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Comparison of Upstream Sequence Requirements for Positive and Negative Regulation of a Herpes Simplex Virus Immediate-Early Gene by Three Virus-Encoded *trans*-Acting Factors

PETER O'HARE†* AND GARY S. HAYWARD

The Virology Laboratories, Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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Using a short-term cotransfection system with recombinant chloramphenicol acetyltransferase (CAT) target genes and intact genes for regulatory proteins, we previously demonstrated that expression from the promoter-regulatory region of the gene for the immediate-early 175,000-molecular-weight (IE175K) protein of herpes simplex virus type 1 was subject to *trans*-acting effects by three different virus-encoded components. In the present work we have attempted to delineate the upstream *cis*-acting requirements within the IE175K promoter-regulatory region for stimulation by the late structural protein Vmw65, stimulation by the IE110K protein, and repression by its own gene product, the IE175K protein. Our results augment previous reports of others by demonstrating that a construct containing only the single TAATGARAT consensus sequence, TAATGGAAT, between −115 and −106 was efficiently induced by Vmw65. Deletion to −108 effectively abolished the response to Vmw65. However, this latter construct remained responsive to IE110K stimulation and was induced as efficiently as the parental construct which contained sequences to −1900. Furthermore, not only basal levels of expression, but also Vmw65 activation of the parental construct and deletion mutants Δ380, Δ330, Δ300, and Δ160 and IE110K-activated expression of the Δ108 construct were all subject to dominant repression by the IE175K protein. Finally, we show that expression from each of the deletions was open to stimulation by linkage to the simian virus 40 enhancer region. Enhancer-stimulated expression from each construct, including the −108 deletion, was efficiently repressed by the IE175K protein. In contrast, expression from the simian virus 40 enhancer when linked to its own promoter was unaffected by IE175K. These results place sequence requirements for both IE110K stimulation and IE175K autoregulation within the minimal promoter region −108 to +30, separate from the major requirements for Vmw65 activation located further upstream.

Selective interactions between different combinations of virus-encoded regulatory factors, components of the host-cell transcriptional machinery, and the virus promoter-regulatory DNA sequences are likely to play a major role in the control of herpes simplex virus (HSV) transcription. One approach in the attempt to identify and analyze the potential multiplicity of interactions between virus-encoded *cis*- and *trans*-acting factors is examination of the expression of recombinant target genes containing appropriate class-specific promoter-reglatory regions and their regulation by defined *trans*-acting factors introduced into the cell by cotransfection with regions of the genome containing the candidate regulatory components (3, 5, 7, 9, 22, 25, 32).

Early results from analyses of virus infection of cell lines which contained selected hybrid target genes controlled by immediate-early (IE) promoter-regulatory regions indicated that IE promoter activity was stimulated by a structural component of the HSV virion (1, 13, 16, 28). Sequence data from upstream regions of IE genes revealed IE-specific TAATGARAT consensus motifs present in two or more copies of the 5′ regions of each of the five HSV IE genes, and these consensus regions were predicted to be involved in the induction of IE transcription (19, 21, 23, 36, 37). This prediction was subsequently confirmed by the demonstration of a requirement for the TAATGARAT consensus sequence in the stimulation of expression from IE hybrid genes after HSV infection (2, 8, 17, 19, 20, 30). However, it has also been demonstrated that a TAATGARAT box may not be sufficient for IE-type regulation, that sequences flanking the TAATGARAT signal influence the magnitude of the stimulation of IE expression induced by virus infection, and that not all TAATGARAT-like sequences may be responsive to virion stimulation (2, 4, 8, 17, 19, 20, 30). The identity of the structural component mediating IE induction was revealed by the cotransfection experiments of Campbell et al. (3) as the late protein Vmw65 (VP16). Results of early work on HSV protein synthesis and virion topology have shown that Vmw65 is a major late infected-cell phosphoprotein which is assembled into the tegument region of the virion at approximately 400 to 600 molecules per particle (12, 33).

Recently, we provided results which indicate that expression from the IE promoter-regulatory region of the gene for the IE 175,000-molecular-weight (175K) protein (ICP4) is subject to *trans*-acting regulation by at least two additional virus-encoded products, the IE175K protein itself and the IE110K protein. Having initially shown (25) that the IE175K protein could independently stimulate expression from delayed-early (DE) promoter regions, results consistent with those of others using similar transient (5, 9, 22, 32) or IE175K-expressing long-term (27) systems, we subsequently demonstrated that the IE175K protein could in addition repress expression from its own promoter-regulatory region (26). Surprisingly, in direct comparisons with the activities

* Corresponding author.
† Present address: Marie Curie Memorial Foundation Research Institute, The Chart, Oxted, Surrey RH8 OTL, United Kingdom.

of the IE110K protein, which also independently stimulated DE expression (25), we found that expression from the IE175K promoter-regulatory region was also subject to a *trans*-acting effect of the IE110K protein but in this case was positively regulated, with overall increases in expression equivalent to those produced on expression from DE promoter regions. Furthermore, we showed that the inhibitory effect of IE175K on expression from its own promoter region was not due to competition and that repression dominated over *trans* activation by either the Vmw65 component or IE110K (26).

In this work we attempt to discriminate between the *cis*-acting sequence requirements within the IE175K regulatory region for the *trans*-acting effects mediated by the three virus regulatory components. Our results on the sequence requirements for induction by the individual Vmw65 protein augment previous results and provide the first evidence that a construct containing the single TAATGARAT consensus signal located at position −115 to −106 (TAATGGAAT) can efficiently respond to Vmw65. In addition we show that the sequence requirements for induction by Vmw65 are separable from those involved in induction by IE110K, which efficiently stimulates expression from the basal promoter of the IE175K gene. Finally, we provide results which indicate that the IE-specific TAATGARAT consensus sequence required for Vmw65 activation is unlikely to be involved in IE175K autoregulation, since a deletion mutant containing virus sequences between 108 base pairs (bp) upstream and 30 bp downstream from the mRNA cap site, and therefore lacking an intact TAATGARAT sequence, was still subject to the dominant inhibitory effect of the IE175K protein.

MATERIALS AND METHODS

Cells and transfection procedures. All transfections were performed in Vero cells plated in cluster dishes (6 by 35 mm; Costar, Cambridge, Mass.) at 4×10^5 to 5×10^5 cells per well by the calcium phosphate precipitation method with a glycerol boost as described in our previous work (24, 25). Amounts of the recombinant chloramphenicol acetyltransferase (CAT) target genes and cotransfected plasmids are indicated in the figures or figure legends.

