

Functional dissection of VP16, the *trans*-activator of herpes simplex virus immediate early gene expression

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Mature virions of herpes simplex virus type 1 contain an activating factor that primes transcription from the five virally encoded immediate early (IE) genes. This activator is specified by a 65-kD polypeptide termed VP16. The action of VP16 is mediated through *cis*-regulatory elements located in regions adjacent to each IE gene. Although VP16 is normally introduced into cells by infecting virions, its *trans*-activating function can also be observed by cotransfecting cells with a plasmid that encodes VP16 along with a reporter gene driven by IE *cis*-regulatory sequences. We have used such an assay to examine the function of mutant forms of VP16. Our results provide tentative identification of two domains of VP16 that are crucial to its role in the induction of IE gene expression. One domain is located within the carboxy-terminal 78 amino acids of VP16 and is characterized by its acidity. Another domain, located in a more amino-terminal region of the protein, appears to tailor the specificity of VP16 for IE genes. According to the results presented in this and the accompanying paper, we predict that VP16 achieves IE gene specificity via protein : protein, rather than protein : DNA, interaction.

[*Key Words*: IE gene activation; VP16; acidic activation domain; HSV-1]

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During lytic infection by herpes simplex virus type 1 (HSV-1), viral genes are expressed in three temporally ordered tiers (Hones and Roizman 1974), designated immediate early (IE), delayed early (DE), and late (L) (Clements et al. 1977). The IE phase of viral gene expression is specifically and potently activated by a component of the infecting virion (Post et al. 1981). The activating polypeptide, first identified by Preston and colleagues (Campbell et al. 1984), is a virus-encoded protein variously termed VP16 or Vmw65.

Work in several laboratories has shown that DNA sequences closely associated with IE genes are required for response to *trans*-activation by VP16. For example, a 220-bp region upstream of the IE gene that encodes ICP4 (infected cell protein 4) acts as a transcriptional enhancer (Lang et al. 1984; Preston and Tannahill 1984; Puga et al. 1985). This 220-bp region contains two distinct *cis*-regulatory motifs that mediate *trans*-activation by VP16. One such motif, identified in several different studies (Mackem and Roizman 1982; Cordingley et al. 1983; Kristie and Roizman 1984), has easily recognized counterparts upstream of each of the viral IE genes and is characterized by the nonanucleotide sequence 5'-TAATGARAT-3' (R = purine). We recently identified a second *cis*-regulatory motif within the ICP4 enhancer that specifies response to VP16 (Triezenberg et al. 1988). The sequence of this second element is purine rich and is typified by direct repeats of the hexanucleotide sequence 5'-

GCGGAA-3'. Our results indicate that either of these two *cis* motifs can act independently to mediate *trans*-activation by VP16 but that maximal induction depends on the presence of both motifs.

The simplest model that might account for VP16 *trans*-activation of IE genes would entail the direct binding of VP16 to one or both of the IE *cis*-response elements. The validity of such a model is contradicted by the observation that purified VP16 displays no substantial affinity for double-stranded DNA (Marsden et al. 1987). Instead, host-cell proteins bind in a sequence-specific manner to the IE *cis*-response elements (Kristie and Roizman 1987; Preston et al. 1988; Triezenberg et al. 1988). In the accompanying paper, we present evidence indicating that two distinct host factors exist that bind specifically to the two *cis* motifs that mediate VP16-dependent induction of IE gene transcription (TAATGARAT and GCGGAA) (Triezenberg et al. 1988). The mechanism by which VP16 utilizes these host DNA-binding proteins to facilitate *trans*-activation remains obscure.

To study the molecular basis of VP16 action further, we have used methods of *in vitro* mutagenesis to delete terminal or internal portions of the VP16 open reading frame (ORF). Mutated VP16 genes were tested in a transient cotransfection assay that measured *trans*-induction of IE gene expression. Our results demonstrate the existence of two functionally distinguishable domains within the VP16 polypeptide. A domain localized within the carboxy-terminal 78 codons of the VP16 ORF, which

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is unusually rich in acidic amino acids, is critical to the transcriptional activating function of VP16. The acidic tail of VP16 may be analogous to the activating domains of the yeast gene activator proteins, GCN4 (Hope and Struhl 1986) and GAL4 (Ma and Ptashne 1987), which are also markedly acidic. A second domain integral to the function of VP16 occurs amino-terminal to its acidic tail. In the absence of the acidic tail, this amino-terminal segment acts to interfere dominantly and specifically with the native inducer. That is, expression of the 'tail-deleted' form of VP16 fully abrogates the capacity of the intact inducer to *trans*-activate IE gene expression but does not interfere with the function of enhancers that are not VP16 responsive. We speculate that this amino-terminal 'interfering' domain of VP16 may represent the aspect of the protein that tailors its exclusive interaction with IE *cis*-regulatory DNA sequences, perhaps via protein:protein interaction with the cellular factors that bind to TAATGARAT and GCCGAA.

Results

Evidence of a transcriptional activation domain in VP16

To investigate the mechanism of *trans*-activation of IE

gene expression, we attempted to identify distinct domains of the inducer protein, VP16, that are necessary for its function. Our strategy was to construct and test deleted versions of the VP16 gene which, when transfected into cultured cells, would encode mutated polypeptides. The parental plasmid for deletion mutagenesis and subsequent expression of VP16 in mammalian cells was termed pMSVP16. This plasmid represents a fusion between transcriptional regulatory sequences of the murine sarcoma virus long terminal repeat (LTR) (Graves et al. 1985) and protein-coding sequences of VP16. The VP16 gene fragment in this plasmid was derived from the KOS strain of HSV-1. The nucleotide sequence of the KOS VP16 gene differs slightly from sequences reported from HSV-1 strain F (Pellett et al. 1985) and HSV-1 strain 17 published previously (Dalrymple et al. 1985). However, the predicted VP16 amino acid sequences of the KOS and F strains are identical for all 490 residues and differ from strain 17 by only two amino acid substitutions (Asp-Asn at position 13 and Ala-Thr at position 124). Figure 1 shows the predicted amino acid sequence of the KOS form of VP16.

