Evidence of DNA : protein interactions that mediate HSV-1 immediate early gene activation by VP16

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The viral genes first expressed upon lytic infection by herpes simplex virus type 1 (HSV-1) encode the five immediate early (IE) proteins. IE gene expression is potently and specifically induced by a virion protein termed VP16. Previous studies have shown that the activating properties of VP16 are IE gene specific and mediated by upstream regulatory elements common to each IE gene. Paradoxically, however, VP16 does not appear to be a sequence-specific DNA-binding protein. To understand the specificity of VP16 activation, we identified the cis-regulatory sequences of an IE gene that mediate VP16 response. Two distinct DNA sequence motifs enable the ICP4 gene to respond to VP16. Biochemical fractionation of nuclear proteins from uninfected cells revealed the existence of cellular proteins that bind directly to each of these VP16 cis-response elements. These observations, in concert with the identification of functional domains of the VP16 protein, lead to the hypothesis that VP16 achieves activation specificity via protein : protein, rather than protein : DNA, interactions.

[Key Words: VP16; IE gene activation; HSV-1; sequence-specific DNA-binding proteins]

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Lytic infection by herpes simplex virus type 1 (HSV-1) involves the temporally regulated expression of three classes of viral genes (Honess and Roizman 1974; Clements et al. 1977). The products of these genes are termed immediate early (IE), delayed early (DE), and late (L) polypeptides. The transcriptional activation of IE genes can occur in the absence of new protein synthesis and is induced in trans by a factor present in mature virus particles (Post et al. 1981). This ‘virion factor’ is specified by the product of an L gene and has been identified as a 65-kD phosphoprotein variously termed Vmw65 or VP16 (Campbell et al. 1984).

The mechanism by which VP16 activates IE gene expression has been considered in a number of previous studies. Activation is known to require cis-acting DNA sequences that occur upstream of IE genes (Post et al. 1981). A variety of different reports have underscored the importance of a nonanucleotide sequence, 5'-TAATGARAT-3' [R = purine], common to each of the five IE genes (Mackem and Roizman 1982a,b; Cordingley et al. 1983; Kristie and Roizman 1984; Gaffney et al. 1985; Bzik and Preston 1986; O'Hare and Hayward 1987). The upstream sequences of at least one IE gene exhibit enhancerlike properties; i.e., they are capable of activating transcription from a heterologous promoter in a distance- and orientation-independent manner (Lang et al. 1984; Preston and Tannahill 1984). Importantly, when fused to a heterologous gene, IE upstream DNA sequences cause that gene to become VP16 responsive (Post et al. 1981; Mackem and Roizman 1982a).

Despite numerous indications of the specificity of VP16 action, attempts to demonstrate direct interaction between VP16 and IE upstream sequences have consistently failed (Marsden et al. 1987; S. Triezenberg, unpubl.). We have addressed this paradox in two ways. First, we have carefully delineated the IE cis-acting DNA sequences that facilitate VP16 response. Second, we have fractionated nuclear extracts derived from uninfected cells with the goal of identifying and isolating proteins that bind selectively to these sequences. These efforts have identified two distinct DNA sequence motifs that mediate VP16 response and have provided evidence for two corresponding proteins that bind to these sequences in vitro.

Results

Two distinct cis-regulatory elements facilitate virus-mediated trans-induction

Several previous studies have shown that DNA sequences located within 400 bp upstream from the IE gene encoding ICP4 (infected cell protein 4; also called VMW175 and a4) specify response to trans-induction by VP16 (Post et al. 1981; Mackem and Roizman 1982a; Cordingley et al. 1983; Bzik and Preston 1986). To study the nature of the regulatory DNA sequences associated with the ICP4 gene, we excised a restriction fragment carrying these sequences and fused it to two derivatives of the HSV-1 thymidine kinase (tk) gene that lacked the
native tk promoter [see Materials and methods]. The two ICP4/tk chimeric genes differed by only 11 bp; one chimera bore the ICP4 regulatory sequences fused to the tk gene at a point 25 bp downstream from the normal start site of tk transcription [pICP4tk25], whereas in the other construct, the two genes were fused 36 bp downstream from the tk mRNA start site [pICP4tk36].

Chimeric ICP4/tk mRNAs transcribed from both templates could be detected by a primer extension assay, using a synthetic oligonucleotide complementary to nucleotides 55–78 of tk mRNA (Graves et al. 1985). Because the fusion points in the two chimeric genes differed by 11 bp, the primer extension products were of distinguishable sizes. Thus, the two ICP4/tk chimeras provided both an experimental template for systematic in vitro [pICP4tk25] mutagenesis and an unaltered control template that served as an internal reference for transient transfection and viral trans-induction assays.

Two systematic sets of deletions extending into ICP4 regulatory DNA sequences were prepared in vitro [see Materials and methods]. Each deletion mutant was transfected, along with an equal amount of the internal reference template, into a single dish of cultured mouse L cells. One day later, the transfected cells were split equally into two fresh culture dishes. Two days posttransfection, one of the dishes was superinfected with a tk-deficient strain of HSV-1 in the presence of cycloheximide. The remaining dish was mock infected. Total RNA was harvested from each dish 2 hr postinfection, and expression from the transfected templates was monitored by primer extension [see Materials and methods].

