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Dissection of Immediate-Early Gene Promoters from Herpes Simplex Virus: Sequences That Respond to the Virus Transcriptional Activators

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The immediate-early promoters of herpes simplex virus give rise to the first series of transcripts after infection. These promoters are composed of compound sequence elements that govern basal level and regulated transcription. The response of three core (truncated) promoters from the herpes simplex virus type 1 IE-4, IE-0, and IE-27 genes to a battery of virus-encoded *trans*-acting proteins was examined in a short-term transient expression assay system. The results of this study reveal (i) a role for a sequence, 5'---GGGGG---3', flanked by 3 to 5 base pairs of symmetry (the G box), which is present in the upstream region of all immediate-early gene promoters, (ii) a requirement for the consensus sequence protected by ICP4 for autoregulation by this immediate-early gene product, and (iii) an alternative, sequence-independent mechanism for the augmentation of alpha gene expression by the virion-associated transcriptional activator Vmw65, now designated as TIF.

Infection of permissive cells with herpes simplex virus (HSV) results in activation of at least three sets of viral genes that are expressed in a coordinately regulated manner (17, 18). Although examples of translational control exist (19, 36), the major point of regulation is at the level of transcription (37). The control signals which define whether a gene is regulated as an alpha, beta, or gamma gene are found in upstream promoter-regulatory and adjacent noncoding sequences (3, 5–9, 21, 26, 32, 33). We are studying the regulated expression of alpha genes. Previous results demonstrated that alpha gene promoters contain two components: a minimal transcription unit which governs basal level expression and an upstream region composed of multiple sequence elements that are required for regulated expression as an alpha gene (3, 6, 21, 26, 33). These *cis*-acting signals which respond to a variety of cellular and viral *trans*-acting factors include: (i) a TATA box located 20 to 25 bases upstream of the mRNA initiation site, (ii) multiple binding sites for the host transcription factor Sp1 (20), (iii) one to three copies of the consensus sequence TAATGARATTC (which mediates transcriptional activation by the virion-associated factor Vmw65, also known as TIF [3, 4, 12, 24, 26, 38]), and (iv) sequences homologous to the core of the simian virus 40 enhancer with enhancer-like activity (25, 26, 34). In addition, the promoters for three of the five alpha genes contain a sequence (consensus: ATCGTCRNNNYGCRC [10, 26, 27]) which probably targets binding of ICP4 to DNA.

Previously, we and others used a transient expression assay system to examine the basal level expression from alpha gene promoters (IE-0, IE-4, IE-27, and IE-22/47) in either HeLa or Vero cells and the response of these promoters to a battery of HSV effectors (8, 13–15, 30, 31). These included DNA encoding the virion-associated factor Vmw65 (4, 32) and the *trans*-acting alpha gene products ICP0, ICP4, and *ts*ICP4 (from *ts*K) (13–15, 30, 31). These studies demonstrated that alpha promoters contain sequence elements that respond to both host and virus transcription factors to mediate constitutive and activated expression. Specifically, we showed that (i) alpha promoters are activated when the molar ratio of ICP0 + ICP4 to target is low but repressed when the ratio of these effectors is high (the "spike" response [14, 15]), (ii) alpha promoters containing the ICP4 recognition consensus sequence, ATCGTCRNNNYCGRC (10) [(found in IE-4, IE-0, and an only slightly degenerate homolog in IE-27: ATTCGTCCTTGTCTGTGC [26]), were repressed by ICP4 (29, 30), and (iii) all alpha promoters which contain the consensus sequence TAATGARATTC were activated by Vmw65 (3, 4, 12, 15, 29, 30). The spike response occurred in HeLa cells with CAT chimeras containing the full alpha promoters for IE-0, -4, -22/47, or a core promoter from IE-22/47 (from HSV type 2 [HSV-2] [14, 15]), and in Vero cells with a CAT chimera containing the complete alpha promoter from IE-27 (15). Cotransfection with a plasmid encoding Vmw65 augmented expression from each of the immediate-early targets in both cell lines, with one exception; CAT activity from the IE-27 promoter was not increased in HeLa cells. Several of the responses were common only to some of the alpha promoters. For example, ICP4 suppressed expression from promoters containing an ICP4 recognition sequence (IE-0, -4, and -27) whereas it slightly stimulated expression from the 22/47 promoter, which lacks this sequence. *ts*ICP4 (a temperature-sensitive product of the IE-4 allele from *ts*K) suppressed expression from the IE-22/47 and IE-27 promoters and suppressed the ability of ICP0 to enhance expression from these promoters. However, although *ts*ICP4 itself had no effect on expression from the IE-0 or IE-4 promoters, it augmented the ability of ICP0 to activate these promoters. The response of the IE-27-CAT chimera was unique. In HeLa cells, it had characteristics of both alpha and beta genes, yet in Vero cells, its response pattern was indistinguishable from that of the IE-4 and IE-0 promoters. These results reveal that HSV effectors influence expression from individual alpha promoters, depending on (i) which effectors are present, (ii) their abundance, and (iii) the presence or absence of cell-type-specific transcription factors.

