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Synergism with the Coactivator OBF-1 (OCA-B, BOB-1) Is Mediated by a Specific POU Dimer Configuration

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Summary

POU domain proteins contain a bipartite DNA binding domain divided by a flexible linker that enables them to adopt various monomer configurations on DNA. The versatility of POU protein operation is additionally conferred at the dimerization level. The POU dimer formed on the PORE (ATTTGAAATGCAAAT) can recruit the transcriptional coactivator OBF-1, whereas POU dimers formed on the consensus MORE (ATGCATATG CAT) or on MOREs from immunoglobulin heavy chain promoters (AT[G/A][C/A]ATATGCAA) fail to interact. An interaction with OBF-1 is precluded since the same Oct-1 residues that form the MORE dimerization interface are also used for OBF-1/Oct-1 interactions on the PORE. Our findings provide a paradigm of how specific POU dimer assemblies can differentially recruit a coregulatory activity with distinct transcriptional readouts.

Introduction

Development of multicellular organisms is characterized by an intricate series of genetic and epigenetic events that generate the complex adult body from the unicellular zygote. A refined and sophisticated regulatory network that is established during embryogenesis reflects the complexity of organisms. Although embryonic development is a multistep process characterized by the sequential activation and repression of many genes, only a relatively small number of transcription factors are responsible for regulating the expression of developmental genes. This diversity in transcriptional control by a limited array of transcription factors is achieved through a complex network of interactions between these proteins and specific DNA sequences found in promoters and enhancers of developmental genes. The primary structure of these DNA elements defines the composition and architecture of the transcriptional activation complexes that ultimately control gene expression in the appropriate temporo-spatial context of the developing organism. For example, nonsteroid members of the nuclear receptor superfamily that possess a zinc-finger DNA binding domain operate by binding to the hormone response elements (HREs). HREs consist of two minimal core hexad sequences, AGGTCA, which can be configured into various functional motifs. The orientation and spacing between these two hexamers as well as subtle differences in their sequence dictate the identity and the mode (monomer, hetero-, or homodimer) of nuclear receptor binding that results in diverse effects on transcription (Mangelsdorf and Evans, 1995).

The operation of members of the POU domain family of transcription factors is also highly dependent on the nature of cognate DNA elements. The 160 amino-acid-long DNA binding domain of these proteins is composed of two structurally independent subdomains: the POU-type homeodomain (POU-homeo or POU_H), and the POU-specific domain (POU_S) that are connected by a flexible linker region (Schöler, 1991; Verrijzer and van der Vliet, 1993). POU domain proteins demonstrate impressive versatility in how they regulate transcription. This is due to several, often interdependent, factors: (1) flexible amino acid–base interaction, (2) variable orientation, spacing, and positioning of DNA-tethered POU subdomains relative to each other, (3) posttranslational modification, and (4) interaction with heterologous proteins (Herr and Cleary, 1995).

POU domain proteins are able to bind to DNA cooperatively, thus conferring additional functional variability. The homo- and heterodimerization of Oct-1 and Oct-2 on immunoglobulin (Ig) heavy chain promoters (V_H) provided evidence of cooperativity, with a yet unknown dimer arrangement (Kemler et al., 1989; LeBowitz et al., 1989; Poellinger et al., 1989). The *cis*-elements are considered to consist of low-affinity heptamer and high-affinity octamer sites separated by two nucleotides (CTCATGAATATGCAAAT).

The pituitary-specific POU domain protein Pit-1 binds to DNA either as a homodimer or as a heterodimer with Oct-1 (Voss et al., 1991). Crystallographic studies determined the structure of a Pit-1 homodimer assembled on the synthetic motif ATGTATATACAT (referred to here as PitD) that had been derived from the natural Pit-1 cognate element within the prolactin gene promoter (ATATATATTCAT) (Jacobson et al., 1997). The structure of the Pit-1 POU_S and POU_H domains, and their docking onto DNA, are very similar to that observed in the cocrystal of the Oct-1 POU domain monomer with the octamer site (ATGCAAAT, Klemm et al., 1994). The Oct-1 POU_S

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domain recognizes the ATGC subsite whereas the Pit-1 POU_S domain binds to the sequence ATAC. However, the latter subsite lies on the opposite strand and, as a consequence, the orientation of POU_S relative to the POU_H domain is inverted (Jacobson et al., 1997).

Another mechanism outlining cooperative DNA binding by POU proteins was recently determined during the course of an Oct-4 target gene characterization (Botquin et al., 1998). The *P*alindromic *O*ct factor *R*ecognition *E*lement (PORE), ATTTGAAATGCAAAT (15 bp), of the *Osteopontin* (*OPN*) enhancer interacts with an Oct-4 dimer, thereby mediating strong transcriptional activation in preimplantation mouse embryos. Homo- and heterodimerization of other Oct factors like Oct-1 and Oct-6 on the PORE has also been demonstrated.

The aforementioned examples provide evidence of the various ways in which POU domain proteins are able to cooperatively bind to substrate DNA. The particular mode of binding employed is primarily defined by the DNA sequence. To address the question of whether diversity in cooperative binding is reflected in transcriptional regulation, we have assessed and compared the ability of two different types of POU dimers to interact with the coactivator OBF-1 (OCA-B, Bob-1). This coactivator synergistically interacts with Oct-1 and Oct-2 monomers bound to the octamer motif (Luo et al., 1992; Gstaiger et al., 1995; Luo and Roeder, 1995; Strubin et al., 1995). We have investigated one type of POU dimer that is formed on the PORE and another that is formed on another palindromic DNA motif called MORE (*M*ore P*ORE*), ATGCATATGCAT. The data presented in this study provide an example of how POU domain molecules that bind to DNA in the same stoichiometry but in different configurations can differentially recruit a transcriptional coactivator to the promoter resulting in differential transcriptional activation.

Results

Dimerization of Oct Factors on the MORE

We asked whether the DNA binding configuration exemplified by the Pit-1:PitD crystal structure (Jacobson et al., 1997) is a conserved property of the POU family. We focused our analysis on POU proteins that had been identified by virtue of their binding to the *oct*amer motif, previously termed Oct factors (Schöler et al., 1989; Schöler, 1991).

In an electrophoretic mobility shift assay (EMSA), both naturally and bacterially expressed Oct-1 and Oct-4 formed monomers and dimers on the PitD site (Figure 1B). We anticipated however that this site was not the ideal substrate for cooperative binding of these Oct factors. Indeed, in the Pit-1:PitD cocrystal, Pit-1 POU_S domains are docked onto two ATAC subsites on both DNA faces (Jacobson et al., 1997 and Figure 1A). The POU_S domain of Oct-1 selects ATGC from a pool of random sequences (Verrijzer et al., 1992) and provides all base contacts within the same sequence in the Oct-1:octamer crystal (Klemm et al., 1994). Based on this, we converted the two POU_S docking subsites within PitD (Figure 1A). Compared to the original motif, the resulted sequence (MORE) mediated assembly of more stable Oct-1 and Oct-4 homodimers and a heterodimer between these two Oct factors (Figure 1B). The identity of these complexes was subsequently confirmed using Oct-1 and Oct-4 antibodies (Figure 1C). Further in vitro analyses demonstrated that Oct-2 and Oct-6 could also bind to the MORE as homodimers (Figure 2).

The Oct proteins studied in this work have overlapping temporo-spatial expression patterns during embryogenesis and in adult tissues (Schöler et al., 1989; Schöler, 1991; Herr and Cleary, 1995; Ryan and Rosenfeld, 1997). Oct-1 is coexpressed with Oct-2 (lymphoid cells and some cell types of central nervous system) and with Oct-4 and Oct-6 (embryonic pluripotent cells). Subsequently, Oct-1/Oct-2, Oct-1/Oct-4, Oct-1/Oct-6, and Oct-4/Oct-6 heterodimers can be formed on the MORE (Figure 2). Thus, the ability to heterodimerize on the MORE is a property shared by all four tested Oct proteins.

