Spacing and orientation of bipartite DNA-binding motifs as potential functional determinants for POU domain factors

Peng Li,^{1,2,6} Xi He,^{1,3,6,7} M. Renee Gerrero,^{1,4} Minsin Mok,¹ Aneel Aggarwal,⁵ and Michael G. Rosenfeld¹

¹Howard Hughes Medical Institute, Eukaryotic Regulatory Biology Program, University of California, San Diego, School and Department of Medicine; ²Biomedical Sciences Graduate Program; ³Biology Graduate Program, ⁴Molecular Pathology Graduate Program, La Jolla, California 92093-0648 USA; ⁵Department of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, New York, New York 10032 USA

Investigation of the large POU domain family of developmental regulators has revealed a molecular mechanism by which highly related transcription factors sharing common DNA-binding motifs act to functionally discriminate their cognate DNA sequences. Studies of two classes of neuron-specific POU domain factors (III and IV) indicate that functional specificity on their native response elements is achieved by accommodating different nucleotide spacing between variably oriented bipartite core DNA-binding motifs. The preferred orientation of the POU-specific domain of the neuronal factors on their native response elements appears to be opposite that of Pit-1 and Oct-1. Members of POU-III (Brn-2) class exhibit remarkable flexibility in DNA site recognition (tolerating core motifs spaced by 0, 2, or 3 nucleotides), whereas POU-IV (Brn-3) class is highly constrained (tolerating core motifs with a spacing of 3 nucleotides). The molecular determinant of the constraint in DNA site selection appears to be imparted by 3 amino acid residues in the amino-terminal basic region in concert, with helix 2 of the POU homeo domain which together are involved in minor groove and possibly phosphate backbone contacts. Similar mechanisms may underlie differential flexibility in spacing and orientation for diverse families of transcription factors.

[Key Words: POU domain factors; DNA-binding motifs; transcription factors; spacing; orientation]

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Critical gene activation events during organogenesis are regulated by families of transcription factors harboring sequence-specific DNA-binding motifs. The genetic analyses in *Drosophila* led to the identification of a large family of proteins containing a highly conserved 60-amino-acid region, referred to as the homeo domain (Gehring 1987; Scott et al. 1989). The structures of the *Antennapedia* (*Antp*) homeo domain (Qian et al. 1989), *engrailed* (*en*) homeo domain (Kissinger et al. 1990), and the MAT α 2 homeo domain (Wolberger et al. 1991) are similar, consisting of three helices with helix 2 and helix 3 forming a helix–turn–helix (HTH) motif.

The structural characterization of three mammalian transcriptional regulators, Pit-1, Oct-1, and Oct-2, and a *Caenorhabditis elegans* developmental modulator, *unc-86* (for review, see Rosenfeld 1991; Ruvkun and Finney 1991), led to the identification of a novel DNA-binding motif, referred to as the POU domain. The POU domain consists of an amino-terminal conserved 75- to 82-amino-acid POU-specific (POU_s) domain, a 15- to 27-amino-acid linker region, and a 60-amino-acid POU ho-

meo domain (POU_{HD}) (Herr et al. 1988; Rosenfeld 1991). On the basis of mutational analyses of the POU_s domain and POU_{HD} of Pit-1 (Ingraham et al. 1990) and Oct-1 (Kristie and Sharp 1990; Verrijzer et al. 1990, 1992), it is apparent that both the POU_s domain and the POU_{HD} are required for site-specific, high-affinity DNA binding. The POU_s domain of Oct-1 contributes to both binding affinity and specificity by direct DNA contacts (Verrijzer et al. 1990, 1992). Presumably, POU_{HD} has a HTH structure similar to that of classic homeo domain factors. Recently, the solution structure of the POU_s domain of Oct-1 has been solved by nuclear magnetic resonance (NMR) spectroscopy (Assa-Munt et al. 1993; Dekker et al. 1993). It appears that the POU_s domain consists of a four α -helix cluster that is strikingly similar to the DNA-binding domains of λ and 434 repressors, and 434 Cro protein (for review, see Harrison and Aggarwal 1990; Assa-Munt et al. 1993), with the second and third helices of the POU_s domain form the HTH motif.

Oct-1 and Oct-2 bind to an A/T-rich sequence known as the octamer motif, ATGCAAAT, which is found upstream of a number of tissue-specific and ubiquitously expressed genes (Staudt et al. 1986). Indirect evidence suggests that the POU_s domain binds to the upstream ATGC, with major groove contacts on the GC of the ATGC (Staudt et al. 1986; Verrijzer et al. 1992). Pit-1 also

⁶The first two authors contributed equally to this work.

⁷Present address: Mammalian Genetics Laboratory, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702.

binds and functions on a series of highly related A/T-rich response elements found in the prolactin, growth hormone, and Pit-1 genes (Rosenfeld 1991; Rhodes et al. 1993). These elements contain the consensus sequence ATGAATAT, in which the ATG serves as the putative POU_s domain recognition sequence (Ingraham et al. 1988, 1990), with major groove contact in the G residue of ATG (Elsholtz et al. 1989). Therefore, the DNA recognition elements for Pit-1, Oct-1, and Oct-2 are fundamentally similar, with a homeo domain (AAAT or ATAT) and putative POU_s domain (ATGC or ATGA) recognition motifs.

Large numbers of additional POU domain gene products have been identified (for review, see Rosenfeld 1991; Ruvkun and Finney 1991; Wegner et al. 1993), and these have been subdivided into six classes (POU-I to POU-VI) according to their primary amino acid similarity in the linker region of the POU_{HD}, as well as sequence similarity in the amino terminus to the POU domain. In situ hybridization analyses suggest that members of the class III POU domain proteins, such as Brn-1, Brn-2, Brn-4, and Tst-1/SCIP/Oct-6 (for review, see Wegner et al. 1993), and class IV factors, such as Brn-3.0 and Brn-3.1 (He et al. 1989; Guerrero et al. 1993), are expressed in distinct spatial and temporal patterns in the brain and peripheral nervous system. By analogy with the functions of Pit-1 (Li et al. 1990), *unc-86* (Finney and Ruvkun 1990), and Oct-2 (Corcoran et al. 1993), it is likely that these neuron-specific POU domain proteins selectively control differentiation events in specific neuronal phenotypes.

