The oct-1 homeo domain contacts only part of the octamer sequence and full oct-1 DNA-binding activity requires the POU-specific domain

C. Peter Verrijzer, Arnoud J. Kal, and Peter C. van der Vliet

Laboratory for Physiological Chemistry, University of Utrecht, NL-3521 GG Utrecht, The Netherlands

The ubiquitous octamer-binding protein oct-1 contains a POU domain required for DNA binding, which can be subdivided into a POU-specific domain and a POU homeo domain. We have overproduced the POU domain and the POU homeo domain in a vaccinia expression system, purified both polypeptides to near homogeneity, and compared their DNA-binding properties. In contrast to the POU domain, the homeo domain protects only part of the octamer sequence in the Ad2 origin against breakdown by DNase I or hydroxyl radicals. Analysis of purine contacts by DMS and DEPC interference assays shows that the Ad2 octamer can be divided into two regions: one that is recognized both by the POU domain and the homeo domain in an identical fashion, and one that is only recognized by the POU domain. This suggests that the POU-specific domain is responsible for the additional contacts located at one side of the octamer. In agreement with this, mutating the first 3 nucleotides (ATG) of the octamer affected binding by the POU domain but not by the homeo domain. The apparent binding affinities to different octamer sites were compared. The homeo domain binds 600-fold less efficiently to the canonical octamer sequence (ATGCAAAT) than the POU domain. The difference is only sevenfold for the Ad2 octamer, whereas both K_d values are almost identical for the HSV ICP4 TAATGARAT motif. Both the POU and homeo domains recognize target sequences for mammalian homeo box proteins. We conclude that the octamer can act as a bipartite recognition sequence for oct-1 and that the POU-specific domain contributes to the binding affinity, as well as to the specificity, by providing additional contacts.

[Key Words: POU domain; homeo box; transcription factor; octamer sequence; contact points]

Received June 14, 1990; revised version accepted August 20, 1990.

Crucial cellular processes like DNA replication and transcription are regulated by sequence-specific DNA-binding proteins. One of these, the ubiquitous transcription factor oct-1, also referred to as NFIII, OTF-I, OBP100, or NF-A1, is a 90- to 95-kD nuclear protein that binds to the octamer element (Pruijn et al. 1986; Singh et al. 1986; Fletcher et al. 1987; Sturm et al. 1987; O'Neill and Kelly 1988). The octamer is an essential component of promoters and enhancers of several cellular genes like histone H2B, U1 and U2 small nuclear RNAs (snRNAs), and immunoglobulin light and heavy chains. It is also present in the adenovirus origin of DNA replication, where it mediates a four- to sixfold enhancement of initiation by oct-1 (Pruijn et al. 1986; Wides et al. 1987). The contacts between oct-1 and its binding sites were examined in some detail (Baumruker et al. 1988; Pruijn et al. 1988). Both minor and major groove base contacts were detected, as well as many backbone contacts at both sides of the DNA helix. Comparison of several degenerated binding sites indicated that specific binding may reflect the sum of many independent interactions between protein and DNA in agreement with

promiscuous sequence recognition of oct-1 (Baumruker et al. 1988). Analysis of the oct-1 cDNA sequence (Sturm et al. 1988) has indicated the presence of a conserved region involved in DNA binding, the POU domain. This 150- to 160-amino-acid-long region was first recognized in the transcription factors Pit-1 (Bodner et al. 1988; Ingraham et al. 1988), oct-1 (Sturm et al. 1988) and oct-2 (Clerc et al. 1988; Ko et al. 1988; Müller-Immerglück et al. 1988; Scheidereit et al. 1988), and the nematode *Caenorhabditis elegans unc-86* gene product (Finney et al. 1988; for review, see Herr et al. 1988). A search for other POU domain-containing proteins led to the discovery of several other POU proteins forming a family of transcription factors all recognizing the octamer sequence. So far, five different classes can be distinguished, based on sequence homology (He et al. 1989; Schöler et al. 1990). They are present in murine tissue at different stages of development (Lenardo et al. 1989; Schöler et al. 1989a,b; Okamoto et al. 1990), in adult human and rat brain (He et al. 1989), chicken (Petryniak et al. 1990), *Xenopus laevis* (Smith and Old 1990), *C. elegans* (Bürglin et al. 1989), and *Drosophila* (Johnson

and Hirsh 1990), where they may function as neurospecific transcription factors.

Apart from DNA binding, the oct-1 POU domain is also sufficient for adenovirus DNA replication (Verrijzer et al. 1990). Furthermore, the homeo domain directs the interaction with the herpes simplex virus (HSV) *trans*-activator VP-16 or Vmw65 (Stern et al. 1989; for review, see Goding and O'Hare 1989). The POU domain can be subdivided into a POU-specific domain (amino acids 279–354) and a homeo domain (amino acids 379–439) connected by a variable nonconserved linker region. The oct-1 homeo domain is homologous to the homeo domains found in many homeotic genes (for review, see Scott et al. 1989). Although an intact homeo domain is required for DNA binding, the oct-1 homeo domain does not represent the full DNA-binding activity of the oct-1 protein (Sturm and Herr 1988; Verrijzer et al. 1990). This is in contrast to the classical homeo box genes (Hall and Johnson 1987; Mihara and Kaiser 1988; Müller et al. 1988). Based on the failure to detect DNA binding of the oct-1 homeo domain in the absence of an intact POU-specific domain, a bipartite DNA-binding structure has been proposed (Sturm and Herr 1988) in which, in addition to the homeo domain, the POU-specific domain is required for binding, for example, by providing additional contacts. An alternative explanation for these experiments, suggested by García-Blanco et al. (1989), is that binding to the octamer sequence would be specified solely by the homeo domain. In this view, the POU-specific domain could enhance the binding by stabilizing existing protein–DNA interactions of the homeo domain.