Deletion mutants of the cloned VP16 gene were constructed in vitro (see Materials and methods). Deletion endpoints, determined by DNA sequencing, are indicated in Figure 1. For the carboxy-terminal deletions,

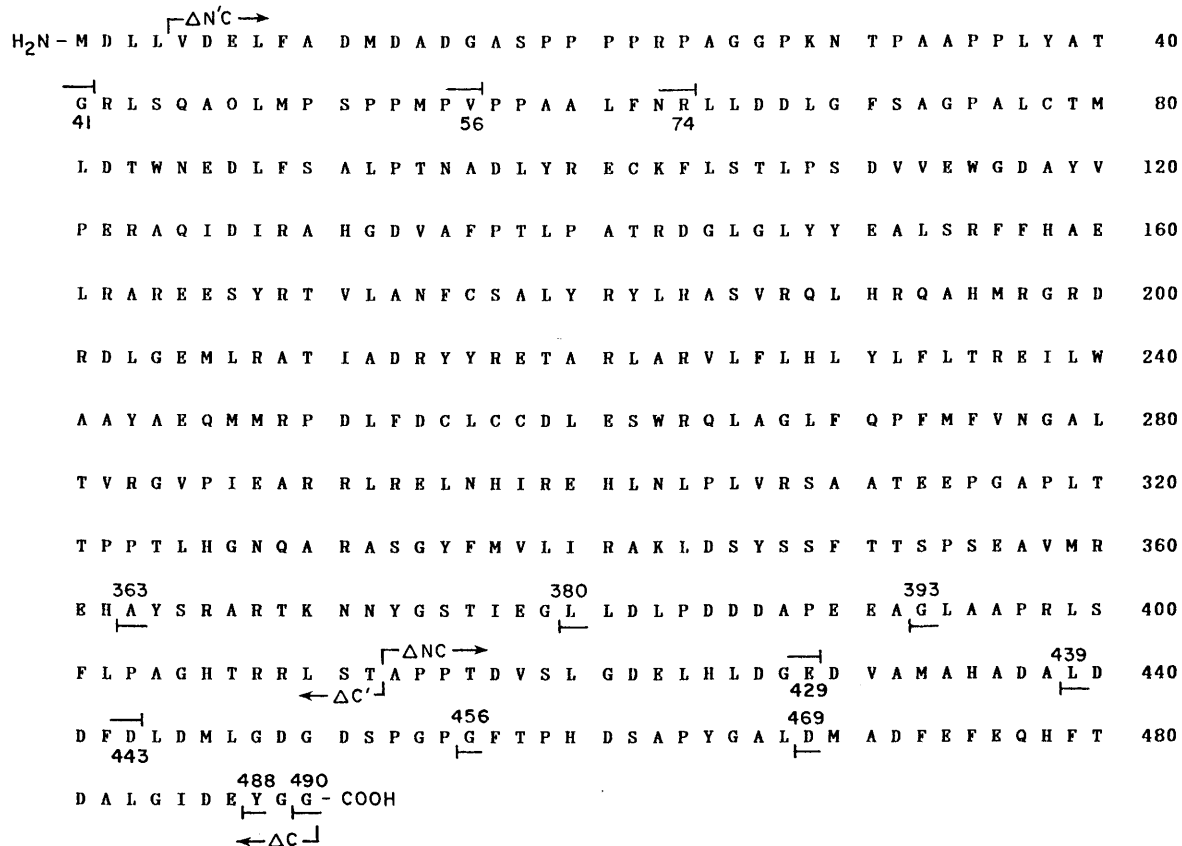


Figure 1. Amino acid sequence of VP16 deduced from the nucleotide sequence of the VP16 gene. Single-letter abbreviations are used for amino acids. The deletion endpoints for three sets of mutants are shown along the amino acid sequence. ΔC deletions begin at position 490 and extend in the carboxy-to-amino direction to endpoints indicated by bars (below sequence) and amino acid position numbers (above sequence). $\Delta N'C$ and ΔNC deletions begin at positions 5 and 413, respectively, and extend in the amino-to-carboxy direction to endpoints indicated by bars (above sequence) and amino acid position numbers (below sequence).

termed C mutants, translation termination codons covering all three potential reading frames were provided by a synthetic oligonucleotide, and polyadenylation signals were provided by a DNA fragment derived from the 3' terminus of the HSV-1 thymidine kinase (*tk*) gene. Internal deletions localized near the carboxyl terminus of the protein (termed Δ NC mutants) retained the native VP16 translation termination codon and polyadenylation signals. Internal deletions localized near the amino terminus of the protein (termed Δ N'C mutants) retained 76 bp of DNA 5' to the VP16 ORF, as well as the first four codons of the ORF.

A transient expression assay was used to monitor the ability of mutant forms of VP16 to *trans*-activate IE gene expression. In this assay, three plasmids were cotransfected into cultured mouse L cells. The indicator plasmid, designated pICP4*tk*, contained *cis*-regulatory sequences from the HSV-1 ICP4 gene (-396 to +30, relative to the ICP4 mRNA cap site), fused to the HSV-1 *tk* gene at nucleotide +25 relative to the *tk* mRNA cap site. The second plasmid included in the transfection assay was pMSV*tk* (Graves et al. 1985). This recombinant, which contains the LTR of Moloney murine sarcoma virus (MSV) fused to the HSV-1 *tk* gene, is not activated by VP16 (Triezenberg et al. 1988) and was intended to serve as an internal control for transfection efficiency, RNA recovery, and primer extension. The third plasmid included in the transfection assay was the test plasmid bearing either a native or deleted version of the VP16 structural gene. The three plasmids were introduced into cultured mouse L cells by the DEAE-dextran technique (Lopata et al. 1984). Two days later, total cellular RNA was isolated, and *tk*-specific expression was quantitated by primer extension. Because transcription from both the indicator plasmid (pICP4*tk*) and the reference plasmid (pMSV*tk*) read directly into *tk*-coding sequences, expression from each template could be monitored by primer extension using the same synthetic *tk* primer (Graves et al. 1985).

Transient expression results for a set of six carboxy-terminal truncations of VP16 are presented in Figure 2. Panel a shows an autoradiogram of primer extension products that were sized on a 9% polyacrylamide gel. Aside from six deletions that removed carboxy-terminal codons, two additional VP16 templates were assayed. One, termed C+119bp, contained the entire VP16 ORF, as well as the native VP16 polyadenylation signals. The other, termed C+7bp, contained the intact VP16 ORF but lacked the VP16 polyadenylation signals. C+7bp, as well as all six mutants that were deleted for carboxy-terminal codons, contained polyadenylation signals derived from the HSV-1 *tk* gene (see Materials and methods). Both C+119bp and C+7bp potently *trans*-activated expression from the pICP4*tk* template. Similarly high levels of *trans*-activation were observed for mutants that lacked one or three carboxy-terminal codons. The next two mutants in the series, designated Δ C469-490 and Δ C456-490, which were missing 21 and 34 residues, respectively, were slightly impaired in their *trans*-activating function. In the experiment shown in Figure 2,

the ratio of ICP4*tk* expression to MSV*tk* expression was reduced slightly. In other trials, the reductions resulting from the Δ C469-490 and Δ C456-490 truncations were more severe (data not shown). The final two truncations, Δ C439-490 and Δ C413-490, inactivated VP16 com-