The MORE and PORE Mediate Different Domain Arrangements of POU Factor Dimers

The MORE arrangement is depicted schematically in Figure 3A (left) on the basis of a crystal structure of the Oct-1 POU domain bound to the MORE as a dimer recently solved at 1.9 Å resolution (A. R. et al., unpublished). The study revealed that the POU_S domains interact mainly with the ATGC sequences of the palindromic half-sites of the MORE, whereas the POU_H domains bind to the AT sequences. The dimer interface between the two POU molecules is formed by a loop region within the POU_S domain (between helices 3 and 4) of one molecule interacting with the C-terminal end of the recognition helix of the POU_H of a second molecule. The arrangement of the POU subdomains is that reported for the Pit-1/PitD cocrystal (Figure 3A, left; Jacobson et al., 1997), the arrangement of the POU:PORE dimeric complex is based on mutagenesis analysis and computer modeling (Figure 3A, right; Botquin et al., 1998).

In the MORE complex, one half-site binds POU_S and POU_H domains from two different Oct molecules. In the PORE model, the two subdomains bound to one half-site originate from the same protein molecule. The POU dimers assembled on the PORE and MORE should behave differentially in terms of their tolerance to base-pair insertions between the half-sites. Insertions in the center of the PORE alter the relative positions of POU domains belonging to two different POU molecules (Botquin et al., 1998). This is an indication that the altered arrangement on the DNA abolishes dimer formation due to a loss of interface contacts. In contrast, extra base-pair insertions in the center of the MORE (Figure 3B) should not affect dimerization. The length of the flexible linker connecting the POU_S and POU_H domains within each monomer, however, would impose one possible limitation to the extent of spacing. In agreement with this notion, Oct-4 dimerization can tolerate up to two base-pair insertions between the half-sites in the MORE (Figure 3C).

The MORE Can Mediate Transcriptional Activation by Oct Factors

Next, the MORE was compared to the PORE for the ability to mediate transcriptional activation by Oct factor in transient transfection assays. The PORE was used as

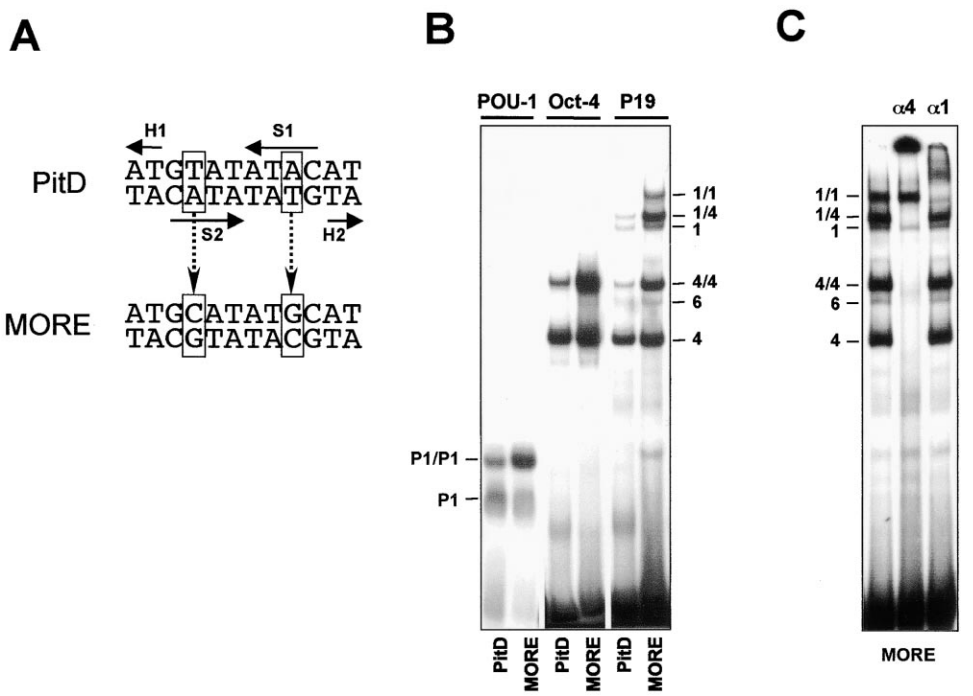

Figure 1. Two Replacements within a Pit-1 Dimer Binding Site Increase Cooperative Binding of Oct Factor

(A) Sequences of PitD motif and the MORE. Solid arrows indicate the relative orientation (from N to C terminus) and the positioning of two POU_S (S) and two POU_H (H) subdomains of Pit-1 (Jacobson et al., 1997). The MORE was derived from the PitD site by replacing the base pairs shown in boxes.

(B) An EMSA performed to compare the ability of PitD- and MORE-containing oligonucleotides to bind to the bacterially expressed POU domain of Oct-1 (POU-1), recombinant Oct-4 (Oct-4), and natural Oct-1 and Oct-4 proteins present in a whole-cell extract of P19 embryonic carcinoma (EC) cells.

(C) EMSA of P19 cell extracts using the MORE oligonucleotide as a probe. Anti-Oct-4 (α4) or anti-Oct-1 (α1) antibodies were included in the binding reaction before applying it onto the gel to prove the identity of the complexes. The α1 had only limited effect on Oct-1-containing complexes, which may have been due to a low affinity of this antibody. The protein–DNA complexes are denoted as follows: P1 and P1/P1, bacterial POU-1 monomer and homodimer, respectively; 1 and 1/1, natural Oct-1 monomer and homodimer, respectively; 6, Oct-6 monomer; 4 and 4/4, monomer and homodimer of both recombinant and natural Oct-4; and 1/4, Oct-1/Oct-4 heterodimer.

a reference, because it had been shown to be highly active in EC cells (Botquin et al., 1998). Transient transfection of the reporters into P19 EC cells demonstrates that the MORE can mediate transcriptional activation as efficiently as the PORE (Figure 4A). The Oct-1 and Oct-4 factors present in P19 extracts form the predominant complexes with the MORE in vitro (Figure 1), suggesting that these proteins provide the observed transcriptional stimulation in P19 EC cells.

To study the effect of specific Oct proteins, the same reporter plasmids were cotransfected into 293 cells along with four different expression vectors (Figure 4B). First, comparing two dimer binding sites shows that the MORE mediates transcriptional activation two to three times more efficiently (Oct-2, Oct-4), or at least as good as, the PORE (Oct-6). Second, comparing different Oct factors shows that Oct-4 is the most potent transactivator in this particular cellular context. In contrast, Oct-1 was barely able to stimulate either reporter in our transfection experiments (data not shown). Thus, the Oct factors exhibit different strengths in stimulating transcription although they can all bind to the MORE equally well (Figure 2 and Botquin et al., 1998). One possible reason is that transcriptional coactivators that act in conjunction with Oct-1, Oct-2, and Oct-6 are not expressed in 293 cells. The ability of Oct-4 to efficiently activate transcription via the MOREs and POREs may be due to the presence of E1A protein in 293 cells, a transcriptional coactivator that can enhance Oct-4 monomeric activity but not that of Oct-1 or Oct-2 (Schöler et al., 1991; Pesce et al., 1998; Brehm et al., 1999; Pesce and Schöler, 2000). MORE and PORE can be also activated by coexpressing Oct-4 and E1A (data not shown), indicating that E1A recognizes both dimeric configurations of Oct-4.

