Differential Control of Transcription by Homologous Homeodomain Coregulators†

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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 (POU5 domain), which recognizes the ATGC portion of the octamer sequence, tethered by a hypervariable linker to a carboxy-terminal POU-type homeodomain (POU4 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 (ATCCTAATGARAT), 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
Oct-2 homeodomains in the same manner. In contrast, however, they recognize and activate transcription from different VP16 response elements by disrupting 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(\textsuperscript{O−}) and H-TAAT(O\textsuperscript{−}) were described previously as ICP\textsubscript{0} (33) and (OCTA\textsuperscript{+})TAAT (6), respectively. B-TAAT, B-TAAT(\textsuperscript{5′})\textsuperscript{+}, and H-TAAT(\textsuperscript{5′})\textsuperscript{+} were cloned into the pUC119 BamHI site by ligating each an un-}

nealed pair of complementary oligonucleotides: B-TAAT, GATGCGCTCTAT TATAATGACGTGCGGACG and GATGCGCCGAAGTCATTTAAATATAT GGACG (the IE-1 probe in reference 22); B-TAAT(\textsuperscript{5′})\textsuperscript{+}, GATGCGCTCTAT TCTTAATGACGTGCGGACG and GATGCGCCGAAGTCATTTAAATATAT TGG and GATCCCGAGAATATCATTTAAATAGGACG.

All of the other mutant DNA-binding sites were created by oligonucleotide-directed mutagenesis (42), using the above-described constructs as parent templates. H-TAAT(\textsuperscript{O−}) was used to generate (i) the TA-to-GC substitution in the H-TAAT(O\textsuperscript{−}) GARAT swap, (ii) the CT-to-GG substitution in the H-TAAT(D\textsubscript{2}) D segment swap, and (iii) the T-to-G substitution for H-TAAT(B\textsuperscript{+}), B-TAAT(D\textsubscript{2}) D segment swap, and (iv) the TA-to-GC substitution in the B-TAAT(D\textsubscript{2}) D segment swap. B-TAAT(\textsuperscript{5′})\textsuperscript{+} was used to generate the CT-to-GC substitution in the B-TAAT(\textsuperscript{5′})\textsuperscript{+} D segment swap. H-TAAT\textsuperscript{−} and B-TAAT\textsuperscript{−} sites were similarly used to generate the single-base-pair substitutions described in Fig. 5.

Reportor plasmid constructs. For in vivo transcription assays, 66-nucleotide single-stranded oligonucleotides, containing two tandem copies of each VP16 base-pair substitutions described in Fig. 5.

The H-TAATP and B-TAATP sites were similarly used to generate the single-base-pair substitutions described in Fig. 5.

