal activity of IE68(−760/+9)-CAT was diminished dramatically (25- to 70-fold) in the presence of either the HCMV or SCMV IE gene products (Fig. 2a). Similarly, the IE94(−300/+30)-CAT target gene was inhibited by both effector plasmids, although to a reduced extent (10- and 5-fold), whereas the HSV-1 delayed-early promoter in 38K-CAT was stimulated 60-fold in a parallel sample. We also tested a derivative of the IE94(−69/+30)-CAT 5′-deletion construction which contains only the minimal promoter elements from the SCMV IE gene but has the resulting reduced basal expression compensated for by the addition in *cis* of the 340-bp SV40 upstream promoter plus enhancer region. This SV2/MIN(S)-CAT target gene also displayed a down-regulation phenotype, giving 8- and 14-fold-reduced expression in the presence of the HCMV or SCMV IE gene products (Fig. 2a).

In a second related experiment, the same three target gene plasmids, plus an SV2-CAT negative control and a minimal HCMV promoter construction with the IE68(−17/+9)-CAT placed at position +58 downstream from the 340-bp promoter-enhancer region from SV40 (pMP23b), were cotransfected with an effector plasmid containing only the HCMV exon 5 coding sequences under the transcriptional control of the SCMV promoter-enhancer region (pRL51; 30). This IE2 COOH-terminal domain expression vector gave a four- to sixfold shutoff of all four CMV IE promoter targets compared with a slight activation of the parent SV2-CAT target DNA (Fig. 2b).

In other experiments (not shown) a series of 5′ deletions of the IE94 promoter, including the minimal IE94(−69/+30)-CAT gene prepared without added *cis*-acting enhancer sequences (plasmid pDG2; Fig. 1), were also all down-regulated, both at the basal level and after positive *trans* activation by the IE110 effector gene from HSV-1 (plasmid pIGA15; 26). Therefore, we conclude that both the *cis*- and *trans*-acting elements that mediate IE2 autoregulation are conserved between the human and simian versions of the CMV major IE genes. Although the SV40-enhanced minimal promoter in IE94(−67/+30)-CAT was strongly down-regulated in a number of separate experiments, this result was not consistently observed with the SV40-driven minimal IE68(−17/+9)-CAT version. However, since the latter construction lacks the HCMV-derived TATAA-box element and is therefore effectively located far downstream in the SV40 leader sequence, it would not be expected to have yielded a fully responsive phenotype. Certainly the data suggest that the *cis*-acting autoregulation signal does not specifically require upstream CMV-derived enhancer elements.

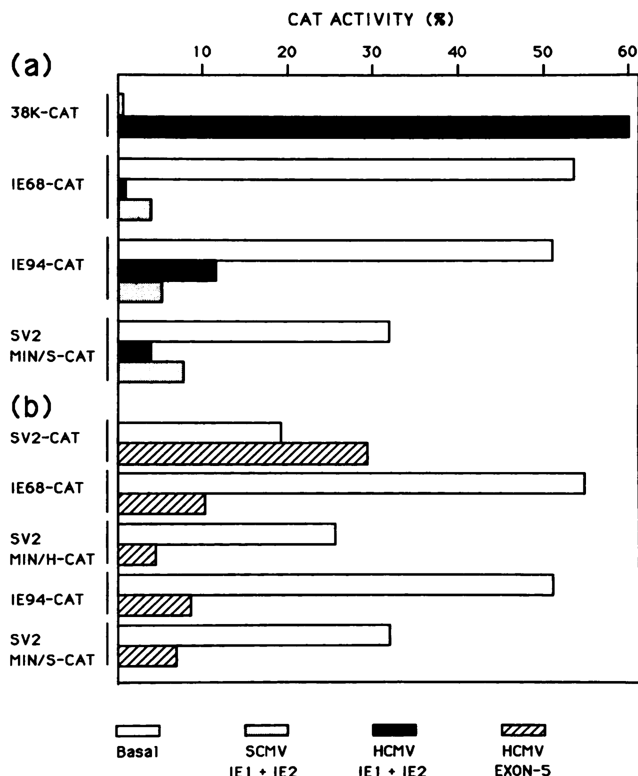

FIG. 2. Down regulation of IE68 and IE94 promoters by both HCMV and SCMV IE functions. The histogram presents the results of CAT assays expressed as percent conversion of [^{14}C]chloramphenicol to 1′- or 3′-acetyl chloramphenicol per hour in extracts obtained from Vero cells after transient DNA transfection. (a) Cotransfection with intact IE1 plus IE2 genes derived from HCMV or SCMV. Target DNAs included the HSV 38K-CAT gene (plasmid pPOH1, 2 μg), the HCMV IE68(−760/+9)-CAT gene (pCATwt760, 0.5 μg), the SCMV IE94(−340/+30)-CAT gene (pTJ279, 2 μg), and the SV2/IE94(−67/+30)-CAT gene (pGH146b, 2 μg). Effector DNAs included 3 μg of carrier pBR322 plasmid DNA (Basal), pRL45 (HCMV IE1 + IE2), or pTJ148 (SCMV IE1 + IE2). (b) Cotransfection with an HCMV IE2 plasmid lacking the NH_2-terminal *trans*-activation domain. Target DNAs included 2 μg each of plasmids containing the pSV2-CAT gene—SV2/IE68(−17/+9)-CAT [SV2/MIN(H) promoter in pMP23b], IE94(−340/+30)-CAT (parent SCMV promoter in pTJ279), and SV2/IE94(−67/+30)-CAT [SV2/MIN(S) promoter in pGH146b]—or 0.5 μg of IE68(−760/+9)-CAT (parent HCMV promoter in p760wt-CAT). Effector DNAs were 3 μg of either carrier pBR322 plasmid DNA (Basal) or pRL51 DNA (HCMV exon 5).

A specific negative autoregulation signal is located near the major IE mRNA start site. On the basis of the demonstration that the minimal promoters described above retained the autoregulation signals, we created a 3′-deleted IE68-CAT construction using a convenient *Sac*I site located at position −14 just upstream from the mRNA start site. This construction possesses the normal HCMV TATAA-box element from IE68 but has replaced 19 bp from the cap site (positions −10 to +8) with polylinker sequences. Importantly, the high-level basal expression obtained from the HCMV IE promoter was not significantly altered in the deleted IE68(−760/−11)-CAT construction. A comparison of the responses of wild-type and 3′-deleted target genes to cotransfection with various HCMV IE effector plasmids is shown in Fig. 3a. We have reported previously that plasmids containing either the intact IE1 gene alone or IE1 plus a truncated IE2 gene give nonspecific shutoff effects in Vero cells (30), and this phe-

