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Herpes Simplex Virus Immediate-Early Promoters Are Responsive to Virus and Cell *trans*-Acting Factors

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The promoters for each of the immediate-early genes from herpes simplex virus type 1 were cloned and fused to a chloramphenicol acetyltransferase cassette. These chimeric genes were used as targets in a transient expression assay to determine how the immediate-early gene products ICP4 and ICP0 and the virion-associated stimulatory protein Vmw65 affected their expression in HeLa and Vero cells. The basal level of expression from these cassettes differed significantly depending on the extent of 5′-flanking sequence and the cell line that served as host. The promoters from IE-4 and IE-0 behaved in a qualitatively similar fashion independent of the host cell. However, the promoter for ICP27 had a unique response pattern: in Vero cells it acted as an α gene promoter, whereas in HeLa cells its response was more like that of a β gene promoter. The promoter sequences for ICP22 and ICP47 behaved as the IE-4 and IE-0 promoters did in HeLa cells, but their response to the effector molecules in Vero cells was unlike that of other α gene promoters we have studied. Evidence is also presented for a role for ICP27 in autoregulation.

Regulation of transcription in eucaryotes is effected by the interaction of *cis*-acting regulatory sequences (promoters) and *trans*-acting proteins that modulate the level of gene expression. In many instances the promoters are known to be composed of multiple elements that interact with different *trans*-acting factors. We are studying the immediate-early genes from herpes simplex virus (HSV) which are transcribed shortly after infection by host RNA polymerase II (8) in conjunction with a virion-associated *trans*-acting factor (1, 5, 44). These genes encode infected-cell polypeptides (ICP) 0, 4, 22, 27, and 47. At least three and perhaps four of the proteins are thought to be involved in regulating the cascade of macromolecular synthesis that occurs in cells infected with HSV (11, 17, 20, 21, 37, 41, 42, 45, 48, 49, 52). The promoters for these genes are contained within upstream regions of various lengths. Previous studies have demonstrated that these upstream regions can be dissected into at least two components: a minimal transcription unit that governs basal-level expression and upstream sequences that are required for their regulation as α genes (4, 7, 30, 35, 46). These upstream regions are extremely complex. They are composed of multiple *cis*-acting elements which respond to a variety of cellular and viral *trans*-acting factors. Five specific *cis*-acting regulatory sequences have been identified in these promoters. Each of the promoters contains (i) a TATA box located 25 to 30 base pairs (bp) 5′ to the mRNA start site, (ii) multiple SP1 binding sites (28), (iii) at least one and frequently multiple copies of the sequence TAATGARATTC (which is responsive to the presence of the virion-associated α gene activator, Vmw65) (5, 19, 35), and (iv) sequences homologous to the core of the simian virus 40 enhancer (34, 35). Three of the five genes also contain a binding site or a homolog of the sequence known to be protected by ICP4, the major immediate-early regulatory protein (18).

There is genetic and biochemical evidence that at least two of these gene products are required to complete the replication cycle. Thus, extensive analysis of mutations in the gene encoding ICP4 have demonstrated that this protein is required for transition from the synthesis of α to β gene polypeptides and for continued progression through the cascade (11, 37, 45). In contrast, a temperature-sensitive (*ts*) mutation in ICP27 appears normal until late in the infectious cycle, when alterations in the amount and temporal appearance of some γ gene products are seen (49).

Studies with viruses from which portions of the IE-0 gene have been deleted demonstrate that they can be propagated without helper virus. However, it is clear that the yield of virus and its growth rate in cells infected with these mutants are aberrant (50, 54). Therefore, the role of this immediate-early gene product is not clearly established in lytic infection. Recent studies in this laboratory have suggested a possible role for ICP0 in activation and repression of transcription from HSV type 1 (HSV-1) promoters (20, 21). A transient transfection assay system was used to demonstrate that the distribution of ICP0 in the nucleus of cells depended on the presence of functional ICP4 and that model α and β promoters, when linked to reporter sequences (targets), responded differently to the presence of these gene products (effectors). Thus, the response of a target driven by a β gene promoter increased in response to increasing amounts of either effector gene sequence. Cotransfection of a plasmid containing sequences encoding both of these gene products resulted in enhanced expression from the β target above what would be expected from the sum of activities of either gene alone. The response of a model α gene promoter was more complicated. The level of expression from an IE-0 promoter-driven target increased in response to increasing levels of ICP0. In contrast, at corresponding ratios of IE-0 plasmid to target, the signal decreased below the basal level of expression in response to increasing molar gene equivalents of effectors. When both plasmids encoding *trans*-activating proteins were present, we detected an initial high level of expression from the reporter, which decreased in response to increasing molar ratios of effector to target. This pattern of expression from the α target is qualitatively similar to how α genes are expressed during the course of infection with HSV-1 (26).

In our previous study we hypothesized that the gene products encoded by IE-0 and IE-4 functionally interact. In the present study we pursued this hypothesis by examining

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the response of each of the immediate-early gene promoters in the transient transfection assay system to determine (i) what effect cellular environment has on qualitative and quantitative aspects of expression from these promoters and (ii) how they respond to the presence of immediate-early *trans*-acting gene products from HSV-1.

MATERIALS AND METHODS

Growth and transfection of animal cells. HeLa and Vero cells were propagated as described previously (20). HeLa or Vero cells were seeded at 1×10^6 to 3×10^6 per 10-cm dish in Dulbecco modified Eagle medium plus 10% calf serum and transfected the next day with a carrier-free calcium phosphate DNA precipitate containing equimolar amounts of effector or indicator plasmid DNAs as previously described (21, 57). The cells were incubated at 37°C, except those receiving pGX164 (*ts*IE-4), which were incubated at 39°C as described previously (21). The actual amounts or ratios of plasmid DNA used are described in each figure legend. The level of expression from the target gene (chloramphenicol acetyltransferase [CAT] activity) was analyzed at 36 to 48 h after transfection.

Recombinant DNAs. The plasmids pGX58, pGX164, and pGR212b, which encode HSV-1 ICP4, *ts*ICP4 (from *ts*K), and Vmw65, respectively, have been described (21). pSS7, which encodes HSV-1 ICP0, is a subclone of pIGA-15 (20) lacking a 2-kilobase (kb) *Kpn*I-*Eco*RV fragment downstream of the IE-0 3′ terminus. pIGA-42 contains a *Bam*HI-*Sac*I fragment encoding HSV-1 ICP27. All IE-CAT chimeras were constructed by the same protocol. Restriction endonuclease fragments containing IE promoters were cloned into pIC-20R or -20H vectors (38) between the *Bam*HI and *Bgl*II sites in the polylinker. Depending on the orientation of the promoter, these constructs were opened at either the *Bam*HI or the *Bgl*II site, and a *Bgl*II-*Bam*HI *cat* cassette fragment derived from pIGA-53 (pIGA-53 contains the *Hin*dIII-*Bam*HI fragment from pRSVCAT [23]) was ligated to this vector. The orientation of the *cat* cassette was determined by restriction endonuclease digestion, and both orientations were tested in transient expression assays. When the *cat* gene was inserted in the opposite direction relative to the promoter, the CAT activity was indistinguishable from that of pIGA-53. The constructs, their designations, and the 5′ and 3′ boundaries of their promoters are shown in Table 1. All plasmids were propagated in *Escherichia coli* DH5α, and DNA was extracted as described (2).

