MOLECULAR AND CELLULAR BIOLOGY, May 1994, p. 3484–3493
0270-7306/94/$04.00+0

Vol. 14, No. 5

# Transcriptional Activation by Herpes Simplex Virus Type 1 VP16 In Vitro and Its Inhibition by Oligopeptides

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Received 7 December 1993/Accepted 31 January 1994

**VP16 is a herpes simplex virus (HSV)-encoded transcriptional activator protein that is essential for efficient viral replication and as such may be a target for novel therapeutic agents directed against viral gene expression. We have reconstituted transcriptional activation by VP16 in an in vitro system that is dependent on DNA sequences from HSV immediate-early gene promoters and on protein-protein interactions between VP16 and Oct-1 that are required for VP16 activation in vivo. Activation increased synergistically with the number of TAATGARAT elements (the *cis*-acting element for VP16 activation in vivo) upstream of the core promoter, and mutations of this element that reduce Oct-1 or VP16 DNA binding reduced transactivation in vitro. A VP16 insertion mutant unable to interact with Oct-1 was inactive, but, surprisingly, a deletion mutant lacking the activation domain was ~65% as active as the full-length protein. The activation domains of Oct-1 were necessary for activation in reactions containing the VP16 deletion mutant, and they contributed significantly to activation by full-length VP16. Addition of a GA-rich element present in many HSV immediate-early gene enhancers synergistically stimulated VP16-activated transcription. Finally, oligopeptides that are derived from a region of VP16 thought to contact a cellular factor known as HCF (host cell factor) and that inhibit efficient VP16 binding to the TAATGARAT element also specifically inhibited VP16-activated, but not basal, transcription. Amino acid substitutions in one of these peptides identified three residues that are absolutely required for inhibition and presumably for interaction of VP16 with HCF.**

Herpes simplex virus type 1 (HSV-1) and HSV-2 package a protein, VP16, also known as Vmw65 or α-TIF, that activates transcription of the viral immediate-early (IE) genes and is essential for efficient virus replication (1, 2, 7, 13, 24, 61). Two features of this 65-kDa protein have made it the object of much interest in the study of eukaryotic gene regulation. Its highly acidic C-terminal activation domain, which is absolutely required for IE gene activation in vivo (22, 59, 62), is one of the most potent known and has been fused to heterologous DNA binding domains to create novel transactivators for both in vivo and in vitro studies (8, 10, 50). Investigations of wild-type and mutant forms of this domain have begun to shed light on its key structural features (14, 17, 22, 49, 59, 62). Despite such intensive study, the mechanism of action of this prototypic activation domain—whether it acts directly on a basal transcription factor (27, 39, 40, 57), indirectly on basal factors via an intermediary molecule (4, 5, 18, 30, 63), or via displacement of histones (15, 36)—remains hotly debated.

The other intriguing feature of VP16 is its mechanism of DNA binding. Unlike most other well-characterized transactivators, VP16 does not bind DNA efficiently. Instead, considerable evidence suggests that VP16 interacts with two cellular proteins, Oct-1 and HCF (host cell factor; also known as C1 factor, VCAF, or CFF), which direct its binding to a sequence element, the TAATGARAT motif, found in all HSV-1 IE enhancers (20, 23, 29, 32, 33, 46, 56). Oct-1, which stimulates the transcription of a number of cellular genes (52), binds to a sequence known as the octamer motif (consensus ATGC AAAT) that overlaps with the 5′ (TAAT) portion of the TAATGARAT element. Although Oct-1 and VP16 can form a low-affinity ternary complex with TAATGARAT (32, 55, 56), HCF stimulates complex formation by several orders of magnitude (32). HCF has no apparent sequence-specific DNA binding activity, but it does interact with VP16 in solution (32, 34, 55, 66). The most highly purified preparations of HCF contain multiple related polypeptides with molecular weights of 100,000 to 135,000 that can interact independently with VP16 (34). A recent report on the cloning of a cDNA encoding HCF (64) indicates that the primary translation product is a single 2,035-amino-acid polypeptide which is rapidly processed to a family of polypeptides similar to that described by Kristie et al. (34). In the context of the VP16–HCF–Oct-1–DNA complex, which can be visualized in electrophoretic mobility shift assays and is referred to as the C1 complex, VP16 protects the GARAT portion of the element (33).

DNA binding and transfection studies such as those mentioned above have revealed much about the DNA and protein sequence requirements for C1 complex formation and IE gene activation. Although there have been numerous reports of transactivation in vitro by fusion proteins containing the VP16 activation domain fused to heterologous DNA binding domains, there have been no reports of activation by native VP16 in vitro. Such a system would provide a powerful approach for simultaneously studying the requirements for formation of the C1 complex and the effect of this complex on the structure and function of the basal promoter and associated factors. In this report, we describe an in vitro transcriptional activation system for VP16 that displays key features known to be essential for IE gene activation during HSV-1 infection. We also show that peptides derived from VP16 that inhibit C1 complex formation specifically inhibit VP16-activated transcription. From studying the effects of amino acid substitutions in one of these peptides, which are derived from a region of VP16 that likely contacts HCF, on inhibitory activity, we have identified three

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amino acids in VP16 that probably form a critical contact interface with HCF.

## MATERIALS AND METHODS

**DNA templates.** The TATA box of pGCG17 (65) was excised by digestion with *Eco*RI and *Sst*I and replaced with an oligonucleotide containing the TATA element and flanking sequences from the adenovirus major late promoter and *Eco*RI and *Sst*I sites (italicized) (*GAATTC*CGGGGGCTA TAAAAGGGGGTG*GAGCTC*) to generate pGCAdML. An oligonucleotide containing the HSV-1 ICP0 TAATGARAT element and surrounding sequences flanked by *Bam*HI sites (GGATCCCGTGCATGCTAATGATATTCTTTGGGATCC) was inserted into the *Bam*HI site of pGCAdML to generate the template with one TAATGARAT, designated pG C($O^+G^+$). The TAAT mutant oligonucleotide in pGC ($O^-G^+$) contained the sequence GGATCCCGTGCATG CGACTGATATTCTTTGGGATCC (mutant bases are underlined). The GARAT mutant oligonucleotide in pGC ($O^+G^-$) had the sequence GAGGATCCCGTGCATGCTA ATGCTGTTCTTTGGGATCCGG. To generate plasmids with three TAATGARAT motifs, an oligonucleotide having two TAATGARAT elements with an intervening *Bgl*II site (italicized), or mutants described above (sequence CTGCA GCGTGCATGCTAATGATATTCTTTG*AGATCT*GGGGTT CCCGTGCATGCTAATGATATTCTTTGTCTAGA), was inserted between the *Pst*I and *Xba*I sites of pGC($O^+G^+$), pGC($O^-G^+$), and pGC($O^+G^-$). The resultant plasmids were designated pGC($O^+G^+$)$_3$, pGC($O^-G^+$)$_3$, and pGC($O^+G^-$)$_3$, respectively. To create plasmids with five TAATGARAT elements, an oligonucleotide containing two TAATGARAT elements flanked by *Bam*HI sites was cloned into the *Bgl*II site of the *Pst*I-*Xba*I fragment described above. Plasmids containing the GA-rich motif were constructed by inserting an oligonucleotide containing the GA-rich motif of ICP4 flanked by *Pst*I and *Hin*dIII sites (italicized) (*AGCTT*GCATGCGCGGAACGG AAGCGGAAACCGGATATC*CTGCA*) between those sites in plasmids having either no TAATGARAT (pGCAdML) or three copies of the wild-type or mutant TAATGARAT elements described above. The RNA product of these plasmids was ~400 nucleotides long. pGC340 was constructed by BAL 31 digestion of the G-less cassette of pML($C_2$AT) (51) and religation. The RNA product of this plasmid was ~200 nucleotides long.

