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POU-specific domain of Oct-2 factor confers 'octamer' motif DNA binding specificity on heterologous Antennapedia homeodomain

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The bipartite DNA binding domain of the POU family of transcription factors contains a 'POU-specific' domain unique to this class of factors and a 'POU homeodomain' homologous to other homeodomains. We compared DNA binding of the Oct-2 factor POU domain and the Antennapedia (Antp) homeodomain with a chimeric Oct-2/Antp protein in which the distantly related Antp homeodomain was substituted for the Oct-2 POU homeodomain. The Oct-2/Antp chimeric protein bound both the octamer and the Antp sites efficiently, indicating that DNA binding specificity is contributed by both components of the POU domain.

POU domain; DNA binding protein; Homeodomain; Octamer factor

1. INTRODUCTION

The bipartite DNA binding POU domain [1] contains two subdomains, both of which are required for high-affinity DNA binding [2–4] (Fig. 1). The POU homeodomain is related to other homeodomains [5], while the POU-specific domain is unique to this class of proteins. Structural studies have shown that homeodomains contact DNA via a helix–turn–helix motif similar to that found in certain bacterial DNA binding proteins [6–8]. Although homeodomain-containing proteins have rather specific activities in vivo, this specificity is apparently only partly attributable to DNA binding, because different homeodomains exhibit overlapping DNA binding specificities in vitro [9].

POU proteins appear to bind DNA with greater specificity, probably due to the greater structural complexity of their DNA binding domains, although individual POU proteins also bind multiple sequences [3,10]. Analysis of chimeric constructs between the POU factors, Oct-1 and Pit-1, revealed that the POU-specific domain is important for differentiating between octamer and Pit-1 recognition sites [11]. However, other studies showed that both the POU-specific domain and the POU homeodomain can influence DNA binding specificity [12]. The POU homeodomain, but not the POU-specific domain, can bind DNA independently, but, as detailed structural information is not yet available, it is not clear whether the subdomains of the POU domain interact independently with the DNA or whether the POU-specific domain acts through the POU homeodomain by inducing a conformational change. Previous studies of the POU subdomains have relied exclusively on chimeric constructs made from portions of related POU proteins. We tested the functional autonomy of the POU-specific domain from Oct-2 by fusing it to the distantly related Antennapedia (Antp) homeodomain, and found that the chimeric protein acquired the ability to recognize both the Antp and octamer sites, suggesting that each domain contributes to DNA binding specificity.

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2. MATERIALS AND METHODS

2.1. *Expression plasmids*

The Oct-2 POU domain expression plasmid, pLXPOU, was constructed by total synthesis of the the coding region of the Oct-2 POU domain, codons 194–356 [13], and ligation into the pET3c expression vector [14] at the *Nde*I site (described in Rigoni et al., submitted). Both cysteines were changed to serines, which did not, however, alter DNA binding specificity (Rigoni et al., submitted). The chimeric Oct-2/Antp expression plasmid pEB1 was constructed by excising the Antp homeodomain coding region from pAop2 [15] with *Eco*RI (site subsequently filled in with Klenow DNA polymerase) and *Kpn*I. This fragment was ligated with a bridging oligonucleotide 5′ CGGGTGTATGTCTGCCTTCCGCGTTTGCGACGT 3′ and 5′ CGCAAACGCGGAAGGCAGACATACACCCGGTAC 3′ to pLXPOU, previously digested with *Aat*II and *Ssp*I, resulting in a fusion of the Oct-2 POU-specific domain and linker region codons 194–296 [13] to the Antp homeodomain codons 297–363 [15].

2.2. *Expression of proteins*

Expression plasmids were transformed into *E. coli* BL21 pLysS [14], bacteria were grown (37°C, LB broth) to A_{600} 0.5, IPTG added to 0.4 mM, and cultures were incubated 2 h. For the chimeric protein, bacteria were frozen and lysed in buffer (20 mM Tris-Cl, pH 8, 10 mM DTT, 1 mM EDTA, 100 mM NaCl) by sonication. After centrifugation, the protein was found in the soluble fraction and was used

without further treatment. For the POU domain protein, bacteria were lysed with lysozyme in buffer (50 mM Tris-Cl, pH 8, 1 mM EDTA, 25% w/v sucrose), centrifuged, and the protein was recovered from the pellet by treatment with 6 M guanidine HCl in buffer (30 mM Tris-Cl, pH 7.6, 0.1 mM EDTA, and 1 mM DTT), renaturation (20°C), and dialysis in the same buffer. The Antp homeodomain [15] was a gift from W. Gehring.