PORE and MORE Have Different Potential to Mediate Synergism between Oct-1 and OBF-1

An Oct-1 and Oct-2 specific auxiliary activity was discovered in lymphoid cells, the B cell-specific coactivator OBF-1 (OCA-B, Bob-1). This coactivator interacts and transcriptionally synergizes with octamer site bound Oct-1 or Oct-2, but neither with Oct-4 nor Oct-6 (Luo et al., 1992; Gstaiger et al., 1995; Luo and Roeder, 1995; Strubin et al., 1995). We investigated whether OBF-1 can serve as a bridging factor also for Oct-1 and Oct-2 dimers. As revealed by transient transfection, this coactivator failed to stimulate MORE-mediated transcription alone or in conjunction with either Oct-1 or Oct-2 in 293 cells (Figure 5A). In contrast, PORE-mediated

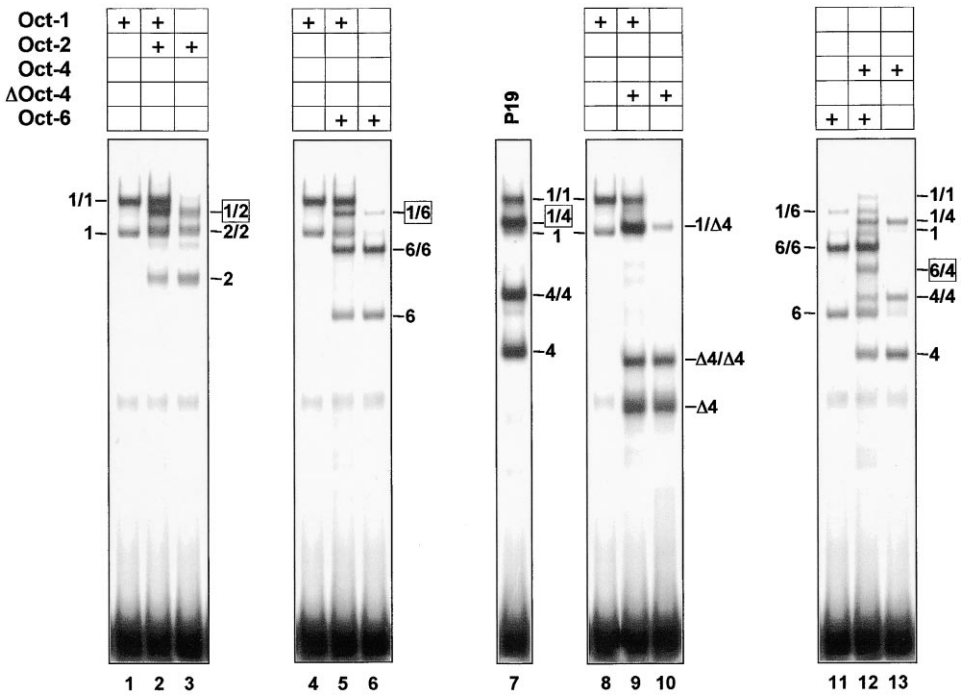

Figure 2. Heterodimerization of Oct Factors on the MORE

293 cells (a human kidney epithelium cell line) were transfected with cytomegalovirus (CMV) promoter-based plasmids directing the expression of full-length Oct-1 (lanes 1, 4, 8), Oct-2 (lane 3), Oct-6 (lanes 6, 11), Oct-4 (lane 13), and truncated Oct-4 (Δ4, lane 10). The extracts were mixed in pairs, as indicated above each panel, and subjected to EMSA using the consensus MORE (Figure 1) as a probe. The whole cell extracts from nontransfected P19 EC cells are also included (lane 7) to show the heterodimer comprised of the endogenous Oct-1 and Oct-4 proteins, seen in Figure 1. The mobility of DNA-protein complexes formed on this gel is marked as per Figure 1.

transcription was significantly stimulated by OBF-1 cointroduced with either Oct-1 or Oct-2. The level of activation, ranging from 10- to 35-fold, depended on the ratio of the Oct factor and the OBF-1 protein. The observed dependence on the stoichiometry is consistent with OBF-1 acting as a bridging factor binding between the upstream activator (here Oct-1 or Oct-2) and the basal transcription machinery (Schöler et al., 1991). Lower amounts of exogenous Oct-1 are required to achieve maximum synergy with OBF-1, which could be explained by the fact that 293 cells express endogenous Oct-1 protein. Furthermore, the Oct-2/OBF-1 pair stimulated transcription about 2-fold more than the Oct-1/OBF-1 pair. This may be due to different inherent potentials of the Oct-1 and Oct-2 transactivation domains (Babb et al., 1997).

In order to determine whether OBF-1 prefers monomer or dimer configuration of the POU domain, we included two PORE-derived sites in the analysis, PORE^D and PORE^M (Figure 5A and Botquin et al., 1998). The association between OBF-1 and Oct-1 or Oct-2 monomer requires adenosine at the fifth position within the classical octamer (ATGCAAAT), or within derivatives thereof (Cepek et al., 1996; Gstaiger et al., 1996). Even though the PORE^M represents the canonical octamer sequence with the required adenosine (ATgTGAAATG CAAAT), this site exhibits virtually no enhancer activity (Figure 5A). This result is reminiscent of the failure of OBF-1 to stimulate the histone 2B promoter octamer (Luo and Roeder, 1995). Thus, both sets of data indicate that the promoter context in OBF-1 recruitment is important. On the other hand, the octamer-mutated PORE^D mutant (ATTTGAAAgGCAAAT) is indistinguishable from the natural PORE with regards to OBF-1 recruitment. Thus, the PORE represent a new class of OBF-1-responsive DNA elements that efficiently recruit this coactivator through corresponding dimers of Oct-1 (Oct-2) in an octamer-independent fashion.

Further EMSAs revealed a good correlation between the ability of the PORE and PORE^D (versus MORE and PORE^M) to mediate synergism in transcriptional activation in vivo and the ability of these sites to mediate the interaction between OBF-1 and Oct-1 in vitro (Figure 5B). Consistent with the reported OBF-1 specificity to POU domains (Luo and Roeder, 1995; Sauter and Matthias, 1998), OBF-1 neither interacted nor synergized with Oct-4 bound to the PORE (data not shown).

The PORE and derivatives thereof were compared to the octamer site from the immunoglobulin κ light chain promoter (Vκ, Bergman et al., 1984) that is likely to be a natural target of OBF-1 (Gstaiger et al., 1995; Strubin et al., 1995). Strikingly, the EMSA revealed that the Vκ octamer recruits OBF-1 less efficiently than the PORE and PORE^D and only slightly better than the PORE^M (Figure 5C). Also unexpected, the mobility of the Vκ complex is lower compared to those formed on the PORE. It is possible that conditions in our EMSA assay were favorable for the binding of an extra OBF-1 or Oct-1 molecule to the complex with the Vκ octamer. Another explanation might be that the PORE-mediated complex induced

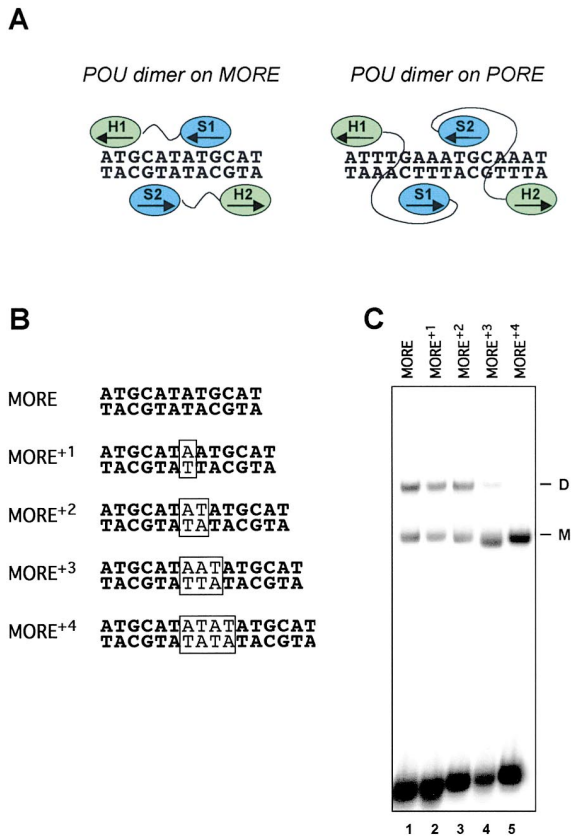

Figure 3. Distinct Configuration of POU Dimers on the MORE and PORE

(A) Scheme summarizing the overall arrangements of the POU subdomains within the Oct-1:MORE crystal structure and in the Oct-1:PORE model. The POU_S domain (S) and the POU_H (H) are indicated in blue and green, respectively. The POU subdomains belonging to one polypeptide chain have the same numbering and are connected by a linker in black. Arrows indicate the direction of the chain from the N to C terminus.

