The VP16 Paradox: Herpes Simplex Virus VP16 Contains a Long-Range Activation Domain but within the Natural Multiprotein Complex Activates Only from Promoter-Proximal Positions

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Upon infection of permissive cells, e.g., epithelia, by herpes simplex virus type 1 (HSV-1), the viral genes are expressed according to their function in a strict temporal order beginning with the immediate-early (IE or α) genes followed by the early (E or β) and late (L or γ) classes (reviewed in reference 72). α Gene activation is brought about by a viral pre-IE factor, the key transactivator VP16 (also known as Vmw65, αTIF, or ICP25), a 490-amino-acid phosphoprotein (5, 10, 62, 67). Mutations in VP16 that compromise its α gene transactivation function lead to severely impaired viral replication and even avirulence in certain animal models (1, 80). The VP16 gene is itself a member of the γ gene class and, as such, a structural component of the virion being highly abundant in the viral tegument (24). The structural function of VP16 is strictly required for HSV multiplication (65, 87). Upon fusion of the viral envelope with the cell membrane, the VP16 protein is released into the cytoplasm of the newly infected cell together with other tegument components. After import into the nucleus, VP16 is corecruited onto so-called TAATGARAT response elements found in all α gene regulatory regions (50), in association with the cellular protein HCF (host cell factor, also known as VCAF, CFF, or C1 factor [37, 40, 92, 95]), by the ubiquitous cellular transcription factor Oct-1 (also termed NF III, OBP100, OTF-1, or POU2F1 [17, 60, 68, 83; reviewed in reference 59]). In this trimeric protein complex, Oct-1 provides the DNA-binding activity via its conserved bipartite POU domain, which consists of an N-terminal POU-specific domain and a C-terminal homeodomain (29). Oct-1 is involved in the transcriptional regulation of a number of cellular genes like the histone H2B gene or the U2 small nuclear RNA gene (reviewed in reference 28). Furthermore, in B lymphocytes, Oct-1 and another closely related POU family member, the B-cell (and neuron)-specific Oct-2 (also termed OTF-2 or POU2F2 [9, 57, 74]), activate the transcription of B-cell-specific genes via complex formation with a B-cell-specific coactivator termed Bob-1 (also known as OCA-B or OBF-1 [20, 48, 82]). Both these Oct factors exhibit a very similar, if not identical, DNA sequence specificity and bind with comparable affinity to an 8-bp DNA motif known as the “octamer” motif of consensus ATGCAAAT (12, 14, 61, 79), which in some cases overlaps with the 5′ half site of the HSV TAATGARAT response elements (59). Although Oct-1 and Oct-2 exhibit roughly 90% identity on the amino acid level within their conserved POU domain, only Oct-1 is capable of interacting with VP16 and HCF and thus forming an activating complex, due to key amino acid differences in the POU homeodomain (44, 64). Neither VP16 nor HCF exhibits efficient sequence-specific DNA-binding activity on its own; instead, they interact with each other in solution (37, 42, 51, 95). The cellular function of HCF is still enigmatic, but during HSV α gene activation, it promotes Oct-1–VP16–HCF complex formation (17, 40, 42). The third component, VP16, contains a potent acidic activation domain which is located within the C-terminal 78 amino acids and is indispensable for HSV α gene activation in vivo (15, 19, 85). This activation domain has been most intensely studied in the context of protein fusions with heterologous DNA-binding domains. Fusion to the DNA-binding domain of the yeast activator GAL4 results in one of the strongest known transcriptional activators (21, 73, 76). Cell-free transcription
studies indicate that it functions primarily by increasing the rate of preinitiation complex assembly (7, 34, 46), most probably due to direct protein-protein interactions with some of the general transcription factors, such as TFIIA (46, 47, 69, 71), TFIIF (39), and the TATA-binding protein and TAFII40 components of the multisubunit TFIID (33, 38, 81). Other studies also implicate VP16 in recruiting the RNA polymerase II holoenzyme (25); in facilitating transcriptional elongation (96), perhaps via interactions with the general transcription factors TFIIF (97) and TFIIF (94); and/or in recruiting an RNA polymerase II C-terminal domain kinase to the preinitiation complex (30). Previously, we have functionally characterized various activation domains based upon their ability to activate transcription from promoter-proximal and remote enhancer positions. Thereby, “proximal” activation domains, like the glutamine-rich activation domains of Sp1 or Oct-1, were shown to activate transcription significantly only from promoter-proximal positions in response to an additional enhancer. By contrast, “general” activation domains, like the acidic domain of VP16 or the serine/threonine-rich domain of ITF-2, were able to activate transcription on their own both from proximal and distal positions (76) (Table 1).