Recently, we showed that the isolated oct-1 homeo domain is able to recognize the octamer but with a reduced affinity compared to the intact POU domain (Verrijzer et al. 1990). Here, we investigate the reason for this reduction by an analysis of the contacts between DNA and the homeo domain. We present evidence that the POU-specific domain contributes to the binding affinity of the POU domain by making additional DNA contacts, thereby influencing the specificity of binding. We propose that although the homeo domain is the DNA-binding core, the POU domain is a true bipartite DNA-binding structure recognizing two distinct regions of the octamer sequence.

Results

DNase I footprint borders of the POU domain and the homeo domain differ

We overproduced the oct-1 POU domain and the homeo domain employing recombinant vaccinia viruses constructed as described before (Verrijzer et al. 1990). The two polypeptides were isolated from infected HeLa cells and purified by standard chromatographical procedures (see Materials and methods). From 6 liters of HeLa cells, 400–500 μ g highly purified protein was obtained, as determined by SDS–gel electrophoresis (Fig. 1). We compared the DNase I footprint borders of the POU domain

Figure 1. (A) Silver-stained polyacrylamide gel of purified vaccinia-expressed POU domain and homeo domain. (Lane 1) 1.4 μ g POU domain (P). (Lane 2) 0.8 μ g homeo domain (H). (Lane 3) Marker lane with bovine milk α -lactalbumin (14 kD), bovine milk β -lactoglobulin (18.4 kD), bovine pancreas trypsinogen (24 kD), ovalbumin (45 kD), and bovine serum albumin (66 kD). (B) Structure of the oct-1 protein and the domains that were expressed. The numbering of the oct-1 amino acids is derived from Sturm et al. (1988).

with those of the homeo domain, using the octamer sequence present in the Ad2 origin of DNA replication. Figure 2 shows that the regions protected by the homeo domain footprint are shorter on both strands. On the top strand, phosphate bonds 36 (the bond on the 3' side of nucleotide 36) to 56 are protected by the POU domain, and phosphate bonds 40–56 are protected by the homeo domain. On the bottom strand, the footprint extended from phosphate bonds 54 (3' side of nucleotide 54) to 34 for the POU domain and from 54 to 39 for the homeo domain, making the homeo domain footprint 4 nucleotides shorter on the top strand and 5 nucleotides shorter on the bottom strand. Remarkably, the difference was located at one side only, defined here as the left side of the recognition sequence proximal to the molecular end, whereas the borders at the right side coincided completely. It should be emphasized that the footprint borders of the POU domain are identical to those determined previously for the intact protein using the same probe (Pruijn et al. 1986).

Contact points within the Ad2 origin binding site

To compare contact points of the POU domain and the homeo domain with their recognition sequence, we employed hydroxyl radical footprinting and chemical modification interference assays.

Figure 2. DNase I footprint analysis of the POU and homeo domain interaction with both strands of the Ad2 octamer motif. The marker lane (M) shows the location of the purines numbered according to their position in the Ad2 origins. (Lanes 2,6) No protein; (lanes 3,7) 60 ng homeo domain; (lanes 4,8) 40 ng POU domain. The protected regions are indicated.

Hydroxyl radical footprinting

Hydroxyl radicals, generated by iron(II)-promoted reduction of hydrogen peroxide, attack the deoxyribose residues in the DNA backbone. Sugars in close contact with protein are completely or partially protected against cutting. This enables precise mapping of the backbone contacts made by a DNA-binding protein (Tullius and Dombroski 1986). Figure 3 shows the hydroxyl radical footprints of the POU domain and the homeo domain on the Ad2 origin. Densitometric scanning of the results of several independent experiments reveals that the POU domain partially protects two regions on both strands: a small, weakly protected region around position 38 on the top strand and position 36 on the bottom strand, and a larger, strongly protected region, mainly from position 43 to 48 on the top strand and from 42 to 45 on the bottom strand. Between these two protected regions, weak hypersensitivity is observed at position 41 on the top strand and positions 38 and 39 on the bottom strand. The homeo domain only protects the larger region. In that part of the octamer, protection is almost identical to that by the POU domain. In contrast to the POU domain, the homeo domain does not induce any hypersensitivity. At the top of the gel an additional footprint by the homeo domain can be seen. The protected sequence $^9\text{ATAATA}^{14}$ is very similar to the right part of Ad2 octamer (ATAATG).

DMS and DEPC interference

Interference assays are based on the observation that modification of bases, which are contacted by a bound protein, will reduce binding. Bound and unbound DNA can be separated by gel retardation and analyzed on sequence gels after specific strand cleavage at the modified

nucleotide. Under-representation of a band in the bound fraction of DNA is interpreted as a contact.