(B) Oligonucleotide probes used to assess the effect of phasing mutations on the MORE upon dimerization. Inserted base pairs are shown in boxes.

(C) EMSA using the MORE plus its phasing mutants (B) as probes, and recombinant Oct-4 as a testing protein. M and D indicate, respectively, the monomeric and homodimeric Oct-4 complexes with the oligonucleotides.

conformational changes in the DNA resulting in an increased mobility evident in the EMSA.

To determine the number of POU molecules within the complexes supershifted by OBF-1, we performed an EMSA using two differently sized versions of Oct-1 (Figure 5D). OBF-1 supershifted POU-1 and ΔOct-1 to different positions, reflecting the different sizes of these two Oct-1 species. After mixing all three proteins, a new band between these complexes appeared that was likely to be a complex of OBF-1 with the POU-1/ΔOct-1 heterodimer (Figure 5D). Thus, this mixing experiment suggests that the OBF-1 complex assembled on the PORE comprises a POU dimer.

The Heptamer/Octamer Motif Is a MORE Variant

A nucleotide database search using MORE and its spacing variant sequences (Figure 3) revealed a significant number of genes with these motifs in intronic and promoter regions. For example, the MORE within the γ-actin gene first intron (GenBank accession number U20365) matches the consensus MORE (ATGCATATGCAT). The MOREs in the Hsp84 gene promoter (ATGCATATGCAa, number U47056) and in a Bmp4 intron (ATGCATATG CAg, number D14814) are slightly divergent from the consensus MORE sequence within the POU_H docking subsites AT. All three motifs were able to support Oct factor dimerization in EMSAs (data not shown).

One of the identified homologies, ATGCATATGCAa, is located within the human Ig V_H promoter LR35 (Figure 6A). Strikingly, this MORE lies within a nucleotide stretch (CTCATGCATATGCAaAT), which differs only in one position from the sequence referred to for more than a decade as the heptamer/octamer motif (CTCATGaA TATGCAaAT; Kemler et al., 1989; LeBowitz et al., 1989; Poellinger et al., 1989). Further database searches, using the LR35 MORE plus adjoining promoter sequences as a query, found the consensus heptamer/octamer motif itself (e.g., BCL1, Poellinger et al., 1989) and slightly divergent sequences like B9c (Figure 6A). All these sequences are located within Ig V_H promoters at the same distance from the TATA box (Figure 6A). The BCL1- and B9c-type MOREs occur in numerous human and mouse V_H promoters, whereas the LR35-type MORE appears to be unique. When these V_H MOREs were subsequently used to search the database, we found MOREs within the promoter regions of crucial genes like the human RNA polymerase II gene (ATGAATATGCAg, number Z54152) or in an intron of the human β-globin gene (ATGAATATGCAa, number U01317.1).

We focused our further analysis on the three distinct Ig V_H promoters. One of these promoters, the BCL1, had been studied extensively and claimed to be one of the major *cis*-elements recruiting OBF-1 through octamer motif bound Oct-1 or Oct-2 (Luo et al., 1992; Luo and Roeder, 1995). The double mutation, Ile159Asp and Asn160Ala, was introduced into the POU domain of Oct-1. According to the crystallographic data, the residues are located at the C-terminal part of the α helix 3 in the POU_H domain that forms the MORE-type dimer interface (Figure 7; A. R. et al., unpublished). The indicated mutation had little effect on the monomer or PORE-type dimer binding, but abolished dimerization on the consensus MORE (data not shown). It also abolished dimerization on all three natural MOREs from the V_H promoters (Figure 6B), suggesting the same arrangement of the POU subdomains.

None of the three V_H MOREs was able to efficiently mediate an interaction with OBF-1 in EMSAs (Figure 6C). A weak OBF-1 binding can be attributed to the classical monomeric POU-1 complex (Klemm et al., 1994) formed on the overlapping octamer subsite. Indeed, the residual complex with OBF-1 was eliminated by destroying this subsite (mutant $BCL1^M$). In contrast, a significant gain of OBF-1 binding occurred upon conversion of the MORE to the PORE (mutant $BCL1^P$, Figure 6C), consistent with the previous data on the *OPN* PORE (Figure 5B).

The in vitro data were further correlated with transient transfection, performed as described in Figure 5A. In 293 cells, the V_H MORE-containing promoters (LR35, B9c, and BCL1) can respond to OBF-1 only weakly, but even this weak activation is due to the octamer submotif

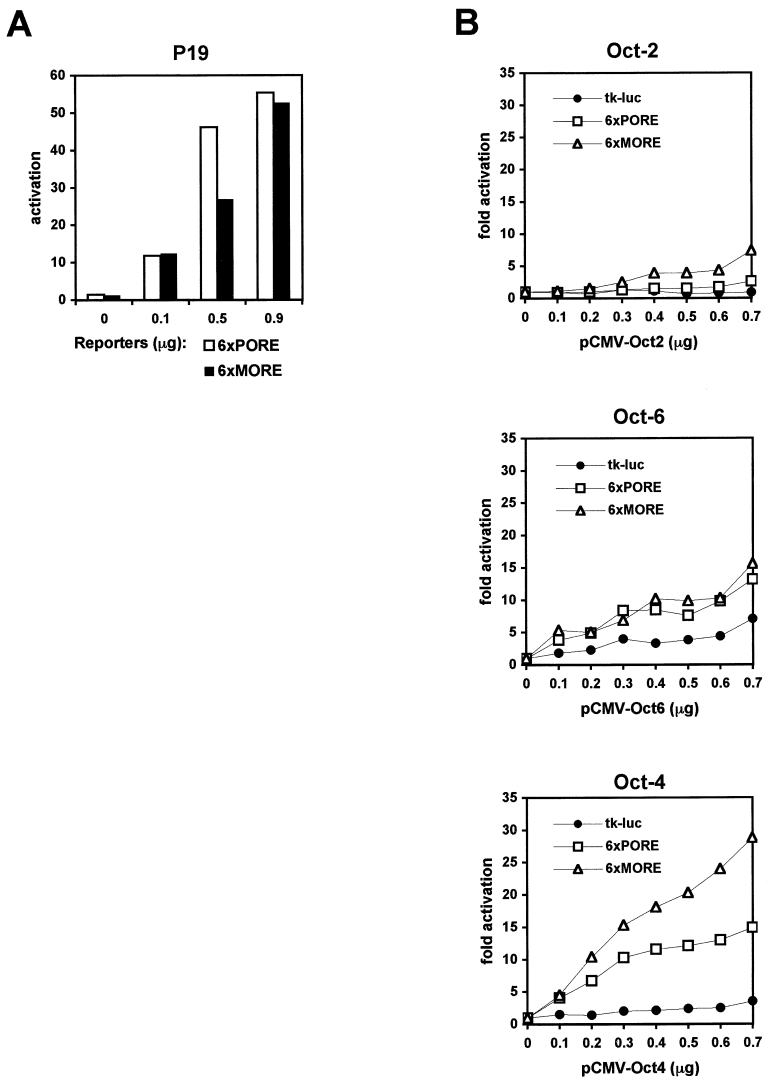

Figure 4. MORE-Mediated Transcriptional Activation

(A) Comparison of enhancer activities of the MORE and PORE in transient transfection experiment. P19 EC cells were transfected with different amounts of luciferase reporter plasmids (X axis) containing hexamers arranged in tandem. The hexamers were copies of the MORE or PORE plus 10–15 bp flanks from each side, inserted 37 bp upstream of the TATA box promoter of the *thymidine kinase* (*tk*) gene. Human β-actin LacZ vector (0.1 μg) was included as an internal control of the transfection efficiency. Y axis: activation of transcription, expressed as a ratio of luciferase to β-galactosidase activities.