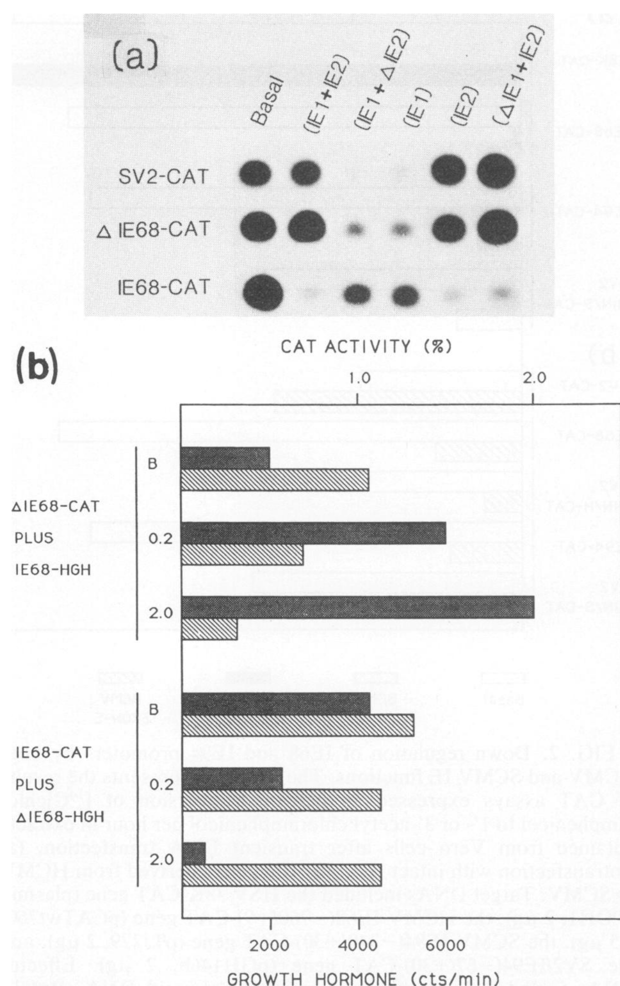

FIG. 3. Removal of IE68 cap site sequences abolishes the down-regulation phenotype. (a) Comparison of the effects of DNA cotransfection on different target promoters in Vero cells. The autoradiograph shows only the 3′-acetyl [^{14}C]chloramphenicol spots from the enzyme assay. The three target DNAs contained the negative control SV40 early promoter (SV2-CAT plasmid, 2 μg), the 3′-deleted IE68(−760/−10)-CAT promoter (ΔIE68-CAT in plasmid pMP21, 0.5 μg), and the wild-type positive control IE68(−760/+9)-CAT promoter (IE68-CAT in plasmid pCATwt760, 0.5 μg]. The six cotransfected effector DNA samples used were 3 μg of pBR322 carrier DNA (Basal) or 3 μg each of pMP17 (IE1), pMP18 (IE2), pMP25 (IE1 + ΔIE2), pMP10 (ΔIE1 + IE2), and pRL45 (IE1 + IE2) DNA. (b) Comparison of target promoters containing HGH (▧) or CAT (■) reporter genes. The histogram shows the results of reciprocal joint cotransfection experiments with cap site plus and minus IE68 target promoter constructions in Vero cells. Target DNA samples contained either a mixture of 0.5 μg of the wild-type IE68(−760/+9)-CAT plasmid DNA (IE68-CAT, pCATwt760) plus 0.5 μg of deleted IE68(−760/−11)-HGH plasmid DNA (ΔIE68-HGH, pMP28) or a mixture of 0.5 μg of deleted IE68(−760/−10)-CAT plasmid DNA (ΔIE68-CAT, pMP21) plus 0.5 μg of wild-type IE68(−760/+9)-HGH plasmid DNA (IE68-HGH, pMP29). Effector DNA sample groups consisted of carrier pBR322 only for basal expression and two different input doses (0.2 or 2.0 μg) of pRL45 DNA containing the intact HCMV IE1 plus IE2 genes. The data are averages from two samples each. In this experiment, 1,500 cpm in the radioimmunoassay represented 1 ng of HGH per ml.

notype was displayed here also with both of the HCMV IE promoter targets and with the control SV2-CAT gene. In contrast, plasmids containing IE2 alone, an intact IE2 gene plus truncated IE1 gene, or both the IE1 and IE2 genes intact all specifically down-regulated only the wild-type IE68-CAT target and had no additional effects on either the ΔIE68-CAT or SV2-CAT targets. The magnitude and reproducibility of the different responses observed for the IE68-CAT target gene compared with ΔIE68-CAT and SV2-CAT control genes in Vero cells in a number of experiments covering a wide range of basal expression levels are summarized in Table 1. Clearly, the pattern of response by the ΔIE68-CAT target gene differed dramatically from that of the wild-type IE68-CAT and closely resembled that of the SV2-CAT control. Therefore, we conclude that a specific *cis*-acting signal for the IE2 negative response must lie near the cap site in the HCMV major IE promoter.

IE2-mediated down-regulation is not reporter gene specific. To further emphasize the different properties of the cap site deletion construction, we prepared an HGH reporter gene driven by the wild-type IE68 promoter and cotransfected low input doses of this DNA together with the ΔIE68-CAT plasmid as target genes in the presence of increasing amounts of IE1 plus IE2 effector DNA (Fig. 3b). This protocol permitted both HGH and CAT levels to be measured from exactly the same DNA-transfected cell cultures. The results demonstrated that expression of IE68-HGH was also down-regulated up to sixfold by the IE effector genes, whereas in the same cells, ΔIE68-CAT expression was actually stimulated fourfold. Thus, after removal of the HCMV cap site sequences, the IE68 promoter in effect behaved like all other heterologous promoters that we have tested by responding positively to the nonspecific *trans*-activating function of IE2.

Negative autoregulation also occurs in permissive HF cells. Since infected Vero cells are nonpermissive for HCMV IE gene expression from the input viral genome, we asked whether these properties would also be exhibited in diploid HF cells. Usually expression of DNA-transfected genes is much easier to accomplish in Vero cells than in HF cells, and the lower basal levels in HF cells make down-regulation difficult to demonstrate convincingly. However, all cotransfected plasmids containing either the IE1 or IE2 genes intact gave three- to fourfold down-regulation of CAT expression from the parent wild-type IE68-CAT target, and the IE1 effector plasmids also reduced expression from the ΔIE68-CAT gene (Fig. 4). In contrast, all effector plasmids containing an intact IE2 gene yielded a three- to fivefold stimulation rather than inhibition of ΔIE68-CAT. Therefore, although the magnitude of the effects was reduced, all three functions that we have demonstrated in Vero cells, namely, nonspecific IE1 shutoff, specific IE2 down-regulation, and IE2 *trans* activation in the absence of the target autoregulation signal, were confirmed to occur also in HF cells.

