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OCA-B Is a Functional Analog of VP16 but Targets a Separate Surface of the Oct-1 POU Domain

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OCA-B is a B-cell-specific coregulator of the broadly expressed POU domain transcription factor Oct-1. OCA-B associates with the Oct-1 POU domain, a bipartite DNA-binding structure containing a POU-specific (POU_S) domain joined by a flexible linker to a POU homeodomain (POU_H). Here, we show that OCA-B alters the activity of Oct-1 in two ways. It provides a transcriptional activation domain which, unlike Oct-1, activates an mRNA-type promoter effectively, and it stabilizes Oct-1 on the Oct-1-responsive octamer sequence ATGCAAAT. These properties of OCA-B parallel those displayed by the herpes simplex virus Oct-1 coregulator VP16. OCA-B, however, interacts with a different surface of the DNA-bound Oct-1 POU domain, interacting with both the POU_S and POU_H domains and the center of the ATGCAAAT octamer sequence. The OCA-B and VP16 interactions with the Oct-1 POU domain are sufficiently different to permit OCA-B and VP16 to bind the Oct-1 POU domain simultaneously. These results emphasize the structural versatility of the Oct-1 POU domain in its interaction with coregulators.

In eukaryotes, the activities of transcriptional activators can be modified by coregulators, thus providing flexibility and diversity in transcriptional regulation. For example, the broadly expressed human POU domain transcription factor Oct-1 acquires cell-specific promoter activation properties through association with both viral and cellular coregulators. In herpes simplex virus (HSV)-infected cells, HSV virion protein VP16 (also referred to as Vmw65 and αTIF) activates HSV immediate-early gene transcription by directing the formation of a multiprotein-DNA complex with Oct-1 and another cellular protein, HCF (also referred to as C1, VCAF, and CFF) on VP16-responsive TAATGARAT elements (for reviews, see references 18, 36, and 54). In B cells, Oct-1 associates with the cellular transactivator OCA-B (also referred to as OBF-1 and Bob-1) to activate transcription from octamer (ATGCAAAT) elements in immunoglobulin (Ig) gene promoters (17, 29, 30, 48).

Oct-1 binds to a diverse range of *cis*-regulatory DNA sequences through its DNA-binding POU domain, a bipartite structure consisting of an amino-terminal POU-specific (POU_S) domain joined by a flexible linker to a carboxy-terminal POU homeodomain (POU_H) (for reviews, see references 18 and 42). High-affinity Oct-1-binding sites include the octamer sequence found in a number of cellular promoters, such as the broadly expressed histone H2B and small nuclear RNA (snRNA) promoters. Consistent with the presence of Oct-1-binding sites in snRNA promoters, Oct-1 contains activation domains which activate transcription from snRNA promoters preferentially; in contrast, many activation domains activate transcription from mRNA promoters preferentially (10, 53).

Both VP16 (24, 47) and OCA-B (17, 30, 48) interact with Oct-1 through its POU domain. VP16 recognizes residues on the solvent-exposed surface of the DNA-bound Oct-1 POU_H domain (25, 40, 47) and alters the transcriptional activity of Oct-1 in two ways, (i) by providing a transcriptional activation domain which activates mRNA promoters preferentially (10) and (ii) by stabilizing Oct-1 on VP16-responsive elements (5).

OCA-B also stimulates the activation of mRNA-type promoters by Oct-1 (17, 29, 30, 48), but the requirements for OCA-B interaction with Oct-1 and the nature of the effects of this interaction on the activities of Oct-1 have not been extensively characterized. Here, we analyzed the functional properties of OCA-B. The results show that OCA-B functions analogously to VP16. OCA-B contains an activation domain which preferentially activates mRNA-type promoters, and OCA-B can stabilize Oct-1 binding to a *cis*-regulatory site. In contrast to VP16, however, OCA-B recognizes a separate surface of the Oct-1 POU domain.

MATERIALS AND METHODS

Reporter plasmids. Reporter constructs pc-*fos*/-197/4xG17M and pU2/-198/4xG17M have been described previously (10). For the in vivo corecruitment assay, two tandem copies of wild-type or mutant $A_5 \rightarrow T$ (ATGCTAAT) Ig(κ) octamer sites (38) separated by *Xho*I recognition sequences, GAGGAGCATG CAAATCATTGGCTC, were inserted into the *Sma*I site of pU2/β (5).

Effector plasmids. Full-length human OCA-B coding sequences were amplified from B-JAB cell RNA by reverse transcriptase PCR with primers, 5′ CAT CTAGACTCTGGCAAAAACCCACAGC (OBFNXBA) and 3′ CAGGATCC TAAAAGCCTTCCACAGAG (OBFCBAM), containing *Xba*I and *Bam*HI sites (underlined), respectively. These sequences were inserted between the *Xba*I and *Bam*HI sites of the hemagglutinin epitope tag-cytomegalovirus promoter expression vector pCGN (52), creating pCGN-OCA-B. pCGN-OCA-BΔC was prepared via PCR amplification of sequences encoding OCA-B residues 1 to 119. pCGN-Oct-1-POU is a derivative of pCG-Oct-1-POU (25) that contains the hemagglutinin epitope tag. pCGN-POU–VP16 was created by the insertion of sequences encoding the VP16 activation domain (residues 413 to 490), flanked by 5′ *Xba*I and 3′ *Nhe*I sites, into the *Xba*I site of pCGN-Oct-1-POU 5′ of the Oct-1 POU domain coding sequences. pCGN-$POU_{S\text{-}E7R}$–VP16 was created by oligonucleotide-mediated site-directed mutagenesis (32). The GAL4 fusion activators have been previously described (10), except for pCG-GAL4(1-94)–OCA-$B_{120–256}$, which contains the sequences encoding OCA-B residues 120 to 256. All effector constructs were verified by DNA sequence analysis.

POU domain mutagenesis. The Oct-1 and Pit-1 POU_S domain, linker, and POU_H domain chimeras PPOO, OOPO, and OOOP (1); POU_S domain chimeras EIR-1, NEV-1, TNE-2, D5E, L6I, E7R, and Pit-1 R7E (32); single POU_S alanine substitution R49A (6); and POU_H alanine substitution E22A (25) have been described previously. Mutant POU domain proteins were generated by oligonucleotide-mediated site-directed mutagenesis of plasmid pET11c.G.POU-1 (25), which encodes the Oct-1 POU domain fused to the glutathione *S*-transferase (GST) gene product. Double-mutant POU domain proteins ($POU_{S\text{-}E7R}$ $POU_{H\text{-}E22A}$ and $POU_{S\text{-}S48C/A52N}$) were made by two suc-

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cessive rounds of site-directed mutagenesis. These mutations are named according to the identity of the wild-type amino acid, followed by its position in the POU_S or POU_H domain and the identity of the amino acid substitution; thus, the replacement of the Oct-1 POU_S domain glutamic acid at position 7 with arginine is referred to as $POU_{S\text{-}E7R}$. Each mutation carries a cosegregating restriction site polymorphism, whose identity is available upon request. Mutations were verified by DNA sequence analysis.

Wild-type and mutant DNA-binding sites. The H2B octamer site has been described previously (5). The mutant site ICP0 perfect octamer (CATGCAAA TGATATT) was created by oligonucleotide-mediated site-directed mutagenesis of ICP0/TAAT-1 (6). The probes used for electropheretic mobility retardation analysis were generated by PCR amplification with ^{32}P-end-labeled primers as previously described (6). The DNA probes used (see Fig. 4) were made by annealing ^{32}P-end-labeled complementary oligonucleotides, 5′ GGGGAGAGT TCGAGGCTTATGCAAATAAGGTGCTCGAGACCTGCGC 3′ (H2BWT1) and 5′ GCGCAGGTCTCGAGCACCTTATTTGCATAAGCCTCGAACTCTC CCC 3′ (H2BWT2). H2B octamer mutant sites G_3→T, C_4→T, A_5→T, A_6→T, IC_5, and IC_6 were generated by annealing complementary mutant H2BWT1- and H2BWT2-related oligonucleotides.

To generate DNase I footprinting probe pUC119-H2B/OCTA+Igκ/OCTA, wild-type or mutant A_5→T Ig(κ) octamer sites were inserted into the *Sma*I site of pUC119-H2B/OCTA (5) in such a way that both octamer sites were in the same orientation and separated by 33 bp. Probes were generated by PCR amplification with a ^{32}P-end-labeled universal sequencing primer and an unlabeled primer, RSP-100 (5′-TGTGAGTTAGCTCACT 3′), which anneals to sequences approximately 100 bp upstream of the reverse sequencing primer.

