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POU Domain Factors of the Brn-3 Class Recognize Functional DNA Elements Which Are Distinctive, Symmetrical, and Highly Conserved in Evolution

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To better understand the diversity of function within the POU domain class of transcriptional regulators, we have determined the optimal DNA recognition site of several proteins of the POU-IV (Brn-3) subclass by random oligonucleotide selection. The consensus recognition element derived in this study, ATAATTAAT, is clearly distinct from octamer sites described for the POU factor Oct-1. The optimal POU-IV site determined here also binds Brn-3.0 with significantly higher affinity than consensus recognition sites previously proposed for this POU subclass. The binding affinity of Brn-3.0 on its optimal site, several variants of this site, and several naturally occurring POU recognition elements is highly correlated with the activation of reporter gene expression by Brn-3.0 in transfection assays. The preferred DNA recognition site of Brn-3.0 resembles strongly the optimal sites of another mammalian POU-IV class protein, Brn-3.2, and of the *Caenorhabditis elegans* Brn-3.0 homolog Unc-86, demonstrating that the site-specific DNA recognition properties of these factors are highly conserved between widely divergent species.

The POU domain transcription factors are characterized by a bipartite DNA recognition domain containing a POU homeodomain and an adjacent POU-specific domain, both of which contribute to site recognition (2, 12). Six subclasses of POU proteins have been described, and various members of these subclasses exhibit tissue-specific expression in the skin, pituitary, and particularly the central nervous system (CNS), where several POU proteins have been shown to play important roles in control of cellular development and function.

The specific DNA sites at which the POU factors can bind and activate or repress transcription constitute a transcriptional genetic code which remains poorly defined for these and many other structural classes of transcription factors. The POU proteins as a class have been described as octamer binding proteins based on their affinity for the octamer element ATGC(A/T)AAT, and the recognition element of one POU protein, Oct-1, has been thoroughly characterized (23). How the functional DNA recognition sites of the other POU classes may differ from that of Oct-1 is not as well understood.

We have been engaged in studies of the Brn-3, or POU-IV, subclass of factors, which includes in mammals Brn-3.0, Brn-3.1, and Brn-3.2 (also designated Brn-3a, Brn-3c, and Brn-3b, respectively) (6, 10, 11, 20–22, 26, 27). These factors are expressed in a highly specific pattern in the developing and adult peripheral sensory system and CNS and exhibit strong sequence similarity within the POU domain to the invertebrate neural transcription factors Unc-86 and I-POU (7, 19). Recent targeted mutations in mice have shown that Brn-3.1 is necessary for correct development of the auditory system and that Brn-3.2 is required for differentiation of some retinal ganglion cells (5, 9). Null mutations at the *brn-3.0* locus result in neonatal death, with defects in sensory ganglia and in some of the CNS nuclei that express Brn-3.0 (16, 28).

The Brn-3 class proteins have been shown to bind with high affinity to a recognition element derived from the rat corticotropin-releasing hormone (CRH) gene promoter, GCATAAA TAAT, and to activate transcription via this site (10, 20). However, none of the Brn-3 factors are detectable in hypothalamic CRH-expressing neurons, ruling out these factors are regulators of CRH expression in vivo. In previous studies (15), it has been suggested that this CRH site represents a general POU domain recognition site of the class $GCATN_3TAAT$, where N represents any base pair, and that for the Brn-3 protein class, high-affinity binding requires that N = 3. A Brn-3.2 binding site containing the conserved core sequence TTAATGAG has previously been determined by random oligonucleotide selection (27), but this recognition element is quite distinct from the CRH site.

To better understand DNA recognition and transcriptional activation by the POU-IV class proteins, we have determined the optimal DNA binding site for Brn-3.0 by random oligonucleotide selection. The site derived contains a core ATAATT AAT sequence and exhibits >1,000-fold higher affinity for Brn-3.0 than the previously described Brn-3 family consensus recognition elements. The optimal DNA recognition site for Brn-3.0 and several derivatives of this site mediate activation of transcription from reporter gene constructs at levels which correlate well with their in vitro binding affinities. The DNA binding properties of Brn-3.0 are extremely similar to those of Brn-3.2 and the *Caenorhabditis elegans* Brn-3 homolog Unc-86, indicating that the evolutionary divergence of the POU-IV class proteins has occurred for reasons other than to permit distinctive DNA recognition.