**Expression and purification of recombinant proteins.** The coding sequence of full-length VP16 was recovered from plasmid pMSVP16 (14) (generously provided by S. Ludmerer, Merck Research Laboratories) by PCR using a primer containing the 5′ end of the gene and a *Bam*HI site (sequence GGGATCCCCCATGGACCTCTTGGTCGACGA GC; the VP16 initiator methionine codon is underlined), a primer containing the 3′ end of the gene and an *Eco*RI site (sequence GGGAATTCCTACCCACCGTACTCGTCAATT CC; the terminator anticodon is underlined), and *Pfu* polymerase (Stratagene) as instructed by the manufacturer. VP16 without the activation domain (truncated VP16) was amplified by using the same upstream primer and reaction conditions and a primer complementary to a region ending with codon 412 that also contains a stop codon and an *Eco*RI site (sequence GGGAATTCCTACGTCGACAGTCTGCGCGT GTGTCC; the terminator anticodon is underlined). The amplified product was digested with *Bam*HI and *Eco*RI and inserted between those sites in pGEX-3X (Pharmacia). The glutathione *S*-transferase (GST) fusion proteins encoded by these plasmids were expressed in *Escherichia coli* DH5α and purified by glutathione-Sepharose chromatography as instructed by the manufacturer. The affinity-purified fraction was applied to a Mono Q fast protein liquid chromatography resin (Pharmacia), and GST-VP16 was eluted with a linear gradient of 0.1 to 1.0 M KCl in buffer D (16). The resultant proteins were >95% homogeneous, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

The Oct-1 POU domain was amplified from plasmid pBS-Oct-1 (gift of S. Ludmerer, Merck Research Laboratories), using the protocol described above and primers with the sequences GTGGGATCCATATGACACCAAAGCGAATT GATAC and CCGGAATTCCTAGCTTGGTGGGTTGAT TC (the initiator and terminator codons are underlined). Cloning, expression, and purification of the GST–Oct-1 POU fusion protein were done as described for the VP16 fusion proteins.

**Nuclear extract preparation and oligonucleotide affinity chromatography.** Nuclear extracts were prepared by a modification of the procedure of Dignam et al. (16). After dialysis against buffer D (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.9, 4°C], 0.2 mM EDTA, 0.1 M KCl, 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.4 μg of pepstatin A per ml), 2 volumes of saturated $(NH_4)_2SO_4$ was added, and the precipitated protein was pelleted, collected with 1/10 volume buffer D10 (buffer D containing 10% glycerol), and dialyzed against two changes of 50 volumes of buffer D10.

Octamer DNA binding activity was depleted from nuclear extracts essentially as described previously (47). Following incubation with the oligonucleotide resin, the extract, which had been depleted of ~90% of its octamer DNA binding activity, as determined by electrophoretic mobility shift assay (data not shown), was concentrated with saturated $(NH_4)_2SO_4$ and dialyzed as described above. Bound octamer DNA-binding proteins were eluted with buffer D containing 1 M KCl and concentrated by the same procedure as used for nuclear extract. The eluted fraction contained 100% of the octamer DNA binding activity that had been depleted from the extract and, together with the depleted extract, was as competent as undepleted extract for reconstitution of C1 complex formation, as measured by an electrophoretic mobility shift assay (data not shown).

**In vitro transcription.** In vitro transcription reactions were carried out in two steps. A preincubation reaction mixture (16 μl) containing 10 mM HEPES-NaOH (pH 7.9), 0.1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 25 ng of test plasmid containing the indicated HSV regulatory sequences, 100 ng of pGC340, 275 ng of pUC19, 30 to 50 μg (2 μl) of nuclear extract, and the indicated amount of full-length or truncated GST-VP16 protein was incubated at 30°C for 30 min. Then 4 μl of a mixture containing 2 mM ATP, 2 mM CTP, 25 μM UTP, 1.75 mM 3′-*O*-methyl-GTP (all Pharmacia), 10 μCi of [α-$^{32}$P]UTP (ICN), 4 mM creatine phosphate (pH 7.9), 50 mM $MgCl_2$, and 5 U of RNase $T_1$ (Calbiochem) was added, and incubation was continued at 30°C for another 30 min. Reactions were stopped by proteinase K digestion and processed for gel electrophoresis as described elsewhere (65). Labeled RNA bands were visualized and quantitated by using a PhosphorImager and ImageQuant software (Molecular Dynamics).

**Electrophoretic mobility shift assay.** Radioactive DNA probe was prepared by digestion of pGC($O^+G^+$) with *Hin*dIII and *Sma*I and 3′ end labeling of the TAATGARAT-containing fragment with [α-$^{35}$S]dATP and dCTP (New England Nuclear). Twenty-microliter reaction mixtures contained 10 mM HEPES-NaOH (pH 7.9), 0.1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 4%