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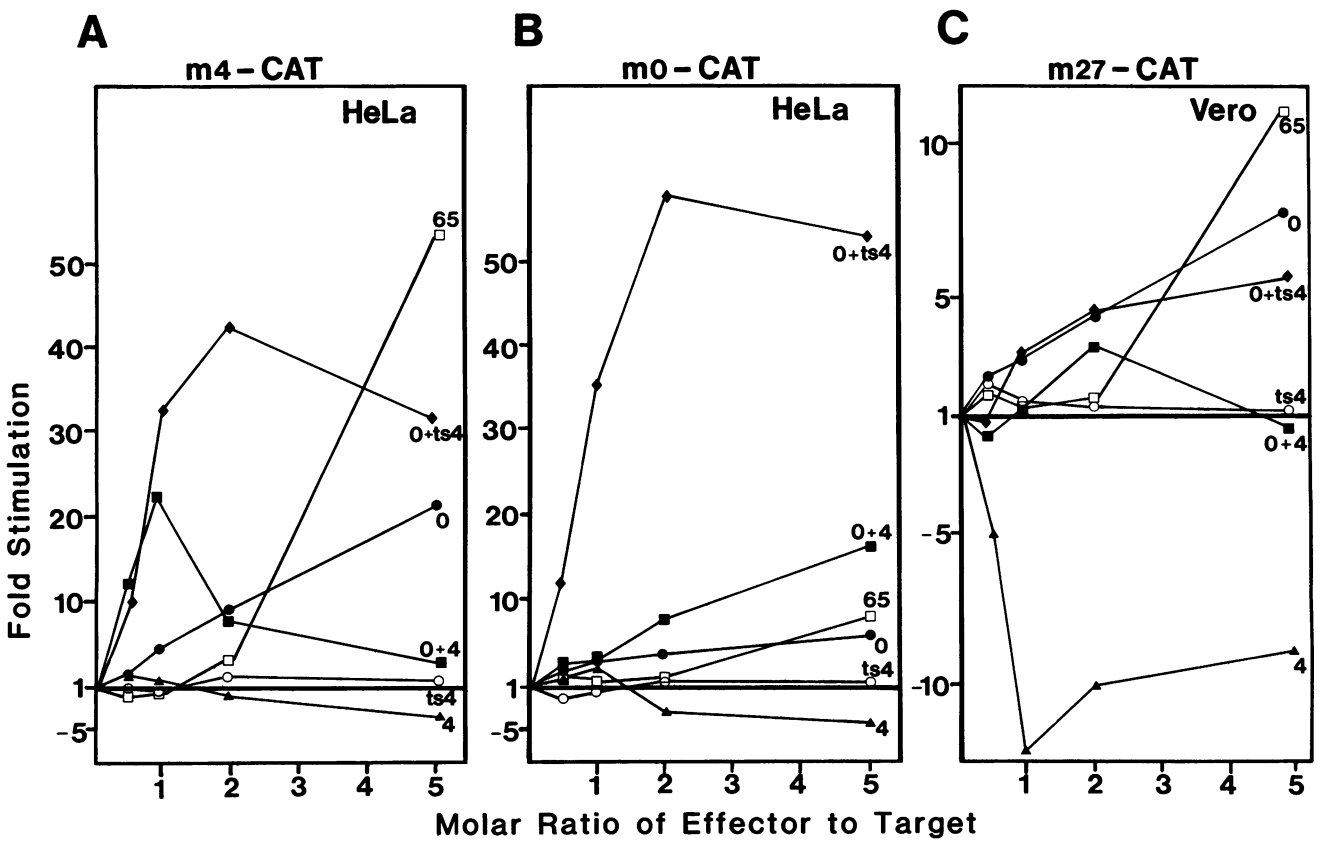