(B) Cotransfection of 293 cells with 0.2 μg of the same reporter plasmids and varying amounts of CMV-based plasmids (X axis) expressing Oct-2, Oct-6, or Oct-4. The −37*tk*-luc enhancerless vector served as a negative control in this experiment; the pCMV-lacZ (50 ng) was used for normalization. Y axis: fold activation, which refers to the background activity of reporter vectors in cells transfected with no effector plasmids (the latter taken as 1 for each effector series). The correlation between protein levels and activation of the luciferase gene was verified in the EMSA using extracts of transfected cells (data not shown).

(cf. BCL1^{M}, Figure 6D). OBF-1 responsiveness was achieved by converting the BCL1 MORE to the PORE within the same promoter context (BCL1^{P}). Very similar results were obtained with the LR35^{M} and LR35^{P} derivatives of the corresponding V_H promoter (data not shown). Thus, the outcome of the transfection experiment (Figure 6D) is in agreement with the obtained in vitro results (Figure 6C). Our data show that the coactivator OBF-1 cannot be recruited efficiently to the V_H promoter bound Oct-1, which is in contrast to a view commonly accepted so far (Luo et al., 1992; Luo and Roeder, 1995).

Discussion

MORE-Mediated Dimerization Is Universal for POU Domains

The consensus MORE used for crystallographic studies appears to be an affiliate of a broad class of similar DNA elements. This class includes the prolactin/PitD site (Jacobson et al., 1997) and, likely, the motif ATG(C/A)AT $(A/T)_{0-2}$ATTCAT that is the optimal dimerization substrate for the neuronal POU factor Brn-2 (Rhee et al., 1998). The POU_S domains of these POU proteins exhibit distinct sequence requirements. Moreover, the POU_S domain of a given POU protein can alter its interaction with DNA in the MORE dimeric configuration, allowing recognition of divergent sequences. For example, the POU_S domain of Oct-1 can bind the ATGC (LR35 MORE) and ATTC (BCL1 MORE) subsites equally well (Figure 6B). The specificity of DNA binding by the POU_H subdomains in the MORE dimeric configuration is relaxed as well: besides the AT subsites, Oct-1 POU_H can recognize the AA (V_H and Hsp84 MOREs) or AG (Bmp4 MORE). Finally, the MORE-type configuration tolerates variable spacing between POU_S docking subsites (Figure 3 and Rhee et al., 1998). Taken together, these data suggest that there is a wide range of possible in vivo complexes where divergent POU domains assemble on divergent DNA sequences in a MORE-like fashion.

Heterodimerization on the MORE

A remarkable feature of the MORE resides in its ability to enable homo- and heterodimerization of a variety of

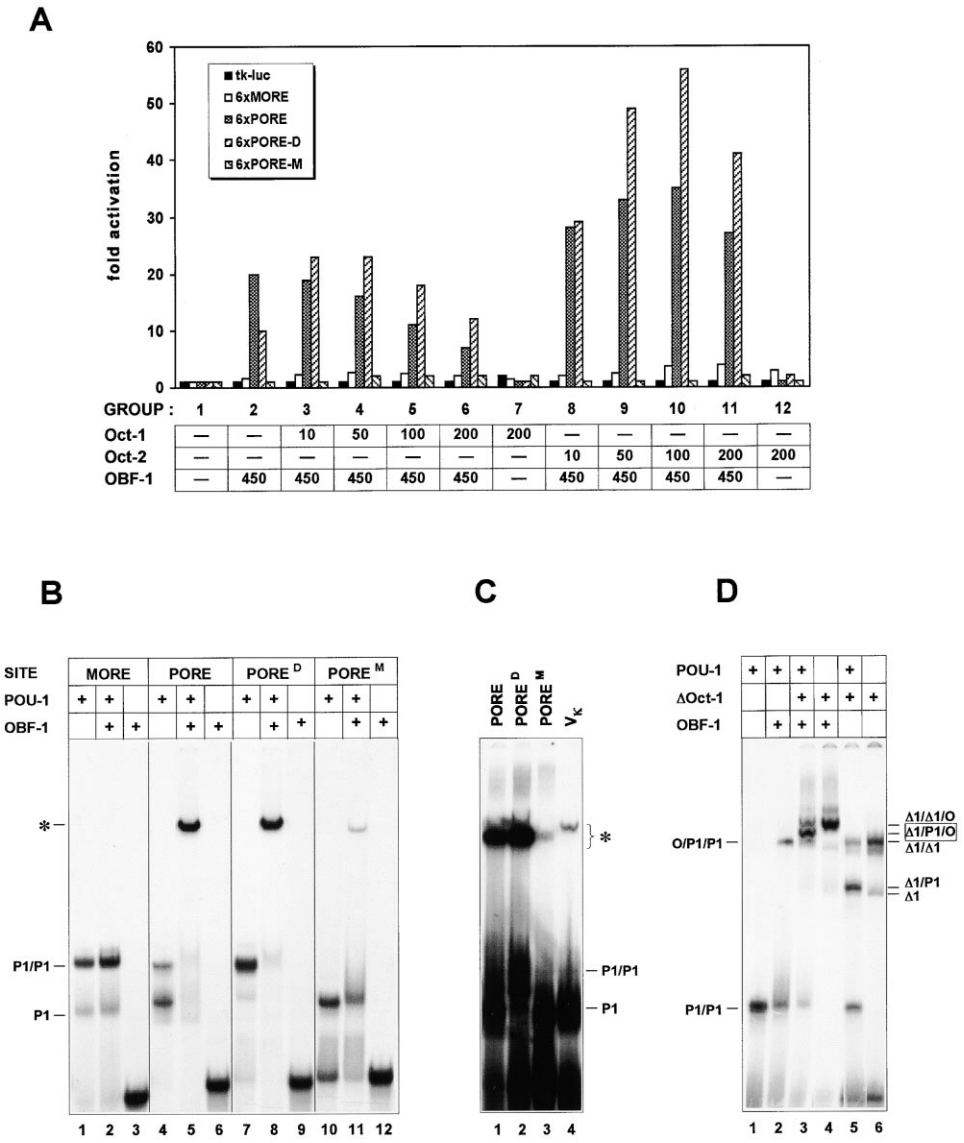

Figure 5. Selective Recruitment of the Coactivator OBF-1 to the POU Dimer Formed on the PORE

(A) Transient transfection of 293 cells. The luciferase reporter plasmids 6xMORE and 6xPORE were described in Figure 4. The PORE^D (ATTTGAAAgGCAAAT) and PORE^M (ATgTGAAATGCAAAT) are mutants of the PORE that were purposely designed to selectively bind Oct factor dimers and monomers, respectively (Botquin et al., 1998). Cells were cotransfected with CMV-based Oct-1, Oct-2, and OBF-1 effector plasmids (nanograms, X axis). Fold activation (Y axis) refers to the luciferase activity in cells transfected with no effector plasmids (group 1). The pCMV-lacZ vector (50 ng) was used for standardization.

(B) The bacterially produced POU-1 (see Figure 1B) and OBF-1 proteins were tested in EMSA using the ^{32}P-labeled MORE, PORE, and mutated versions thereof (PORE^D and PORE^M) as probes. P1 and P1/P1 refer to the POU-1 monomer and dimer, respectively, and the POU-1:DNA complex that is supershifted by OBF-1 is marked by asterisk.

(C) PORE and its mutants were compared in EMSA to the octamer site of the immunoglobulin kappa light chain promoter (V_K). The POU-1:DNA complexes that are supershifted by OBF-1 are denoted by asterisk without specifying the number of POU-1 and OBF-1 molecules therein. The abbreviations used are the same as in (B).