Assuming B-DNA conformation, dimethylsulfate (DMS) methylates guanine residues at the N7 position in the major groove and adenine residues at the N3 position in the minor groove. Alkylation also adds a positive charge and thus changes the resonance state of the purine ring, which could obscure the assignment of minor groove contacts. DMS is especially sensitive for identification of guanine contacts, whereas adenine contacts are often more difficult to detect. Sturm et al. (1987) introduced diethyl pyrocarbonate (DEPC) as a powerful tool for the identification of adenine contacts. DEPC modifies the N7 position of free purines with a preference for adenine.

Figure 4A shows the results of a DMS interference assay. Methylation of G42 at the top strand clearly aborted binding of the POU domain but not the homeo domain. Adenine residues were hardly detectable for the top strand. On the bottom strand, methylation of adenines 39, 41, 44, and 47 affected POU domain binding, whereas only methylation of adenines 44 and 47 impaired binding for the homeo domain.

DEPC interference (Fig. 4B) identified POU domain contacts at G42 and adenines 38, 40, 43, 45, and 46 on the top strand and adenines 39, 41, 44, and 47 on the bottom strand, which agrees completely with the contacts of intact oct-1 (OBP100) with the Ad2 origin, identified by Baumruker et al. (1988). A smaller number of adenine contacts were made by the homeo domain, at

Figure 3. Hydroxyl radical footprints of the POU domain and homeo domain on the Ad2 origin. DNA fragments labeled at top or bottom strand were treated with hydroxyl radicals after incubation with 40 ng POU domain (P, lanes 1,7), 100 ng homeo domain (H, lanes 4,10), or without protein (lanes 2,5,8,11). Products were analyzed on an 8% polyacrylamide gel in parallel with A + G sequence reaction (M). Numbers indicate positions in the Ad2 origin. The protected regions, as determined by scanning, are indicated.

Figure 4. DMS and DEPC modification interference analysis. The Ad2 origin DNA was partially methylated with DMS in *A* and with DEPC in *B*. Modified end-labeled DNA fragments were incubated with the POU domain or the homeo domain. Bound and free DNAs were separated by gel retardation and recovered in a POU domain-free fraction (lanes 2,7), a POU domain-bound fraction (lanes 3,8), a homeo domain-free fraction (lanes 4,9), and a homeo domain-bound fraction (lanes 5,10). After cleavage at modified residues the fractions were analyzed on an 8% polyacrylamide gel in parallel with an A + G sequence reaction (M). Numbers indicate positions in the Ad origin. The regions of interference are indicated with a bar.

positions 43, 45, and 46 on the top strand and only 47 on the bottom strand. Again, this shows the absence of contacts at the left part of the octamer. Generally, the interference of modifications with homeo domain binding seems to be weaker than with binding of the POU domain. Possibly, when present in the POU domain, the homeo domain is forced deeper into the DNA and is therefore more sensitive to modification of the bases.

The results obtained for A44 are interesting, because DEPC modification (carbethoxylation at N7, major groove) does not interfere with homeo domain binding but DMS modification (methylation at N3, minor groove) does interfere. This suggests that this nucleotide is contacted in the minor groove only. However, POU binding is hampered both by DMS and DEPC modification of A44, implying that the POU-specific domain is responsible for the major groove contact.

The POU-specific domain is responsible for protein-DNA contacts

In Figure 5 we have summarized all contacts that were identified. The backbone contacts of the POU domain consist of two regions, enclosing some nucleotides with weak hypersensitivity for hydroxyl radicals. This might reflect the induction of structural changes in this region due to binding of the protein. Base contacts made by the POU domain are observed for every base pair in the core octamer sequence (40–47), as well as at two positions in

front of the octamer. An impression of the spatial distribution of these contacts is given. Both sides of the DNA helix are contacted, and the total contacted region spans 14 bp. The homeo domain contacts span 9 bp, all located in the right part of the octamer, both in the minor and the major groove.

Oct-1 POU and homeo domains differ in binding specificity

We studied the specificity of sequence recognition by comparing binding of the POU and homeo domains to several octamer motifs. Figure 6 shows that a canonical octamer (Ad4, AATATGCAAATAAGGC), which is optimal for oct-1 binding (Prujin et al. 1987), hardly binds the homeo domain. In contrast, the herpes simplex ICP4 TAATGARAT motif (AGGCGGTAATGAGAT; for review, see Goding and O'Hare 1989), which deviates at five of eight positions from the canonical octamer, has a strong affinity for the homeo domain and is suboptimal for POU binding (Fig. 6A, lanes 8 and 9).

Figure 5. Summary of the contacts that were identified for the POU domain and the homeo domain on the Ad2 origin. Nucleotides whose deoxyribose is protected from attack by hydroxyl radicals are indicated with arrows, with the lengths of the arrows indicating the level of protection. Arrows pointing away from the bases show hypersensitive sites. Values were calculated from the scanning of five independent experiments. The maximum protection level obtained was 95%. Bases whose modification by DMS or DEPC interferes with complex formation are in boldface type. All contacts indicated results from an at least twofold reduction in band intensity in the complexed sample, as determined by densitometric scanning. Contacts are also mapped onto a scheme of B-DNA. The direction from which we view the DNA is arbitrary and is not meant to indicate the side of the helix where the protein is situated. Solid circles along the DNA backbone represent deoxyriboses protected from hydroxyl radical attack. Circled purines represent bases whose modification by DEPC or DMS interferes with binding. A44 is boxed to indicate that only DMS methylation interferes with homeo domain binding, while both DMS and DEPC modification at this position abort POU domain binding.