To functionally characterize the transcriptional activation by VP16 in its natural context, i.e., within the trimERIC Oct-1–VP16–HCF complex, we have analyzed this complex in a model system by cotransfection of various VP16 forms with different reporter constructs (see Fig. 1) in HSV-permissive human HeLa cells. In these reporter constructs, either isolated and multimerized TAATGARAT response elements or complete a gene regulatory regions were inserted at a promoter-proximal or remote downstream “enhancer” position. We show that the wild-type VP16 protein, when part of the Oct-1–VP16–HCF complex, is not able to stimulate transcription significantly over long distances despite its potent general acidic activation domain. The complex is, however, highly active from a promoter-proximal position even in the absence of an additional downstream enhancer. When a second general activation domain was fused onto VP16, the complex displayed typical long-range enhancer activity and activation from a proximal position was potentiated. These seemingly paradoxical findings indicate that the VP16 activation domain in its natural context is fully active only when the ternary complex is assembled in close proximity to a transcriptional start site. Thus, the Oct-1–VP16–HCF complex seems to be particularly suited to selectively and strongly activate proximal a genes within the densely packed HSV genome.

MATERIALS AND METHODS

Construction of plasmids. All reporter plasmids are based on the OVEC-1 vector (91). Oligonucleotides were synthesized with SacI-SalI protruding ends and multimerized with I-SceI. They were cloned either in the promoter-proximal or remote downstream “enhancer” position. We have described previously (12). For the corresponding downstream reporter constructs, the same fragments have been inserted in the EcoRI site as described above, and the orientation has been verified by sequencing. All downstream reporter constructs contained two Sp1-binding sites in the promoter-proximal position (Sac-I-SalI) (76).

The reporter constructs bearing GALA-binding sites and most of the GAL4 fusion constructs have been described previously (76). For pSCTEV-GAL16(413–490)–VP16(413–490), which contained the duplicated C-terminal VP16 activation domain, a 250-bp SalI-BglII fragment from pBS SK–VP30 (see below) and a 1.6-kb BglII-BglII fragment from pCGN-VP16–VP16(413–490) (see below) were cloned into the 4.0-kb BglII-SalI vector fragment from pSCTEV-GAL4(1–93) (76). The same 4.0-kb BglII-SalI vector fragment from pSCTEV-GAL4(1–93) was also used to construct pSCTEV-GAL16(413–490)–VP16(413–490)–ITF(2–2–452) by inserting a 2.4-kb BglII-SalI fragment from pCGN–VP16–VP16(413–490)–ITF(2–2–452) (see below), C-terminal to the VP16 activation domain, pSCTEV–GAL16(413–490)–ITF(2–2–452) contained amino acids 2 to 452 of the helix-loop-helix transcription factor ITF2 (26, 27).

The expression plasmids encoding the various VP16 fusion proteins were all based on pCR-VP16 (8). For the C-terminal VP16 deletion mutants, an Xhol–BamHI fragment from pCR-VP16 encompassing the full-length VP16 coding sequence was cloned into pBS SK– (Stratagene) to yield pBS SK–VP16wt. After BamHI-PstI linearization, the clone was subjected to exonuclease III digestion by exonuclease III (New England Biolabs), both ends were blunted with Klenow DNA polymerase and/or Klenow DNA polymerase (Pharmacia) and/or Klenow DNA polymerase (New England Biorlabs) to create blunt ends. Where indicated, the promoter-proximal reporter constructs contained the 196-bp simian virus 40 (SV40) enhancer in the EcoRI site (91).

The oligonucleotides used are as follows:

ICP0 TAATGARAT

5’ CGAGGCGTCTGATCTAATTGATATCTTCGG 3’

3’ TCGAATCCGACACTCCTTAATCTAACATCATGGCC 5’

ICP4 TAATGARAT

5’ CGAGGGATCCGGGCTTGATGATTTCATGCGG 3’

3’ TCGAATCCGACACTCCTTAATCTAACATCATGGCC 5’

The reporter constructs containing the natural ICP0 and ICP4 regulatory regions (without their endogenous TATA boxes) in the promoter-proximal Sac-I-SalI sites [designated n-ICP0(TATA)– and n-ICP4(TATA)–, respectively have been described previously (12). For the corresponding downstream reporter constructs, the same fragments have been inserted in the EcoRI site as described above, and the orientation has been verified by sequencing. All downstream reporter constructs contained two Sp1-binding sites in the promoter-proximal position (Sac-I-SalI) (76).

