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Conformational Alteration of Oct-1 upon DNA Binding Dictates Selectivity in Differential Interactions with Related Transcriptional Coactivators

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VP16 (termed VP16-H here) of herpes simplex virus (HSV) belongs to a family of related regulatory proteins which includes VP16-B of bovine herpesvirus (BHV). We show that VP16-B, while also being a powerful transactivator of transcription dependent on Oct-1 binding sites in its target promoters, has virtually no activity on a defined VP16-H-responsive, octamer-containing target promoter. While Oct-1 binds equally well to the VP16-B-responsive and -nonresponsive sites, VP16-B interacts with Oct-1 only when Oct-1 is bound to the BHV octamer site and not when it is bound to the HSV site. We show from the analysis of chimeric proteins that the ability of VP16-B to discriminate between the Oct-1 forms depends on features of its N-terminal region. We also show from an analysis of chimeric DNA motifs that sequences that lie 3′ to the POU domain-contacting region of the HSV octamer site play a role in making it unresponsive to VP16-B. Finally, we show by high-resolution hydroxyl radical footprint analysis that the conformation of Oct-1 is different on the two sites. These results augment our previous report on an allosteric effect of DNA signals on the conformation of bound proteins and indicate that different conformations of the same DNA binding protein can be recognized selectively by related members of interacting regulatory proteins. The possible implications of our observations for selective gene regulation by Oct-1, a ubiquitous transcription factor, and other multimember transcription families are discussed.

A fundamental aspect of the proper orchestration of coordinate and differential gene expression is the assembly of multicomponent transcription complexes involving different combinations and spatial arrangements of transcription factors. Given the multiplicity of potential interactions between regulatory proteins and the identification of numerous large families of DNA binding proteins with related if not identical binding sites, an understanding of precisely how the appropriate combinations are selected and assembled is crucial to the elucidation of the basis of gene control.

An example of a large multimember family of transcription factors is the POU domain class of proteins. Members of this class of proteins have been demonstrated to play an important role in a diverse range of regulatory processes including the control of gene expression in ontogenic development and cellular differentiation pathways (for reviews, see references 15, 47, and 49). One member of the family, the protein Oct-1, is ubiquitous and is present in most cells during all stages of the cell cycle. In agreement with its broad distribution, Oct-1 is a regulator of housekeeping genes such as those of small nuclear RNA (2, 4). However, octamer recognition sites for the protein are widely distributed in vertebrate genomes and Oct-1 also appears to regulate genes that selectively respond to developmental, cell cycle, or hormonal signals (9, 13, 14, 18, 21, 26, 29, 32, 46, 53). The mechanism by which a ubiquitous factor can differentially activate gene transcription is not well understood. Oct-1 may act in a selective manner by interacting with coactivators or other transcription factors that may themselves be activated or synthesized in response to specific stimuli (4, 6, 13, 18). To date, direct evidence for and identification of Oct factor coactivators is limited (see below). Furthermore, the presence of coactivators does not explain how Oct-1 bound to similar cognate sites in many promoters is able to exert a selective effect on gene regulation. Coactivators that are dependent on Oct-1 for DNA binding (14, 24, 43) would bind simultaneously to Oct-1 associated with several promoters leading to coordinate rather than selective expression.

In addition to regulating cellular gene expression, Oct-1 plays an important role in the initiation of viral gene expression in cells infected by herpes simplex viruses (HSVs) (33). The transcription of HSV immediate-early (IE or α) genes is activated by the virion protein VP16 (Vmw65 or αTIF), and the assembly of the multicomponent complex containing Oct-1 and VP16 serves as a paradigm for the combinatorial control of gene expression by selective protein-protein interactions. Unlike most other transcription activators, VP16 does not bind directly to DNA but is recruited to IE gene promoters by its association with Oct-1 (1, 10, 20, 25, 28, 34, 37, 42). At least one additional cellular protein, host cell factor (HCF) or complex forming factor (CFF), is required for this interaction (10, 17, 19, 50, 52). The promoters of HSV IE genes contain several copies of variants of the Oct-1 binding site. The best-studied IE element (see −167 in Fig. 1b) contains a consensus octamer motif flanked by a GARAT element, where R is purine, and has been termed the octamer-GARAT site. Although the GARAT portion of the sequence is not needed for binding of Oct-1, it is critical for VP16 recognition of Oct-1. Thus, while Oct-1 is involved in the transcriptional regulation of a variety of genes, the GARAT signal flanking the octamer site defines

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which genes respond to the combination of Oct-1, HCF, and VP16. Recently, we provided evidence for how this selectivity takes place (48). The presence of the GARAT element, while having little effect on binding or stability, causes a conformational change in the nature of the POU domain-DNA interaction, altering it from that seen with, for example, the octamer site of the H2B promoter. Substitution of bases within the GARAT or of amino acids predicted to contact the GARAT element caused a reversion of binding to that seen on the H2B motif and, at the same time, inefficient recognition by VP16. These results provided evidence for a mechanism whereby selectivity in transcription complex assembly was dictated by a DNA element acting akin to an allosteric effector of ligand (VP16 in this case) binding to a target protein.

Homologs of VP16 have now been identified in other herpesviruses, including varicella-zoster virus (7, 27), equine herpesviruses (EHV) 1 and 4 (23, 38, 39), and bovine herpesvirus 1 (BHV-1) (3). The primary structural attributes of VP16, demonstrated to be important for interaction with Oct-1 and HCF, are retained in these homologs. In agreement with the conservation of these features, the promoters of BHV-1 and EHV IE genes also contain octamer-GARAT sequences which are critical for transactivation (8, 30). Despite the previous demonstration that these homologs are potent transactivators which function through octamer sites, we report here that the VP16 homolog from BHV-1 (termed VP16-B for ease of reference) interacts with Oct-1 only when Oct-1 is bound to a BHV octamer site and not when it is bound to the HSV site. We show that while VP16-B efficiently transactivates a reporter gene linked to an octamer site from the main BHV-1 IE promoter, it was virtually inactive on a target promoter which contained an octamer site from an HSV type 1 (HSV-1) IE promoter and was efficiently activated by VP16-H. We show that while both of these sites bind Oct-1, they selectively promote recognition by the VP16 homologs; one site promotes complex formation with VP16-B and not VP16-H, while the converse is true for the other site. Furthermore, we demonstrate by high-resolution hydroxyl radical footprinting that Oct-1 binds in a qualitatively distinct way to each site. The results provide evidence for a model of selectivity that has had little direct experimental support, although it has been proposed in one form or another. The data indicate that one DNA binding factor may adopt distinct conformations on related sites and that these conformations can be selectively recognized by individual members of a family of related regulatory proteins which target that factor. The results are discussed with regard to the utilization of particular DNA binding proteins in diverse pathways of transcriptional regulation and general aspects of protein selectivity in differential gene control.