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 BHV-1 and H-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 panel of Fig. 1 shows the sequences of the two HSV-derived TAATGARAT elements, an (OCTA\textsuperscript{−})TAATGARAT site [called H-TAAT(O\textsuperscript{−}) or simply H-TAAT in later experiments] and an (OCTA\textsuperscript{−})TAATGARAT site [called H-TAAT(O\textsuperscript{+})] 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 GARKAT-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\textsuperscript{−}) site than to the H-TAAT(O\textsuperscript{+}) 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\textsuperscript{−}) 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 contained 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-VPH16- and B-VP16-induced complex formation with a bovine cell extract (data not shown). These results suggest that the two different VP16 proteins can...
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 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(5′) 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′) and B-TAAT(5′)] constructs (Fig. 3A, lanes 7 to 12)], exchange of the perfect ATGC Oct-1 POU-biding 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′) site, and B-VP16 recognizes the B-TAAT(5′) 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 G4 and G9 for the HSV-1 H-TAAT(5′) site and G9 for the B-TAAT site. The 5-bp G4 and G9 segments differ at two positions (GAT in G4 versus GAGCT in G9). Like the 5′ swap, the GARAT swap—sites H-TAAT(G4) and B-TAAT(G9)—affects the affinity of Oct-1 for these two sites, except that here the HSV-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(G4) site containing the BHV-1 GARAT segment (Fig. 3A, lane 15), and B-VP16 bound to the BHV-1 B-TAAT(G9) 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: CT in the HSV-1 site and GCC 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(5′) and B-TAAT(5′) 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 H-TAAT(D5′) site (Fig. 3A, lane 22) but not the H-TAAT(D9) site (lane 21), whereas B-VP16 bound the H-TAAT(D5′) site (lane 23) but not the B-TAAT(D9) 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(5′) site [H-TAAT(5′)] and the B-TAAT(5′) site, as shown in Fig. 4. Two of these sites contain identical sequences 5′ of the shared TAAT sequence; they each differ, however, from the consensus GARAT sequence at one position [GAGCT in the H-TAAT(5′) site and GAGCT in the B-TAAT(5′) 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 p and B-TAAT p 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; TGGG...
in the H-TAATp site and GGGCG in the B-TAATp 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-TAATp site (Fig. 5A), exchange of any one of the three D1 segment residues for its corresponding D2 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-TAATp site, exchanges of single base pairs at positions 1 and 2 of the D2 segment (G1 → C and G2 → T)
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 G1 → C well (compare samples 1 and 3 in Fig. 5B), whereas they both bind the position 2 exchange G2 → T poorly (see sample 4 in Fig. 5B). Thus, no one D segment residue is completely responsible for the discrimination of the D1 and D2 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(0+) and B-TAAT sites and the matched consensus TAATGARAT H-TAATp and B-TAATp sites and their respective D segment swaps, H-TAATp(D1) and B-TAATp(D1) (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 TAATp 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-TAATp and B-TAATp 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-TAATp site and of B-VP16 on the H-TAATp 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-TAATp site than on the B-TAATp site (compare lanes 3 and 6 in Fig. 6A), and B-VP16 is still twofold more active on the HSV-related B-TAATp site than on the H-TAATp 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 D1-containing H-TAATp site than from the D2-containing H-TAATp(D1) site (compare lanes 3 and 4 in Fig. 6A) and does so 7-fold better from the D1-containing B-TAATp(D2) site than from the D2-containing B-TAATp site (compare lanes 7 and 6). Conversely, albeit less dramatically, B-VP16 activates transcription two- to threefold better from the D2-containing H-TAATp(D2) site than from the D1-containing H-TAATp site (compare lanes 4 and 2 in Fig. 6B) and does so twofold better on the D2-containing B-TAATp site than on the D1-containing B-TAATp(D1) 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. 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 electrophoretic 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 proteins: the H-VP16 and B-VP16 proteins both bind the position 2 exchange G2 → T (Fig. 5B), whereas they both bind the position 1 exchange G1 → C well (compare samples 1 and 3 in Fig. 5B). The Oct-1-induced and VP16-induced (VIC) complexes are shown in lane 11 and 12, whereas the excess free probe is not shown. The sequences of the H-TAAT(WT) [H-TAAT(0+)] and related H-TAATp and H-TAATp(D1) probes and of the B-TAAT(5′) and related B-TAATp and B-TAATp(D1) probes are shown at the bottom. BHV-1-derived sequences are shaded.
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
is not critical for VP16-induced complex formation: the Oct-1 POU\(_3\) 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), Moriiuchi 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 a2 homeodomain protein with a1 and MCM1 (reviewed in reference 13). In this case, a2 associates with a1 to repress haploid-specific genes and with MCM1 to repress a2-specific genes. The interaction of Oct-1 with the VP16 proteins contrast with the interactions between a2 and its a1 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 a1 and MCM1 proteins are nonorthologous: a1 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 a1/a2 (10) and MCM1/a2 (14, 15) sites differ more in sequence. Last, the two VP16 proteins recognize the same surface of Oct-1, the
homeodomain, whereas a1 and MCM1 recognize very different regions of a2 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 lyrically 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.

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