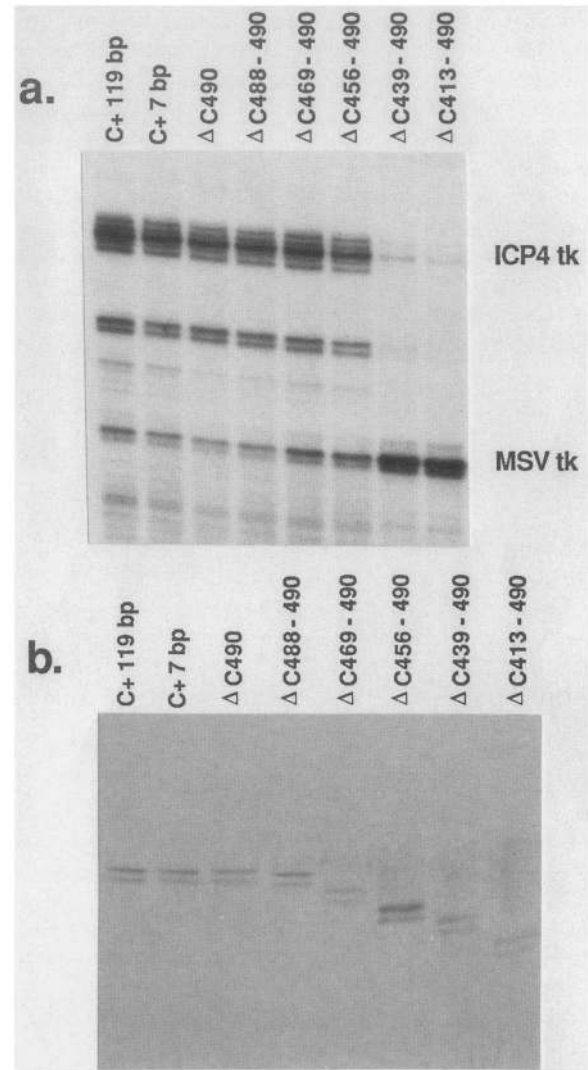


Figure 2. *Trans*-induction of IE gene expression by carboxy-terminal deletion mutants of VP16. (a) Mouse L cells were transfected with 1 μ g each of the signal plasmid pICP4*tk*, the control plasmid pMSV*tk*, and a VP16 deletion mutant, as indicated above each lane. Total cellular RNA was isolated 2 days later. The steady-state levels of *tk*-specific transcripts were measured using primer extension assays. The positions of primer extension products representing ICP4/*tk* and MSV/*tk* transcripts, after fractionation on a 9% polyacrylamide gel, are indicated next to the autoradiogram. (b) Western blot analysis of VP16 polypeptides produced in cells transfected with VP16 deletion mutants. L cells were transfected with plasmids bearing VP16 deletion mutants, as indicated above each lane. Two days later, total cellular proteins were solubilized and fractionated on an 8% polyacrylamide-SDS gel. After transfer to nitrocellulose, VP16 polypeptides were detected using polyclonal anti-VP16 serum and an immunoperoxidase visualization system (Vector Labs).

pletely. Although we have noted some quantitative variation in the effect of the Δ C469-490 and Δ C456-490 truncations, we consistently observed the carboxy-terminal truncations to fall into three categories: fully active (Δ C490 and Δ C488-490), partially active (Δ C469-490 and Δ C456-490), and inactive (Δ C439-490 and Δ C413-490).

One unexpected outcome of the initial assays should be noted. Contrary to expectation, VP16 did influence the expression of our internal reference plasmid (pMSVtk). Transcription from the pMSVtk template was inhibited reproducibly by the presence of plasmids expressing the native VP16 protein. Moreover, the effects of carboxy-terminal deletions of VP16 on the inhibition of pMSVtk expression correlated inversely with their effects on *trans*-activation of pICP4tk. Mutants partially defective in activation of pICP4tk were less able to inhibit pMSVtk expression. Mutants incapable of activating pICP4tk allowed high levels of expression from pMSVtk (see Fig. 2). Although we can only speculate as to the basis of this phenomenon (see Discussion), we do recognize that it complicates quantitation of VP16 mutant phenotypes. As such, we have chosen to confine mutant phenotypes to three categories: fully active (+), partially active (+/-), and inactive (-).

We wished to determine whether deletion mutants of VP16 lost *trans*-induction activity simply as a result of inadequate expression or acquired instability. Soluble protein extracts from cells transfected with each deletion mutant were electrophoresed on a denaturing polyacrylamide gel and transferred to nitrocellulose. The filter was then probed with a polyclonal rabbit anti-serum directed against purified VP16 protein (see Materials and methods). The results of these assays, shown in Figure 2b, indicated that roughly equivalent amounts of VP16 protein were present in cells transfected with functional, semifunctional and nonfunctional carboxy-terminal truncations of VP16. These assays also confirmed the prediction that the mutated VP16 DNA templates specify the synthesis of truncated polypeptides. Apparently, the loss of *trans*-activation function caused by carboxy-terminal truncations cannot be attributed to the absence of the VP16 polypeptide.

During the evaluation of the aforementioned results, we noted two facts. First, the carboxy-terminal region of VP16 is strikingly acidic. Of the 78 carboxy-terminal amino acids, 21 have acidic side chains and only 3 have basic side chains. Moreover, all three that have basic side chains are weakly basic (histidines). Second, work on two yeast regulatory proteins, GCN4 and GAL4, had identified generalized acidity as a property common to 'transcriptional activation' domains (Hope and Struhl 1986; Ma and Ptashne 1987). Given these observations, we adopted the hypothesis that the acidic tail of VP16 might constitute its transcriptional activating domain.

To map the amino-terminal boundary of this hypothetical activating domain, we constructed deletion mutants progressing in an amino- to carboxy-terminal direction, starting at amino acid 412. The two mutants that were evaluated maintained a fixed amino-terminal

boundary at residue 412. One mutant, termed Δ NC413-429, lacked 17 amino acids, whereas a second mutant, termed Δ NC413-443, was missing 31 amino acids. Both mutants were tested *in vivo* for *trans*-induction activity. As shown in Figure 3a, Δ NC413-429 retained full *trans*-induction activity, and Δ NC413-443 retained no activity. These differences are probably not related to differences in either the synthesis or stability of the polypeptide products encoded by the mutated templates. The 'western blot' presented in Figure 3b shows that both of these intragenic deletion mutants specified the synthesis of stable, immunoreactive VP16 protein (see Fig. 3).