MATERIALS AND METHODS

Tissue culture. Details of passage of Vero and COS-7 cells, transfection by the calcium phosphate coprecipitation technique, preparation of cell extracts, and chloramphenicol acetyltransferase (CAT) assays have been described elsewhere (11, 30).

Plasmids. The plasmids pAB5 and pAB2 (36) were constructed from pPO49, which contains the entire promoter-regulatory sequence of the IE110 gene from HSV-1 strain KOS, as described previously (34). pAB5 contains a single octamer-GARAT motif (position −167 relative to the mRNA start site), while pAB2 does not contain this motif and is unresponsive to HSV VP16. To insert octamer-GARAT motifs into pAB2, sense and antisense oligonucleotides bracketed by *Hin*dIII and *Xba*I sites and spanning the motif as well as flanking sequences were synthesized without 5′ terminal phosphate groups. The oligonucleotides were annealed and cloned between the *Hin*dIII-*Xba*I sites of pAB2. The resulting plasmids were designated pAB-31 and pAB-167, etc., where the numbers at the end indicate the locations of each sequence in relation to the beginning of the transcripts of the BHV IE-1 or HSV IE110 gene (see Fig. 1). The construction of chimeric proteins between VP16-H and VP16-B has been recently described (31).

Gel retardation. Nuclear extracts of COS-7 cells transfected with VP16-expressing plasmids were prepared by the technique described by Wu (51) as described previously (11, 12). For preparation of extracts, cells in 100-mm-diameter plates were transfected with 50 μg of the expression plasmids. Details of the gel retardation assays were exactly as described previously (48). Essentially, extracts from cells transfected with either VP16-expressing plasmids or pcDNA1 (the empty expression vector [Invitrogen]) were incubated in 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid HEPES (pH 7.9)–10% glycerol–200 mg of bovine serum albumin (BSA) per ml–0.05% Nonidet P-40–1 mM dithiothreitol–1 mM EDTA–25 mM NaCl. HeLa cell nuclear extract and salmon sperm DNA were incubated at 20°C for 10 min before the addition of ^{32}P-labeled double-stranded probes, and incubation continued for an additional 10 min. The complexes were then separated on 4% nondenaturing polyacrylamide gels in 0.5× Tris-borate-EDTA. For competition experiments, unlabeled homologous or heterologous probes or an equivalent volume of buffer was added to the reaction mixtures before addition of the labeled probe. In experiments designed to assess the ability of antibodies to the POU domain to inhibit complex formation, 2 μl of immunoglobulin G fractions of either anti-POU antiserum (48) or preimmune serum was added to the reaction mixtures before the addition of labeled oligonucleotides.

Footprinting. Details of the purification of Oct-1 and its POU domain used in the footprinting experiments have been described elsewhere (48). The orthophenanthroline copper [$(OP)_2Cu^+$] assay was performed on DNA binding complexes separated in 10% native polyacrylamide gels. After electrophoresis, the gels were exposed to 1,10-phenanthroline copper as described elsewhere (48). Complexes identified by autoradiography were excised and eluted overnight in elution buffer (0.5 M ammonium acetate and 1 mM EDTA). Chemical sequencing of the DNA fragments used as probe was performed by the method described by Maxam and Gilbert, and sequences were analyzed along with the footprinting reactions. For hydroxyl-radical [$Fe(EDTA)]^2$ footprinting, binding reactions were performed in 50 μl of binding buffer (20 mM HEPES-KOH [pH 7.9], 1 mM EDTA, 0.025% Nonidet P-40, 4% Ficoll, 125 mg of BSA per ml, 50 mM NaCl) with 100 ng of poly(dI-dC) · poly(dI-dC) and 1 ng of radiolabeled probe. Cleavage was initiated by the addition of 9 μl of digestion solution (0.13 mM EDTA, 0.07 mM ferrous ammonium sulfate, 2% H_2O_2, 6.7 mM sodium ascorbate). The reaction was allowed to proceed for 3 min before termination by the addition of 5 μl of 0.2 M thiourea. Complexes were separated from unbound DNA by native polyacrylamide gel electrophoresis, and DNA was analyzed as for $(OP)_2Cu^+$ footprinting. In experiments in which the Oct-1 POU domain was used, 0.3 ng of purified protein was incubated with the target DNA.

RESULTS

Differential transactivation of octamer-containing target promoters by VP16-H and VP16-B. We previously demonstrated that the VP16 homolog of BHV, VP16-B (BHV-αTIF), was a potent activator of transcription. VP16-B induces activity of the BHV IE1 promoter with an efficiency at least as great as VP16-H and this induction requires an octamer binding site located at approximately position −31 (30). This site binds Oct-1 efficiently and promotes complex formation with VP16-B. A schematic diagram summarizing the relationship between VP16-H and VP16-B is illustrated in Fig. 1a. The N-terminal domains of the proteins are very similar, exhibiting 37% identity plus 13% similarity over 390 amino acids, and many blocks of between 10 and 16 residues exhibit 80 to 94% identity. The C-terminal region of VP16-B is dissimilar to that of VP16-H in that it is not noticeably rich in acidic residues; however, like VP16-H, this C-terminal region is required for potent *trans*-inducing activity. To dissect further the structure-function relationships within both proteins, we began by using the target CAT construct pAB5 (Fig. 1c). This promoter has a single very well-conserved octamer site (Fig. 1b [−167 motif]), which has been characterized and analyzed in detail in a number of studies, and is required for induction by VP16-H through the formation of the Oct-1–VP16 complex (34, 35). As expected, cotransfection of this target promoter with VP16-H resulted in induction by more than 30-fold in CAT activity (Fig. 2a). Deletion of the octamer site abolishes the response (34, 35). Unexpectedly, in parallel assays VP16-B was virtually inactive on the target pAB5, with cotransfection resulting in essentially no significant increase in CAT activity above background (Fig. 2a). The absence of activity of VP16-B was not due to a lack of synthesis. Western blot (immunoblot) analyses