MATERIALS AND METHODS

Expression of POU proteins. DNA inserts for expression of the Brn-3.0, Brn-3.2, and Unc-86 POU domains and the Brn-3.0 POU homeodomain were generated by PCR using the following oligonucleotides: Brn-3.0 POU, GCGCC GGTCGACGACTCGGACACAGACCCCCGAGAG and GGCCAGGCCTG GAGTGCAGCGACGGGTAC; Brn-3.0 POU homeodomain, GGCCGTCGA CGGCGCGCAGCGTGAGAAAATGAAC and AGAGAGAGGCGGCCGCA AGCTTTCAGTAAGTGGCAGAGAATTTCAT; Brn-3.2, GCGCCGGTCGA CGACGTGGATGCAGACCCGCGGGA and GGCCAAGCTTCTAAATGCC GGCAGAGTATTTCATT; and Unc-86, GCGCCGGTCGACGATATGGACA CTGATCCGAGACAA and GGCCAAGCTTCTAATCAAAGAATCCAGGT

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AGCCC. The template Unc-86 cDNA clone was a gift of G. Ruvkun. Inserts were cut with *Sal*I and *Hin*dIII and inserted into the glutathione *S*-transferase (GST) fusion vector pGEX-KG, modified to include (i) an in-frame Flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) encoded by the sequence (GACTACAA GGACGACGATGACAAG) at the carboxy terminus of the GST domain and (ii) a *Sal*I cloning site. The coding portions of the expression constructs were sequenced on both strands prior to protein expression. Fusion protein expression was induced in bacterial cultures by using isopropythiogalactopyranoside, bacterial pellets were lysed by sonication, and the GST fusion proteins were isolated by glutathione-agarose affinity chromatography, using standard methods (3).

Random oligonucleotide selection. In a majority of past studies, agarose affinity chromatography or electrophoresis has been used to separate fusion protein-oligonucleotide complexes from unbound random oligonucleotides. In the current study, we improved the method of isolation of the protein-oligonucleotide complexes by the incorporation of a Flag epitope (above) between the GST and POU domains of the bacterially expressed POU proteins. Bound nucleotides were separated by immunoprecipitation using mouse monoclonal antibodies to the Flag epitope, followed by anti-mouse secondary antisera immobilized on paramagnetic beads and subsequent magnetic separation. This modification greatly reduced background oligonucleotide binding, presumably due to the inability of the oligonucleotide to bind or penetrate the impermeable polystyrene magnetic particles.

Random oligonucleotide selection was performed by using a double-stranded oligonucleotide with an internal 16-bp degenerate region containing equal proportions of all four nucleotides, flanked by 17-bp invariant sequences which included *Xho*I restriction sites. Prior to selection, the template random oligonucleotide, CCAGGCTCGAGGTCTCG N_{16}GCACGCTCGAGGAGTCC, was annealed to a 17-bp primer complementary to the 3′ invariant sequence (R2, GGACTCCTCGAGCGTGC) and filled with Klenow DNA polymerase.

For the initial round of selection, binding reaction mixtures were prepared in a final volume of 50 μl with the following components: 20 mM Tris (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mg of poly(dI-dC) per ml, 1 mg of bovine serum albumin (BSA) per ml, 10% glycerol, 1 mM dithiothreitol, and 0.2 μg of the Flag-POU fusion protein (selection rounds 3 to 6 used 0.02 μg of protein). After incubation for 30 min at room temperature, 0.8 μg of anti-Flag M2 antibody (0.4 μg for rounds 3 to 6; Kodak) was added, and the mixture was allowed to incubate for another 30 min at room temperature. After the second incubation, this binding mixture was added to approximately 10^7 Dynabeads M-280 (sheep anti-mouse immunoglobulin G [IgG]; Dynal A.S.), and the bead-binding mix was incubated with frequent mixing for 30 min at 4°C, followed by magnetic separation of the DNA-protein-antibody-bead complexes from the unbound oligonucleotides. After being quickly washed two times with a cold solution consisting of the binding buffer without dI-dC or fusion protein, the beads were resuspended in 42 μl of water and 6 μl of 10× PCR buffer. This solution was heated at 85°C for 10 min to free the selected oligonucleotide from the complex, followed by magnetic removal of the beads.

The supernatant containing the selected oligonucleotide was transferred to a PCR mixture for amplification of the selected product. The PCR mixture included 0.05 nmol of a 17-mer primer identical to the 5′ end of the random template (R1, CCAGGCTCGAGGTCTCG), an equimolar amount of R2 primer, and *Taq* DNA polymerase (Perkin-Elmer/Cetus), nucleotide triphosphates, and PCR buffer as recommended by the manufacturer. PCR was performed for 30 cycles of 94°C for 30 s and 45°C for 30 s with a hot start, followed by addition of excess primers and fresh *Taq* polymerase and a final cycle of 94°C for 2 min, 45°C for 2 min, and 72°C for 2 min to ensure a fully complementary double-stranded oligonucleotide product. The PCR product was separated in a 4% agarose gel and after purification was used in the next round of selection.

