MOLECULAR AND CELLULAR BIOLOGY, June 1996, p. 2967–2976 Vol. 16, No. 6
0270-7306/96/$04.00+0

Differential Control of Transcription by Homologous Homeodomain Coregulators†

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Received 11 January 1996/Returned for modification 29 February 1996/Accepted 14 March 1996

The human herpes simplex virus type 1 (HSV-1) transactivator VP16 and its homolog from bovine herpesvirus 1 (BHV-1) can each recruit the human homeodomain protein Oct-1 into a transcriptional regulatory complex. Here, we show that these two Oct-1 coregulators possess similar, if not identical, homeodomain recognition properties but possess different virus-specific *cis*-regulatory specificities: the HSV-1 VP16 protein activates transcription from the HSV-1 VP16 response element, and the BHV-1 VP16 protein activates transcription from the BHV-1 VP16 response element. A distinct 3-bp segment, the D segment, lying 3′ of the canonical TAATGARAT motif (where R is a purine) in the VP16 response element, is responsible for the differential *cis* element recognition and transcriptional activation by these two homeodomain coregulators. These results demonstrate how a single homeodomain protein can direct differential transcriptional regulation by selective association with homologous homeodomain coregulators.

Homeodomain proteins are transcription factors that regulate many developmental processes in eukaryotes. An interesting feature of homeodomain proteins is that they effect different transcriptional regulatory programs even though, on their own, they often display similar, if not identical, DNA-binding specificities. An important question, therefore, about the transcriptional control of developmental processes is how homeodomain proteins acquire these different transcriptional regulatory specificities.

Examples of two homeodomain proteins that display the same DNA-binding specificities but activate transcription differently are the broadly expressed Oct-1 and the cell-specifically expressed (e.g., B cells) Oct-2 transcription factors (reviewed in reference 11). These two proteins recognize the 8-bp octamer sequence ATGCAAAT and contain very similar POU DNA-binding domains. The POU domain is a bipartite DNA-binding domain containing an amino-terminal POU-specific domain (POU_S domain), which recognizes the ATGC portion of the octamer sequence, tethered by a hypervariable linker to a carboxy-terminal POU-type homeodomain (POU_H domain), which recognizes the AAAT portion of the octamer sequence (reviewed in reference 12).

Studies of Oct-1 and Oct-2 have revealed at least two mechanisms by which these two similar proteins can activate different promoters. One mechanism is through activation domains that display different promoter-selective properties (7, 36). The second mechanism is through the selective association of Oct-1, but not Oct-2, with the herpes simplex virus (HSV) transactivator VP16 (also known as Vmw65, VF65, or αTIF) (9, 16, 33), resulting in recruitment to a new regulatory site (6).

VP16 is an HSV virion protein that upon infection associates with Oct-1 and a second cellular factor, called HCF (or C1, VCAF, or CFF), to form a multiprotein-DNA complex—the VP16-induced complex—that activates transcription of HSV immediate-early (IE) promoters (reviewed in references 25 and 37). VP16 alters the transcriptional regulatory properties of Oct-1 in at least two ways: it provides Oct-1 with a potent activation domain for mRNA-type promoters (6, 38), and it recruits Oct-1, but not Oct-2, to promoters that are otherwise responsive to neither Oct-1 nor Oct-2 (6). Thus, the HSV VP16 protein is a homeodomain coregulator that imparts transcriptional regulatory specificity to proteins that, on their own, display the same DNA-binding specificity.

The selective association of Oct-1 with VP16 is directed by the Oct-1 homeodomain (33) and results primarily from a single amino acid difference on the exposed surface of the DNA-bound Oct-1 and Oct-2 homeodomains (19, 27). HCF greatly stabilizes formation of the complex (9, 16), but it is not known to provide any selectivity to the association of VP16 with Oct-1 and DNA (27, 32, 40).

VP16 provides DNA-binding specificity to formation of the VP16-induced complex because it associates with Oct-1 only on certain Oct-1-binding sites (9, 16, 26). These sites generally conform to the sequence motif TAATGARAT, where R indicates a purine, and fall into two categories (1): those that contain an overlapping Oct-1-binding octamer sequence (**AT GCTAAT**GARAT), which we refer to as ($OCTA^+$)TAATGARAT sites, and others that lack the overlapping octamer sequence, which we refer to as ($OCTA^-$)TAATGARAT sites. The details of how VP16 provides DNA-binding specificity are not known, but it is evident that the GARAT sequence plays a central role because mutations in the GARAT sequence can disrupt VP16-induced complex formation without evidently disrupting the affinity of Oct-1 for the same site (9, 16, 26).

Recent analyses of herpesviruses have revealed a number of VP16 homologs with similar IE promoter activation properties. For example, VP16 homologs from bovine herpesvirus 1 (BHV-1), varicella-zoster virus (VZV), and equine herpesvirus 1 transactivate IE promoters from the cognate virus and can form VP16-containing regulatory complexes on TAATGARAT-like elements (8, 22–24, 29).

Here, we have compared the ability of HSV type 1 (HSV-1) VP16 (H-VP16) and its BHV-1 homolog, called BHV-1 αTIF (referred to here as B-VP16), to form VP16-induced complexes on and to stimulate transcription from HSV-1- and BHV-1-derived VP16 response elements. We have found that these two VP16 homologs differentiate between the Oct-1 and

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† Dedicated to the memory of Yakov Gluzman, a colleague and friend.

Oct-2 homeodomains in the same manner. In contrast, however, they recognize and activate transcription from different VP16 response elements by discriminating a novel regulatory element that lies outside the TAATGARAT sequence. These results exemplify how, through selective association with homologous but differing homeodomain coregulators, a single homeodomain protein can direct differential transcriptional regulation.

MATERIALS AND METHODS

Wild-type and mutant DNA-binding sites for electrophoretic mobility retardation assay. H-TAAT(O^+) and H-TAAT(O^-) were described previously as ICP0 (33) and ($OCTA^-$)TAAT (6), respectively. B-TAAT, B-TAAT($5'_H$), and H-TAAT($5'_B$) were cloned into the pUC119 *Bam*HI site by ligating each annealed pair of complementary oligonucleotides: B-TAAT, GATCGCTCCTAT TATAATGAGCTTGGCGGC and GATCGCCGCCAAGCTCATTATAATA GGAGC (the IE-1 probe in reference 22); B-TAAT($5'_H$), GATCCCGTGCA TGCTAATGAGCTTGGCGGC and GATCGCCGCCAAGCTCATTAGCA TGCACGG; and H-TAAT($5'_B$), GATCGCTCCTATTATAATGATATTCTT TGG and GATCCCAAAGAATATCATTATAATAGGAGC.

All of the other mutant DNA-binding sites were created by oligonucleotide-directed mutagenesis (18, 42), using the above-described constructs as parental templates. H-TAAT(O^+) was used to generate (i) the TA-to-GC substitution in the H-TAAT(G_B) GARAT swap, (ii) the CTT-to-GGC substitution in the H-TAAT(D_B) D segment swap, and (iii) the T-to-G substitution for H-TAAT$_P$. B-TAAT was used to generate (i) the GC-to-TA substitution in the B-TA AT(G_H) GARAT swap and (ii) the GGC-to-CTT substitution in the B-TA AT(D_H) D segment swap. B-TAAT($5'_H$) was used to generate the C-to-A substitution in B-TAAT$_P$. H-TAAT$_P$ was used to generate the CTT-to-GGC substitution in the H-TAAT$_P$(D_B) D segment swap, and B-TAAT$_P$ was used to generate the GGC-to-CTT substitution in the B-TAAT$_P$(D_H) D segment swap. The H-TAAT$_P$ and B-TAAT$_P$ sites were similarly used to generate the single-base-pair substitutions described in Fig. 5.