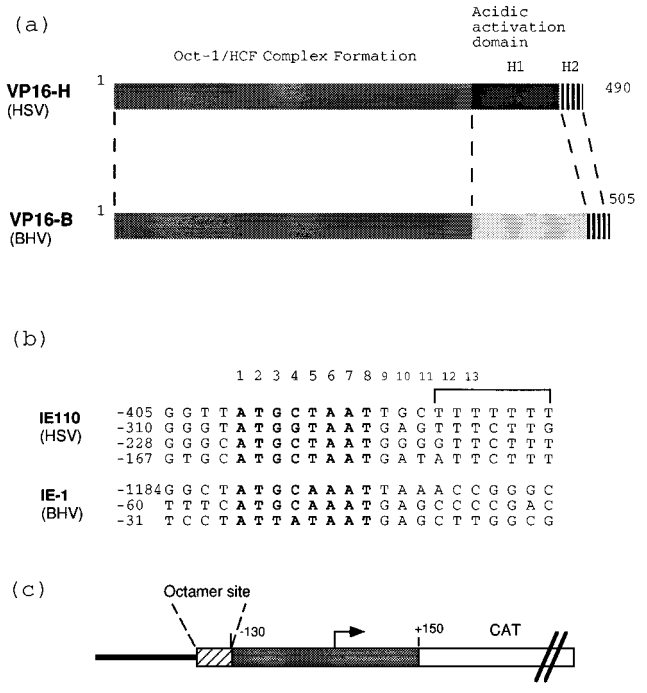

FIG. 1. Schematic comparison of VP16-H and VP16-B (a) and the octamer-GARAT elements in the HSV-1 IE110 and BHV-1 IE-1 promoters (b). H1 and H2, the activating subdomains of VP16-H. The carboxyl terminus of VP16-B does not contain a region homologous to H1, although a segment similar to H2 is present. The numbering system for the octamer motifs in panel b refers to the first position of each octamer site upstream of the characterized start sites of the genes. The bracket indicates the strong conservation of a 3′ T string. (c) Summary of the test basal promoter from the IE110 gene, deleted to −130, into which the octamer sites were inserted.

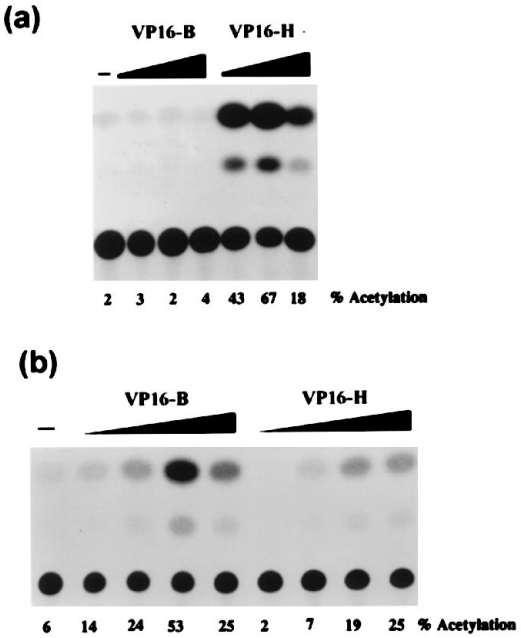

FIG. 2. Activation of target promoters containing different octamer sites by VP16-H and VP16-B. COS-7 cells were transfected with either pAB5 (a), which contains a single octamer-GARAT motif from IE110 (−167), or pAB31 (b), which contains the single octamer site from IE1 (−31) together with various amounts (1, 10, or 100 ng [a] and 0.1, 1, 10, and 100 ng [b]) of VP16-H- or VP16-B-expressing plasmids or the control expression vector (−). Cells were harvested and assayed for CAT 48 h after transfection. CAT activities in each sample are expressed as percentages of input chloramphenicol acetylated and are given at the bottom of each panel.

confirmed that VP16-B was synthesized in equivalent amounts to VP16-H (reference 31 and data not shown). Furthermore, VP16-B was active on the appropriate target (see below).

To demonstrate further that this surprising result was not due to a quantitative effect, but reflected a qualitative difference in the N-terminal domains required for complex formation we constructed a chimeric protein in which the N-terminal domain of VP16-B was fused to the C-terminal acidic domain of VP16-H. This chimeric protein VP16-BH is an extremely potent activator of BHV IE transcription. In control experiments VP16-BH induced CAT activity from a target promoter containing the complete BHV IE1 promoter regulatory region by up to 100- to 200-fold (Fig. 3b). In fact, the chimeric activator was at least as powerful if not more so than VP16-H itself (Fig. 3b [cf. BH and HH]). Despite this, in parallel assays VP16-BH, like VP16-B, was unable to activate pAB5, which again demonstrated strong responsiveness to VP16-H (Fig. 3a). This series of experiments contains two types of positive controls. The target promoter pAB5 is clearly responsive to VP16-H, and the proteins VP16-B or VP16-BH are expressed, are potent activators of transcription of the BHV IE promoter, and require an octamer site (30; also see below). Despite this, neither VP16-B nor VP16-BH was able to activate the target promoter pAB5. In Fig. 3, results from the corresponding chimera, VP16-HB, are not included since, as we previously demonstrated, it lacks a functional activation domain and is inactive on all promoters tested (31).

The promoter and regulatory regions of the HSV IE110 and BHV IE1 genes contain several putative octamer-GARAT elements (Fig. 1b). We have demonstrated that the motif located at −31 in the BHV IE1 is necessary for activation by VP16-B, since mutations in this sequence inhibit transactivation (30). In addition, introduction of the −31 octamer site motif into a basal promoter is sufficient to allow activation by VP16-B (30). Similarly, the herpes simplex virus −167 motif is sufficient for activation by VP16-H (34). It is noteworthy that the core octamer, ATGCTAAT, is strongly conserved in the HSV motifs and that the flanking GARAT region although less conserved shows good conservation. In addition, a striking feature of the HSV motif is the very strong conservation of a 3′ T string (Fig. 1b). In the BHV motifs, although the octamer-GARAT sites are reasonably well conserved, there is less conservation in the 3′ region and a notable lack of a T-string conservation among the sites. We have previously shown that Oct-1 binding to different octamer sites can be qualitatively distinct and that differences in the nature of binding have a profound effect on the interaction with VP16-H (48). Therefore, on the basis of this, it was possible that VP16-B and VP16-H differed in target specificity in that although both are able to be recruited by Oct-1 into a complex, they may have a selective preferences for Oct-1 based on conformational specificity at the two sites. We therefore compared pAB5, with pAB-31, in which the octamer-GARAT in pAB5 was replaced with the corresponding sequences from BHV IE1 −31 site. Cells were transfected with either pAB5 or pAB-31 as well as various concentrations of plasmids expressing VP16-H or VP16-B. In contrast to the result with pAB5 (Fig. 2a), replacement of the IE110 −167 site with the IE1 −31 motif made the promoter more responsive to VP16-B than to VP16-H (Fig. 2b). Although there was still activation by VP16-H, considering the virtual inactivity of VP16-B on pAB5, this result represented a major change in target preference in favor of VP16-B.