After six rounds of selection and amplification, a 1/100 aliquot of the product was reamplified for cloning. This PCR product was gel purified and digested with *Xho*I, the product of the digest was separated on an 4% agarose gel, and the cut oligonucleotide was purified and ligated into pBKS digested with *Sal*I. Following the ligation, the vector was recut with *Xho*I and religated to ensure single inserts, and the ligation product was used to transform competent *Escherichia coli* (XL1-blue; Stratagene).

Affinity screening of selected sites. Bacterial colonies from the transformation of the ligated selection product were used to prepare plasmid minipreps. Plasmids were checked for single inserts by restriction digest at flanking sites and analysis on 3% agarose gels. To produce insert DNA fragments for competition electrophoretic mobility shift assays (EMSAs), the plasmid samples were used as templates for PCR amplification using primers corresponding to the pBKS vector sequence flanking the cloning site. One of the primers was 5′ end labeled with [^{32}P]ATP and polynucleotide kinase at a low specific activity (~10^4 cpm/pmol) to allow the molar concentration of the purified PCR product to be easily measured. The PCR products were gel purified and used to compete against binding of a ^{32}P-labeled CRH oligonucleotide (~2×10^6 cpm/pmol) to the POU protein used in the selection process. These EMSAs used 0.125 pmol of the selected sequence to compete with 0.025 pmol of a 5′-end-labeled CRH oligonucleotide. Each competition EMSA binding reaction consisted of a 20-μl total volume containing 20 mM Tris (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 100 μg of poly(dI-dC) per ml, 100 μg of BSA per ml, 10% glycerol, and 1 mM dithiothreitol (EMSA cocktail). The POU domain protein was added to the cocktail after the competitor and labeled CRH oligonucleotide and allowed to incubate for 30 min. The concentration of the radiolabeled CRH oligonucleotide in the competition assays was at least 10-fold in excess of the concentration of POU protein. Immediately following the incubation, the bound complexes were separated from free probe by electrophoresis in 6% nondenaturing acrylamide gels and visualized by autoradiography. Clones that competed similarly or better than the same concentration of unlabeled CRH oligonucleotide were sequenced and used to derive the consensus sites.

Comparison of the affinity of the optimal Brn-3.0 binding site to a series of variant sites (sites 2 to 12) by competition EMSA was done under the conditions described above, with a range of concentrations of unlabeled variant oligonucleotide competing with 2.5×10^{-9} M ^{32}P-labeled site 1 oligonucleotide. The concentrations of the competitor oligonucleotides used in these assays varied from 2.5×10^{-9} to 2.5×10^{-6} M and were selected for each site to include points near half-inhibition of site 1 binding. Following electrophoresis, the bound complexes were localized by autoradiography and cut from the dried gel for scintillation counting. The radioactivity contained in the gel slices was measured in a scintillation counter and compared to a standard curve generated by using an unlabeled site 1 competitor (self-competition).

Determination of equilibrium dissociation and rate constants. EMSAs used to determine the equilibrium binding of Brn-3.0 to site 1 and the dissociation and association rate constants for the Brn-3.0–site 1 complex were conducted in the EMSA cocktail described above except that poly(dI-dC) was omitted from the reaction mixture and 200 μg of BSA per ml was used. For these assays, electrophoresis was conducted in 10% polyacrylamide gels. Each method used Brn-3.0–POU domain fusion protein and site 1 oligonucleotide labeled with ^{32}P to a specific activity of 2×10^6 to 5×10^6 cpm/pmol. The amount of active Brn-3.0 protein used in these assays was estimated by counting of the shifted band in the presence of a saturating concentration of site 1 oligonucleotide.