Plasmids and deletion constructs. The parental target construct p175KCAT contains the 1.9-kilobase *Bam*HI-*Hin*dIII fragment (coordinates 0.865 to 0.876) of HSV-1 [MP] and was constructed as described previously (24). This plasmid contains the CAT gene under the control of the IE175K promoter-regulatory region and includes approximately 1,900 bp upstream of the mRNA start site. The downstream *Bam*HI site used for construction the recombinant gene is located approximately 30 bp 3′ of the mRNA start site and before the translational start site of the IE175K protein.

The upstream and coding sequences of the HSV-1 IE175K gene have been determined in other laboratories (19, 23), and a number of restriction enzyme sites in the 5′ region have been identified. We constructed a series of deletion mutants (Fig. 1A) by using enzyme sites unique to the HSV-1 DNA sequences in the recombinant plasmid. The parent plasmid, p175KCAT, was digested with *Hin*dIII and *Bss*HII, *Hin*dIII and *Xma*III, *Hin*dIII and *Sph*I, or *Hin*dIII and *Sst*II, and the ends were made flush with DNA polymerase I (Klenow fragment) and blunt-end ligated to give deletion mutants Δ380, Δ330, Δ300, and Δ160, respectively. To construct the Δ108 mutant, plasmid pGH12 (24) was digested with *Eco*RI and religated to give pPO22. The only HSV sequences located in pPO22 are those from −108 to +30 with the upstream HSV sequences to −1900 and the short *Eco*RI-*Hin*dIII segment of pBR322 deleted. The Δ108.CAT construct was then made by inserting the *Bam*HI-*Sal*I CAT-containing fragment of pCATB′ between the *Bam*HI and *Sal*I sites of pPO22 in a fashion similar to that used for construction of the parent plasmid p175KCAT (24).

To place the simian virus 40 (SV40) enhancer upstream of p175KCAT, the enhancer-containing *Hin*dIII C fragment (nucleotides 5171 to 1046) was inserted into the unique *Hin*dIII site in p175KCAT. The fragment was inserted with SV40 late orientation towards the IE175K CAT gene, and this orientation placed the enhancer approximately 2.7 kilobases upstream of the IE175K mRNA start site to give the plasmid p175KCAT.SV.L. (Fig. 1B). Although this construction places the SV40 late promoter upstream of the IE175K CAT gene, Keller and Alwine (15) constructed an SV40 late promoter-CAT recombinant gene by using the *Hin*dIII C fragment and showed that the late promoter was virtually inactive to CV-1 cells. Moreover, the SV40 major late start site and progressively greater regions of the IE175K upstream region were deleted by digestion of p175KCAT.SV.L with *Kpn*I and *Bss*HII, *Kpn*I and *Sma*I, or *Kpn*I and *Sst*II, filling in with DNA polymerase I Klenow fragment and blunt-end ligation to give constructs Δ380.SV.L, Δ330.SV.L, and Δ160.SV.L, respectively. These constructions effectively place the SV40 enhancer in the late orientation but without the major late transcription start site, upstream of the various deletion mutants in the IE175K regulatory region. To construct Δ108.SV.L, plasmid pPO22 (see above) was modified by the insertion of an *Hin*dIII linker at the unique *Eco*RI site. The *Bam*HI-*Sal*I CAT coding region fragment of pCATB′ was then inserted, followed by the SV40 *Hin*dIII C fragment in the late orientation.

The structure and location of the virus sequences in additional plasmids pXhoI-C, pIGA-15, pGR135, and pGR212 have been given previously (25, 26). The only intact IE gene in pXhoI-C is that for the IE175K protein of HSV-1, and in pIGA-15 the only IE gene is that for the IE110K protein. Plasmids pGR135 and pGR212 encompass the HSV-2 and HSV-1 genes, respectively, for Vmw65.

CAT assays. All harvesting and CAT assay procedures were done as described previously (24, 25).

RESULTS

Upstream sequences augment basal levels of expression of the IE175 promoter region. A series of constructs (Fig. 1) containing specific deletions in the IE175K promoter-regulatory region linked to the CAT coding region were generated by using restriction enzyme sites identified from sequence data from other laboratories (19, 23). Representative results of a series of experiments examining the effects of these deletions on constitutive levels of CAT expression are shown in Fig. 2. In this experiment the various constructs were transfected in equimolar concentrations at three different amounts equivalent to 0.5, 2.5, and 7.5 μg of p175KCAT. The total amount of DNA transfected in each case was made up to 7.5 μg by the addition of pBR322 DNA. While CAT activity was detected with all of the constructs, the highest levels of expression were observed with the Δ380 and Δ330 constructs, which gave 2- to 4-fold-higher activity than the Δ300 construct and approximately 10-fold-higher activity than the Δ160 and Δ108 constructs. One curious