FIG. 1. Expression from immediate-early core promoter-CAT chimeras. The target genes (2.5 μg) mIE-4-CAT (A), mIE-0-CAT (B), and mIE-27-CAT (C) were cotransfected into either HeLa or Vero cells along with increasing molar ratios of plasmids encoding ICP0 (●), ICP4 (▲), *ts*ICP4 (○), ICP0 + ICP4 (■), ICP0 + *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 is arbitrarily assigned a value of unity.

In this study, the response of three minimal alpha promoters, each lacking one or more known alpha promoter-regulatory sequence elements, to a battery of HSV effectors is examined. Our results suggest that (i) a sequence found in the promoters of all alpha genes, GGGGG, flanked by 3 to 5 base pairs of symmetry (G box), is required but not sufficient for the spike response, (ii) the ICP4 response sequence was required for repression by ICP4, and (iii) alpha promoters lacking the consensus TAATGARATTC were activated by high levels of Vmw65 only, suggesting an alternate, sequence-independent mechanism for activation by Vmw65.

MATERIALS AND METHODS

Growth and transfection of animal cells. HeLa and Vero cells were propagated as previously described (13). HeLa or Vero cells were seeded at 1×10^6 to 3×10^6/10-cm dish and transfected the next day with a carrier-free calcium phosphate-DNA precipitate containing 2.5 μg of target plasmid DNA plus various molar ratios of effector plasmid DNAs as previously described (14, 39). The actual ratios of plasmid DNAs used are described in the legend to Fig. 1. The cells were incubated at 37°C, except those receiving pGX164 (*ts*IE-4), which were incubated at 39°C (12). The level of expression from the target gene (CAT activity) was analyzed at 40 h after transfection. Each experiment was repeated at least two times, and although there were variations in the absolute CAT activity from one experiment to the next the profile of the response was always consistent.

Recombinant DNAs. The plasmids pGX58, pGX164, and pGR212b, which encode HSV-1 ICP4, *ts*ICP4 (from *ts*K), and Vmw65, respectively, have been described previously (14). pSS7, which encodes HSV-1 ICP0, is a subclone of pIGA-15 (13) lacking the 2-kilobase *Kpn*I-*Eco*RV fragment downstream of the IE-0 3′ terminus. All minimal IE-CAT chimeras were subcloned into the same plasmid background as described previously (15). Thus, identical plasmid sequences were juxtaposed to the minimal promoter-CAT construct. The constructs, their assignations, as well as the 5′ and 3′ boundaries of their promoters are found in Table 1. All plasmids were propagated in *Escherichia coli* DH5alpha, and DNA was extracted as described previously (1).