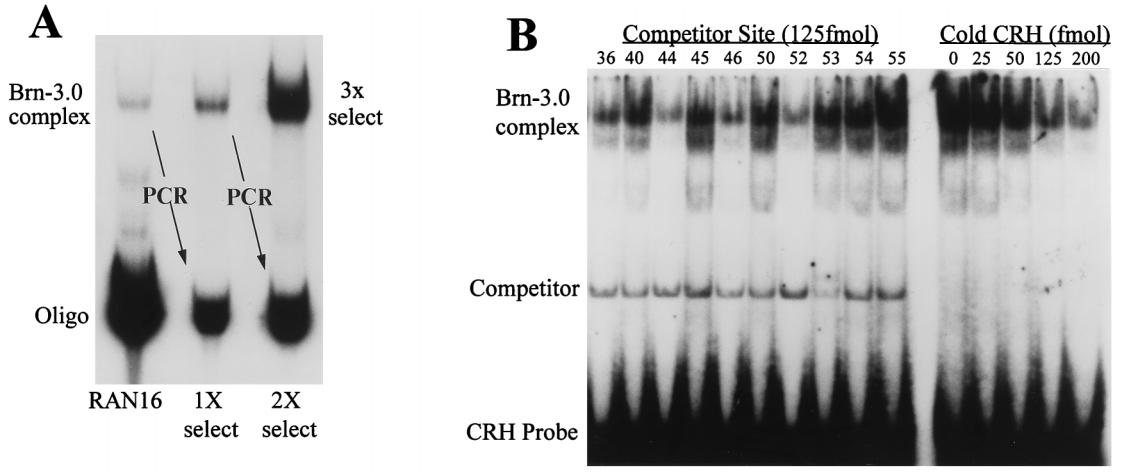
For determination of the Brn-3.0–site 1 equilibrium dissociation constant (K_D), various labeled oligonucleotide concentrations (5×10^{-13} to 10^{-10} M) were mixed with a fixed amount of POU protein (10^{-11} M) in the modified EMSA cocktail. After a 1-h incubation, free and bound complexes were separated electrophoretically, the gel was dried, and the oligonucleotide complexes were quantitated in a Bio-Rad phosphorimager. In all phosphorimager assays, the zero or background values for the Brn-3.0 complexes were determined by the integration of an area equal to the band area from a gel lane containing free oligonucleotide probe only. The bound DNA/free DNA ratio was plotted against the concentration of bound DNA, and the K_D was determined from the slope of a least-squares fit of these data (slope = $-1/K_D$).

For the determination of the association rate constant (k_a), Brn-3.0 POU protein (2.5×10^{-11} M) was added to labeled site 1 oligonucleotide (2.5×10^{-12} M) in the modified EMSA cocktail. At time points from 0 to 3 min after mixing, the reaction was quenched by addition of a 400-fold excess of unlabeled oligonucleotide and directly loaded onto a running 10% nondenaturing polyacrylamide gel. After the gel was dried, the shifted complex was quantified on a Bio-Rad phosphorimager. Using the method of Fried and Crothers (8, 23), we plotted the experimental values for $1/([P]_0 - [D]_0)\ \ln\{[D]_0([P]_0 - [PD])/[P]_0([D]_0 - [PD])\}$ as a function of time, where $[P]_0$ and $[D]_0$ are the concentrations of free protein and free labeled DNA at time 0 and $[PD]$ is the concentration of the shifted protein-DNA complex at time t. The slope of this linear plot, determined from a least-squares fit, yielded the k_a value.

To determine the dissociation rate constant (k_d) of the Brn-3.0–site 1 complex, Brn-3.0 protein (5×10^{-11} M) was added to site 1 oligonucleotide (1.25×10^{-9} M) in the modified EMSA cocktail and allowed to incubate at room temperature for 5 min and then at 4°C for 1 h. At indicated time points (0 to 3 h) prior to electrophoresis, a 100-fold molar excess of unlabeled site 1 oligonucleotide was added, and the samples were incubated at 20°C until the start of electrophoresis in 10% acrylamide gels. The complex remaining at various time points was measured by phosphorimaging and plotted semilogarithmically versus time. The dissociation rate constant was calculated from a least-squares fit of the equation: $-k_d t = \ln[PD]/[PD]_0$, where $[PD]_0$ represents the amount of shifted complex present immediately after addition of unlabeled DNA.

RESULTS

Determination of the optimal Brn-3.0 binding site. A number of prior studies have determined the DNA recognition sites of various classes of transcription factors by selection from a pool of oligonucleotides containing random internal sequences. Typically, several rounds of selection and amplification of the selected sequences by PCR are followed by the sequencing of plasmid clones containing individual selected oligonucleotides. Figure 1A illustrates the application of this method to the enrichment of an oligonucleotide pool for specific Brn-3.0 recognition sequences through the initial three rounds of selection and amplification. Following six to eight rounds of selection and amplification, the oligonucleotides which bound to Brn-3.0 were ligated into the cloning vector



C

```
                 11
          12345678901
P22    gtgcACCCATTAATAATGTAcgag
P23        gtgcATAATAAATGAGTTGGcgag
P25      ctcgTATAAATAATTGATGGgcac
P26     gtgcTGCATAATTTATGGGCcgag
P27       cgtgCATAATTAATCACCCCcgag
P28        gtgcATAATTAATTAGTTCCcgag
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P34        gtgcATAATTAATTAGCTGT
P36         tgcATAATTTATGAGCCCCcgag
P37        gtgcATATTTAATTACGTCCcgag
P38       gcCCCATTAATAATTCCCcgag
P43        cgGCATTAATTATTCATGgcac
P51        gtgcATAATTAATGAGTGCCcgag
P52      ctcgGCATAAATTATTAATGGgcac
P53      ctcgCCATTAATAATTTATGGgcac
P54    gtgcACTCATCAATTATTTAcgag
P55        gtgcATAATTTATGACCCCCcgag
Consensus    gcATAATTAAT
                T A T
```