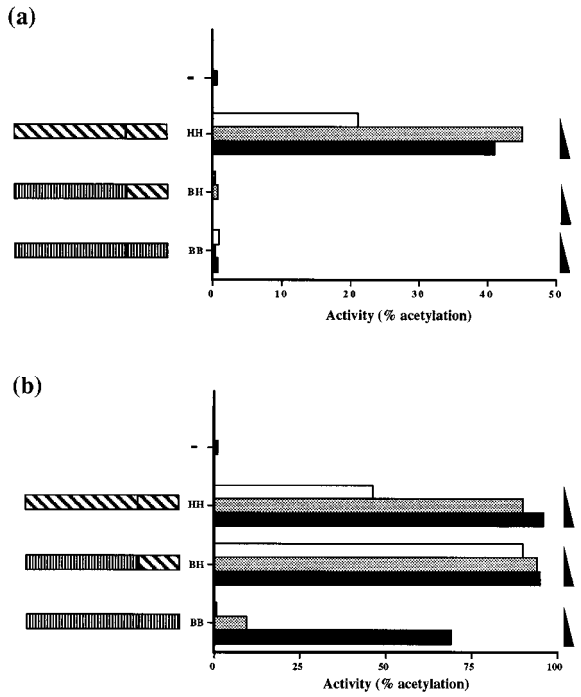

FIG. 3. The N-terminal domain of VP16-B is responsible for site selectivity. Activation of IE-1 and pAB5 by VP16 and various chimeras. COS-7 cells were transfected with pAB5 (a) or IE-1 (b) and 0, 1, 10, or 100 ng of expression vectors for native VP16-B (BB), native VP16-H (HH), or chimeras containing the N-terminal region of VP16-B fused to the C-terminal activation domain of VP16-H (BH). Schematic representations of the constructs are given to the left. Boxes with vertical lines, VP16-B sequences; boxes with diagonal lines, VP16-H sequences. Cells were harvested 48 h after transfection and were assayed for CAT. CAT activities are expressed as percentages of input chloramphenicol acetylated.

We have previously demonstrated that sequences flanking the core octamer affect the conformation of the POU domain (48). To attempt to identify sequences outside the core octamer site that may be involved in selective recognition by VP16-B, we examined various permutations of the IE1 −31 and IE110 −167 octamer-GARATs and their flanking sequences for their ability to respond to VP16-B. Oligonucleotides constituting the various combinations were cloned into the basal IE110 promoter in the plasmid pAB2 and assayed for transactivation by VP16-H and VP16-B in transient-expression experiments. As shown in previous experiments (Fig. 2 and 3), VP16-B failed to activate a promoter containing the HSV IE110 −167 sequence (Fig. 4a, line 1). Replacement of the 5′ flanking sequences of the motif with sequences from BHV IE1 −31 did not change the responsiveness of the sequence to VP16-B (Fig. 4a, line 2). However, replacement of the 3′ flanking sequences or both 5′ and 3′ flanking sequences made the promoter responsive to VP16-B (Fig. 4a, lines 3 and 4). Conversely, replacement of the 3′ but not the 5′ sequences flanking the BHV IE1 −31 motif with those from HSV IE110 −167 reduced the ability of VP16-B to activate the promoter (Fig. 4b; compare lines 6 and 7). VP16-H exhibited less specificity than VP16-B and activated each of these promoters to approximately similar levels. The most striking difference between the HSV IE110 and BHV IE1 motifs is that all of the IE110 octamer-GARATs are flanked at their 3′ ends by several T residues, while 3′ sequences flanking the BHV IE1 motifs are made up largely of G and C bases (Fig. 1c). These results suggest that the T string plays a role in selectively making the motif nonresponsive to VP16-B but not to VP16-H.

Target selectivity represents differential complex formation with Oct-1. It was possible that the inactivity of VP16-B on the pAB5 target compared with potent activation on pAB-31 (Fig. 2) reflected its selective recruitment into complexes with Oct-1 on the different sites. Therefore, we examined the ability of oligonucleotides representing the IE1 −31 and the IE110 −167 sites to bind Oct-1 and to recruit VP16-H and VP16-B into ternary complexes. Radioactive oligonucleotides representing the two motifs were incubated with extracts from cells transfected with plasmids expressing intact or carboxyl-terminus-truncated variants of VP16-H or VP16-B. The complexes were then analyzed on nondenaturing polyacrylamide gels (Fig. 5). Binding of Oct-1 to the IE110 −167 site has been well characterized previously (20, 35). The IE1 −31 site bound a component of identical mobility which was cross-inhibited by the IE110 −167 site, but not by an oligonucleotide containing a mutated octamer motif (data not shown). The IE1 binding factor was also disrupted by the inclusion of antibody to the POU-specific domain of Oct-1, and footprinting studies demonstrated binding of the purified POU domain to the −31 site (data not shown and see below).

As predicted, while intact VP16-B and a truncated form lacking the carboxyl terminus were able to promote the formation of a ternary complex (TRF.C) on the BHV IE1 −31 site (Fig. 5) complex formation on the HSV IE110 −167 site was weak or undetectable. Also as expected, VP16-H and a truncated version of the protein formed a complex with the IE110 −167 site (the reduced complex formation on the IE110 site by VP16-H lacking the acidic tail was not typical; see reference 12). Surprisingly, although VP16-H activates promoters containing the IE1 −31 site, it was not recruited into a ternary complex at this site. These results suggest that both VP16-B and VP16-H display selectivity in recognizing Oct-1 in association with distinctive DNA motifs and that mobility shift assays may be a more sensitive method of detecting the subtle selectivity displayed by VP16-H.

Distinct conformations of Oct-1 on the BHV IE1 sites and on the HSV IE110 sites. These results suggest a mechanism whereby these two proteins, VP16-H and VP16-B, although they are clearly recognizable as related members of a family of regulatory proteins and although both interact with a target factor Oct-1, nonetheless discriminate between Oct-1 bound to different DNA sites. One possible explanation for this selectivity is that the DNA binding domain of Oct-1 adopted altered conformations on the different sites from the two virus target genes and that the different VP16 proteins exhibited selectivity in recognizing and interacting with these conformations. To establish whether the selectivity in complex formation described above could be explained by DNA binding conformation, we examined POU domain binding to the IE1 −31 site by a series of chemical interference assays and high-resolution footprinting techniques, in comparison with binding on the IE110 −167 site (48). Figure 6 shows the results of dimethyl sulfate (DMS) and diethyl pyrocarbonate (DEPC) interference assays. DMS reacts with the N-7 atom of guanine and less efficiently with the N-3 atom of adenine residues, while DEPC reacts efficiently with the N-7 atoms of adenine bases and less efficiently at guanine bases. For ease of discussion and comparison, the bases within the octamer motif at the −31 site have been numbered 1 to 8. For the top strand, methylation at the adenines at positions 1, 4, 6, and 7 interfered with binding, with the strongest interference being at positions 6 and 7 (Fig. 6a). On the bottom strand (Fig. 6b), interference was observed at positions 5 and 8, with little interference at positions 2 and