M13-CAT is a recombinant phage that contains a 326-base *Sph*I-*Pvu*II fragment spanning the cap site of pRSVCAT (23) cloned into M13mp19 (*Sph*I-*Sma*I). M13-CAT was propagated in *E. coli* JM109, and a single-stranded template was purified as described (27).

Preparation and analysis of RNA. Total cellular RNA was isolated and purified as described previously (21). Cytoplasmic RNA was purified as described previously (14).

RNA species were analyzed by blot hybridization after separation by electrophoresis through 1.0% (wt/vol) agarose gels in 2.2 M formaldehyde (43) and transfer to GeneScreen Plus (New England Nuclear Corp., Boston, Mass.). The blots were dried, prehybridized, and hybridized at 42°C with nick-translated DNA probes as specified by the manufacturer.

Primer extension analysis. Total cellular RNA (50 μg) purified from cells transfected with IE-CAT DNAs was added to 5 μl of ^{32}P-labeled, single-stranded M13-CAT primer DNA, 10 μl of 5× RT buffer (250 mM Tris hydrochloride, pH 7.4, 375 mM KCl, 50 mM dithiothreitol, and 15 mM $MgCl_2$), 5 μl of deoxynucleotide triphosphate (dNTP) solution (5 mM each dATP, dTTP, dGTP, and dCTP), 1 μl (200 U) of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.), and water to a final volume of 50 μl. The contents were vortexed lightly and incubated for 1 h at 37°C. Then, 100 μl of 400 mM $NaC_2H_3O_2$ containing 5 μg of RNase A per ml was added, and the tubes were incubated at 65°C for 5 min to partially hydrolyze the RNA. The extended primer was extracted once with phenol-chloroform-isoamyl alcohol (50:48:2), and the nucleic acids were precipitated by adding 2 volumes of ethanol and incubating at −70°C for 30 min. The nucleic acid was collected by centrifugation, and the pellets were dried and suspended in formamide containing 0.04% bromophenol blue and xylene cyanole at 65°C for 15 min. The samples were electrophoresed through a 6% acrylamide (19:1, acrylamide-bisacrylamide)–50% urea sequencing gel at 1,500 V for 2 to 3 h. The gels were exposed to Kodak XAR film for 1 week at −85°C.

TABLE 1. Basal-level activity of IE-CAT chimeras[a]

Chimera		Promoter boundaries		CAT activity[b]		Relative activity[c] (V/H)
Promoter	pIGA	5′	3′	HeLa	Vero	
pIC	53			0.05	0.68	14
IE-0	65	−585	+150	0.81	59.16	73
IE-0	96	−125	+150	0.79	27.81	35
IE-4	102	−790	+33	0.19	37.53	197
IE-4	104	−330	+33	0.20	10.28	51
IE-4	72	−290	+33	2.19	73.89	34
IE-4	91	−108	+33	0.05	5.89	118
IE-27	95	−240	+1	0.24	2.91	12
IE-27	98	−84	+1	0.12	20.03	167
IE-22/47	101	−715	+100	0.14	1.37	10
IE-22/47	106	−400	+100	0.09	37.25	413

[a] The 5′ and 3′ restriction sites used to clone the IE promoter fragments were: pIGA-65 (*Stu*I-*Nco*I), pIGA-96 (*Sma*I-*Nco*I), pIGA-102 (*Nru*I-*Bam*HI), pIGA-104 (*Sma*I-*Bam*HI), pIGA-72 (*Sph*I-*Bam*HI), pIGA-91 (*Eco*RI-*Bam*HI), pIGA-95 (*Bam*HI-*Eag*I), pIGA-98 (*Apa*I-*Eag*I), pIGA-101 (*Bam*HI-*Nru*I), and pIGA-106 (*Sph*I-*Nru*I).

[b] CAT activities are normalized for protein content in each lysate; the data are summarized from Fig. 1.

[c] Amount of CAT expressed by a given IE-CAT plasmid in Vero cells (V) relative to that in HeLa cells (H).

RNA slot blots. Cytoplasmic RNA (25 μg) was diluted to 100 μl with sterile water and then added to 300 μl of SSCF (10× SSC [1.5 M NaCl, 150 mM sodium citrate, pH 8.0], 18.5% formaldehyde). The RNA was denatured by incubation at 65°C for 15 min and then loaded onto a nylon membrane with a slot-blotting device (Schleicher & Schuell, Inc., Keene, N.H.). The RNA was slowly filtered and then washed once with 400 μl of 10× SSC and dried by suction. The filters were processed identically to the way Northern blots were prepared. The hybridization signal was determined by scanning multiple exposures of the same autoradiograph.

CAT assays. Cell lysates were analyzed for CAT activity at 36 to 48 h after transfection by the technique of Gorman et al. (23) as previously described (21). The concentration of protein in CAT extracts was determined by the method of Bradford (3). We defined the CAT specific activity as percent acetylation of chloramphenicol (i.e., [counts per minute of acetylated [^{14}C]chloramphenicol/total counts per minute] per microgram of protein).

Probe preparation. Double-stranded DNA fragments were labeled as described by Maniatis et al. (36), with all four

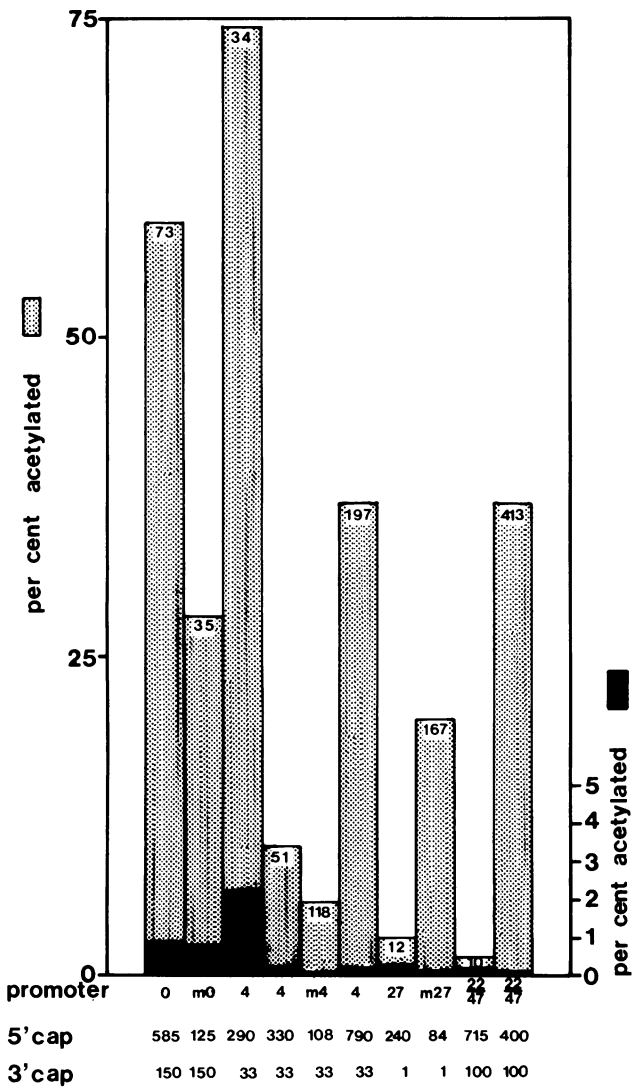

FIG. 1. Basal-level expression of IE-CAT chimeras. HeLa cells (black) or Vero cells (stippled) were transfected with 2.5 μg of each chimeric gene. Cell extracts were prepared at 36 h posttransfection, and 20 μl of extract was incubated to assay for CAT activity. The m refers to minimal promoter and the numbers to the immediate-early gene from which it was cloned. 5′ and 3′ cap refer to the boundaries of each construct. The numbers at the top of each bar indicate the relative ratio of CAT activity in Vero versus HeLa cells.