D

	1	2	3	4	5	6	7	8	9	10	11
G	15	0	0	0	0	0	0	0	0	0	0
A	1	0	18	0	13	17	5	1	12	18	0
T	1	1	0	18	4	1	13	17	6	0	18
C	2	17	0	0	1	0	0	0	0	0	0
	g	c	A	T	A/T	A	T/A	T	A/T	A	T

E

```
                 11
          12345678901
L11    ctcgGGCCGGCATCAATTATgcac
L21       gtgcAGCGATGAATTATTTAcgag
L24       gtgcCCACATCAATTATGTAcgag
L27    ctcgGGGCGCCATCAATAATGCATGTgcac
L29          gtgcATAATTAATGTTCCCCcgag
L35       cgtgctgcATAATTTATGGCGTCCcgag
L36    ctcgGGGGGACATCAATTATgcac
L40          gtgcATAATTAATCCCGCCCcgag
L44          gtgcATAATTAATCGTTTGCcgag
L46    ctcgGCAGCACATCAATTATgcac
L52    ctcgGGCACACATCAATTATgcac
Consensus       gcATNATTTAT
                   A A
```

FIG. 1. Oligonucleotide selection by Brn-3.0 POU and full-coding proteins. (A) A pool of oligonucleotides containing an internal 16-bp random sequence (RAN16) was enriched for Brn-3.0 recognition sites through three rounds of selection with GST–Brn-3.0 POU domain fusion protein. (B) Cloned oligonucleotide binding sites selected by a GST–Brn-3.0 full-coding fusion protein were individually screened for affinity. Each assay was performed with GST–Brn-3.0 full-coding fusion protein with 0.025 pmol of radiolabeled CRH site plus 0.125 pmol of PCR-generated competitor site or the stated concentration of unlabeled CRH oligonucleotide. Free competitor DNA is labeled at low specific activity for purposes of quantitation and appears as a faint band. Only cloned sites which competed as well as or better than an equimolar concentration of CRH oligonucleotide were used to derive a consensus sequence. (C and D) Results of six rounds of selection and subsequent affinity screening for a GST–Brn-3.0 POU domain fusion protein. The initial GC residues of the consensus site are shown in lowercase to indicate that they were often contributed by the flanking invariant sequence. (E) Oligonucleotide sequences selected by Brn-3.0 full-coding protein under conditions slightly less stringent screening than those used for the POU domain fusion protein.

pBKSII, and the individual selected oligonucleotides were characterized.

In most prior applications of this technique, all of the sequences selected after an arbitrary number of selection cycles have been aligned to derive a consensus binding site. In the present study, to ensure that only the highest-affinity sites were used to derive a consensus, we added an intermediate step in which the cloned sequences derived from the selection process were screened individually for their binding affinities. Because one site from the rat CRH gene promoter which binds Brn-3.0 with high enough affinity to mediate transcriptional activation has previously been identified (20), this CRH site was used as a competition assay standard (Fig. 1B), and only selected sites with affinity equal to or higher than that of the CRH site were used to derive the consensus binding sequence.

The high-affinity sites derived from oligonucleotide selection by the Brn-3.0 POU domain fusion protein are shown in Fig. 1C. The Brn-3.0 POU domain selection results suggest a consensus recognition site GCATAATTAAT, with relatively interchangeable use of A or T at positions 5, 7, and 9 (Fig. 1D). Because the initial G and C were frequently contributed by the nonrandom flanking sequence, the significance of these two initial base pairs was not clearly determined by this method. Although slightly more sequence variation was observed when

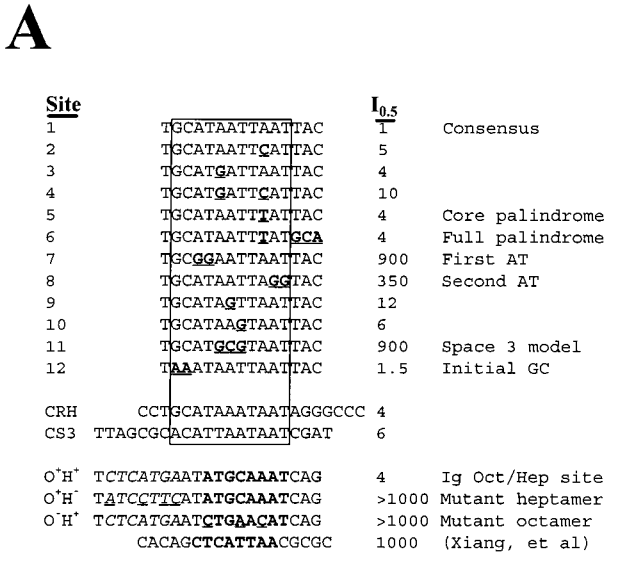
random oligonucleotide binding was performed with the full-coding Brn-3.0 protein, these selected sites exhibit the same core invariant bases as those selected by the POU domain alone (Fig. 1E).