In this paper we provide evidence that neuron-specific POU domain factors preferentially recognize bipartite DNA binding elements in which the POU_s domain recognition motif appears to be in an orientation opposite that of Pit-1, Oct-1, and Oct-2 sites. Furthermore, the POU_s domain of Brn-2 appears to be capable of switching orientations on binding to structurally distinct Pit-1 DNA response elements. Examination of the two classes of neuron-specific POU domain proteins has revealed

that they have fundamental differences in flexibility with respect to DNA sites to which they can bind effectively. Three amino acids in the amino-terminal basic cluster of the POU_{HD}, in conjunction with helix 2 of the POU_{HD}, appear to dictate the class-specific difference in DNA-binding flexibility. These data suggest that the base contact in the minor groove and the sugar phosphate backbone contacts on one edge of the major groove by the POU homeo domain are the critical determinants of class-specific DNA site recognition. These data provide a molecular explanation for the variable flexibility of DNA-binding proteins on their cognate DNA sites.

Results

Sequence-specific binding and transcriptional activation of Brn-2 on the corticotropin-releasing hormone promoter

A functional analysis of Brn-2 was initiated by isolating two independent rat cDNA clones (2.2 and 1.6 kb) with identical open reading frames, encoding a 445-amino-acid polypeptide with a predicted molecular mass of 47 kD (Fig. 1A). RNA blot analyses using a series of tissues revealed that Brn-2 was expressed selectively at high levels in the brain (Fig. 1B). In situ hybridization was used to examine the expression of Brn-2 in the rat hypothalamus, where precise patterns of neuropeptide biosynthesis have been described extensively (Vale et al. 1983). As shown in Figure 1C, Brn-2 transcripts were found in the dorsomedial paracellular (mpd) region of the paraventricular nucleus (PVH), which synthesizes and secretes corticotropin-releasing hormone (CRH). CRH cooperates with vasopressin in control of ACTH release, and plays a critical role in regulating the stress response (Vale et al. 1983). In addition, high levels of Brn-2 expression were also detected in the medial posterior magnocellular neurons (pmm), which synthesize both oxytocin and CRH

Figure 1. Expression and function of Brn-2 in CRH-expressing neurons of the PVH. (A) Schematic diagram of the Brn-2 protein and the deduced amino acid (single-letter code) sequence. G₂₀, Q₂₆, and Hr represent reiterated regions of glycine and glutamine, and a histidine-rich region, respectively. (B) RNA blot analysis of Brn-2 in rat tissues using the Brn-2 cDNA as probe. The sources of the poly(A)⁺ RNA (2 µg) are Brain (Brn); spleen (Spl); heart (Hrt); testis (Tst); trigeminal (TG). (C) Autoradiography and schematic diagram of in situ hybridization of Brn-2 in the adult PVH. CRH neurons are centered in the dorsal mpd of the nucleus, whereas magnocellular neurosecretory neurons projecting to the posterior pituitary are centered at this level in the medial pmm (oxytocin [Oxy]) and lateral posterior magnocellular (pml; vasopressin) parts of the nucleus. Most, but not all, neurons in the dorsal (dp) and ventral medial (mpv) parvicellular part of the PVH, which contain only scattered hybridized neurons, project to autonomic-related cell groups in the brainstem and spinal cord and to the reticular formation. The periventricular part (pv) contains a large population of neuroendocrine somatostatin neurons. The slide is the frontal section with the left PVH slightly rostral to the right PVH: dark-field illumination of emulsion-coated section. (D) Footprint analysis of the CRH promoter by bacterially expressed Brn-2. Both strands are shown (left, sense; right, antisense). (G + A) G + A sequencing ladder; (0, 0.2, 0.5, and 1) The amount of the bacterial Brn-2 protein (µg). The regions (I–V) correspond as follows: I (–64 to –78 bp); II (–119 to –133 bp); III (–200 to –186 bp); IV (–216 to –202 bp); and V (–271 to –301 bp), respectively. (E) Schematic diagram of the CRH promoter (–337 to +25 bp) and fold induction in cotransfection assay. Brn-2-binding regions are boxed (I–V), a cAMP response element (CRE) and the TATA region in the CRH promoter are also indicated. The promoters for rat CRH (–337 to +25 bp) and tyrosine hydroxylase gene (TH, –497 to +26 bp) were inserted in front of the luciferase gene. Three copies of CRH II site and the ERE were inserted into the *Bam*HI site of the p36 promoter. Transient cotransfection was performed in HeLa cells, and the fold induction by Brn-2 was determined by comparing the luciferase activity of HeLa cells transfected with each reporter plasmid and either the pCMV–Brn-2 or pCMV plasmid. The basal luciferase activity for CRH and TH promoters was ~80 U/µl of lysate, and the basal level for CRH II and ERE sites was 8 U/µl.

(Swanson and Simmons 1989), and in lateral posterior magnocellular neurons (pml), which produce vasopressin

as well as CRH when stimulated. Thus, Brn-2 is expressed in the hypothalamus in distinct, adjacent neural

Figure 1. (See facing page for legend.)

cell types that are capable of expressing the CRH gene product.

The colocalization of Brn-2 and CRH suggested that CRH gene expression might be regulated by Brn-2. To test this hypothesis, we first examined the CRH promoter for Brn-2-binding sites by DNase I footprint analysis (Fig. 1D). Bacterially expressed Brn-2 protein bound specifically to five distinct elements in the rat CRH gene (from -337 to +25), exhibiting higher affinity for CRH II and CRH V sites (-135 to -112 and -290 to -278). The dissociation constant (K_d) for the highest affinity element (-135 to -112 bp) CRH II site was ~0.5 nM (data not shown). Brn-2 specifically and effectively activated reporters under control of the CRH promoter (40-fold), or the CRH II element (30-fold), but failed to activate the tyrosine hydroxylase promoter or the prolactin basal promoter (p36), including the estrogen response element (p36-ERE, Fig. 1E). The ability of Brn-2 to bind and activate the CRH promoter confirmed that this factor is able to function as a transcriptional activator on a potential neuronal target gene.