CAT assays. Cell lysates were analyzed for CAT activity at 40 h after transfection according to the technique of Gorman et al. (16) as previously described (14). The concentration of protein in CAT extracts was determined by the method of

TABLE 1. Minimal IE-CAT chimeras

mIE-CAT (promoter)	Plasmid	Promoter boundaries (5′/3′)	Cloning sites (5′/3′)
mIE-4	pIGA-91	−108/+33	*Eco*RI/*Bam*HI
mIE-0	pIGA-96	−125/+150	*Sma*I/*Nco*I
mIE-27	pIGA-98	−84/+1	*Apa*I/*Eag*I

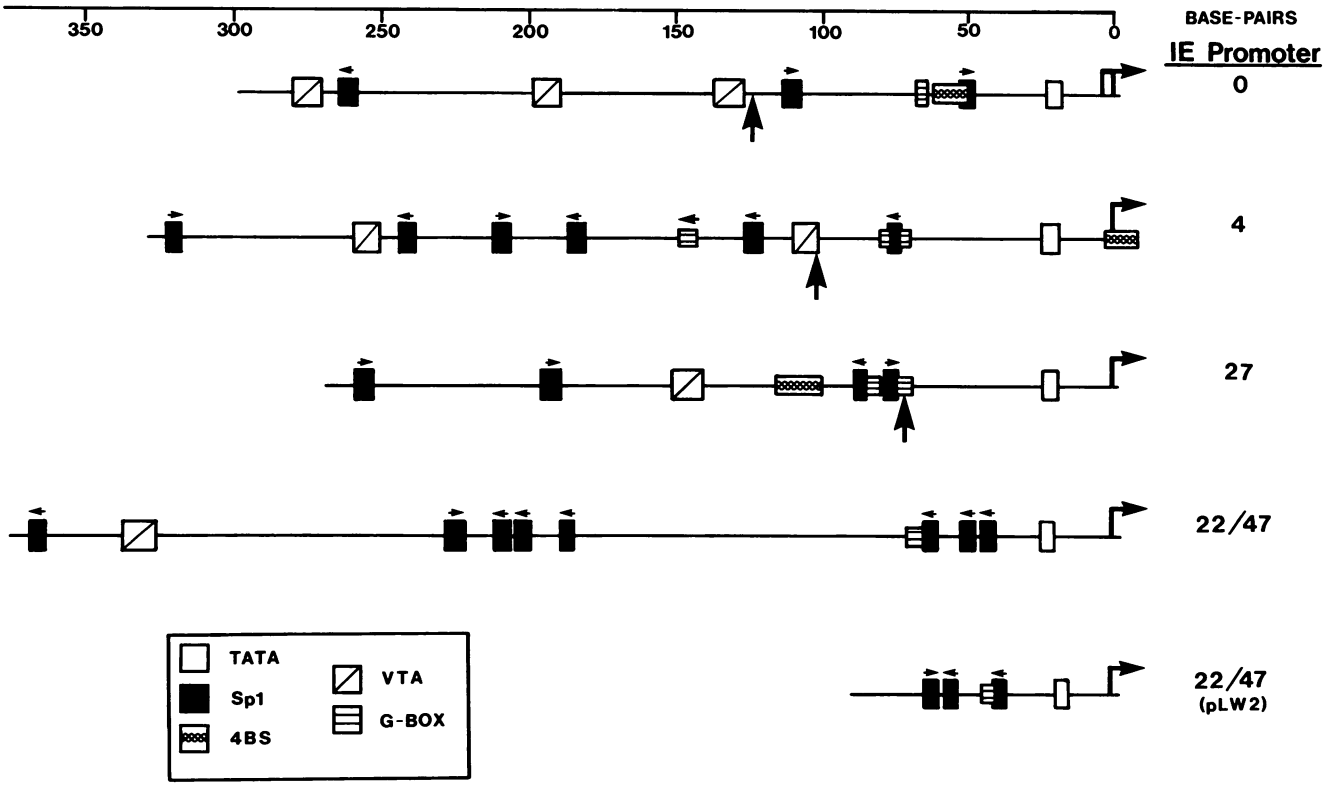

FIG. 2. Schematic representation of the distribution of promoter-regulatory elements in the upstream region of alpha genes. The location and orientation of five sequence motifs present in the promoters of each of the HSV-1 alpha genes are displayed relative to the 5′ end of the mRNA (large rightward-pointing arrow) that is transcribed from them. The nucleotide sequences of these promoters were taken from: IE-4 (26), IE-0 (26), IE-27 (26, 38), and IE-22/47 (28). These elements are the: TATA box, SP1 binding site (5′ GGGCGG 3′ [20]), 4BS (the site protected by ICP4, consensus: 5′ ATCGTCRNNNYGCRC 3′ [10, 22, 27]), VTA (the site recognized by Vmw65 in conjunction with a host protein [24], consensus: 5′ TAATGARATTC 3′ [4, 12, 26]), and the G box (5′---GGGGG---3′; the dashes refer to 3 to 5 flanking base pairs, which are present as either a palindrome or a dyad). All elements not capped by arrows are oriented towards the CAP site. The vertical arrows represent the 5′ endpoint of each of the minimal promoters examined in this study. pLW2 refers to the HSV-2 minimal IE-22/47 promoter (12) that we previously studied (14).

Bradford (2). We define specific activity of CAT enzyme as percent acetylation of chloramphenicol (i.e., counts per minute of acetylated [^{14}C]chloramphenicol/total counts per minute) per microgram of protein.

RESULTS

Activation and repression of minimal IE-promoters. The promoters of immediate-early (alpha) genes are compound and composed of core sequences which govern basal level expression and sequences often overlapping the core sequences which are responsive to *trans*-acting viral and cellular factors (3, 6, 21, 26, 30, 31, 33). In previous studies, we examined the response of IE-CAT chimeras (containing each of the alpha gene promoters fused to a CAT cassette) to a battery of HSV effectors in two cell lines (14, 15). To attempt to separate alpha promoter from alpha regulatory sequences, we