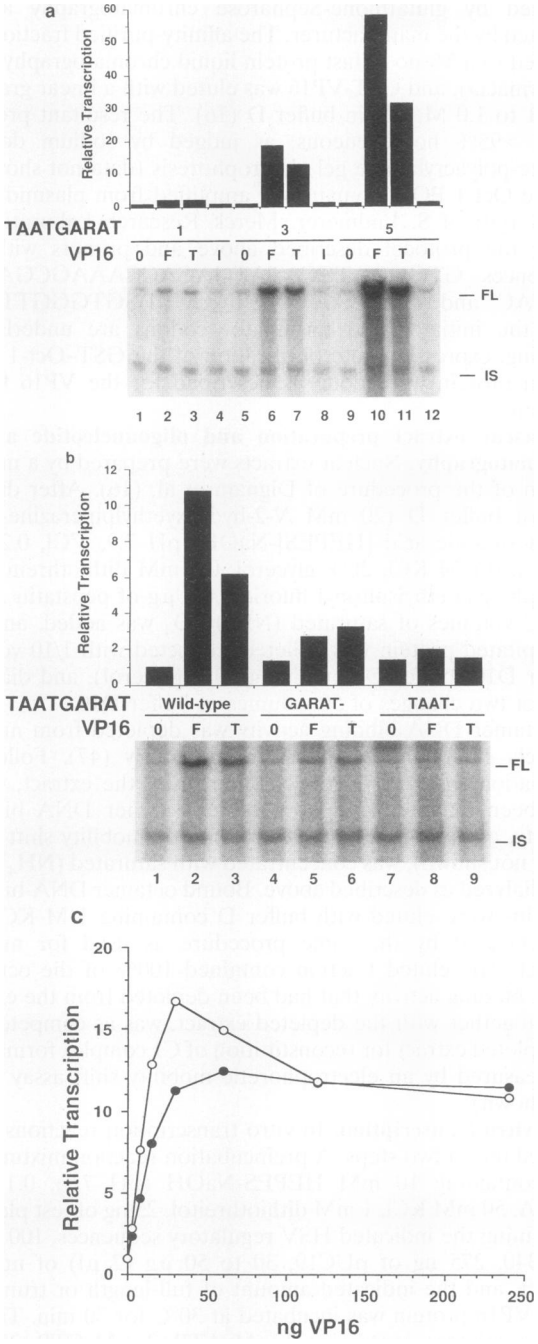



FIG. 1. Transcriptional activation of templates bearing multiple TAATGARAT elements by wild-type and mutant VP16. (a) Activation requires multiple TAATGARAT elements. Reaction mixtures containing template DNA with either one, three, or five TAATGAR AT elements as indicated on the top line and HeLa cell nuclear extract were unsupplemented (0) or supplemented with 30 ng of full-length (F), truncated (T), or *in*14 (I) VP16 that had been expressed in and purified from *E. coli*, and transcription was carried out as described in Materials and Methods. FL indicates the position of the full-length transcript synthesized from templates containing HSV IE promoter sequences, and IS indicates the position of the internal standard transcript synthesized from a template lacking HSV IE sequences. The amount of RNA produced in each reaction, expressed relative to the amount produced in a reaction containing no VP16 and a template with one TAATGARAT and normalized on the basis of the amount of IS RNA, is shown in the bar graph above each lane. (b) Activation requires wild-type octamer and GARAT motifs. Templates having three copies of either a wild-type TAATGARAT element or one with point mutants in the TAAT (TAAT-) or GARAT (GARAT-) portion were added to reaction mixtures containing either no VP16 (0), 30 ng of full-length VP16 (F), or 30 ng of truncated VP16 (T). The bar graph above indicates relative transcription in each lane, where 1 equals the amount of transcript produced in reactions containing three wild-type TAATGARAT elements and lacking VP16. (c) Titration of full-length and truncated VP16. The indicated amounts of either full-length (open symbols) or truncated (closed symbols) VP16 were added to reaction mixtures containing DNA templates with three copies of the wild-type TAATGARAT element. Results were quantitated as described for panel a but are expressed relative to results from reaction mixtures containing a template with three TAATGARAT elements and no VP16.

Ficoll-400, and 10 fmol of DNA probe; 7 μg of nuclear extract, prepared as described above, 60 ng of GST-VP16, and the indicated peptides were added as described in the figure legends. Following a 30-min incubation at 30°C, the reaction mixture was loaded on a 4% acrylamide gel (37.5:1, acrylamide/bisacrylamide) prepared in 0.5× Tris-borate-EDTA and electrophoresed at 8 V/cm for 2 h with 0.5× Tris-borate-EDTA as the electrode buffer. Gels were dried, and radioactive bands were visualized and quantitated with a PhosphorImager as described above.

**Synthetic peptides.** Peptides D31-134 (REHAYSRARTK NNY) scrambled (RSYAHREYNNKTAR), CP192-1 (SEA VMREHAYSRAR), and CP436-3 (EAVMREHAYSR) were synthesized and purified to ≥97% purity by Chiron Mimotopes (Clayton, Victoria, Australia) and supplied as dried powder. They were resuspended with 0.1 M $(NH_4)HCO_3$, lyophilized, and resuspended at a final concentration of 10 mg/ml with 20 mM HEPES-NaOH (pH 7.9) containing 50 mM KCl. Composition and purity were verified by quantitative amino acid analysis.

## RESULTS

**Synergistic transcriptional activation in vitro by VP16 through multiple TAATGARAT elements.** The DNA templates used in these studies consisted of HSV-1 IE regulatory sequences inserted upstream of the adenovirus major late TATA element fused to a G-less cassette (65). Wild-type and mutant VP16 proteins were expressed in *E. coli* as fusions to GST and purified to homogeneity by affinity and ion-exchange chromatography. Because glycerol significantly inhibited C1 complex formation (43b), nuclear extracts prepared by standard methods (16) were concentrated by ammonium sulfate precipitation to allow smaller volumes to be used in the reactions. C1 complex formation was also inhibited by $Mg^{2+}$ and required ~20 min to reach equilibrium under transcription assay conditions (43b), so reactions consisted of two consecutive incubations: preincubation of nuclear extract (the source of basal transcription factors, Oct-1, and C1 factor), VP16, and template DNA to allow C1 complex formation followed by addition of nucleoside triphosphates and $Mg^{2+}$ to initiate transcription. A second DNA template with no HSV IE regulatory sequences and a shortened G-less cassette was included as an internal standard.

Using conditions described above, we assayed transcriptional activation of DNA templates having one, three, or five copies of the HSV-1 ICP0 TAATGARAT (−144 to −170 [41]) by recombinant VP16. In accordance with previous observations that multimerization of transactivator binding sites synergistically enhances transactivation (9, 37), we found

that whereas a single TAATGARAT yielded only a weak response to VP16 (Fig. 1a), insertion of three or five TAATG ARAT elements dramatically increased VP16 responsiveness (Fig. 1a, lanes 5, 6, 9, and 10). Point mutations in the TAAT portion of the TAATGARAT element known to block Oct-1 DNA binding and C1 complex formation (33) completely eliminated activation (Fig. 1b, lanes 7 to 9). A double point mutation in the GARAT motif (33) which reduces C1 complex formation ~5-fold in an electrophoretic mobility shift assay (43a) had the same effect on transactivation (Fig. 1b, lanes 4 to 6), indicating that the affinity of VP16 for this element is directly related to its ability to activate transcription. A VP16 insertion mutant incapable of interacting with Oct-1 or forming the C1 complex (1, 2, 55) did not activate transcription (Fig. 1a, lanes 4, 8, and 12). Surprisingly, VP16 lacking the C-terminal activation domain, which is fully active in C1 complex formation in vitro but is transcriptionally inactive in vivo, activated transcription ~65% as well as full-length VP16 in vitro (Fig. 1a and b). Although the difference in activation between the two proteins was relatively small, it was repeatable with four different preparations of each protein and over the course of more than 80 assays. $His_6$ and maltose-binding protein fusions to full-length and truncated VP16 gave identical results (data not shown), indicating that the GST moiety neither contained a cryptic activation domain nor selectively inhibited the activation domain of VP16.