As summarized in Figure 4, deletion studies of VP16 have demonstrated the importance of a 61-amino acid segment located at the carboxyl terminus of the protein. When evaluated most literally, our results suggest that the single most crucial aspect of this segment is located between residues 429 and 456. That is, deletions in the carboxy-to-amino direction beyond residue 456 eliminated *trans*-activation potential completely; similarly, deletions in the amino-to-carboxy direction beyond residue 429 were also fully debilitating. This 26-amino acid segment contains 8 amino acids bearing acidic side chains (all aspartate) and only 1 basic amino acid (histidine). One other region appeared to contribute to *trans*-activating potential. A substantial drop in activity was observed upon deletion of amino acids between the endpoints of Δ C488-490 and Δ C469-490 (see Fig. 2). This 19-amino acid segment contains seven acidic amino acids (four aspartate and three glutamate) and again only one histidine. Finally, it should be noted that other than the most terminal 3 amino acids, the only segment within the carboxy-terminal 61 amino acids that could be deleted without noticeable phenotypic effect was the 13-amino acid segment between the deletion endpoints of Δ C469-490 and Δ C456-490. This region has only one acidic amino acid and one histidine. In summary, these data highlight the importance of acidic segments at the carboxyl terminus of VP16 in the protein's ability to *trans*-activate IE gene expression.

Evidence of a specificity domain in VP16

The results presented thus far allow the tentative identification of a transcriptional activating domain at the carboxyl terminus of VP16. If this acidic domain is indeed related to the activating domains of the prototypical gene regulatory proteins of yeast, one might expect VP16 to maintain a specificity domain, i.e., a domain that tailors its activation domain for HSV-1 IE genes. In the case of the yeast activator proteins, specificity is achieved via domains of the proteins that facilitate sequence-specific interaction with DNA (Giniger et al. 1985; Hope and Struhl 1986). In contrast, purified VP16 does not bind double-stranded DNA (Marsden et al. 1987). To search for additional activities in the VP16 protein, with the goal of resolving its activation specificity, we tested whether nonactivating deletions such as Δ C439-490 might act in a dominant manner to inhibit *trans*-activation by the native protein.

Cultured mouse L cells were transfected with the indicator plasmid (pICP4*tk*), the internal control plasmid (pMSV*tk*), and varying amounts of Δ C439-490. This derivative of the parental VP16 template encodes a truncated polypeptide that is incapable of *trans*-activating

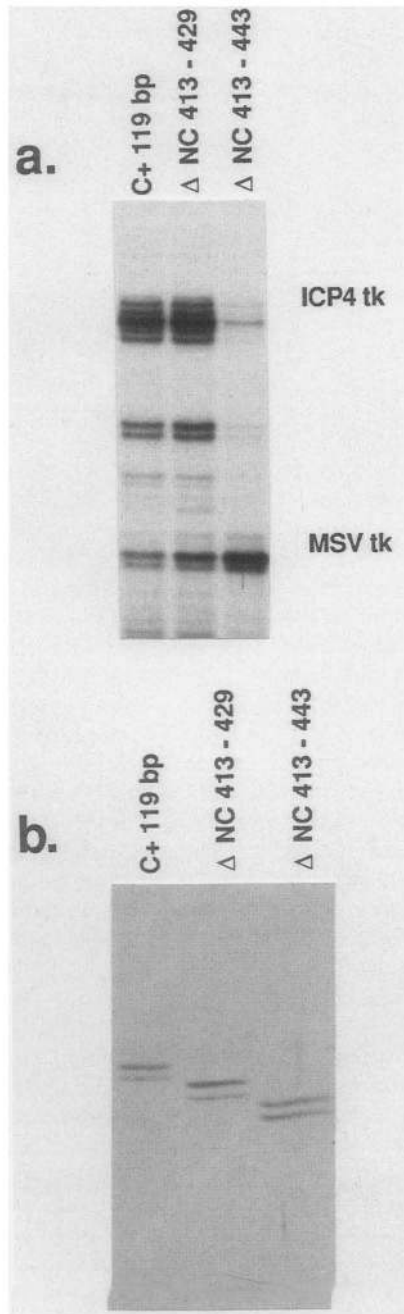


Figure 3. Effects of internal deletions near the carboxyl terminus of VP16 upon *trans*-induction of IE gene expression. (a) *Trans*-induction by internal deletion mutants Δ NC413-429 and Δ NC413-443 was tested, as described in the legend to Figure 2. The positions of primer extension products from ICP4/*tk* and MSV/*tk* transcripts are indicated. (b) Western blot, showing VP16 polypeptides produced from mutants Δ NC413-429 and Δ NC413-443, was performed as described in the legend to Figure 2.

pICP4*tk* (see Fig. 2). Two days later, the transfected cells were superinfected with HSV-1, as a means of introducing native, virus-encoded VP16. RNA was then purified from each culture plate and assayed by primer extension for expression from pICP4*tk* and pMSV*tk* (Fig. 5). As the amount of Δ C439-490 was increased, expression from pICP4*tk* decreased. Indeed, VP16-mediated *trans*-induction was eliminated when as little as 1 μ g of Δ C439-490 was included in the transfection mix. Furthermore, the inhibitory effects of Δ C439-490 were specific. Inclusion of as much as 3 μ g of Δ C439-490 did not cause any change in the expression of the internal reference plasmid pMSV*tk*.

The results outlined in the preceding paragraph raised the possibility that some portion of the polypeptide encoded by Δ C439-490 caused a specific inhibition of VP16-mediated *trans*-induction. To investigate this phenomenon in more detail, we constructed additional deletion mutants of VP16 as a means of defining the inhibitory domain. Our strategy was to begin with a fully inhibitory molecule (lacking the carboxy-terminal 78 amino acids of VP16), pare it down by *in vitro* mutagenesis, and test the ability of mutated variants to inhibit the activity of the native inducer. Deletion of the starting template (Δ C413-490) was initiated from two locations. In one case, additional carboxy-terminal codons were removed (termed Δ C' mutants). Although 12 such mutants were constructed to evenly cover 30 kD of the protein, only the least truncated mutants proved to be useful. Δ C'393-490 inhibited the *trans*-activating potential of native VP16 to an intermediate degree, whereas Δ C'380-490, Δ C'363-490, and all other mutants failed to interfere (Fig. 6a). Western blot analysis of the products of the mutagenized templates (Fig. 6b) revealed that only the starting plasmid (Δ C413-490) and its least truncated variant (Δ C'392-490) produced immunoreactive protein. Apparently, truncation beyond codon 392 leads either to destabilization of VP16 or to loss of an epitope necessary for recognition by our anti-VP16 serum (see subsequent results).

A second series of deletions was prepared from Δ C413-490 starting near the amino terminus of the VP16 ORF (see Fig. 1). In this series, termed Δ N'C, each mutant retained a fixed boundary four codons downstream from the first methionine codon of the ORF and a variable boundary extending toward the carboxyl terminus of the protein. Twelve mutants were prepared to evenly span \sim 30 kD of the protein. Again, only the least deleted mutants provided useful information (Fig. 6c). Δ N'C5-41, which encoded a protein lacking 36 amino acids near the amino terminus (as well as to the 78 carboxy-terminal amino acids), interfered potently with VP16 action. Δ N'C5-56, which lacks 51 codons, showed an intermediate capacity to interfere. Finally, Δ N'C5-74, and all additional members of the Δ N'C deletion series, were unable to interfere with VP16-mediated *trans*-induction.