Characterization of Brn-2 DNA recognition elements

To explore potential downstream target genes for Brn-2, we investigated the nature of the Brn-2 recognition element by performing mobility shift and random oligonucleotide selection assays. Sequence comparison of a series of characterized homeo domain and POU domain recognition elements bound by Brn-2, and the Brn-2 recognition elements on the CRH promoter revealed the consensus CATnTAAT ($n=0,2,3$; Fig. 2B), with CRH II being the highest affinity site. One round of random oligonucleotide selection for Brn-2 also revealed similar recognition sequences C/AATnTAAT ($n=0,2,3$). These data suggested that the Brn-2 binding site might consist of two conserved core motifs separated by a variable spacer region, with n representing the number of nucleotides in this "spacing" region. As expected, deletion of POU_S domain of Brn-2 drastically decreased binding to all sites tested (Fig. 2A). A bipartite consensus sequence for Brn-2 binding sites is consistent with the bipartite structure of the POU domain-binding sites that has been described for Oct-1 (Verrijzer et al. 1990, 1992), Oct-2 (Kristie and Sharp 1990), and Pit-1 (Ingraham et al. 1990).

The orientation of the POU_S domain recognition motif for Brn-2 on the CRH II site

To provide direct evidence for the potential preference of the Brn-2 POU_S domain or the POU_{HD} for binding to upstream or downstream core motifs, a single BrdU residue was introduced in or adjacent to the major groove contacts of each core motif (Kurokawa et al. 1993; Fig. 3A) and UV cross-linking was performed. A thrombin proteolytic cleavage site introduced into the linker region of Brn-2 permitted distinction between POU_S-DNA (46 kD) and POU_{HD}-DNA (18 kD) cross-linked products in the presence of thrombin. As shown in Figure 3A,

Figure 2. DNA-binding activity of Brn-2 on various DNA sites plus sequence alignment. (A) Mobility shift assay of Brn-2 using radiolabeled random oligonucleotides. (Random) A double-stranded DNA fragment with fully 16-bp random sequences; (en) *engrailed* site; (ftz) *fushi tarazu* site; (CRH-II) CRH-II site; (P₀) Tst-1 recognition element; (HSV-OCT and IgH-OCT) Oct-1 and Oct-2 recognition elements. Binding reactions containing probe alone, probe with lysate (lysate), probe with in vitro translated Brn-2 whole protein (Brn-2), and probe with mutated Brn-2 in which the POU_S domain was deleted (Brn-2ΔPs) were performed for each site and electrophoresed on 5% native polyacrylamide. (B) Sequence alignment for Brn-2 recognition elements. The boxed region in the DNA sites represents the sequence homologous region. (ROB) The random oligonucleotide selection assay. (CRH-II, CRH-IV, and CRH-V) The three regions detected by footprint analysis on the CRH promoter (Fig. 1D). Mec 3 (1) and Mec 3 (2) are the *unc-86* recognition elements in the *mec-3* promoter (Way et al. 1991). CE₂ is Brn-3 recognition element in the POMC promoter (Gerrero et al. 1993). CRH I (CATTTGCCTAATAAGC) and CRH III (TTGATATAAT-TGGA) exhibited very low binding activity by mobility shift assay. The cfl a site is a recognition element in the *ddc* promoter (Johnson and Hirsch 1990). (ROB) Random oligonucleotide blot.

when a single thymidine residue was substituted with BrdU at position 1 (BrdU-1), a labeled cross-linked protein-DNA complex migrated at 18 kD, consistent with binding of the POU_{HD} domain to the TAAT site. Conversely, a cross-linked protein-DNA product of ~46 kD was obtained when a T was substituted by BrdU at position 2 (BrdU-2) consistent with binding of the POU_S domain to the GCAT core motif. These cross-linked products were not observed when Brn-2 bound to the wild-type oligonucleotide [BrdU(-)], or in the absence of thrombin. These results provided direct evidence that the POU_S domain contacted the upstream core motif

Figure 3. Polarity of the POU_S domain and POU_{HD} of Brn-2 on the two DNA core motifs of CRH II site and DNA-binding activity of wild-type and mutant (Q⁴⁴→A) Brn-2 on the mutated CRH II site. (A) UV cross-linking assay of Brn-2 on the CRH II site. (Top) The schematic diagram of the GST-Brn-2 fusion protein; the LVPR is the thrombin recognition site. The 37- and 9-kD products represent the proteolytic products of GST-POU_S and POU_{HD} after thrombin cleavage, their DNA-protein cross-linking complexes migrate at 46 and 18 kD, respectively. The DNA sites for the cross-linking assay and the position substituted by BrdU is shown in the middle. The ¹⁴C protein markers were included in the SDS protein gel, and their relative positions are indicated on the left side. (Lane 1) Wild-type site [BrdU(-)]; (lanes 2,3) BrdU (1 and 2) sites in the absence of thrombin; (lanes 4,5) BrdU sites in the presence of thrombin. The doublets of the cross-linking products for POU homeo domain (BrdU-1, lane 4) are the result of nonspecific thrombin cleavage in the POU_{HD} region of Brn-2. The radiolabeled cross-linking products (GST-POU_S and POU_{HD}) were identified according to their size on the SDS gel. (B) (Top) (λ Rep) The amino acid sequences of the helix 3 of λ repressor; and the putative recognition helix of the POU_S domain of Brn-2. The core DNA sequence of recognition elements for λ repressor (O_R1) and Brn-2 (CRH II) are also shown. The solid and broken arrows indicate the established or proposed contact at the adenine residue (circle) by Gln-44 of λ repressor or Brn-2, respectively. The boxed region is the putative POU_S domain recognition motif for Brn-2. (Bottom) The DNA-binding activity of wild-type Brn-2 and mutant (Q⁴⁴→A) on the wild-type CRH II (CRHII-A) site, on sites in which the circled residue is substituted by G (CRHII-G), C (CRHII-C), or T (CRHII-T). The complementary strand changed accordingly. (C) Competition experiments of wild-type and mutant (Q⁴⁴→A) Brn-2 on the CRH II site. The CRH II wild-type element was labeled by ³²P and different fold excess of cold DNA elements of CRHII-G, CRHII-C, or CRHII-T was added to the binding reaction.