Figure 6. Binding of the POU and homeo domains to degenerate octamer motifs and cellular homeo box protein recognition sequences. (A) Probes containing the octamer motifs from the Ad4 origin (lanes 1–3), the Ad2 origin (lanes 4–6), the HSV ICP4 TAATGARAT motif (lanes 7–9), the *Antp* BS-2 site (lanes 10–12), or the *Hox-2.3* promoter region (lanes 13–15) were incubated with a cytoplasmic extract containing the POU-specific domain (PS), 2 ng purified POU domain (P), and 10 ng purified homeo domain (H) and separated on a 15% native polyacrylamide gel (lanes 1–12). Samples 13–15 were assayed in a separate experiment and analyzed on a 12% polyacrylamide gel. (B) Footprint analysis of the homeo and POU domains on the *Hox-2.3* β site. The marker lane (M) shows the locations of the purines numbered according to their positions in the *Hox-2.3* promoter region. Partial DNase I digestion was in the absence of protein (lane 2) or in the presence of 200 ng homeo domain (lane 3) or POU domain (lane 4). The protected regions are indicated.

The part of the Ad2 octamer contacted by the oct-1 homeo domain (ATAATGA) shows a clear similarity to homeo box protein recognition motifs (Desplan et al. 1988; Hoey and Levine 1988; Scott et al. 1989). The *Drosophila Antennapedia* (*Antp*) sites, for example, all contain a conserved TAATG motif (Müller et al. 1988). To test directly whether the oct-1 homeo domain binds to such a sequence, we performed a band-shift assay with the *Antp* BS2 site (Affolter et al. 1990). Despite its high similarity to the Ad2 octamer (see Fig. 8, below), the *Antp* BS2 site was not bound by the oct-1 homeo domain nor the POU domain (Fig. 6, lanes 10–12). Apparently, mutations from ATAATGA to CTAATGG abolish binding. In the murine *Hox-2.3* promoter region, two different sites, α and β , are recognized by the *Hox-1.3* homeo box protein (Odenwald et al. 1989) and probably also by the *Hox-2.3* protein itself (R. de Jong and F. Meijlink, pers. comm.). We tested a radiolabeled fragment containing both *Hox-2.3* sites (α , CAGTAATGA; β , TCAATAATGA). The POU and homeo domains bind both sites, as indicated by the formation of two complexes (Fig. 6A, lanes 14 and 15). Comparison of DNase I footprints of the POU and homeo domains on the *Hox-2.3* β site (Fig. 6B) shows that, like on the Ad2 octamer, the homeo domain footprint is shorter at only one side of the recognition site. The extension of the additional sequence protected by POU is in the same direction as on the Ad2 octamer sequence, suggesting an identical orientation of the POU domain. None of the probes tested gave any binding of the POU-specific domain (Fig. 6, lanes 1, 4, 7, 10, and 13). The presence of

the POU-specific domain in recombinant vaccinia-infected HeLa cell extracts was verified by polyacrylamide gel electrophoresis.

The contribution of the POU-specific domain to the binding affinity depends on the recognition sequence

We used the band shift assay for a quantitative comparison of the POU and homeo domain-binding affinities by increasing the protein concentration at a fixed probe concentration of 2×10^{-10} M in the absence of competitor DNA (Fig. 7A). As shown in Figure 7B, the POU domain concentration required for half-saturation of the Ad4 probe was 1.9×10^{-10} M, whereas a much higher value was obtained for the homeo domain (5.4×10^{-8} M). From these data we calculate that the K_d of the homeo domain is 5.4×10^{-8} M, approximating the protein concentration at 50% binding. For the POU domain we corrected for the DNA concentration (see Materials and methods) and calculated a K_d of 9×10^{-11} M. For proteins having a high binding affinity, Scatchard analysis, varying the recognition site concentration at constant protein concentration, is a more accurate method to determine the K_d . Using this technique, we found a K_d of 7×10^{-11} M and 4×10^{-8} M for the POU and homeo domains, respectively. Thus, both methods gave almost identical results.

The POU and homeo domain-binding curves come closer together when the Ad2 oligonucleotide (AATATGATAATGAGGG) is used. This is caused both by a 20-fold increase of the homeo domain affinity and a 3-fold

decrease of the affinity for the POU domain (Fig. 7C). When we mutated the left side of the Ad2 octamer (ATG to CGC), homeo domain binding was unaffected, but the binding affinity for the POU domain decreased to approximately that of the homeo domain (Fig. 7D). This suggests that mutation of the left side of the octamer aborts the contacts made by the POU-specific domain. With the ICP4 TAATGARAT motif (GGGCGGTAATGAGAT), a similar observation was made. The POU domain binds only 1.7-fold more efficiently than the homeo domain (Fig. 7E). Previous experiments suggested that the oct-1 homeo domain has a considerably weaker affinity for the ICP4 TAATGARAT sequence than the POU domain (Sturm and Herr 1988), in contrast to our results. This difference might be explained by the use of poly[d(I-C)]/[d(I-C)]. We observed that the isolated homeo domain is more sensitive to nonspecific competitors like poly[d(I-C)]/[d(I-C)] than the POU domain. This leads to a reduction of specificity and apparently weak binding of the homeo domain in the presence of competitor.