The first series of mutants to be examined included deletions that removed ICP4 regulatory sequences starting at a point 325 bp upstream of the native ICP4 mRNA cap site [termed 5′-deletions]. The results of transient expression assays of the 5′-deletion mutants are shown in Figure 1a. The top panel shows the expression level of each deletion mutant in cells that had been superinfected with HSV-1 (conditions we term ‘induced’). The bottom panel shows the expression level of the same mutants in mock-infected cells (conditions that we term ‘basal’). The autoradiogram shown in the bottom panel was exposed fivefold longer than that shown in the top panel. We estimate the HSV-1 superinfection stimulates transcription of the unmutated ICP4/tk templates by 15- to 30-fold.

Deletion of ICP4 sequences to a point 290 bp upstream from the mRNA cap site resulted in no deleterious effect on either the induced or basal levels of expression. The two subsequent deletions, bearing endpoints 270 and 256 bp upstream from the mRNA cap site, were clearly impaired when tested under induced conditions. The deletion mutant that retained 270 bp of DNA flanking the ICP4 mRNA cap site was expressed only 20% as efficiently as the internal control template, whereas expression from mutant retaining 256 bp was at only 5% of the control. The behavior of these deletion mutants was quite different when monitored under conditions of basal expression, both mutants were expressed at an efficiency equivalent to the internal control template. Basal expression declined only after deletion of sequences up to or beyond a point 241 bp from the mRNA cap site.

Inspection of the ICP4 DNA sequence in intervals separating the aforementioned deletion endpoints revealed three putative cis-regulatory DNA sequence motifs. The hexanucleotide 5′-GGGCGG-3′, the canonical sequence specifying interaction with the transcription factor Sp1 [Briggs et al. 1986], is located between the endpoints of mutants 5′-256 and 5′-241. Five additional GC hexanucleotides occur within the ICP4 regulatory element [indicated by black boxes in Fig. 1]. The interval between mutants 5′-256 and 5′-270 contains the sequence 5′-TAATGAGAT-3′. Previous reports have pointed out that this sequence is common to each of the HSV-1 IE genes [Mackem and Roizman 1982b]. Moreover, in independent studies, Preston and colleagues have shown that deletion of the TAATGAGAT [R = purine] consensus sequence interferes with ‘virus factor’ response [Coringley et al. 1983; Preston et al. 1984; Bzik and Preston 1986]. Aside from its location in the −256 to −270 interval, closely related derivatives of this same sequence occur at sites 360 and 110 bp upstream from the ICP4 mRNA cap site [designated by open boxes in Fig. 1]. Finally, the interval between mutants 5′−270 and 5′−290 contains three direct repeats of the sequence 5′-GCCGAA-3′ [designated by a hatched box in Fig. 1]. The possible involvement of this ‘GA’-rich element has been noted previously for the ICP4 [Bzik and Preston 1986] and ICP27 [Kristie and Roizman 1984] regulatory regions. This hexanucleotide is present in the 5′-flanking region of all IE genes and is generally found in close proximity to at least one TAATGAGAT motif. Contrary to the relatively common occurrence of GC hexanucleotides within transcriptional regulatory elements [McKnight and Tjian 1986], neither TAATGAGAT nor the GCCGAA sequence motif is known to play a role in the expression of other viral or cellular genes.

The regulatory sequences associated with the ICP4 gene were analyzed further by the construction and assay of a series of 3′ deletion mutants. In this case, DNA sequences were removed progressively in a 3′ → 5′ direction [relative to the orientation of transcription] starting at a naturally occurring EcoRI restriction site 110 bp upstream from the ICP4 mRNA cap site. Results of transient expression assays using 3′-deletion mutants are shown in Figure 1b. As with the series of 5′-deletion mutants, induced expression was eliminated in two discernible steps. In this case, however, the two steps were separated by a great distance (~150 bp). A substantial drop in expression occurred upon deletion of sequences between the EcoRI site and a point 131 bp upstream from the mRNA cap site. The second notable decrease did not occur until the endpoint of 3′ deletion reached residue −292. Neither of these stepwise drops in expression was observed when the same deletion mutants were assayed under conditions of basal expression. The two incremental reductions in induced expression observed from the analysis of 3′-deletion mutants correlated with the locations of TAATGAGAT [positioned at
Figure 1. Effects of deletion mutations upon expression from the ICP4 promoter. ICP4 upstream sequences (−335 to +30 relative to the ICP4 mRNA cap site) were fused to the HSV-1 tk gene at nucleotide +25 (experimental) or nucleotide +36 (control). Deletion mutants of the experimental template were constructed in vitro. The deletion endpoint of each mutant that was assayed is indicated above its respective gel lane. Experimental and control templates (1 μg each) were transfected into mouse L cells. One day after transfection, the cells of each 60-mm plate were trypsinized and split equally onto two 60-mm plates. Two days after transfection, one plate of each pair was infected with HSV-1 in the presence of 100 μg/ml cycloheximide, and the other plate was mock infected. Two hours after infection, cells were lysed, and total cellular RNA was isolated. Steady-state levels of tk-specific mRNA were quantitated by primer extension, gel electrophoresis, and autoradiography. The control template yielded primer extension products 11 nucleotides shorter than the experimental template. Autoradiograms in panels representing mock-infected cells (lower panels of both a and b) were exposed four to five times longer than autoradiograms representing HSV-1 infected cells. [a] Primer extension products from transient expression assays, using 5′-deletion mutants. [b] Primer extension products from transient expression assays, using 3′-deletion mutants.
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residue −115) and GCGGAA (positioned at residue −280) motifs.