(D) The OBF-1/Oct-1:PORE complex contains two POU domain molecules. ΔOct-1 protein (Δ1), described in the legend of Figure 3, was introduced in the analysis in addition to POU-1 (P1) and OBF-1 (O). The proteins were mixed in different combinations, as indicated above the panels, with the labeled PORE^D probe and subjected to EMSA. Notice the appearance of an intermediate species (Δ1/P1/O, lane 3), which likely represents the POU-1/ΔOct-1 heterodimer (Δ1/P1, lane 5) that is supershifted by OBF-1.

Oct factors (Figure 2). This is surprising considering that some amino acids making up the MORE-type dimerization interface are quite variable among the Oct factors (for alignment of the POU domains see Herr and Cleary, 1995). Such are, for example, the two last amino acids of the α helix 3 in the POU$_H$ domains of Oct-1, Oct-4, and Oct-6 (positions 159 and 160). Nevertheless, a computer modeling of corresponding subdomains into the coordinates of the Oct-1:MORE crystal demonstrates that these amino acids can fit into the conserved hydrophobic pocket of the interacting POU$_S$ domains (Figure 7B and A. R. et al., unpublished).

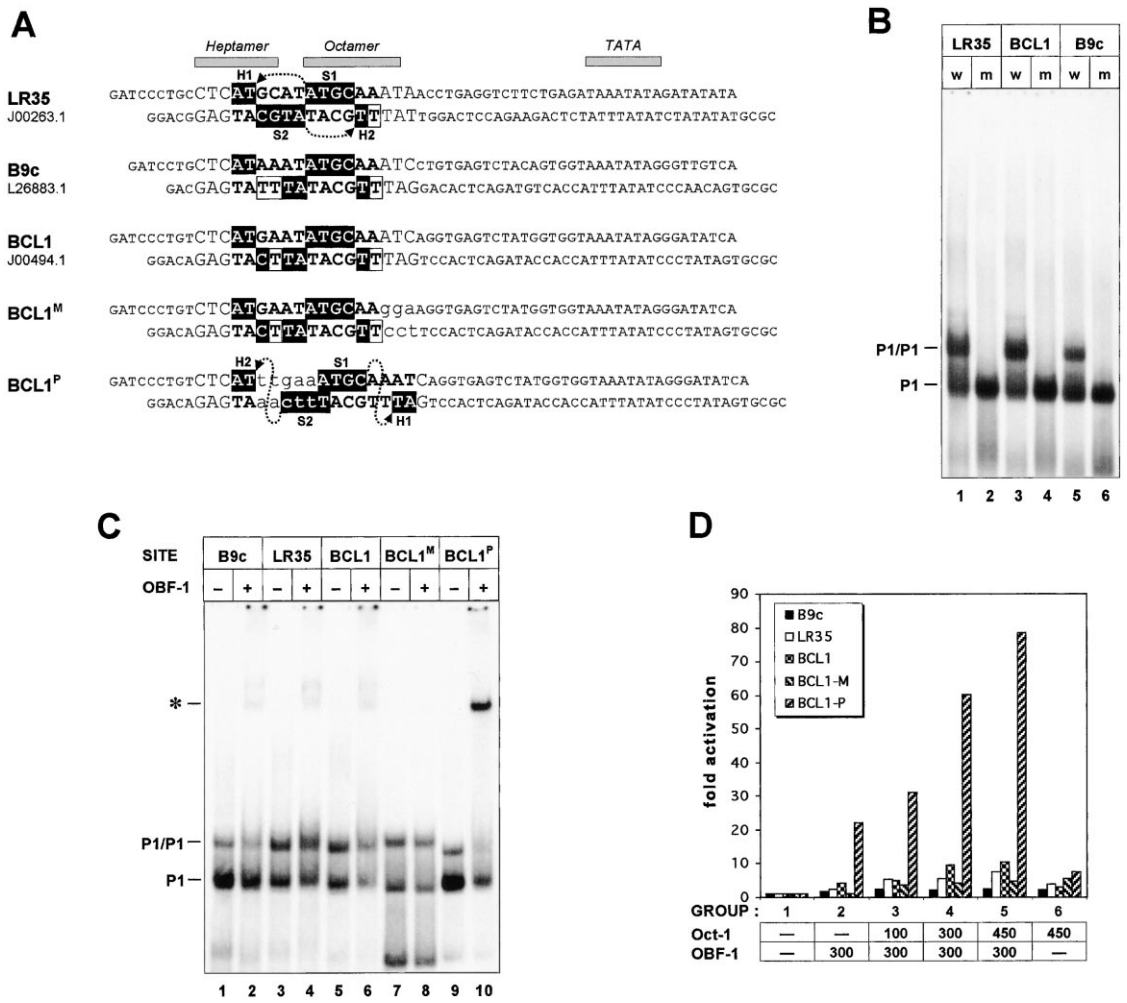

Figure 6. OBF-1 Cannot Be Recruited to the Oct-1 Dimers Bound to Natural MOREs from the Immunoglobulin Heavy Chain Promoters (V_H)

(A) Three representative V_H promoters containing slightly different MOREs (upper three sequences). Numbers under the names specify GenBank entries these sequences were retrieved from. LR35 is unique, whereas the BCL1 is the most abundant type of the MOREs occurring in V_H promoters family. In the MOREs (shown in bold) and PORE (last lane), the docking sites for the POU subdomains are in filled boxes; open boxes in the V_H MOREs designate positions, respectively, matching to and divergent from the consensus MORE (ATGCATATGCAT). The mutations were introduced in the BCL1 promoter fragment (two bottom sequences): in the BCL1$^{M\ (MORE)}$ the octamer part was destroyed (lower case) without affecting the MORE itself, and in the BCL1$^{P\ (PORE)}$ mutant, the MORE was converted to the PORE. Note the distinct arrangements of the POU subdomains on the MOREs and PORE (see also Figure 3A). The nucleotide stretches previously referred to as heptamer and octamer motifs, and the TATA boxes are denoted on the top; the BamHI and MluI half-sites at the ends were introduced to facilitate cloning.

(B) EMSA analysis of bacterially produced wild-type (w) and mutated (m: Ile159Asp, Asn160Ala) forms of the Oct-1 POU domain (P1) using ^{32}P-labeled V_H promoter fragments (A) as probes.

(C) EMSA with the wild-type Oct-1 POU domain and OBF-1 proteins. Oligonucleotide probes are described in (A); the asterisk points to the OBF-1/POU-1:DNA complex.

(D) Transient transfection of the 293 cells. By cloning the V_H promoter fragments (A) upstream of the transcription start of the *luc* gene, the reporter plasmid series was created. The assay conditions, effector plasmids, and abbreviations are as in Figure 5A.

MORE Dimerization Prevents OBF-1 Recruitment to V_H Promoters: Correlation with OBF-1 Deficiency in Mice

The consensus MORE used for X-ray crystallography (ATGCATATGCAT, A. R. et al., unpublished) and the MOREs from the Ig V_H promoters (AT[G/a][C/a]ATATG CAa, Figure 6A) bind the Oct-1 dimer in the same configuration (Figure 6B) hampering the recruitment of the coactivator OBF-1 (Figures 6C and 6D). The V_H MOREs are included in the well-known heptamer/octamer motifs (Kemler et al., 1989; LeBowitz et al., 1989; Poellinger et al., 1989), and one of them (from the BCL1 V_H promoter) was the first regulatory DNA element reported to mediate interaction and synergism between Oct-1 (Oct-2) and OBF-1 (Luo et al., 1992). This apparent discrepancy can be explained considering the deletion of the heptamer subpart and consequently, the ablation of MORE-mediated dimerization of Oct-1 (Oct-2) in the DNA constructs used in those studies (Luo et al., 1992; Luo and Roeder, 1995). Although this deletion allowed isolation and characterization of OBF-1, it also led to the conclusion that this protein activated Ig gene transcription via the octamer motif within the V_H promoter. To illustrate this situation, we mutated the BCL1 MORE