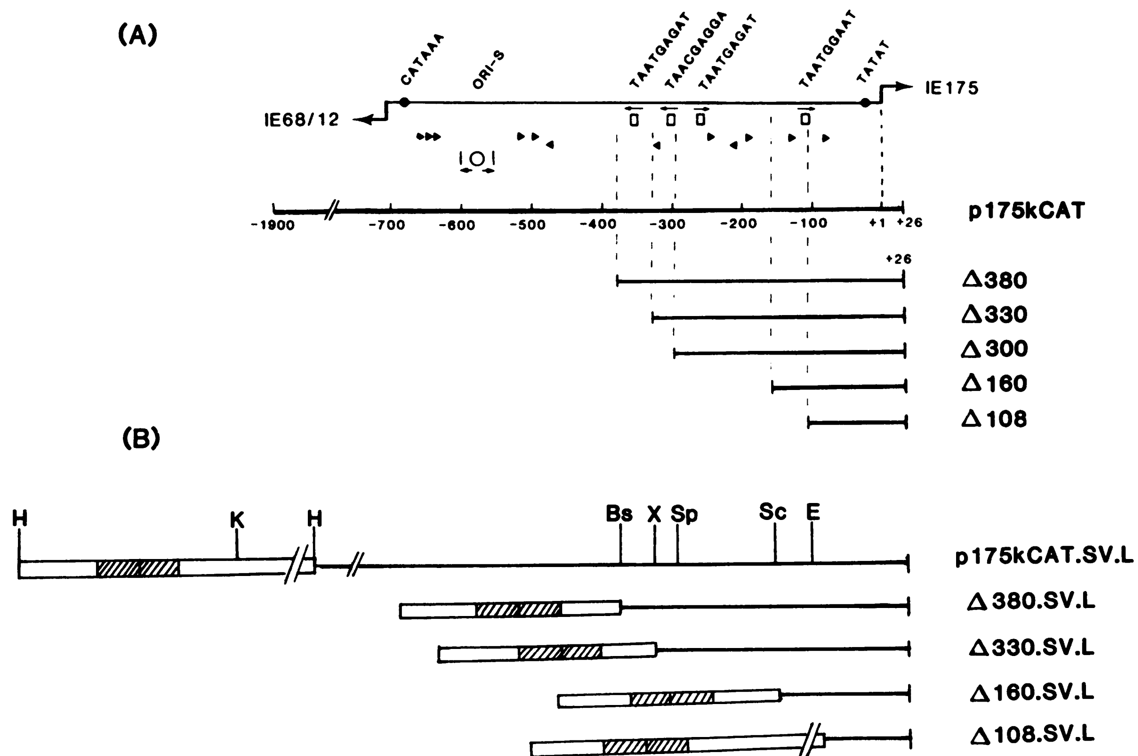

FIG. 1. Summary of the virus sequences present in the parent plasmid p175KCAT, the series of 5′ deletions, and the set of constructs containing the SV40 enhancer. (A) Major regulatory features between the divergently transcribed genes for the IE175K and IE68K proteins are illustrated. Numbers refer to the extent of the sequences upstream (−) or downstream (+) from the cap site (+1) of the gene for the IE175K protein. The TAATGARAT consensus motifs and SpI-binding sites are indicated by open boxes and solid triangles, respectively, with arrows indicating orientation. The SpI-binding sites indicated are those which have been identified by the DNase I protection studies of Jones et al. (14). The TATA box consensus sequences are indicated by solid circles, and the large open circle labeled ori-S indicates the DNA replication origin located in the short repeat region. The endpoints of the parental plasmid and 5′ deletion constructs in relation to these features are indicated in the lower part of the diagram. (B) Structure of the parental plasmid p175KCAT and 5′ deletion series linked to the SV40 enhancer region. The SV40 sequences in the constructs are shown in boxes, with the shaded area indicating the 72-bp tandem repeats. The constructs p175KCAT.SV.L and Δ108.SV.L. contain the complete *Hin*dIII C fragment (nucleotides 5171 to 1046), while the remainder contain the *Hin*dIII-*Kpn*I subfragment (nucleotides 5171 to 294). Restriction enzyme sites: H, *Hin*dIII; K, *Kpn*I; Bs, *Bss*HII; X, *Xma*III; Sp, *Sph*I; Sc, *Sst*II; E, *Eco*RI.

finding over the course of this work was that activity from p175KCAT (which contains sequences up to −1900) was two- to fivefold lower than that from the Δ380 and Δ330 constructs (see also Fig. 3 and 4A). This result was reproducibly observed and obtained with several independent CAT constructs containing these deletions. With the exception of this observation, our results are consistent with the results of other workers who have previously shown that upstream sequences augment basal levels of expression for the IE175K promoter (2, 17, 19, 30). Our results place these sequences largely between −380 and −160 relative to the mRNA cap site and indicate that sequences further upstream than −380 may have an inhibitory effect on expression from the IE175K promoter region.

Sequence requirements for induction by the virion component Vmw65. To assay the effect of deletions in the IE175K promoter-regulatory region or specific induction by the HSV structural component Vmw65, in the absence of any potential complicating influence of the effect of virus infection per se, Vmw65 was introduced by cotransfection with an appropriate cloned segment of virus DNA (pGR135). Analysis of subclones and selective inactivation by restriction enzymes showed that the only gene product encoded within this clone which had any effect on expression from the IE175K promoter region was the Vmw65K protein encoded by a 1.7-kilobase mRNA mapping between coordinates 0.675 and 0.69 on the virus genome (3, 11, 26; P. O'Hare et al., manuscript in preparation).

To rigorously examine the effect of deletions in the IE175K promoter-regulatory region on Vmw65 induction in a quantitative manner, dose-response experiments were performed in which each of the deletion constructs was transfected in constant amounts together with increasing amounts of pGR135. Typical results from a series of such experiments are shown in Fig. 3A, in which the target constructs were transfected in equimolar amounts (equivalent to 1 μg of p175KCAT) together with pGR135 DNA in increasing amounts up to 3 μg. The control for increase in overall amounts of DNA is shown in the bottom row of Fig. 3A , in which the target plasmid was transfected with 3 μg of pBR322 DNA. The nonspecific increase (two- to fourfold) caused by higher total input DNA concentration has been accounted for in the quantitative illustration of these results shown in Fig. 3B. The data demonstrate that in addition to the parental construct, the Δ380, Δ330, Δ300, and Δ160 mutants were all strongly induced by cotransfection with pGR135, while the Δ108 construct showed little response.

Deletion from −380 to −330 had no effect on the response to Vmw65, and although levels of CAT expression induced from these two constructs were greater than those from Δ300

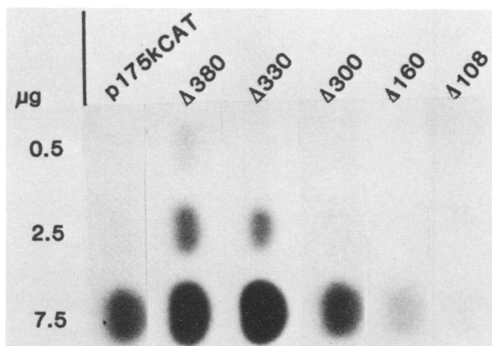

FIG. 2. Effect of 5′ deletions on constitutive expression. Equimolar amounts of each of the deletion constructs equivalent to 0.5, 2.5, and 7.5 μg of p175KCAT were transfected together with appropriate amounts of pBR322 as carrier DNA to make the amount transfected 7.5 μg in each case. Cells were harvested 45 h after transfection, and equal amounts of extract were assayed for CAT activity. The major product, Cm-3-Ac, is shown in each case.