Transfer of the autoregulation signal. Final confirmation of the identification of a putative regulatory response signal usually requires positive evidence that the isolated sequence conveys the appropriate phenotype to a heterologous promoter. Although the SV40 promoter-enhancer-driven minimal IE68(−17/+9)-CAT construction provided some supportive evidence of this type, we chose to prepare a more direct and definitive construction in which a double-stranded synthetic oligonucleotide containing sequences from −17 to +9 was placed close to the cap site in the IE175 promoter from HSV-1. The IE175 promoter also displays specific down-regulation mediated by its own gene product acting

TABLE 1. Specific inhibition of HCMV major IE promoter by cotransfection with IE2 gene

Effector gene	Plasmid name	CAT activity with target genes								
		IE68-CAT			ΔIE68-CAT[a]			SV2-CAT		
		Basal[b] (%)	Trans[c] (%)	Fold change[d]	Basal (%)	Trans (%)	Fold change	Basal (%)	Trans (%)	Fold change
IE1 + IE2	pRL45	87	5.4	−16				30	50	−1.7
	pRL45	52	0.8	−66				19	9.9	−1.9
	pRL45	29	0.8	−36	13.6	18	+1.3	5.8	4.9	+1.2
	pRL45	22	1.6	−14				9.9	15.3	+1.6
	pRL45	21	0.45	−47	11.5	24	+2.1			
	pRL45	16	2.1	−7.5						
	pRL45	8.4	0.7	−11.5	8.2	12.8	+1.6			
IE2 only	pMP18	29	0.8	−35	13.6	9.9	−1.4	5.8	8.7	+1.5
	pMP18	22	5.7	−3.8				9.9	37	+3.8
	pMP10	22	2.7	−8.1				9.9	66	+6.6
	pMP18	16	1.1	−15						
IE1 only	pMP17	29	3.9	−7.4	13.6	2.3	−5.9	5.8	0.7	−8.2
	pMP25	29	3.7	−7.8	13.6	2.7	−6.3	5.8	0.6	−9.7
	pMP17	22	10.7	−2.0				9.9	8.2	−1.2
	pMP12	22	5.0	−4.4				9.9	3.5	−2.8

[a] pMP21(Δ−10/+8).
[b] Percent conversion after cotransfection with vector DNA only.
[c] Percent conversion after cotransfection with effector DNA.
[d] −, Down-regulation; +, stimulation.

through a negative autoregulation response signal at the cap site (27, 28, 32), but these two viral down-regulation systems do not complement one another (30, 32). In essence, the experiment was designed to convert the IE175 promoter from a target that responds negatively to the HSV IE175 protein into a target that responds negatively to the HCMV IE2 protein. The parent plasmid was a 3′-deleted version of IE175(−380/+32)-CAT which had a *Bam*HI linker inserted in place of the HSV cap site sequences from −16 to +32. Insertion of the putative HCMV autoregulation signal oligonucleotide (Iwt−17/+9) resulted in a construction in which the normal IE68 start site was now located 37 bp downstream from the IE175 TATAA-box element (i.e., equivalent to position +10 in the IE175 promoter; Fig. 5b). Basal expression from the IE175-CAT construction was unaltered by insertion of the oligonucleotide.

Two types of cotransfection experiments were carried out. In the first, the effects of cotransfected HCMV IE1 and IE2 effector plasmids were assessed directly in Vero cells. In the second, the relatively low-level basal expression from the IE175-CAT genes was first boosted by introduction of the HSV IE110 nonspecific transactivator gene (plasmid pIGA15; 26). At the input doses used in the first experiment, expression from the standard wild-type IE68-CAT target DNA was down-regulated ninefold by IE2- or IE1-plus-IE2-containing plasmids and reduced two- to threefold by both the IE1 effector plasmid and a ΔIE1-plus-ΔIE2 promoter competition control plasmid (Fig. 6a). As expected, expression from the parent ΔIE175-CAT was stimulated between 7- and 11-fold by the IE2 plasmids, but a 3-fold shutoff effect occurred in the presence of the IE1 or ΔIE2-plus-ΔIE2 control plasmids. In contrast, the ΔIE175-CAT target gene with the inserted oligonucleotide [ΔIE175(Iwt)-CAT] failed to respond to IE2 activation and was in fact inhibited 10-fold. Thus, the two IE175 target genes differed 70- to 100-fold in their responses to cotransfection with the IE2 or combined IE1 plus IE2 effector genes. In this experiment, the nonspecific shutoff by the IE1 plasmid appeared to be largely explained by promoter competition effects, which were greater on the relatively weak heterologous IE175 constructions than on the homologous enhancer-containing IE68 promoter. However, this phenomenon accounted for no more than a twofold difference between the IE175 promoter containing the HCMV cap site oligonucleotide and that without it.

A similar result was obtained when the levels of CAT expression from the IE110-activated ΔIE175-CAT promoter constructions were compared in the presence or absence of the IE2 gene products (Fig. 6b). In our experience, the results of cotransfection of heterologous target genes with both IE110 and IE2 together can range from synergistic to highly antagonistic depending on the relative amounts of the two plasmids used. At the ratios used here there was only a mild 2- to 3-fold antagonistic effect on the parent ΔIE175-CAT plasmid, whereas a large 30- to 90-fold down-regulation effect was obtained with the version containing the IE68 cap site oligonucleotide [ΔIE175(Iwt)-CAT]. Again, the IE1 and promoter competition controls gave between three- and eightfold shutoff effects that were of essentially the same magnitude for both target genes. Therefore, the conclusion appears inescapable that introduction of the CMV cap site not only transfers the IE2-specific down-regulation phenotype to the previously nonresponsive IE175 promoter but also leads to dominant inhibition over the nonspecific IE2 *trans*-activation phenotype.

Restoration and inactivation of the autoregulation signal with mutant oligonucleotides. To more precisely map the sequences contributing the HCMV autoregulation signal and to begin to address the mechanism of IE2-mediated down-regulation, we synthesized two sets of oligonucleotides that contained deletions or point mutations around the cap site and inserted them into the nonresponsive ΔIE68-CAT reporter gene construction (Fig. 5b). In the first set of four new target genes, nucleotides −17 to +9 were placed at the *Sma*I site of ΔIE68(−760/−11)-CAT in both the forward and backward orientations. Addition in the forward orientation