(5'-GCAT-3') and the POU_{HD} bound to the downstream 5'-TAAT-3' motif, corresponding to the consensus-binding sequence of classic homeo domain protein (Gehring 1987).

Because cross linking data revealed that the Brn-2 POU_S domain contacted a core motif (CAT) that appeared to be in an inverted orientation compared with that of the ATG core motif in the Pit-1-binding element

(Prl-1P site; Elsholtz et al. 1989), we evaluated the methylation interference pattern of Brn-2 on the CRH II site and the Prl-1P site. Methylation interference assays indicated that POU_S domain makes major groove contacts with GC of GCATAAATAAT (CRH II) and G of CAT-GNATAT (Prl-1P), which was comparable with that of Pit-1 on Prl-1P site (data not shown; Elsholtz et al. 1989). Furthermore, using a site in which both potential orien-

tations of the CAT sequence were simultaneously presented (CATGCGTAAT) revealed that both were used (data not shown). Therefore, the orientation of the POU_S domain on its high affinity site (CRH II) appears to be opposite that when it is bound to the Pit-1 DNA-recognition element.

The orientation of the POU_S domain relative to the POU_{HD} for Brn-2 on the CRH II site was actually evaluated on the basis of the structural similarity between the POU_S domain and λ and 434 repressors and 434 Cro protein (Assa-Munt et al. 1993; Dekker et al. 1993). Gln-44, at the beginning of the putative DNA recognition helix (helix 3) of the POU_S domain is conserved among all POU domain proteins and the DNA recognition helices of λ and 434 repressors and 434 Cro protein. On the basis of the cocrystal structures of λ and 434 repressors, and 434 Cro protein (for review, see Harrison and Aggarwal 1990) and the existing model based on the NMR solution structure of the POU_S domain of Oct-1 (Assa-Munt et al. 1993; Dekker et al. 1993), Gln-44 in helix 3 of the POU_S domain of Brn-2 might make direct contact with the adenine (A) in the antisense strand of the position T of the CAT (Fig. 3B, top). To test this hypothesis, we adopted a strategy used previously to determine the direct amino acid and base pair contacts of λ repressor (Hochschild and Ptashne 1986) by replacing Gln-44 with a much smaller amino acid, alanine (Q⁴⁴→A). As shown in Figure 3B, when the T of GCAT was substituted with A, G, or C, the binding activity of wild-type Brn-2 was decreased drastically. Competition experiments (Fig. 3C) confirmed that the relative affinity of Brn-2 for these altered sites was 50-fold less than that of CAT (calculated by EC₅₀). However, the Gln-44→Ala mutation in the Brn-2 POU_S domain resulted in a loss of preference for binding in the T position of the CAT motif. The affinity of the mutant protein for each of the four bases at the T position (CAT) was relatively similar (fivefold difference between T and C; Fig. 3C, bottom). Wild-type and mutant Brn-2 demonstrated similar binding preference when A of CAT was substituted with G, C, or T (data not shown). Thus, the alteration of Gln→Ala in the DNA recognition helix of the Brn-2 POU_S domain perturbed the ability of Brn-2 to discriminate between different base pairs at the T position (GCAT_TTAAT), suggesting that the Gln-44 residue of the Brn-2 POU domain specifically contacted the A residue in the antisense strand of CAT. The binding activity of wild-type and mutant protein was also evaluated on the Prl-1P site, where A of ATG was substituted with each of the other three nucleotides, and the results of this analysis was consistent with the model proposed by Assa-Munt et al. (1993), that A of ATGNATAT is the base contacted by Gln-44 in the case of Oct-1 (data not shown). Because Gln-44 of the Brn-2 POU_S domain appears to make the identical base pair contact on CRH II or Prl-1P sites, which exhibit opposite orientations of the POU_S core recognition element (CAT or ATG), the orientation of the POU_S domain relative to the POU_{HD} appears to be altered from a direct to an inverted position, respectively.

Brn-2 binds to DNA elements with a spacing of 0, 2, or 3 nucleotides

To investigate further the possibility that the Brn-2 recognition element is composed of two conserved core-binding motifs separated by variable spacing, a systematic investigation was initiated using the modified CRH II site so that the spacing nucleotides between the core motifs GCAT and TAAT were GCG instead of AAA (GCATGCGTAAT); substitution of GCG with AAA did not change the binding affinity for Brn-2 (data not shown). A series of mutations across the two conserved half-sites, the putative spacer region and the 5'- or 3'-flanking regions, were evaluated for their ability to bind to Brn-2. As shown in Figure 4A, no binding activity was detected when the core motifs GCAT or TAAT were mutated (M2,3,5,6,7). In contrast, altering sequences 5' (M1) or 3' (M8) of the core motifs, or within the spacer region (M4), did not alter the DNA binding dramatically. This analysis suggested that nucleotide sequences within the two distinct core motifs were crucial for sequence-specific, high affinity binding. DNA sites in which the putative spacing region GCG was further substituted with various combinations of two or three nucleotides (Fig. 4A, bottom) revealed that Brn-2 was able to accommodate alterations in both the sequence and the number of nucleotides separating the two core motifs. The Brn-2 relative binding affinity for each of these sites depended on the precise nucleotide sequence of the spacer region.