^{32}P-labeled dNTPs as substrate. Probes prepared by this procedure routinely had specific activities of 2×10^8 to 5×10^8 cpm/μg. Uniformly ^{32}P-labeled, single-stranded M13-CAT probe of mRNA antisense was synthesized by the procedure of Hu and Messing (27) as described previously (21).

Immunofluorescence. Vero and HeLa cells seeded on cover slips were transfected with plasmid DNAs coding for ICP0 and thymidine kinase (TK). They were fixed and stained 40 h after transfection as described previously (21). ICP0 was identified by a mouse monoclonal antibody, H1083, graciously provided by Lenore Pereira. Bound antibodies were visualized after incubation with goat anti-mouse immunoglobulin antibody conjugated to fluorescein isothiocyanate (FITC). Preparations were viewed on a Leitz Dialux fluorescent microscope and photographed for 1 min with Kodak Tri-X film pushed to 800 ASA.

RESULTS

Basal-level activity of immediate-early gene promoters. The putative promoter regions lying upstream of each of the immediate-early genes have been cloned and sequenced (35, 40). Their response to *trans*-activation by the virion-associated transcriptional activator (Vmw65) was analyzed by monitoring the response of chimeric genes, composed of promoter-regulatory sequences from α genes fused to *tk* reporter sequences, in biochemically transformed cells to superinfection with the appropriate virus (4, 7, 19, 35, 44). These studies revealed that the promoters were compound elements composed of a proximal sequence responsible for basal-level transcription and a distal element that was required for regulating expression of the gene (30). We assessed the basal-level activity of these immediate-early promoters by measuring CAT activity from gene fusions. Each immediate-early gene promoter was cloned and fused to an identical *cat* cassette (the boundaries and cloning sites of each construct are indicated in Table 1). Each of the IE-CAT constructs directed transcription from bona fide HSV IE cap sites, as determined by primer extension analysis with a ^{32}P-labeled, single-stranded primer complementary to the 5′ end of *cat* RNA (data not shown). An equivalent amount of each chimera was introduced into both HeLa and Vero cells, and the amount of CAT activity that accumulated by 36 h posttransfection was scored and compared with the basal-level activity of a *cat* cassette lacking a eucaryotic promoter. Accumulation of CAT differed depending on which 5′ sequence was juxtaposed to the *cat* cassette and the cell type which served as host (Fig. 1). Interpretation of these data is not straightforward: in certain instances the addition of sequences 5′ to the proximal element resulted in increased basal-level activity, whereas the addition of still more 5′ sequence resulted in a diminution of the response. For example, the minimal IE-4-CAT construct contained sequences from −108 to +33 (40); addition of sequences including −290 resulted in a sixfold stimulation in CAT activity in both HeLa and Vero cells. However, the further addition of only 40 bp caused a marked decrease in the basal-level activity from this cassette. Higher levels of activity were restored, in Vero cells only, when additional sequences from −330 to −790 were present. This complex response was compatible with the findings of other groups. Their results suggested that this promoter is composed of multiple elements that govern both basal-level and regulated expression of this gene (4, 30, 35, 46). Moreover, we noted that this sequence-dependent response was seen in the absence of any virus-specified *trans*-acting factors and varied in different cell types. For example, addition of sequences from −400 to −715 within the IE-22/47 promoter to pIGA-106 resulted in a 27-fold decrease in CAT activity in Vero cells only. Similarly, a sevenfold decrease in CAT activity, only in Vero cells, was seen when sequences between −84 and −240 in the promoter for IE-27 were added to pIGA-98. The observation that there was more CAT in Vero than in HeLa cells was reflected in the abundance of immediate-early mRNAs that accumulated in these cells after infection with the same amount of HSV-1 (unpublished observations).

Although it was conceivable that the variation in basal-level expression resulted from differences in transfection efficiency, immunofluorescence analysis of HeLa and Vero

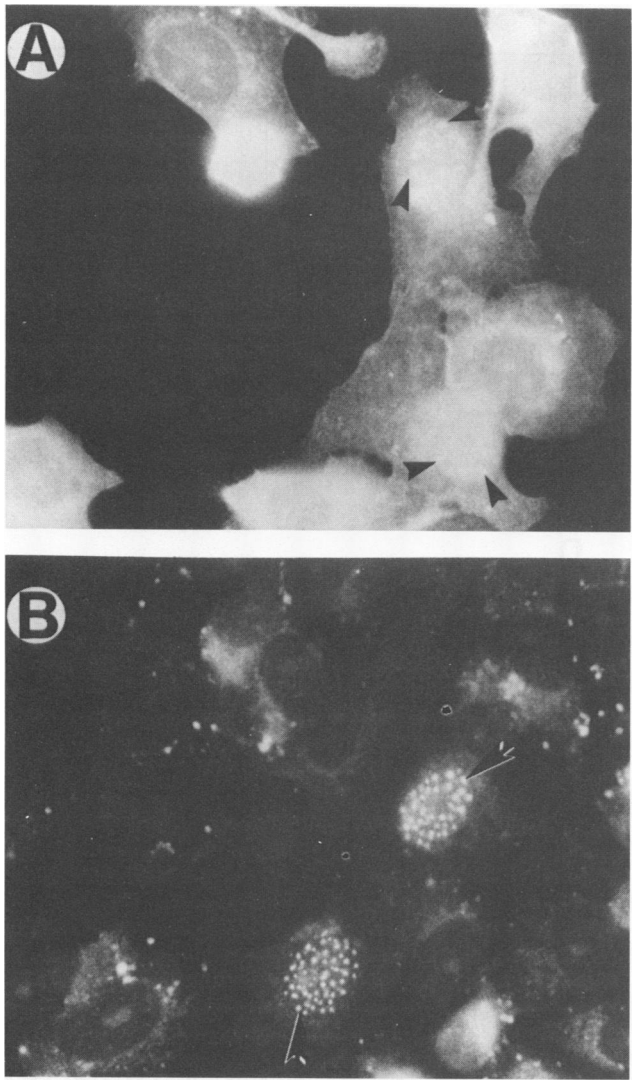

FIG. 2. Immunofluorescence analysis of gene expression. HeLa (A) and Vero (B) cells were transfected with the same calcium phosphate precipitate containing 10 μg of a plasmid (pSS-7) encoding ICP0. After 48 h the cells were fixed and incubated with antisera specific for ICP0 and subsequently visualized by the addition of goat anti-mouse immunoglobulin conjugated with FITC. The cells were photographed for identical periods (1 min). The arrows point to ICP0-specific granules.

cells transfected with an identical calcium phosphate precipitate revealed no difference in the percentage of cells that expressed the immediate-early gene product ICP0. Of 1,168 HeLa and 1,172 Vero cells counted, 79 (6.8%) and 81 (6.9%), respectively, were positive in the immunofluorescence assay. However, staining in Vero cells was consistently more intense than in HeLa cells (Fig. 2). This finding suggests that the gene products are either more stable or expressed to higher levels in Vero cells.