2.3. *Gel mobility shift assays*

Proteins were tested in gel mobility shift assays as described [16] with 5 fmol of oligonucleotide and 8% non-denaturing polyacrylamide gels. The octamer oligonucleotide hep$^-$oct$^+$ [16] contains a single octamer binding site derived from the 70Z IgH promoter, the Antp oligonucleotide 5' CGAGTATCCTGCTGAGAAAAAGCCATTAGAGTGG 3' and 5' TCGACCACTCTAATGGCTTTTTCTCAGCAGGATACTCGAGCT 3' contains an Antp binding site derived from that described in [17]. The Sp1 oligonucleotide competitor was 5' TCGACTGGGCGGGGCCTCGAGCT 3' and 5' CGAGGCCCCGCCCAG 3'.

2.4. *Methylation interference*

Prior to gel shift analysis, DNA was methylated by treatment with dimethylsulfide [19]. Gel shift complexes were eluted with Tris-Cl, pH 8, 300 mM NaCl, 1 mM EDTA, and 2% SDS, precipitated, chemically sequenced [18] and analyzed on 15% sequencing gels.

2.5. *DNAse I footprinting*

A 1.2 kb *Bam*HI/*Hin*dIII fragment from p70Zhep$^+$oct$^+$ containing a heptamer/octamer binding site derived from an IgH promoter [16] was end-labeled by filling in the *Bam*HI site. Proteins were bound to DNA (4 fmol) in 10 μl of 12.5 mM HEPES, pH 7.9, 12.5% glycerol, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 10 μg BSA, 300 ng poly dGdC·dGdC (Pharmacia) and 70 mM KCl. After incubation at 37°C for 30 min, reactions were started by addition of 1 μl (80 ng) DNAse I (Sigma, D-4527) and 9 μl 7.8 mM $CaCl_2$, 16.7 mM $MgCl_2$. After two min, 20 μl of 1% SDS, 20 mM EDTA were added, and samples were extracted with phenol/dichloromethane, precipitated, and run on 5% sequencing gels.

3. RESULTS AND DISCUSSION

Previous studies of the determinants of POU domain specificity involved subdomain exchanges between different POU domain proteins, all of which have related homeodomains distinct from other homeodomain proteins [11,12]. From these relatively conservative changes, it was not possible to distinguish whether the POU-specific domain and POU homeodomains act relatively independently, both providing important DNA contacts, or whether the POU-specific domain acts through the POU homeodomain. We reasoned that a more severe test of the putative independent action of the POU-specific domain would be the substitution of the homeodomain from the distantly related Antp homeodomain protein for the Oct-2 homeodomain (35% sequence identity between these domains) [19]).

The DNA binding specificity of the chimera was compared to that of the wild-type Oct-2 POU domain and the Antp homeodomain (Fig. 2) using an octamer and an Antp binding site. A clear hierarchy of binding affinity was discernible, which was quantified by comparing band intensities in the Scintillation counter. Under the conditions of our experiment the octamer oligonucleotide was bound 30-fold more efficiently by the POU domain than by the Antp domain, while the Antp oligonucleotide was bound 60-fold better by the Antp homeodomain than by the POU domain (Fig. 2, lanes 6 vs. 10 and 16 vs. 17). The chimeric Oct-2/Antp protein had intermediate properties. Most remarkably, addition of the POU specific domain yielded a protein with high affinity for the octamer site (lane 2), but reduced affinity for the antennapedia site (lanes 10 vs. 17). Competition of the complexes with octamer, Antp or Sp1 oligonucleotides demonstrated the specificity of binding and confirmed the different relative affinities of the constructs. Similar results were obtained with an alternative histone H2B octamer oligonucleotide (data not shown, also see oligonucleotide used in Fig. 4).