To contribute to DNA binding, the POU-specific domain must be covalently bound to the homeo domain. Coexpression of the POU-specific domain and the homeo domain does not lead to enhancement of homeo domain binding or to complex formation (not shown; also see Sturm and Herr 1988). The data are summarized in Figure 8.

Discussion

The octamer as a bipartite recognition sequence

The results of contact point analysis, as well as the observation that the left part of the POU target sequence can be mutated without affecting homeo domain binding, can be explained by a model in which the Ad2 octamer sequence is divided into two parts: one recognized by the homeo domain, and the other, by the POU-specific region. When ascribing sequence recognition to the POU-specific region, some reservations should be made, because such a function is only inferred from a comparison between the POU and homeo domains. Thus, we cannot exclude a role of the linker sequence. However, this is unlikely, considering the lack of conservation in this part of the protein and the observation that insertions in the linker do not change DNA binding (Sturm and Herr 1988). Using several probes, we and others (Sturm and Herr 1988) were not able to show binding of the POU-specific region directly. This may be caused by an aberrant conformation of the POU-specific

Figure 7. Comparison of the binding affinities of the POU and homeo domains for several octamer motifs. Labeled oligonucleotides were incubated with increasing amounts of POU or homeo domains and assayed by gel retardation. As an example, A shows the result for the homeo domain and the Ad2 oligonucleotide. The results were quantitated by Cerenkov counting of gel slices, and the amount of free DNA was plotted against the concentration of the POU domain (●) or the homeo domain (□). Probes contained the Ad4 octamer (B), the Ad2 octamer (C), the Ad2ml mutant site (D), or the HSV ICP4 TAATGARAT motif (E).

Figure 8. Comparison of equilibrium dissociation constants (K_d) of oct-1 POU and homeo domains for different sites. Sequences were aligned according to the octamer motif. The oct-1 homeo domain recognition sequence, deduced from the Ad2 contact point analysis is centered. Sequences are from transcription regulatory elements: U2 snRNA enhancer (Mattaj et al. 1985), the V_H promoter (Prujñ et al. 1987), the HSV ICP4 TAATGARAT motif (O'Hare and Goding 1988), and the *Hox-2.3* promoter region (Verrijzer et al. 1988) or the Ad2 and Ad4 origins; Ad2ml is an Ad2 octamer mutant (O'Hare and Goding 1988). The K_d values were calculated from the curves in Fig. 7. All experiments were performed in triplicate, and the deviation was never more than 10%, except for the two *Hox-2.3* sites for which the K_d was measured less exactly with a deviation of 25%. In the last column, the ratio ($1/K_d$) of the POU domain to the homeo domain is given as a measure of the relative affinity to the various recognition sites.

					K _D (M)	1/K _D (M ⁻¹)
	5	10	15	POU	homeo	POU/homeo
U2	GCTATG	CAAATAG	GGT	3.5×10 ⁻¹²	>1×10 ⁻⁷	>280
V _H , Ad4	AATATG	CAAATAA	GGC	9×10 ⁻¹¹	5.4×10 ⁻⁹	600
Ad2	AATATG	ATAATGA	GGG	3.7×10 ⁻¹⁰	2.7×10 ⁻⁹	7.3
Ad2ml	AATCGC	ATAATGA	GGG	2.5×10 ⁻⁹	2.6×10 ⁻⁹	1
ICP4	AGGGCG	GTAATGA	GAT	1.9×10 ⁻⁹	3.2×10 ⁻⁹	1.7
Antp	CT	CTAATGG	CTT	>1×10 ⁻⁷	>1×10 ⁻⁷	-
Hox2.3α	CTCCCA	GTAATGA	GGA	3.1×10 ⁻⁹	2.9×10 ⁻⁹	0.9
Hox2.3β	TGATCA	ATAATGA	ATG	1.8×10 ⁻⁹	2.6×10 ⁻⁹	1.4

region, when present without the remainder of the POU domain, leading to a loss of the DNA-binding capacity. More likely, it is due to a low DNA-binding affinity, as the POU-specific region is only responsible for a limited number of contacts (see below).

At present, we cannot exclude that the structure of the homeo domain or the DNA contacts made by it are influenced by the POU-specific domain. However, the latter is difficult to reconcile with our observation that the contacts in the right part of the octamer do not change upon deletion of the POU-specific domain. Moreover, an octamer site mutated in the left part (Ad2ml) has the same affinity for the complete POU domain as for the homeo domain, making it less likely that the POU-specific domain affects the binding properties of the homeo domain.

An alternative explanation for the role of the POU-specific domain, suggested by García-Blanco et al. (1989), is stabilization of the contacts made by the homeo domain. We consider this less likely in view of the difference in specificity and in DNA contacts between the POU and homeo domains.