Although we have not systematically examined the other IE promoters as thoroughly as IE-4, it is clear from the data in Fig. 1 and Table 1 that their basal-level activities were also determined by the amount of 5′ sequence and the cell type into which they were transfected. From these results we can conclude that the promoters vary in their ability to direct the synthesis of CAT and that they are utilized to different extents in these two cell types. Thus, the relative ratios of expression in the two cell types vary widely for the different constructs.

Activation and repression of immediate-early gene promoters by their own gene products. The presence of the IE-4 and IE-0 gene products can affect the level of transcripts that accumulate from either α or β gene promoters (16, 17, 20, 21, 41, 48). In this experiment we sought to determine what effect the various immediate-early gene products would have on expression of their own promoters. Previously we demonstrated a direct correlation between accumulation of RNA and CAT activity. Therefore, we assume that the accumulation of CAT activity in a transient transfection assay system measures expression. IE-CAT chimeras containing previously defined promoter-regulatory regions from each of the immediate-early genes were constructed and introduced into HeLa and Vero cells. Both the ratio of effector to IE target and the combination of plasmids were systematically varied, and the effect of these permutations was determined by measuring the amount of CAT which accumulated after 48 h. We have previously reported on the response of the IE-0 promoter in HeLa cells; here we extend this study to examine the response of this target to the same effector molecules in Vero cells. The pattern of response was qualitatively similar in the two cell lines (Fig. 3A). Thus, in the presence of increasing concentrations of a plasmid encoding ICP0, more CAT activity accumulated from the IE-0-CAT target. A similar response was seen with the virion-associated *trans*-activator Vmw65. The response to other effectors was more complex and has been described previously. Thus, despite the 73-fold difference in the basal-level activity, the target sequence behaved similarly in the two cell lines.

Others have studied IE-4-CAT chimeras in Vero cells and used constructs that contain differing amounts of 5′-flanking sequences (10, 41). These studies demonstrated that the IE-4-CAT gene is negatively regulated by ICP4 and that cotransfection with a *ts* allele results in increased accumulation of CAT as the ratio of effector increased over a 100-fold range (41). In one instance the 5′ boundary was at approximately −1600 (41), while in the other it was at −790 (10). In each instance there were multiple *cis*-acting regulatory sequences that might affect the level of expression from this promoter, including promoter-regulatory regions for another immediate-early gene (IE-22/47) as well as an origin of replication. We systematically examined the basal-level CAT activity of constructs containing various amounts of 5′ sequence (Fig. 1) and chose to study pIGA-104 (Table 1) because it lacked the IE-22/47 promoter and *ori* sequences and was likely to contain all of the sequences required for regulation. In agreement with other studies, we found that the signal from this target was unresponsive or slightly depressed when ICP4 was the effector molecule. In response to increasing molar gene equivalents (MGEq) of IE-0, we detected an initial increase in the amount of CAT activity followed by a leveling off, as though the target were saturated by effector. This response differed slightly from that of the IE-0 chimera. The level of CAT initially increased in response to a low MGEq of both IE-4 and IE-0. At higher ratios only low levels of CAT accumulated. This response was very similar to that of the IE-0 target. The qualitative response of this target to *ts*ICP4, ICP4, and the combination of ICP0 and *ts*ICP4 was also the same as that of the IE-0 chimera (Fig. 3B). Furthermore, this response correlated with the finding that both ICP0 and ICP4 were overexpressed in cells infected with *ts*K at 39°C (37).

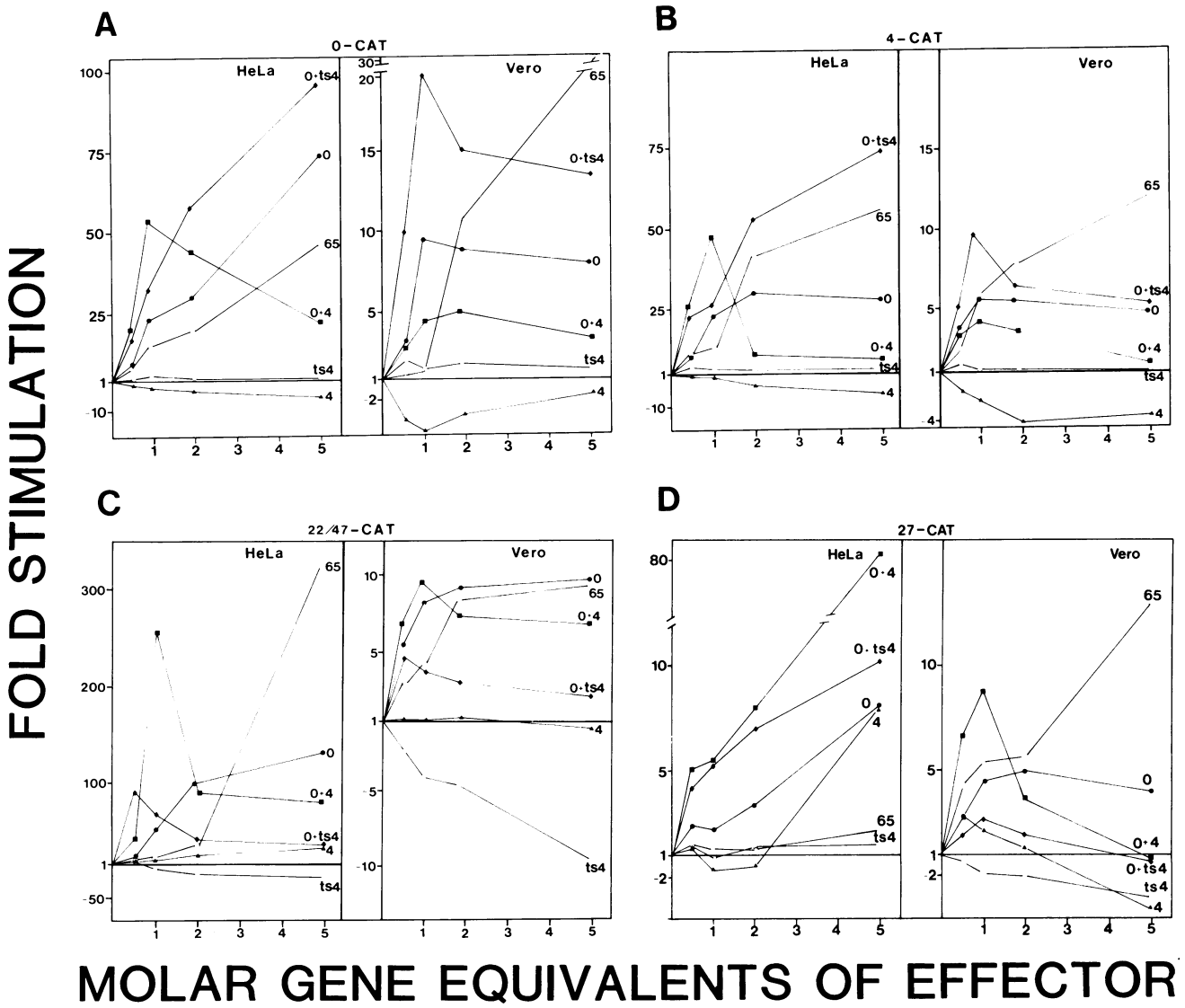

FIG. 3. Expression from IE-CAT chimeras cotransfected with immediate-early genes or Vmw65. The target gene (2.5 μg) was cotransfected into HeLa and Vero cells along with increasing molar ratios of plasmids encoding ICP0 (●), ICP4 (▲), *ts*ICP4 (○), ICP0 plus ICP4 (■), ICP0 plus *ts*ICP4 (◆), or Vmw65 (△). At 40 h posttransfection, extracts were prepared and incubated for 30 min to assay for CAT activity. The basal level of expression of the target sequence in the absence of effector was arbitrarily assigned a value of 1.