and Δ160, the difference in activity decreased as the amount of pGR135 increased. At 0.2 μg of pGR135, significant levels of CAT activity (representing a four- to sixfold total increase) were expressed from Δ380 and Δ330, while at the same dose, little increase in expression was detected from Δ300 or Δ160 and CAT activity was three- and eightfold lower, respectively. At 1 μg of pGR135, when stimulation of Δ300 and Δ160 was first detected, Δ380 and Δ330 were stimulated maximally, and no significant increase in CAT activity was observed after the addition of higher amounts of pGR135. Activity from Δ300 and Δ160 was increased by adding more pGR135 and at 3 μg of pGR135 (the highest amount tested), attained levels only 2-fold lower than those from Δ380 and Δ330, representing nearly 50-fold stimulation of expression over basal levels for both constructs. In additional dose-response experiments which examined stimulation by pGR135 in multiple doses up to 1 μg (data not shown), certain levels were obtained which discriminated between the responsiveness of Δ300 and Δ160, i.e., Δ300 was significantly induced but Δ160 was unaffected. However, there was clearly a qualitative difference between these constructs and the Δ108 mutant. Even with 3 μg of pGR135, expression from Δ108 was stimulated only four- to sixfold, and absolute levels of activity were less than 5% of those obtained with Δ380 and Δ330 (Fig. 3). Over the course of this work, cotransfection of Δ108 with a range of concentrations of pGR135 up to 5 μg never resulted in increases of expression of more than sixfold and routinely little or no activation was observed. Absolute levels of activity ranged from 1 to 8% of those obtained with constructs containing upstream regions.

In addition, a notable feature of our results was that while the parent plasmid p175KCAT clearly responded to activation by pGR135, it was less efficient than Δ380 and Δ330 and only slightly more responsive than Δ300 and Δ160. Levels of CAT expression induced by Vmw65 from p175KCAT were routinely 2- to 10-fold lower than the levels observed from Δ380 and Δ330.

Our data indicate that in assessing the effect of sequence alteration on the inducibility of IE target-regulatory regions, the amount of the input *trans*-acting component must be taken into consideration. While sequences upstream from −160 may augment inducibility with relatively low levels of Vmw65, sequences within 160 bp upstream of the cap site can suffice for efficient induction. Additional deletion of

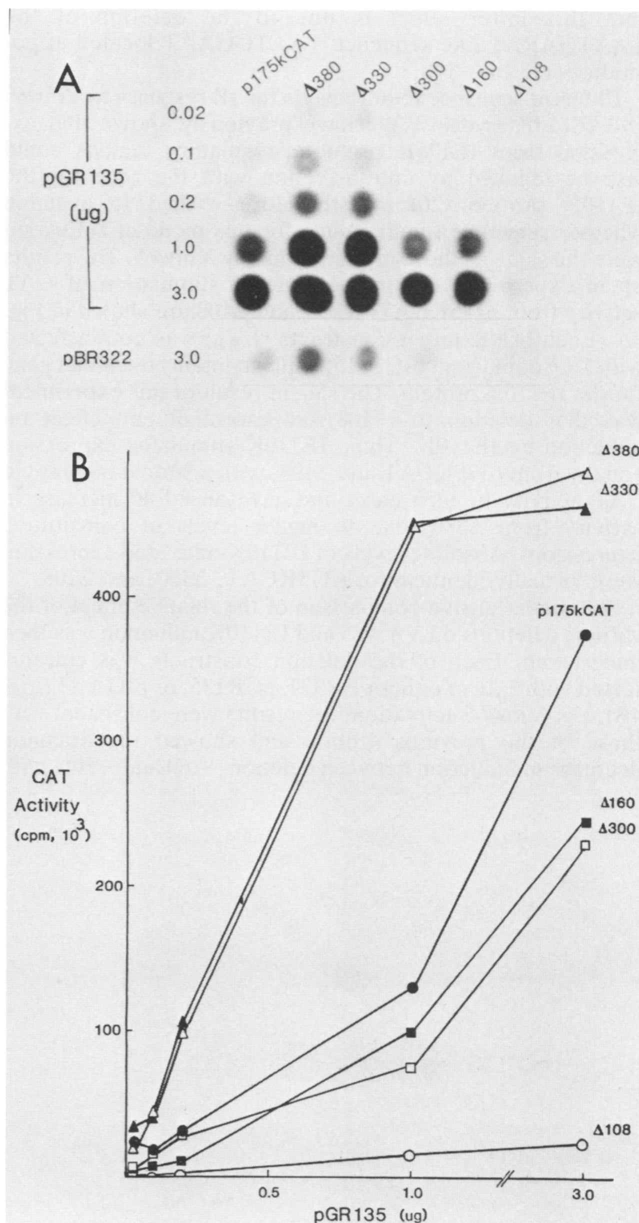

FIG. 3. Dose-response curves of each of the 5′ deletion mutants to increasing amounts of the gene for Vmw65. (A) Approximately 1 μg of p175KCAT or equimolar amounts of the 5′ deletion mutants were cotransfected with various amounts of pGR135 as indicated. As a control for the effect of overall increase in DNA concentration, each mutant was cotransfected with 3.0 μg of pBR322 carrier DNA, equivalent to the highest amount of pGR135 tested. Cells were harvested 42 h after transfection, and equal amounts were assayed for CAT activity. The major product, Cm-3-Ac, from the assays is aligned for comparison of activity of each of the 5′ deletions at a given concentration of pGR135 and for the effect of increasing concentrations of pGR135 on a single deletion construct. (B) Quantitative illustration of the dose-response results, with the amount of cotransfected pGR135 shown on the abscissa. The nonspecific increase in activity due to the effect of DNA concentration has been accounted for in plotting CAT activity for each of the constructs.

sequences from −160 to −108 essentially abolished specific induction by Vmw65 at all levels tested. We think it probable that this latter effect is due to the deletion of the TAATGARAT-like sequence TAATGGAAT located at position −115 to −106.

Different sequence requirements for IE response to Vmw65 and IE110K proteins. We have previously shown that expression from IE175K promoter-regulatory regions could also be induced by cotransfection with the gene for the IE110K protein (26) and therefore wished to examine whether sequence requirements for this mode of activation were the same as those for activation by Vmw65. The results of an experiment comparing IE110K stimulation of CAT activity from p175KCAT, Δ380, and Δ108 are shown in Fig. 4A. Each of the target constructs (1 μg) was cotransfected with 1 μg of plasmid pIGA-15, which contains the intact gene for the IE110K protein. The salient result of this experiment was that deletion to −108 had essentially no effect on induction by IE110K. Thus, IE110K stimulated expression equally from p175KCAT and Δ108, with a 20-fold increase in CAT activity in each case, and gave an 8-fold increase in activity from Δ380 (due to higher levels of constitutive expression). Absolute levels of IE110K-activated expression were virtually identical for p175KCAT, Δ380, and Δ108.