**High concentrations of full-length, but not truncated, VP16 inhibit activation.** At least two factors may contribute to the modest differential between full-length and truncated VP16 in this in vitro system: weak response to the VP16 activation domain and the presence of a second activation domain in the C1 complex that is active in vitro but not in vivo. Evidence consistent with the former possibility was obtained when full-length and truncated VP16 were titrated into the in vitro reaction. Activation increased linearly for both proteins up to ~30 ng (Fig. 1c), at which point all TAATGARAT sites should be occupied, as judged from gel shift analysis (data not shown). Further addition of truncated VP16 gave no change in the level of transcript produced, but higher concentrations of full-length VP16 inhibited transcription. This autoinhibition may partially attenuate the ability of full-length VP16 to activate transcription relative to truncated VP16.

**Activation domains on Oct-1 contribute to transactivation in vitro.** The unexpected activation by truncated VP16 may be due to the presence of another activation domain in the C1 complex that is more active in our in vitro conditions than in vivo on HSV IE promoters, reminiscent of the case of the cryptic activation domain reported in a truncated version of the yeast GAL4 protein (38). Two candidates for this second activation domain are the N- and C-terminal activation domains of Oct-1 (52). To test this hypothesis, we replaced the endogenous Oct-1 with a truncated protein containing only the POU domain, which is fully competent to reconstitute C1 complex formation but which lacks the N- and C-terminal activation domains (29, 33, 56). Nuclear extracts depleted of >90% of their Oct-1 by DNA affinity chromatography did not support VP16 activation, although basal transcription was unaffected (Fig. 2). Addition of Oct-1 eluted from the affinity matrix had no significant effect on basal transcription, but it quantitatively restored activation by full-length and truncated VP16 to levels observed with the undepleted extract (compare lanes 7 to 9 with lanes 1 to 3). When an equal amount (in terms of C1 complex forming activity) of the Oct-1 POU domain was added instead, weak activation was observed in response to full-length, but not truncated, VP16 (lanes 10 to 12). Moreover, the level of activation by full-length VP16 in the presence

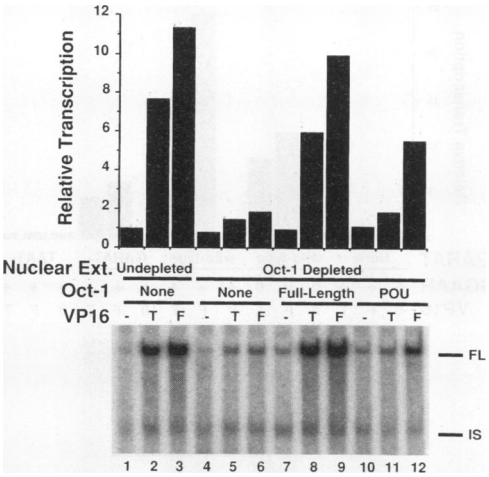



FIG. 2. Activation domains on Oct-1 are required for maximal activation in vitro. Nuclear extract was depleted of octamer DNA binding activity by oligonucleotide affinity chromatography and tested for transcriptional activity in the absence of VP16 (0) or in the presence of 30 ng of full-length (F) or truncated (T) VP16 as indicated (lanes 4 to 6). Activation under these conditions was also tested in the presence of a 1 M KCl eluate of the affinity matrix (designated Oct-1; lanes 7 to 9) or in the presence of the Oct-1 POU domain (designated POU) expressed in and purified from *E. coli* (lanes 10 to 12). Reactions carried out with the starting undepleted extract are shown in lanes 1 to 3. The amount of RNA produced in each reaction, quantitated as described in the legend to Fig. 1 and expressed relative to the amount of transcript in lane 1, is shown at the top. FL and IS are as in Fig. 1.

of Oct-1 POU, while reduced, was equivalent to the difference between the levels of transcription obtained by full-length and truncated VP16 in the presence of endogenous Oct-1 (compare lane 12 with lanes 2 and 3 or lanes 8 and 9). Thus, the transactivation by truncated VP16 appears to be due to activation domains on Oct-1.

**Addition of a GA-rich element found in many HSV IE promoters synergistically enhances VP16 activation through the TAATGARAT element.** In addition to one or more TA ATGARAT motifs, many HSV IE enhancers also contain a GA-rich sequence, the CGGAAR element, that stimulates VP16-activated, but not basal, transcription in vivo (6, 35, 54, 58, 60). As shown in Fig. 3, the CGGAAR element from the HSV-1 ICP4 enhancer increased VP16-activated, but not basal, transcription three- to fourfold, in agreement with in vivo observations (6, 54, 60). The presence of this element did not change the relative response to full-length or truncated VP16. Mutations of the octamer and GARAT motifs had the same effect on this promoter as they did on templates having the TAATGARAT element alone (compare Fig. 1b and 3).

**Oligopeptides derived from VP16 specifically inhibit VP16-activated transcription in vitro.** Recently, O'Hare and colleagues described several VP16 peptides that inhibit C1 complex formation (25, 26). In vitro DNA binding studies with purified components indicate that these peptides interact with HCF and block its ability to facilitate complex formation between Oct-1, VP16, and the TAATGARAT element (25, 26, 43b, 45). If the C1 complex is a necessary intermediate for VP16 activation and if the interaction targeted by the peptide is unique to this complex, then these peptides should inhibit VP16-activated, but not basal, transcription in vitro. As shown in Fig. 4, a 14-mer described previously (25, 26), which we