The promoters for ICP22 and ICP47 are identical because they are derived from sequences located within the repeats that bound the short unique region of virus DNA (55). The response of this promoter to immediate-early gene products was examined. In HeLa cells this target accumulated more CAT in response to increasing MGEqs of a plasmid encoding ICP0. In Vero cells the response reached a plateau when the ratio of effector to target exceeded 2. This target was stimulated to a low degree by ICP4 in HeLa but not in Vero cells. Its behavior in the presence of both ICP4 and ICP0 was qualitatively similar to that of the IE-4 and IE-0 chimeras in HeLa and Vero cells. In addition, this target exhibited novel behavior in the presence of *ts*ICP4 in that it was repressed in both Vero and HeLa cells. In each instance the amount of CAT that accumulated was decreased by 10-fold from the basal level. Finally, we noted that this was the only one of the four immediate-early promoters that was not increasingly stimulated by exposure to increasing MGEqs of IE-0 and *ts*IE-4. These analyses reveal that the IE-22/47 promoter-regulatory element responds differently in the two cell types and that the qualitative response pattern to the various combinations and differing MGEqs of immediate-early gene products is distinct from that seen with either IE-4 or IE-0 targets (Fig. 3C).

The fourth promoter we studied was from the IE-27 locus. This is the only immediate-early gene present entirely within unique-sequence DNA. The response of this target to the battery of effectors was distinct from those of other IE promoters. It was the only one of the promoters we have studied that was continuously stimulated in the presence of increasing levels of ICP4 and ICP0 in HeLa cells. This response was similar to that of the *tk* gene, which contains a β-class promoter. Its response to ICP4 alone was also unique, as it was unaffected at low ratios of effector to target but stimulated at high levels. It is unclear whether ICP4 can interact with sequences in this promoter. However, there is

a homolog of the consensus ATCGTCNNNNYCGRC (35, 36) protected by extracts containing ICP4, ATTCGTCCT TGTCTGTGC. Moreover, it was the only sequence examined which was stimulated less than fivefold by Vmw65 (Fig. 3D). While it is clear from our studies that cell type can affect both the basal level of activity of a particular construct and the quantitative aspects of its response, we were surprised at the response of this target to the various effectors in Vero cells. In these cells the chimera responded to the presence of ICP0, ICP4, and ICP4 plus ICP0 in a qualitatively similar fashion to both the IE-4 and IE-0 targets. However, it was not responsive to the combination of ICP0 and *ts*ICP4 as these other targets were. This result was unexpected, because ICP27 is overexpressed in Vero cells infected with mutants carrying mutations in the IE-4 locus at the nonpermissive temperature (9). This suggests a role for one of the other virus immediate-early gene products, or Vmw65, in fully activating the IE-27 promoter under these experimental conditions. Thus, the pattern of expression from IE-27 appears to be distinct from that of the other immediate-early gene promoters.

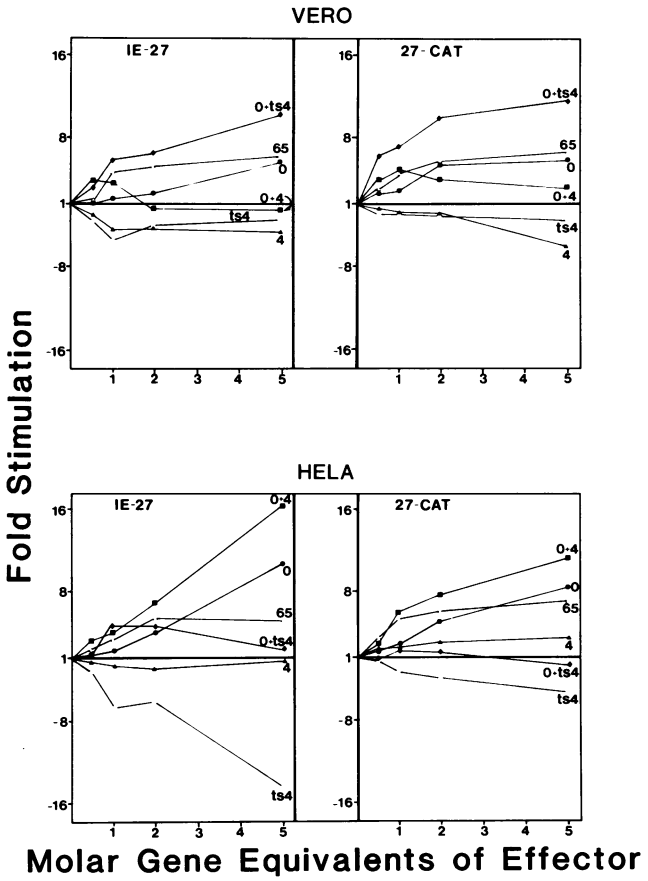

FIG. 4. Analysis of RNAs that accumulated from wild-type and IE-27-CAT constructs. HeLa and Vero cells were cotransfected with 2.5 μg each of pIGA-42 and pIGA-95 and various molar ratios of plasmids encoding ICP0 (●), ICP4 (▲), *ts*ICP4 (○), ICP0 plus ICP4 (■), ICP0 plus *ts*ICP4 (◆), and Vmw65 (△). At 48 h posttransfection, cytoplasmic RNAs were prepared, and 25 μg of each sample was bound to duplicate nylon membranes and hybridized with ^{32}P-labeled probes specific for the IE-27 or CAT mRNA. The filters were exposed to X-ray film, and the resulting autoradiographs were scanned. The areas under the peaks were quantitated and graphed, setting the value of IE-27 or IE-27-CAT RNA that accumulated in the absence of additional effector at 1.

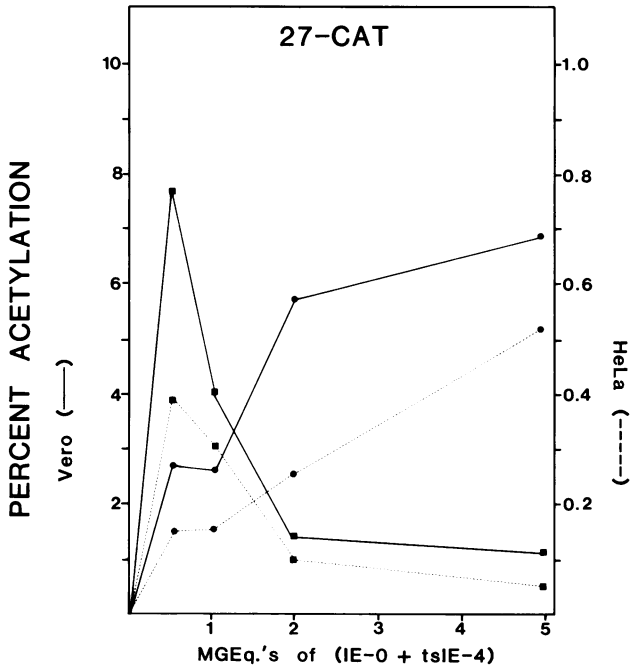

FIG. 5. Response of an IE-27-CAT chimera to the presence of immediate-early gene products. HeLa (-----) and Vero (——) cells were cotransfected with 2.5 μg of an IE-27-CAT target along with increasing MGEqs of the effector genes IE-0 and IE-4 in the presence (●) or absence (■) or 1 MGEq of an IE-27 plasmid. After 48 h of incubation at 39°C, cells were harvested and lysates were prepared to analyze the amount of CAT present.