Unfortunately, the polyclonal antibody reagent that we raised in rabbits against purified VP16 protein was not useful for analyzing Δ N'C mutants. Although raised

		AA ^S DELETED	INDUCTION ACTIVITY
Δ C 413 - 490		78	-
Δ C 439 - 490		52	-
Δ C 456 - 490		35	+/-
Δ C 469 - 490		22	+/-
Δ C 488 - 490		3	+
Δ C 490		1	+
<p>H₂N COOH</p>			
Δ NC 413 - 429		18	+
Δ NC 413 - 443		32	-

Figure 4. Summary of effects of carboxy-terminal deletions of VP16 upon *trans*-induction of IE gene expression. (Left) Deletion mutants tested, next to schematic diagrams representing VP16-coding sequences retained by each mutant. (Right) The number of amino acids deleted and the relative activity in transient *trans*-induction assays.

against an intact VP16 polypeptide, this antiserum is incapable of recognizing mutated forms of the protein that lack the amino-terminal 23 amino acids (S. Triezenberg, unpubl.). The fact that all of the ΔN'C mutants lack 19 of these 23 amino acids may account for the failure of the antibody to recognize the products of mutants that retain interference activity (ΔN'C5-41 and, to a lesser extent, ΔN'C5-56). Whether stable polypeptides are produced by the ΔN'C mutants that fail to function in the interference assay remains unresolved.

The deletion studies described in the preceding paragraphs indicated that an extended region of the protein must remain intact in order to support the interference phenomenon. Near the amino terminus, a boundary critical to interference occurred between codons 56 and 74. Near the carboxyl terminus, a boundary was observed between codons 380 and 393. If these assignments are valid, one might predict that they would hold true in a positive assay of VP16 function. That is, if each of the ΔC' and ΔN'C mutants was reattached to the acidic activating domain, the fully interfering mutant (ΔN'C5-41) should activate fully. Likewise, the mutants that interfered to an intermediate degree (ΔN'C5-56 and ΔC'393-490) should activate to an intermediate level, and those that fail to interfere should fail to activate. Such predictions were fulfilled by the data shown in Figure 7. When reattached to the acidic tail, ΔN'C5-41

activated potently, ΔN'C5-56 and ΔC'393-490 activated to an intermediate degree, and all other mutants failed to activate IE gene transcription. These results, which are summarized schematically in Figure 8, lend support to the legitimacy of the dominant interference assay.

Discussion

We have analyzed deletion mutants of the HSV-1 transcriptional activator protein VP16, using a transient co-transfection assay. Our results provide indirect evidence for two distinguishable domains of VP16 that are critical for its *trans*-inducing function. One domain is located within the carboxy-terminal 78 amino acids of the protein. When this region is removed, VP16 loses its ability to *trans*-activate IE gene expression. Moreover, deletion of this region of the protein results in a dominant interfering activity. We believe that this interfering activity reveals a second functional domain of VP16 and speculate that this second domain imparts IE gene specificity.

Our interpretations are influenced, to a large measure, by recent work that has focused on two yeast activator proteins (Hope and Struhl 1986; Ma and Ptashne 1987). Such efforts have shown that GCN4 and GAL4, which regulate amino acid biosynthesis and galactose metabolism respectively, in *Saccharomyces cerevisiae*, consist of at least two functional domains. Each of these pro-

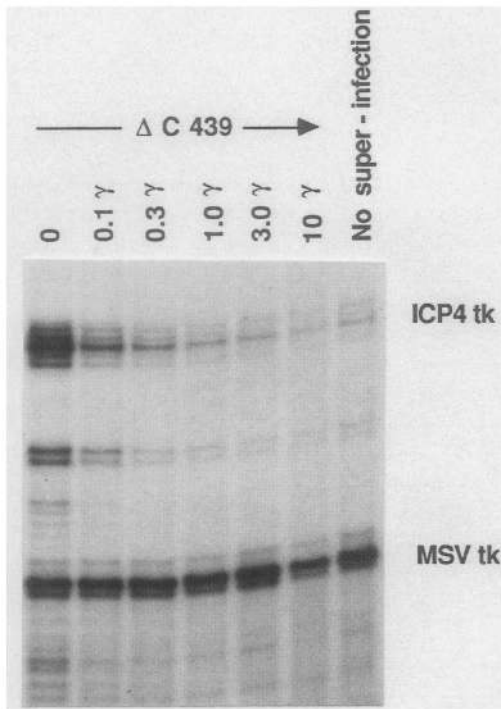


Figure 5. Induction of IE gene expression by HSV-1 infection is inhibited by a nonactivating deletion mutant of VP16. Mouse L cells were transfected with the signal plasmid pICP4tk, the control plasmid pMSVtk, and varying amounts of the nonactivating deletion mutant Δ C439-490 (as indicated above each lane). A fourth plasmid, pMSVcat (Triezenberg et al. 1988), was also included to equalize the amount of DNA in each transfection. Two days after transfection, cells were superinfected with HSV-1 to introduce wild-type VP16 protein. One sample, which received 10 μ g of Δ C439-490 (right lane), was not superinfected to show basal levels of expression from pICP4tk and pMSVtk. Expression was measured by primer extension as described in Figure 2.

teins has a DNA-binding domain that tailors its specificity for the appropriate target genes, as well as an activating domain that plays an ill-defined role in the process of transcriptional induction. Although the activating domains of GCN4 and GAL4 show no overt similarity in amino acid sequence, they are both highly negatively charged. The importance of acidity to the function of the GAL4 analog is supported by the elegant genetic experiments of Gill and Ptashne (1987). Amino acid substitutions that improve the activating properties of GAL4 invariably create a greater net negative charge in its activating domain, whereas those that block activation reduce net negative charge.

We have presented evidence that VP16 also uses an acidic activating domain to induce gene expression. The density of amino acids bearing acidic side chains, relative to basic residues, is unusually high in the putative activating domain of VP16. We speculate that this acidity imparts potency to the activating function of VP16. Recall that expression of VP16 in transfected cells, while activating IE gene expression, caused a marked reduction in transcription from the cotrans-

fected pMSVtk template. This reduction in pMSVtk expression is neutralized in a manner that correlates with the stepwise removal of the VP16 acid tail (see Fig. 2). We predict that the reduction is related to the ability of VP16 to saturate the host-cell transcription apparatus under our assay conditions, committing all available capacity for gene expression to IE enhancers.