A more extensive comparison of the relative effect of the various deletions on Vmw65 and IE110K induction was then undertaken. Each of the deletion constructs was cotransfected with 2 μg of either pBR322, pGR135, or pIGA-15 (Fig. 4B). For Vmw65 activation, the results were consistent with those of the previous section and showed the dramatic decrease in induction between deletion −160 and −108, with

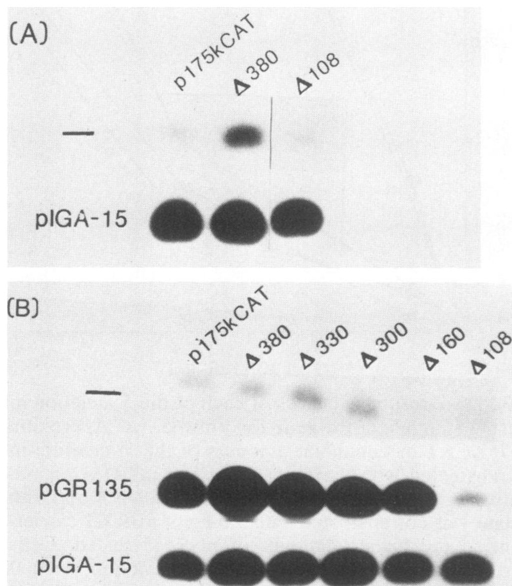

FIG. 4. Differential effect of deletions in the upstream regulatory region on *trans* activation by Vmw65 and IE110K. (A) Approximately 1 μg of p175KCAT or equimolar amounts of Δ380 and Δ108 were cotransfected with 1 μg of pBR322 (—) or 1 μg of pIGA-15. Assays were performed on cells harvested 44 h after transfection. (B) The target plasmid p175KCAT (1 μg) or equimolar amounts of each of the 5′ deletion mutants were cotransfected with approximately 2 μg of pBR322 (—), 2 μg of pGR135, or 2 μg of pIGA-15. Cells were harvested 46 h after transfection, and equal amounts were assayed for CAT activity. The figure shows the amount of the major product, Cm-3-Ac, in each case.

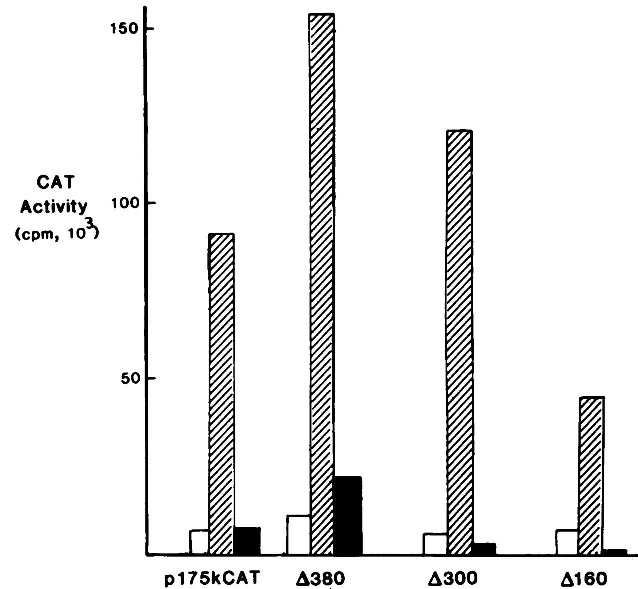

FIG. 5. Vmw65 *trans* activation of each deletion mutant to −160 is subject to dominant repression by the IE175K protein. Approximately 2 μg of p175KCAT or equimolar amounts of Δ380, Δ330, Δ300, and Δ160 were cotransfected with 2 μg of pBR322 (open bars), 1 μg of pGR135, (hatched bars), or 1 μg of pGR135 together with 1 μg of pXhoI-C (solid bars). Cells were harvested 44 h after transfection, and equal amounts were assayed for CAT activity.

a resulting 18-fold reduction in CAT activity. In this experiment the higher response of Δ380 and Δ330 compared with p175KCAT was particularly noticeable. Maximum levels of CAT activity were observed with Δ380 (100-fold induction) and were approximately 70-fold higher than those observed with the Δ108 construct.

In contrast, deletion to −108 had little effect on IE110K activation, with Δ108 expression being stimulated with equal efficiency (ca. 18-fold) as that from Δ160 and p175KCAT and only slightly lower than that from Δ380. Over the course of a number of additional experiments, IE110K stimulation of Δ108 was at least as efficient as stimulation of the parental construct p175KCAT (15- to 30-fold), with equal or greater increases resulting in approximately equal absolute levels of CAT activity.

These results indicate that while upstream sequences may augment absolute levels of activity, promoter sequences downstream from −108 are sufficient for efficient response of the IE175K promoter-regulatory region to the IE110K protein. Thus, the *cis*-acting sequences involved in response to Vmw65 and IE110K can be differentiated, with the former requiring an intact TAATGARAT consensus regulatory sequence and the latter requiring only the IE175K promoter sequences between −108 and +30.

Sequence requirements for autoregulation by the IE175K protein. Previous work from this and other laboratories demonstrated that the IE175K promoter-regulatory region was the target of direct negative regulation (5, 26). Our cotransfection experiments demonstrated that the IE175K protein inhibited basal levels of expression and exerted a dominant inhibitory effect on Vmw65- or IE110K-activated levels of expression from its own promoter-regulatory region (26). These results provided the first demonstration that the IE175K protein directly repressed expression from its own transcription unit. Experiments in this section were designed