Reporter plasmid constructs. For in vivo transcription assays, 66-nucleotide single-stranded oligonucleotides, containing two tandem copies of each VP16 response element linked by an *Xho*I recognition site and ending with sequences compatible for ligation to an upstream (relative to the transcriptional start site) *Kpn*I site and a downstream *Xba*I site, were ligated into *Kpn*I- and *Xba*I-digested p$\beta\Delta^{36}$, an enhancerless (enh^-) β-globin reporter construct (35). The VP16 response element sequences were as follows: H-TAAT, CTAGCCAAAGAATATCATTA GCATGCACGGCTCGAGCCAAAGAATATCATTAGCATGCACGGGTAC; H-TAAT$_P$, CTAGCCAAAGAATCTCATTAGCATGCACGGCTCGAGCCAA AGAATCTCATTAGCATGCACGGGTAC; H-TAAT$_P$(D_B), CTAGCCAGCC AATCTCATTAGCATGCACGGCTCGAGCCAGCCAATCTCATTAGCAT GCACGGGTAC; B-TAAT, CTAGGCCGCCAAGCTCATTATAATAGGAG CCTCGAGGCCGCCAAGCTCATTATAATAGGAGCGTAC; B-TAAT$_P$, CT AGGCCGCCAATCTCATTAGCATGCACGGCTCGAGGCCGCCAATCT CATTAGCATGCACGGGTAC; and B-TAAT$_P$(D_H), CTAGGCCAAGAAT CTCATTAGCATGCACGGCTCGAGGCCAAGAATCTCATTAGCATG CACGGGTAC.

H-VP16, B-VP16, and Oct-1 expression vectors. The H-VP16 expression vector for protein purification from *Escherichia coli* was pET11c-GST-VP16ΔC (19). The wild-type Oct-1 POU domain (Ho1 wild type) was expressed from pET11c.G.POU-1 (2), and the constructs to express the POU domain mutants Ho1(E22A), Ho2, and Ho2(A22E) were as described by Lai et al. (19). For B-VP16 expression in *E. coli*, an *Spe*I-*Bam*HI fragment from pcBTIF (3) was cloned between the same *Xba*I and *Bam*HI sites of the pET11c-GST vector as for the other constructs to create pET11c-GST-BTIF. The glutathione *S*-transferase (GST) fusion proteins were expressed in *E. coli* BL21(DE3) cells and purified by affinity chromatography over glutathione-agarose beads (2). The effectors for the in vivo transcription assays were pCGNBTIF for B-VP16, in which the same *Spe*I-*Bam*HI B-VP16 fragment was cloned into the *Xba*I and *Bam*HI sites of pCGN, and pCGNVP16 (6) for H-VP16.

Electrophoretic mobility retardation assay for VP16-induced complex formation. Protein-DNA binding reactions were performed as previously described (19). The DNA probes were prepared by PCR as previously described (2). A fractionated human HeLa cell nuclear extract enriched for HCF and Oct-1 (the HCF/Oct-1 wheat germ agglutinin fraction) was a kind gift from A. C. Wilson (41). The HCF/Oct-1 fraction (~5 ng) was mixed in a 10-μl reaction mixture [10 mM Tris-HCl (pH 7.9), 80 mM KCl, 1 dithiothreitol, 1 mM EDTA, 0.1% Nonidet P-40, 2% glycerol, 2% Ficoll, 3% fetal bovine serum, and 1 μg of poly(dI-dC)] in the absence or presence of purified GST-VP16ΔC (H-VP16) or GST-BTIF (B-VP16) (~20 ng). After incubation at 30°C for 30 min, samples were loaded on a 4% native polyacrylamide gel (acrylamide/bisacrylamide ratio, 39:1) in 1× TGE (20 mM Tris, 0.2 M glycine, 1 mM EDTA [pH 8.3]). After electrophoresis, the gel was dried and subjected to autoradiography. Levels of complex formation were quantitated on a Fuji BAS2000 PhosphorImager. For the experiment with Oct-1- and Oct-2-related POU domains, whose results are shown in Fig. 2, a purified fraction of human HCF free of human Oct-1 (the DNA-cellulose fraction [41]), a kind gift from M. Cleary, was used.

In vivo transcription assays. HeLa cells were seeded at about 5×10^5 cells per 10-cm-diameter dish and transfected after 24 h by calcium phosphate coprecipitation as described previously (34). In cells in which H-VP16 or B-VP16 was coexpressed, 0.5 μg of pCGNVP16 (H-VP16) or pCGNBTIF (B-VP16) was cotransfected with 2 μg of reporter constructs and the internal reference plasmid pα4x (A+C) (100 ng with pCGNVP16 or 25 ng with pCGNBTIF). To assay reporter gene expression, cytoplasmic mRNA was collected by cell lysis with Nonidet P-40 and subsequently analyzed by RNase protection (36). The probes used for RNA hybridization were the α-globin α98 and β-globin β134 probes (6). For pCGNVP16-transfected samples, the α98 and β134 probes had equal specific activities. For pCGNBTIF-transfected samples, the α98 probe had an eightfold-lower specific activity than the β134 probe. Unprotected RNA was digested by RNases A and T_1, and the protected fragments were visualized after electrophoresis through a 6% denaturing gel. Levels of reporter gene expression were quantitated on a Fuji BAS2000 PhosphorImager and normalized to the level of expression of the internal reference α-globin RNA.

RESULTS

Misra et al. (22) showed that B-VP16 (BHV-1 αTIF) can activate transcription of a BHV-1 IE gene, and they identified a B-VP16-responsive *cis*-regulatory element bearing similarity to the HSV TAATGARAT motif. Furthermore, they showed that, like H-VP16 on HSV-1 TAATGARAT sites, B-VP16 can induce a multiprotein-DNA complex on the BHV-1 TAATGARAT-like element. To study the structure and function of VP16, we compared the activities of the related (33% identical) H-VP16 and B-VP16 proteins. Our initial experiments failed, however, because B-VP16 expressed in and purified from *E. coli* did not form a VP16-induced complex on HSV-1 TAATGARAT sites. We therefore assayed B-VP16-induced complex formation on the BHV-1 TAATGARAT-like site described by Misra et al. (22).

Differential VP16-induced complex formation by H-VP16 and B-VP16 on different VP16 response elements. Figure 1 shows a comparison of B-VP16- and H-VP16-induced complex formation on two HSV-1-derived VP16 response elements and one BHV-1-derived VP16 response element. The bottom of Fig. 1 shows the sequences of the two HSV-derived TAATGARAT elements, an ($OCTA^+$)TAATGARAT site [called H-TAAT(O^+) or simply H-TAAT in later experiments] and an ($OCTA^-$)TAATGARAT site [called H-TAAT(O^-)], and the BHV-1-derived site. The three sites are aligned at a shared TAAT sequence, which is a consensus homeodomain binding site, and at a 3′ flanking GARAT-related sequence.

In an electrophoretic mobility retardation assay with human HeLa cell Oct-1 and HCF, Oct-1 bound all three sites, although with different affinities (Fig. 1, lanes 4 to 6). As shown previously (2), Oct-1 bound better to the octamer-containing H-TAAT(O^+) site than to the H-TAAT(O^-) site (Fig. 1, lanes 4 and 5); curiously, although the B-TAAT site does not contain a consensus octamer sequence, it bound to Oct-1 as well as or even better than to the HSV-1 H-TAAT(O^+) site (lane 6). Nevertheless, H-VP16 formed a VP16-induced complex only on the HSV-1-derived sites (Fig. 1, compare lanes 7 to 9). In contrast, B-VP16 formed a VP16-induced complex only on the BHV-1-derived B-TAAT site (Fig. 1, compare lanes 10 to 12).