Aside from generalized acidity, the sequence of amino acids within the carboxy-terminal tail of VP16 is not notably similar to the sequences located within the activating domains GCN4 or GAL4. In terms of function, however, the viral and yeast counterparts do share one additional similarity. When acidic amino acids are removed by stepwise truncation, activation levels fall in a graded manner. Apparently, each of these activating domains contains redundant information.

If we are correct in assuming that VP16 uses an activating domain functionally analogous to the yeast prototype, the protein should also have a means of specifying direct interaction with its target genes. The two yeast proteins achieve target gene selectivity via sequence-specific DNA-binding domains (Giniger et al. 1985; Hope and Struhl 1986). In this regard, VP16 is clearly different. Purified VP16 is not a DNA-binding protein, regardless of whether it is assayed for interaction with nonspecific DNA sequences (Marsden et al. 1987) or IE *cis*-regulatory sequences (S. Triezenberg, unpubl.).

As outlined in the accompanying paper (Triezenberg et al. 1988), uninfected mammalian cells contain two different DNA-binding proteins that interact specifically with the two IE *cis*-regulatory sequences that specify response to VP16 (TAATGARAT and GCGGAA). We predict that VP16 achieves IE gene specificity via protein:protein interactions with one or both of these cellular factors. Although we have not yet tested this hypothesis directly, we believe that it offers the most reasonable explanation for the dominant interfering activity of the tail-truncated form of VP16. The tail-deleted molecule can completely inhibit the activity of native VP16, and this inhibition is specific to the IE regulatory system. That is, the tail-deleted form of VP16 does not interfere with transcription from other enhancers. Our interpretation predicts that truncated VP16 molecules retain the ability to interact with the cellular factors that bind IE *cis*-regulatory DNA sequences. By so doing, truncated molecules competitively inhibit interaction with native VP16.

Although our genetic assay does not identify the level at which tail-truncated molecules interfere, an experiment not shown here argues against the obvious possibility that tail-deleted VP16 simply blocks superinfecting HSV-1 (which provides native VP16 in our interference assay). That is, the acid tail-deleted molecule is also inhibitory in a standard cotransfection assay that includes a plasmid encoding native VP16 (S. Triezenberg and S. McKnight, unpubl.).

It is perhaps instructive to compare the dominant interfering activity of tail-deleted VP16 with the two products of the bovine papilloma virus (BPV) E2 ORF.

Howley and colleagues have shown that the product of the E2 ORF can either activate or repress the use of a BPV enhancer, depending on the presence or absence of an amino-terminal segment (Lambert et al. 1987). A truncated molecule bearing only the carboxy-terminal segment of the ORF represses enhancer function. The carboxyl terminal segment of the E2 ORF represents the DNA-binding domain of the protein. When this domain

is produced in the absence of the amino terminus of the protein, the molecule adopts the properties of a dominant repressor. We interpret the BPV E2 and HSV-1 VP16 phenomena similarly, despite the fact that the two proteins achieve specificity by different molecular mechanisms.

In closing, we make note of coherence between these interpretations and recent work from the laboratories of