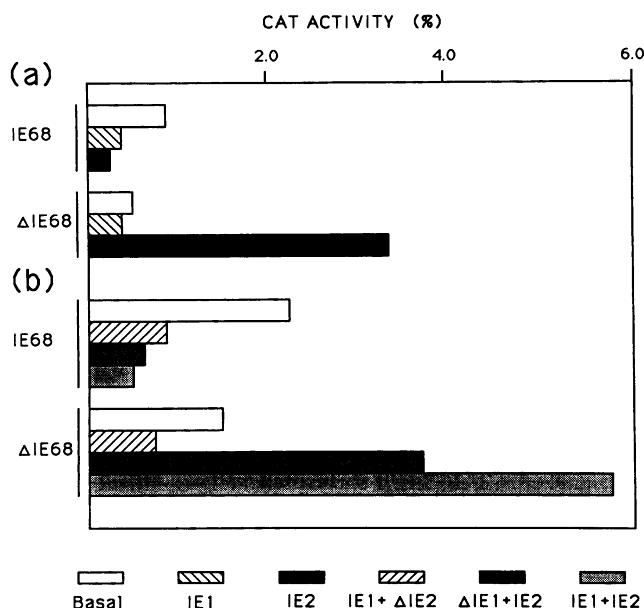

FIG. 4. Demonstration of specific negative autoregulation in permissive diploid HF cells. The histogram shows the results of transient CAT expression assays carried out in HF cell cultures. (a) Effects of cotransfection with single effector genes. (b) Comparison of the use of combinations of intact and truncated IE1 plus IE2 genes. The two different types of target DNA contained the wild-type IE68(−760/+9) promoter (IE68-CAT in plasmid pCATwt760) and the 3′-deleted IE68(−760/−11) promoter (ΔIE68-CAT in plasmid pMP21). The effector DNA samples used were 3 μg each of pUC19 carrier DNA (Basal), pMP17 (IE1), pMP18 (IE2), pRL45 (IE1 + IE2), pMP12 (IE1 + ΔIE2), and pMP10 (ΔIE1 + IE2). Data given in panel b are averages from two separate experiments.

essentially recreated a wild-type IE68 promoter (−760 to +9) except for a 12-bp linker insertion at position −17. A third insertion received the same sequences except for a 6-bp substitution mutation that created a *Kpn*I site at position −3 to +3. The final construction received a shorter oligonucleotide insertion containing nucleotides from positions −7 to +7 only. Assessment of the ability of these target genes to respond to IE2 down-regulation by cotransfection with pRL45 DNA revealed that both orientations of the wild-type sequence from −17 to +9 restored autoregulation, whereas neither the sequence from −7 to +7 nor the linker substitution mutation from −3 to +3 had any effect (Fig. 7). Surprisingly, the backward orientation of the sequence from −17 to +9 inserted at +11 consistently gave severalfold-greater down-regulation than the forward orientation, and this effect was confirmed with pMP18 effector DNA as well (Fig. 8a). These results indicate that the response signal probably acts at the DNA level rather than at the mRNA level and that components of the signal must lie both upstream of −7 and within 3 bp of the normal IE68 mRNA start site.

A second set of oligonucleotide inserts was also placed between the *Sac*I and *Bgl*II sites in ΔIE168-CAT to create a series of adjacent triple-point mutations that all lie within an otherwise unaltered parental DNA sequence and spacing context at positions −11 to +4 (pMP66 to pMP70; Fig. 5b). After cotransfection with pRL45 DNA, all of these mutant target promoters proved to have intermediate effects in restoring the negative autoregulation phenotype (Fig. 8b). In comparison, a deletion of 4 bp from the wild-type promoter at the *Sac*I site between −16 and −13 failed to affect the autoregulation phenotype (pMP62; Fig. 8a). The mutations at −5 to −3 and +2 to +5 had the least effect and those at −8 to −6 and −2 to +1 the greatest effect on reducing the response, especially at lower levels of input IE2 DNA. The negative result obtained with the inserted oligonucleotide containing positions −7 to +7 (Fig. 7) is also significant here. That construction (in plasmid MP51a) can be considered in this context to have substituted CCCCC for TTT at positions −10 to −8. Therefore, provided that the 2-bp positional alteration is not significant, this site also defines a component of the response signal. Note that positions −15 to +5 are highly conserved between both the HCMV and SCMV major IE promoters, although positions +6 to +9 are not (Fig. 5a). Therefore, we conclude that the response signal, which may be either partially redundant or dependent on some form of secondary structure, lies totally within a 17-bp element with the sequence CGTTTAGTGAACCGTCA between position −12 and +5. Interestingly, this sequence encompasses a potential near-palindromic consensus recognition motif of CGTTTNNNNAACCG (which is highlighted in reverse-type format in Fig. 5).

DISCUSSION

We have identified a *cis*-acting target signal in the major IE promoter of HCMV that is necessary for the IE2-dependent down-regulation phenotype in transient cotransfection assays. Initial mapping studies revealed that a key sequence necessary for this response mapped between positions −17 and +9 in the HCMV promoter. A 30-bp double-stranded oligonucleotide carrying this sequence was sufficient both to restore the negative autoregulation properties to a deleted HCMV IE promoter and to transfer the response signal to the heterologous IE175 promoter from HSV. In addition, this signal functioned efficiently in either the forward or reverse orientation and also when moved 12 and 14 bp downstream from its normal position relative to the TATAAA element. Therefore, specific interactions with factors binding to nearby regulatory elements are either unlikely to be of critical importance or must occur in a spatially independent manner.

Our attempts to define the precise position and DNA sequences of the response element revealed that it lies close to or overlapping the cap site. A linker scanner mutation at −3 to +3 completely abolished the phenotype, but this still left open the question of whether the response signal might be in the mRNA. However, several triple-point mutations upstream from the cap site partially reduced the response, and a mutation between positions −10 and −8 eliminated autoregulation. Therefore, we infer that the signal is probably recognized at the DNA level. No significant secondary structural features are obvious other than the possible palindromic character of the recognition motif.

The negative autoregulation phenomenon that we have described here for HCMV may mechanistically resemble the interactions of procaryotic repressor DNA-binding proteins with their operator motifs. There are also three other similar negative autoregulation systems that have been defined in some detail in mammalian DNA viruses, and all of them are known to act through specific DNA-binding interactions occurring near the cap site. In SV40 and polyomavirus, binding of large T antigen to sites I and II in the cap site and leader region for the early-gene transcripts produces a negative down-regulation effect in vitro that is presumed to involve steric blocking of access of RNA polymerase II to