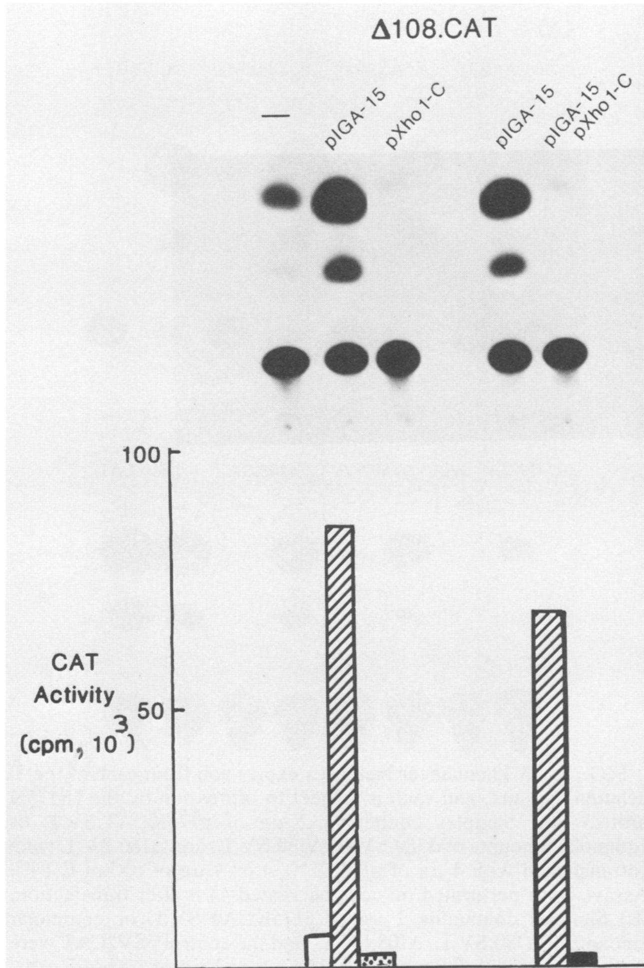

FIG. 6. Basal expression and IE110 K-*trans*-activated expression of Δ108 deletion mutant are both subject to IE175K repression. Approximately 4 μg of Δ108. CAT was cotransfected with 4 μg of pXhoI-C, 2 μg of pIGA-15, or 2 μg of pIGA-15 together with 2 μg of pXhoI-C. Assays were performed on cells harvested 48 h after transfection. Quantitative illustration of the counts present in the major product, Cm-3-Ac, of each assay is given in the lower part of the diagram. Each bar corresponds to the lane shown directly above it.

to examine the *cis*-acting sequence requirements for IE175K autoregulation and particularly to examine whether the IE-specific TAATGARAT consensus sequences were involved.

Although inhibition of basal p175KCAT expression by the IE175K protein was assayable directly, more sensitive measurements of IE175K repression were obtained when expression from the test constructs was considerably higher. Thus, in initial experiments, we examined the ability of the IE175K protein in pXhoI-C to repress Vmw65-induced CAT activity from p175KCAT and each of the deletion mutants to −160. Each of the constructs was cotransfected with 2 μg of pBR322, 1 μg of pGR135, or 1 μg of pGR135 together with 1 μg of pXhoI-C (Fig. 5). The addition of pXhoI-C clearly repressed Vmw65 *trans* activation of each of the deletion mutants, with the relative inhibition of the Δ160 construct (20-fold reduction from activated levels) being as efficient as inhibition of the parent plasmid p175KCAT.

Since the induction of Δ160 by Vmw65 was mediated by sequences between −160 and −108 and therefore most likely required the intact consensus TAATGARAT sequence at −115 to −106, it was possible that the negative regulation by IE175K protein also required this sequence. However, because expression from the Δ108 construct was not efficiently stimulated by Vmw65, we asked instead whether the IE175K protein would repress expression when Δ108 was activated by the IE110K protein. The Δ108 CAT target gene was cotransfected with pBR322, pIGA-15, pXhoI-C, or both pIGA-15 and pXhoI-C (Fig. 6). Consistent with our previous results, the IE110K protein stimulated expression from Δ108 approximately 15-fold. In contrast, cotransfection with pXhoI-C inhibited expression, resulting in a three- to four-fold reduction from basal levels. Moreover, the inhibitory effect of pXhoI-C dominated over the stimulatory effect of pIGA-15 when both plasmids were added together. The combination of pIGA-15 and pXhoI-C resulted in levels of CAT activity from the Δ108 construct that were approximately 30-fold lower than those with pIGA-15 alone. Over the course of this work the addition of pXhoI-C repressed IE110K-activated levels of expression from the Δ108.CAT construct as efficiently as from deletion mutants containing upstream sequences.

IE175K repression of enhancer-mediated stimulation of expression from the IE175K promoter. We wished to rule out

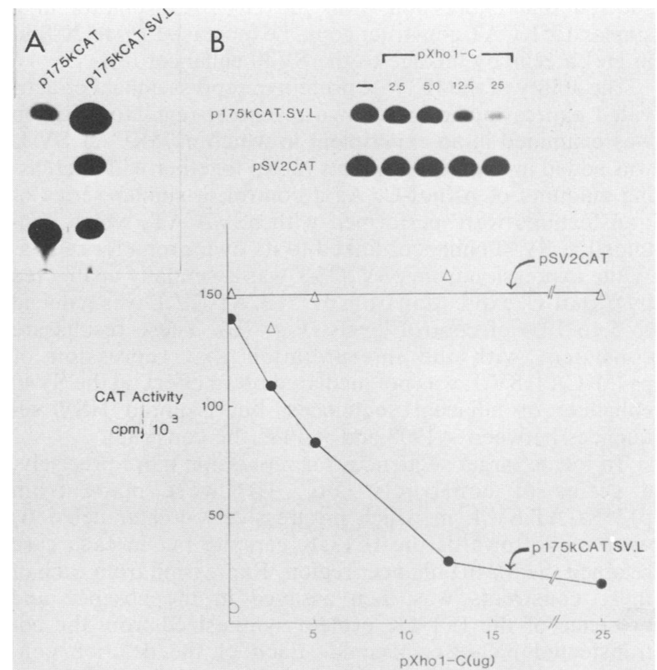

FIG. 7. Linkage of the parent IE175K promoter-regulatory region to the SV40 enhancer region results in elevated levels of expression which are specifically repressed by the IE175K protein. (A) Approximately 2 μg of p175KCAT or equimolar amounts of p175KCAT.SV.L were transfected in the absence of carrier DNA. Cells were harvested 48 h after transfection, and equal amounts were assayed for CAT activity. (B) Samples containing 2 μg of p175KCAT.SV.L or equimolar amounts of pSV2CAT were cotransfected with 2 μg of pBR322 (—) or increasing amounts of pXhoI-C (in micrograms), as indicated above the lanes. Cells were harvested 44 h after transfection, and equal amounts were assayed for CAT activity as shown by the formation of the major product, 3′-monoacetylated chloramphenicol. Quantitative illustration of these results is given in the lower part of the figure. The basal level of expression from 2 μg of the parent plasmid p175KCAT (O) is shown for comparison.