The observations outlined in the preceding paragraph, coupled with results obtained from the analysis of 5′-deletion mutants, raised the possibility that two different DNA sequence motifs specify response by the ICP4 gene to the trans-inducing effects of HSV-1 superinfection. One motif, characterized by the nonanucleotide sequence TAATGARAT (R = purine), is present at three locations in the ICP4 regulatory element. The other motif, characterized by the hexanucleotide sequence GCGGAA, occurs as three direct repeats at one location within the regulatory element.

The identification of two cis-regulatory elements required for virus-mediated trans-induction was deduced from the effects of deletion mutations. Deletions create new and artificial DNA sequence arrangements and, thus, can affect the function of transcriptional regulatory elements in spurious and unanticipated ways. To test the roles of the TAATGARAT and GCGGAA motifs more rigorously, we introduced clustered point mutations into ICP4 DNA sequences in a manner that led to the selective elimination of these elements (see Materials and methods).

Four contiguous transversions were introduced into each of the TAATGARAT motifs. By recombining these clustered point mutants at naturally occurring restriction sites located between TAATGARAT motifs, we were able to construct each possible double mutant, as well as the triply mutated variant that bore three disrupted TAATGARAT motifs. Results of transient assays using these clustered point mutants are shown in Figure 2. Mutation of TAATGARAT site 1 or 2, alone or in combination with mutation of site 3, led to a modest decrease in induced expression. Mutation of all three TAATGARAT sites reduced expression markedly, yet not down to the basal level of uninduced expression. Consistent with the results of deletion mutagenesis, elimination of any or all of the TAATGARAT motifs in no way altered basal expression (Fig. 2, bottom panel).

We considered two hypotheses to account for the fact that mutation of all three TAATGARAT motifs failed to completely eliminate response of ICP4 regulatory sequences to virus-mediated trans-induction. On the one hand, residual response could be due to the activity of a different cis-regulatory motif (namely, the GCGGAA motif). Alternatively, the clustered base changes that were introduced into each TAATGARAT motif may not have fully neutralized TAATGARAT function. To test the first hypothesis, we introduced clustered point mutations into the GCGGAA motif of ICP4 regulatory elements containing either native or mutated TAATGARAT motifs. As shown in the left-hand panel of Figure 3, mutation of the GCGGAA motif alone had a minor but reproducible effect on virus-mediated trans-induction. The variant mutated at all three TAAT-

![Figure 2](image-url)
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Figure 3. Clustered point mutations in TAATGARAT and GCGGAA sequence motifs affect trans-induction by VP16. Mutant templates were constructed that contained clustered point mutations in all three TAATGARAT sites [TAAT"-; see Fig. 2], in the promoter-proximal GCGGAA repeat [GA"-], or in both the TAATGARAT and GCGGAA sites [TAAT"/GA"-]. Experimental and control templates were transfected into L cells, as described in Figure 1. Trans-induction was achieved by infection with HSV-1 [left panel] or by inclusion of 1 μg of a VP16 expression plasmid in the transfection mix [right panel]. Expression of ICP4/tk-specific mRNAs was quantitated by primer extension, as described in Figure 1.

GARAT motifs, as well as at the GCGGAA motif, did not respond to the trans-inducing effects of HSV-1 superinfection.

It is notable that the variant carrying clustered point mutations at the GCGGAA motif alone [leaving all three TAATGARAT motifs intact] is substantially more responsive to virus induction than the 5'-deletion mutant that removed the GCGGAA motif without impinging on the site-2 copy of TAATGARAT [5'-270]. This disparity may be accounted for by the fact that the 5'-deletion mutant [5'-270] had removed all three copies of the GCGGAA motif, whereas the clustered point mutant had disrupted only the most gene-proximal motif [see Materials and methods]. Alternatively, or additionally, the deletion mutant 5'-270 had removed TAATGARAT site 1, whereas the clustered point mutant at the GCGGAA motif did not.