The greater affinity for the octamer site of the Oct-2/Antp chimera relative to the Antp homeodomain alone demonstrates that the Oct-2 POU-specific domain can contribute to binding specificity even outside of its normal context. This result suggests that the POU specific domain acts autonomously by directly contacting the DNA, as has been previously proposed [3,12]. We cannot exclude an indirect effect, whereby the POU specific domain would modulate the binding specificity of the homeodomain [5]. In the latter case one would have to assume that the POU specific domain not only changes the structure and specificity of the associated homeodomain (as observed for the Pit-1 factor [11]), but also the homeodomain of the highly divergent antennapedia factor. The differences in affinities between the chimera and the Antp homeodomain are due to differences in specific binding rather than merely overall affinity for

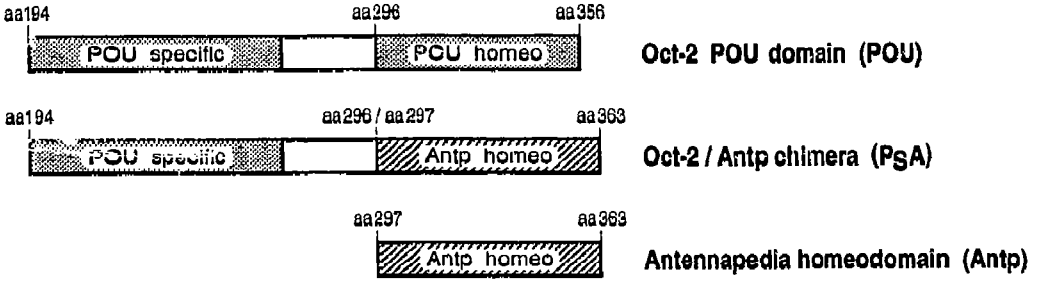

Fig. 1. Wild-type and chimeric proteins tested for DNA binding specificity.

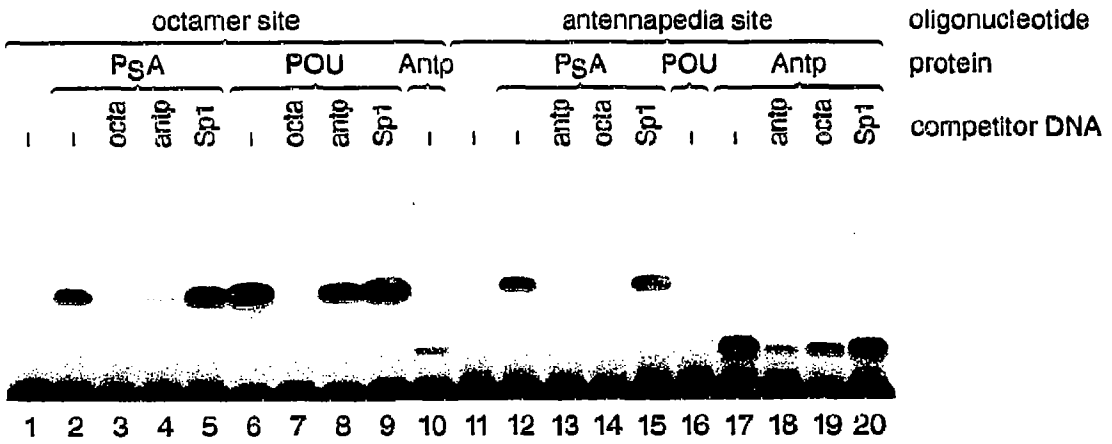

Fig. 2. Gel mobility shift assay with Oct-2 POU domain (POU), Oct-2 POU domain/Antp homeodomain chimera (P_SA) and Antennapedia homeodomain (Antp). Lanes 1 and 11, free oligonucleotide. Competitions were performed with 10 pmol of unlabelled oligonucleotide. No competitions were performed for complexes which were only inefficiently formed (lanes 10 and 16).

DNA, since, depending on the oligonucleotide used, either the Antp or the Oct-2/Antp protein formed complexes preferentially (lanes 2 vs. 10 and 12 vs. 17).