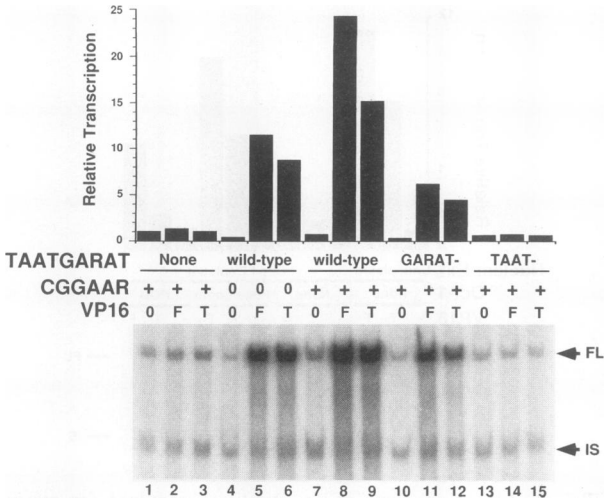



FIG. 3. The GA-rich (CGGAAR) element from the HSV ICP4 promoter augments VP16-mediated transactivation. DNA templates containing either the ICP4 CGGAAR element (lanes 1 to 3), three TAATGARAT elements (lanes 4 to 6), or the CGGAAR element together with three copies of the wild-type (lanes 7 to 9), GARAT point mutant (lanes 10 to 12), or TAAT point mutant (lanes 13 to 15) TAATGARAT element were tested for transcription in the absence of VP16 (0) or in the presence of 30 ng of full-length (F) or truncated (T) VP16. The amount of transcript relative to lane 1 is displayed graphically above each lane. FL and IS are as in Fig. 1.

designate D31-134, selectively inhibited VP16 transactivation. When tested at the same concentrations, a scrambled variant of this sequence did not significantly inhibit transcription (lanes 3 to 5), indicating that primary sequence and not composition per se was important for inhibitory activity. By assaying other peptides derived from the same region of VP16, we identified a 14-mer (CP192-1) and an 11-mer (CP436-3) that overlap with D31-134 but are 100- and 30-fold more active, respectively (Fig. 4). None of the peptides affected basal-level transcription from the internal standard template, nor did they reduce transcription from the TAATGARAT-containing template to below the uninduced levels, indicating that inhibition was specific for VP16-activated transcription. In all cases, inhibitory activity in the in vitro transcription assay paralleled inhibitory activity in DNA binding assays for C1 complex formation (see below), strong evidence that this complex is a necessary intermediate in VP16 transactivation.

**Identification of residues critical for peptide inhibitory activity.** The peptides described above are derived from a region of VP16 that is thought to interact with HCF (23, 26). To identify which amino acids are critical for the inhibitory activity of the peptides described above (and hence possibly for VP16 interaction with HCF), each nonalanine residue of CP436-3 was individually changed to alanine, and the resultant peptides were tested for inhibition of C1 complex formation (Fig. 5) and transcriptional activation by VP16 (Fig. 6). Alanine was chosen because it minimally perturbs secondary structure, and thus any change in activity would likely be due to loss of a critical R group rather than a gross change in peptide secondary structure (11). As shown in Table 1, the patterns of inhibitory activity in the two assays were very similar, consistent with C1 complex formation being essential for VP16 transactivation. Of the substitutions introduced, only three, E-6→A-6, H-7→A-7, and Y-9→A-9, completely abolished activity. Interestingly, E-1 could be changed to alanine with no loss of activity, and substitution of V-3 or M-4 each had less than a 10-fold effect on activity, whereas deletion of these residues dramatically reduced activity (Fig. 4). Changing either arginine had a modestly diminished inhibitory activity, and substitution of S-10 did not diminish activity, suggesting that these R groups do not play a major role in the interactions targeted by the peptide.

## DISCUSSION

Combinatorial regulation appears to be the principal mechanism whereby a limited number of regulatory factors that can activate many genes work to coordinately regulate the expression of smaller number of genes under very specific circumstances. The key to combinatorial regulation is that maximal expression of a given gene depends on a specific combination of proteins either binding to distinct *cis*-acting sequences or forming a complex that binds a single element. The activation of HSV IE genes by VP16 is one of the best-characterized examples of combinatorial regulation. In vivo transactivation studies and in vitro DNA binding assays have revealed much about the protein and DNA sequence requirements for this process. In this report, we describe the first in vitro reconstitution of activation by native VP16 through HSV IE regulatory sequences. This system recapitulates many diverse aspects of

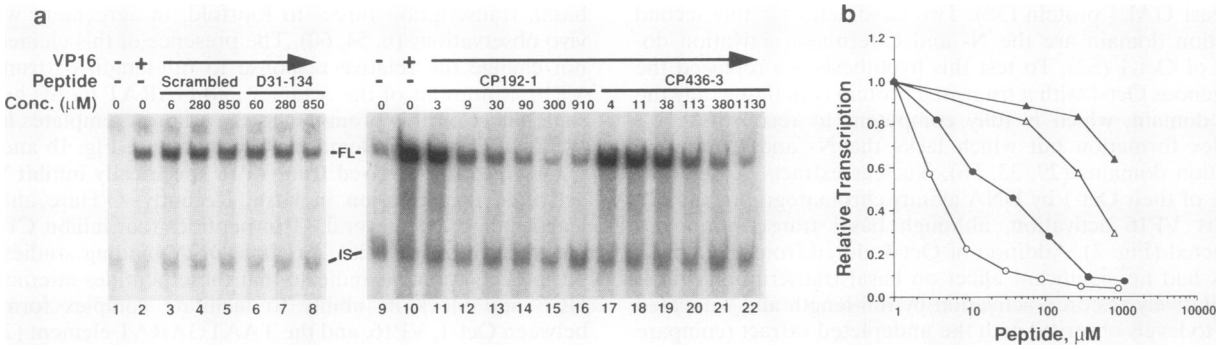
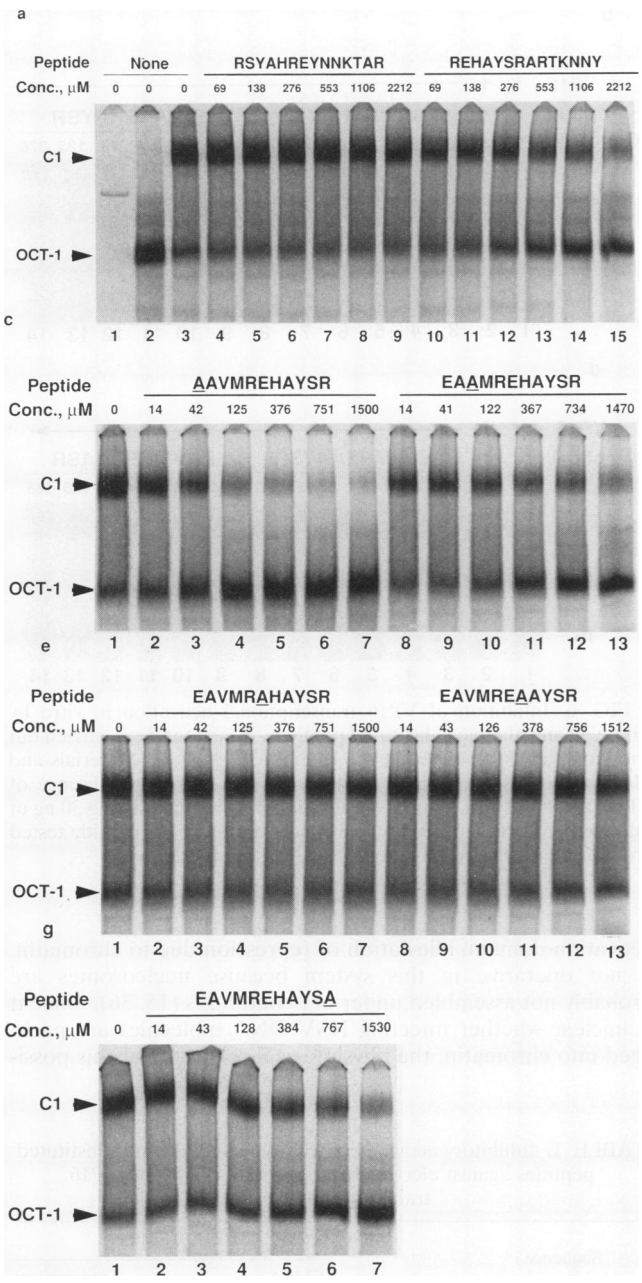