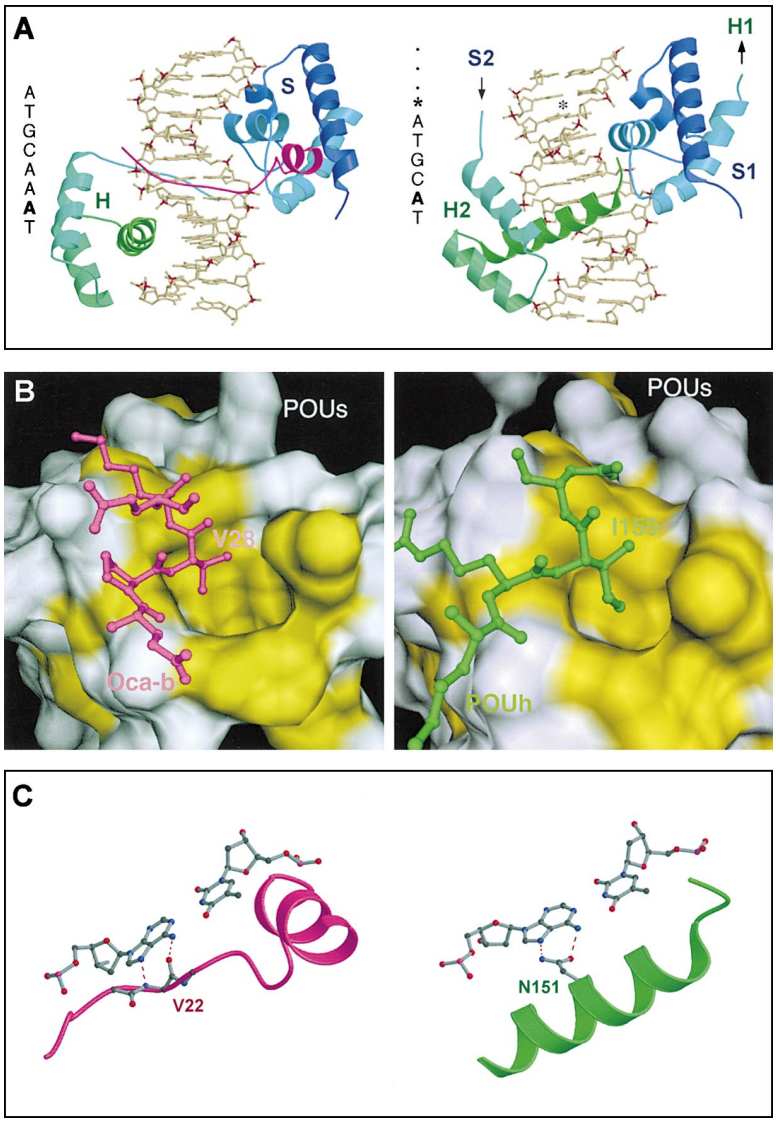

Figure 7. The Recognition Helix of the POU_H in the Oct-1:MORE Crystal Structure and the OBF-1 Peptide in the Ternary Oct-1/OBF-1:Octamer Motif Complex Occupy the Same Position in the Major Groove of the DNA

(A) Overall structure of the Oct-1:MORE and the Oct-1/OBF-1:octamer complexes. Left panel: the crystal structure of the ternary complex between the Oct-1 POU domain, the OBF-1 peptide, and the octamer binding site. The coordinates of Chasman et al. (1999) were used for this figure. In this structure, the Oct-1 POU domain is bound as a monomer; the linker (not shown) connects POU_S (S) and POU_H (H) subdomains. Right panel: The crystal structure of the Oct-1:MORE dimer complex. Only one half of the complex bound to one half-site of the palindromic MORE is shown. The POU_S (S1) and POU_H (H2) subdomains in this half complex originate from two different polypeptide chains. The complete dimer image is generated by a 2-fold rotation along an axis positioned between the two half-sites of the palindromic MORE that is perpendicular to the plane of the figure (ATG CAT*ATGCAT, indicated by asterisk). Color code: POU_S domain, blue; POU_H domain, green; OBF-1 fragment, magenta. The brightness of the colors is ramped from the N terminus to the C terminus of each domain. The DNA motifs of the two complexes are given to the left of each panel. The base pair in the 5th position is highlighted in bold (see C). In the ternary Oct-1/OBF-1:octamer complex the OBF-1 peptide makes extensive protein–protein interactions with the POU_S domain and contacts the POU_H via the major groove in the DNA (left). There is a change in spacing, by two base-pairs, in the DNA binding of the POU_H domain in the Oct-1:MORE dimeric structure compared to that of the Oct-1/OBF-1:octamer complex.

(B) The intermolecular hydrophobic interactions in the Oct-1/OBF-1:octamer and in the Oct-1:MORE complex are similar in nature. The protein–protein interface in the Oct-1/OBF-1:octamer motif (left panel) and in the Oct-1:MORE complex (right panel) are compared. The interface of POU_S is shown as a surface presentation. The hydrophobic amino acids that are involved in the interface are colored in yellow. Segments of OBF-1 (magenta) and POU_H (green) are shown in ball-and-stick presentation. Both Val28 (OBF-1) and Ile159 (POU_H) bind to the same hydrophobic pocket of the POU_S domain.

(C) The A:T base pair in the 5th position plays a key role in the protein–DNA interface in the ternary Oct-1/OBF-1:octamer complex (left) and in the Oct-1:MORE complex (right). The backbone of the OBF-1 peptide (Val22) provides a pair of hydrogen bonding interactions with the adenine base in the 5th position of the octamer motif (left). The same base in the MORE binding site is hydrogen bonded with the side chain of Asn151 from the recognition helix of the POU_H domain (right).

(cagggTATGCAAAT) with the purpose to eliminate the dimer assembly without disturbing the monomer binding to the octamer site. The indicated mutation created a strong (similar to the V_K octamer) OBF-1 responsive enhancer, although transcriptional activity is weaker than for the PORE (data not shown).

The phenotype of OBF-1-deficient mice eventually challenged the idea about direct recruitment of OBF-1 to the V_H promoters. It was shown that the transcription of Ig genes in OBF-1-deficient mice was largely unaffected (Kim et al., 1996; Schubart et al., 1996). Our study provides a rationale for this: the dimerization of Oct-1 on the V_H MOREs does not allow the recruitment of OBF-1 to the corresponding promoters (Figures 5 and 6; see next section for the underlying structural aspects). However, it is possible that, under certain physiological conditions, dimerization is prevented, e.g., by phosphorylation, and an Oct-1 or Oct-2 monomer binding to the high-affinity octamer sequence becomes accessible to OBF-1.

Deficiency of OBF-1 does have an impact on Ig gene transcription, but only subsequent to immunoglobulin class switch in antigen-responding B cells. Secondary heavy chain Ig isotypes are expressed at severely reduced levels in OBF-$1^{-/-}$ mice (Kim et al., 1996; Schubart et al., 1996). The immunoglobulin class switch, characterized by the recombination of the VDJ to C region, brings the 3′-IgH enhancer into proximity to the V_H pro-

moters. Remarkably, this enhancer contains no MORE-like sequences but the consensus octamer motif. As opposed to the V_H promoters, the latter appears to be a bona fide genomic target for OBF-1 (Stevens et al., 2000).

The Structural Basis for Differential Recruitment of OBF-1

The crystal structure of a POU complex in the PORE dimer configuration without OBF-1 became available just recently (A. R. et al., unpublished). Preliminary crystallographic data revealed an arrangement of the POU subdomains very similar to that predicted by computer modeling (Figure 3 and Botquin et al., 1998), providing an idea of the structural basis of this coactivator interaction in the PORE dimer. Since the PORE structure is based on the monomer configuration in the Oct-1:octamer crystal structure (Klemm et al., 1994), we assume that the observed binding surface of OBF-1 in the monomer (Chasman et al., 1999) is the same in the PORE dimer.