assayed the response of minimal IE-CAT chimeras, containing primarily core IE promoter sequences, to a battery of HSV effectors. The 5′ and 3′ cloning boundaries of m4-CAT (pIGA-91), m0-CAT (pIGA-96), and m27-CAT (pIGA-98) are listed in Table 1. Specifically, we sought to determine (i) whether a sequence common to each of the alpha promoters, the G box, is involved with the spike response, (ii) whether the consensus sequence TAATGARATTC is stringently required for activation of alpha gene expression by Vmw65, and (iii) whether the consensus sequence in alpha gene promoters protected by ICP4, or a close homolog of this sequence, is required for suppression of alpha gene expression by ICP4 (30).

The response of m4-CAT (Fig. 1A), containing sequences from +33 to −108, to the effectors ICP0, ICP0 + ICP4, and ICP0 + *ts*ICP4 was similar to that of an IE-4-CAT chimera containing all of the IE-4 promoter-regulatory sequences (15, 21, 33). Thus, the spike response of m4-CAT to ICP0 + ICP4 is maintained in a construct that contains a TATA box, a known SP1-binding site (20), the consensus sequence protected by partially purified ICP4 (10, 27), and the G-box sequence that overlaps the footprinted SP1 site. Moreover, the response of the m4-CAT target to ICP0 + *ts*ICP4 is saturated at 2 molar gene equivalents of effector, whereas the response of the complete IE-4-CAT promoter (15) increases in response to increasing levels of plasmid DNA encoding these two effectors. This suggests that sequences upstream of −108 potentiate rather than directly mediate the response to ICP0 + *ts*ICP4. Furthermore, though this promoter contains an ICP4 recognition sequence (Fig. 2), ICP4 repressed the expression of m4-CAT only slightly (about twofold). Thus, the m4 promoter contains most of the characterized, *cis*-acting recognition elements that interact with known cell and viral transcription factors although it lacked a TAATGARATTC signal. This cassette remains

TABLE 2. Immediate-early gene G boxes

Virus and IE gene	Sequence	Position relative to CAP site	Symmetry	Strand[a]
HSV-1				
IE-0	attGGGGGaat	−75/−65	Palindrome	Top
IE-4	ggcGGGGGcgg	−78/−68	Dyad	Top
IE-4	gccGGGGGggc	−86/−76	Palindrome	Top
IE-4	gcccGGGGGccg	−152/−141	Dyad	Bottom
IE-27	ccgGGGGGcgg	−78/−68	Palindrome	Top
IE-27	gggcGGGGGccc	−93/−82	Palindrome	Top
IE-22/47	ggccccGGGGGcgggcc	−77/−61	Palindrome	Top
HSV-2				
IE-22/47	cgacGGGGGgcg	−52/−41	Palindrome	Top