VP16 trans-induction is highly gene specific

The inducing function of VP16 displays strict specificity with respect to the array of HSV-1 genes that it activates; it induces the expression of IE genes to the exclusion of DE and L genes [Post et al. 1981]. Certain other viral regulatory proteins, such as the ElA gene product of adenovirus [Nevins 1987], or the immediate early gene products of various herpesviruses [Treisman et al. 1983], activate gene expression with less specificity. Members of this latter class of viral regulatory proteins tend to activate gene expression via promoter-proximal cis-regulatory sequences [e.g., see Graves et al. 1985] and, in a number of instances, have been shown to play an interfering role in expression guided by promoter-distal elements such as enhancers [e.g., see Borrelli et al. 1984]. In contrast, VP16 activates the expression of its IE genes via cis-acting sequences that exhibit enhancer-like properties. Perhaps VP16 represents a general activator of enhancer function, and the TAATGARAT and GCGGAA motifs that specify response by HSV-1 IE genes represent only a subset of the repertoire of sequences through which VP16 can act. To test this possibility, we examined the ability of VP16 to activate gene expression via the enhancers of four other viruses.

Expression of the chloramphenicol acetyltransferase (CAT) gene was directed by the enhancers of SV40, polyoma virus, Rous sarcoma virus, Moloney murine sarcoma virus, or the ICP4 gene. The enhancer–CAT plasmids were transfected into mouse L cells. Parallel culture dishes were either infected with HSV-1 or mock infected. CAT enzymatic activity in protein extracts of transfected cells was monitored by thin layer chromatography. The results of these assays, presented in Figure 4, showed that HSV-1 infection actually reduced the level of CAT expression generated by four of the en-
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Figure 4. HSV-1 infection does not trans-induce other viral enhancers. Plasmids were constructed or obtained that express the bacterial CAT gene, driven by enhancers and promoters from SV40, polyoma virus (Py), Rous sarcoma virus (RSV), Moloney murine sarcoma virus (MSV), or the HSV-1 ICP4 gene. The parental plasmid pSVO-CAT lacks eukaryotic enhancer and promoter sequences. Mouse L cells were transfected and subsequently replated onto duplicate plates, as described in Figure 1. Cells were mock infected (-) or HSV-1 infected (+) in the presence of cycloheximide (100 μg/ml). Three hours postinfection, cycloheximide-containing medium was replaced with medium containing 10 μg/ml actinomycin-D. Two hours later, cells were lysed and assayed for CAT enzyme activity (Gorman et al. 1982). The autoradiogram shows reaction products reflecting the CAT enzyme activity present in equivalent amounts of extract from each sample.

Host cell proteins bind directly to the TAATGARAT and GCGGAA cis-regulatory elements

The simplest mechanism by which VP16 might achieve trans-activation is through direct binding to IE cis-regulatory sequences. Were this to be the case, the data presented thus far would require VP16 to interact with two different DNA sequence motifs (TAATGARAT and GCGGAA). Although DNA-binding proteins have been identified that are capable of recognizing more than one recognition site on DNA (Johnson et al. 1987; Pfeifer et al. 1987), this does not appear to be a property of VP16. Indeed, purified VP16 appears to be incapable of binding DNA (Marsden et al. 1987). Given such observations, we reasoned that VP16 must operate indirectly, perhaps via cellular factors that bind directly to the TAATGARAT and GCGGAA motifs.

We chose to examine rat liver nuclei for cellular proteins that might bind to IE cis-regulatory DNA sequences. Several considerations favored this decision. First, rat liver provides an ample source of nuclear protein. Because we expected the putative TAATGARAT and GCGGAA binding proteins to be relatively rare and because our ultimate goal was to purify the proteins to homogeneity, our efforts required large amounts of starting material. Second, HSV-1 is capable of infecting a broad range of host cells [Whitley 1982], thus, if any cellular proteins are required to facilitate VP16 action, they should exist in liver. Finally, procedures for the isolation of sequence-specific DNA-binding proteins from rat liver were currently in use in our laboratory (Graves et al. 1986; Johnson et al. 1987).

Soluble extracts prepared from ~10^10 rat liver nuclei were fractionated by standard methods of column chromatography. Sequence-specific DNA-binding activities were assayed by DNase I footprinting (Galas and Schmitz 1978), using an end-labeled ICP4 DNA fragment that contained TAATGARAT sites 1 and 2, the GCGGAA motif, and three GC hexanucleotides. The fractionation scheme that was employed is diagramed in Figure 5a. Rat liver nuclear extract was initially subjected to anion exchange chromatography using DEAE-cellulose. Because the DEAE flow through was presumed to contain cationic proteins, subsequent fractionation was conducted using a cation exchange resin [Bio-Rex 70]. Finally, affinity chromatography was performed using double-stranded salmon sperm DNA that had been coupled to cyanogen-bromide-activated Sepharose 4B. Two chromatographically separable, cationic DNA-binding activities were detected. One activity eluted from the DNA Sepharose column at 0.3 M KCl (fraction A) and produced a footprint over each of the GC hexanucleotides (Fig. 5b). Although we have not characterized this activity in detail, we assume that it represents the rat equivalent of the HeLa transcription factor, Sp1 (Briggs et al. 1986). Indeed, Jones and Tjian (1985) have shown that purified Sp1 binds to this same set of ICP4 GC hexanucleotides.