Analysis with HCF (41)- and Oct-1 (20)-specific antibodies showed that, like the H-VP16-induced complex, the B-VP16-induced complex contains both HCF and Oct-1 (data not shown). Thus, the bovine viral B-VP16 protein can form a VP16-induced complex with human Oct-1 and HCF, but formation of the complex is specific to the BHV-1-derived TAATGARAT-like element. Consistent with this result, we observed the same patterns of H-VP16- and B-VP16-induced complex formation with a bovine cell extract (data not shown). These results suggest that the two different VP16 proteins can

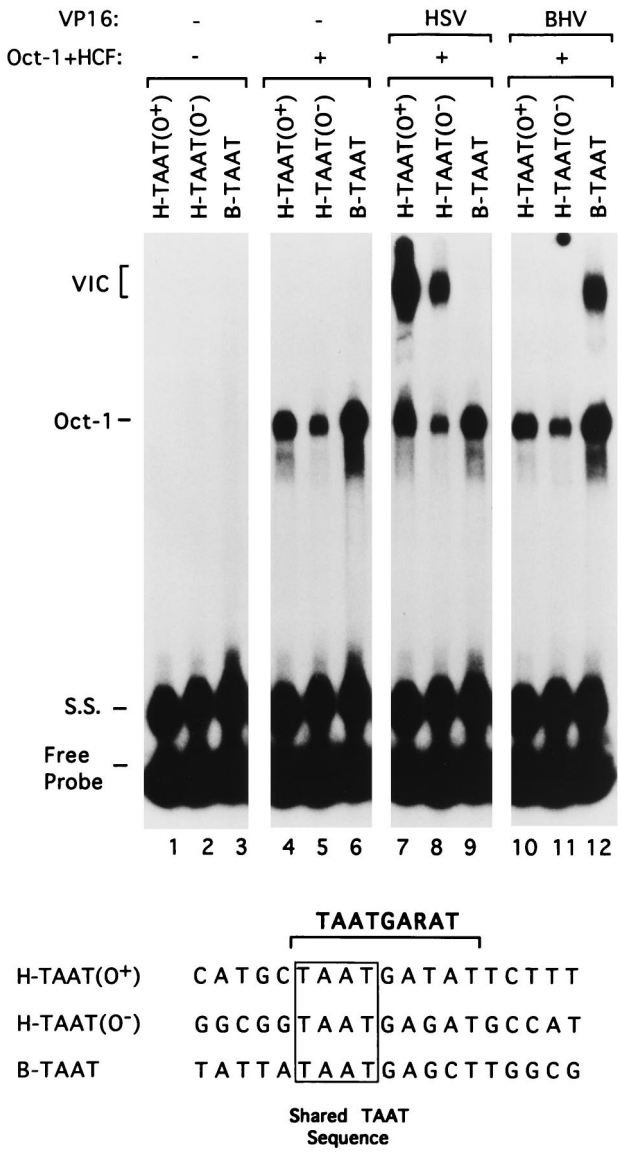


FIG. 1. HSV-1 (H-VP16) and BHV-1 (B-VP16) VP16-induced complex formation on HSV-1- and BHV-1-derived VP16 response elements. An electrophoretic mobility retardation assay was performed with the probes listed at the top of each lane in the absence (lanes 1 to 3) or presence (lanes 4 to 12) of partially purified human Oct-1 and HCF and of GST–H-VP16 (lanes 7 to 9) or GST–B-VP16 (lanes 10 to 12). The positions of the VP16-induced complex (VIC), the Oct-1–DNA complex (Oct-1), the free probes, and denatured single-stranded DNAs (S.S.) are indicated at the left. The sequences of the two HSV-1-derived probes [H-TAAT(O^+) and H-TAAT(O^-)] and the BHV-1-derived probe (B-TAAT) are aligned at the bottom, with the TAATGARAT and shared TAAT (box) sequences indicated.

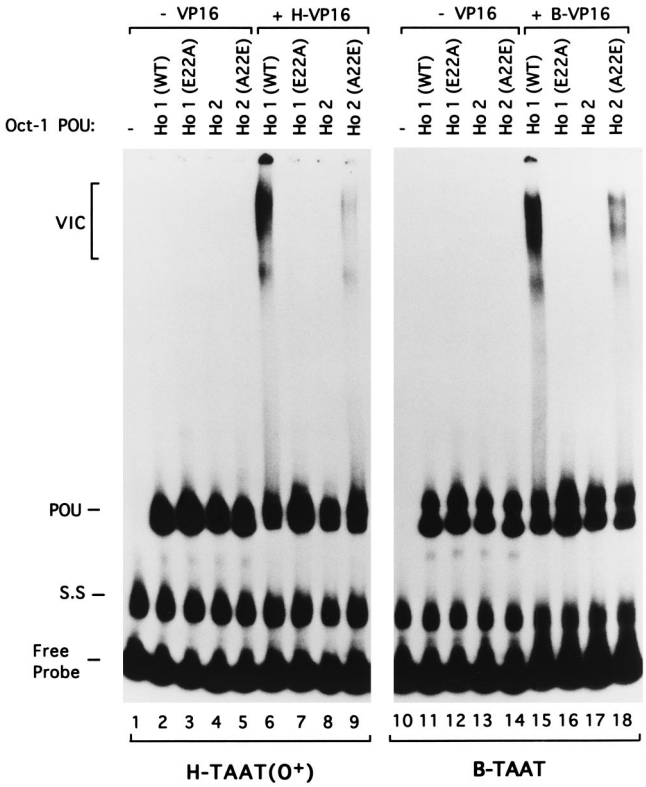


FIG. 2. B-VP16 discriminates between a single amino acid difference on the surface of the Oct-1 and Oct-2 homeodomains. An electophoretic mobility retardation assay was performed with H-TAAT(O^+) (lanes 1 to 9) or B-TAAT (lanes 10 to 18) DNA probes, purified human HCF (DNA-cellulose fraction), and either no POU protein (lanes 1 and 10), the wild-type Oct-1 POU domain (lanes 2, 6, 11, and 15), the Oct-1 POU domain with the E22A amino acid substitution (lanes 3, 7, 12, and 16), the Oct-1 POU domain carrying the Oct-2 homeodomain (lanes 4, 8, 13, and 17), or the corresponding A22E amino acid substitution (lanes 5, 9, 14, and 18), either in the absence of a fusion protein (lanes 2 to 5 and 10 to 14) or in the presence of the GST–H-VP16 (lanes 6 to 9) or GST–B-VP16 (lanes 15 to 18) fusion protein. The positions of the VP16-induced complex (VIC), the POU domain-DNA complex (POU), the free probes, and denatured single-stranded DNAs (S.S.) are indicated at the left. The reason for the POU domain and VIC doublets with the B-TAAT probe is not known.

each associate with human and bovine Oct-1 and HCF but differ in their VP16 response element recognition properties.

Like the HSV-1 VP16 protein, the BHV-1 VP16 protein discriminates between the Oct-1 and Oct-2 homeodomains. The H-VP16 protein forms a complex with Oct-1, but not Oct-2, largely because of one of seven amino acid differences on the surfaces of the Oct-1 and Oct-2 homeodomains: a glutamic acid residue at position 22 in the Oct-1 homeodomain in place of an alanine residue at this position in the Oct-2 homeodomain (19, 27). Because B-VP16 differs from H-VP16 in its recognition of different VP16 response elements, we asked whether the B-VP16 protein differs from the H-VP16 protein in its discrimination of the Oct-1 and Oct-2 homeodomains.