Protein expression and purification. Wild-type and $POU_{S\text{-}E7R}$ GST–Oct-1 POU domain proteins were expressed in *Escherichia coli* BL21(DE3) and purified as described previously (25). Wild-type and mutant Oct-1 POU domains and OCA-B used in electrophoretic mobility retardation assays were synthesized in vitro as described previously (6). OCA-B for DNase I footprint analysis was prepared by overexpression after calcium phosphate coprecipitation-mediated transfection (51) of 10^6 HeLa cells with 16 μg of pCGN-OCA-B, followed by whole-cell nuclear extract preparation as previously described (53). VP16ΔC was expressed in and purified from *E. coli* as previously described (57), and HCF_{450} (HCF amino acids 1 to 450) (58) was prepared by in vitro translation.

In vivo transcription assays. Transcriptional activities were assayed in vivo by calcium phosphate coprecipitation-mediated transfection of HeLa cells and RNase protection of isolated RNAs (51) with α98 and β134 probes (5), the c-*fos* probe (50), and U2 probe A (28). In GAL4 activation domain fusion assays (10), the expression of GAL4 fusion activators was monitored by electropheretic mobility retardation analysis. All activators were expressed at similar levels as single, apparently full-length species, except for GAL4-VP16; it was expressed at lower levels, but this lower-level expression did not prevent transcriptional activation. In the in vivo Oct-1 POU domain–OCA-B recruitment assay, 640 ng of pCGN-Oct-1-POU or a derivative was cotransfected with 480 ng of pCGN-OCA-BΔC, 4 μg of reporter plasmid, and 160 ng of internal reference plasmid pα4x(A+C) (51). The specific activities of the β-globin and c-*fos* probes were half those of the α-globin and U2 probes. Unprotected RNA was digested by RNases A and T_1, and protected fragments were visualized by denaturing polyacrylamide gel electrophoresis (6% polyacrylamide). The levels of reporter gene expression were quantified on a Fuji BAS1000 phosphorimager.

DNase I protection analysis. Protein-DNA binding was performed in 50-μl reaction mixtures containing 2×10^4 cpm of DNA probe, 10 mM Tris-HCl (pH 7.9), 50 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 0.1% Nonidet P-40, 2% glycerol, 2% Ficoll, 3 mg of fetal bovine serum per ml, 50 ng of fish sperm DNA, 3.2 μg of unsonicated poly(dI-dC) (Pharmacia), and 2% polyvinyl alcohol. Purified recombinant GST–Oct-1 POU domain fusion protein (13 ng) and 5 μl of either mock- or OCA-B-transfected HeLa cell extract were used as indicated. Prior to DNase I digestion, protein-DNA reaction mixtures were incubated on ice for 20 min. Reactions were carried out as described previously (5), except that more DNase I (230 ng; Worthington) was used per reaction.

Electrophoretic mobility retardation assays. Protein-DNA binding reactions (10 μl) were performed as follows. In vitro-translated Oct-1 POU domain (1 μl), OCA-B (2 μl), HCF_{450} (0.5 μl), and *E. coli*-expressed VP16 (40 ng) were incubated with 2×10^4 cpm DNA probe in 10 mM Tris-HCl (pH 7.9)–50 mM KCl–2 mM dithiothreitol–1 mM EDTA–0.1% Nonidet P-40–2% glycerol–2% Ficoll–3 mg of fetal bovine serum per ml–10 ng of fish sperm DNA–640 ng of unsonicated poly(dI-dC) (Pharmacia) for 30 min at 30°C. After incubation, reaction mixtures were loaded onto a 6.5% polyacrylamide gel (acrylamide/bisacrylamide ratio, 19:1) in 0.25× TBE (22.5 mM Tris-borate, 0.5 mM EDTA), which had been subjected to electrophoresis for 1 h at room temperature. For the experiment shown in Fig. 4, full-length HeLa cell Oct-1 was 200-fold enriched by passage over wheat germ agglutinin (57).

RESULTS

To analyze the functional properties of human OCA-B, we assayed the promoter-selective properties of the OCA-B activation domain and the ability of OCA-B to stabilize the Oct-1 POU domain on DNA. We subsequently probed the surfaces of the DNA and Oct-1 POU domain required for OCA-B association with Oct-1 on an octamer site.

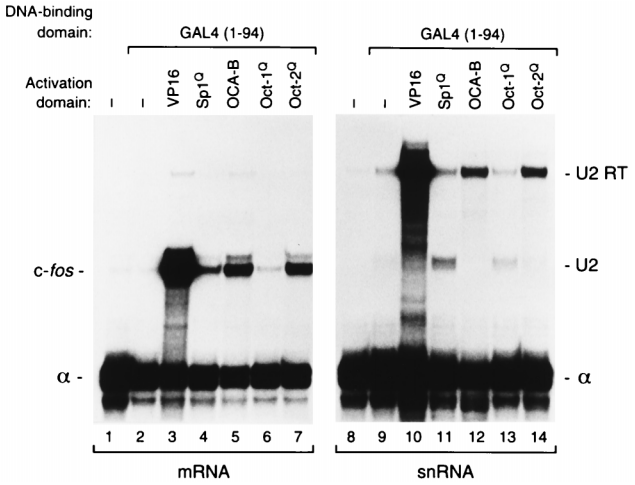

FIG. 1. Comparison of the activation domain properties of the Oct-1 cellular coregulator OCA-B with the viral coregulator VP16. c-*fos* mRNA (lanes 1 through 7) or U2 snRNA (lanes 8 through 14) reporter plasmids and an α-globin internal reference plasmid were transiently transfected into HeLa cells along with the appropriate activator expression plasmid, and RNA expression was monitored by RNase protection analysis as described in Materials and Methods. The GAL4 DNA-binding domain–activation domain fusion used in each sample is indicated above the corresponding lane. The positions of correctly initiated α-globin (α), c-*fos*, U2, and U2 read-through (U2 RT) transcripts are indicated. −, absent.

OCA-B contains an mRNA-type promoter-selective activation domain. Transcriptional activation domains can display promoter selectivity, distinguishing for example between mRNA- and snRNA-type promoters (10, 53). Figure 1 shows a comparison of the activation domain properties of OCA-B (residues 120 to 256) (17) with the mRNA-type promoter-selective activation domains from VP16 and the Oct-1-related B-cell POU domain activator Oct-2 and with the snRNA promoter-selective activation domains from Oct-1 and a second snRNA promoter activator, Sp1 (10). These activation domains were fused to the GAL4 DNA-binding domain (amino acids 1 to 94) and assayed with c-*fos* mRNA and U2 snRNA promoters containing GAL4-binding sites by transient expression in HeLa cells. An α-globin gene was used as a reference for transfection efficiency, and correctly initiated transcripts were measured by RNase protection.

Consistent with previous results (10), the VP16- and Oct-2-derived activation domains were better activators of the c-*fos* promoter (and of an mRNA-type read-through transcript [U2 RT] [10] evident with the U2 snRNA promoter construct [Fig. 1, lanes 8 through 14]) than were the Oct-1- and Sp1-derived activation domains (compare lanes 3 and 7 with lanes 4 and 6). The OCA-B activation domain was also an effective activator of the c-*fos* promoter; it was as active as the Oct-2-derived activation domain (Fig. 1; compare lanes 5 and 7). With the U2 snRNA promoter, however, the Oct-1- and Sp1-derived activation domains were more active than were the VP16- and Oct-2-derived activation domains (Fig. 1; compare lanes 10 and 14 with lanes 11 and 13) and the OCA-B activation domain (lane 12). Thus, like the activation domain of VP16, the OCA-B activation domain displays mRNA promoter selectivity, which provides one explanation as to how, by association with Oct-1, OCA-B can enhance Oct-1 activation of an mRNA promoter (17, 29, 30, 48).