[a] Top, mRNA sense strand; bottom, antisense strand.

responsive to ICP0 and ICP4. Surprisingly, this chimera responded to high levels (5 molar gene equivalents) of a plasmid encoding Vmw65, resulting in a large increase (50-fold) in CAT accumulation. Low levels of effector, though, did not alter the level of m4-CAT expression.

m0-CAT contains promoter sequences from +150 to −125. Thus, it contains an ICP4 recognition sequence (10, 22, 27), a G box, and several potential Sp1 binding sites (20); however, it lacks the TAATGARATTC motif (Fig. 2). The expression of m0-CAT in HeLa cells was repressed by ICP4. However, cotransfection of increasing molar gene equivalents of ICP0 + *ts*ICP4 resulted in increased accumulation of CAT (Fig. 1B). This response was similar but not identical to that of a construct containing the complete IE-0 promoter (15). As with the m4-CAT target, the expression from m0-CAT was augmented only when the molar ratio of Vmw65 effector was high. However, its response was nine- to tenfold less than that of m4-CAT to this effector. Moreover, expression from m0-CAT is suppressed by ICP4 and only slightly stimulated by ICP0 (fivefold). Unlike the response of the complete IE-0-CAT target, the accumulation of CAT activity from m0-CAT continued to increase in response to increasing molar ratios of IE-0 + IE-4; thus, there was no spike response. Therefore, additional upstream sequences contribute to the response to ICP0 and appear to be required for the spike response. The pattern of response to ICP0 + *ts*ICP4, however, was similar to that of the complete IE-0-CAT construct; i.e., it was stimulated in the presence of these two effectors. This result suggests that specific sequences which respond to the presence of these effectors are retained in the m0-CAT chimera.

The m27-CAT chimera contains promoter sequences from +1 to −84. Thus, it lacks the potential ICP4 recognition sequence, a G-box sequence, putative Sp1 binding sites, and the TTAATGARATTC motif (Fig. 2). The response of a CAT chimera containing the complete IE-27 promoter-regulatory region to increasing ratios of a battery of HSV effectors was previously shown to have characteristics of both alpha and beta promoters in HeLa cells but was only alpha-like in Vero cells (15). In addition this chimera did not direct the synthesis of sufficient amounts of CAT activity in HeLa cells to permit us to evaluate its response to the presence of different effector molecules. Therefore, the m27-CAT construct was tested for an alpha-like pattern of response in Vero cells. In comparison to an IE-27-CAT chimera containing a complete promoter-regulatory region (15), the response of m27-CAT (Fig. 1C) differed. Although both ICP4 and *ts*ICP4 repressed expression from a complete 27-CAT chimera (15), the expression from m27-CAT was only suppressed by ICP4. The loss of suppression by ICP4 correlated with the absence of the homolog to the ICP4 recognition sequence in the minimal IE-27-promoter. Also, the standard spike response (activation at relatively low levels, 1:1, and a decreased response at higher ratios of effector to target) to ICP0 + ICP4 was absent. This correlated with the removal of the IE-27 G box. As expected, ICP0 mildly stimulated the expression of m27-CAT. Finally, as with the other minimal IE-CATs lacking TAATG ARATTC signals, CAT expression from this target was stimulated only at high molar gene equivalents of Vmw65, and lower ratios of effector to target had no effect.

DISCUSSION

The responses of three minimal IE-CAT constructs, each lacking one or more alpha promoter-regulatory sequences, to a battery of HSV effectors were examined. The focus of these experiments was on three characteristics of alpha gene expression, (i) the spike response (augmentation of expression at low molar ratios of ICP0 + ICP4 to target; suppression at high ratios of these effectors), (ii) suppression by ICP4, and (iii) activation by Vmw65, and how they correlate with the presence or absence of specific alpha-regulatory sequence elements: a G box, the recognition sequence protected by ICP4 (10, 11, 22, 27), and the Vmw65 response signal.