As shown in Fig. 2, we compared the abilities of H-VP16 (lanes 1 to 9) and B-VP16 (lanes 10 to 18) to form a VP16-induced complex with four related POU domains: the Oct-1 POU domain carrying either the wild-type Oct-1 (Ho1) or Oct-2 (Ho2) homeodomain, or Oct-1 or Oct-2 homeodomains in which the critical residue at position 22 has been exchanged [Ho1(E22A) and Ho2(A22E), respectively]. In this experiment, to study H-VP16 and B-VP16 association with the recombinant Oct-1- and Oct-2-related POU domains, we used purified HCF free of endogenous Oct-1 protein. In the absence of either H-VP16 (Fig. 2, lanes 2 to 5) or B-VP16 (lanes 11 to 14), the four POU domains recognize the H-TAAT(O^+) and B-TAAT probes similarly, consistent with the similar DNA-binding properties of Oct-1 and Oct-2 (2, 31). As previously described (19, 27), H-VP16 recognizes the wild-type Oct-1 homeodomain (Ho1; Fig. 2, lane 6) and, with somewhat reduced efficiency, the Oct-2 homeodomain carrying the Oct-1 glutamic acid residue at position 22 [Ho2(A22E); lane 9)], but it does not recognize the other two homeodomains, Ho2 and Ho1(E22A) (lanes 7 and 8, respectively).

These four POU domains display the same abilities to form a VP16-induced complex with B-VP16 on the B-TAAT site as they do with H-VP16 on the H-TAAT(O^+) site: the proteins carrying the Oct-1 homeodomain (Fig. 2, lane 15) and, to a lesser extent, the Oct-2 homeodomain with the critical glutamic acid residue (lane 18) associate with B-VP16, but the other Oct-2-related homeodomains do not (lanes 16 and 17). These results indicate that, although their *cis*-regulatory element recognition properties differ, the homeodomain recognition properties of these two VP16 proteins are similar, if not identical.

Sequences 3′ of the TAATGARAT-like consensus sequence determine differential VP16 response element recognition by the H-VP16 and B-VP16 proteins. To identify the parts of the HSV- and BHV-derived VP16 response elements that are responsible for their differential recognition by the H-VP16 and B-VP16 proteins, we exchanged three regions of the two different VP16 response elements: sequences 5′ of the shared TAAT sequence, the GARAT segment, and a 3-bp segment 3′ of the GARAT segment. Figure 3 shows the results of such an experiment, in which, as in Fig. 4 (discussed below), only the Oct-1–DNA and VP16-induced complexes are shown. Figure 3B shows the sequences of the different elements and summarizes the averaged quantitative results of two experiments, including those of the one shown in Fig. 3A.

When we exchanged the sequences 5′ of the shared TAAT sequence in a 5′-half swap [the H-TAAT($5'_B$) and B-TAAT ($5'_H$) constructs (Fig. 3A, lanes 7 to 12)], exchange of the perfect ATGC Oct-1 POU_S-binding site in the HSV-1 H-TAAT site with the ATTA sequence in the BHV-1 B-TAAT site improved Oct-1 binding to the B-TAAT site (compare lanes 2 and 8) and slightly impaired Oct-1 binding to the H-TAAT site (compare lanes 1 and 7; see also the quantitation in Fig. 3B). Although the Oct-1 affinities for these sites change, the specificity of VP16-induced complex formation remains the same: H-VP16 recognizes the H-TAAT($5'_B$) site, and B-VP16 recognizes the B-TAAT($5'_H$) site (compare lanes 3 to 6 and lanes 9 to 12). The changes in the levels of the VP16-induced complex probably reflect the altered affinities of these sites for Oct-1 (Fig. 3B). Thus, these two VP16 proteins apparently do not discriminate between the sequences 5′ of the shared TAAT sequence. We therefore asked whether the two VP16 proteins discriminate between sequences 3′ of the TAAT sequence.

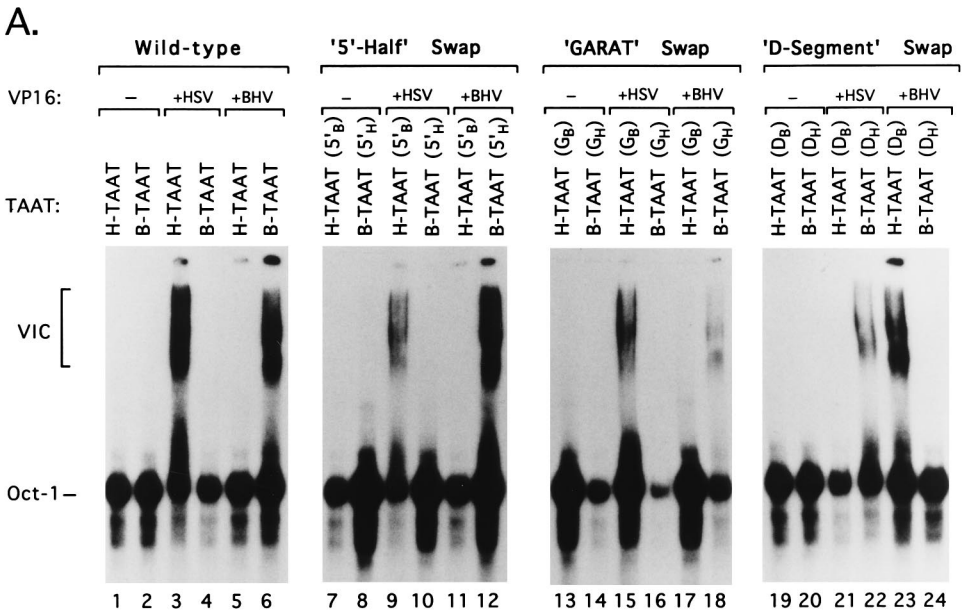
We first exchanged the GARAT segments, which are known to be important for HSV-1 VP16-induced complex formation (9, 16, 26). We refer to the GARAT segments of these two VP16 response elements as G_H for the HSV-1 H-TAAT(O^+) site and G_B for the B-TAAT site. The 5-bp G_H and G_B segments differ at two positions (GA<u>T</u>AT in G_H versus GA<u>G</u><u>C</u>T in G_B). Like the 5′ swap, the GARAT swap—sites H-TAAT(G_B) and B-TAAT(G_H)—affects the affinity of Oct-1 for these two sites, except that here the BHV-1-derived GARAT segment improves binding to the H-TAAT site (Fig. 3A, compare lanes 1 and 13) and the HSV-1-derived GARAT segment impairs binding to the B-TAAT site (compare lanes 2 and 14; see also Fig. 3B). To our surprise, however, the exchange of the important GARAT sequence did not alter the general pattern of VP16-induced complex formation: H-VP16 bound to the HSV-1 H-TAAT(G_B) site containing the BHV-1 GARAT segment (Fig. 3A, lane 15), and B-VP16 bound to the BHV-1 B-TAAT(G_H) site containing the HSV-1 GARAT segment (lane 18). The only differences we observed are quantitative differences in the levels of VP16-induced complex formation that follow the changes in affinity for Oct-1 (Fig. 3B). Thus, although the GARAT segments influence the precise affinities of the VP16 proteins for these two sites, they are not the major determinant for the selective association of these two VP16 proteins with these two sites.

We next exchanged a 3-bp sequence 3′ of the GARAT segment that differs in the HSV-1 and BHV-1 sites: CTT in the HSV-1 site and GGC in the BHV-1 site. As shown in Fig. 3B, we refer to this 3-bp sequence as the D (for determinant) segment. Figure 3A (lanes 19 to 24) shows the effects of exchanging the 3-bp D segment. In contrast to the other exchanges, the D segment exchange in sites H-TAAT(D_B) and B-TAAT(D_H) had little, if any, effect on Oct-1 binding affinity (compare lanes 1 and 2 and lanes 19 and 20; see also Fig. 3B), which is consistent with their position 7 bp distal to the Oct-1-binding octamer sequence. There was, however, a dramatic effect on the binding of the VP16 proteins: H-VP16 bound the B-TAAT(D_H) site (Fig. 3A, lane 22) but not the H-TAAT(D_B) site (lane 21), whereas B-VP16 bound the H-TAAT(D_B) site (lane 23) but not the B-TAAT(D_H) site (lane 24) (see also Fig. 3B). Thus, in their natural context, sequences outside the TAATGARAT sequence can have a profound influence on the selectivity of VP16-induced complex formation.