the possibility that IE175K repression of Vmw65 and IE110K *trans* activation of the parent and deletion constructs was due to an indirect effect on the *trans*-activating components. Therefore, in a second series of experiments we examined the ability of the IE175K protein to repress CAT expression from each of the deletion constructs when activated in *cis* by linkage to the SV40 enhancer. For the experiments with the SV40 enhancer region, it was first necessary to show that the intact IE175K promoter-regulatory region could be activated in *cis* by the enhancer and that this enhancer-mediated activator could be overcome by the addition of IE175K protein. The *Hin*dIII C fragment of SV40, containing the virus enhancer sequences, was placed in the late orientation at the *Hin*dIII site in p175KCAT, approximately 1,900 bp upstream from the mRNA start site. Basal expression from this construct, p175KCAT.SV.L, was up to 20-fold higher than that from the parent p175KCAT (Fig. 7A) and almost as efficient as that from the intact SV40 enhancer-promoter construct pSV2CAT. Although other workers have indicated that the upstream sequence of the IE175K gene may have enhancer-like properties (18, 31), clearly it does not promote gene expression as efficiently as the prototype enhancer in SV40, and moreover the IE175K promoter-regulatory region is open to activation in *cis* by the SV40 enhancer. These results are consistent with recent data from Gaffney et al., who showed that expression from the IE175K promoter in a similar 175KCAT construct could be increased 15- to 20-fold in HeLa cells by linkage to the SV40 enhancer (8).

The ability of the IE175K protein to repress enhancer-activated expression from its own promoter-regulatory region was examined in an experiment in which p175KCAT.SV.L was added in constant amounts (2 μg) together with increasing amounts of pXhoI-C. As a control, a similar series of transfections were performed with pSV2CAT, which contains the SV40 enhancer linked to its own promoter region. While expression from pSV2CAT was essentially unaffected by pXhoI-C, expression from p175KCAT.SV.L was reduced to 5 to 10% of control levels (Fig. 7B). These results are consistent with the interpretation that repression of p175KCAT.SV.L was not mediated by an effect on the SV40 enhancer or adjacent sequences, but required HSV sequences between −1900 and +30 in the construct.

To locate target sequences for repression more precisely, a series of constructs (Fig. 1B) were made from p175KCAT.SV.L in which progressively greater deletions were made towards the IE175K cap site but in each case retained the SV40 enhancer region. Expression from each of these constructs was then assayed in the absence and presence of the IE175K protein synthesized from the cotransfected pXhoI-C plasmid. Each of the deletion constructs containing the SV40 enhancer expressed high levels of CAT activity, and each was repressed by the addition of pXhoI-C. Thus, levels of expression from Δ380.SV.L, Δ330.SV.L, and Δ160.SV.L were at least as high as those from p175KCAT.SV.L, and each was inhibited by pXhoI-C by approximately 30- to 40-fold, to levels less than 4% of those expressed in the absence of pXhoI-C (Fig. 8A). A separate experiment included analysis of Δ108.SV.L and, as a control, plasmid pSV2CAT (Fig. 8B). High levels of expression were also observed from Δ108.SV.L, and the addition of pXhoI-C inhibited expression by approximately 20-fold. Although the absolute amount of input pXhoI-C required for repression varied in different experiments, dramatic inhibition of expression (10- to 40-fold reduction) from the parent and deleted SV.L series, including Δ108.SV.L, was observed at doses of pXhoI-C which had no significant effect on expression from pSV2CAT (Fig. 7 and 8).

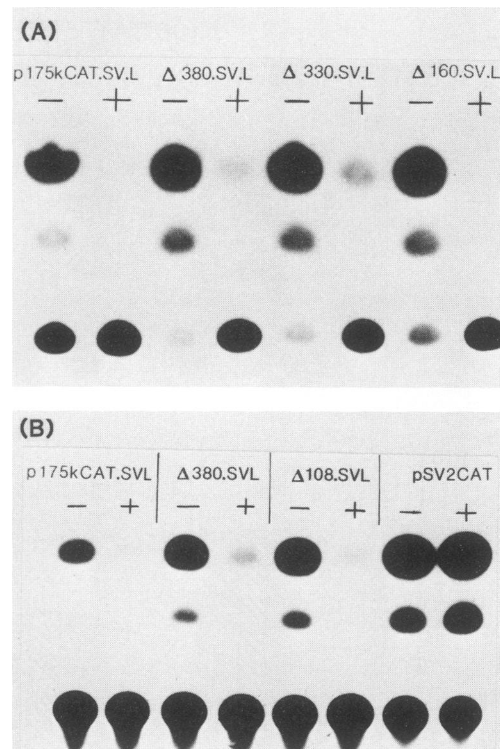

FIG. 8. SV40 enhancer increases expression from each of the 5′ deletion mutants, and each is subject to repression by the IE175K protein. (A) Samples containing 2 μg of p175KCAT.SV.L or equimolar amounts of Δ380.SV.L, Δ330.SV.L, and Δ160.SV.L were cotransfected with 4 μg of pBR322 (−) or 4 μg of pXhoI-C (+). Assays were performed on cells harvested 44 h after transfection. (B) Samples containing 2 μg of p175KCAT.SV.L or equimolar amounts of Δ380.SV.L, Δ108.SV.L, and the control pSV2CAT were cotransfected with 2 μg of pBR322 (−) or 2 μg of pXhoI-C (+). Cells were harvested 46 h after transfection, and equal amounts were assayed for CAT activity.

We conclude from these experiments that sequences located between −108 and +30 are sufficient for the autoregulatory activity of the IE175K protein and that in the experiments examining the effect of IE175K on Vmw65 and IE110K *trans* activation, repression was mediated via *cis*-acting sequences in the IE175KCAT constructs and was probably not due to some indirect effect on the *trans*-activating components.

DISCUSSION

We have previously shown (26) that expression from the IE175K promoter-regulatory region is subject to *trans*-acting regulation by three virus-encoded proteins, i.e., stimulation by the late structural component Vmw65, stimulation by the IE110K protein, and repression by its own gene product, the IE175K protein. In this work we have attempted to delineate the *cis*-acting requirements within the IE175K promoter-regulatory region for the different interactions by analyzing the effect of a series of upstream deletions on the response to each of the three components. Our results indicate that sequences within the basal promoter region from −108 to +30 are sufficient to mediate both stimulation by IE110K

and autoregulation by IE175K. However, we cannot presently exclude the possibility that additional upstream signals may exist for either of these interactions. Consistent with previous reports (8, 17, 19, 21, 30), we show that the basal promoter is not sufficient for induction by Vmw65, which exhibits an absolute requirement for upstream sequences, including one intact TAATGARAT motif.