The homeo domain recognition sequence within the octamer

Contact point analysis identified the ATAATGA sequence within the Ad2 octamer as the oct-1 homeo domain target sequence. This motif shows a clear similarity to several homeo box protein sequences, which all contain a TAAT motif (Desplan et al. 1988; Hoey and Levine 1988; Müller et al. 1988; Scott et al. 1989). Furthermore, most backbone contacts made by the oct-1 homeo domain are accessible from one side of the helix. In this respect it resembles other homeo box proteins like yeast Mat α 2 (Sauer et al. 1988) and *Drosophila* Antp (Affolter et al. 1990). Here, we show that the oct-1 POU and homeo domains can bind two murine homeo box protein target sites, *Hox-2.3* α and β (Figs. 6 and 8). Previously, it was demonstrated that oct-2 also binds to recognition sequences of several homeo box proteins (Ko et al. 1988; Scheidereit et al. 1988; Thali et al. 1988).

Somewhat unexpectedly, the high-affinity Antp protein-binding site BS2 (CTAATGG; Affolter et al. 1990)

does not bind the oct-1 homeo domain. This is probably due to the A-C mutation at position 7 (Fig. 8), which is a homeo domain contact in the Ad2 octamer. From the limited amount of quantitative data, we deduce RTAATGA (R = purine) as the optimal oct-1 homeo domain target site.

Oct-1 binding to the HSV ICP4 TAATGARAT sequence is mainly directed by the homeo domain

VP16 or VMw65 is an HSV protein that *trans*-activates the HSV immediate early (IE) genes, mediated by the TAATGARAT motif in promoters of various IE genes. For *trans*-activation, VP16 must complex to the oct-1 protein (Gerster and Roeder 1988; O'Hare and Goding 1988; O'Hare et al. 1988), which is directed by the homeo domain (Stern et al. 1989). The VP16 TAATGARAT target sequence overlaps in some promoters, like ICP0 (IE110), with the octamer sequence. In others, like ICP4 (IE175), no such overlap exists. Nevertheless, weak binding of oct-1 can be observed, suggesting that the TAATGARAT motif is a degenerate octamer (Baumrucker et al. 1988; O'Hare and Goding 1988; Aprhys et al. 1989). From DEPC interference studies, Baumrucker et al. (1988) concluded that oct-1 contacts the entire TAATGARAT motif. Using purified proteins, we observed that mutations in the "RAT" part of the TAATGARAT sequence hardly affect the binding affinity of the complete POU domain (Fig. 8, cf. Ad2ml, ICP4, *Hox-2.3* α , and *Hox-2.3* β). Also, deletion of the POU-specific domain has only a very limited effect on binding to the TAATGARAT motif. Therefore, the additional contacts identified by Baumrucker et al. (1988) may not be an important determinant for the binding affinity.

Contribution of the POU-specific domain to DNA binding

It has been proposed that in a protein-DNA complex, a large part of the binding energy is derived from electrostatic interactions with the DNA backbone (von Hippel and Berg 1986). Because they are generally independent of base sequence, these interactions do not contribute to

specificity of site recognition. Base contacts determine the sequence specificity of a DNA-binding protein while they provide only a small part of the binding energy. Our results are consistent with such a model. The POU-specific domain makes half of the base contacts identified in the Ad2 octamer, while the majority of the backbone contacts are made by the homeo domain. This probably explains why the contribution of the POU-specific domain to the binding energy is relatively weak and no independent binding is observed. Also the high affinity of the homeo domain for nonspecific DNA ($K_d = \pm 10^{-6}$ M) is in agreement with this model. Thus, even in the absence of a homeo domain recognition sequence, the contribution of the homeo domain to DNA binding of the complete POU domain is considerable.

Although an intact homeo domain is required for DNA binding for all POU proteins studied so far, the contribution to DNA binding of the various POU-specific domains seems to differ. The POU-specific domain does not contain a recognizable DNA-binding motif. Therefore, it is difficult to ascribe the additional contacts to a particular region of this domain. Triple alanine substitutions and deletion studies indicated that the complete oct-1 POU-specific domain is involved in DNA binding (Sturm and Herr 1988). Deletion of 11 amino acids at the amino-terminal part of the oct-2 POU-specific domain impairs transcription but only weakly affects binding to the canonical octamer. Upon deletion of the first 15 amino acids of the POU-specific domain, DNA binding could not be detected anymore (Müller-Immerglück et al. 1990). For GHF-1 (Pit-1), deletion of the amino-terminal half of the POU-specific domain results in 2.5- to 3-fold lower binding activity, whereas the DNase I footprints remained similar (Theill et al. 1989). In the case of oct-4, DNA binding still occurs when the POU-specific domain is almost completely deleted (Schöler et al. 1990).

By comparison of these data it is not yet clear which part of the POU-specific domain is involved in DNA binding. Moreover, we show that the contribution of the oct-1 POU-specific domain to DNA binding depends on the target sequence. If this also applies to the other POU proteins it could complicate comparisons. In addition to DNA binding, the conservation of the POU-specific domain might reflect other functional constraints. The oct-2 POU-specific domain is involved in transcriptional activation (Müller-Immerglück et al. 1990), and the oct-1 POU-specific domain is absolutely required for stimulation of DNA replication (Verrijzer et al. 1990).