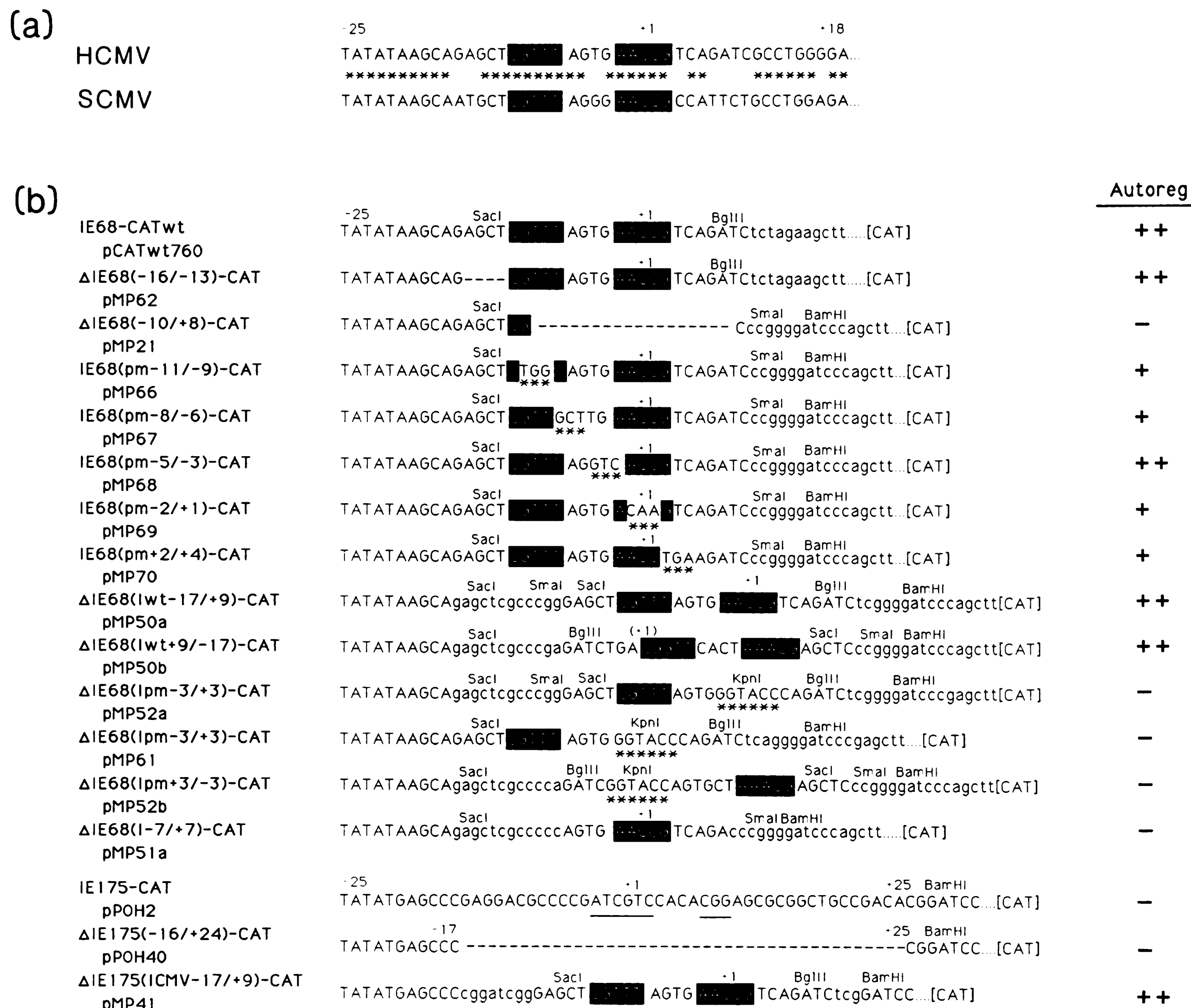

FIG. 5. Evaluation of sequence requirements for HCMV autoregulation. (a) Comparison of nucleotide sequences in the proximal leader regions of the major IE genes of HCMV(IE68) and SCMV(IE94). Matched bases are denoted by asterisks, and the proposed palindromic consensus autoregulation signal elements are indicated by the bases in reverse type. The data come from Thomsen et al. (41) and Jeang et al. (15). (b) Summary of the relevant DNA sequences and autoregulation properties of a series of mutated and deleted target IE68-CAT and IE175-CAT gene constructions. Names, gene designations, and DNA sequences on the noncoding strands of appropriate plasmids are given, together with the phenotypes observed in transient CAT assays (right-hand column). Examples of the experimental results from which these data were derived are illustrated in Fig. 6, 7, and 8. The predicted consensus signal sequence is shown in reverse type, and non-HCMV or HSV nucleotides derived from linker or plasmid sources are shown in lowercase. Mutated bases are indicated by the asterisks. Dashes denote missing bases, and the consensus IE175 autoregulation signal is underlined. The positions of naturally occurring *SacI* and *BamHI* sites and other (introduced) restriction enzyme cleavage sites are indicated above each sequence, and the positions of the nucleotides that represent the normal major mRNA start site in the wild-type CMV and HSV IE promoters are indicated by a +1. The precise identification of the IE94 mRNA start site was described by K.-T. Jeang (Ph.D. Thesis, Johns Hopkins University, Baltimore, 1984).

the initiation site or interference with the formation of initiation complexes (31). However, SV40 autoregulation is less dependent on the large-T-antigen-binding sites in vivo, and the demonstrated ability of large-T-antigen protein to complex with the cellular AP-2 transcription factor and thus to prevent AP-2 binding to functionally important upstream sites (in a T-antigen-binding-site-independent manner) appears to contribute to down-regulation of early-region transcripts (22). Recent studies with the bovine papillomavirus E2 protein show that it stimulates transcription at most upstream consensus binding sites but inhibits transcription at one particular consensus element near an mRNA start site (38). This down-regulation appears to be dependent on an interaction of the bound E2 protein with a cellular factor that recognizes an overlapping binding site. The IE175(ICP4) transactivator protein of HSV also produces a specific negative autoregulation response in cotransfection assays through binding to a conserved consensus element located near the cap site in its own promoter (27, 32). However, the significance of this effect in regulation of the lytic infection cycle or the latent state for HSV has yet to be clarified.

The DNA sequence of the HCMV negative autoregulation signal does not resemble that of any of these other viral response elements, and we have presented evidence previ-