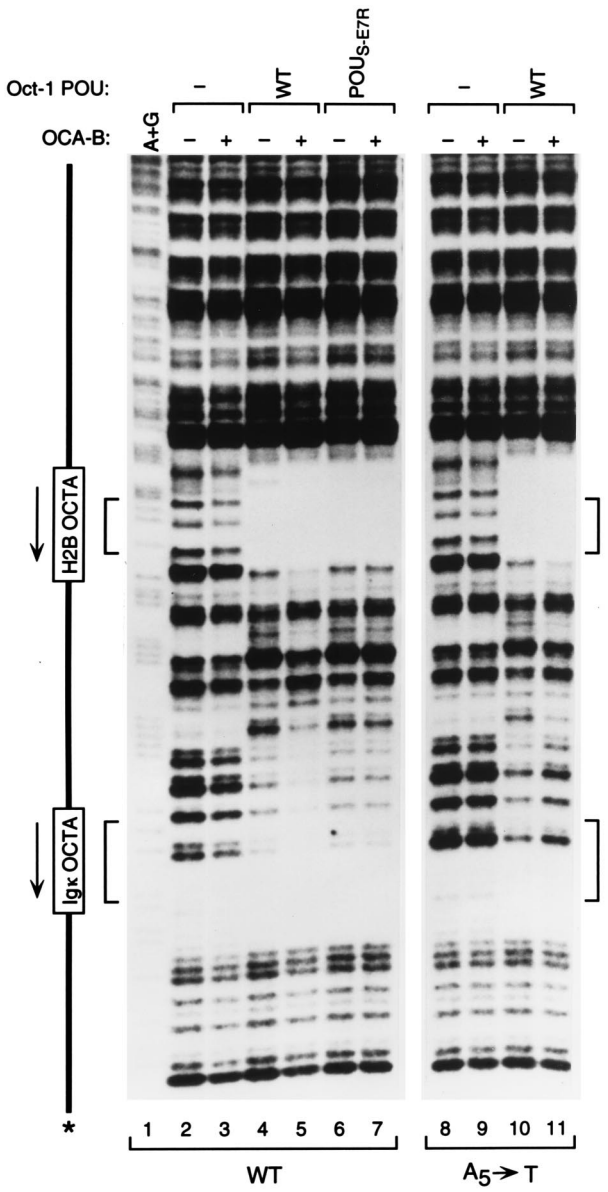

FIG. 2. OCA-B recruits the Oct-1 POU domain to the Ig(κ) octamer site in vitro. DNase I protection analysis of Oct-1 POU domain binding was performed with a DNA probe containing the histone H2B (H2B OCTA) and Ig(κ) (Igκ OCTA) promoter octamer sites. Recombinant wild-type (WT; lanes 4, 5, 10, and 11) and POU$_{S-E7R}$ mutant (lanes 6 and 7) GST–Oct-1 POU domain proteins were assayed in either the presence (+; lanes 3, 5, 7, 9, and 11) or absence (−; lanes 2, 4, 6, 8, and 10) of OCA-B on a probe containing either WT (lanes 2 through 7) or mutant (A$_5$→T; lanes 8 through 11) Ig(κ) octamer sites. Lane 1, chemical sequencing of the same probe (A+G). Asterisk, labeled end of probe.

OCA-B enhances binding of the Oct-1 POU domain to an octamer site both in vitro and in vivo. We assayed the ability of OCA-B to stabilize Oct-1 on an OCA-B-responsive element both in vitro and in vivo. To assay stabilization in vitro, we examined the effects of OCA-B on Oct-1 POU domain binding to a previously described octamer site from an Igκ promoter (38) by DNase I protection analysis. As a positive control for Oct-1 binding to the DNA probe used for DNase I protection analysis, we placed nearby to the Ig(κ) octamer site an octamer site with a higher affinity for Oct-1, the histone H2B promoter octamer site (38). Figure 2 shows Oct-1 POU domain binding to these two octamer sites in the presence and absence of full-length OCA-B.

In the absence of the Oct-1 POU domain, OCA-B had no evident effect on the DNase I cleavage pattern of the probe (Fig. 2; compare lanes 2 and 3). At a concentration of Oct-1 POU domain that affords full protection of the histone H2B octamer site but only partial protection of the Ig(κ) site (Fig. 2, lane 4), the addition of OCA-B enhanced but otherwise did not change Oct-1 POU domain protection of the Ig(κ) site (compare lanes 4 and 5). The enhanced protection was dependent on OCA-B association with Oct-1 because it was not observed when the Oct-1 POU$_S$ domain (POU$_{S-E7R}$) (Fig. 2, lane 7) or the octamer site (A$_5$→T) (lane 11) contained single point mutations that had (see below) little or no effect on Oct-1 binding alone but specifically disrupted OCA-B association with Oct-1. Thus, as is the case with VP16 on a TAATGARAT element (5), OCA-B can stabilize Oct-1 on an octamer site in vitro.

To measure OCA-B recruitment of Oct-1 to an OCA-B-responsive element in vivo, we fused the potent mRNA promoter activation domain of VP16 to the Oct-1 POU domain and assayed the activity of this fusion protein in the presence and absence of OCA-B in a transient-expression assay. The transcriptional activity of this fusion protein should reflect its relative occupancy of the octamer response element. For this assay, we created a reporter containing two tandem copies of the Ig(κ) octamer site upstream of a minimal β-globin promoter. To ensure that any transcriptional readout reflected recruitment of the Oct-1–VP16 fusion protein rather than direct activation by OCA-B, we removed the carboxy-terminal activation domain (amino acids 120 to 256) from OCA-B; the remainder of OCA-B (OCA-BΔC) retains the ability to interact with the Oct-1 POU domain (16, 48).

Figure 3 shows the results of such an in vivo Oct-1 recruitment assay. When OCA-BΔC was transiently expressed either alone (Fig. 3, lane 2) or with the Oct-1 POU domain (lane 4), there was no evident activation of transcription, as expected. To our surprise, the Oct-1 POU domain–VP16 fusion protein alone did not activate transcription from the octamer site effectively (Fig. 3, lane 5); in the presence of OCA-BΔC, however, high levels of transcription were achieved (lane 6). The enhanced activation of the reporter by the Oct-1 POU domain–VP16 fusion protein in the presence of OCA-BΔC likely results from its recruitment to the octamer site because (i) immunoblot analysis showed that its expression was not elevated in the presence of OCA-BΔC (data not shown) and (ii) OCA-BΔC enhancement of transcription was disrupted by the POU$_{S-E7R}$ Oct-1 POU domain (lane 8) and octamer site (A$_5$→T; lane 14) point mutations introduced above. Indeed, when the POU$_{S-E7R}$ Oct-1 POU domain and octamer site (A$_5$→T) point mutations were combined, OCA-BΔC had no evident effect on the activation of transcription by the Oct-1 POU domain–VP16 fusion protein (Fig. 3; compare lanes 5 and 16). These results can be explained by OCA-B recruitment of the Oct-1 POU domain fusion protein to the octamer site in vivo or by OCA-B enhancement of the activity of the VP16 transcriptional activation domain tethered to DNA by the Oct-1 POU domain; although we cannot exclude the latter, we favor the former hypothesis because it is consistent with the in vitro studies described above. Thus, these results suggest that as is the case with VP16 on a TAATGARAT element (5), OCA-B association with the Oct-1 POU domain results in Oct-1 recruitment to DNA in vivo. To our surprise, in the case of OCA-B, these effects were seen even with a consensus octamer site.

Together, the characterization of the OCA-B activation domain and the effects of OCA-B on Oct-1 binding to an octamer

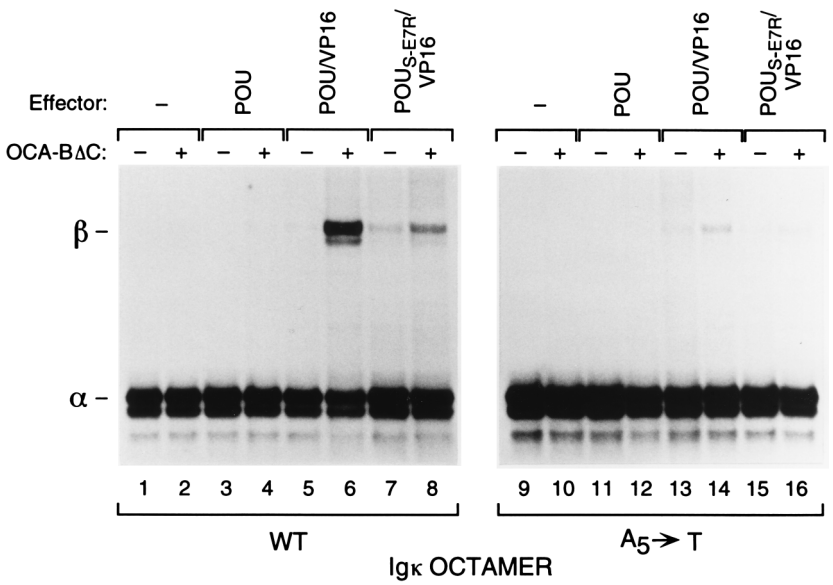

FIG. 3. OCA-B recruits the Oct-1 POU domain to the Ig(κ) octamer site in vivo. Transcriptional activation was measured by RNase protection analysis of RNA isolated from transiently transfected HeLa cells as described in Materials and Methods. Wild-type (WT; lanes 1 through 8) and mutant (A_5→T; lanes 9 through 16) Ig(κ) octamer-containing reporter plasmids were cotransfected with (+; even-numbered lanes) or without (−; odd-numbered lanes) an OCA-BΔC (amino acids 1 through 119) expression vector and the Oct-1 POU domain expression vector indicated above each pair of lanes. The positions of correctly initiated α-globin (α) and β-globin (β) transcripts are indicated.

site in vitro and in vivo reveal that OCA-B is a functional analog of VP16; it alters both the transcriptional activation and DNA-binding properties of Oct-1 in a manner analogous to that of VP16.