Proximal sequences coding for ICP27 mRNA not required for basal-level or regulated expression from this promoter. Promoters for both the gC (25) and *tk* (6, 25) genes use adjacent sequences which comprise the 5′ noncoding region of the RNA as part of their promoter-regulatory domains. Because the IE-27 chimeras were constructed by fusing 3′ sequences terminating at an *Eag*I site at +1, regulatory signals might have inadvertently been excluded from the targets. To control for this possibility, we compared the level of RNA that accumulated from an IE27-CAT chimera with that in a wild-type IE-27 gene. Cells were cotransfected with pIGA-42 (wild-type IE-27) and pIGA-95 (IE27-CAT chimera) and effector molecules. Cytoplasmic RNA was isolated at 48 h posttransfection, and probes specific for *cat* and ICP-27 sequences were prepared and hybridized to filters containing RNA isolated from each transfection. Autoradiographs of the filters were scanned, and the areas under the peaks were integrated and plotted as a function of the MGEqs of effector sequence. RNAs from the wild-type gene and the chimera accumulated to very similar levels (under the conditions of this assay), and their responses to the different effectors were qualitatively indistinguishable (Fig. 4). Therefore, we conclude that sequences comprising the 5′ transcribed portion of this gene are not required for either basal-level or regulated transcription from this promoter and that the IE27-CAT chimera is a valid target for these measurements.

However, we noted that the response from this promoter to the effects of increasing MGEqs of IE-0 and *ts*IE-4, in Vero cells, differed from the results shown in Fig. 3D. In this

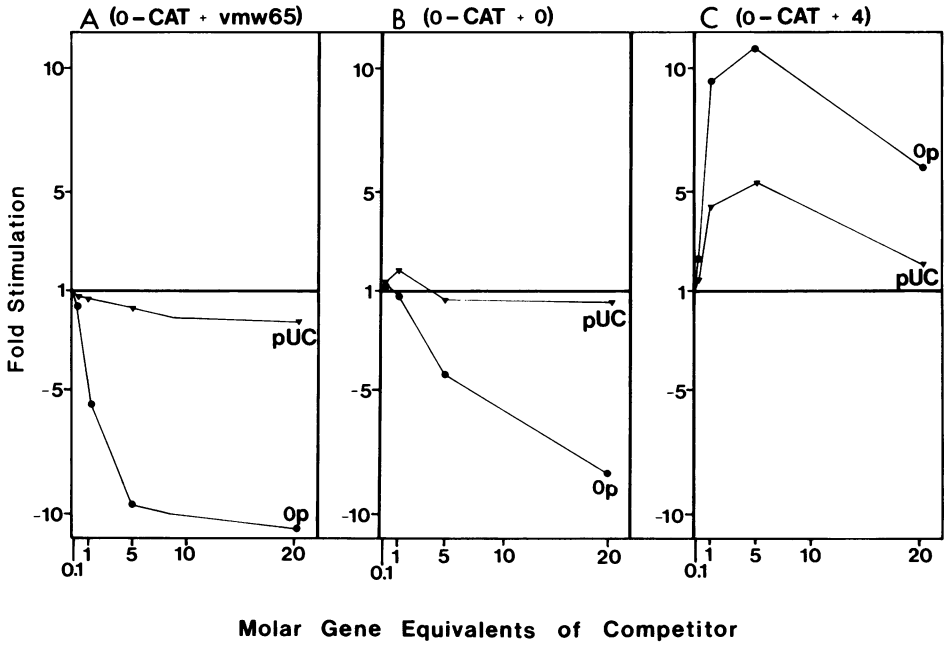

FIG. 6. Competition by IE-0 promoter constructs for cell and virus factors. HeLa cells were cotransfected with a 1:1 ratio of IE-0-CAT and plasmids encoding Vmw65 (A), ICP0 (B), or ICP4 (C) along with 0.5, 1, 5, and 20 MGEq of either pUC or a plasmid containing the IE-0 promoter (pOP; see Table 1 for boundaries). At 48 h posttransfection, cell lysates were prepared and extracts were assayed for CAT activity. The amount of CAT activity that accumulated in the absence of competitor was arbitrarily set at 1.

latter experiment, *ts*ICP4 augmented the ability of ICP0 to activate expression from the IE-27 promoter when a plasmid encoding ICP27 was present. This result might be expected if ICP27, in concert with ICP4 and ICP0, plays a role in autoregulation of expression from its own promoter, as previously suggested (49). This possibility was addressed by transfecting cells with an IE27-CAT target along with effector DNA in the presence and absence of a plasmid encoding ICP27. The response of the target differed depending on the presence of the IE-27 plasmid (Fig. 5). We conclude that ICP27 participates in the regulation of its own expression.

Competition experiments with an immediate-early gene promoter. Previous studies in this laboratory have established that the β promoter for the *tk* gene is stimulated in proportion to the level of cotransfected effector (20). This study suggested that the immediate-early gene products ICP4 and ICP0 interacted in a functional way to regulate transcription from both α and β promoters. In these studies we have shown that the response of α gene promoters is multifactorial and depends on the relative molar ratio of effector(s) to target, the host cell, and the information contained within the sequences that flank the 5′ end of the reporter gene. A series of competition experiments were performed to determine whether the activation and suppression phenomena were in part a result of interaction between specific promoter-regulatory regions and virus-encoded immediate-early gene products.

In the first of these experiments a plasmid containing only the IE-0 promoter was tested for its ability to compete for cell- and virus-encoded transcription factors. Cells were cotransfected with an IE-0-CAT chimera and a plasmid encoding Vmw65, along with increasing amounts of either pUC or a construct containing the IE-0 promoter. The pUC plasmid did not effectively compete even when present at a 20-fold excess over the target sequence (Fig. 6A). This observation suggests that the cells were not saturated in terms of their ability to take up DNA. When CAT was prepared from cells cotransfected with increasing amounts of the IE-0 promoter clone, we noted a precipitous decline in CAT activity. This result suggests that Vmw65 can effectively be saturated by low levels of competitor. Moreover, the finding that the level of CAT activity declined only to the basal level of activity at high concentrations of competitor argues that cellular transcription factors which recognize the IE-0 promoter and govern basal-level transcription are not saturated even when the ratio of competitor to target is 20:1.

These studies were extended to assay competition for ICP0 under similar conditions. A 1:1 molar ratio of a plasmid containing the IE-0 gene as effector, the IE0-CAT target, and increasing amounts of either pUC or the IE-0 promoter clone were transfected onto HeLa cells, and CAT was extracted and assayed for activity. The effect was similar to what was seen with Vmw65, except that the level of CAT activity did not decrease as rapidly or to the same level and did not reach a plateau (Fig. 6B). This result suggests that ICP0 was in excess under the conditions of this experiment.