The nucleotides that are contacted directly by Brn-2 were defined by potassium permanganate (KMnO₄) interference assays (Truss et al. 1990). As expected, modifications at thymine residues within each core recognition motif (GCAT_TTAAT) markedly reduced or abolished binding of Brn-2 (Fig. 4B). The interference patterns in sites containing a spacing of either 2 or 3 nucleotides were identical (Fig. 4B), consistent with the model that major groove contacts between Brn-2 and DNA elements remained invariant, irrespective of the presence of 2 or 3 spacing nucleotides.

A series of DNA elements were designed to investigate whether there were restrictions in spacing that would accommodate high affinity binding by Brn-2. Brn-2 exhibited high affinity with a spacing of 2, moderate affinity when spacing was 0 and 3, and low activity or no binding activity on sites with spacing of 1, 4, 5, 10, or 13 nucleotides (Fig. 5A). The relative affinity of Brn-2 on DNA sites in which the core motifs were spaced by 0, 2, 3, or 4 was further assessed by competition analysis (Fig. 5A, top left), revealing that Brn-2 had the highest affinity for sites with a spacing of 2 (EC₅₀ = 0.25 nM), moderate affinity for a spacing of 3 (EC₅₀ = 1.5 nM) or 0 (EC₅₀ = 2.3 nM), and exhibited no effective binding activity with a spacing of 4 nucleotides.

Class-specific spacing preferences of neuron-specific POU domain proteins

Because Brn-2 exhibited preference for DNA sites with spacing of 0, 2, or 3 nucleotides, it was of interest to

To investigate the underlying molecular basis for class-specific spacing preferences in core DNA-binding motifs and to identify the regions responsible for the selective binding characteristics of Brn-2 and Brn-3, a series of POU domain chimeras of Brn-2 and Brn-3 were generated, on the basis of the observation that the POU domain itself was sufficient to confer the same binding profile as the holoprotein (Fig. 6C). A sequence comparison between the POU domains of Brn-2 and Brn-3 [Fig. 6A] illustrated that the linker regions are entirely divergent and that significant differences are observed throughout the POU_S domain and POU_{HD}, except in the DNA recognition helices. The structures of the chimeric proteins and their binding activity on DNA sites with spacing of 0, 2, or 3 nucleotides are shown schematically in Figure 6B. As shown in Figure 6, B and C, chimeric POU domains including the POU_S domain (2/3/3) or the linker region (2/2/3) of Brn-2 appeared to function equivalently to the Brn-3 wild type, selectively recognizing DNA sites with a spacing of 3 nucleotides, indicating that the POU_S domain and the linker region are not critical in determining the distinct spacing choices by Brn-2 and Brn-3. Therefore, the critical region regulating the spacing preference apparently resided in the POU homeo domain. To define the precise regions within the POU_{HD}

examine the spacing preferences for other neuron-specific POU domain factors. In striking contrast with Brn-

Figure 5. (See facing page for legend.)

Figure 6. DNA-binding activity of chimeric POU domains of Brn-2 and Brn-3. (A) Amino acid comparison between the POU domains of Brn-2 and Brn-3, with identity indicated by a hyphen. Boundaries of the four α helices for the POU_S domain and three α -helices for the POU_{HD} were deduced from the solution structure of Oct-1 POU_S domain (Assa-Munt et al. 1993; Dekker et al. 1993) and the *en* homeo domain structure (Kissinger et al. 1990). The three different amino acids in the basic region of the POU_{HD} of Brn-2 and Brn-3 are highlighted. (B) Schematic diagram of the chimeric structure and their binding activity. The open and solid bars represent the Brn-2 and Brn-3 sequences and the position of the basic region (B), the linker region (L), helix 1 (H1), helix 2 (H2), its preceding loop (Lo), and helix 3 (H3) of the POU_{HD} are indicated in the relative position. (C) DNA-binding activity of representative chimeras. The DNA-protein complex was resolved on 8% native polyacrylamide gel.

that conferred spacing discrimination, we first considered the amino-terminal basic region known to make minor groove contacts in the *en* homeo domain (Kissinger et al. 1990). The Brn-2 POU domain containing the basic region of the Brn-3 POU domain [2/2/3_(b)2] exhibited a marked decrease in its binding activity on DNA sites spaced by 2 bp (75%) and by 0 bp (90%), compared with the wild type Brn-2 POU domain, but retained wild-

type activity on the spacing 3 site. This switch involved an alteration of only 3-amino-acid residues distinct between Brn-2 and Brn-3 [R→E in position 3 and RK→KR in position 5,6; Fig. 6A]. A further alteration to include the Brn-3 helix 1 [2/2/3_(b+H1)2] or helix 1 and the preceding loop of helix 2 [2/2/3_(b+H1+Lo)2] did not alter the binding profile (Fig. 6B). However, a Brn-2 chimera containing the basic region of Brn-3 POU_{HD} and both helix

Figure 5. DNA-binding activity and *trans*-activation function of Brn-2 and Brn-3 on DNA sites with different nucleotide spacing. (A) DNA-binding activity of Brn-2 on sites with different spacing nucleotides. A primer (5'-TCAGAGATCTGGCGC-3') was labeled with ³²P, annealed to its complementary strand (5'-TGACAGGATCCACGCATnTAATGCGCCAGATCTCTGA-3'; n is 1, G; 2, GC; 3, GCG; 4, GCGC; 5, GCGCG; 10, GCGCGCGCGC; and 13, GCGCGCGCGCGCGC), and filled in with Klenow fragment (BRL). (B,F) The bound and free probes, respectively. (Top right) Results of the competition assay. Brn-2 was bound to a radiolabeled site with a spacing of 2 nucleotides in the presence of 0, 5, 10, 50, 100, and 500-fold molar excess of unlabeled sites that have spacing of 0, 2, 3, and 4 nucleotides. (Bottom right) The fold induction by Brn-2 in cotransfection assay. (B) DNA-binding activity of Brn-3 on DNA sites with a spacing of 0, 1, 2, 3, or 4 nucleotides and fold induction of Brn-3 on reporters that contain DNA elements with a spacing of 0, 1, 2, or 3 nucleotides. (C) Summary of the identified DNA recognition elements for members of POU-III and POU-IV classes according to their spacing preferences.