OCA-B makes multiple DNA contacts in the major groove at the center of the octamer sequence. On a VP16-responsive TAATGARAT site containing an overlapping octamer sequence [(OCTA$^+$)TAATGARAT], residues 3′ of the octamer sequence are important for VP16 association with Oct-1 (15, 20, 24, 31, 35). OCA-B association with Oct-1 is also DNA sequence dependent; the fifth position of the octamer sequence ATGCAAAT is important for OCA-B binding to Oct-1 (4, 16). To characterize the DNA sequence requirements for OCA-B association with Oct-1 in greater detail, we investigated the effects of individual substitutions of the central four residues of a perfect octamer site (positions 3 through 6 [ATGCAAAT]) with thymine on OCA-B–Oct-1 complex formation in an electrophoretic mobility retardation assay (Fig. 4). Because the structure of the Oct-1 POU domain–octamer site complex was determined on the histone H2B octamer site (23), we used this site (Fig. 4A) to study OCA-B association with DNA-bound Oct-1.

All of the thymine substitutions in the core of the octamer sequence affected Oct-1 binding but to various degrees (see the legend to Fig. 4). To determine the effects of thymine substitutions on OCA-B binding, we titrated the concentration of Oct-1 to generate similar levels of octamer site binding to all of the thymine-substituted sites (Fig. 4B, lanes 1 through 5; only the Oct-1–octamer site complex is shown). The addition of in vitro-translated OCA-B to these binding reactions retarded the mobility of Oct-1 on the wild-type site (Fig. 4B; compare lanes 1 and 7 with lanes 8 and 14). OCA-B, however, failed to associate with Oct-1 on the two sites carrying thymine substitutions at positions 5 and 6 (Fig. 4B; lanes 11 and 12, respectively) and had intermediate effects on the mobilities of Oct-1 complexes on the two sites with substitutions at positions 3 and 4 (lanes 9 and 10, respectively). The reason for the intermediate effects of the substitutions at positions 3 and 4 is not known but may reflect partial interference with OCA-B association with Oct-1. Together, the results with these thymine substitutions indicate that OCA-B interacts directly or indirectly with the four central residues of the octamer sequence.

In the Oct-1 POU domain–octamer site cocrystal structure, the major groove of the DNA is accessible at positions 5 and 6 of the octamer sequence (ATGCAAAT). To test whether the strong effects of the thymine substitutions at positions 5 and 6 on OCA-B association with the Oct-1–DNA complex reflected interaction with the major groove of DNA, we individually replaced these two AT base pairs with IC base pairs, substitutions that selectively alter the major groove (45). Although they permitted binding by Oct-1 (Fig. 4C, lanes 2 and 3), both of these IC substitutions interfered with OCA-B association with Oct-1 severely (lanes 7 and 8), indicating that OCA-B indeed recognizes the major groove of DNA at these two positions. The effect of the substitution at position 5 is consistent with a parallel disruption of OCA-B binding by a 5-methyl IC base pair at this position (4). In contrast to the other substitutions we analyzed, the IC substitution at position 6 did not affect Oct-1 binding to the octamer site (see the legend to Fig. 4), suggesting that at least at this position OCA-B interacts directly with the major groove of DNA.

In summary, as is the case with VP16, OCA-B association with Oct-1 depends on multiple DNA residues in the regulatory site; however, unlike VP16, which depends on residues 3′ of the octamer sequence, OCA-B depends on residues within the octamer sequence.

OCA-B fails to interact with the Pit-1 POU domain due to amino acid differences in both the POU_S and POU_H domains. In contrast to VP16 (24, 47), OCA-B does not discriminate between the POU domains of Oct-1 and the closely related B-cell POU domain protein Oct-2 (4, 17, 30, 48). The more distantly related N-Oct-3 (Brn-2), Oct-3/4, and Oct-6 (SCIP) POU domains, however, fail to interact with OCA-B (17, 30, 48). Here, we studied OCA-B interaction with the pituitary

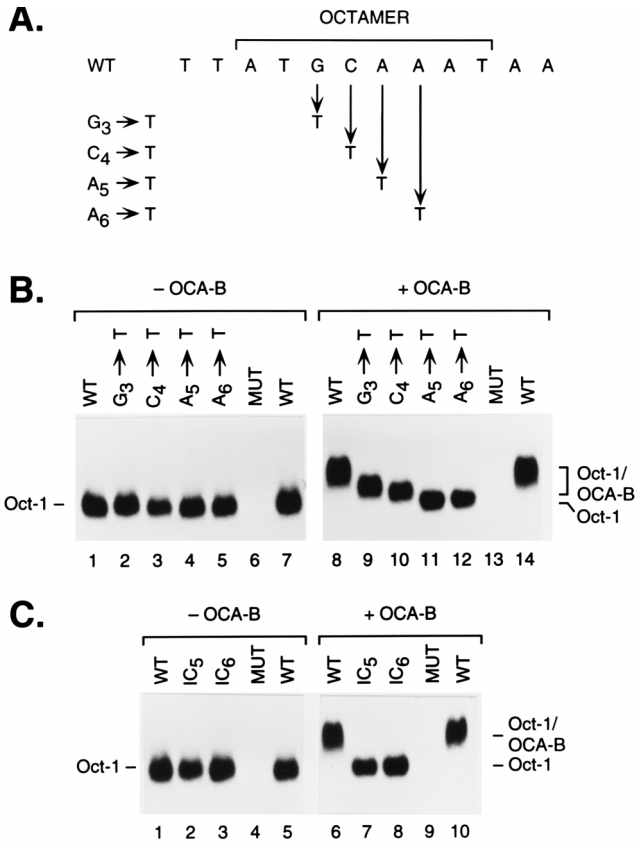

FIG. 4. Changes in the major groove of the octamer site disrupt OCA-B association with Oct-1 and DNA. (A) Wild-type (WT) and mutant H2B octamer sites. Each mutation is numbered according to its position within the octamer sequence. (B) Electrophoretic mobility retardation analysis of Oct-1 bound to the octamer sites indicated in the absence (−; lanes 1 through 7) or presence (+; lanes 8 through 14) of OCA-B. MUT, a double-base pair substitution at the first and second positions of the octamer site described previously (H2B/OCTA*dpm1* [6]). To obtain equal Oct-1 DNA binding to $G_3 \rightarrow T$, $C_4 \rightarrow T$, $A_5 \rightarrow T$, and $A_6 \rightarrow T$ sites, more Oct-1 protein (4-, 1.6-, 1.6-, and 16-fold, respectively) compared to the amount of the WT site sample was used. MUT samples contained the maximum (16-fold) enrichment of Oct-1 protein. Oct-1, Oct-1–DNA complex; Oct-1/OCA-B, Oct-1–OCA-B–DNA complexes. Free DNA is not shown. (C) Electrophoretic mobility retardation analysis of Oct-1 to IC substitutions at positions 5 and 6 in the absence (−; lanes 1 through 5) or presence (+; lanes 6 through 10) of OCA-B. The identity of each probe is indicated above each lane. To obtain equal Oct-1 DNA binding to the IC_5 site, fourfold more Oct-1 protein compared to the amount of the WT site sample was used. The IC_6 sample contained the same level of Oct-1 as did the WT site sample, and MUT samples contained 16-fold more protein. Oct-1, Oct-1–DNA complex; Oct-1/OCA-B, Oct-1–OCA-B–DNA complex. Free DNA is not shown.