A role for ICP4 in repressing expression from immediate-early promoters has been proposed (11, 45). More recently, ICP4 was shown to interact with sites in the promoters for IE-0 and IE-4 (18, 32). To provide further in vivo evidence in support of the model proposing that ICP4 interacts directly with specific promoter-regulatory sequences to negatively regulate transcription, cells were cotransfected with a constant ratio of an IE-0-CAT chimera as target and a plasmid encoding ICP4 along with increasing molar ratios of pUC or the IE-0 promoter clone as competitors. If ICP4 works by binding to sequences within or to cellular proteins that recognize the promoter, then competing DNA should effectively dilute the negative regulator and increase the signal

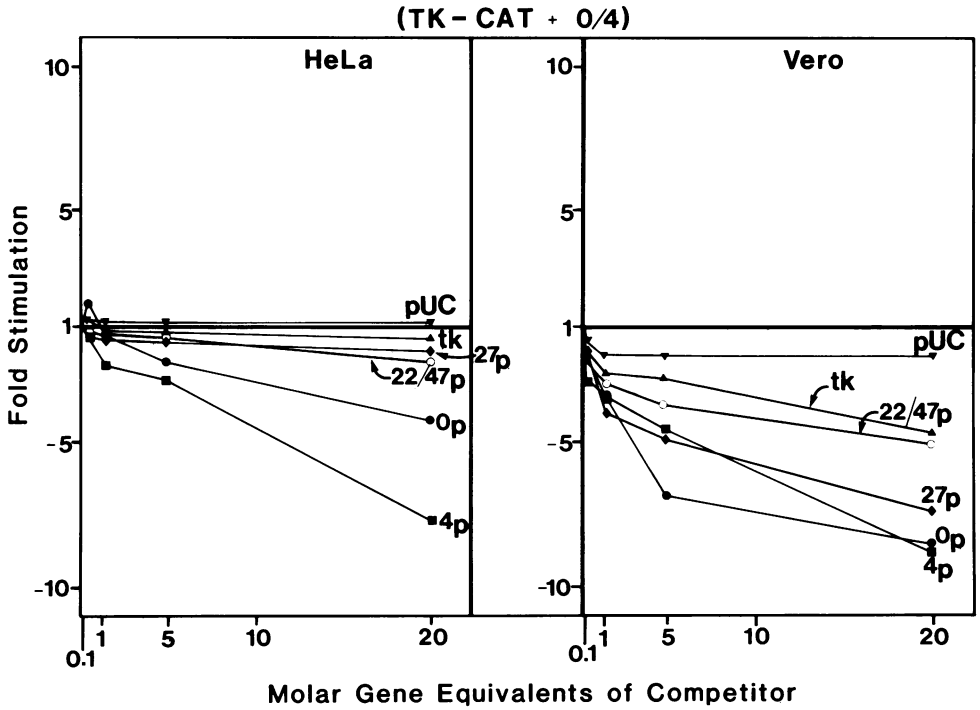

FIG. 7. Competition between the *tk* and immediate-early gene promoters. HeLa and Vero cells were cotransfected with a 1:1 ratio of TK-CAT, IE-0, and IE-4 and 0.5, 1, 5, or 20 MGEq of either pUC or plasmids containing only the promoters for the IE genes (p4P, pOP, p22/47P, p27P, or pTK). At 48 h posttransfection, cell lysates were prepared and extracts were assayed for CAT activity. The amount of CAT activity that accumulated in the absence of competition was arbitrarily set at 1.

from the target sequence. This was exactly what occurred when the IE-0 promoter construct was used as the competitor (Fig. 6C). Curiously, we noted a similar pattern of stimulation, albeit at lower levels, when pUC was the competitor. This could occur if a weak binding site for ICP4 was present within pUC. In support of this observation, a weak binding site for ICP4 within the Amp^r gene of pUC has recently been identified (K. Wilcox, personal communication). These competition assays provide evidence in support of in vitro footprinting and in vivo gel retardation assays with extracts from infected cells. We postulate a negative role for regulation of IE-0 and other immediate-early genes with ICP4 binding sites by direct interaction of protein with specific DNA sequences.

We previously suggested that there might be more than one mechanism of gene activation by the immediate-early genes of HSV. This hypothesis was primarily based on two observations: there is no apparent binding site for either ICP0 or ICP4 in the promoter for the *tk* gene (an early gene promoter [31]), and ICP4 can either positively or negatively regulate the expression of α genes depending on its abundance, the target sequence, and the presence of ICP0 (10, 21, 41, 42). Thus, sequences with which ICP4 is known to interact should be capable of acting as sinks to deplete functional transcription factors and thereby decrease the signal from a reporter target. To test this hypothesis HeLa and Vero cells were cotransfected with a 1:1:1 ratio of *tk-cat* reporter and plasmids encoding both ICP4 and ICP0 and increasing amounts of either pUC or plasmids containing the promoters for *tk* or IE 4, 0, 27, or 22/47. Cell lysates were prepared after 48 h of incubation, and CAT activity was determined. Neither pUC nor plasmids containing the promoters for *tk*, IE-27, or IE-22/47 were effective competitors in HeLa cells (Fig. 7). However, in the presence of increasing amounts of both the IE-0 and IE-4 promoter clones, less CAT activity accumulated. These latter two sequences are protected by ICP4 (18, 32). This result suggests that these promoters compete by depleting ICP4 and ICP0. We repeated this experiment in Vero cells and found that the promoters for both IE 0 and 4 appeared to be effective competitors, whereas pUC was not. The IE-27 promoter plasmid acted as a competitor in these cells. This result is compatible with our finding (Fig. 3D) that this promoter behaved as though it were a β gene in HeLa cells and an α gene in Vero cells. Both the *tk* and IE-22/47 clones were weak competitors. The lack of sequences known to interact with ICP4 in either of the promoters for these genes suggests that these genes compete for factors other than ICP4 in Vero cells.

DISCUSSION

The program of macromolecular synthesis in cells infected with HSV is orchestrated by a complex interaction of *cis*- and *trans*-acting elements. In the present study we cloned the promoters from each of the immediate-early genes and fused them to a *cat* cassette. The response of these targets to three activators of transcription (ICP4, ICP0, and Vmw65) was examined in a short-term transient expression assay system in HeLa and Vero cells. These studies confirmed and extended previous observations that the immediate-early gene products ICP4 and ICP0 can each activate transcription from β promoters and that ICP4 can either activate or repress expression from α promoters (10, 16, 20, 21, 41, 42, 48). When present together these immediate-early gene products appear to function in a cooperative manner to regulate expression of HSV-1 genes. They can synergistically activate expression from the *tk* promoter and either

activate or repress expression from α promoters depending on the relative concentration of effector to target and the length of promoter sequence that is appended to the reporter cassette in the chimeric construction.