1 and helix 2 and its preceding loop [2/2/3_(b+H1+H2)2] exhibited a complete switch of the binding profile from that of Brn-2 to that characterized by Brn-3, exclusively binding to the spacing 3 site. When only helix 1 and helix 2 sequences of Brn-2 found were replaced with those of Brn-3 [2/2/3_(H1+H2)2], in the absence of altering the Brn-2 basic region, the chimeric protein demonstrated a 60% decrease in the ability to use the spacing 2 site. Therefore, the 3 residues in the basic cluster of Brn-3 POU_{HD} and helix 2 together transfer a complete switch in the spacing preference of Brn-2.

Discussion

Distinct orientation and spacing of core-binding motifs in neuronally expressed POU domain proteins

On the basis of our analyses, the preferred Brn-2 recognition sequences consists of two distinct half-sites **GCA**Tn**TAAT** separated by a nonconserved spacer region (*n*) of 0, 2, or 3 nucleotides. UV cross-linking experiments establish the polarity of the DNA recognition site with the POU_S domain selectively contacting the **GCA**T core motif and the POU_{HD}, the **TAAT** motif. Random DNA site selection using isolated POU_S and POU_{HD} suggests that Oct-1 exhibits a comparable polarity on its cognate DNA site (Verrijzer et al. 1992). However, the POU_S domain binding motif of the CRH II site (**GCA**Tn**TAAT**) unexpectedly appears to be oriented opposite to the POU_S domain recognition motif in Oct-1, Oct-2, and Pit-1 DNA-binding elements (e.g., **ATG**-CAAAT and **ATGAATAT**, respectively). On the basis of the striking similarity of the solution structure of the POU_S domain of Oct-1 determined by NMR to the λ and 434 repressors, and 434 Cro protein, some of the contacts between the POU_S domain and its core recognition motif can be predicted (Assa-Munt et al. 1993). In the λ and 434 repressors, and 434 Cro protein, Gln-1- α 3, which is the first residue in helix 3, forms two hydrogen bonds with the adenine of base pair 2, whereas a glutamine residue (Gln-1- α 2) at the amino terminus of the helix 2 plays an important role in positioning the Gln-1- α 3 interaction for its with DNA (Pabo et al. 1990). On the basis of methylation interference assays and binding analyses of a Gln-44 $\rightarrow$ Ala mutation in Brn-2, it appears that Gln-44 in the POU_S domain of Brn-2 plays a role similar to Gln-1- α 3 by contacting the antisense adenine residue at the T position in the CRH II site (**GCA**Tn**TAAT**). Therefore, the orientation of the Brn-2 POU_S domain on binding to its recognition motif is likely to be opposite that of the POU_S domain of Oct-1 or Pit-1 on their response elements (Fig. 7A,B). These data are consistent with the orientation of the POU_S domain for Oct-1 suggested by Assa-Munt et al. (1993). However, it appears that the POU_S domain of Brn-2 is also capable of "switching" orientation when it binds to the Pit-1 recognition element (Prl-1P), with the POU_S domain now in an inverted orientation relative to the POU_{HD}, as is the case for Pit-1 (Fig. 7A). The organization of the bipartite DNA site for Brn-2 binding (CRH II) is in a sense analogous to the

bipartite sites used by retinoic X receptor (RXR), retinoic acid receptor (RAR), thyroid hormone receptor (T₃R), or vitamin D receptor (VDR) heterodimers, in which core-binding motifs are in a direct repeat orientation (Näär et al. 1991; Umesono et al. 1991; Kurokawa et al. 1993), but they also bind to sites in which core-binding motifs are in an inverted palindrome orientation (Näär et al. 1991).

The fact that Brn-2 is able to recognize DNA elements with different spacing (0, 2, or 3) nucleotides, further reveals the remarkable flexibility of the Brn-2 POU domain, because in B-form DNA, altering of spacing from 0 to 3 represents ~ 10 Å linear distance and 108° rotation between the two core-binding motifs. These data imply either a difference in the precise conformation of Brn-2 on DNA elements with different spacing, or a tolerance conferred by a bending of the DNA sites, as has been reported on binding of the Oct-1 POU domain to its site (Verrijzer et al. 1991). Although Brn-2 (POU-III) binds effectively to DNA sites with spacing of 0, 2, or 3 nucleotides, Brn-3, a member of class IV, binds with high affinity only to DNA sites with a spacing of 3 nucleotides, suggesting that the structure of its POU domain is highly constrained when it binds to DNA sites. It is possible that other spacing choices (such as 1 and 4) may be used by other, as yet unidentified, POU domain proteins. Spacing nucleotides can potentially serve as a binding site for other factors, a strategy exploited in the case of the yeast MAT α 2 homeo domain protein in which MCM1 binds between MAT α 2 subunits, permitting proper spacing and cooperative binding of the MAT α 2 subunits (Smith and Johnson 1992). Spacing was also able to serve as a determinant for C6 zinc cluster proteins (Reece and Ptashne 1993). Random DNA site selection confirmed that each steroid receptor heterodimeric pair exhibits a distinct spacing preference on DNA sites containing direct repeat core motifs (Kurokawa et al. 1993).