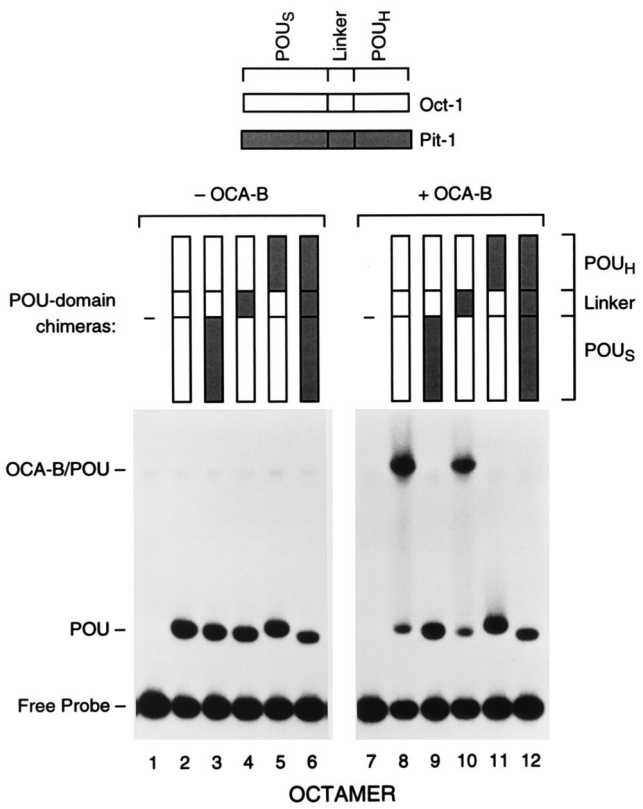

FIG. 5. OCA-B distinguishes between both the Oct-1 and Pit-1 POU_S and POU_H domains. An electrophoretic mobility retardation assay was performed with the histone H2B octamer probe. The identity of each Oct-1–Pit-1 chimeric protein assayed is indicated above the corresponding lane. White boxes represent segments derived from the Oct-1 POU domain, and gray boxes represent segments derived from the Pit-1 POU domain. Wild-type and chimeric POU domains were produced in vitro and assayed in either the absence (−; lanes 1 through 6) or presence (+; lanes 7 through 12) of in vitro-translated OCA-B. POU, POU domain-DNA-containing complexes; OCA-B/POU, POU domain–OCA-B–DNA-containing complexes.

POU domain protein Pit-1 (3, 21), which is about 50% identical to the Oct-1 POU domain. Figure 5 shows the results of an electrophoretic mobility retardation assay of OCA-B interaction with in vitro-translated Oct-1, Pit-1, and Oct-1–Pit-1 POU domain chimeras. All of the POU domains assayed bound the histone H2B octamer site effectively (Fig. 5, lanes 1 through 6); in contrast to the Oct-1 POU domain, however, the Pit-1 POU domain failed to interact with OCA-B (compare lanes 8 and 12). Determinants in the Pit-1 POU_S and POU_H domains are responsible for preventing OCA-B association with this POU domain, because when the Oct-1 POU_S domain, linker, and POU_H domain were individually replaced with the corresponding region from Pit-1, only the linker swap retained the ability to associate with OCA-B (Fig. 5, lanes 9 through 11). Thus, OCA-B recognizes the Oct-1 POU_S and POU_H domains. In contrast, VP16 recognizes only the Oct-1 POU_H domain (41, 47).

OCA-B and $SNAP_C$ interactions with the Oct-1 POU_S domain overlap. To probe the determinants of the Oct-1 POU_S domain interaction in more detail, we analyzed OCA-B association with an extended series of Oct-1 POU domains carrying one to six amino acid substitutions from the Pit-1 POU_S domain sequence across the entire Oct-1 POU_S domain (Fig. 6). This analysis revealed that Pit-1 sequences in two regions of the POU_S domain, in the amino-terminal region of α-helix 1 (EIR-1) and within α-helix 4 (Pα4), disrupted OCA-B association (Fig. 6).

The EIR-1 substitution also affects Oct-1 POU domain association with the basal snRNA promoter complex $SNAP_C$/PTF (32). In that case, of three substitutions in EIR-1 (D5E, L6I, and E7R), only one (E7R) affects Oct-1 POU domain interaction with $SNAP_C$ (32). Similar to the effect on $SNAP_C$ interaction, only the E7R swap in the amino-terminal region of α-helix 1 affected OCA-B association (Fig. 6). In contrast to $SNAP_C$ (32) and consistent with the effects of the Pα4 substitution, the complementary R7E substitution in the Pit-1 POU_S domain was not sufficient to activate the Pit-1 POU domain for association with OCA-B (Fig. 6). Thus, OCA-B, a B-cell Oct-1 coregulator, and $SNAP_C$, a basal snRNA promoter complex, recognize the same precise difference within the amino-termi-

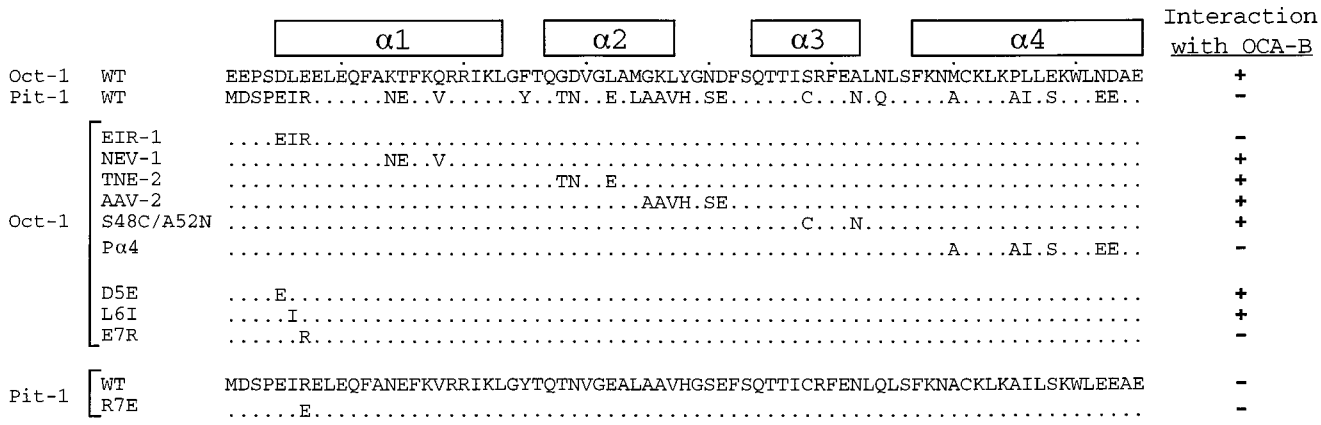

FIG. 6. OCA-B recognizes multiple amino acid differences between the Pit-1 and Oct-1 POU_S domains. The top line shows the sequence of the wild-type (WT) Oct-1 POU_S domain; the next line shows the WT Pit-1 POU_S domain sequence, with identities with the Oct-1 sequence indicated by dots. Labeled boxes above the Oct-1 sequence delineate α-helices in the POU_S domain. The Pit-1-derived sequences in EIR-1, NEV-1, TNE-2, AAV-2, S48C/A52N, Pα4, D5E, L6I, and E7R Oct-1 POU domain derivatives are shown. The sequence of the Pit-1 POU domain and the single Oct-1-derived amino acid difference in the R7E Pit-1 POU domain derivative are shown at the bottom. The ability (+) or inability (−) of each WT and substituted POU domain to associate with OCA-B, as assayed by electrophoretic mobility retardation analysis, is shown on the right.

nal regions of the Oct-1 and Pit-1 POU_S domains. However, OCA-B recognized additional differences in both the POU_S domain (i.e., Pα4 [Fig. 6]) and the POU_H domain (Fig. 5).