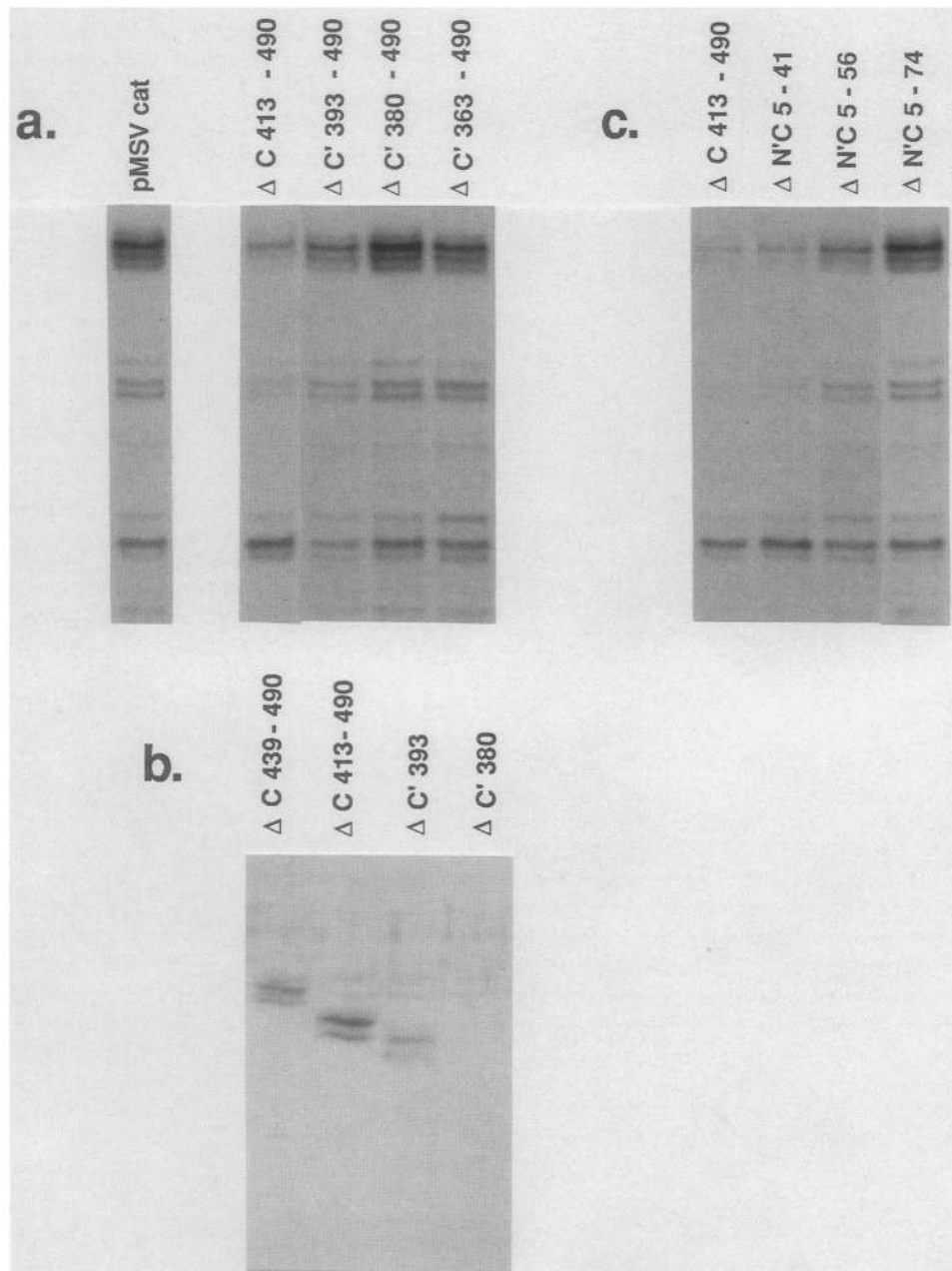


Figure 6. Effects of nonactivating VP16 deletion mutants upon induction by HSV-1 infection. (a,c) Mouse L cells were transfected with 1 μ g of both pICP4*tk* and pMSV*tk*, and 3 μ g of the VP16 deletion mutant indicated above each lane. Two days later, cells were superinfected with HSV-1. RNAs were isolated and assayed by primer extension as described in Figure 2. Carboxy-terminal deletions (Δ C') are shown in panel a; internal deletions near the amino terminus (Δ N'C') are shown in panel c. The left lane of panel a shows that the addition of 3 μ g of pMSVcat does not interfere with VP16-mediated *trans*-induction. (b) Western blot showing VP16 polypeptides produced in cells transfected with carboxy-terminal deletion mutants. The experiment was performed as described in Figure 2b. Similar blots using Δ N'C' series showed no immunoreactive material (data not shown).

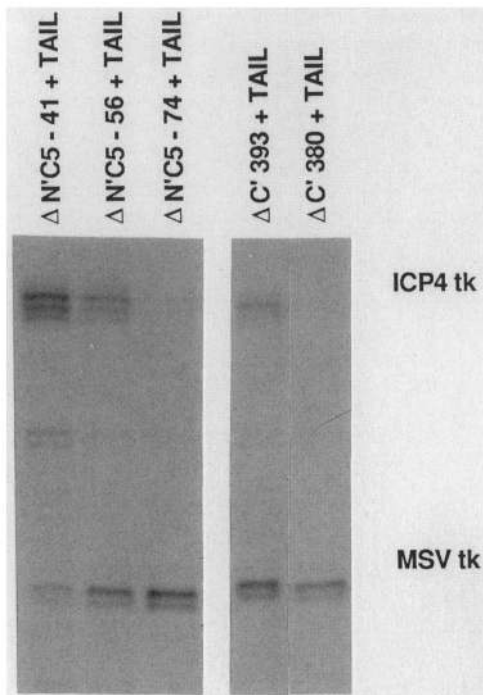


Figure 7. Effects of internal deletions of VP16-coding sequences upon induction of IE gene expression. Deletion mutants tested in the inhibition assay (Fig. 6) were reattached to the carboxy-terminal activating domain (amino acids 413–490). The reconstructed plasmids were transfected into L cells along with pICP4tk and pMSVtk, as described in Figure 2. The autoradiogram shows primer extension products corresponding to ICP4/tk and MSV/tk transcripts, as indicated on the right.

Chris Preston and Bernard Roizman (McKnight et al. 1987; Preston et al. 1988). Using gel mobility shift assays, these investigators have provided evidence for the existence of a ternary complex among VP16, IE *cis*-regulatory DNA sequences, and HeLa cell DNA-binding proteins. If our interpretations and predictions are generally correct, they indicate that acidic activating domains may represent an evolutionarily conserved component of the eukaryotic transcription apparatus. They further offer the unanticipated prediction that activator proteins may achieve target gene specificity via protein : protein linkage to transcriptional regulatory elements.

Materials and methods

Plasmid construction and mutagenesis

The parental plasmid for deletion mutagenesis and expression of VP16 in mammalian cells was termed pMSVP16 Δ 1D3. This plasmid included a 2.5-kbp VP16 gene fragment comprising 76 bp of 5' untranslated sequence, 1470 bp spanning the VP16 ORF, and ~950 bp of 3' flanking sequence. This fragment was derived from a clone of the *Eco*RI fragment I of HSV-1 (KOS) provided by R. Sandri-Goldin (Goldin et al. 1981). Expression of the cloned VP16 gene was driven by an adjacent fragment containing the LTR of the MSV. The 430-bp LTR fragment, provided by B. Graves (Graves et al. 1985), included a transcriptional enhancer, promoter, mRNA cap site, and 27 bp of 5' untranslated sequence.

Several sets of deletion mutations were introduced into the cloned VP16 gene. In one set, sequences encoding the carboxy-terminal domain of VP16 were progressively deleted. The parental plasmid pMSVP16 Δ 1D3 was prepared for deletion by di-

		AA ^S DELETED	INTERFERENCE ACTIVITY	INDUCTION ACTIVITY
Δ N'C 5 - 41		38	+	+
Δ N'C 5 - 56		53	+/-	+/-
Δ N'C 5 - 74		71	-	-
H ₂ N COOH				
Δ C' 393 - 490		21	+/-	+/-
Δ C' 380 - 490		34	-	-
Δ C' 363 - 490		51	-	-

Figure 8. Summary of effects of internal deletions on dominant inhibition of VP16 *trans*-induction. (Left) Deletion mutants tested, next to schematic diagrams representing VP16-coding sequences retained by each mutant. (Right) The number of amino acids deleted and relative interfering activity. Each mutant was also tested for *trans*-induction after the addition of the carboxy-terminal 80 amino acids of VP16 (see Figure 7).

gestion with *Pst*I at the 3' end of the VP16 gene fragment. The 3'-overhanging end was trimmed by digestion with T4 DNA polymerase in the absence of nucleotide triphosphates. Deletions were created by sequential digestion with exonuclease III and nuclease S1 (Sakonju et al. 1980). *Bam*HI linkers were then ligated to the ends of deleted molecules. Digestion with *Bam*HI and an appropriate second restriction enzyme released fragments that were subsequently fractionated by agarose gel electrophoresis. The recipient vector for the deleted fragments contained translation stop codons in all three reading frames, followed by polyadenylation signals. The termination codons were encoded by a synthetic oligonucleotide that was positioned adjacent to the cloning site for deletion endpoints, so that either one, three, or four additional amino acids (depending on the reading frame used) would be added to deleted versions of VP16. The polyadenylation signals resided in a 390-bp fragment from the 3'-flanking sequences of the HSV-1 *tk* gene (McKnight 1980). This fragment was positioned next to the terminating oligonucleotide. The purified restriction fragments bearing deletions of the VP16 gene were cloned into complementary sites in the recipient vector. Deletion endpoints were determined by DNA sequencing (Maxam and Gilbert 1980). Plasmids of this series were designated ΔC , with mutant nomenclature corresponding to the amino acids removed from the VP16 ORF (see Fig. 1).