The ability of Brn-2 to recognize various DNA sites with a wide range of affinity by altered spacing and orientation could expand the number of target genes that may be modulated differentially by POU-III class (Brn-2) as compared with POU-IV class (Brn3.0). Three potential downstream targets for POU-III class have been identified: dopa-decarboxylase (*ddc*) gene (*cf1a*; Johnson and Hirsch 1990), the cell surface adhesion molecule P0 (Tst-1; He et al. 1991), and CRH (Brn-2), and the high affinity binding sites for these proteins correspond to the selected sites with spacing 0, 2, or 3 nucleotides (Fig. 5C). A potential target gene for Brn-3 has been identified (Gerrero et al. 1993); and we noted that this response element (CE-2; Fig. 5C) can be characterized as a site with a spacing of 3 nucleotides, although previous studies suggested that Brn-3.0 binds to the octamer site with low affinity (Gerrero et al. 1993). Another member of POU-IV class *unc-86* has a genetically identified regulatory target, *mec-3*, a homeo domain factor controlling neuronal differentiation (Way et al. 1991). Examination of the *unc-86* recognition elements in the *mec-3* promoter suggests that the *unc-86* recognition elements can be defined as a spacing 3 site. (Xue et al. 1992; Fig. 5C). Thus, the orga-

Figure 7. Models of the POU domain DNA complex. (A,B) The schematic illustrates the orientation of the helix 3 of POU_S domain relative to helix 3 of the POU_{HD} of Pit-1 on Prl-1p and Brn-2 on CRH II sites. Critical contacts by the POU_{HD} of Pit-1 and Brn-2 on their consensus sites and the relationship of the three α -helices were deduced from the crystal structure of the *en*-DNA complex (Kissinger et al. 1990). The relationship of the four α -helices of the Pit-1 and Brn-2 POU_S domains was based on the striking similarity of the solution structure of the Oct-1 POU_S domain to the λ and 434 repressors (Assa-Munt et al. 1993; Dekker et al. 1993). The arrows in the helix 3 of the POU_S domain and POU_{HD} indicate the direction from amino terminus to the carboxyl terminus. Cylinders are used to show the positions of the α -helices, ribbons are used to show the sugar-phosphate backbone of the DNA, and bars indicated the base pairs. The amino acid sequence of the helix 3 of the POU_S and POU_{HD} of Pit-1 and Brn-2 and the detailed contacts in the major groove by these helices are shown at bottom. The numbers on the top of the amino acids corresponds to the numbering system described by Rosenfeld (1991) for POU_{HD} and Assa-Munt et al. (1993) for the POU_S domain. (C) The altered major groove position of the POU_S domain when Brn-2 binds to DNA elements with a spacing of 0 ($n = 0$) or 3 ($n = 3$). The broken line indicates the position of the POU_S domain; the arrow shows the direction of the sliding for the POU_S domain. The hatched helix 2 of the POU_{HD} and the KRR amino acids in the basic region of the POU_{HD} that make minor groove contacts are the regions acting to constrain the positions of major groove contacts by the POU_S domain in the class IV POU domain factors.

nization of the limited number of identified native DNA recognition elements for neuronally expressed POU domain factors is consistent with the observed spacing preferences exhibited in our analyses, suggesting that the preferred binding sequences can be used in predicting potential target genes for new members of the POU domain gene family.

Molecular mechanisms determining the spacing preference of Brn-2 and Brn-3

The molecular basis of the fundamental difference in their flexibility when they bind to high affinity DNA sites exhibited by class III and class IV POU domain factors presents an intriguing problem. We have demonstrated that replacing the only three variant amino acids in the amino-terminal basic region of the Brn-2 POU_{HD} with those of Brn-3 is sufficient to produce a marked switch from the Brn-2 spacing profile to that of Brn-3. In concert with helix 2 of the Brn-3 POU_{HD}, a complete switch in the spacing profile of Brn-2 to that of Brn-3 is accomplished. One of the three residues (K^5) that differs between Brn-2 and Brn-3 corresponds to R^3 in the *en*

homeo domain that makes a base pair contact in the minor groove (Kissinger et al. 1990). The other two residues that differ between Brn-2 and Brn-3 (R^2 and R^4) correspond to D^{-1} and K^2 in the disordered region of the *en* homeo domain structure (Kissinger et al. 1990); the same region is also disordered in the MAT α 2 cocrystal structure (Wolberger et al. 1991) and NMR structure of the Antp protein (Qian et al. 1992). This disorder may reflect an inherent flexibility of this region, which could easily lead to different configurations, on the basis of the 2-amino-acid difference between Brn-2 and Brn-3. Therefore, the flexible amino-terminal arm of different POU domain proteins specifies interactions with DNA and contributes significantly to DNA site recognition. These observations lead us to propose a model for spacing preferences of the POU domain on its DNA-binding sites (Fig. 7C). Our studies suggest that minor groove contacts conferred by 3 amino acids in the Brn-3 amino-terminal basic region of the POU_{HD}, and perhaps phosphate backbone contacts by helix 2, may act in concert to constrain the permitted major groove contacts by the POU_S domain, restricting Brn-3 binding to DNA elements in which the core binding motifs are spaced by 3 nucle-

otides. In contrast, the corresponding amino acid residues permit flexibility of minor groove contact for Brn-2; thus Brn-2 is able to bind effectively to DNA sites with spacing of 0, 2, or 3 nucleotides.

Thus, in addition to the defined role of helix 1 and helix 2 in protein-protein interaction (Lai et al. 1992; Pomerantz et al. 1992), specific residues in the amino terminus of the POU_{HD} can act to determine the flexibility of the protein when it binds to DNA. The fact that specific minor groove contacts serve as the molecular mechanism that dictates flexibility or constraint in sequence-specific DNA site recognition has many intriguing implications that can be applied to other proteins. The role of orientation and spacing in specifying DNA recognition elements for functionally distinct POU domain factors is likely to be prototypic for functional specification in other families of transcription factors.

Materials and methods

cDNA screening, DNA sequencing, Northern analysis, and in situ hybridization

An adult rat brain λ gt 11 cDNA library was screened with the POU domain of Brn-2, and the positive DNA inserts were subcloned into pBKS(-) (Stratagene) and sequenced using the Sequenase 2.0 kit (U.S. Biochemical; He et al. 1991). Sequence compilation and amino acid analysis were performed using the DNA star program. Poly(A)⁺ RNA isolation and Northern analysis were performed as described (Ingraham et al. 1988). Poly(A)⁺ (5 μ g) RNAs from rat tissues were used, and the blot was hybridized with 10⁶ cpm of radiolabeled Brn-2 cDNA. In situ hybridization was carried out as described previously (He et al. 1989). The Brn-2 antisense cRNA probe corresponds to a 405-bp region coding for amino acids 182–310. The sense-strand control probes were tested.