Footprint analysis of cationic nuclear proteins revealed a second DNA-binding activity specific for ICP4 regulatory DNA sequences. This second activity eluted from the DNA affinity column between 0.3 and 0.7 M KCl (fraction B). As shown in Figure 5b, the B fraction activity bound at two locations on the ICP4 enhancer. The two sites of DNase I protection established by the B
fraction coincided with TAATGARAT sites 1 and 2.

The protein fraction that bound to DEAE-cellulose was step eluted with 0.4 M KCl, dialyzed to 0.1 M KCl, and subjected to affinity chromatography on DNA Sepharose. Protein that eluted at 0.3 M KCl (fraction C) was tested for binding activity by DNase I footprinting. Surprisingly, this pool of anionic proteins contained a sequence-specific DNA-binding activity. Figure 5b shows that the C fraction activity selectively protected the GCGGAA motif from DNase I digestion.

To examine the specificity of the ICP4 binding activities observed in rat liver nuclear extracts, we conducted DNase I footprint assays in the presence of unlabeled competitor DNA fragments specific to each of the ICP4 cis-regulatory sequences that had been defined by in vitro mutagenesis. Double-stranded oligonucleotides

Figure 5. Nuclear extracts from uninfected cells contain proteins that bind specifically to cis-regulatory elements upstream from the ICP4 gene. RLNE were fractionated, as diagramed in panel a. Procedures for chromatographic separation are described in Materials and methods. The presence of sequence-specific DNA-binding proteins in three fractions (A, B, C) was demonstrated by DNase I footprinting assays, shown in panel b. For this purpose, a DNA fragment derived from the ICP4 regulatory region was radioactively labeled at an NcoI restriction site (nucleotide -211). The purified probe was incubated in the presence (+) or absence (−) of the various protein fractions before digestion with DNase I (as described in Materials and methods). The autoradiogram shows DNase I cleavage products after separation on a 7% polyacrylamide/8 M urea electrophoresis gel. cis-regulatory elements within this region of the ICP4 gene are depicted in the schematic diagram following the conventions of Figure 1.
corresponding to the GC hexanucleotide motif, the TAATGARAT motif, and the GCGGAA motif were synthesized and added to footprint reactions to test for specific competition with the aforementioned DNA-binding activities [see Materials and methods]. To examine the competitive effect of each synthetic binding site on each DNA-binding activity, unfractionated nuclear extract was used as the protein source. Under these conditions, the activity that binds to the TAATGARAT motif was difficult to detect. Instead of occupying a 20-bp area surrounding the TAATGARAT motif at site 2, the region of binding appeared to be limited to only two sites of DNase I cleavage. Although modest in appearance, the site of binding corresponded precisely to the location of TAATGARAT site 2 and was competed specifically and exclusively by the appropriate oligonucleotide [see subsequent Results].

The data presented in Figure 6 show that the synthetic GC hexanucleotide eliminated DNase I protection at the locations of the two GC hexanucleotides within the ICP4 DNA fragment. It did not, however, interfere with either of the two other DNA-binding activities. Likewise, the synthetic GCGGAA motif eliminated the DNase I footprint at its cognate site but interfered with neither the GC hexanucleotide-binding activity nor the TAATGARAT-binding activity. Finally, the synthetic TAATGARAT motif interfered with the activity that footprinted over TAATGARAT site 2 but did not block interaction at either of the two other cis-acting motifs associated with the ICP4 regulatory element.

The activities in the B and C fractions bind, respectively, to the two cis-acting motifs that render the ICP4 gene responsive to VP16. If these cellular DNA-binding activities play a role in the induction of IE gene expression in vivo, one might predict that their capacity to bind to the TAATGARAT and GCGGAA motifs would be eliminated by the clustered point mutations in each of the motifs that had been shown previously to interfere with VP16-mediated gene expression [see Figs. 2 and 3]. This prediction was tested by examining the binding affinities of the partially purified B and C fractions for ICP4 regulatory sequence bearing clustered point mutations in the TAATGARAT and GCGGAA motifs. The results of these assays are presented in Figure 7. Binding of the B fraction activity to TAATGARAT site 1 was eliminated by the cluster of four point mutations that were found to neutralize the functional activity of this cis-regulatory element. Similar results were observed for TAATGARAT site 2 (data not shown).

Figure 7 also shows that the clustered point mutations introduced into the GCGGAA motif interfered with binding of the C fraction activity. In this case, however, a residual level of binding was observed over the gene-distal portion of the motif. This binding might be explained by the fact that the clustered point mutations were restricted to the gene-proximal GCGGAA repeat, leaving the two gene-distal copies of the motif intact [see Materials and methods]. This explanation is consistent with the observation that the phenotypic effect of the clustered point mutations in the GCGGAA motif was less severe than the mutation that deleted all three GCGGAA repeats [see Figs. 1 and 2].