What makes an optimal oct-1 POU recognition sequence? The octamer motif present in the Ad4 origin and the V_H promoter is the strongest oct-1-binding site identified so far (Prujn et al. 1987 and this paper). Mutations at positions 7 and 8 from AT to CA, changing the Ad2 octamer to the Ad4 octamer, lead to a higher affinity for the POU domain (Fig. 8). Paradoxically, binding of the homeo domain is strongly decreased. This shows that an optimal POU-binding site is not created by simply joining a strong POU-specific and homeo domain target sequence. Possibly, flexibility of the DNA,

in addition to direct contacts, also determines the affinity of a particular site. Such a mechanism has been reported for cap (Gartenberg and Crothers 1988) and the bacteriophage 434 repressor (Koudelka et al. 1988). In this respect, it is noteworthy that we recently obtained evidence for bending of DNA by the POU domain (C.P. Verrijzer et al., in prep).

Materials and methods

Recombinant vaccinia viruses

The production of recombinant vaccinia viruses, expressing the oct-1 POU and homeo domains, has been described (Verrijzer et al. 1990). To prepare vaccinia virus containing the cDNA sequence encoding the POU-specific domain, the 370-bp *EcoRI* fragment from mutant 1-23/269-743 (Verrijzer et al. 1990) was cloned in pATA-18-STOP. This leads to a construct encoding amino acids 1–23 and 269–368 to which SSSVPGIL was added carboxy-terminally. The POU-encoding recombinant virus expressed amino acids 1–23 and 269–440 to which TIAE was added carboxy-terminally. For the homeo domain, amino acids 369–440 were expressed and a methionine residue was added at the amino terminus.

Preparation of extracts

Nuclear extracts were prepared from 3×10^9 HeLa cells 24 hr postinfection. The cells were collected by centrifugation at low speed (10 min, 2500 rpm), washed twice with PBS containing 0.5 mM $MgCl_2$, and resuspended in 30 ml hypotonic buffer (20 mM HEPES–KOH at pH 7.5), 5 mM KCl, 0.5 mM $MgCl_2$, 2 mM PMSF, and 0.1% Nonidet P-40). After 20 min, the cells were Dounce-homogenized. The nuclei were collected by low-speed centrifugation and resuspended in 10 ml of the same buffer containing 0.3 M NaCl. After 60 min, the suspension was centrifuged for 10 min at 3500 rpm. The supernatant was centrifuged at 100,000g for 30 min. Glycerol was added to a final concentration of 20%, and aliquots were stored at $-20^\circ C$. All procedures were on ice and centrifugation was at $4^\circ C$. Cytoplasmic extracts were made as described (Verrijzer et al. 1990).

Protein purification

Two buffers were used for further purification: buffer A (25 mM Tris–HCl at pH 8.0, 1 mM PMSF, 0.02% Nonidet P-40, and 20% glycerol) and buffer B, which is almost identical to buffer A, except HEPES (pH 8.0) is used instead of Tris. For the purification of the homeo domain, nuclear extracts were diluted with buffer A to 100 mM NaCl and applied to a 40-ml DEAE column. The flowthrough was fractionated on an 18-ml fast-flow S column in buffer and eluted with a 120-ml linear gradient of B/100–400 mM NaCl and assayed by gel retardation. The homeo domain eluted ~ 300 mM NaCl. Peak fractions were combined, diluted to 30 mM NaCl with buffer A, and loaded onto a 20-ml fast-flow Q column. The flowthrough was further fractionated on a 9-ml heparin–Sephacrose column. The homeo domain was eluted by a linear gradient (B/100–600 mM NaCl) and peaked at 500 mM NaCl. Homeo domain fractions were combined, diluted to 100 mM, and applied to a 5-ml single-stranded DNA–cellulose column. Homeo domain was eluted by a step elution with B/400 mM NaCl. A similar protocol was used for the purification of the POU domain. The POU domain eluted at 220 mM and 300 mM from the fast-flow S and heparin–Sephacrose columns, respectively. All procedures were

on ice or at 4°C. Protein concentrations were determined by using the Bio-Rad dye reagent with BSA as a standard. The yields were ~0.5 mg of the POU domain and 0.4 mg for the homeo domain.

Gel retardation

Oligodeoxynucleotides used for gel retardation were end-labeled with T4 polynucleotide kinase and purified by preparative polyacrylamide gel electrophoresis by using standard procedures (Maniatis et al. 1982). The sequences of the double-stranded oligonucleotides are Ad4, CGAATATGCAAA-TAAGGC; Ad2, AGGCCAATATGATAATGAGGGGGT; Ad2ml, AGGCCAATCGCATAATGAGGGGGT; TAAT8 (ICP4), AGGGCGGTAATGAGATGCCATGT; BS2-18 (*Antp*), CTCTAATGGCTTTTCTC. The DNA concentration of the oligonucleotides used for quantitative analysis was determined both by absorption at 260 nm and by comparison with marker DNA in ethidium bromide-stained polyacrylamide gels. Binding studies with the *Hox-2.3* promoter region were performed with a 150-bp *SalI-EcoRV* fragment (686–836 bp; Verrijzer et al. 1988) containing two *Hox-1.3*-binding sites: α (799–806) and β (741–747; Odenwald et al. 1989).