DNA-binding assays

Proteins for Brn-2 (pMET-Brn-2) and Brn-3 (pBKS-Brn-3; Gerero et al. 1993) were synthesized by TNT transcription-translation-coupled system (Promega). Parallel translation reactions were performed either with [³⁵S]methionine or unlabeled methionine. The quantity of trichloroacetic acid (TCA) perceptible radioactivity contained in each translation reaction was used to normalize the amounts of proteins. All translation products were examined by SDS-PAGE. A ¹⁴C-methylated protein mixture was included for molecular weight standards.

Electrophoresis mobility shift assay (EMSA) was performed by using equivalent molar of in vitro-translated Brn-2 and Brn-3 proteins. Synthetic, double stranded oligonucleotides were labeled with [γ -³²P]ATP. Binding reaction was performed as described by Ingraham et al. (1988). In competition assay, varying amounts of unlabeled sites were added into the binding reaction at the same time as the probe. The free and binding fractions were detected quantitatively by PhosphorImager screen (Molecular Dynamics) and analyzed using a molecular scanner (Molecular Dynamics). The competition curve was generated using the GraphPAD program.

DNase I footprint analysis was performed essentially as described by He et al. (1991), except that double stranded DNA probes were generated by PCR, using plasmids containing CRH promoter (–337 to +25 bp). Primers for PCR were designed according to the sequences at the 5' and 3' termini of the pro-

moters. One of the primers was kinased with [γ -³²P]ATP, and another was unlabeled; thus a directional probe was generated.

Potassium permanganate (KMnO₄) interference was performed essentially as described by Truss et al. (1990). Under this condition, T residues were strongly modified. However, some of A residues can also be modified. Large-scale mobility shift assays were carried out as described earlier using the modified probe. The free and binding fractions were transferred into DE81 paper and eluted [20% EtOH, 1 M LiCl, 10 mM Tris (pH 7.5), 1 mM EDTA]. The isolated DNA was subjected to piperidine cleavage and resolved on a 12% denaturing polyacrylamide gel.

For UV cross-linking assay, two oligonucleotides containing a single BrdU residue were synthesized (Kurokawa et al. 1993); the positions of BrdU residues are illustrated in Figure 3A. A thrombin site was introduced into the linker region of the Brn-2 POU domain by site-directed mutagenesis (Mutagene, Bio-Rad). The insertion of nucleotides CCGCGGAACCAG at position 1092 changed the peptide sequence from SSGS to SSLVPRGS. The DNA fragment containing the mutated POU domain was inserted into pGEX-3X (Pharmacia) vector *Bam*HI and *Eco*RI sites. Glutathione *S*-transferase (GST) fusion protein preparation was performed as described by Assa-Munt et al. (1993). The binding affinity of the mutated protein for the DNA sites was indistinguishable from that of the wild-type protein. The UV cross-linking experiment was performed as described (Kurokawa et al. 1993) except that gels separated from the glass molding plate were exposed directly to a shortwave UV source for 5 min. The gel slices including the binding fraction were incubated in 0.5 $\times$ TBE buffer with or without 1 unit of bovine thrombin (Sigma) for 30 min at room temperature. These slices were then reequilibrated in loading buffer and subjected to 15% SDS-PAGE. After electrophoresis, gels were fixed, dried, and autoradiographed.

Generation of chimeras between POU domains of Brn-2 and Brn-3

In general, chimeras were generated by PCR amplification of fragments from different segments of the POU domain of Brn-2 and Brn-3. The *Bst*EII site was introduced at boundaries of all PCR products to permit in-frame ligation. Replacing Val and Thr by the addition of a *Bst*EII site did not affect the DNA-binding activity for the chimeras. All primers for PCR contained 24-mer completely matched sequence to Brn-2 and Brn-3. The structure diagram of each construct was presented schematically in Figure 6B. 2/3/3 and 2/2/3 were generated by ligating PCR products, such as the POU_S domain or the POU_S domain plus the linker region of Brn-2, to either the linker plus the POU_{HD} of Brn-3 or POU_{HD} alone. To prepare 2/3/2, long primers were designed to include sequences of the linker region of Brn-3. The POU domain of Brn-2 was used as template for PCR amplifying the fragment that consisted of the Brn-3 linker region and Brn-2 POU_{HD}. The chimeras for 2/3/3_b2, and 2/2/3_b2 were constructed by PCR amplifying the Brn-2 POU_{HD}, which contained either the linker and basic regions or the basic region alone of Brn-3, and ligating these fragments to the POU_S domain of Brn-2. Three subsequent rounds of PCR were performed to generate POU_{HD} of Brn-2, which included the basic region and helix 1 of the POU_{HD} of the Brn-3 [3_b+H1]2 (the helix 1 in this construct is 2 amino acids shorter than the boundary indicated in Fig. 6A). The POU domain of 2/2/3_b(+H1+H2) 2 was generated by designing primers that included sequences of the helix 3 of Brn-2 and PCR-amplifying using Brn-3 POU_{HD} as template. The Brn-2 POU domain consisting of the helix 1 and helix 2 of the POU_{HD} of Brn-3, was generated by

PCR amplification 3_(b+H1+H2)2 chimeric POU_{HD} using primers that contained the basic region of Brn-2.

Cotransfection assay

Brn-2 and Brn-3 expression vectors were constructed by inserting the full-length cDNAs into the cytomegalovirus (CMV) expression vector (*KpnI*–*XbaI*). All of the reporter vectors used in the study harbor a single copy of the indicated oligonucleotides at the unique *Bam*HI site upstream of p36–Luc (Ingraham et al. 1988). The identity of the inserted oligonucleotides was confirmed by sequencing. Expression plasmid (1 µg) and luciferase reporter plasmid (3 µg) were cotransfected into HeLa cells by the calcium–phosphate precipitation method; cell extracts were prepared and luciferase assays were performed as described by Ingraham et al. (1988).

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Spacing and orientation of bipartite DNA-binding motifs as potential functional determinants for POU domain factors.

P Li, X He, M R Guerrero, et al.

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