TABLE 1. Functional properties of different types of activation domains

<table>
<thead>
<tr>
<th>Type of activation domain</th>
<th>Proximal activation in vivo (no downstream enhancer)</th>
<th>Proximal activation in vivo (with downstream enhancer)</th>
<th>Remote activation in vivo</th>
<th>Activation in vitro</th>
<th>Activation in S. cerevisiae</th>
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<tbody>
<tr>
<td>Proximal</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>General</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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* Original classification according to reference 76.
* Transcriptional activation from a promoter-proximal position in a standard transcription reaction in Dignam nuclear extract (2, 16).
* From reference 43.

<table>
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<th>Type</th>
<th>Activation in vivo</th>
<th>Activation in vitro</th>
<th>Activation in S. cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>General</td>
<td>+</td>
<td>+</td>
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</table>

In pCAG-VP16(433–490)–VP16(433–490), which contained the duplicated C-terminal VP16 activation domain, a 250-bp SalI-BglII fragment from pBS SK–VP30 (see below) and a 1.6-kb BglII-BglII fragment from pCGN-VP16–VP16(433–490) (see below) were cloned into the 4.0-kb BglII-SalI vector fragment from pSCTEV-GAL4(1–93) (76). The same 4.0-kb BglII-SalI vector fragment from pSCTEV-GAL4(1–93) was also used to construct pSCTEV-GAL16(413–490)–VP16(413–490)–ITF(2–2–452) by inserting a 2.4-kb BglII-SalI fragment from pCGN–VP16–VP16(413–490)–ITF(2–2–452) (see below), C-terminal to the VP16 activation domain, pSCTEV–GAL16(413–490)–ITF(2–2–452) contained amino acids 2 to 452 of the helix-loop-helix transcription factor ITF2 (26, 27).

The expression plasmids encoding the various VP16 fusion proteins were all based on pCR-VP16 (8). For the C-terminal VP16 deletion mutants, an Xhol–BamHI fragment from pCR-VP16 encompassing the full-length VP16 coding sequence was cloned into pBS SK– (Stratagene) to yield pBS SK–VP16wt. After BamHI-PstI linearization, the clone was subjected to exonuclease III digestion by exonuclease III (New England Biolabs), both ends were blunted with 31 nuclease (Sigma) and religated with T4 DNA ligase (Pharmacia), and the length of the reaction was monitored by sequencing. The resulting pBS SK– clones were linearized with EcoRI, and a STOP oligonucleotide was inserted. The STOP oligonucleotide was as follows:

5’ AATTCGACTGCTAGATAGTCG 3’

3’ GATCCAGATCTCGTATTAA 5’

Xhol–EcoRV fragments containing the remaining VP16 coding sequences and the STOP oligonucleotide were inserted into the 5.0-kb Xhol–BamHI pCGN vector fragment, the BamHI end of which had been blunted with Klenow DNA polymerase.

The clone pCAG-VP16(433–490)–VP16(433–490)–ITF(2–2–452) contains amino acids 2 to 452 of the helix-loop-helix transcription factor ITF2 (26). The clone pCAG-VP16wt, 483–490 was inserted into the 5.1-kb fragment of the pCGN-VP16 vector that had been digested with EcoRI and BamHI.

Amino acid sequences of the C-terminal VP16 deletion mutants (amino acids encoded in boldface type are encoded by the VP16 coding sequence, and the ones in normal type are encoded by the polylinker; * indicates a stop):