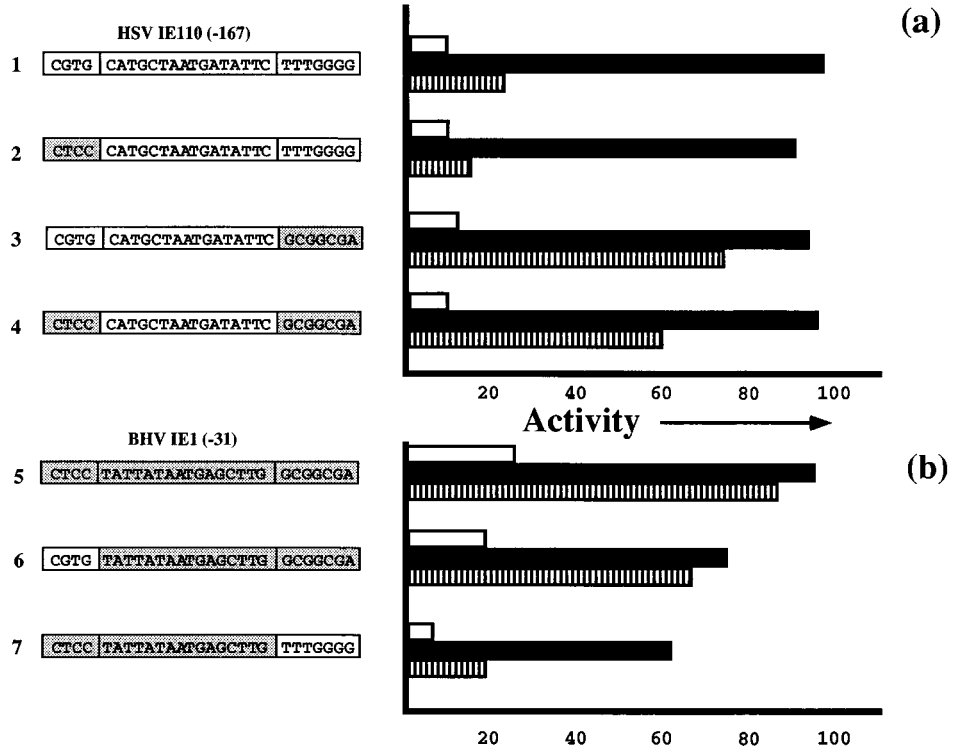

FIG. 4. Effect of swapping octamer-GARAT and flanking sequences of HSV IE110 −167 and BHV IE1 −31 on selectivity of VP16-B. Oligonucleotides representing various permutations of the HSV IE110 −167 (empty boxes) and BHV IE1 −31 (shaded boxes) octamer-GARATs and their flanking sequences were cloned into pAB2. The resultant plasmids (50 ng) were introduced into COS-7 cells with various amounts (0.1, 1, 10, or 100 ng) of plasmids expressing VP16-H (black columns), VP16-B (striped bars), or control plasmid (open bars). Cells were harvested 48 h after transfection and were assayed for CAT. The histograms represent CAT activities at only the optimal concentrations of VP16 expressing plasmid.

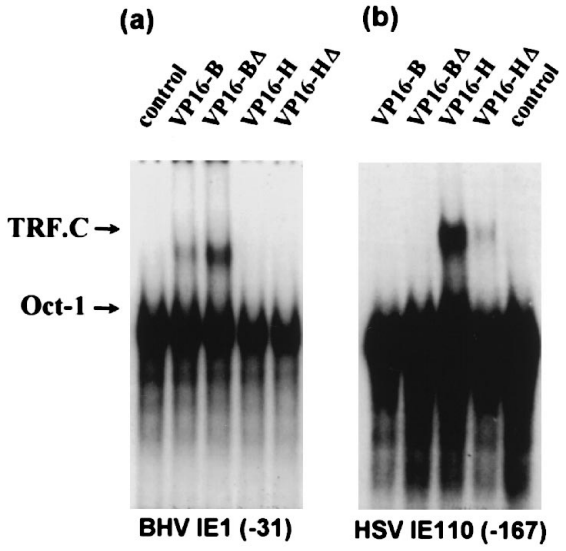

FIG. 5. Differential recruitment of VP16-H and VP16-B to probes containing the IE1-31 and IE110-167 octamer-GARAT motifs. Double-stranded oligonucleotides were labeled with ^{32}P and incubated with extracts from COS-7 cells transfected with plasmids expressing VP16-B, VP16-B truncated at amino acid 417 (VP16-BΔ), VP16-H, or the protein truncated at amino acid 411 (VP16-HΔ). Complexes were separated on nondenaturing polyacrylamide gels. Arrows indicate the complex between independent Oct-1 and the probes or the ternary complex TRF.C.

3. This last result is relevant, since the IE1 −31 site exhibits dyad symmetry and binding of the POU domain could have been in either orientation. Since the strongest interference is seen on the central two adenines of the TAAT or AAAT which bind the POU homeodomain, the result indicates that the POU domain binds to the orientation of the octamer site indicated on the top strand, with the homeodomain contacting bases 4 to 8. Furthermore, it was possible that POU binds to this site as a dimer. However, the results of the interference analysis showing selective binding to the two A's at positions 6 and 7 and little binding at positions 2 and 3 and the results of the electrophoretic mobility shift assays with intact Oct-1 (Fig. 5) or the POU domain showing mobilities identical to those for the HSV −167 site (data not shown) together demonstrate that binding was monomeric. For carbethoxylation interference (Fig. 6c and d), the strongest interference was again observed at the adenines at positions 6 and 7 on the top strand, and weaker but significant interference was observed at positions 1 and 4 (Fig. 6c and d). On the bottom strand, interference was observed at positions 2, 3, 5, and 8. No interference was observed by carbethoxylation at the purine bases on the 3′ side of the octamer core motif (Fig. 6d). One curious feature of the DEPC interference assays was noted. DEPC normally reacts well with adenine residues and less well if at all with guanines. For example, the adenines at positions 6 and 7 of the octamer site are highly reactive and easily seen in the unbound track. However, for the bases immediately on the 3′ side of the IE1 octamer site, the G residues (positions 9 and 11) were highly reactive, while the A at position 10 reacted poorly (Fig. 6c [cf. positions 6, 7, and 10]). The reason for this is unclear, but it may be due to an altered secondary structure of the DNA