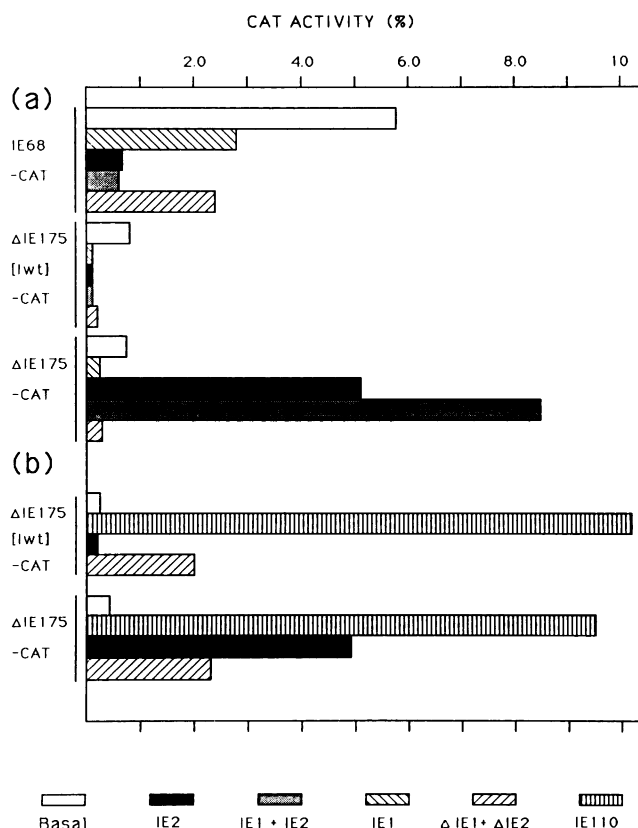

FIG. 6. Transfer of the negative autoregulation signal to the HSV IE175 promoter. The histogram presents results of transient CAT expression assays carried out in Vero cells. (a) Inhibition of IE2-mediated activation. Target DNAs included 0.5 μg of the control IE68(−760/+9)-CAT gene (IE68-CAT in plasmid pCATwt760) and 2 μg each of either the parent IE175(−360/−19)-CAT gene (IE175-CAT in plasmid pPOH40) or the same construction with an inserted 30-mer oligonucleotide containing the putative HCMV autoregulation signal [IE175(Iwt)-CAT in plasmid pMP41]. Effector DNA included 3 μg of pKP54 carrier DNA (Basal), pMP18 (IE2), pRL45 (IE1 + IE2), pMP17 (IE1), or pMP20 DNA (ΔIE1 + ΔIE2). (b) Inhibition of HSV IE110-mediated transactivation. The same two versions of IE175(−360/−19)-CAT described above were used as target DNAs (i.e., pMP41 or pPOH40, 2 μg). The results given are averages from paired test samples receiving 0.5 and 2.5 μg of pRL45 (IE1 + IE2) or 0.5 and 2.5 μg of pMP19 DNA (ΔIE1) in addition to 0.5 μg of pIGA15 DNA. Control samples were cotransfected with pKP54 carrier DNA only (Basal) or 0.5 μg of pIGA15 effector DNA (IE110). All samples received a total of 5 μg of effector plus carrier DNA.

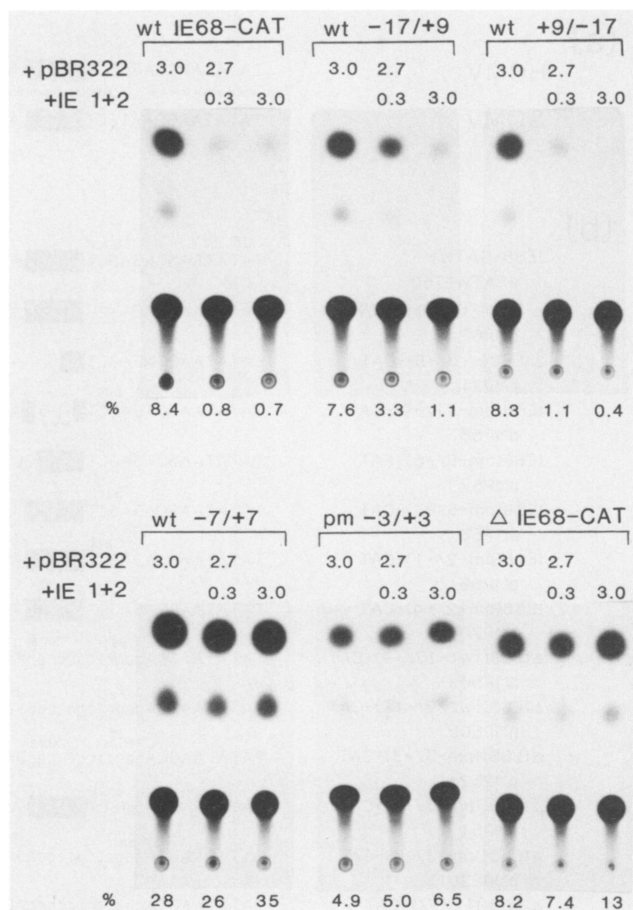

FIG. 7. Negative autoregulation signal restores function when inserted into the deleted IE68 promoter in either orientation. The autoradiographs show the results of transient cotransfection CAT assays with target ΔIE68(−760/−11)-CAT genes containing inserted wild-type or mutant oligonucleotides placed near the cap site. Data for these and similar constructions are summarized in Fig. 5. The amounts of carrier and effector DNA used are given above the lanes, and the resulting levels of CAT measured are given as percent conversion of [^{14}C]chloramphenicol to 1′-acetyl chloramphenicol and 3′-acetyl chloramphenicol per hour. The target DNA plasmids used were 0.5 μg each of pCATwt760(IE68-CAT), pMP50a(wt −17/+9), pMP50b(wt +9/+17), pMP51a(wt −7/+7), pMP52a(pm −3/+3), and pMP21(ΔIE68-CAT).

ously that the HCMV and the HSV systems do not complement or cross-react with each other functionally (30, 32). However, our data clearly show that the HCMV IE2 autoregulation signal is both conserved and functional in the SCMV(Colburn) major IE promoter. Interestingly, there is no recognizable matching homologous DNA sequence at this position in the major IE promoter of MCMV (7), but experiments to look for a phenotypically similar regulatory mechanism have yet to be carried out with that system. Surprisingly, there are no data available to indicate that HCMV IE2 acts as a direct DNA-binding protein. In preliminary experiments, we have been unable to demonstrate binding to HCMV cap site DNA probes with a 40-kDa IE2 COOH-terminal domain produced as an *Escherichia coli* fusion protein, despite the fact that this portion of the protein alone (codons 305 to 579) is sufficient to give specific down-regulation in transient assays (M. C. Pizzorno and G. S. Hayward, unpublished data). Nevertheless, we presume that the HCMV autoregulation mechanism must involve a sequence-specific DNA-binding factor and that IE2 (if it is not that factor itself) will be able to form a bound complex with or modify some cellular factor that recognizes the negative response signal. On the other hand, the lack of any significant alteration in basal expression in the autoregulation-minus promoter mutants suggests that the IE2 cap site response element does not represent a binding site for any positively acting cellular transcription factors. Therefore, we favor a model whereby IE2 acts either directly or indirectly through a DNA-binding mechanism to sterically block access of TFIID or of other general transcription factors in formation of the initiation complex.