A subset of the ΔC plasmids, termed $\delta C'$ + tail, was subsequently modified to restore the last 80 amino acids of the VP16 ORF, thereby generating internal deletions of VP16. To maintain continuous ORFs across deletion endpoints into the carboxyl terminal coding sequences, three versions of the fragment bearing the carboxy-terminal sequences were prepared. A deletion plasmid with an endpoint located 122 bp to the 3' side of the VP16 ORF ($\Delta C + 112$ bp) was used as the source of this fragment. This plasmid was digested with *Sal*I, cleaving at the eightieth codon from the carboxy-terminus. The 5'-extended *Sal*I terminus was partially filled in with zero, one, or two nucleotides, using T4 DNA polymerase and appropriate deoxyribonucleotide triphosphates. The ends were rendered blunt by digestion with mung bean nuclease, and *Bgl*II linkers were ligated in place. Plasmids were then circularized and transfected into *Escherichia coli*. The identities of the three different reading frame versions of this construction were confirmed by DNA sequencing. The three versions of the carboxy-terminal fragment were excised from the respective plasmids and joined to deletion endpoints of selected $\Delta C'$ plasmids, using the cohesive *Bam*HI restriction sites, so that continuous reading frames were maintained.

Deletion mutations were also introduced into the amino-terminal coding sequence of the VP16 ORF. All of these deletions retained the 5'-untranslated region and the first four codons of the VP16 ORF. To construct this series of mutants, the parental plasmid pMSVP16 $\Delta 1D3$ was linearized at a second *Sal*I restriction site near the 5' end of the VP16 ORF and then digested sequentially with exonuclease III and nuclease S1. Synthetic *Xba*I linkers were ligated to the ends of deleted molecules. After digestion with appropriate restriction enzymes, deleted molecules were purified by agarose gel electrophoresis. Three recipient vectors were constructed to ensure continuous reading frames for the amino-terminal internal deletions. The parental plasmid pMSVP16 $\Delta 1D3$ was cleaved with *Sal*I at the fifth codon of the VP16 ORF. The 5' extension was partially filled in and then rendered blunt, as described above. *Xba*I linkers were ligated to the blunt ends, and molecules were recircularized. The identities of recipient vectors carrying *Xba*I linkers in each reading frame were confirmed by DNA sequencing. All of the VP16 deletion fragments were cloned initially into one of the

three vectors. After deletion endpoints were identified by DNA sequencing, VP16 deletion fragments were recloned into the appropriate vector to ensure reading frame continuity. This series of mutants was designated $\Delta N'C$ + tail to indicate amino-terminal deletions with an intact carboxyl terminus.

Some of the amino-terminal deletions were subsequently modified to remove 52 codons from the carboxy-terminal coding sequences. Selected $\Delta N'C$ + tail plasmids were digested with *Eco*RI and *Sal*I to release fragments spanning the MSV LTR and most of the VP16 ORF. These fragments were recloned into a vector prepared from a ΔC deletion mutant lacking the final 52 codons of the VP16 ORF. Translation termination and polyadenylation signals were present, as described for the ΔC plasmids. This series of plasmids is designated $\Delta N'C$, indicating amino terminal deletions lacking the final carboxy-terminal codons.

The final sets of mutant plasmids carried internal deletions within the final 80 codons of the VP16 ORF. Plasmid $\Delta C + 112$ bp, which retained an intact VP16 ORF, was cleaved at the eightieth codon from the carboxyl terminus, using *Sal*I. After digestion with exonuclease III and nuclease S1, *Sal*I linkers were added to the deletion endpoints. Fragments bearing deletions of the carboxy-terminal coding region were purified and recloned into a vector prepared from the parental plasmid. Deletion endpoints were defined by DNA sequencing; only those plasmids with continuous reading frames were selected for study.

Plasmid pICP4*tk*, used as an indicator of IE gene expression, contains regulatory sequences from the HSV-1 ICP4 gene (nucleotides -396 to +30, relative to the mRNA cap site) fused to the body of the HSV-1 *tk* gene. Details of the construction of this plasmid are described elsewhere (Triezenberg et al. 1988). A plasmid containing the MSV LTR fused to *tk*-coding sequences (pMSV*tk*) was a gift from B. Graves (Graves et al. 1985). Plasmid pMSVcat, in which the MSV LTR is fused upstream from the bacterial chloramphenicol acetyltransferase gene, was constructed by inserting the MSV LTR into the *Hind*III site of pSV0cat (Gorman et al. 1982).

Transient expression assay

Mouse L cells (*tk*⁻, *aprt*⁻) were provided by B. Sollner-Webb. One day prior to transfection, 8×10^5 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 μ g of the ICP4-*tk* fusion plasmid (pSJT703), 1 μ g of internal control plasmid (pMSV*tk*), and 1 μ g of *trans*-inducing plasmid (pMSVP16 or a deletion derivative thereof). Control plates did not receive inducing plasmid but were mock infected or infected with HSV-1 $\Delta 35$ virus (Halpern and Smiley 1984) 40 hr after transfection. Infections were carried out at a multiplicity of 5–10 pfu per cell in the presence of 100 μ g/ml cycloheximide. Two hours postinfection or 42 hr after transfection, total RNA was harvested by the proteinase K/DNase I method (Eisenberg et al. 1985). Primer extension assays were performed as described (Eisenberg et al. 1985) to measure expression from the ICP4*tk* and MSV*tk* templates. The primer used was a synthetic oligonucleotide that hybridized to sequences between +50 and +75, relative to the *tk* mRNA cap site. The major extension products observed from transcripts of pSJT703 and pMSV*tk* were 81 bases and 55 bases, respectively.

A transient expression interference assay was performed using deletion mutants lacking 52 or more amino acids from

the carboxyl terminus of VP16. These mutants are incapable of *trans*-activation (see Results). Transfections into L cells were carried out as described above, using pSJT703, pMSVtk, and a given deletion mutant of VP16. Forty hours after transfection, cells were infected with HSV-1 Δ 35, as described above. RNA samples were harvested 2 hr after infection and analyzed by primer extension.

To determine whether deletion mutant plasmids produced stable VP16 polypeptides, transfected cell proteins were analyzed on Western blots. In each case, a 60-mm plate of L cells was transfected with 1 μ g of a given plasmid. Forty hours later, cells were lysed in 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 1% SDS. The lysate was sonicated briefly and precipitated with four volumes of acetone. After centrifugation at 3000g for 15 min, pellets were resuspended in 200 μ l of sample buffer, and incubated at 65°C for 15 min and then at 100°C for 3 min. One fourth of each sample (50 μ l) was loaded onto an 8% polyacrylamide-SDS gel (Laemmli 1970). Following electrophoresis, proteins were transferred to nitrocellulose filters (Towbin et al. 1979) and probed, using polyclonal rabbit serum directed against gel-purified VP16 (S. Triezenberg, unpubl.). Antibody-antigen complexes were detected, using biotinylated goat anti-rabbit antibodies and an avidin : biotin : horseradish peroxidase conjugate from Vector Labs. These materials were used as specified by the supplier, except that 20% horse serum was included in all blocking and antibody solutions.

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