An ATAATTAAT core sequence dictates Brn-3.0 binding. To test the significance of the consensus binding site, a series of specific variant sites were synthesized and used as competitors against the optimal recognition site (site 1) in EMSAs. The sequence of the variant oligonucleotides and the relative concentrations of these competitors which half-inhibited Brn-3.0 binding to site 1 are shown in Fig. 2A. The variant sites can be divided into two affinity classes, those which showed binding affinity reduced 10-fold or less relative to the consensus site, and those which exhibited Brn-3.0 binding affinity reduced 1,000-fold or more. Alteration of the initial (positions 3 and 4) and final (positions 10 and 11) AT elements to GC residues (sites 7 and 8) dramatically decreased binding. In contrast, alteration of the initial GC residues to AA (site 12) had little effect on affinity. Changing individual residues at positions 5, 7, and 9 to G or C had slight effect on affinity (sites 2, 3, and 10), and changing two of these positions simultaneously (site 4) had an additional inhibitory effect on binding. In accordance with the invariant positions observed in the selected oligonucleotides, altering position 6 had a somewhat greater effect than the other single base changes (site 9). Although the central AAT of the consensus sequence (positions 5 to 7) has been described as a variable 3-bp space region in the CRH site in one prior study (15), changing this sequence to GCG (site 11) drastically reduced binding.

These site-specific mutations, combined with the range of oligonucleotides observed in random selection, confirm a core Brn-3.0 consensus binding sequence of AT(A/T)A(T/A)T(A/T)AT, with a slight preference for a GC sequence preceding the initial AT. A greater tolerance for deviations from the consensus sequence at positions 5, 7, and 9 is indicated both by the occurrence of A or T at these positions in the randomly selected sites and by the relatively small decrease in affinity caused by specific mutations to G or C at these sites. This finding is consistent with the high-affinity binding previously observed for the CRH site, which is encompassed by this consensus. Brn-3.0 also bound with moderate to high affinity to similar sites, CS2 and CS3, derived from the promoter of the *C. elegans mec-3* gene, which have been previously identified as binding sites for Unc-86 protein (25). This finding suggests that DNA recognition properties are highly conserved between invertebrate and vertebrate members of the POU-IV class, a hypothesis explored in detail below. In contrast, Brn-3.0 bound very poorly to a minimal octamer element (O^+H^- [Fig. 2A]) or to an Oct-TAATGARAT site (data not shown), clearly distinguishing the Brn-3.0 recognition element from sites described for Oct-1 (23). Brn-3.0 affinity for the octamer site was increased significantly, however, when an adjacent heptamer site was included (O^+H^+). The consensus binding site for Brn-3.2 (Brn-3b) previously derived by oligonucleotide selection (27), which contains a core CTCATTAA sequence, bound the Brn-3.0 POU domain with at least 1,000-fold-lower affinity than the consensus binding site derived in this study.

The affinity of the Brn-3.0 POU domain for its optimum site was calculated from equilibrium binding data by Scatchard analysis and from the rates of association and dissociation of the Brn-3.0–site 1 complex. Equilibrium binding was determined with 5×10^{-13} to 10^{-10} M ^{32}P-labeled site 1 oligonucleotide incubated with 10^{-11} M Brn-3.0 protein. After 1 h, the free oligonucleotide was separated from the Brn-3.0–site 1 complex by electrophoresis. As shown in Fig. 3A, the amounts of bound and free oligonucleotide were quantified (Materials and Methods), and the bound DNA/free DNA ratio was plotted against the concentration of bound DNA. The slope of the plot corresponds to $-1/K_d$ and yielded a K_d of 2×10^{-13} M.