The ternary monomer complex shows the way the OBF-1 fragment binds to the N-terminal part of helix 1 (residues 6–10) and a segment between helices 3 and 4 (residues 49–60) of the POU_S domain (Figure 7A, left). Our new structure of the Oct-1:MORE dimer complex (Figure 7A, right and A. R. et al., unpublished) provides a rationale for why binding of OBF-1 is inhibited in this dimer configuration. The direct comparison of the Oct-1/OBF-1:octamer complex (Figure 7A, left) and the Oct-1:MORE dimer (Figure 7A, right) reveals that the binding site for OBF-1 is identical to the protein–protein POU_S/POU_H interface site in the MORE dimer, in which the same residues of POU_S (helix 1 and the loop between helices 3 and 4) interact with the C terminus of POU_H. The most important contact within this interface is a key–lock type interaction: the side chain of Ile159 of POU_H fits into a hydrophobic cavity of POU_S (Figure 7B, right). The equivalent interaction is observed in the Oct-1/OBF-1:octamer complex where Val28 of OBF-1 fits into the very same pocket of the POUs domain of Oct-1 (Figure 7B, left).

The analogy can be further extended to specific DNA base binding (Figure 7C). The amido group of the Asn151 side chain of POU_H makes two specific hydrogen bonds to the A:T base pair in position 5 of the MORE (Figure 7C, right). This hydrogen bond interaction is regarded as a signature for DNA binding of homeo domains (Brehm et al., 1998). In the Oct-1/OBF-1:octamer complex, the same base is hydrogen-bonded by the amino group and by the carbonyl group of the main chain of Val22 of OBF-1 (Figure 7C, left). From this structural comparison, we conclude that OBF-1 and the POU_H domain compete for binding to the same site of the POU_S domain where in the MORE dimer, the OBF-1 binding site is blocked by POU_H but accessible in the predicted PORE dimer. The specificity of competitive binding of OBF-1 and POU_H is further enhanced by the capability of the two competing domains, POU_H and OBF-1, to specifically interact with the binding motif of the respective DNA. The data also indicate that the POU_S/POU_H binding affinity of the examined MORE dimer complexes is superior compared to the affinity of POU/OBF-1 interaction.

Conclusion

A hallmark of the POU domain family transcription factors is their flexibility in DNA recognition (reviewed by Herr and Cleary, 1995). In this study, we show that the flexibility in POU factor functioning can also be extended to dimerization. We demonstrate the binding of Oct factor family members as homo- and heterodimers to the two high-affinity regulatory elements, the PORE and the MORE. The structural difference between PORE- and MORE-mediated dimerization leads to the differential recruitment of transcriptional coactivators. OBF-1, for example, binds and synergizes in transcriptional activation with the PORE configuration of the Oct-1 dimer, but fails to bind to the MORE-mediated Oct-1 dimer. Thus, our data demonstrate the mechanism by which distinct POU dimer configurations can recruit specific transcriptional coactivators with different effects on gene transcription. In addition, we outline the structural parameters leading to this selectivity in coactivator recruitment.

The Ig V_H promoter fragments, containing the MOREs (Figure 6A), have been shown to be fairly active in B cells or B cell extracts (Kemler et al., 1989; LeBowitz et al., 1989; Poellinger et al., 1989). Since OBF-1 fails to activate these promoters (Figure 6D), it is tempting to speculate that a yet unknown class of transcription coregulators exists. This novel class should have an opposite specificity for dimer assembly specifically binding to the MORE-type configuration of the POU domain.

Experimental Procedures

Oligonucleotides

PORE-, $PORE^D$-, and $PORE^M$-containing oligonucleotides were described by Botquin et al. (1998), named there O, O^{-1}, and O^{-3}, respectively. The consensus MORE, its spacing derivatives, and PitD motif (indicated with X) were placed into the PORE-flanking regions derived from the *OPN* intron (upper strand: 5′-CTGAAAGT TAAAATCTC**XXXXXXX**GGAAAAGCAAG-3′, lower strand: 5′-TCAG CTTGCTTTTCC**XXXXXXX**GAGATTTTAACTT-3′). One base-pair replacement (underlined) in a flanking region was required to eliminate an occasionally created binding site for an unknown protein from the 293 cell extracts. The sequence of the V_κ octamer-containing oligonucleotide is as follows: 5′-CTGACTCCTGCCTTCAGGGT**ATG CAAAT**TATTAAGTCTCGAG-3′ (upper strand), 5′-TCAGCTCGA GACTTAATA**ATTTGCAT**ACCCTGAAGGCAGGAG-3′ (lower strand). The CTGA and TCAG 5′-protruding sequences are of nongenomic origin.

Plasmid Constructs

The POU domain of Oct-1 (POU-1) was amplified from its cDNA by PCR using 5′-TTTCCATGGAGGAGCCCAGTGACCTTGAGGAG-3′ and 5′-TAATGTGCGGCCGCTCAGTTGATTCTTTTCTCCTTCTG GCG-3′ oligonucleotides. The amplified fragment was first cleaved with NcoI and NotI, then cloned into pET9d-NHis6 vector (which is a modified version of pET-9d(+) from Novagen). The resulted construct was used for a site-directed mutagenesis to create a vector expressing the POU-1 mutant Ile159Asp, Asn160Ala. The full-length Oct-4 cDNA was PCR-amplified with oligonucleotides: 5′-TCCATGGCTGGACACCTGGCTT-3′ and 5′-TCCATGGGGAACT CAGAGGGAACCTCCTCTGAG-3′ and ligated into the pCR2.1 vector (TA cloning kit, Invitrogen). The insert was cleaved out by NcoI and NotI and ligated into pET9d-NHis6. The primers 5′-GGGAGGACGT CATGAGCCTCTGGCAAAAATCCACAG-3′ and 5′-AATTATATGCG GCCGCTAAAAGCCCTCCACGGAGAGG-3′ were used to amplify the full-length OBF-1. The generated fragment was cleaved with BspH1/NotI and ligated into NcoI/NotI-linearized pET24-TEV-His6 plasmid, which was a derivative from pET-24d (Novagen). The truncated Oct-1 (ΔOct-1, amino acids 183–508) was generated from cDNA using 5′-CATGCCATGGGTCTTCAGCAGCAAAATCTCAAC-3′ and

5′-TCCCGAGCTCCTACCAGCTGCATCCTCTTCTAA-3′ oligonucleotides. The fragment was cleaved with NcoI/SacI and cloned into pET24d-TEV-His6 at corresponding sites.

The cytomegalovirus (CMV) promoter-driven eukaryotic expression plasmids were obtained from other investigators (pCG-Oct1, W. Herr; pEV-OBF1, P. Matthias), and pCMV-Oct2, pCMV-Oct4, pCMV-16NOct4 (here referred to as ΔOct-4), and pCMV-Oct6 were described previously (Schöler et al., 1990; Brehm et al., 1997). The MORE-containing oligonucleotides (see above) were multimerized and cloned into the −37*tk*-luc enhancerless vector in the same way as the PORE reporter series was generated (Botquin et al., 1998). The B9c, LR35, and BCL1 reporter series were created by replacing the BamHI/MluI promoter fragment of the −37*tk*-luc with the V_H oligonucleotides shown in Figure 6A.

Other Procedures

All recombinant proteins were expressed in BL21(DE3)pLysS *E. coli* strain (Novagen) and subsequently purified on the Ni-NTA agarose columns (Qiagen). The eluted protein solutions were used for the EMSA that was performed as previously described (Sylvester and Schöler, 1994). Oct-1 antibody was purchased from Santa Cruz Biotechnology and the generation of Oct-4 polyclonal antibody was described elsewhere (Palmieri et al., 1994).

Transient transfection experiments were performed in 24 well tissue culture plates using FuGene6 transfection reagent (Roche). The total amount of DNA per well was equalized to 1 μg with a carrier plasmid. After 36–48 hr, cells were washed with PBS, and were lysed directly in the wells in 250 mM Tris-HCl (pH 7.8), 1 mM DTT through three cycles of freezing in liquid nitrogen and quick thawing in a 37°C water bath. Approximately 1/20 of the amount of the crude lysate was used to measure the luciferase and β-galactosidase activities in standard assays.

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