<table>
<thead>
<tr>
<th>Type</th>
<th>Activation in vivo</th>
<th>Activation in vitro</th>
<th>Activation in S. cerevisiae</th>
</tr>
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<tr>
<td>Proximal</td>
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<tr>
<td>General</td>
<td>+</td>
<td>+</td>
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FIG. 1. Schematic drawing of the reporter constructs (A and B) and expression vectors (C and D). (A and B) Reporter constructs are based on the plasmid OVEC-1 (91), where the β-globin reporter gene is transcriptionally regulated by the cis-acting sequences inserted either directly upstream of the core promoter elements at the SacI-SalI sites (promoter reporter constructs [A]) or about 1.8 kb downstream at the EcoRI site (enhancer reporter constructs [B]). Inserted sequences comprise either the complete ICP0 and ICP4 regulatory regions with their endogenous TATA boxes deleted (12), the isolated and multimerized VP16 response elements from the ICP0 or ICP4 regulatory regions (as described in Materials and Methods), or multimerized copies of the GAL4-binding site (76). For some experiments, the 196-bp SV40 enhancer fragment (91) was inserted at the downstream position to further boost transcription driven by the promoter-proximal inserts. All enhancer reporter constructs contain, in addition to their enhancer inserts, two Sp1-binding sites at the promoter-proximal position (76). (C) GAL4 fusion proteins consist of the DNA-binding domain of GAL4, GAL(1–93), and activation domains from various transcription factors. The GAL4 fusions are all based on plasmid pSCTEV (76). (D) The same is shown for VP16 fusion proteins, which are all based on plasmid pCGN (8).
digested with XhoI, and a 2.2-kb fragment was cloned into the 5.3-kb pCGN-VP16-ITF-2(2–452) vector fragment obtained from an XbaI digest.

In pCGN-VP16-VP16(413–490), the VP16 activation domain was duplicated. pBS SK VPA30 was digested with ApaI, the ends were blunted with T4 DNA polymerase, and a further digest with KpnI yielded a 1.0-kb fragment. This fragment was cloned together with a 1.6-kb PvuII-BglII fragment from pSCTEV-GAL-VP16(413–490) (76), into a 4.8-kb BglII-KpnI vector fragment from pCGN-VP16 (8).

pCGN-VP16-PRV IE(1–120) contained the N-terminal acidic activation domain of the pseudorabies virus (PRV) IE protein that was cloned C-terminal to the VP16 coding region (52). pSCT-GAL-PRV IE9 (21) was digested with XbaI, and a 2.2-kb fragment was cloned into the 5.3-kb pCGN–VP16-IE9 vector fragment.

DNA transfections and S1 nuclease mapping. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco/BRL)–2.5% fetal calf serum–2.5% newborn calf serum–100 U of penicillin per ml–50 μg of streptomycin per ml–2 mM L-glutamine. Transfections were performed by cotransfecting 0.5 mg of pCGN-VP16-based expression plasmid and 3 μg of pSCTEV-GAL4 fusion expression plasmid by calcium phosphate coprecipitation (91). Empty expression vectors were cotransfected into HeLa cells together with either wild-type VP16 (wt) or a truncated version of VP16, VP16ΔS, lacking the C-terminal activation domain, and the CMV globin reference plasmid. Cells were harvested 36 h after transfection, and the amount of β-globin transcripts was determined by S1 nuclease mapping (top) and quantified by PhosphorImager analysis (bottom). Both HSV a gene regulatory regions are strongly induced (about 100-fold) by wild-type VP16, whereas the truncated version, VP16ΔS, is virtually inactive. Pinit indicates the correctly initiated β-globin transcripts, and ref indicates the reference signal from the CMV globin reference plasmid. (B) Four copies of the VP16 response elements from either the ICP0 or the ICP4 regulatory region were inserted at the proximal position and tested for VP16 inducibility. Only the high-affinity binding sites from the ICP0 regulatory region are strongly inducible (about 200-fold), whereas the ICP4 low-affinity binding sites show a rather weak induction (about 30-fold; the ICP4 autoradiogram was exposed approximately 8 times longer to clearly see the induction by wild-type VP16). Again, the truncated form of VP16 is essentially inactive.

Preparation of nuclear extracts and gel retardation (bandshift) assays. Small-scale nuclear extracts from transfected HeLa cells were prepared by the method of Schreiber et al. (75). Binding reactions for bandshift analyses were carried out by incubating 5 to 10 μg of nuclear extract with 4 fmol of double-stranded 5955

![Image](http://jvi.asm.org/) on August 28, 2017 by guest
Western blot analyses of nuclear extracts. Nuclear extracts of transfected HeLa cells were prepared by a modified version of the method of Schreiber et al. (75). In brief, pelleted nuclei were resuspended in 50 μl of sample loading buffer (50 mM Tris-Cl [pH 6.8], 2 mM EDTA, 1% sodium dodecyl sulfate, 1% β-mercaptoethanol, 8% glycerol) and boiled for 5 min. A 40-μg sample of total nuclear protein was loaded on a sodium dodecyl sulfate–7.5% polyacrylamide gel and transferred to nitrocellulose. The filter was incubated with LP1, a monoclonal antibody directed against the N terminus of VP16 (55), and the bands were visualized by an alkaline phosphatase-coupled secondary antibody (Dako; color reaction by Sigma).