Multiple residues in the D segment can influence the selectivity of H-VP16- and B-VP16-induced complex formation on idealized TAATGARAT sites. To determine the minimal differences between VP16 response elements that can support differential association of the H-VP16 and B-VP16 proteins with Oct-1, we created a matched set of H-VP16 and B-VP16 complex formation sites, in which the TAATGARAT and 5′ flanking sequences are identical. To do this, we chose the wild-type octamer-containing H-TAAT(O^+) site [H-TAAT (WT)] and the B-TAAT($5'_H$) site, as shown in Fig. 4. These two sites contain identical sequences 5′ of the shared TAAT sequence; they each differ, however, from the consensus GARAT sequence at one position [GA<u>T</u>AT in the H-TAAT(WT) site and GAG<u>C</u>T in the B-TAAT($5'_H$) site]. We therefore first mutated each of these sites to a perfect GARAT consensus sequence (GAGAT) to create the perfect TAATGARAT H-$TAAT_P$ and B-$TAAT_P$ sites, as shown in Fig. 4.

The conversion to the perfect GARAT consensus sequence had only a small positive effect on H-VP16- and B-VP16-induced complex formation on the H-TAAT and B-TAAT sites, respectively (Fig. 4, compare lanes 7 and 8 and lanes 16 and 17). The improved VP16 binding to the H-$TAAT_P$ site may reflect the slight improvement in Oct-1 binding to the H-$TAAT_P$ site (Fig. 4, compare lanes 1 and 2). Importantly, even with idealized TAATGARAT sequences to which Oct-1 binds with similar if not identical affinities (compare lanes 2 and 5), H-VP16 and B-VP16 still discriminate between the two sites (compare lanes 8 and 11 and lanes 14 and 17). Furthermore, exchange of the 3-bp D segment between the H-$TAAT_P$ and B-$TAAT_P$ sites switches the specificity for VP16-induced complex formation (Fig. 4, compare lanes 8, 9, 11, and 12 and lanes 14, 15, 17, and 18) with little obvious effect on Oct-1 binding to these sites on its own (compare lanes 2, 3, 5, and 6). These results indicate that sequences flanking a common homeodomain protein-binding site, in this case the Oct-1-binding site, can selectively influence the binding of homologous homeodomain coregulators, the HSV-1 and BHV-1 VP16 proteins.

We next assayed the influence of individual D segment and neighboring base pairs on H-VP16- and B-VP16-induced complex formation in the context of the closely related H-$TAAT_P$ and B-$TAAT_P$ sites, as summarized in Fig. 5. In this figure, the histograms show the relative binding of each site to H-VP16 and B-VP16. Residues 3′ of the D segment that differ between the H-$TAAT_P$ and B-$TAAT_P$ sites (positions 4 and 6; T<u>G</u><u>G</u>G



B.

Wild Type	Octamer / GARAT / D	Oct-1 Binding	Complex Formation With H-VP16	Complex Formation With B-VP16
H-TAAT	CCGTGCATGCTAATGATATTCTTTGGG	1	1	(-)
B-TAAT	GCTCCTATTATAATGAGCTTGGCGGCG	1.3	(-)	0.9
'5'-Half' Swap				
H-TAAT ($5'_B$)	GCTCCTATTATAATGATATTCTTTGGG	0.6	0.3	(-)
B-TAAT ($5'_H$)	CCGTGCATGCTAATGAGCTTGGCGGCG	3.2	(-)	1.5
'GARAT' Swap				
H-TAAT (G_B)	CCGTGCATGCTAATGAGCTTCTTTGGG	2.2	0.5	(-)
B-TAAT (G_H)	GCTCCTATTATAATGATATTGGCGGCG	0.4	(-)	0.2
'D-Segment' Swap				
H-TAAT (D_B)	CCGTGCATGCTAATGATATTGGCTGGG	1.1	(-)	1.0
B-TAAT (D_H)	GCTCCTATTATAATGAGCTTCTTGGCG	1.2	0.4	(-)

FIG. 3. A 3-bp segment outside the TAATGARAT-like consensus sequence determines differential VP16 response element recognition by the H-VP16 and B-VP16 proteins. (A) An electophoretic mobility retardation assay was performed with the DNA probes listed above each lane and shown in panel B, with enriched human Oct-1 and HCF and either no additional proteins (lanes 1, 2, 7, 8, 13, 14, 19, and 20) or the GST–H-VP16 (lanes 3, 4, 9, 10, 15, 16, 21, and 22) or GST–B-VP16 (lanes 5, 6, 11, 12, 17, 18, 23, and 24) fusion protein. The Oct-1-induced and VP16-induced (VIC) complexes are shown; the excess free probe is not included. (B) Sequences of the H-TAAT [H-TAAT(O^+)]- and B-TAAT-related probes used in panel A, with the positions of octamer, GARAT, and D segment sequences identified by brackets. BHV-1-derived sequences are shaded. Quantitations (averaged from two experiments) of Oct-1 binding relative to Oct-1 binding to the H-TAAT site and of H-VP16- and B-VP16-induced complex formation relative to levels of H-VP16-induced complex formation on the H-TAAT site are shown to the right of each sequence. The two experiments gave very similar results. (−), undetectable.

in the H-$TAAT_P$ site and GGCG in the B-$TAAT_P$ site) do not apparently contribute to the specificity of H-VP16- and B-VP16-induced complex formation (compare samples 1 and 2 in Fig. 5A with samples 2 and 1 in Fig. 5B, respectively). In contrast, however, the three single-base-pair exchanges at each of the positions 1 to 3 affect H-VP16- and B-VP16-induced complex formation (compare samples 3 to 5 with sample 1 in Fig. 5). These results delimit the D segment to these three residues.

The effects of the single-base-pair exchanges differ, however, from those of the triple-base-pair D segment exchanges. For example, in the H-$TAAT_P$ site (Fig. 5A), exchange of any one of the three D_H segment residues for its corresponding D_B segment residue does not switch the VP16 protein-binding specificity; in each case H-VP16 still binds better than B-VP16 (compare samples 3 to 5 with samples 1 and 2 in Fig. 5A). In contrast, with the B-$TAAT_P$ site, exchanges of single base pairs at positions 1 and 2 of the D_B segment ($G_1 \rightarrow C$ and $G_2 \rightarrow T$)