The rates of formation and dissociation of the Brn-3.0 POU domain–site 1 complex were also determined in electro-



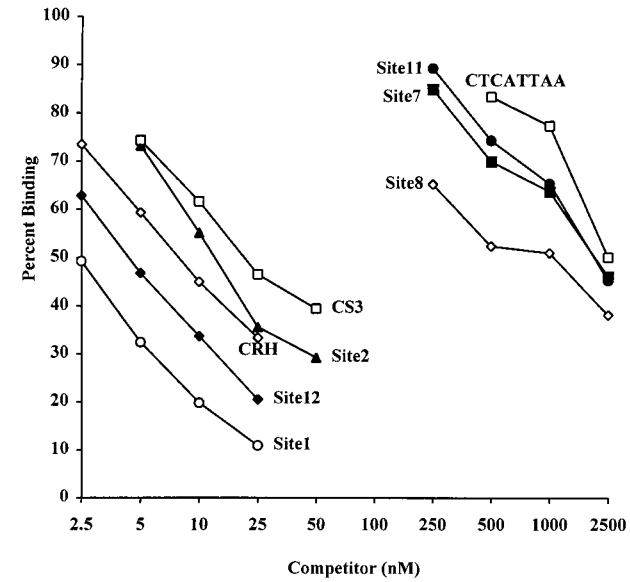


FIG. 2. Relative affinities of Brn-3.0 recognition sequences. To determine the critical features of the consensus Brn-3.0 recognition site (site 1), site 1 was compared to a series of variant oligonucleotides (sites 2 to 12) and naturally occurring POU recognition elements in competition EMSAs. (A) The concentration of competitor oligonucleotide which half-inhibited binding of site 1 to Brn-3.0 ($I_{0.5}$) is expressed as a ratio to the half-inhibitory concentration of site 1 itself; thus, higher values correspond to lower-affinity binding. (B) The full inhibition curves for selected sites. CRH, site from the rat CRH gene promoter; CTCATTAA, consensus Brn-3.2 recognition element derived in a previous study (27); O^+H^+, O^+H^-, and O^-H^+, octamer-heptamer (Oct/Hep) elements containing wild-type, mutant heptamer, and mutant octamer sequences derived from the IgG heavy-chain promoter (18). The sequence of the site 1 oligonucleotide was GATCTCTCCTGCATAATTAATTACGCCCGGATC, and in each of the variant oligonucleotides derived from this site, the underscored sequence was replaced by the sequences shown.