OCA-B interaction surface on the Oct-1 POU domain. To map the precise surface of the Oct-1 POU domain that is targeted by OCA-B, we assayed the activities of a systematic series of alanine substitutions in the Oct-1 POU domain. We used the Oct-1 POU domain–octamer site cocrystal structure (23) to guide the selection of residues for alanine substitutions. An examination of the Oct-1 POU domain–octamer site cocrystal structure revealed that the glutamic acid at position 7 of the Oct-1 POU_S domain, which is critical for OCA-B association, is located on the same face of the Oct-1 POU domain–DNA complex as are the DNA major groove positions that are important for interaction with OCA-B (positions 5 and 6 [Fig. 4]). Therefore, we individually replaced all of the solvent-exposed residues on this surface of the POU domain with alanine. To test the importance of Oct-1 POU domain–octamer site interactions for OCA-B association, we also included two separate alanine substitutions for conserved residues in the POU_S and POU_H domains that contact DNA (POU_S domain residue R49 and POU_H domain residue N51).

Figure 7 summarizes the activities of the alanine substitution mutants and shows the results of electrophoretic mobility retardation assays for a representative set of mutations. Of the 23 alanine substitutions in the Oct-1 POU_S domain assayed, only one (R49A) had a great effect on DNA binding (Fig. 7A, lane 3) (6, 55); consistent with the importance of the POU_S domain for OCA-B interaction, this mutant also failed to associate with OCA-B (lane 3). Nine of the other 22 POU_S alanine substitutions also affected Oct-1 POU domain association with OCA-B (Fig. 7B). Consistent with previous results (16), mutants L53A, L55A, and N59A, but not mutant N54A, failed to associate effectively with OCA-B.

The representative set of POU_S domain substitutions in Fig. 7A includes substitutions in the amino-terminal region of α-helix 1 and in α-helix 4. The results for α-helix 1 substitutions show that as was the case in Oct-1 and Pit-1 swaps, E7 was critical for OCA-B association; in contrast to the results for the L6I substitution in Oct-1 and Pit-1 swaps (Fig. 6), the L6A substitution affected OCA-B association (Fig. 7A, lane 5). These results suggest that positions 6 and 7 are critical for OCA-B association but that a conservative substitution (isoleucine for leucine) can be tolerated at position 6. The results for substitutions in α-helix 4 show that a mutation of the proline at position 65 (P65) which causes a bend in this α-helix (23) did not disrupt OCA-B association with the Oct-1 POU domain (Fig. 7A, lane 9), suggesting that some structural changes in the POU_S domain can be tolerated without necessarily affecting the interaction with OCA-B. Alanine substitution for the methionine at position 60 (M60A), however, affected OCA-B interaction (Fig. 7A, lane 8); this residue lies between the major groove contacts at positions 5 and 6 of the octamer site on one side and POU_S domain residues L6 and E7 on the other side (see below). Together, the substitutions that affect OCA-B interaction form a patch on the surface of the Oct-1 POU_S domain (see below), suggesting that this region of the Oct-1 POU_S domain contacts OCA-B.

We assayed 14 alanine substitutions in the Oct-1 POU_H domain for the ability to interact with OCA-B. Unexpectedly, the substitution for N51, which is conserved in nearly all homeodomains and makes specific base contacts in homeodomain-binding sites (13), had only a twofold effect on binding to the histone H2B octamer site (Fig. 7A; compare lanes 12 and 13). Although it had relatively little effect on DNA binding per se at this site, it had a large effect on OCA-B association (Fig. 7A, lane 13). It is unlikely that OCA-B contacts this asparagine directly because it is not exposed when the POU domain is bound to the octamer site (23); perhaps the precise arrangement of the POU_H domain on the DNA is critical for association with OCA-B.

Only 3 of the other 13 alanine substitutions assayed affected association with OCA-B (Fig. 7B). Consistent with previous results (16), substitutions R13A and E17A did not affect the interaction with OCA-B. Two mutations, E22A and E30A, which prevent VP16 interaction with the Oct-1 POU domain (25, 39, 40) did not affect the ability of the POU domain to interact with OCA-B (Fig. 7A, lanes 15 and 16). All three substitutions that did affect OCA-B association are within the carboxy-terminal region of α-helix 3, the DNA recognition helix (Fig. 7A, lanes 18 and 19, and B).

Figure 8 summarizes these results. It shows three representations of the Oct-1 POU domain–octamer site cocrystal structure (the POU domain is shown in white, and DNA is shown in

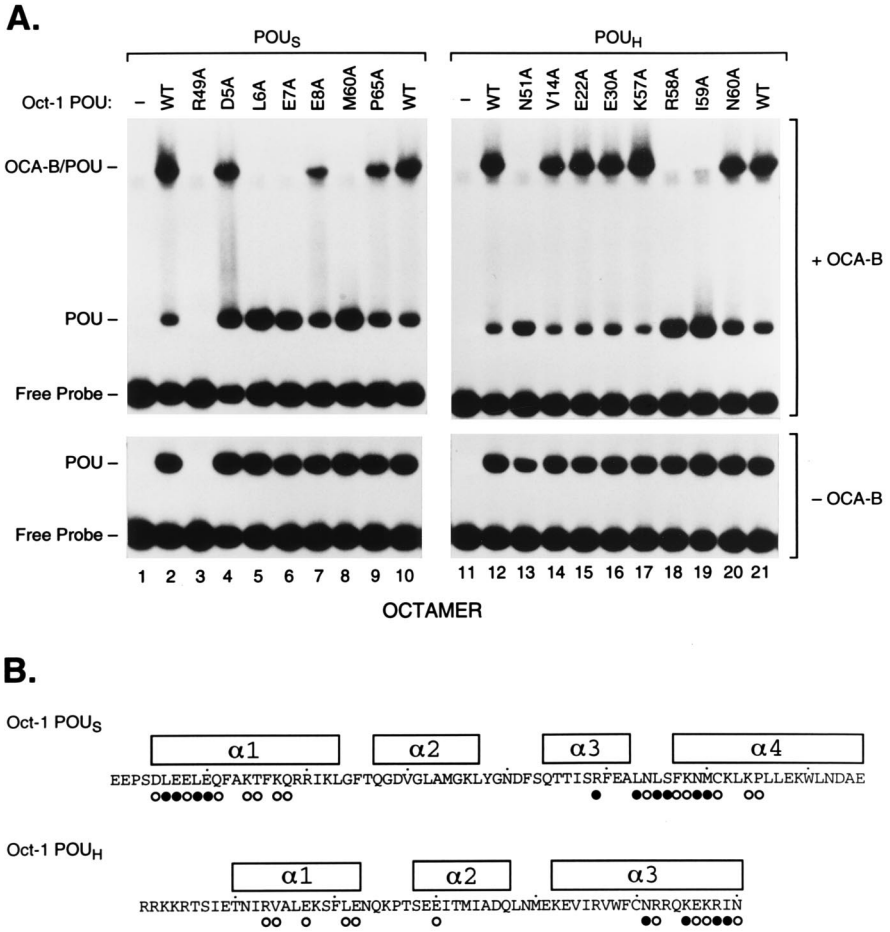

FIG. 7. OCA-B interacts with multiple residues in the Oct-1 POU_S and POU_H domains. (A) Electrophoretic mobility retardation analysis of a representative set of alanine substitutions in the Oct-1 POU_S and POU_H domains. In vitro-translated wild-type (WT) and mutant POU domains were assayed in the absence (−) or presence (+) of in vitro-translated OCA-B with the histone H2B octamer probe. The identity of each POU domain is indicated above the corresponding lane. POU, POU domain-DNA-containing complexes; OCA-B/POU, POU domain–OCA-B–DNA-containing complexes. (B) Summary of the effects of alanine substitutions in the Oct-1 POU domain on Oct-1 POU domain association with OCA-B, as assayed by electrophoretic mobility retardation analysis. The amino acid sequences of the Oct-1 POU_S and POU_H domains are shown, with labeled boxes above each sequence delineating the α-helices in the corresponding POU domain. Filled and open circles indicate alanine substitutions that disrupted or did not disrupt association with OCA-B, respectively.

gray). Positions 5 and 6 of the octamer sequence, where the major groove of DNA is critical for association with OCA-B (Fig. 4), are shown in green. Residues whose substitution with alanine affected OCA-B interaction are highlighted in red, and adjacent residues whose substitution did not affect OCA-B interaction are shown in blue. The alanine substitutions that did not affect OCA-B interaction define the boundaries of the OCA-B interaction surface. Shown in yellow is E22, the glutamic acid residue in the Oct-1 POU_H domain that is critical for VP16 association (25, 40).