FIG. 4. Peptides derived from VP16 inhibit transactivation in vitro. (a) Gel analysis of transcription products. The indicated amounts of peptide were added to reaction mixtures containing 30 ng of full-length VP16 and a DNA template having three wild-type TAATGARAT elements. See Materials and Methods for the sequences of the peptides. FL and IS are as in Fig. 1. (b) Quantitative analysis of data shown in panel a. The amount of transcript produced in the presence of the concentration of peptide designated on the *x* axis is expressed relative to the amount of transcript produced in reactions lacking peptide, which was arbitrarily set at 1. ○, CP192-1; ●, CP436-3; △, D31-134; ▲, scrambled peptide.



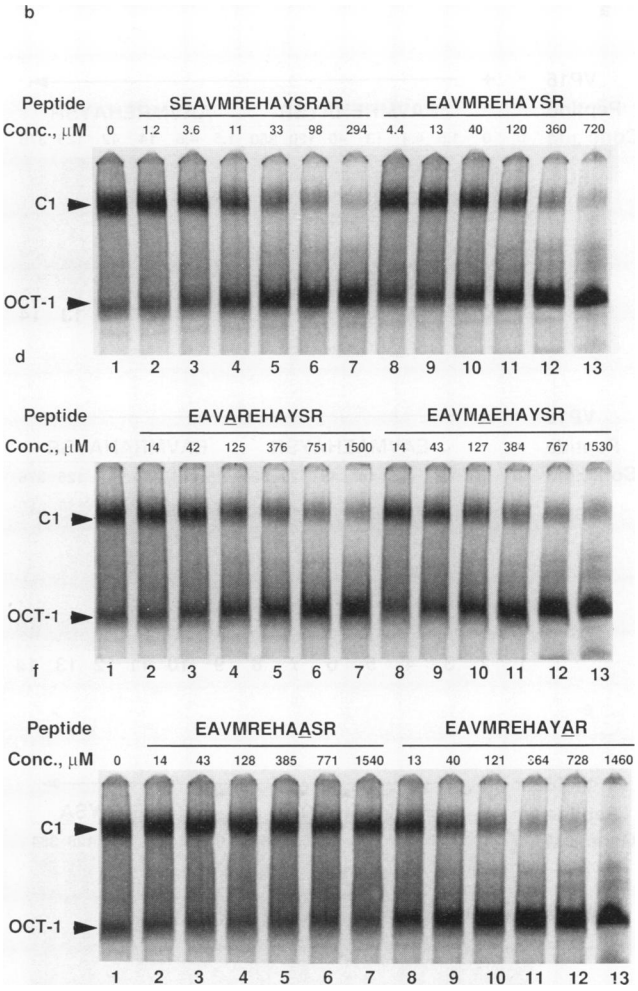




FIG. 5. Inhibition of C1 complex formation by wild-type and alanine substitution mutant peptides. Electrophoretic mobility shift assays were performed as described in Materials and Methods. The positions of the Oct-1–DNA and VP16-induced complexes, verified by shifts with mutant DNA probes (data not shown), are indicated by the arrows to the left of each gel. The concentration of peptide added to each reaction mixture is indicated above each lane. (a) Scrambled peptide (RSYAHREYNNKTAR) and D31-134 (REHAYSRARTK NNY). Reaction mixtures were supplemented with VP16 alone (lane 1), nuclear extract alone (lane 2), or both (lanes 3 to 15) as described in Materials and Methods. (b) CP192-1 (SEAVMREHAYSRAR) and CP436-3 (EAVMREHAYSR). All reaction mixtures contained GST-VP16 and nuclear extract. (c to g) Alanine mutants of CP436-3. The position of the substituted alanine in each peptide, indicated above the gel, is emphasized by underlining. All reaction mixtures contained GST-VP16 and nuclear extract.

this process that had been previously studied in multiple different systems and has the advantage that it unifies, in one assay, the requirements for both DNA binding and transcriptional activation by native VP16.

Several observations that capitalize on the unique advantages of this system are worthy of note. For example, using this assay, we show that in some cases, VP16-dependent activation may require activation domains on Oct-1. Two mechanisms may be envisaged to explain this observation: (i) interaction with VP16 (with or without its activation domain) alters the conformation of one or more activation domains on Oct-1, or (ii) Oct-1 binds DNA more efficiently in the context of the C1 complex. While we cannot rule out the former possibility, we favor the second explanation for the following reasons. In vivo results similar to ours were recently reported by Cleary et al. (12), who showed that VP16-mediated activation of a U2 small nuclear RNA promoter containing multiple TAATGARAT elements is reduced, but not eliminated, by deletion of the VP16 activation domain. They found that although Oct-1 binds to the TAATGARAT element, its affinity is increased significantly upon complexing with VP16 and HCF. We have observed that the C1 complex dissociates much more slowly than the Oct-1-DNA complex (43b). If the interaction of Oct-1 with its downstream target occurs slowly relative to the dissociation of the Oct-1-DNA complex, then any event that stabilizes Oct-1 on the DNA would increase the likelihood that it could interact with its downstream target, resulting in an increase in