As a final comment, we point out that under our assay conditions for DNA binding, mutation of TAATGARAT site 1 did not interfere with the binding of the C fraction activity to the GCGGAA motif, nor did mutation of the GCGGAA motif alter binding of the B fraction activity to TAATGARAT site 1 (Fig. 7). This absence of interac-

Figure 6. Synthetic oligonucleotides compete specifically for binding of nuclear proteins to ICP4 cis regulatory sequences. A DNA fragment spanning the ICP4 regulatory region was labeled at a SalI restriction site [nucleotide -396]. The pattern of DNase I fragments from the unprotected probe is shown in the left lane, adjacent to reaction products obtained after incubation with crude nuclear extract. The locations of the various footprints are indicated schematically at the right side of the figure. Footprints over the GC hexanucleotide and the GCGGAA motif are easily visible. The footprint over the TAATGARAT motif [site 1, according to the numerology of Fig. 2] is more difficult to discern using the crude extract than when partially purified fractions were used (see Fig. 5). Increasing amounts of synthetic double-stranded oligonucleotides representing each of the three sequence motifs were added to extract: probe incubations prior to digestion with DNase I. The competitors used in each reaction [see Materials and methods] are indicated above the autoradiogram.
tion was not simply a result of the biochemical separation of the B and C activities. That is, unfractionated rat liver nuclear extracts also failed to show any evidence of binding cooperativity, even in the case of the two most closely juxtaposed motifs (TAATGARAT site 2 and the GCGGAA site).

Discussion

Detailed mutational analysis of the ICP4 regulatory element has revealed the presence of three distinct cis-regulatory motifs. Two of these motifs, TAATGARAT and GCGGAA, are uniquely required for response to the HSV-1 trans-activator protein, VP16. Mutational elimination of these motifs produced no deleterious effect on basal expression from the ICP4 regulatory element but completely eliminated trans-induction by VP16. The third cis-regulatory motif is specified by GC hexanucleotides that bind Sp1 (Briggs et al. 1986). The Sp1-binding sites appear to be necessary for basal expression from the ICP4 regulatory element (see Fig. 1). In a series of experiments not included in this paper, we have selectively eliminated Sp1-binding sites by the introduction of clustered point mutations. The results of those experiments indicated that Sp1 also plays a role in facilitating response to VP16 (S. Triezenberg, unpubl.). However, Sp1 clearly does not endow VP16 responsiveness to a transcriptional regulatory element, neither the HSV-1 tk promoter (Post et al. 1981) nor the SV40 early promoter (see Fig. 4) is VP16 responsive, despite the fact that they contain multiple Sp1-binding sites (Dynan and Tjian 1983; Jones et al. 1985; McKnight and Tjian 1986).

VP16 is similar to the adenovirus EIA gene product in that it somehow achieves trans-activation, despite its inability to bind directly to DNA. However, in contrast to EIA, VP16 displays strict specificity with regard to its target genes. We believe that we have pinpointed the two cis-regulatory motifs that mediate VP16 action (TAATGARAT and GCGGAA). One might speculate that the exceptional specificity of VP16 is related to the combinatorial use of two different cis-regulatory motifs. However, the data presented herein indicate that both TAATGARAT and GCGGAA are capable of acting independently. We speculate instead that HSV-1 has acquired the ability to achieve stimulation of IE gene transcription via two distinct cellular DNA-binding proteins and that perhaps it is this property that allows HSV-1 to infect a broad range of host cells.

Because purified VP16 does not appear to bind DNA in either a specific or nonspecific manner, it probably acts indirectly through cellular factors that themselves bind to the TAATGARAT and GCGGAA motifs. Several studies (Kristie and Roizman 1987; McKnight et al. 1987; Preston et al. 1988) have reported evidence of a HeLa DNA-binding activity that interacts specifically with IE cis-regulatory sequences. Likewise, we report the existence of liver nuclear proteins that bind to ICP4 cis-regulatory sequences. No direct evidence demonstrates that the cellular proteins that bind to these sites in vitro mediate VP16 action in vivo. However, because mutational disruption of the two VP16-specific motifs

Figure 7. Clustered point mutations prevent binding of nuclear proteins to ICP4 cis-regulatory elements. Three DNA fragments spanning the ICP4 regulatory region were radiolabeled at a SalI site (nucleotide −396). The fragments contained either the native ICP4 sequence (left lanes), clustered point mutations in TAATGARAT site 1 (middle lanes), or clustered point mutations in the promoter-proximal GCGGAA repeat (right lanes). Each fragment was incubated in the presence (+) or absence (−) of nuclear protein fraction B (lower lanes) or fraction C (top lanes) prior to DNase I digestion. The locations of regions of DNase I protection are indicated schematically at the right.
(TAATGARAT and GCGGAA) eliminate both VP16-dependent transcription and sequence-specific binding by the B and C fraction activities, we speculate that the proteins that specify these activities indeed represent the host cell factors that participate in VP16-mediated trans-induction.