A number of workers have demonstrated that upstream sequences in the IE175K promoter-regulatory region augment expression in the absence of *trans*-activating components, an observation consistent with data from our present work. Although this region has been shown to exhibit some of the properties of an enhancer, in that the sequences can function at a distance and in an orientation-independent fashion, disparate results have been obtained on the ability of these sequences to enhance expression when placed at the 3′ end of a gene (18, 31). In addition, the magnitude of enhancement of the HSV sequences at the 5′ end was very much lower than, for example, the prototype enhancer of SV40, given that experiments have been performed in cells fully permissive for HSV growth (e.g., Vero, BHK, HeLa). Finally, our results and the recently published work of Gaffney et al. (8) both show that expression from the IE175K promoter-regulatory region is open to enhancement when the latter is linked in *cis* to the SV40 enhancer. From these considerations, together with the results of Jones and Tijan (14), who demonstrated the presence of approximately six binding sites for the cellular transcription factor SpI in the IE175K upstream sequences, one likely possibility for the augmented constitutive expression imparted by these latter sequences is simply an additive effect of the multiple SpI-binding sites. Similar conclusions have been reached by Gaffney et al. (8) and by Bzik and Preston (2), who recently published a refined analysis of the IE175K upstream sequences augmenting constitutive expression and indicated the involvement of multiple regions, each spanning an SpI-binding site and each imparting two- to threefold stimulation.

Although the TAATGARAT consensus motif apparently does not contribute significantly to constitutive expression (2, 8), it is clearly an essential requirement for induction by Vmw65. Our results add to previous analyses of the requirements for IE175K stimulation by the virion component the first demonstration that a deletion mutant containing only 160 bp upstream of the mRNA cap site efficiently responds to *trans* activation. Since deletion to −108 effectively abolished response to Vmw65, we think it likely that the TAATGARAT consensus sequence TAATGGAAT, located at position −115 to −106, can effectively mediate stimulation by Vmw65 in the absence of sequences further upstream than −160.

Although previous reports of Vmw65 stimulation of IE175K gene expression from Preston and colleagues (2, 30, 31) demonstrated an absolute requirement for the TAATGARAT motif located at positions −265 to −256, their analyses were based on examination of deletions and alterations of sequences upstream from −174 when linked to the basal promoter extending only to −108 and thus lacking the intact TAATGARAT motif between −115 and −106. It is interesting that the sequence CCGAACGGAA is present in an inverted orientation adjacent to this latter TAATGARAT motif. A similar GA-rich region present in the same orientation as, and located 20 to 30 bp upstream of the TAATGARAT motif at −265 to −256, has been shown by Bzik and Preston (2) to augment stimulation by Vmw65 in the presence of the TAATGARAT motif. In addition, previous results of Kristie and Roizman (17) have indicated the requirement for a GA-rich sequence, since a region which contained this latter sequence together with a TAATGARAT motif could confer Vmw65 inducibility while a TAATGARAT motif alone could not. Further analyses of deletions and alterations within the −160 construct will illuminate whether sequences adjacent to the TAATGARAT consensus sequence at −115 to −106 influence the response to Vmw65 in a similar fashion.

An important feature of the results reported here is that a second *trans* activation mechanism also produced high levels of expression from the IE175K promoter region and that this was observed in the absence of both the TAATGARAT motifs and Vmw65. Thus, a construct lacking all of the sequences upstream of −108 and thus unresponsive to Vmw65 was still *trans* activated by the IE110K protein, with resulting stimulation similar to that observed for the intact parental construct p175KCAT. Notable features downstream of −108 are a single SpI-binding site centering on position −80 (14) and a TATA consensus sequence at approximately −25, although interestingly this region of the IE175K promoter lacks a sequence resembling the consensus CAAT box (23). Binding of SpI at the −80 position in a −108 deletion construct is required for transcription in vitro, with the resulting efficiency of expression being roughly equal to in vitro transcription of a construct containing sequences up to −330 (14). One possibility to account for our previous observation that the IE110K protein can transactivate both DE and IE expression (25, 26), and the present results which limit requirements for IE *trans* activation to within −108 bp upstream of the cap site, is that IE110K acts by stimulating the interaction or function of promoter-common SpI- or TATA box-binding factors. However, at present several other mechanisms can be proposed to account for IE110K *trans* activation, and thus it will be of interest to test additional virus and cellular promoters to determine whether stimulation by IE110K correlates with the presence of the consensus TATA box or SpI-binding sites. Internal deletions in the Δ108 CAT target gene and attempts to confer inducibility on noninducible constructs by the addition of consensus binding sites should also help to further refine the sequence requirements for induction by IE110K.

Our results indicate that the *cis*-acting requirements for negative autoregulation of IE175K expression can also be limited to the promoter region between −108 and +30. Thus, although repression by the IE175K protein is specific in that no other promoter-regulatory regions tested were inhibited (26; P. O'Hare and G. S. Hayward, manuscript in preparation), the IE-specific TAATGARAT sequences are not required for autoregulation. One explanation consistent with the observation that IE175K not only repressed basal levels of expression but also exerted a dominant inhibitory effect on expression activated in *trans* by Vmw65 or IE110K and in *cis* by linkage to the SV40 enhancer is that IE175K binds within the −108 to +30 region and inhibits expression by preventing RNA polymerase binding or translocation at the IE175K promoter. Since IE175K stimulates expression from promoters that contain SpI-binding sites and TATA box region (5, 9, 26, 27, 32), it is unlikely that these motifs are involved in the negative autoregulatory function. Analyses of further deleted or altered promoter regions are now in progress to determine whether, by analogy with SV40 T antigen autoregulation, sequences encompassing or adjacent to the cap site are involved in IE175K autoregulation and whether direct binding of IE175K to the promoter region can be demonstrated.

In conclusion, we have provided evidence that the *cis*-acting requirements within the upstream region of the IE175K gene for regulation by two virus-encoded components, stimulation by the IE110K protein and repression by the IE175K protein, are separate from the major requirement for yet a third interaction stimulated by the virion component Vmw65. In addition to its negative autoregulatory function, the IE175K protein is also a positive *trans* activator which is essential (6, 29, 35) and sufficient (5, 9, 25, 27) for the stimulation of expression from DE promoters during a lytic virus infection. Moreover, IE175K has been shown to be required for the establishment of a latent infection (34) and to be expressed in latently infected rabbit ganglia (10). Thus, further dissection of the multiplicity of *cis*- and *trans*-acting interactions influencing IE175K promoter activity should provide a useful model system for studies of complex control mechanisms whereby alternative interactions of several regulatory factors on a promoter-regulatory region may direct different pathways of eucaryotic gene expression.

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