Figure 8A shows a typical projection of the Oct-1 POU domain–octamer complex, with the POU_H domain to the upper right and the POU_S domain to the lower left, revealing the amino-terminal arm of the POU_H domain. In this projection, VP16 interacts with the upper right surface of the POU_H domain (25, 40, 47). In contrast, OCA-B does not interact with this surface of the Oct-1 POU domain. Rotating the complex along the horizontal axis (Fig. 8B) reveals the OCA-B interaction surface. In this projection, the major groove octamer site and Oct-1 POU domain substitutions that affect OCA-B association form a continuous surface that links the POU_S and POU_H domains via DNA, suggesting that OCA-B contacts the two POU subdomains and the octamer sequence simultaneously. Rotating the complex shown in Fig. 8A along the vertical axis (Fig. 8C) shows how the two POU_S and POU_H domain surfaces that interact with OCA-B jut out from the Oct-1–DNA complex, emphasizing their accessibility for interaction with a coregulator such as OCA-B.

OCA-B and VP16 can associate with the Oct-1 POU domain simultaneously. An analysis of the Oct-1 POU domain–OCA-B interaction surface suggested that OCA-B and VP16 target separate surfaces of the POU domain. To test this hypothesis, we determined whether OCA-B and VP16 can interact with the Oct-1 POU domain simultaneously. For this purpose, by a single base pair substitution, we changed an ($OCTA^+$)TAATGARAT VP16-responsive element from the HSV ICP0 promoter to contain a consensus overlapping octamer sequence, thus creating the ICP0 perfect octamer site (ATGCAAATGATAT). We tested the ability of OCA-B and VP16 to interact with the Oct-1 POU domain on this site in an electrophoretic mobility retardation assay (Fig. 9).

Consistent with previous results (4, 12, 16), the wild-type Oct-1 POU domain associated with either OCA-B (Fig. 9, lane 6) or VP16 (lane 7) on the ICP0 perfect octamer site. When

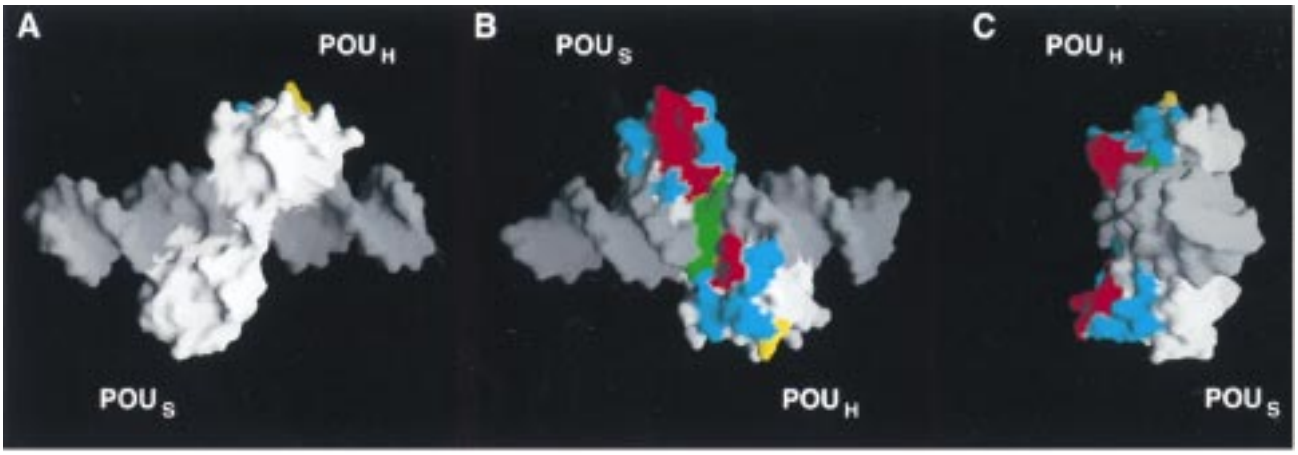

FIG. 8. Graphic representation of the OCA-B interaction surface of the Oct-1 POU domain–octamer site complex. The molecular modeling program GRASP (34) was used to generate three projections of the Oct-1 POU domain–octamer site complex (23). (A) In this projection, the octamer sequence ATGCAAAT is shown from left to right, with the POU_H domain to the upper right and the POU_S domain to the lower left. (B) Shown is a 180° rotation along the horizontal axis of the projection in panel A, with the POU_H domain to the lower right and the POU_S domain to the upper left. (C) Shown is a 90° counterclockwise rotation, looking down the vertical axis of the projection in panel A in such a way that the octamer sequence ATGCAAAT goes away from the viewer, the POU_H domain is on top, and the POU_S domain is on the bottom. In this projection, the POU domain linker is predicted to lie to the right of the DNA. In all three projections, the POU domain is shown in white and DNA is shown in gray. Residues whose substitution by alanine disrupted OCA-B association are shown in red, and neighboring residues whose substitution by alanine did not disrupt OCA-B association are shown in blue. The two base pairs that disrupted OCA-B association selectively when they were replaced by IC are shown in green. Shown in yellow is E22, the glutamic acid residue in the Oct-1 POU_H domain that is critical for VP16 association (25, 40).

VP16 and OCA-B were added together in either the presence (Fig. 9, lane 8) or absence (data not shown) of HCF, a novel complex of reduced mobility was evident. This novel complex was affected by both the $POU_{S\text{-}E7R}$ mutation, which prevented OCA-B but not VP16 association (Fig. 9, lanes 9 through 12), and the $POU_{H\text{-}E22A}$ mutation, which prevented VP16 but not OCA-B association (Fig. 9, lanes 13 through 16). Thus, the novel complex probably represents simultaneous association of

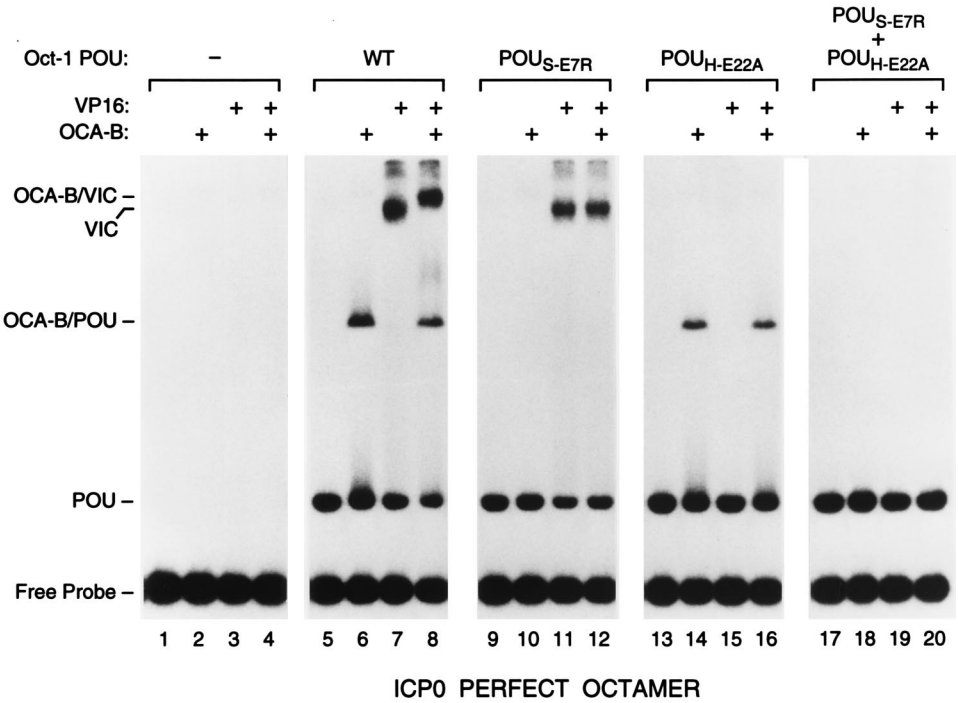

FIG. 9. OCA-B and VP16 interact with separate surfaces of the Oct-1 POU domain. The results of electrophoretic mobility retardation analysis of wild-type (WT) and mutant Oct-1 POU domains for OCA-B and VP16 association on the ICP0 perfect octamer site are shown. Binding reactions were performed in the absence (−; lanes 1 through 4) or presence of the WT Oct-1 POU domain (lanes 5 through 8), the $POU_{S\text{-}E7R}$ (lanes 9 through 12) or $POU_{H\text{-}E22A}$ (lanes 13 through 16) single mutant Oct-1 POU domain, or the $POU_{S\text{-}E7R}$ + $POU_{H\text{-}E22A}$ double mutant Oct-1 POU domain (lanes 17 through 20). The presence (+) or absence of OCA-B or VP16ΔC and HCF_{450} (VP16) in each sample is indicated above the corresponding lane. OCA-B, Oct-1 POU domains, and HCF_{450} were synthesized by translation in vitro, and VP16ΔC was synthesized in *E. coli*. POU, POU domain-DNA-containing complexes; OCA-B/POU, POU domain–OCA-B–DNA-containing complexes; VIC, VP16-induced complex; OCA-B/VIC, OCA-B-containing VP16-induced complex.