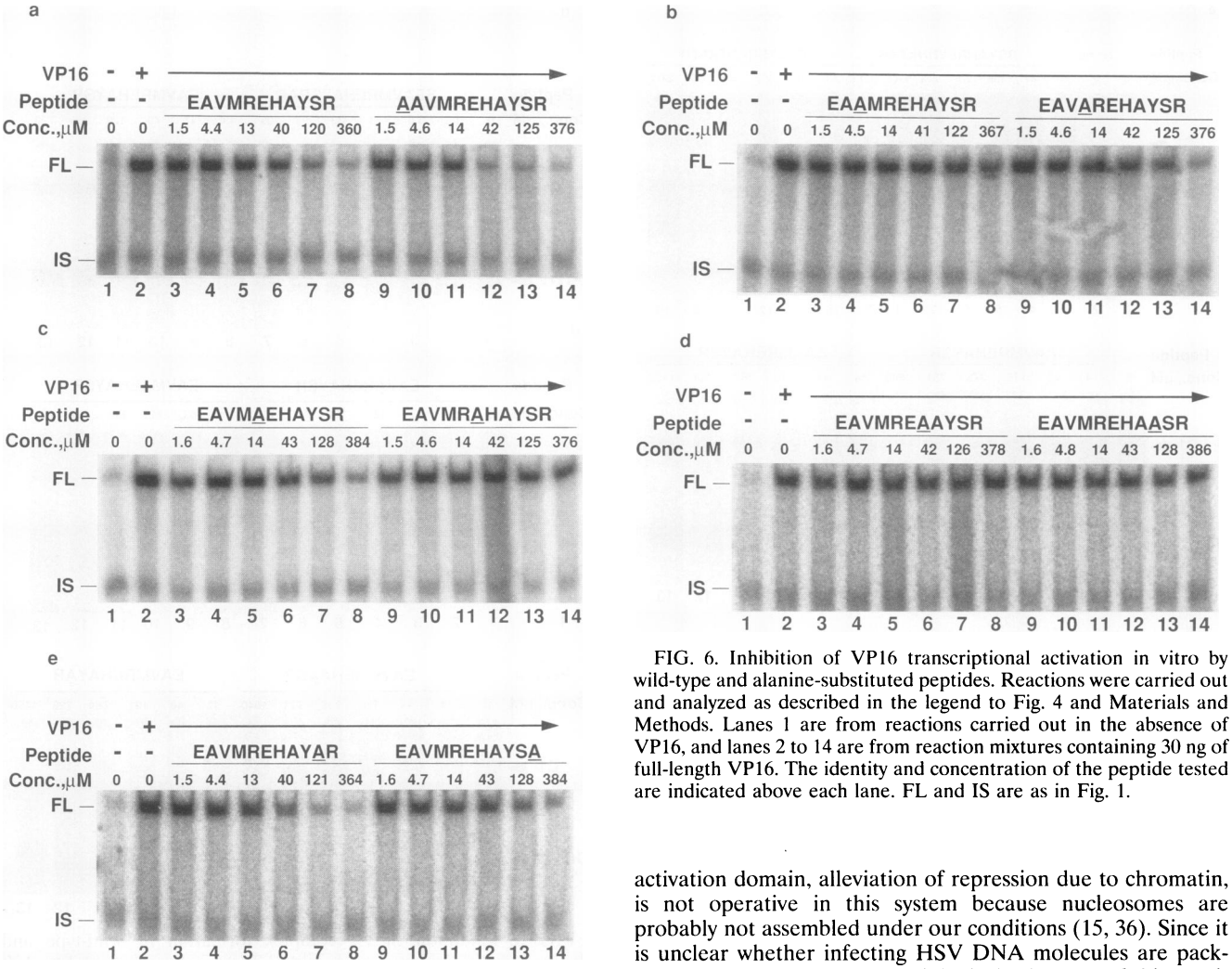



FIG. 6. Inhibition of VP16 transcriptional activation in vitro by wild-type and alanine-substituted peptides. Reactions were carried out and analyzed as described in the legend to Fig. 4 and Materials and Methods. Lanes 1 are from reactions carried out in the absence of VP16, and lanes 2 to 14 are from reaction mixtures containing 30 ng of full-length VP16. The identity and concentration of the peptide tested are indicated above each lane. FL and IS are as in Fig. 1.

its apparent transactivation potential. Since the activation domain of VP16 is not required for C1 complex formation, this would provide a mechanism whereby VP16 without its activation domain would apparently activate transcription.

While the activity of Oct-1 activation domains may explain the higher-than-expected activity of truncated VP16 in vitro, the observation that high levels of full-length VP16 inhibited activated transcription suggests that the VP16 activation domain may not be functioning to its full potential. Numerous examples of this latter phenomenon, often referred to as squelching, have been observed in vivo and in vitro and are thought to result from the sequestration of a limiting factor necessary for activated transcription (4, 21, 30, 42). Similarly, we suggest that a key factor required by the activation domain may be severely limiting in our nuclear extracts and that the activation by full-length VP16 is actually the net result of activation and squelching. It is noteworthy that very high levels of full-length VP16 do not reduce transcription to the basal level but instead reduce it to the plateau level obtained with truncated VP16, suggesting that if there is a second activation domain in the C1 complex (e.g., the Oct-1 activation domains), it differs mechanistically from the VP16 C-terminal VP16 activation domain. Another explanation that is consistent with our observations is that one proposed activity of the VP16 activation domain, alleviation of repression due to chromatin, is not operative in this system because nucleosomes are probably not assembled under our conditions (15, 36). Since it is unclear whether infecting HSV DNA molecules are packaged into chromatin, the physiological relevance of this possi-

TABLE 1. Inhibitory activities of wild-type and alanine-substituted peptides against electrophoretic mobility shift and VP16 transactivation assays

| Sequence | $IC_{50}$ (μM)[a] | |
|---|---|---|
| | EMSA | Transcription |
| REHAYSRARTKNNY | 276 | 300 |
| SEAVMREHAYSRAR | 12 | 4 |
| EAVMREHAYSR | 31 | 25 |
| AAVMREHAYSR | 34 | 21 |
| EAAMREHAYSR | 184 | 119 |
| EAVAREHAYSR | 130 | 85 |
| EAVMAEHAYSR | 81 | 75 |
| EAVMRAHAYSR | >1,000 | >1,000 |
| EAVMREAAYSR | >1,000 | >1,000 |
| EAVMREHAASR | >1,000 | >1,000 |
| EAVMREHAYAR | 38 | 28 |
| EAVMREHAYSA | 127 | 102 |