RESULTS

VP16 is a very potent transcriptional activator from promoter-proximal positions. The majority of previous studies, including our own, characterizing transcriptional activation by the VP16 activation domain was carried out with fusion proteins in which the acidic activation domain of VP16 was fused to a heterologous DNA-binding domain. Therefore, we decided to characterize the activation properties of full-length VP16 within the HSV α gene-activating complex. As previously shown, VP16 strongly activates transcription from complete HSV α gene regulatory regions when inserted close to the transcriptional start site (Fig. 2A) (12, 22, 67). The same is true for a high-affinity binding site from the ICP0 regulatory region (Fig. 2B) (12, 41), an effect that was also observed in an in vitro transcription system (2, 93). The major TAATGARAT response element from the ICP4 promoter is much less inducible by VP16 due to a severely reduced affinity of the VP16 complex (Fig. 2B) (12). VP16-mediated transcriptional activation is strictly dependent on its C-terminal activation domain since C-terminal deletion mutants (VPΔ16S = VP16 core) strongly compromise the transactivation capacity (Fig. 2) (19, 85, 90). As expected, when the VP16-mediated induction from the ICP0 TAATGARAT element (or the complete ICP0 and ICP4 regulatory regions) is compared to the results of GAL fusion experiments, the VP16 complex is clearly classified as bearing a general activation domain (Fig. 3A) (76) in that the strong induction (typically >100-fold) is independent of an additional downstream enhancer. Thus, it behaves similarly to the respective GAL-VP16(413–490) fusion protein (compare Fig. 2B to Fig. 3A). On the other hand, VPΔ16S still shows a small residual activity and behaves similarly to GAL fusions with “proximal” activation domains, e.g., the glutamine-rich activation domains of Sp1 or Oct-1 (Fig. 3A). This finding is consistent with earlier observations demonstrating the ability of VPΔ16S to activate transcription in vitro to the same extent as wild-type VP16 (reference 93 and data not shown) and with the fact that VPΔ16S is still able to respond to a downstream SV40 enhancer (data not shown), a property also seen with other proximal activation domains like the ones of Oct-1, Oct-2, and Sp1 (76).

The VP16 complex is unable to significantly stimulate transcription from a downstream enhancer position. We next tested the activation property of the VP16 complex when bound downstream of the transcription start site in a typical enhancer position. To our surprise, the complex was not able
to stimulate transcription strongly when bound to isolated binding sites or complete α gene regulatory regions (Fig. 4) (five- to eightfold induction with respect to the transcriptionally compromised deletion mutant VPΔ16S). Comparison with GAL fusion proteins indicated that under these conditions, the VP16 complex behaved like a proximal activator, such as GAL-Sp1 or GAL–Oct-1, which also yield an approximately fivefold induction (Fig. 3B, lanes GAL-Sp1 and GAL-Oct-1). The most obvious feature is the very clear-cut and unexpected difference between the VP16 complex formed on HSV α gene response elements and the respective GAL-VP16 fusion protein when bound at a distal enhancer position (compare Fig. 4, left panel, lane VP16wt, to Fig. 3B, lane GAL-VP16), which is in striking contrast to the behavior from a promoter-proximal position (compare Fig. 2B, left panel, lane VP16wt, to Fig. 3A, lane GAL-VP16). Thus, the VP16 complex behaves differently depending on whether it is bound close to or at a distance from a preinitiation complex. This result implies a more sophisticated mechanism of regulation than was originally anticipated on the basis of results obtained with GAL fusion proteins.