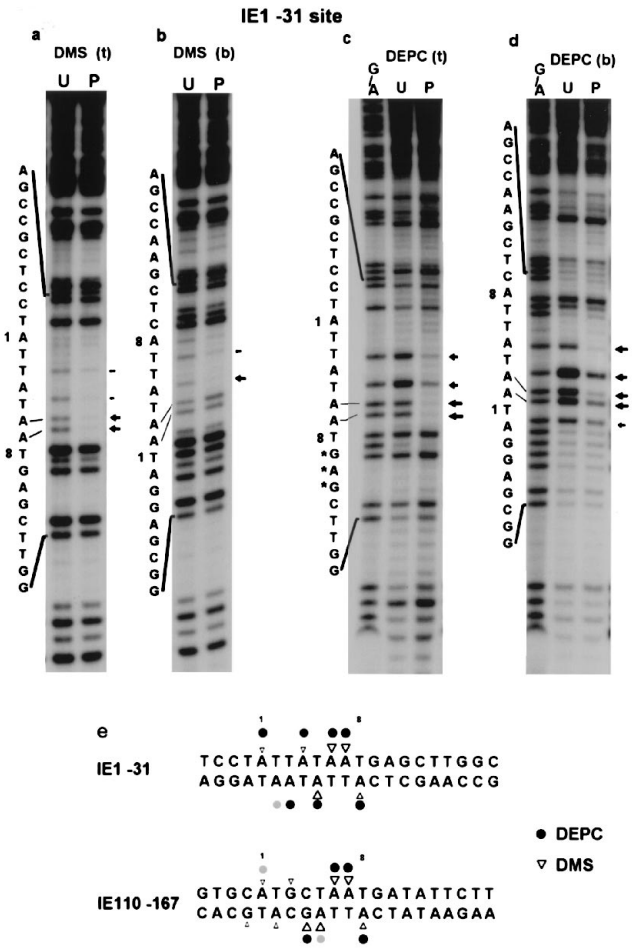

FIG. 6. Analysis of base specific contacts at the IE1 −31 octamer site by DMS and DEPC interference analysis. Fragments containing the IE1 −31 octamer motif were modified by DMS (a and b) or by DEPC (c and d) and were incubated with 0.3 ng of the POU domain of Oct-1. The complexes were then identified by electrophoresis in nondenaturing gels, and the DNA was purified and cleaved with NaOH at 90°C for 30 min. The sequences for the region analyzed are given to the left of each panel, the first and last bases of the core octamer motif are indicated by 1 and 8, respectively. In panel c, asterisks indicate apparent reactivities of G and A residues on the 3′ side of the octamer. (e) A summary of the contact points (arrows) in comparison to those that we have previously identified for the IE110 −167 motif (48) is illustrated below. The sizes of the triangles (for DMS) and the shading of the dots (for DEPC) indicate the relative importance of the contacts by their underrepresentation in the bound fraction.

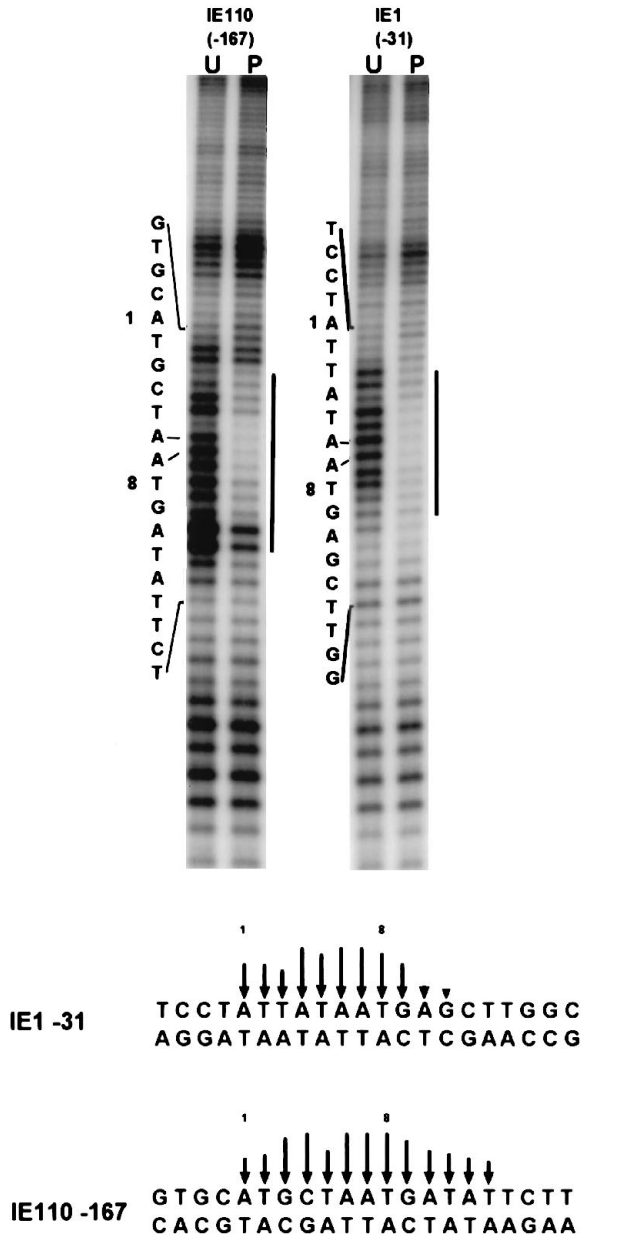

FIG. 7. Comparison of the $(OP)_2Cu^+$ footprint by the POU domain at the IE110 and IE1 octamer sites. The extents of the motifs protected from $(OP)_2Cu^+$ cleavage in the bound (P) versus the unbound (U) fraction are illustrated by the vertical bars. 1 and 8, the first and last bases of the core octamer sites, respectively. A semiquantitative comparison of the contacts at each site is shown at the bottom.

around this octamer motif. A semiquantitative summary of the interference results, together with a comparison of those for the IE110 −167 octamer site, is illustrated in the Fig. 6e. Overall, bearing in mind the difference in informative bases at positions 2 and 3, the results were reasonably similar for each of the sites.

We then compared POU binding to the sites by footprinting analyses. The results of $(OP)_2Cu^+$ footprinting are illustrated in Fig. 7. For the IE1 −31 site on the top strand, protection beginning at position 1 and continuing to position 8 was observed, with weak protection on the 3′ side. The strongest protection was observed across the central TAAT region (Fig. 7 [A's at positions 6 and 7 indicated]). Again, the footprinting pattern (also on the bottom strand; data not shown) of POU on this site was similar to that on the IE110 site (Fig. 6a) (48), with the exception that on the IE1 site the footprint was somewhat shorter on the 3′ side on the core motif. These results are summarized semiquantitatively in the bottom panel of Fig. 7. While these results showed slight differences in the POU binding to the two motifs, there was no strong indication of a significant qualitative difference between the two sites. However, we have previously demonstrated that although $(OP)_2Cu^+$ represents a very-high-resolution reagent, it may not be the appropriate reagent to detect a particular alteration in the nature of a protein-DNA interaction (48), and that a distinct shift in binding can be observed with higher-resolution reagents. To probe further for an alteration in the nature of