[a] The $IC_{50}$ (concentration of peptide required to give 50% inhibition of C1 complex formation [see Fig. 5] or VP16 transactivation in vitro) was determined from duplicate experiments of the type shown in Fig. 5 and 6. In each case, 100% inhibition corresponds to the signal observed in either the C1 complex region of the electrophoretic mobility shift assay (EMSA) gel or full-length transcript in the transcription assay gel in the absence of VP16. The position of substituent alanine is indicated by an underlined A.

| | |
|---|---|
| VZV ORF10 | R H V R A D H P Y A K |
| EHV-1 gene 12 | L F P L A E H S Y S K |
| HSV-1 VP16 | E A V M R E H A Y S R |

FIG. 7. Comparison of amino acid sequences VZV ORF10 and EHV-1 gene 12 protein with the sequence of VP16 inhibitory peptide. Portions of the sequences from the ORF10 (residues 366 to 376) and gene 12 (residues 399 to 409) proteins previously suggested to be homologous to the region of HSV-1 VP16 that contains the inhibitory peptides (44) are shown along with the sequence of the inhibitory VP16 peptide CP436-3. The conserved E/D, H, and Y residues are boxed.

ble activity remains to be determined. In any event, the identification of factors required to increase responsiveness to the VP16 activation domain should further our understanding of how it stimulates transcription in its native context.

The degree of stimulation of VP16 activation by the CGG AAR motif agrees fairly well with in vivo observations on the effect of this element on IE gene expression (6, 35, 54, 58, 60). This element binds an interesting heterodimeric protein, termed GABP (35, 58). The α subunit of this protein contains sequences homologous to the Ets family of transcriptional activators, and the β subunit contains repeats of a motif, the ankyrin/cdc10/SWI6 repeats, found in a number of proteins, including transactivators (35, 58). It has been suggested that this factor cooperatively enhances C1 complex formation (3), providing a mechanism for the synergistic stimulation of VP16 activation. The effect of mutation or deletion of the TAATG ARAT element on VP16 activation of promoters containing the CGGAAR element (Fig. 3) suggests that the TAATGAR AT element is the primary site of VP16 interaction and that if VP16 interacts with the CGGAAR element or its associated proteins, the interaction is insufficient to elicit a response.

We have shown that peptides directed against a key protein-protein interaction in this system specifically inhibited VP16-activated, but not basal, transcription. These results also strongly support the proposal that the C1 complex characterized by gel mobility shift assays is indeed a necessary intermediate for native VP16 DNA binding and transactivation. Furthermore, by systematically changing each nonalanine residue in the inhibitory 11-mer to alanine, we have identified three residues (E-6, H-7, and Y-9) whose R groups are absolutely essential for inhibition and four others (V-3, M-4, R-5, and R-11) whose R groups enhance inhibition, but which in and of themselves are not absolutely required for inhibition. Since the region of VP16 from which the peptides are derived is thought to interact directly with HCF (23, 26), we suggest that the three essential amino acids are critical contact points between these two proteins.

The importance of the three essential residues is underscored by their conservation in two other alphaherpesvirus transactivators: the varicella-zoster virus (VZV) ORF10 protein and the equine herpes virus type 1 (EHV-1) gene 12 protein. Like VP16, both of these proteins are packaged in the virus tegument and transactivate viral IE gene promoters (44, 48). They have also been shown to be capable of activating transcription through a TAATGARAT element (44, 48). As shown in Fig. 7, the only amino acids conserved among all three proteins over the region defined by the inhibitory peptides are E/D, H, and Y at the same relative positions. These observations suggest that these proteins, which perform similar functions in the life cycles of their respective viruses, all interact with HCF or a similar protein via a common structural motif.

In spite of the similarities noted above and the recent observation that VZV ORF10 can rescue an HSV-1 mutant with a defective VP16 protein (44), efforts to demonstrate the formation of a complex between ORF10 and cellular factors in electrophoretic mobility shift assays with a TAATGARAT element have not been successful (43). This result might suggest that ORF10 does not form a complex with HCF and Oct-1. Alternatively, we suggest that the ORF10 C1-like complex is simply more difficult to detect because of amino acid changes (e.g., R-5→A-5) that our results predict would have a destabilizing effect (Table 1). More detailed mutational studies of the relevant regions of VP16 and ORF10 would resolve this issue.

VZV ORF10 and EHV-1 gene 12 proteins are also interesting because despite their relatively good sequence homology with HSV-1 VP16 over most of their length, they diverge significantly from VP16 at their C termini, the location of VP16's potent activation domain (44). In particular, ORF10 terminates shortly after the proposed region of interaction with Oct-1 and HCF. How then do these proteins activate transcription? One possibility is that they have activation domains elsewhere in their structure, or alternative C-terminal activation domains, that remain to be identified. Another possibility is that they activate transcription by a mechanism similar to that used by truncated VP16 in our in vitro system, by stabilizing the binding of Oct-1, which provides its own activation domains. Again, more detailed mutational studies should readily resolve these possibilities.

One goal of studying the mechanism of activation by VP16 is to facilitate the discovery and design of novel antiherpetic agents. The observation that overexpression of truncated VP16 in cultured cells confers resistance to HSV-1 infection while having no effect on cell viability (19) suggests that it should be possible to develop compounds that disrupt the interaction of VP16 with cellular proteins but do not significantly affect cellular gene expression. Peptides are becoming increasingly useful as model structures and lead compounds in the drug discovery process (28, 31, 45, 53). The results from the peptide inhibition experiments described in this report have provided an important clue to the contact points between VP16 and HCF. By eliminating problems of cell permeability and catabolic processing, this in vitro transcription system and the information gleaned from it should facilitate the identification and development of more potent inhibitors and the investigation of their modes of action. Such small molecules may lead the way for the development of novel therapeutic agents directed at modulating the expression of key disease-related genes and in addition will provide powerful tools for probing the molecular interactions that drive transcriptional activation.

## ACKNOWLEDGMENTS

We thank S. Parent, S. Ludmerer, T. Kristie, E. O'Neill, and S. Buratowski for many valuable discussions and suggestions and S. Parent, S. Ludmerer, E. O'Neill, M. Tocci, S. Buratowski, and R. Lafemina for reviewing the manuscript. We also thank S. Triezenberg and C. Preston for generously providing plasmids encoding wild-type and mutant forms of VP16.

## ADDENDUM IN PROOF

During review of the manuscript, Arnosti et al. published a report (D. N. Arnosti, C. M. Preston, M. Hagmann, W. Schaffner, R. G. Hope, G. Laughlin, and B. F. Luisi, Nucleic Acids Res. **21**:5570–5576, 1993) describing in vitro transcriptional activation by VP16 through an HSV-1 ICP0 enhancer. Our results are in good agreement with these previously published observations.

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