**Fusion of general activation domains onto VP16 confers the capacity for long-distance activation to the VP16 complex.** From the data obtained so far, we considered it possible that the VP16 activation domain within a distantly bound complex was not accessible for interactions with the general transcription factors at the core promoter. Thus, in the framework of such a model, fusion of a second general activation domain on top of VP16 might alleviate the steric constraints and permit activation from remote positions. To test this, we fused to VP16 three different general activation domains which had previously been shown to be active from a distance, namely, those of ITF-2 (27, 76), VP16(413–490) yielding a VP16 protein with a duplicated activation domain (73), and PRV IE (21, 32, 52). Indeed, when these VP16 fusion proteins were tested from a downstream position, they behaved as bona fide enhancer factors; i.e., they could activate transcription, though to various extents, from both isolated high-affinity binding sites and the complete ICP0 regulatory region (Fig. 5). Comparison with respective GAL fusion proteins carrying two activation domains revealed, however, that transcriptional activation from a remote enhancer position by these factors is potentiated compared to GAL fusions with a single activation domain (Fig. 3B; compare lanes GAL–VP16–ITF-2 and GAL-VP16–VP16 to lanes GAL-VP16 and GAL–ITF-2). Thus, it is obvious that in these GAL fusions, both activation domains contribute to the overall activity. In contrast, in the VP16 fusion proteins, only one of the activation domains seems to be active whereas the other does not seem to contribute significantly to remote activation; this is compatible with the idea that it is not accessible to the transcription apparatus. The activity of the VP16 fusion proteins cannot, however, be attributed exactly to either one of the two activation domains; either the second domain is directly activating transcription or it somehow helps to unmask the endogenous VP16 activation domain which, in turn, activates transcription. Yet when random linker peptides of either 15 or 72 amino acids (which were unable to activate transcription by themselves) were inserted between the VP16 core domain and its endogenous activation domain, the resulting fusion proteins did not exhibit an increased activity compared to wild-type VP16 when tested from an enhancer position (data not shown).

The preference of the VP16 wild-type protein for promoter-proximal activation was directly demonstrated by a side-by-side comparison with VP16 fusion proteins in which the endogenous VP16 activation domain was replaced by a heterologous one, such as ITF-2 or PRV IE–ITF-2 (Fig. 1D). As shown in Fig. 6, wild-type VP16 was about fivefold stronger than VP16 core–ITF-2 from a proximal position whereas the situation was reversed from a distal position, where the induction was relatively weak (Fig. 6A). Similarly, while being equally potent from a promoter-proximal position, VP16 core–PRV IE–ITF-2 was about four- to fivefold stronger from an enhancer position (Fig. 6B). Thus, when incorporated in the VP16 complex, the endogenous VP16 activation domain shows a clear preference for activation from proximal positions compared to other general activation domains that were fused to the VP16 core domain. Wild-type VP16 therefore seems perfectly suited to elicit a strong transcriptional induction of proximal HSV α genes without undesired activation of more distal genes.

**Both activation domains of the VP16 fusion proteins contribute to transactivation from a promoter-proximal position.** To determine whether both activation domains of the various VP16 fusions would be active from a proximal position (as observed in the case of the GAL fusion proteins in Fig. 3A [13]), we tested these fusion proteins for transactivation from promoter-proximal high-affinity ICP0-binding sites. Consistent with our previous findings, all three VP16 fusion proteins activated transcription significantly over wild-type VP16, making use of both their activation domains (Fig. 7). The inhibition of a single VP16 activation domain at a distal position prompted us to perform additional experiments. One way of functionally compromising an activation domain might be by intramolecular inhibitory interactions that must be allosterically alleviated for transactivation to occur. We reasoned that such an inhibition within the VP16 protein would be maintained when fusing the full-length VP16, rather than just the activation domain (as done before), to the DNA-binding domain of GAL4. This fu-
tion generated a very potent transactivator [GAL-VP16wt(5–490)], which, however, activated transcription not only from promoter-proximal positions but also from distal positions (Fig. 8). Even though this construct performed slightly better from a proximal position than the standard GAL-VP16(413–490) construct did (Fig. 8), it seems that the full extent of proximal-distal regulation of VP16 requires a further component of the ternary complex, i.e., Oct-1 and/or HCF. The activation by the full-length VP16 GAL4 fusion also depended on the C-terminal activation domain: when the C terminus was omitted in the fusion protein, i.e., when only the VP16 core domain was fused to GAL4, activation was virtually abolished from either position (Fig. 8) (56).

A trivial though not very likely explanation for the different behaviors of various VP16 fusion proteins would have involved differences in the binding affinities of the respective VP16 complexes for the TAATGARAT sequence. However, when the various VP16 forms were tested for complex formation on the ICP0 TAATGARAT element in a gel retardation assay, all three fusion proteins showed a reduced capacity to form a supershifted complex with Oct-1 and HCF (Fig. 9). This was particularly obvious for VP16-VP16(413–490), which consistently showed the highest transactivation capacity (Fig. 5 and 7). Thus, the stronger transactivation we observed was not simply due to a higher affinity of the modified VP16 complexes for the ICP0 response element. The expression levels of the various VP16 fusion proteins were analyzed by Western blot analyses of nuclear extracts of transfected cells. As shown in Fig. 10, there were no major differences in the expression level (variation by a factor of 2 to 3) except for the transcriptionally