While the POU-specific domain is important for binding specificity, the POU homeodomain also appears to contribute to binding specificity. Substitution of the Antp homeodomain for the Oct-2 homeodomain simultaneously increased affinity for the Antp site and decreased affinity for the octamer site (lanes 12 vs. 16 and 2 vs. 6). This result is in apparent contrast to the findings of Ingraham et al. [11], in which the POU-specific domain was found to be the important determinant of binding specificity. In those studies, however, the homeodomain changes were relatively conservative, between Oct-1 and Pit-1, which have similar recognition sequences and POU homeodomains with similar structure (57% identity, 92% in the putative DNA recognition helix).

One difficulty with previous studies of chimeric POU proteins is that DNA interactions were characterized only by gel mobility shift assays. Therefore, we used DNAse I protection and methylation interference to characterize the DNA binding properties of the Oct-2/Antp chimera and the wild-type POU domain. Both proteins gave similar DNAse I protection patterns on an IgH promoter heptamer/octamer binding site (Fig. 3), suggesting that both bind on the DNA in similar orientations and can bind to this site as a dimer, as does the wild-type Oct-2 factor [16]. (Both proteins can also form dimers on DNA in gel mobility shift assays; data not shown.)

Methylation interference analysis of complexes formed on a single octamer site (Fig. 4) demonstrated that both proteins were similarly strongly affected by methylation of the G in the 'lower' strand of the octamer motif in the ATGC portion of the octamer site (lanes 5–8), which has been indirectly implicated in contacting the POU-specific domain [3]. Interference patterns were similar for both proteins on the initial A and the G residue of the 'top' strand (lanes 1–4). Methylation of the three consecutive A's in the top strand was strongly inhibitory to binding of the POU domain but less so to the chimera. This portion of the octamer motif is similar to the concensus TAAT homeodomain binding site [20], and is bound by an isolated POU homeodomain [3], so it is likely that the Antp homeodomain portion of the chimera contacts the octamer here, apparently with fewer critical contacts than with the wild-type Oct-2 homeodomain.

Our studies show that the Oct-2 POU-specific domain can confer octamer motif binding specificity on a chimeric protein containing a distantly related homeodo-

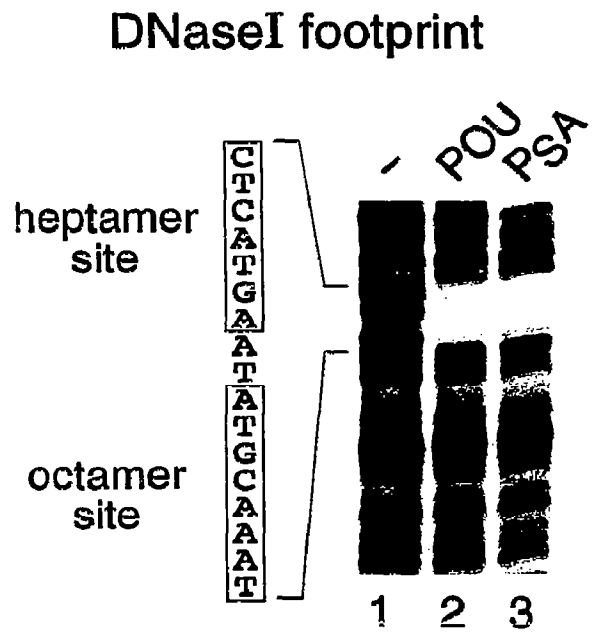

Fig. 3. DNAse I protection pattern of Oct-2 POU domain and Oct-2/Antp chimera (P_SA) on heptamer/octamer binding site derived from immunoglobulin 70Z heavy chain promoter.