Binding reactions were carried out in 20 μ l binding buffer (20 mM HEPES–KOH at pH 7.5, 1 mM EDTA, 1 mM DTT, 0.025% Nonidet P-40, 4% Ficoll, and 125 μ g/ml BSA). After 60 min on ice, bound and free DNAs were resolved on a 15% polyacrylamide gel run in 0.5 $\times$ TBE at 4°C and quantified by Cerenkov counting of gel slices. The equilibrium dissociation constant (K_d) in a bimolecular reaction is given by the equation $1/K_d = D_f/D_i(P_i - D_b)$. The concentrations of free DNA (D_f) and bound DNA (D_b) were calculated from the gel quantification. The concentration of input DNA was 2×10^{-10} M. The total protein concentration (P_i) was calculated by using a deduced molecular mass of 26.7 and 10.6 kD for the POU and homeo domains, respectively. At half-saturation, the equation becomes $K_d = P_b - D_b$ in which $D_b = 10^{-10}$ M. For Scatchard analysis, the equation was rearranged into $D_b/D_i = (P_i - D_b)/K_d$. The K_d value was obtained from the slope of the plot of D_b/D_i versus D_b .

DNase I and hydroxyl radical footprinting

Binding reactions were performed on ice in 50 μ l binding buffer in the presence of 100 ng poly[d(I-C)]/[d(I-C)]. Approximately 40 ng POU and 60 ng homeo domain were added to initiate the binding reaction. After 1 hr, 5 μ l 50 mM $MgCl_2$ and 0.02 units DNase I were added. Digestion was allowed for 90 sec at 25°C and terminated by addition of 3 μ l 0.2 M EDTA, 10% SDS. For hydroxyl radical footprinting (Tullius and Dombroski 1986), 9 μ l of a fresh mixture of 0.13 mM EDTA, 0.07 mM ferrous ammonium sulfate, 2% H_2O_2 , and 6.7 mM sodium ascorbate was added. After 3 min, the cleavage reaction was stopped by addition of 30 μ l 0.2 M thiourea. One microgram of calf thymus DNA was added, followed by phenol–chloroform extraction and ethanol precipitation, and the samples were analyzed on an 8% denaturing polyacrylamide gel. The DNA used in the footprint assays was a 110-bp *EcoRI-XbaI* fragment from pHRI, containing the Ad2 origin (Hay 1985). The bottom strand was labeled at the 5' end of the *XbaI* site by T4 polynucleotide kinase. The top strand was labeled with [α - ^{32}P]dCTP at the *XbaI* site by DNA polymerase I (Klenow fragment). Approximately 2 ng probe (30,000 cpm) was used for each reaction. Sequencing reactions were performed as described (Maxam and Gilbert 1980).

Interference experiments

For methylation interference experiments, labeled DNA fragments were methylated with DMS by the method of Siebenlist and Gilbert (1980). The DEPC modification was performed according to Sturm et al. (1987). Probes were purified on a 6% polyacrylamide gel. The band shift assay was as described above; only 80,000 cpm modified probe DNA was used per assay and 20 ng of the POU domain or 50 ng of the homeo domain. DNA was eluted from the gel (Maxam and Gilbert 1980), phenol-extracted, and ethanol-precipitated twice with 1 μ g carrier calf thymus DNA. Pellets were dissolved in 100 μ l H_2O , and 10 μ l 100 mM KPO_4 (pH 7.0) was added. Samples were heated for 15 min at 90°C, cooled on ice, and 10 μ l 1 M NaOH was added, followed by incubation at 90°C for 30 min. After cooling on ice, 13 μ l 1 M HAc and 8 μ l 3 M NaAc were added. Samples were phenol–chloroform-extracted once and ethanol-precipitated twice. Pellets were dissolved in 2 μ l sample buffer (80% formamide, 20 mM NaOH), and products were analyzed on an 8% denaturing polyacrylamide gel. The results of footprint and interference experiments were quantified by densitometric scanning of the autoradiographs with a laser densitometer (Ultrosan XL, Pharmacia LKB). Calculated levels of interference were normalized for regions outside the protected area.

Acknowledgments

We are grateful to Winship Herr for providing pBS oct-1⁺ and pBS oct-⁺ Δ BH DNA. We thank Drs. Affolter and Gehring for the *Antp* BS2-18 DNA and for making data available before publication, Peter O'Hare for TAAT8, Ad2, and Ad2ml, and Frits Meylink for *Hox-2.3* promoter DNA. Thanks are also due to Wim van Driel for useful discussions and to Erik de Vries and Yvonne Mul for critical reading of the manuscript. This work was supported, in part, by the Netherlands Foundation for Chemical Research (SON), with financial support from the Netherlands Organization for Scientific Research (NWO).

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The oct-1 homeo domain contacts only part of the octamer sequence and full oct-1 DNA-binding activity requires the POU-specific domain.

C P Verrijzer, A J Kal and P C van der Vliet

Genes Dev. 1990, **4**:

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