OCA-B and VP16 with the Oct-1 POU domain. By combining the OCA-B-specific $POU_{S\text{-}E7R}$ mutation and the VP16-specific $POU_{H\text{-}E22A}$ mutation, we created a variant Oct-1 POU domain (Oct-1 $POU_{S\text{-}E7R}$ + $POU_{H\text{-}E22A}$) that recognized DNA effectively (Fig. 9, lane 17) but failed to interact with either OCA-B or VP16 (lanes 18 through 20). The ability of OCA-B and VP16 to associate with the Oct-1 POU domain simultaneously on a single DNA site indicates that although OCA-B and VP16 affect the function of Oct-1 similarly, they interact with separate protein and DNA surfaces of the DNA-bound Oct-1 POU domain.

DISCUSSION

We analyzed the function of the B-cell-specific Oct-1 coregulator OCA-B. The results show that OCA-B shares functions with the viral Oct-1 coregulator VP16. Both change the transcriptional activation potential of Oct-1 and recruit Oct-1 to *cis*-regulatory sites. They achieve these functions, however, through completely different interactions with the Oct-1 POU domain and DNA. OCA-B interacts with a continuous surface of the DNA-bound Oct-1 POU domain, encompassing the POU_S domain, the central region of the octamer sequence, and the POU_H domain, whereas VP16 interacts with a different surface of the POU_H domain and with sequences 3′ of the octamer sequence. These two surfaces are sufficiently different that these two Oct-1 coregulators can associate with Oct-1 simultaneously.

The POU domain offers flexibility in both protein-DNA and protein-protein interactions. POU domain proteins display considerable flexibility in their interactions with DNA (18). Mutagenesis studies of Brn-2 and Oct-1 (6, 27, 56) and DNA cross-linking studies (7) have revealed that individual POU domains can adopt different conformations on different *cis*-regulatory sites. Indeed, even on a single *cis*-regulatory site, the Oct-1 POU domain can adopt more than one conformation. When it is bound to a VP16-responsive TAATGARAT site containing an overlapping octamer sequence, the Oct-1 POU_S domain can bind both 5′ and 3′ of the POU_H domain; surprisingly, both of these conformations are compatible with VP16 association (7).

The POU domain also displays great flexibility in its interactions with other proteins. Previous analyses of Oct-1 POU domain mutations that disrupt interactions with Oct-1 coregulators have shown that VP16 interacts with the POU_H domain (41, 47) and that $SNAP_C$ interacts with the POU_S domain (32). Although it is not known whether $SNAP_C$ also interacts with the Oct-1 POU_H domain, our study has shown that OCA-B interacts with both the POU_S and POU_H domains. The simultaneous interaction of OCA-B with the Oct-1 POU_S and POU_H domains and with the DNA between the two POU DNA-binding structures suggests that the Oct-1 POU domain and OCA-B form a linked structure surrounding the octamer site (Fig. 8C). Such a ring-like structure may form a stable complex for high-level and long-term activation of Ig gene transcription in differentiated B cells.

The bipartite pattern of OCA-B interaction with the Oct-1 POU domain suggests that in contrast to VP16, which can associate with Oct-1 when the Oct-1 POU_S domain is in different positions (7), the precise positioning of the two POU domain DNA-binding structures is critical for OCA-B association. Consistent with such differences in how VP16 and OCA-B interact with the Oct-1 POU domain, VP16 can associate with Oct-1 when the POU_S domain carries the R49A POU_S domain DNA recognition helix mutation, which disrupts POU_S domain binding to DNA (6), whereas this mutation prevented OCA-B association with the Oct-1 POU domain on the octamer site (Fig. 7). Surprisingly, although the N51A substitution in the POU_H domain had little effect on POU domain binding to the histone H2B octamer, it had a major effect on OCA-B association (Fig. 7), suggesting that the precise configuration of the POU_H domain on DNA is also critical for Oct-1 POU domain association with OCA-B. Whichever Oct-1 POU domain conformation OCA-B recognizes, the ability of the Oct-1 POU domain to associate simultaneously with OCA-B and VP16 (Fig. 9) demonstrates that these two Oct-1 coregulators can recognize the same conformation of the Oct-1 POU domain.

In contrast to the independent interactions of OCA-B and VP16 with the Oct-1 POU domain, the associations of OCA-B and $SNAP_C$ with the Oct-1 POU domain are sensitive to the same single amino acid substitution, the E7R Oct-1–Pit-1 exchange in the POU_S domain. Consistent with overlapping OCA-B and $SNAP_C$ interactions with the Oct-1 POU domain, in a transient-expression assay, OCA-B is able to interfere with octamer-dependent activation of U2 snRNA gene transcription (2). How do these competing interactions not interfere with one another on an snRNA promoter in a B cell? Probably, under natural conditions, promoter-specific interactions (e.g., those of promoter-selective activation domains) stabilize the appropriate Oct-1 POU domain interaction and prevent competition.

Together, the versatility and flexibility displayed by the Oct-1 POU domain in both DNA sequence recognition and coregulator association reveal a DNA-binding domain which can adapt itself to many promoter contexts. This versatility and flexibility may result from the unusual structural characteristics of the POU domain, in which two independent DNA-binding structures, the POU_S and POU_H domains, are joined by a flexible linker. This structural arrangement provides not only flexibility in how the POU domain binds DNA but also diverse surfaces for interaction with coregulators.

Two mechanisms by which an octamer motif can achieve B-cell-specific gene transcription. In the context of a typical mRNA-type promoter, the octamer motif displays B-cell-specific activation of transcription (11, 14, 26, 59). One explanation for this specificity is the expression of the Oct-1-related POU domain protein Oct-2 (8, 33, 43, 49), which has the same octamer motif-binding specificity (46) but, in contrast to Oct-1, contains activation domains which more effectively activate mRNA-type promoters (52, 53). B-cell-specific OCA-B, with its mRNA-type promoter-selective activation domain (Fig. 1), provides a second mechanism by which the octamer motif can display B-cell-specific transcription. Its association with Oct-1, a generally ineffective activator of mRNA-type promoters (52), allows activation of mRNA-type promoters (4, 16, 17, 29, 30, 48), very similar to what VP16 does on HSV promoters (36, 54). These two mechanisms by which B-cell-specific octamer motif function is achieved apparently evolved separately. Oct-1 and Oct-2 arose through gene duplication (19), whereas OCA-B probably arose by a different mechanism.

Why have two mechanisms for B-cell-specific octamer motif-dependent transcription arisen? Perhaps they provide flexibility in the regulation of B-cell-specific transcription. For example, although OCA-B association with Oct-1 provides for activation of an mRNA-type promoter, OCA-B association with Oct-2 may provide even higher levels of mRNA-type promoter transcription or transcriptional activation from more distal regulatory elements, such as enhancers (37), because of the combination of mRNA-type promoter-selective activation domains provided by both OCA-B and Oct-2. Furthermore, OCA-B stabilizes the Oct-1 POU domain (and probably the

Oct-2 POU domain) on low-affinity octamer sites (Fig. 2 and 3), allowing for activation of transcription from promoters that would be silent in the absence of OCA-B despite the presence of Oct-2. Thus, in the presence of OCA-B, promoters with low-affinity octamer sites can respond to either Oct-1 and Oct-2; however, in the absence of OCA-B, only mRNA-type promoters with high-affinity octamer sites should be active, because of the mRNA-type transcriptional activation domain activity of Oct-2.

Consistent with the functional redundancy of an OCA-B–Oct-1 complex and Oct-2 alone, gene disruption of OCA-B (22, 44) or Oct-2 (9) does not impair Ig gene transcription in early B cells. However, mice deficient for either OCA-B or Oct-2 do display different phenotypes, and some of these differences may result from the different activities of OCA-B and Oct-2 in transcriptional activation. These results reveal that through the generation of families of related transcription factors, such as Oct-1 and Oct-2, or by using coregulators, such as OCA-B, flexibility and complexity in cell-specific transcription can be achieved.

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