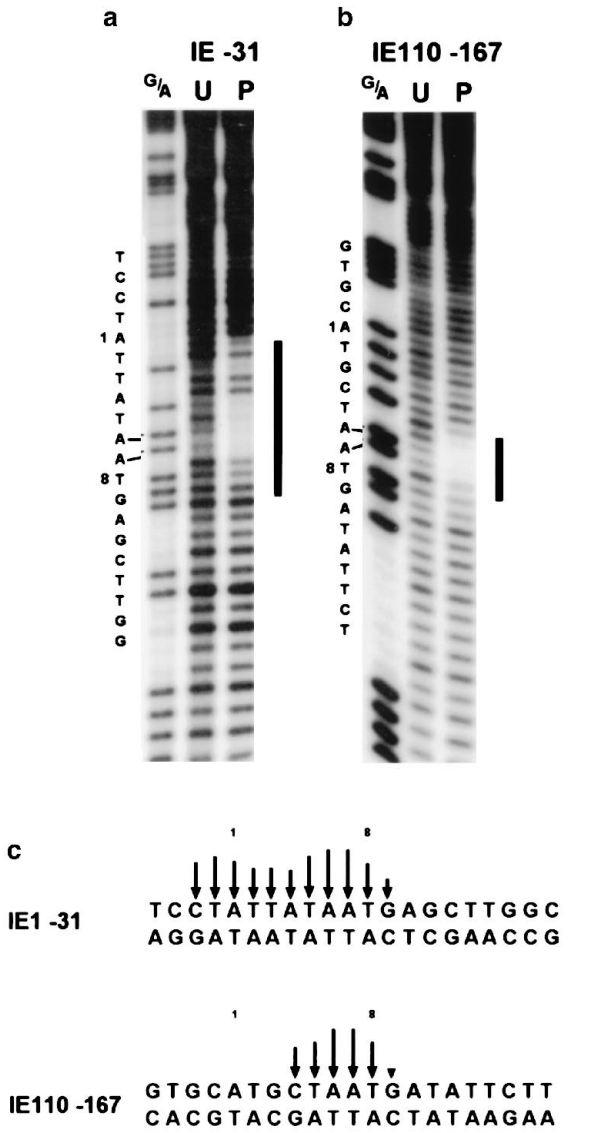

FIG. 8. Alteration in the contacts made by the POU domain at the IE1 site compared with the IE110 site detected by $[Fe(EDTA)]^2$ footprinting. As in Fig. 6, the sequences for the region analyzed are given to the left of the analysis for DEPC (lanes labeled G/A). P and U, the patterns for cleavage by $[Fe(EDTA)]^2$ in the bound and unbound fractions, respectively.

POU binding to the two sites which might underpin the selective recognition by the VP16 proteins, we employed hydroxyl radical footprinting by using $[Fe(EDTA)]^2$. Unlike $(OP)_2Cu^+$, $[Fe(EDTA)]^2$ does not bind to DNA, and it is the neutral hydroxyl radical acting as a reactive water molecule which is the probe of protein binding (44, 45). $[Fe(EDTA)]^2$ footprints are, therefore, the highest-resolution maps of protein-DNA interactions. As demonstrated previously, the POU domain produced a smaller $[Fe(EDTA)]^2$ footprint on the IE110 octamer motif, where protection was limited to the TAAT region which contacts the POU homeodomain (Fig. 8). Although a footprint was observed on the 5′ side on the TAAT bases when $(OP)_2Cu^+$ was used and DMS or DEPC interference was observed for this region, no $[Fe(EDTA)]^2$ footprint was observed. By contrast, strong protection was observed for POU binding to the IE1 −31 site, in both the central TAAT and the 5′ ATTA bases (Fig. 8a). In fact, the $[Fe(EDTA)]^2$ footprint extended to bases further 5′ to positions −1 and −2 of the octamer site. This pattern clearly represented one qualitatively distinct from that seen on the IE110 site. These results provide a strong correlation with those from the complex formation assays and a basis for selective interaction. Thus, the footprinting data indicate that the POU domain of Oct-1 binds in a conformationally distinct manner to two octamer sites within the IE110 or IE1 target genes. The mobility shift assays indicate that while Oct-1 binds to each of the sites, VP16-H selectively recognizes Oct-1 on one site (IE110) and poorly recognizes it on the other, while the converse is true for VP16-B. Despite their relationship in primary sequence and mechanism of action, the two VP16 polypeptides recognize distinct conformational isomers of Oct-1.

DISCUSSION

Differential regulation of gene transcription is a fundamental control mechanism in most eucaryotic processes, including, for example, development, differentiation, the cell cycle, and responses to environmental stimuli or infectious agents. A fundamental aspect of the proper orchestration of coordinate and differential gene expression is the assembly of multicomponent transcription complexes involving different combinations and spatial arrangements of transcription factors. Given the multiplicity of potential interactions between regulatory proteins and the identification of numerous large families of DNA binding proteins with related if not identical binding sites, an understanding of precisely how the appropriate combinations are selected and assembled is crucial to the elucidation of the basis of gene control.

From this work, together with our previous data on the VP16-H homolog VP16-B, a number of conclusions can be made which lead to the proposal for a mechanism of selectivity in the protein-protein interactions between DNA-bound targeting factors and coactivator type regulatory factors which has implications for eucaryotic transcriptional control in general. (i) VP16-B is a potent activator of IE1 transcription functioning like its counterpart VP16-H through Oct-1 and octamer sites. (ii) Transfer of a single octamer site from IE1 to a nonresponsive gene is sufficient to confer VP16-B responsiveness (30). (iii) This site binds and footprints Oct-1, and VP16-B forms a ternary complex on this site with Oct-1 (reference 30, this work, and data not shown). Despite this, VP16-B was completely inactive on an identical target promoter which also contained an octamer site and, importantly, which was also strongly activated by the related protein VP16-H. We demonstrate the basis for this selectivity. VP16-B recognizes and is recruited into a complex with Oct-1 on the IE1 octamer site but not on the IE110 octamer site. Our results suggest that two factors contribute to the selective recruitment of VP16-B by Oct-1 bound to the IE1 octamer site—features of the amino portion of VP16-B itself and sequences that flank the octamer site at its 3′ end. Furthermore, we provide an explanation from high-resolution physical analyses of the DNA binding complexes, in that distinct conformational difference can be detected in the nature of Oct-1 binding to each of these sites. In our previous work, we demonstrated that conformational differences in POU binding profoundly affect VP16-H recruitment into a ternary complex, and our results together with those described by Cleary and Herr (5) demonstrated conformational flexibility in POU binding to related sites. We propose that the mechanism of selectivity by the VP16 proteins is based on the conformational difference in the Oct-1 DNA complex at each site. Formal proof of the precise nature of