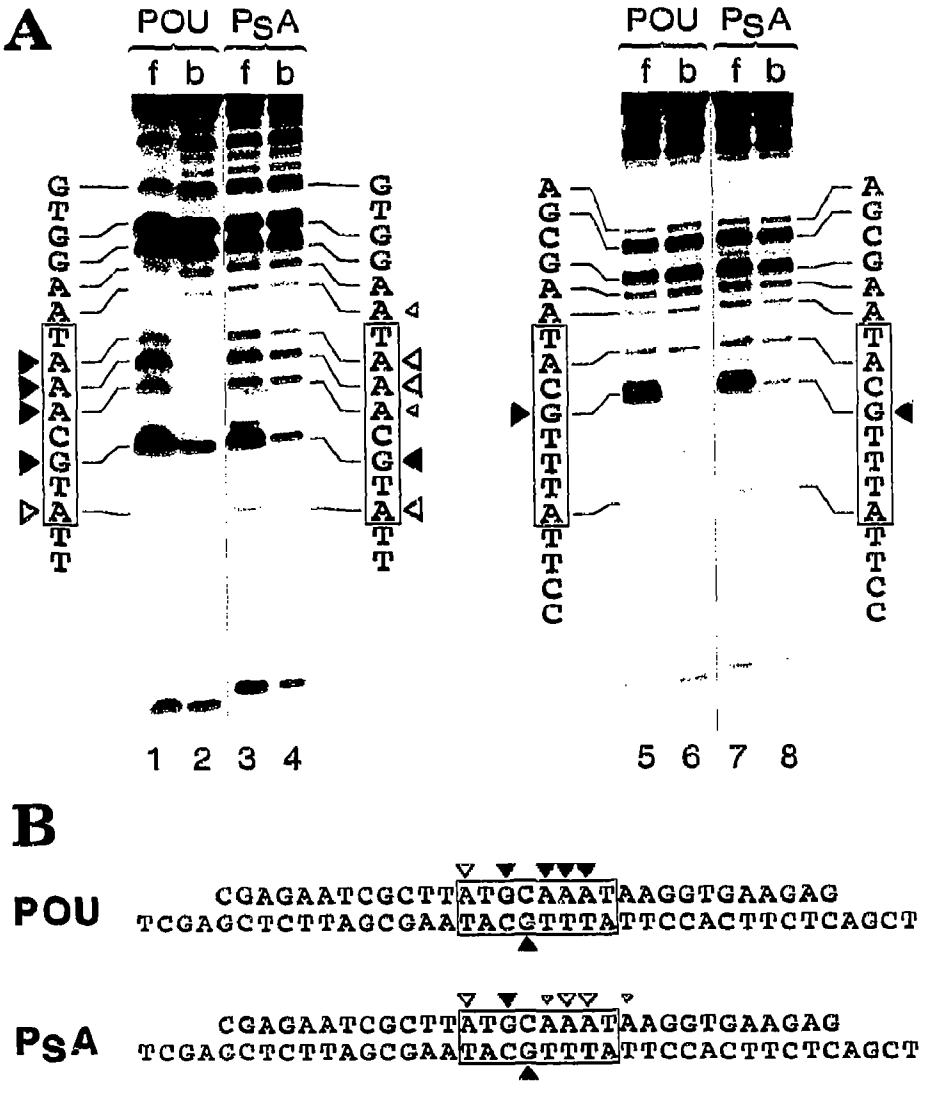

Fig. 4. Methylation interference analysis with octamer site derived from histone H2B promoter. Complexes were formed as in Fig. 2 on methylated oligonucleotides, then free (lanes 1,3,5,7) and bound (lanes 2,4,6,8) DNA was analyzed by chemical sequencing. Filled or open triangles indicate complete or partial interference with protein binding, respectively. The pattern of interference with the POU protein is very similar to that obtained with Oct-1 [16].

main from Antp. While the POU-specific domain crucially contributes to binding specificity, by itself it does not bind DNA to an appreciable extent, certainly with at least 3 000-fold lower affinity as compared to the POU domain (data not shown). The Oct2/Antp chimera also retains specificity for the Antp binding site, which suggests an autonomy of action for the subcomponents of the POU domain. The simplest interpretation for these results is that both domains contribute specific DNA contacts. Consistent with this model, a single change in helix 3 of *Drosophila* homeodomains changes binding specificity [21,22], while modifications in the same residue in POU homeodomains do not affect DNA binding ([12], Rigoni et al., submitted) suggesting that POU domain DNA binding specificity is dictated by multiple determinants. A definitive picture of POU domain–DNA interaction will, however, have to await high-resolution structural studies.

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