It is evident that the same promoter sequences were expressed differently in the two cell types (Table 1). This finding was unexpected because the virus grows to equivalent titers in the two cell types and the temporal patterns of RNA synthesis was indistinguishable. However, immediate-early mRNAs were consistently more abundant in Vero cells than in HeLa cells (unpublished observations). These two cell lines are of different origins (Vero cells are kidney fibroblasts, and HeLa cells are epidermal cells from a cervical carcinoma), and it is well documented that transcriptional factors responsible for recognizing and activating promoters expressed only in certain tissues or at specific stages during differentiation exist (12, 15, 22, 24, 33, 39, 47, 51). The promoters for the immediate-early genes of HSV are composed of multiple regulatory elements which may interact with virus and cellular factors (7, 30). These include regions required for constitutive basal-level expression, response to the immediate-early gene products, and activation by the virion-associated activator of α gene transcription, Vmw65. Our analyses of the basal-level activity of mutants carrying deletions in these promoters in both HeLa and Vero cells suggest that they also contain silencers and other sequences that are recognized by cell-specific transcription factors to differentially regulate their expression. Transcription factors such as SP1 are known to bind to both immediate-early and early gene promoters (28, 29). However, it is clear that SP1-binding sites alone are insufficient to account for the complex nature of the response of these promoters. Moreover, the difference in the response of a given IE-CAT chimera in the two cell lines suggests that other, as yet undefined, cell-specific factors are involved in regulating basal-level expression from these promoters.

Transfection experiments with isolated genes coding for ICP4 and ICP0 have defined roles for these gene products in the regulated expression from both α and β promoters. Recent studies suggest that ICP47 may augment the action of other immediate-early gene products (42) and that ICP27 may participate with ICP4 and ICP0 in activating the expression of certain γ genes (17). In the experiments reported in this manuscript, we examined the regulatory effects of ICP4, *ts*ICP4, ICP0, and Vmw65 on each of the α gene promoters in both HeLa and Vero cells. Whenever possible we chose to use reporter cassettes linked to sequences containing the complete promoter-regulatory region without other overlapping transcription or replication signals. Our results demonstrate that the promoters for IE-4 and IE-0 respond in a very similar fashion to all combinations of transcriptional activators tested. Although the response was more apparent in HeLa than in Vero cells, in each instance both activation and repression were seen. Moreover, the pattern of response was similar. The response of these promoters to the presence of plasmids encoding ICPs 4, *ts*4, 4 plus 0, and *ts*4 plus 0 is compatible with the biology of virus infection with both wild-type and *ts*K virus. Nevertheless, it is difficult to dissect the responses when both IE-4 and IE-0 or *ts*IE-4 and IE-0 were present because of the effects of the proteins encoded by these sequences on both their own and the others' promoters. Both of these promoters respond to increasing amounts of Vmw65 in a positive manner, as each contains the consensus sequence that is responsive to the virion-associated factor.

The response of the other immediate-early gene promoters was different. The dual promoter for IE-22/47 did not contain a consensus sequence protected by ICP4, yet in HeLa cells it responded to the presence of ICPs 4 and 0 in the same fashion as to IE-4 and IE-0 chimeras. Moreover, its response to activating factors was more pronounced than the other α gene promoters tested. Unlike the other α gene promoters this promoter was activated at low MGEqs of *ts*ICP4 plus ICP0, but this response did not continue at higher molar ratios. This response, coupled with the unusual threshold response to increasing amounts of a plasmid encoding Vmw65, suggests that in HeLa cells this promoter is not recognized in precisely the same manner as the promoter for IE-4 and IE-0. In Vero cells it behaved in a conservative fashion; the magnitude and pattern of its response were similar to those detected with the other two promoters.

The response of the IE-27 promoter in HeLa cells had characteristics of both α and β genes. Unlike the other α genes, its expression increased in response to increasing amounts of ICP4 and ICP4 plus ICP0. Previously, we demonstrated that the *tk* promoter, a β promoter, was only marginally active in the presence of *ts*ICP4 plus ICP0 (21). Thus, a functional ICP4 is required for synergistic activation of β promoters by the combination of ICP4 and ICP0. However, cotransfection of the IE-27 chimera along with an increasing MGEq of genes encoding *ts*ICP4 and ICP0 resulted in the accumulation of more CAT. This behavior differentiated it from a true β promoter. Moreover, this promoter was not very responsive to the presence of Vmw65. This was unexpected because there is a degenerate homolog (TAATTAAAT) of the TAATGARAT motif present in this promoter between −152 and −141, and this sequence confers α-like regulation when linked to reporter molecules (35, 56). Although the identical 5′ endpoint was used in both studies, it is conceivable that the difference in response that we detected is a consequence of the way that the chimeric genes were constructed or a result of strain-specific sequence differences. The result shown in Fig. 4 argues that sequences 3′ to the cap site do not alter the magnitude or qualitative response of this promoter. However, two different reporter molecules were present in this experiment. The results in Fig. 5 indicate that ICP27 participates with ICP4 and ICP0 in regulating the expression of both targets.

The competition experiments we described demonstrate the multifactorial nature of transcriptional activation of HSV promoters. It is clear from these experiments that gene products such as Vmw65 (Fig. 6A) are limiting under the conditions of this assay. Thus, the IE-0 promoter construct readily competed for and depleted the pool of activating molecules, depressing the CAT activity level to baseline. In another analysis we demonstrated that CAT activity levels from an IE-0 chimera were not as readily decreased by the IE-0 promoter competitor when ICP0 was the effector molecule. One interpretation of this result is that neither cellular factors nor ICP0 is depleted under the conditions of this assay. The IE-0-CAT promoter competition experiment with ICP4 as the effector molecule was straightforward to interpret because ICP4 is known to act as a negative regulator of both IE-0 and IE-4 promoters (10, 21, 26, 41, 42). Thus, we confirmed the predictions of genetic experiments (which used viruses with *ts* mutations in the IE-4 allele) that suggested the gene product was autoregulatory (11, 45) and biochemical analyses which demonstrated that the protein protects sequences contained within the promoters for IE-0 and IE-4 (18, 31, 32).

Similar competition analyses with *tk* as the target and

ICP4 plus ICP0 as the effectors revealed that in HeLa cells, in which IE-4 and IE-0 are negatively regulated by ICP4 and IE22/47 and IE27 are not, addition of *tk*, IE-22/47, or IE-27 promoter DNA did not result in competition. This result is consistent with a hypothesis which suggests multiple modes of activation of HSV promoters that depend on the abundance of ICP4, the target sequence, and the presence of ICP0. The results in Vero cells emphasize the multifactorial nature of the response. In these cells all of the immediate-early chimeras accumlated slightly lower levels of CAT in the presence of ICP4. Accordingly, each IE promoter, to various degrees, was an effective competitor resulting in lower levels of CAT activity. Curiously, the *tk* promoter also competed under the conditions of this assay. Clearly, it will be necessary to examine this response in greater detail to ascertain the nature of this specificity and to determine whether cell or virus factors are being depleted. We are presently investigating this phenomenon by competitive footprint analysis.

In summary, we have demonstrated that activation of immediate-early gene promoters is a complex phenomenon involving cellular and viral factors that interact to modulate the response of virus genes. These findings are not unexpected because in the natural course of infection the virus is sequestered and enters a latent state after the initial lytic phase. How might this occur? One obvious control point lies at the level of expression of immediate-early genes. Thus, a lack of cellular transcription factors in nerve cells might prevent the initiation of and progression through the cascade and result in establishment of the latent state. In response to external stimuli the cell may derepress a gene(s) encoding a factor(s) that activates transcription of virus immediate-early genes and results in reinitiation of the cascade.

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