How, in a mechanistic sense, might VP16 activate IE gene expression? Perhaps upon entry into the host cell cytoplasm, VP16 triggers a cascade of catalytic events that leads ultimately to activation of the B and C DNA-binding activities. Alternatively, VP16 may be transported to the nucleus of the infected cell where it interacts directly with the B and C fraction proteins. Two recent discoveries offer support to the latter model. In an elegant study, Preston and colleagues [Preston et al. 1988] have shown that VP16, the TAATGARAT motif, and a cellular DNA-binding activity form a ternary complex that is stable to electrophoresis in low ionic strength buffer. Additionally, Roizman and colleagues [Kristie and Roizman 1987; McKnight et al. 1987] have reported two separable HeLa protein fractions that contain activities that bind ICP4 cis-regulatory DNA sequences. Antibody reagents were used to show that VP16 is capable of forming a complex with one of the two binding activities.

The accompanying paper also provides support for a model involving direct interaction between VP16 and host-cell proteins that bind to ICP4 regulatory DNA sequences [Triezenberg et al. 1988]. Mutational studies of the VP16 gene product have revealed several functional domains. One of these domains is embedded within the carboxy-terminal 78 amino acids of the VP16 polypeptide and is required for the transcriptional activating function of the protein. The carboxy-terminal region of VP16 is highly acidic, thereby resembling the transcriptional activating domains of two yeast regulatory proteins, GCN4 [Hope and Struhl 1986] and GAL4 [Ma and Ptashne 1987]. Because GCN4 and GAL4 achieve transcriptional activation only after binding to cis-regulatory sequences adjacent to their target genes, we predict that VP16 must make intimate, if not direct, contact with the cis-regulatory elements of HSV-1 IE genes. As outlined in the accompanying paper, we predict that VP16 achieves this task via specific protein:protein interactions with cellular factors that bind directly to the TAATGARAT and GCGGAA motifs.

**Materials and methods**

**Plasmid construction and mutagenesis**

Cloning vectors pEMBL18 + and pEMBL19 + were derived from pEMBL8 + and pEMBL9 + [Dente et al. 1983] by S. Lazarowitz, using the polylinker from M13mp19 [Norrander et al. 1983]. Plasmid pICPtk25, used as an indicator of IE gene expression, contained regulatory sequences of the ICP4 gene fused to the HSV-1 tk gene. A Smal–BamHI fragment spanning nucleotides –332 to +27, relative to the ICP4 mRNA cap site [Cordinley et al. 1983; McGeoch et al. 1986], was cloned into pEMBL18 + following addition of an XbaI linker to the Smal terminus of the fragment. The IE sequences were then excised from the pEMBL vector, using SalI and BamHI. The protein-coding segment of the HSV-1 tk gene was derived from a 5'-deletion mutant that contains a BamHI linker at nucleotide +25 relative to the tk mRNA cap site [McKnight and Kingsbury 1982]. A BamHI–HindIII fragment carrying the tk gene was inserted along with the SalI–BamHI ICP4 promoter fragment into pBR322. Plasmid pICPtk36, used as an internal control in expression assays, was constructed in an identical manner, except that the tk gene fragment began at nucleotide +36 relative to the tk mRNA cap site. A plasmid containing additional ICP4 upstream sequences (to nucleotide –396), designated psIT703, was constructed from pICPtk25 by insertion of a Smal fragment (–396 to –333) bearing SalI and XbaI linkers at its distal and proximal termini, respectively.

Deletion mutations were derived from pICPtk25 by removing sequences from either end of the ICP4 upstream regulatory region. To construct 5’-deletion mutants, pICPtk25 was linearized at the XbaI site (nucleotide –332) and then digested sequentially with exonuclease III and nuclelease S1 [Sakonju et al. 1980]. Synthetic XbaI linkers were ligated to the ends of deleted molecules. Following digestion with XbaI and KpnI [which cleaves within the tk gene], fragments were size fractionated by agarose gel electrophoresis. Purified fragments were ligated to an XbaI–KpnI vector fragment derived from pICPtk25. The 3’-deletion mutants were constructed by digesting pICPtk25 with BamHI, followed by exonuclease III and nuclease S1. EcoRI linkers were ligated to the deleted molecules, which were then digested with EcoRI and HindIII. Appropriately sized fragments were purified after agarose gel electrophoresis and ligated to an EcoRI–HindIII fragment derived from pICPtk25. Deletion endpoints for individual mutants were identified by DNA sequencing, using the chemical cleavage method [Maxam and Gilbert 1980].

Clustered point mutations were introduced at selected sites within the ICP4 upstream regulatory region by oligonucleotide-directed mutagenesis. Oligonucleotides 24 or 25 residues in length were designed to introduce three or four transversion mutations, as shown below:

1. 5’-TAATGARAT-3’
2. 5’-CCGT-3’
3. 5’-GGCGAGCGGAAACGCGGAAAC-3’
4. 5’-A-T-C-3’

Oligonucleotides were synthesized using an Applied Biosystems automated synthesizer, purified by HPLC, and phosphorylated using polynucleotide kinase and ATP. Single-stranded template DNA was prepared from an M13 recombinant clone containing the SalI–BamHI ICP4 fragment of pSIT703. Each mutagenic oligonucleotide, together with the M13 sequencing primer [Boehringer Mannheim], was annealed to the template, extended, and ligated, using the conditions of Zoller and Smith [1982]. The reaction products were digested with BamHI and SalI. Fragments corresponding to the ICP4 regulatory sequences were purified by gel electrophoresis before ligation into a BamHI–SalI vector fragment prepared from pSIT703. Bacterial transformants were screened by colony hybridization, using the corresponding mutagenic oligonucleotide as a probe. The identity of mutant plasmids was confirmed by chemical sequencing.