The HCMV IE2 protein is also a nonspecific *trans* activator of gene expression in almost all heterologous promoters

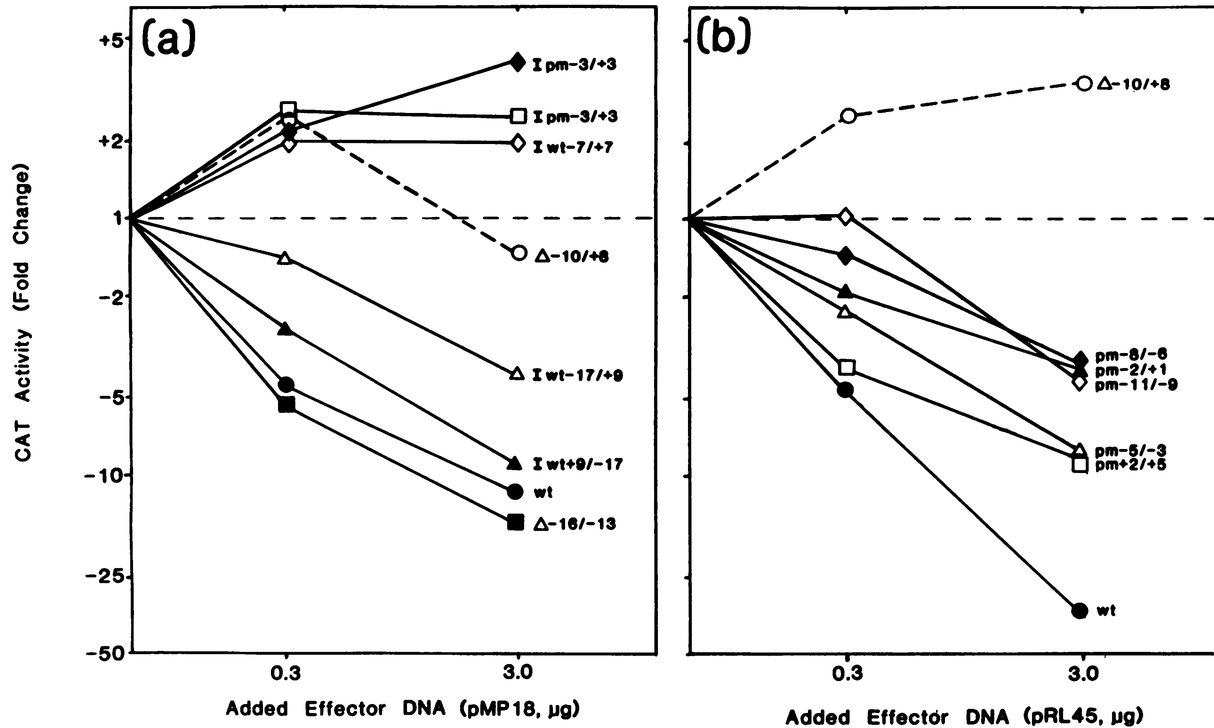

FIG. 8. Effects of mutagenesis and spacing changes on the ability of oligonucleotides to restore the autoregulation phenotype. Results of dose-response CAT assays with various target IE68-CAT genes containing inserted oligonucleotides are plotted as fold induction (+) or fold repression (−) of CAT activity after cotransfection with IE1 only (pMP18) (a) or IE1 plus IE2(pRL45) effector DNA (b). Target plasmid DNA samples (0.5 μg) were as follows: (a) pMP21 (○), pCATwt760 (●), pMP50a (△), pMP50b (▲), pMP51 (◇), pMP52 (◆), pMP61 (□), and pMP62 (■); (b) pMP21 (○), pCATwt760 (●), pMP66 (◇), pMP67 (◆), pMP68 (△), pMP69 (▲), and pMP70 (□). All samples were brought to 3.5 μg total DNA with added pKP54 carrier DNA.

that we have tested. Therefore, we anticipated that after mutation of the autoregulation signal, the response of the HCMV IE promoter to cotransfection with IE2 might be converted from a negative into a positive effect. Indeed, in many of our experiments this was the case. Therefore, one could argue that the negative effects on the wild-type promoter are actually much greater than the response that we have measured here, because of the superimposed nonspecific positive influences of IE2. Alternatively, one could argue that the negative effects simply dominate any positive ones, which is the case for the HSV IE175 autoregulation system in similar assays and was also the case here when we examined the influence of IE2 on the levels of IE110-activated gene expression.

The known propensity of the powerful HCMV upstream enhancer domain to produce promoter competition complications and of the HCMV IE1 protein to nonspecifically down-regulate gene expression in Vero cells (30) made it necessary to carry out a number of controls for these effects, especially when the combined-IE1-plus-IE2 class of effector gene plasmids was used. We conclude that in many of our experiments in which HCMV IE promoter targets were cotransfected with the intact HCMV IE genes, the overall yield of reporter gene product represented a summation of four distinct functional activities, i.e., IE promoter competition, IE1 nonspecific shutoff, IE2 nonspecific *trans* activation, and IE2-mediated sequence-specific down-regulation. There is also the possibility that IE1-specific positive effects were being exerted through the upstream NFkB sites as described by Cherrington et al. (4) in HF cells and Jurkat cells. However, at this point, nothing is known about the mechanism or target signals for either the IE1 or IE2 nonspecific effects, and we have seen no evidence for NFkB-mediated positive effects in DNA-transfected Vero cells.

The negative autoregulation phenomenon that we have described here provides a possible explanation for the lack of IE1 mRNA expression seen after HCMV infection of nonpermissive transformed human cell types (17, 18). In our experience, SCMV IE2 mRNA is synthesized within 1 h after virus infection and before any significant level of IE1 mRNA is produced (Y. N. Chang and G. S. Hayward, unpublished data). Furthermore, the high constitutive basal expression of a DNA-transfected IE94-CAT gene in human teratocarcinoma cells can be completely shut off after superinfection by SCMV(Colburn) virus (D. Gay and G. S. Hayward, unpublished data). Conceivably, in the appropriate cellular environment, initial expression of IE2 in the absence of IE1 could lead to dominant inhibition of both IE transcription and all subsequent viral gene expression. Knowledge of the target signal for this autoregulation should now make it possible to test these ideas in future studies.