**FIG. 6.** VP16 wild-type protein preferentially activates transcription over short distances. When compared to other activation domains fused to the VP16 core domain, VP16 (amino acids 5 to 405), the endogenous VP16 activation domain shows a clear preference to activate transcription from a promoter-proximal position rather than from a distal enhancer position. (A) The VP16 wild-type protein is more potent from a proximal position than is VP16-ITF-2 (Fig. 1D) (about fivefold), whereas VP16-ITF-2 is stronger from a distal enhancer position (about three- to fourfold). (B) Both VP16 wild-type protein and GALΔ16-PRV IE-ITF-2 are equally strong from a proximal position, but VPΔ16-PRV IE-ITF-2 is clearly stronger from an enhancer position (about four- to fivefold).

**FIG. 7.** From a promoter-proximal position, the various VP16 fusion proteins make use of both their activation domains. In contrast to the downstream enhancer position, a proximal position enables the VP16 fusion proteins to use both activation domains, resulting in synergistic stimulation of transcription up to levels comparable to the ones seen in the GAL4 fusion context (about three- to fivefold over wild-type VP16 [compare to Fig. 3A]).
inactive VPΔ16S, which was expressed at about a 10-fold-higher level.

**DISCUSSION**

Most DNA-binding transcription factors and also coactivators contain domains which are specifically engaged in the activation of transcription. We have shown previously that such activation domains can be divided into different classes based on their activation properties. General activation domains which are often acidic/hydrophobic and in some cases also serine/threonine-rich, are capable of activating transcription not only from promoter-proximal positions but also over long distances from a remotely located enhancer (76). In fact, the C-terminal acidic activation domain of the HSV transactivator VP16 is considered a paradigm of such a general activation domain (6, 73). On the other hand, glutamine-rich activation domains, as exemplified by those of octamer factors Oct-1 and Oct-2, or Sp1, as well as the proline-rich activation domains of CTF or AP2, preferentially activate transcription from a promoter-proximal position in response to a downstream enhancer (76) (Table 1). These experiments were performed with chimeric proteins, whereby the activation domains were fused onto heterologous DNA-binding domains, e.g., the one of the yeast transcription factor GAL4 or the bacterial LexA repressor.