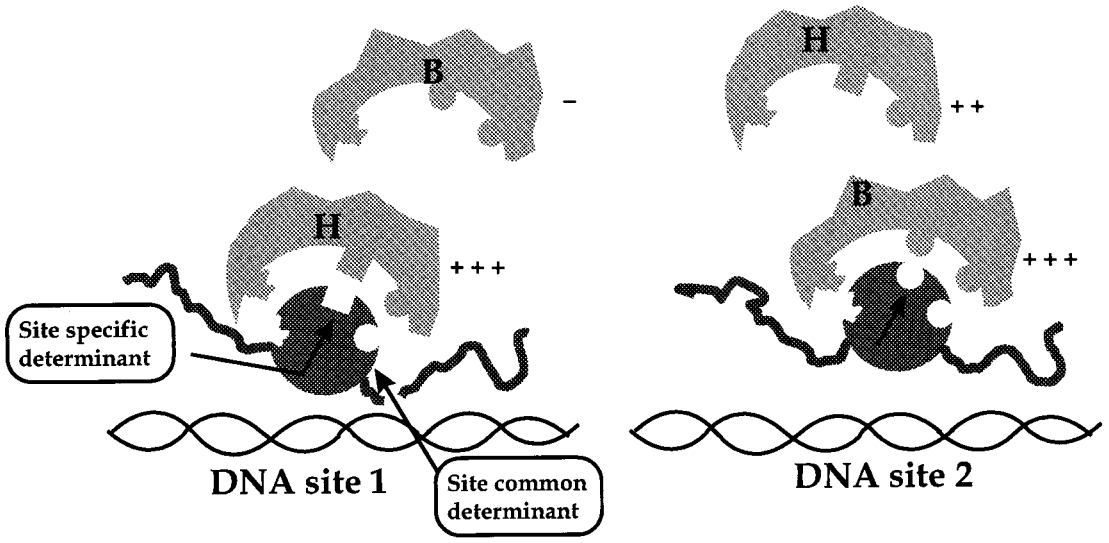

FIG. 9. Model for selective recognition and interaction of a DNA-bound factor by individual members of a family of related coactivators such as, in this case, VP16-H and VP16-B (H and B, respectively). Overall, the DNA binding factor (shaded circles) appears similar on two binding sites, and several determinants (shaded patterns) on the surface remain unchanged. However, other determinants (outlined square to circle, marked by an arrow) appear different on the two sites. The interacting proteins, H and B, have recognition determinants (reciprocal shapes) for the DNA binding factor, some of which are conserved between the two proteins but some of which are different (shaded square to circle). These last determinants are involved in recognition of the site-specific conformational alteration in the DNA-bound protein. VP16-B exhibits more sensitivity to the differences in the Oct-1 binding conformation and does not function on DNA site 1, while VP16-H is less selective and retains activity on site 2. In other situations, the reciprocal nature of the selectivity may be more pronounced.

these differences may require crystal structural analysis of the different sites in association with the POU domain but could include swivelling of the POU-specific domain through the flexible linker region or rotation of the POU-specific domain with respect to the homeodomain. In a similar manner, identification of the region(s) in VP16 involved in conferring selectivity will require the construction of appropriate chimeric proteins and demonstration of the contribution of appropriate alterations to site preference. Such work is under way, but it is clear from electrophoretic mobility shift assays that the VP16 proteins are selectively recruited into ternary complexes at each site.

By constructing chimeric proteins, we show that the determinant(s) for selective Oct-1 recognition lies in the N terminus of VP16-B, which is the best-conserved region with VP16-H, exhibiting approximately 50% identical or similar residues over a 390-amino-acid region. A model for the interactions between VP16-H and VP16-B and Oct-1 is illustrated in Fig. 9. While certain regions of VP16 are critical for complex formation, it is reasonable to propose (and for this there is evidence) that multiple determinants are involved. We propose that certain of these determinants involved in interacting with features of the Oct-1–DNA complex which are common at the two octamer sites will themselves be conserved between the two VP16 proteins. In addition, a specific determinant in VP16 which is altered in the two species is involved in recognizing the difference in the Oct-1–DNA complexes. The construction of chimeric proteins with refined crossovers between the two VP16 species should help define the precise region involved in recognition of Oct-1 conformational specificity.

The POU domain proteins constitute an expanding family of general and cell-type-specific regulators of gene expression. They are involved in a broad range of processes, particularly in the developing nervous system, including organogenesis and determination of cell identity and in the cell-type-specific functions in fully differentiated cells and have been shown to have important roles in a number of other processes (for reviews, see references 40, 47, and 49). Even Oct-1, because of its ubiquitous nature, was thought to be a general transcription factor with a limited role in selective activation. However, it has recently become apparent that Oct-1 is also responsible for activating genes in response to external and intracellular signals (4, 6, 13, 18). One mechanism by which selectivity is achieved is clearly in DNA binding site preference itself. However, site selectivity does not explain many aspects of transcriptional selectivity in response to the candidate protein. For example, concerning the POU domain family itself, a B-cell-specific protein which is thought to determine the expression of immunoglobulin genes by binding to Oct-1 (or Oct-2) has recently been identified (14, 24, 43). This factor (OBF-1, BOB1, or OCA-B) and its interaction with Oct-1 have certain similarities to the situation characterized for VP16. OBF1 does not independently bind DNA but will form a complex with Oct-1 on the octamer site in the promoters of immunoglobulin genes, and it is required for the activation of transcription. Yet while Oct-1 binds the octamer site in the H2B promoter, OBF1 does not stimulate transcription of H2B. Although it has not been directly shown, it is possible that one mechanism to explain this is that OBF1 will form a complex with Oct-1 only when Oct-1 is bound to the immunoglobulin site and not the H2B site. Many other examples in which DNA binding specificity per se does not explain functional selectivity have been demonstrated, many of which come from studies of the homeodomain proteins in development regulation. The DNA binding specificity of the homeodomain protein fushi tarazu (ftz) can be switched to that of another homeodomain protein, bicoid (bcd); however, this does not convert the ftz variant into a functional bcd protein (41). It is possible that for functional specificity to be recapitulated, DNA binding is not sufficient, and that it must also induce a conformational alteration in these proteins to expose regions involved to further protein-protein interactions. Although no direct evidence such as shifts in DNA contact patterns was demonstrated, recent studies of mutants of the glucocorticoid receptor indicated that confor-

mational alterations in the DNA binding domain after binding to a specific site were involved in recognition by not yet identified target proteins for transcriptional activation (22). As a final example, Hill et al. (16) concluded that DNA-induced conformational changes in the serum response factor may be involved in its interaction with additional regulatory factors, although again there was no direct evidence for a conformational change. Our studies provide the first demonstration of selective interaction between members of a coactivator class of proteins (VP16) and a single DNA binding target, correlated with a conformational difference in the nature of the DNA-bound complex.

The coordinate activation of HSV IE genes by VP16 in association with Oct-1 has long been regarded as a paradigm for the combinatorial control of gene expression by selective protein-protein interactions. It is intriguing to hypothesize that selective recognition of conformational differences in Oct-1 by the various VP16 proteins may mimic mechanisms in the cell by which specific coactivators bind to a ubiquitous DNA binding factor. In this model, coactivators activated in response to particular stimuli would recognize only specific conformations of Oct-1 bound to the appropriate octamers. The specificity of gene expression would be regulated by the coactivators rather than Oct-1. This may explain the mechanism of Oct-1 involvement in constitutive and coordinate as well as specific gene regulation.

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