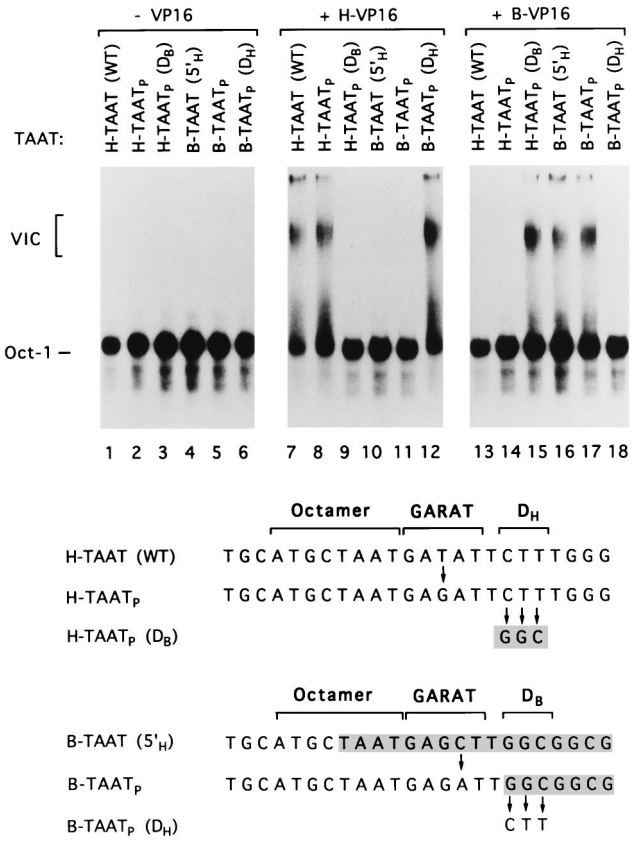


FIG. 4. Comparison of VP16-induced complex formation by H-VP16 and B-VP16 proteins on matched sites that differ only at the D segment. An electophoretic mobility retardation assay was performed with the probes listed at the top of each lane in the presence of partially purified human Oct-1 and HCF alone (lanes 1 to 6) or with added GST–H-VP16 (lanes 7 to 12) or GST–B-VP16 (lanes 13 to 18). The Oct-1-induced and VP16-induced (VIC) complexes are shown; the excess free probe is not shown. The sequences of the H-TAAT(WT) [H-TAAT(O^+)] and related H-TAAT$_P$ and H-TAAT$_P$(D_B) probes and of the B-TAAT(5'$_H$) and related B-TAAT$_P$ and B-TAAT$_P$(D_H) probes are shown at the bottom. BHV-1-derived sequences are shaded.

result in the loss of discrimination by the H-VP16 and B-VP16 proteins: the H-VP16 and B-VP16 proteins both bind the position 1 exchange $G_1 \rightarrow C$ well (compare samples 1 and 3 in Fig. 5B), whereas they both bind the position 2 exchange $G_2 \rightarrow T$ poorly (see sample 4 in Fig. 5B). Thus, no one D segment residue is completely responsible for the discrimination of the D_H and D_B sequences by H-VP16 and B-VP16. Curiously, H-VP16 and B-VP16 appear to be most sensitive to exchanges at different positions in the D segment: H-VP16 is most sensitive to the exchange at position 1 (compare the black bars in samples 3 to 5 in Fig. 5), whereas B-VP16 is most sensitive to the exchanges at position 2 (compare the shaded bars in samples 3 to 5 in Fig. 5).

The D segment of VP16 response elements regulates the differential response to transcriptional activation by H-VP16 and B-VP16. The studies described above demonstrate that the differential abilities of H-VP16 and B-VP16 to bind to VP16 response elements are determined by the D segment. To determine whether these effects on binding correspond to changes in transcriptional activation, we assayed the in vivo response of a set of VP16 response elements to H-VP16 and B-VP16. We chose three HSV-1-related and three BHV-1-related VP16 response elements: the wild-type H-TAAT(O^+) and B-TAAT sites and the matched consensus TAATGARAT H-TAAT$_P$ and B-TAAT$_P$ sites and their respective D segment swaps, H-TAAT$_P$(D_B) and B-TAAT$_P$(D_H) (described in Fig. 4).

Two tandem copies of each TAATGARAT element were placed upstream of a minimal TATA box-containing β-globin promoter (35). To assay the transcriptional response to VP16, we transfected HeLa cells with the β-globin reporter plasmids together with an α-globin gene-containing internal reference plasmid and H-VP16 or B-VP16 expression vectors. The results of such an experiment are shown in Fig. 6. Transcriptional activities from this experiment were normalized to activation of the wild-type H-TAAT site with H-VP16 in Fig. 6A and to activation of the wild-type B-TAAT site with B-VP16 in Fig. 6B. In the absence of a VP16 expression vector, none of these seven reporters was active (data not shown), and in the presence of either H-VP16 or B-VP16, the reporter lacking VP16 response elements (enh^-) was essentially inactive (Fig. 6, lanes 1).

On the wild-type HSV-1 and BHV-1 VP16 response elements, the H-VP16 and B-VP16 proteins display selective activation. H-VP16 is active on the wild-type H-TAAT site (Fig. 6A, lane 2) but not on the wild-type B-TAAT site (lane 5), whereas B-VP16 is active on the wild-type B-TAAT site (Fig. 6B, lane 5) but relatively inactive on the wild-type H-TAAT site (lane 2). Thus, the two homologous VP16 proteins display different *cis*-regulatory specificities in vivo.

Conversion of the wild-type H-TAAT and B-TAAT sites to the corresponding consensus TAAT$_P$ site generally improved the response to both VP16 proteins, albeit to differing extents. Thus, both H-VP16 and B-VP16 are more active on both the H-TAAT$_P$ and B-TAAT$_P$ sites than on the wild-type H-TAAT and B-TAAT sites (compare lanes 2 and 3 and lanes 5 and 6 in Fig. 6). The activity of H-VP16 on the B-TAAT$_P$ site and of B-VP16 on the H-TAAT$_P$ site contrasts with the lack of VP16 binding observed with these combinations in Fig. 4 (lanes 11 and 14), suggesting that the in vivo VP16 response assay is more sensitive than the in vitro complex formation assay, particularly with respect to a perfect GARAT sequence. With both VP16 proteins, however, the specificity of transcriptional activation does not change: H-VP16 is fivefold more active on the HSV-related H-TAAT$_P$ site than on the B-TAAT$_P$ site (compare lanes 3 and 6 in Fig. 6A), and B-VP16 is still twofold more active on the BHV-related B-TAAT$_P$ site than on the H-TAAT$_P$ site (compare lanes 6 and 3 in Fig. 6B).

The determinant for this selective transcriptional activation is the D segment. H-VP16 activates transcription 16-fold better from the D_H-containing H-TAAT$_P$ site than from the D_B-containing H-TAAT$_P$(D_B) site (compare lanes 3 and 4 in Fig. 6A) and does so 7-fold better from the D_H-containing B-TAAT$_P$(D_H) site than from the D_B-containing B-TAAT$_P$ site (compare lanes 7 and 6). Conversely, albeit less dramatically, B-VP16 activates transcription two- to threefold better from the D_B-containing H-TAAT$_P$(D_B) site than from the D_H-containing H-TAAT$_P$ site (compare lanes 4 and 3 in Fig. 6B) and does so twofold better on the D_B-containing B-TAAT$_P$ site than on the D_H-containing B-TAAT$_P$(D_H) site (compare lanes 6 and 7). Thus, small changes in very similar VP16 response elements, as small as 3 bp, can confer differential responses by these two homologous homeodomain coregulators.

DISCUSSION

In this study, we have shown that the VP16 proteins from HSV-1 and BHV-1 possess different *cis*-regulatory binding-site specificities. Our results suggest that the different specificities