In our investigation of herpesvirus promoter elements, we have come across a seeming paradox. While the VP16 activation domain activates transcription strongly over both short and long distances when tested as a GAL4 fusion protein, the wild-type VP16 protein is highly active only from a promoter-proximal position. It hardly activates from a remote enhancer position once assembled with Oct-1 and HCF into HSV α gene-activating complexes on the TAATGARAT response elements. There are several possible explanations for these findings. (i) The VP16 complex binds avidly to response elements located at promoter-proximal positions but not at distal enhancer positions. This explanation can be excluded based on activation properties of VP16 fusion proteins carrying two activation domains. When a second general activation domain was fused to VP16, the respective complex was able to bring about significant activation from a downstream enhancer position also, even though the affinities of these enhancer-active complexes for an isolated ICP0 TAATGARAT element, as indicated by bandshift analyses, were reduced to 50% compared to that of the VP16 wild-type complex. This indicates that even in the presence of a second activation domain, which lowers the affinity of the complex for its binding site, the VP16 complex is indeed capable of binding to distal response elements. (ii) The C-terminal VP16 activation domain has a weaker overall activity than those of the PRV IE protein and the cellular helix-loop-helix factor ITF-2. Thus, the failure to activate from a downstream position would be merely a quantitative rather than a qualitative effect. However, in the GAL fusion context, GAL-VP16(413–490) was at least as strong as the other activation domains when assayed from a proximal position. (iii) The strong transcriptional activity of the VP16 acidic activation domain represents an artifact of GAL fusion proteins, and the activation observed from a promoter-proximal position is mediated by another activation domain within the natural VP16 complex. There is indeed evidence for additional activation functions within the VP16 complex (56, 66, 93; see also below). This possibility can, however, be ruled out by the fact that the acidic activation domain is absolutely required for strong promoter-proximal activation in our hands (Fig. 2A and B) (85). (iv) Based on the observation that all naturally
occurring TAATGARAT elements in HSV α gene-regulatory regions exhibit the same 5'-to-3' polarity, VP16 complex-mediated transcriptional activation could be dependent on the orientation of the cis-acting response elements. However, activation was not influenced even when all of the multimerized TAATGARAT elements were inverted, irrespective of their location (data not shown). (v) The C-terminal VP16 activation domain in its natural configuration is somehow masked by inhibitory interactions within the VP16 complex when the complex is bound at a remote position, but it is being displayed in an active form when VP16 is binding in proximity to the TATA box/transcriptional initiation site. This might be due to conformational changes upon protein-protein interactions with the close-by preinitiation complex, as depicted schematically in Fig. 11. This is the most likely explanation. In the framework of such a concept, it has been shown that in a cell-free system, the VP16-TFIIB interaction can induce a conformational change in TFIIB which, in turn, drives preinitiation complex assembly forward, leading to activated transcription (70). Although we do not provide direct experimental data, this change in the three-dimensional structure might well be mutual, thus “pulling out” the VP16 activation domain. Thereby, the structurally altered VP16 activation domain could be primed to stimulate postinitiation steps in the activation process (4, 18). In general, activation domains seem to be largely unstructured in solution and can be induced to fold into various conformations (78; reviewed in references 23 and 84), and a conformational change within the VP16 activation domain has indeed been observed upon interaction with general transcription factors, leading to a more constrained conformation (77). Moreover, structural changes induced by protein-protein interactions have been reported for other activation domains, e.g., c-myc (54), as well as for DNA-binding domains, like the ones of GCN4 (89), c-myc (58), ets-1 (63), and NF-κB p50 (53). Conversely, inhibitory intra- or intermolecular interactions that might render the activation domain inaccessible have been found for several other transcription factors, e.g., GAL4/GAL80 (35, 49), AP-1/GR (11, 36), E2F (31, 88), c-myc (3), and ATF-2 (45). The putative inhibitory interactions in the VP16 complex would, however, be intermolecular rather than intramolecular; i.e., they would not be confined to the VP16 protein alone. This is implied by the fact that full-length VP16, when fused to the GAL4 DNA-binding domain, activated transcription strongly from a remote position (Fig. 8). Hence, it seems likely that further components of the ternary complex, notably Oct-1 and HCF, are also required to block transcriptional activation at a distance in the VP16 complex. Since HCF has been shown to initially interact with VP16 in a dimeric complex (37, 42, 95), this interaction should, in principle, also occur when the full-length VP16 is present as a GAL fusion protein. Therefore, it is unlikely that a simple protein-protein interaction between HCF and VP16 is responsible for the observed block of enhancer activity within the VP16 complex. Rather, the exact three-dimensional geometry of the trimeric DNA-bound complex appears to be crucial for the downregulation of the VP16 activation domain at distal positions, thus limiting its activity to proximal promoters.

This conformational model is further supported by the fact that heterologous general activation domains, like ITF-2(2–452) or PRV IE(1–120), which can replace the endogenous VP16 domain, do not follow this pattern. While they may be even less active from a proximal position, they perform significantly better from a remote enhancer position when complexed with Oct-1 and HCF on TAATGARAT motifs (Fig. 6); i.e., they are less selective for promoter-proximal positions than is wild-type VP16. Thus, the VP16 wild-type protein, with the endogenous activation domain, seems to be best suited to restrict its strong transcriptional activity to proximal α genes. It is worth noting that on TAATGARAT response elements, VP16 is not merely converted to one of the typical promoter-active factors like Oct-2 or AP2 (76). Unlike what is seen with

FIG. 11. Schematic model of the proposed conformational change within the VP16 complex. (A) When bound in a promoter-proximal position, the C-terminal VP16 activation domain (shaded) is capable of interacting with components of the preinitiation complex. For reasons of simplicity, only some of the VP16-interacting general transcription factors are depicted, like TFIIB and TFIID (shown as TBP and TAFs). Upon this protein-protein interaction(s), a conformational change occurs, rendering the activation domain readily accessible for subsequent steps. Finally, a productive preinitiation complex is formed, resulting in a strong transcriptional induction of HSV α genes in response to VP16. (B) In contrast, when the VP16 complex is bound at a distal enhancer position, no conformational change is induced. As a consequence, the activation domain remains in an inaccessible conformation within the VP16 complex. The latter is therefore unable to strongly activate remote genes.
these latter factors, VP16 activity from proximal TAATGA RAT motifs does not require the help of an additional enhancer. We think that this sophisticated regulation of VP16 activity makes biological sense: VP16 is harnessed to strongly activate only herpesvirus α promoters over relatively short distances without fortuitous, undesired activation of other genes located further away in the HSV genome, in which some 75 protein coding sequences and regulatory regions are densely packed (86).

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9562 HAGMANN ET AL. J. VIROL.
9563