The plasmid used to express VP16 in transient cotransfection experiments contained 76 bp of the VP16 5’-untranslated region, the entire VP16 open reading frame, and ~900 nucleotides of 3’-flanking sequences. The long terminal repeat (LTR) of Moloney murine sarcoma virus [Graves et al. 1985], was fused to VP16-coding sequences to provide transcriptional regulatory sequences (including the LTR enhancer, promoter, mRNA cap
site, and 27 bp of 5’-untranslated region). This VP16 expression plasmid was termed pMSVP16 Δ1D3.

Transient expression assay

Mouse L cells (tk−, aprt−) were obtained from B. Sollner-Webb. A tk− mutant of HSV-1 which produces no tk mRNA, termed Δ35, was provided by J. Smiley [Halpern and Smiley 1984]. One day prior to transfection, 8 × 10^6 L cells were plated per 60-mm plastic culture dish in Dulbecco’s modified Eagle’s medium (GIBCO), supplemented with 10% fetal calf serum (Hyclone) and antibiotics. CsCl-purified DNAs were transfected into the cells, using the DEAE–dextran method [Lopata et al. 1984]. Each plate received 1 μg of an ICP4-tk test plasmid (pICP4tk25, pSJ703, or a mutated derivative) and 1 μg of the internal control plasmid pICP4tk36. Twenty-four hours after transfection, the cells receiving any given test DNA were trypsinized, pooled, and replated onto duplicate 60-mm plates to ensure uniform transfection efficiency. Forty-four hours after transfection, one plate was mock infected and the other plate was infected with HSV-1 Δ35 virus at a multiplicity of 5–10 pfu per cell, in the presence of 100 μg/ml cycloheximide. Two hours postinfection, total RNA was harvested by the proteinase K/DNase I method [Eisenberg et al. 1985]. Primer extension assays were performed to quantitate expression from the IE-tk chimeric plasmids [Eisenberg et al. 1985]. The primer used was a synthetic oligonucleotide complementary to sequences between +55 and +70, relative to the tk mRNA cap site. The major extension products observed from transcripts of pICP4tk25 and pICP4tk36 were 81 bases and 70 bases, respectively.

Preparation of rat liver nuclear extract

Crude rat liver nuclear extracts (RLNE) were prepared from Sprague-Dawley rats, as described by Gorski et al. [1986], with minor modifications. Phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), benzamidine, spermine, and spermidine were added to buffers immediately prior to use in the concentrations specified below. All manipulations were carried out at 0–4°C. Minced liver tissue [125 g] was resuspended in 150 ml homogenization buffer [10 mM HEPES (pH 7.6), 25 mM KCl, 1 mM EDTA, 2 mM sucrose, 10% glycerol, 0.15 mM spermine, 0.5 mM spermidine, 0.2 mM PMSF, and 2 mM benzamidine]. Tissue was mixed in a Waring blender for 15 sec and homogenized with a Teflon–glass homogenizer until >90% of the cells were disrupted. The homogenate (~100 ml) was diluted with homogenization buffer to 500 ml, and 29-ml aliquots were layered over 10-ml pads of the same buffer containing 1 mM sucrose and centrifuged at 24,000 rpm for 30 min in an SW 28 rotor. Pelleted nuclei were resuspended in nuclear lysis buffer [100 mM HEPES (pH 7.6), 100 mM KCl, 3 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol], aliquoted into 30-ml Beckman ultracentrifuge tubes, and lysed by the addition of 0.4 M ammonium sulfate. The tubes were rocked for 2 min at 50°C. DNA was separated from protein by centrifugation, and boiled for 3 min in 3-5 μl of DNase I stop buffer (1% SDS, 100 μg/ml tRNA, 200 mM NaCl, 20 mM EDTA, and 200 μg/ml protease K). Proteinase K digestion was allowed to proceed for 20 min at 50°C. DNA was separated from protein by phenol–chloroform extraction, recovered by ethanol precipitation, and boiled for 3 min in 3–5 μl of 99% formamide containing appropriate tracking dyes. The samples were electrophoresed on 7% polyacrylamide/8 M urea gels, which were subsequently dried and autoradiographed with Kodak XAR-5 film. For the oligomer competition assays, footprint reactions were performed as described above, except that they were carried out in the presence of 15–150 ng of one of the following double-stranded oligomers:

TAATGAGAT:
5′-CATCCGATCCGATATGAGTACGGCAGATG-3′
3′-GCCAACCCATTTACTCTATGCTTCG-5′
GA:
5′-CGGAAAGCGGGAGCGGAAACCCGCGGATCGGCGGACG-3′
3′-CGCTTTGGCCCTCCCTTGGCCGCGTACCCG-5′
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3'TAGTCCCCGCCCCTAACCCCGCCCCATCC-5'

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