G box. The presence of a G box in the minimal IE-4 promoter and its absence in the minimal IE-27 promoter correlate with the presence and absence, respectively, of a spike response (Fig. 1). Although the minimal IE0 promoter contains a G box, its response to increasing molar gene equivalents of ICP0 + ICP4 is to direct the synthesis of more CAT. However, this promoter is relatively insensitive to ICP0 and therefore appears to lack 5′ sequences which contribute to the spike response. These results suggest that the G box is required but not sufficient for the spike response of alpha promoters to ICP0 + ICP4. Recently; a systematic analysis of more than 40 footprinted sites protected by SP1 revealed that the sites are potentially more degenerate than previously described. This analysis suggests that the consensus recognition site is composed of the sequence GGggCG GGGC, in which the lower case g's appear to be invariant whereas there is some flexibility with regard to substitution at the other sites (T. Evans, T. DeChiara, and A. Efstratiadis, submitted for publication). Six of the seven G boxes shown in Table 2 adhere to this new consensus, and thus the G box might represent potential sites of interaction with SP1.

ICP4 recognition consensus sequence ATCGTCRNNNYG CRC. The consensus sequence ATCGTCRNNNYGCRC is

protected from nuclease digestion by nuclear extracts from infected cells or partially purified ICP4 in vitro (10, 22, 23, 27), probably as a consequence of interaction of ICP4 with a host protein (11). Previous studies from this laboratory suggested that ICP4 could regulate transcription by at least two independent mechanisms (14). In one, ICP4 interacts with host polymerase and virus and/or cellular factors to recognize specific sequences in herpesvirus promoters to regulate transcription. This model does not require that ICP4 bind directly to either the protected consensus or other unidentified specific nucleotide sequences. Rather it, as Vmw65, might interact under the aegis of a host protein (24). Previous experiments which demonstrated an autoregulatory function for ICP4 did not examine the effect of deletion of this sequence on negative regulation by ICP4 (30, 31). The different points of control mediated by the action of ICP4 might occur independent of specific nucleotide sequences. The results of these experiments indicate that expression from minimal IE-0 or -4 promoters, which retain the ICP4-binding consensus sequence, is suppressed in the presence of ICP4. ICP4 also suppresses expression from the minimal IE-27 promoter, which lacks this consensus. However, the absence of this sequence from the minimal IE-27 promoter correlates with the inability of *ts*ICP4 to down regulate. These data indicate that the ICP4 recognition sequence can serve a role in regulation of alpha gene expression by either ICP4 or *ts*ICP4 but is not in and of itself sufficient.

The consensus sequence TAATGARATTC. Activation of the minimal alpha promoters occurred only at high ratios of Vmw65 to target. This result was unexpected because these targets lack the TAATGARATTC motif (Fig. 1) (12, 26). Previous studies showed that Vmw65 activated promoters containing this motif (3, 4, 12, 26, 30). Only low molar ratios of effector were used in these analyses; therefore, it is possible that the primary mechanism of Vmw65 action is mediated through recognition of this sequence. However, our results suggest the existence of an alternative activation pathway. Vmw65 may activate transcription by recognizing other unidentified sequences present in these minimal promoters which respond only at high molar ratios of Vmw65 to target or by interaction with cell transcription factors. In support of this model, Kristie and Roizman (24) recently demonstrated that the action of Vmw65 on TAATG ARATTC signals is mediated through a host protein which protects these sequences from nuclease digestion. This factor is likely to be NFIII, a protein which binds to and protects a homolog of the TAATGARATTC motif from nuclease digestion (35). Thus, there appear to be multiple pathways for how virus *trans*-acting proteins can regulate transcription. These depend on the presence of host factors and specific promoter-regulatory sequences.

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