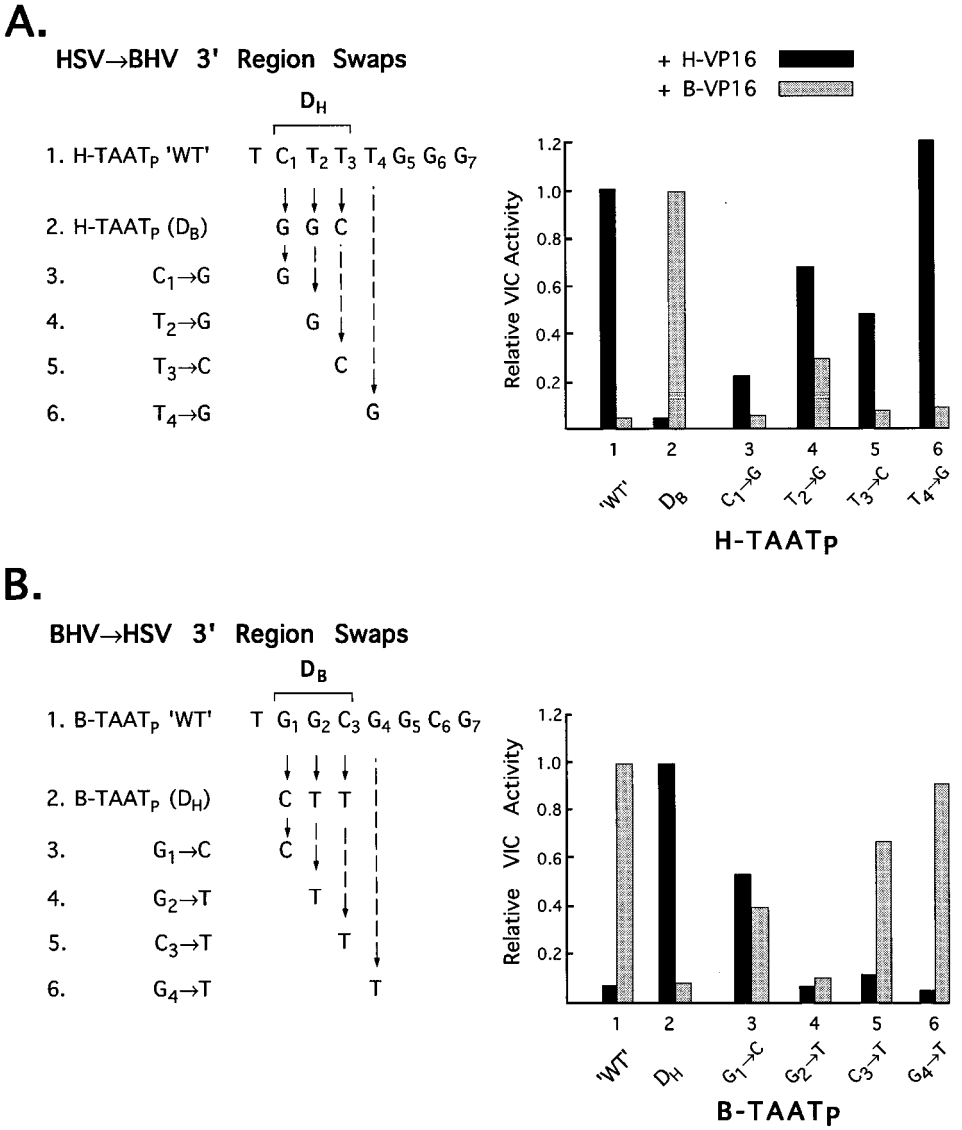


FIG. 5. All three D segment residues influence the selectivity of H-VP16- and B-VP16-induced complex formation. Relative levels of H-VP16 and B-VP16-induced complex formation on H-TAAT$_P$ (A)- and B-TAAT$_P$ (B)-related sites with single-base-pair exchanges are shown. Only the 3′ region sequences of the H-TAAT$_P$ and B-TAAT$_P$ sites are shown; the 5′ sequences that are not shown are identical (compare with Fig. 4). Base pair positions 1 to 7 are indicated in the H-TAAT$_P$ and B-TAAT$_P$ 3′ sequences, and the base pair exchanges are indicated below by arrows. The levels of VP16-induced complex formation by H-VP16 (solid bars) and B-VP16 (shaded bars) are relative to those of H-VP16-induced complex formation on the H-TAAT$_P$′WT′ site. The results shown are the averages of those from three independent electophoretic mobility retardation assays; the results were similar in each assay.

reflect differences in recognition of the *cis*-acting DNA elements rather than differences in how they recognize the Oct-1 homeodomain and discriminate between Oct-1 and Oct-2. Unexpectedly, the DNA sequence responsible for the different VP16 responses does not reside within the TAATGARAT sequence of the VP16 response element but rather is a short 3-bp sequence 3′ of the GARAT sequence; we refer to this 3′ distal determinant segment as the D segment. The different *cis*-regulatory binding-site specificities observed in vitro are also reflected in the qualitative ability of these two VP16 homologs to activate transcription in vivo. These results exemplify how a single transcription factor (e.g., Oct-1) can acquire distinct transcriptional activation properties through association with different but related coregulators.

VP16 molecules can provide different DNA-binding specificities to the VP16-induced complex. The different VP16 response element recognition properties of the HSV-1 and BHV-1 VP16 proteins demonstrate that VP16 can provide different DNA-binding specificities to VP16-induced complex formation. How VP16 provides such specificity, however, is not known with certainty, partly because, on its own, VP16 binds DNA very weakly and the specificity of this weak binding has not been carefully analyzed (17, 32). Two possible explanations for how VP16 provides DNA sequence specificity to VP16-induced complex formation are (i) that VP16 recognizes particular DNA-binding-site-specific conformations of the Oct-1 POU domain, as suggested by Walker et al. (40), and (ii) that VP16 binds DNA directly and discriminates among different DNA sequences. We favor the latter explanation because the precise conformation of the DNA-bound Oct-1 POU domain

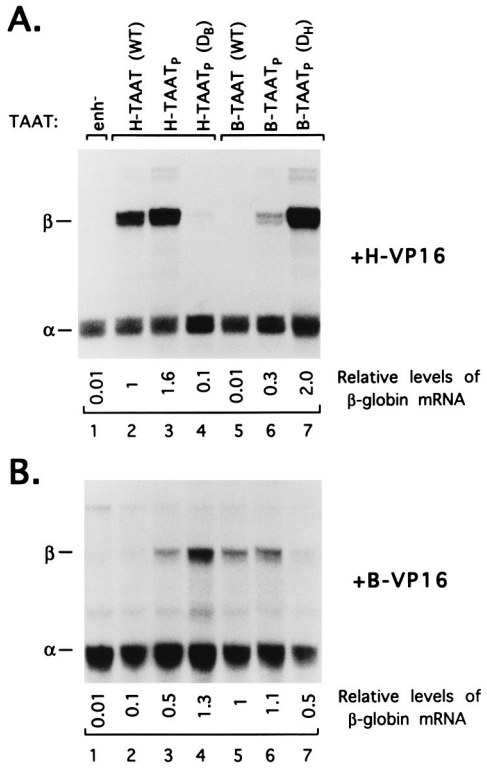


FIG. 6. The VP16 response element D segment regulates the differential response to transcriptional activation by H-VP16 and B-VP16. An in vivo transcription assay was performed with β-globin reporter plasmids carrying either no VP16-response element (enh$^-$) or two tandem copies of the VP16 response element indicated at the top of each lane, an α-globin internal reference plasmid, and either H-VP16 (A) or B-VP16 (B) expression vectors. RNAs were collected at 36 h posttransfection and probed as described in Materials and Methods. The positions of the α-globin (α) and β-globin (β) mRNA-protected probe fragments are shown at the left. Quantitation of the relative β-globin RNA levels is shown below each lane relative to H-TAAT(WT) for H-VP16 and B-TAAT(WT) for B-VP16 after normalization to the α-globin internal reference.

is not critical for VP16-induced complex formation: the Oct-1 POU_S domain can be replaced by a completely different DNA-binding domain (e.g., a zinc finger domain) and still support VP16-induced complex formation and a response to VP16 in vivo (28). Furthermore, direct DNA binding by VP16 can easily explain how VP16 molecules may discriminate among VP16 response elements that differ in D segment sequences—sequences that lie distal to the Oct-1-binding octamer site.

The BHV-1 VP16 protein is not the only VP16 protein to possess a VP16 response element specificity different from that of HSV-1 VP16. As we have shown for the BHV-1 VP16 protein (Fig. 1), Moriuchi et al. (23) have shown that the VZV VP16 homolog (called ORF10) also has DNA-binding-site preferences different from those of HSV-1 VP16. These results suggest that different DNA-binding specificities are a common feature of herpesvirus VP16 proteins. It will be interesting to determine whether, as shown here for the HSV-1 and BHV-1 VP16 proteins, the VZV VP16 protein also differentiates among D segment sequences.