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LITERATURE CITED

1. **Boshardt, M., F. Weber, G. Jahn, K. Dorsch-Hasler, F. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41**:521–530.
2. **Chang, Y.-N., S. Crawford, D. Rawlins, K.-T. Jeang, and G. S. Hayward.** 1990. A series of palindromic repeats in the simian cytomegalovirus major immediate-early promoter behave as both strong basal enhancers and cyclic AMP response elements. J. Virol. **64**:264–277.
3. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
4. **Cherrington, J. M., and E. S. Mocarski.** 1989. Human cytomegalovirus IE1 transactivates the α promoter-enhancer via an 18-base-pair repeat element. J. Virol. **63**:1435–1440.
5. **Davis, M. G., S. C. Kenney, J. Kamine, J. S. Pagano, and E.-S. Huang.** 1987. Immediate-early gene region of human cytomegalovirus trans-activates the promoter of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **84**:8642–8646.
6. **Depto, A. S., and R. M. Stenberg.** 1989. Regulated expression of the human cytomegalovirus pp65 gene: octamer sequence in the promoter is required for activation by viral gene products. J. Virol. **63**:1232–1238.
7. **Dorsch-Hasler, K., G. M. Keil, F. Weber, M. Jasin, W. Schaffner, and U. H. Koszinowski.** 1985. A long and complex enhancer activates transcription of the gene coding for the highly abundant immediate early mRNA in murine cytomegalovirus. Proc. Natl. Acad. Sci. USA **82**:8325–8329.
8. **Ghazal, P., H. Lubon, B. Fleckenstein, and L. Hennighausen.** 1987. Binding of transcription factors and creation of a large nucleoprotein complex on the human cytomegalovirus enhancer. Proc. Natl. Acad. Sci. USA **84**:3658–3662.
9. **Gorman, C. M., L. F. Maffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
10. **Hermiston, T. W., C. L. Malone, P. R. Witte, and M. F. Stinski.** 1987. Identification and characterization of the human cytomegalovirus immediate-early region 2 gene that stimulates gene expression from an inducible promoter. J. Virol. **61**:3214–3221.
11. **Hunninghake, G. W., M. M. Monick, B. Liu, and M. F. Stinski.** 1989. The promoter-regulatory region of the major immediate-early gene of human cytomegalovirus responds to T-lymphocyte stimulation and contains functional cyclic AMP-response elements. J. Virol. **63**:3026–3033.
12. **Jeang, K.-T., G. Chin, and G. S. Hayward.** 1982. Characterization of cytomegalovirus immediate-early genes. I. Non-permissive rodent cells overproduce the IE94K protein from CMV (Colburn). Virology **121**:393–403.
13. **Jeang, K.-T., M.-S. Cho, and G. S. Hayward.** 1984. Abundant constitutive expression of the immediate-early 94K protein from cytomegalovirus (Colburn) in a DNA-transfected mouse cell line. Mol. Cell. Biol. **4**:2214–2223.
14. **Jeang, K.-T., and W. Gibson.** 1980. A cycloheximide-enhanced protein in cytomegalovirus-infected cells. Virology **107**:362–374.
15. **Jeang, K.-T., D. R. Rawlins, P. J. Rosenfeld, J. H. Shero, T. J. Kelly, and G. S. Hayward.** 1987. Multiple tandemly repeated binding sites for cellular nuclear factor I upstream from the promoter enhancer region for the major immediate-early gene of cytomegalovirus(Colburn). J. Virol. **61**:1559–1570.
16. **LaFemina, R. L., and G. S. Hayward.** 1983. Replicative forms of human cytomegalovirus DNA with joined termini are found in permissively infected human cells but not in non-permissive Balb/c-3T3 mouse cells. J. Gen. Virol. **64**:373–389.
17. **LaFemina, R. L., and G. S. Hayward.** 1986. Constitutive and retinoic acid-inducible expression of cytomegalovirus immediate-early genes in human teratocarcinoma cells. J. Virol. **58**:434–440.
18. **LaFemina, R. L., and G. S. Hayward.** 1988. Differences in cell-type specific blocks to immediate-early gene expression and DNA replication in human, simian and murine cytomegalovirus. J. Gen. Virol. **69**:355–374.
19. **LaFemina, R. L., M. C. Pizzorno, J. D. Mosca, and G. S. Hayward.** 1989. Expression of the acidic nuclear immediate-early protein (IE1) of human cytomegalovirus in stable cell lines and its preferential association with metaphase chromosomes. Virology **172**:584–600.
20. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
21. **Markovitz, D. M., S. Kenney, J. Kamine, M. S. Smith, M. Davis, E.-S. Huang, C. Rosen, and J. S. Pagano.** 1989. Disparate effects of two herpesviruses immediate-early gene trans-activators on the HIV-I LTR. Virology **173**:750–754.
22. **Mitchell, P. J., C. Wang, and R. Tjian.** 1987. Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell **50**:847–861.
23. **Mosca, J. D., D. P. Bednarik, C. A. Rosen, J.-G. Sodroski, W. A. Haseltine, G. S. Hayward, and P. M. Pitha.** 1987. Identification of the viral gene product and target sequence responsible for herpesvirus induction of HIV LTR. Proc. Natl. Acad. Sci. USA **84**:7408–7412.
24. **Mosca, J. D., K.-T. Jeang, P. M. Pitha, and G. S. Hayward.** 1987. Novel induction by herpes simplex virus infection of hybrid interferon gene transcripts driven by the strong cytomegalovirus IE94 promoter. J. Virol. **61**:819–828.
25. **O'Hare, P., and G. S. Hayward.** 1984. Expression of recombinant genes containing herpes simplex virus delayed-early and immediate-early regulatory regions and transactivation by herpesvirus infection. J. Virol. **52**:522–531.
26. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role of both the 175K and 110K immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
27. **O'Hare, P., and G. S. Hayward.** 1985. Three *trans*-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. **56**:723–733.
28. **O'Hare, P., and G. S. Hayward.** 1987. Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early promoter by three virus-encoded *trans*-acting factors. J. Virol. **61**:190–199.
29. **O'Hare, P., J. D. Mosca, and G. S. Hayward.** 1986. Multiple trans-activating proteins of herpes simplex virus that have different target promoter specificities and exhibit both positive and negative regulatory functions. Cancer Cells **4**:175–188.
30. **Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S. Hayward.** 1988. *trans*-Activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. J. Virol. **62**:1167–1179.
31. **Rio, D. C., and R. Tjian.** 1983. SV40 T antigen binding site mutations that affect autoregulation. Cell **32**:1227–1240.
32. **Roberts, M. S., A. Boundy, P. O'Hare, M. C. Pizzorno, D. Ciufo, and G. S. Hayward.** 1988. Direct correlation between a negative autoregulatory response element at the cap-site of the herpes simplex virus type 1 IE175 (α4) promoter and a specific binding of the IE175(ICP4) protein. J. Virol. **62**:4307–4320.
33. **Spector, D. J., and M. J. Tevethia.** 1986. Identification of a human cytomegalovirus DNA segment that complements an adenovirus 5 immediate-early mutant. Virology **151**:329–338.
34. **Staprans, S. I., D. K. Rabert, and D. H. Spector.** 1988. Identification of sequence requirements and *trans*-acting functions necessary for regulated expression of a human cytomegalovirus early gene. J. Virol. **62**:3463–3473.
35. **Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson.** 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. J. Virol. **63**:2699–2708.
36. **Stenberg, R. M., and M. F. Stinski.** 1985. Autoregulation of the human cytomegalovirus major immediate-early gene of human cytomegalovirus. J. Virol. **49**:676–682.

37. **Stenberg, R. M., P. R. Witte, and M. F. Stinski.** 1985. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. J. Virol. **56**:665–675.
38. **Stenlund, A., and M. R. Botchan.** 1990. The E2 trans-activator can act as a repressor by interfering with a cellular transcription factor. Genes Dev. **4**:123–136.
39. **Stinski, M. F., and T. J. Roehr.** 1985. Activation of the major immediate-early gene of human cytomegalovirus by *cis*-acting elements in the promoter-regulatory sequence and by virus-specific *trans*-acting components. J. Virol. **55**:431–441.
40. **Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein.** 1983. Organization and expression of the immediate-early genes of human cytomegalovirus. J. Virol. **46**:1–14.
41. **Thomsen, D. R., R. M. Stenberg, W. F. Goins, and M. F. Stinski.** 1984. Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81**:659–663.