A new element is responsible for selective VP16-induced complex formation. We initially expected that the GARAT element would be the key determinant for selective VP16 association. This expectation was based on the knowledge that the GARAT element is crucial for HSV-1 VP16-induced complex assembly (9, 16, 26) and that the GARAT sequence in the BHV-1 VP16 response element we analyzed differs from that in the HSV-1 TAATGARAT sites we analyzed (Fig. 1). To our surprise, however, although the GARAT element had quantitative effects on VP16 binding in vitro and VP16 response in vivo, it did not prove to be responsible for the qualitative difference in the selectivities of HSV-1 and BHV-1 VP16-induced complex assembly in vitro and activation in vivo; instead, it is the 3-bp D segment that lies 3′ of the GARAT sequence which is responsible for the qualitative difference.

We have defined the D segment by those residues that determine the selective association of the HSV-1 and BHV-1 VP16 proteins with the two VP16 response elements we have characterized. Of the 3 bp thus defined, the most critical residue for HSV-1 VP16-induced complex formation is the cytosine at the first position (CTT) (Fig. 5). Although this residue lies outside the GARAT sequence, it has been shown previously to be conserved among TAATGARAT sites in HSV-1 IE promoters (1, 21) and to be important for HSV-1 VP16-induced complex formation (4, 5). The remaining two residues of the HSV-1 D segment were not previously implicated, however, in VP16-induced complex formation.

Why should sequence determinants for selective H-VP16- and B-VP16-induced complex formation lie outside the TAATGARAT sequence? One explanation is that these sequences are not recognized by Oct-1, the cellular DNA-binding protein with which all of the herpesvirus VP16 proteins seem to interact. By using sequences not recognized by Oct-1, new combinations of VP16 proteins and *cis*-regulatory elements could evolve without affecting the intrinsic DNA-binding properties of the shared cellular DNA-binding protein.

The HSV-1 and BHV-1 VP16 proteins confer differential transcriptional regulatory specificity to a single homeodomain protein. The association of the HSV-1 VP16 protein with Oct-1 but not Oct-2 has served as a model for how two very similar homeodomain proteins can differentially activate transcription (11). The differential association of the HSV-1 and BHV-1 VP16 proteins with the Oct-1 homeodomain shows how differential transcriptional activation by the same homeodomain protein can be achieved through selective association with related coregulators. Figure 7 summarizes these findings: HSV-1 VP16 (H-VP16) associates with Oct-1 on an HSV-1 VP16 response element (H-VRE), but not on a BHV-1 VP16 response element (B-VRE), and activates transcription from the H-VRE-containing promoter more effectively (Fig. 7, top); in contrast, BHV-1 VP16 (B-VP16) associates with Oct-1 on a B-VRE, but not on an H-VRE, and activates transcription from the B-VRE-containing promoter more effectively (Fig. 7, bottom).

Another example in which a single transcription factor can acquire different transcriptional regulatory specificities through selective association with coregulators is the interaction of the yeast α2 homeodomain protein with **a**1 and MCM1 (reviewed in reference 13). In this case, α2 associates with **a**1 to repress haploid-specific genes and with MCM1 to repress **a**-specific genes. The interaction of Oct-1 with the VP16 proteins contrasts with the interactions between α2 and its **a**1 and MCM1 coregulators because the differential transcriptional specificities are achieved through more subtle differences in coregulators.

In contrast to the homologous HSV-1 and BHV-1 VP16 proteins, the **a**1 and MCM1 proteins are very different: **a**1 is a homeodomain protein, and MCM1 is a MADS domain protein. Additionally, whereas the two VP16 proteins recognize just a 3-bp difference between binding sites, the known **a**1/α2 (10) and MCM1/α2 (14, 15) sites differ more in sequence. Last, the two VP16 proteins recognize the same surface of Oct-1, the

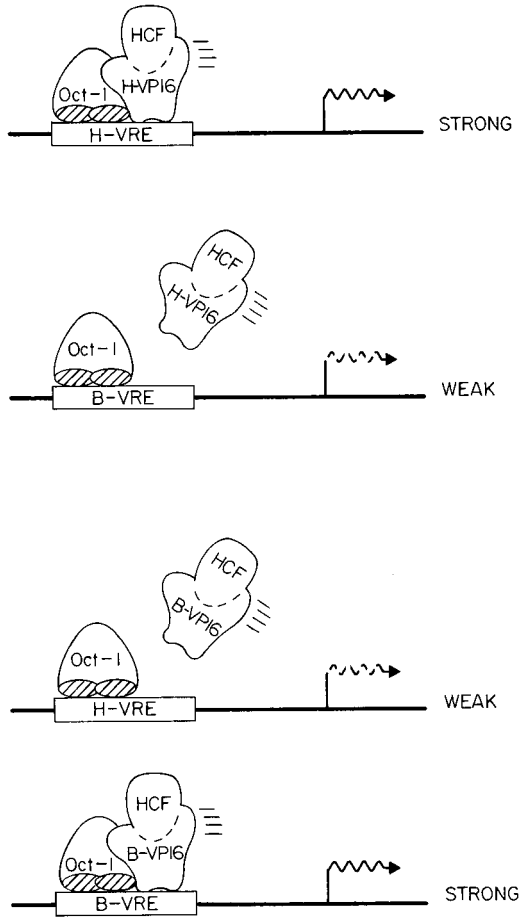


FIG. 7. The HSV-1 and BHV-1 VP16 proteins confer differential transcriptional regulatory specificity to the human Oct-1 homeodomain protein. A graphic representation of HSV-1 VP16 (H-VP16)-induced and BHV-1 VP16 (B-VP16)-induced complexes on HSV-1-derived (H-VRE) and BHV-1-derived (B-VRE) VP16 response elements (open boxes), showing the relative inabilities of an H-VP16–HCF complex to associate with human Oct-1 on a B-VRE and of a putative B-VP16–HCF complex to associate with human Oct-1 on an H-VRE, is shown. The sets of four parallel bars indicate transcriptional activation domains in the VP16 molecules. The relative levels of transcription, either weak or strong, are indicated by the broken or solid wavy arrows, respectively. The hatched ovals in Oct-1 represent the POU_S and POU_H domains.

homeodomain, whereas **a**1 and MCM1 recognize very different regions of α2 that lie outside and on opposite sides of the homeodomain (30, 39). Thus, the association of the Oct-1 homeodomain with the VP16 homologs demonstrates that more closely related coregulators can provide different specificities for transcriptional regulation through differential recognition of even closely related DNA-binding sites.

Why do herpesvirus VP16 proteins display different *cis*-regulatory element specificities? To date, three herpesvirus VP16 proteins, the HSV-1, BHV-1, and VZV VP16 proteins, have been analyzed, and all have been found to interact with Oct-1 and HCF but to differ in their DNA recognition properties (reference 23 and this study). These results suggest that the herpesvirus VP16 proteins are specifically maintaining their specificity for cellular proteins while diverging in their specificity for the viral DNA. We have hypothesized previously that VP16 interacts with the cellular proteins Oct-1 and HCF before initiating the program of viral gene expression because these cellular proteins act as sensors through which the viruses can determine whether the cellular environment is favorable for them to grow lytically or to enter a latent phase (41). By maintaining VP16 interactions with Oct-1 and HCF, the herpesviruses can maintain this gauging mechanism. By changing DNA-binding-site specificities, however, VP16 activators may provide their cognate viruses with a selective advantage by remaining wedded to their specific viral genome.

ACKNOWLEDGMENTS

We are grateful to V. Misra for providing the pcBTIF construct and to M. Cleary, J.-S. Lai, M. Tanaka, and A. Wilson for providing essential reagents and for valuable suggestions. We thank M. Cleary, N. Hernandez, and R. Li for critical readings of the manuscript.

These studies were supported by PHS grant CA13106 from the National Cancer Institute.

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