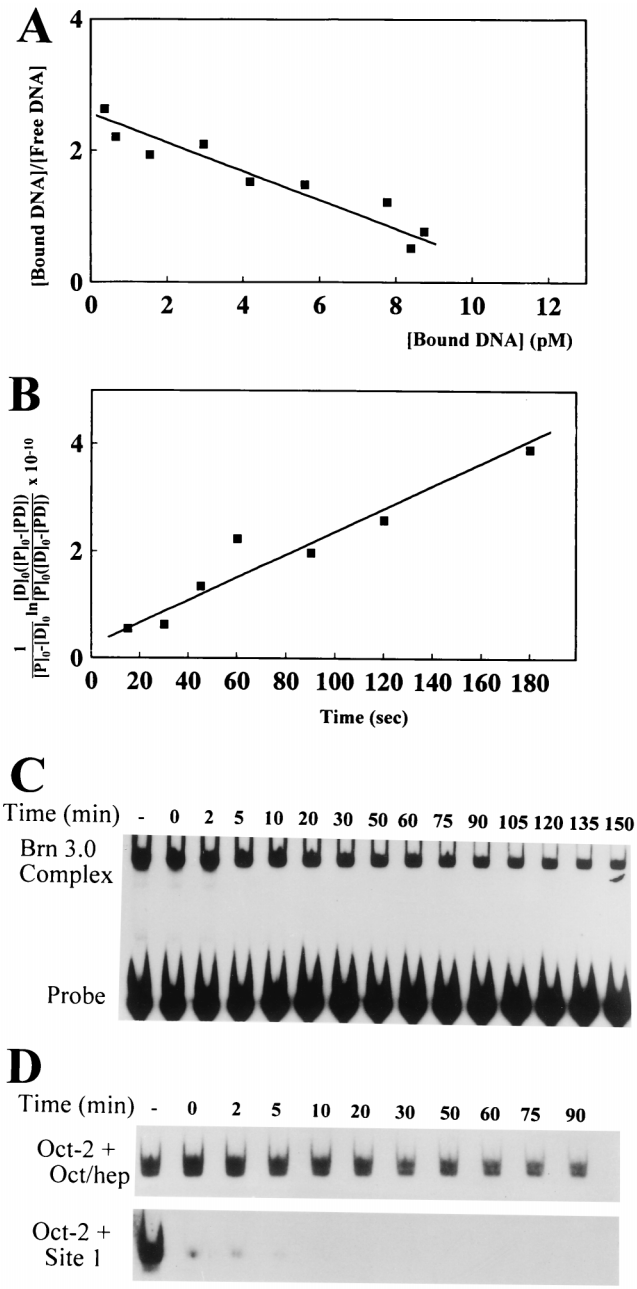


FIG. 3. Equilibrium and kinetic analysis of the Brn-3.0–site 1 complex. (A) Scatchard analysis of the interaction of Brn-3.0 POU protein and site 1 oligonucleotide at equilibrium. (B) Association kinetics of Brn-3.0 and site 1. The association rate constant was calculated from the slope of this linear plot as described in Materials and Methods. (C) To test the kinetics of dissociation of the Brn-3.0 complex with its optimal site, Brn-3.0 POU domain protein was preincubated for 1 h with ^{32}P-labeled site 1 oligonucleotide probe under modified EMSA conditions (Materials and Methods). A 100-fold molar excess of unlabeled site 1 oligonucleotide was then added to each sample mixture at the stated times prior to the start of electrophoresis, and the Brn-3.0–oligonucleotide complexes were separated from the unbound probe in a 10% polyacrylamide gel. Lane − contains no competitor oligonucleotide. (D) Dissociation of an Oct-2 POU domain complex with O^+H^+ and site 1 oligonucleotides. Oct-2 exhibits stability on its high-affinity site similar to that observed for Brn-3.0 on site 1, but the Oct-2–site 1 complex dissociates too quickly for dissociation kinetics to be measured accurately in the EMSA.

phoretic assays. For determination of the association rate constant, 2.5×10^{-11} M Brn-3.0 protein and 2.5×10^{-12} M ^{32}P-labeled site 1 oligonucleotide were mixed, and at time points from 0 to 3 min after mixing, the binding reaction was quenched by the addition of a 400-fold excess of unlabeled DNA. The protein-DNA complexes were separated electrophoretically and quantified in a phosphorimager, and the association rate constant of 2×10^8 M^{-1} s^{-1} was calculated from the time course of the reaction (Fig. 3B) as described in Materials and Methods.

The k_d of the Brn-3.0–site 1 complex was determined by preincubation of 5×10^{-11} M Brn-3.0 POU protein and 1.25×10^{-9} M ^{32}P-labeled site 1 oligonucleotide for a minimum of 1 h. At times from 0 to 180 min prior to electrophoresis, a 100-fold molar excess of DNA was added. As shown in Fig. 3C, the Brn-3.0–site 1 complex was very stable, exhibiting a half-life of approximately 60 min. The k_d of 1.85×10^{-4} s^{-1} was calculated from a semilogarithmic plot of the remaining complex against time (Materials and Methods). The K_d calculated from the kinetic data, $K_d = k_d/k_a$, yields a value of 9×10^{-13} M, in fair agreement with the equilibrium data. The greatest error was associated with the measurement of the very rapid association kinetics, and the values derived kinetically should thus be considered estimates.

Given the high affinity of Brn-3.0 for its optimal site, and the remarkable stability of the Brn-3.0–site 1 complex, we examined for comparison purposes the stability of the related POU protein Oct-2 on its previously characterized O^+H^+ recognition site (18). The Oct-2–O^+H^+ complex (Fig. 3D, top) exhibited a k_d of 1.12×10^{-4} s^{-1}, corresponding to a half-life of approximately 100 min. This result indicates that the stability of the complex of Brn-3.0 with its optimal site is likely to be characteristic of the POU proteins as a class.

In contrast, the dissociation of an Oct-2–site 1 complex was too fast to be accurately measured by the EMSA method, with nearly complete dissociation of the complex by the time the products of the binding reaction entered the polyacrylamide gel (Fig. 3D, bottom). In spite of the instability of the Oct-2–site 1 complex, however, significant complex formation was observed in EMSAs in the absence of competitor oligonucleotide. This result illustrates that EMSAs alone do not discriminate between the stable protein-DNA complexes observed for POU proteins on their optimal sites and interactions which exhibit 100- to 1,000-fold more rapid dissociation and thus lower affinity.

Brn-3.0 recognizes a highly symmetrical site. The POU protein Oct-1 has been demonstrated to interact with an asymmetrical site in a preferred orientation with respect to the POU-specific domain and POU-homeodomain (13, 14, 23). However, examination of the core consensus binding sequence for the Brn-3.0 POU domain and the individual clones obtained by oligonucleotide selection suggests that the Brn-3.0 recognition site may be nearly symmetrical or palindromic. The core consensus binding site contains the sequence AT(A/T)A(T/A)T(A/T)AT, and the reverse complement of this site, AT(T/A)A(A/T)T(T/A)AT, still yields an acceptable recognition element, particularly considering that the differences between the core site and its complement occur at positions 5, 7, and 9, where changes have the least effect on binding (Fig. 1D and 2A). Symmetrical sites sometimes indicate DNA recognition by dimeric proteins, but Brn-3.0 dimer formation was not observed on the consensus binding element.

To test the effect of site symmetry on Brn-3.0 binding, we generated two variants of the consensus binding oligonucleotide (sites 5 and 6 [Fig. 2A]) which include nearly palindromic 9- and 13-bp recognition sequences. These sites cannot be fully

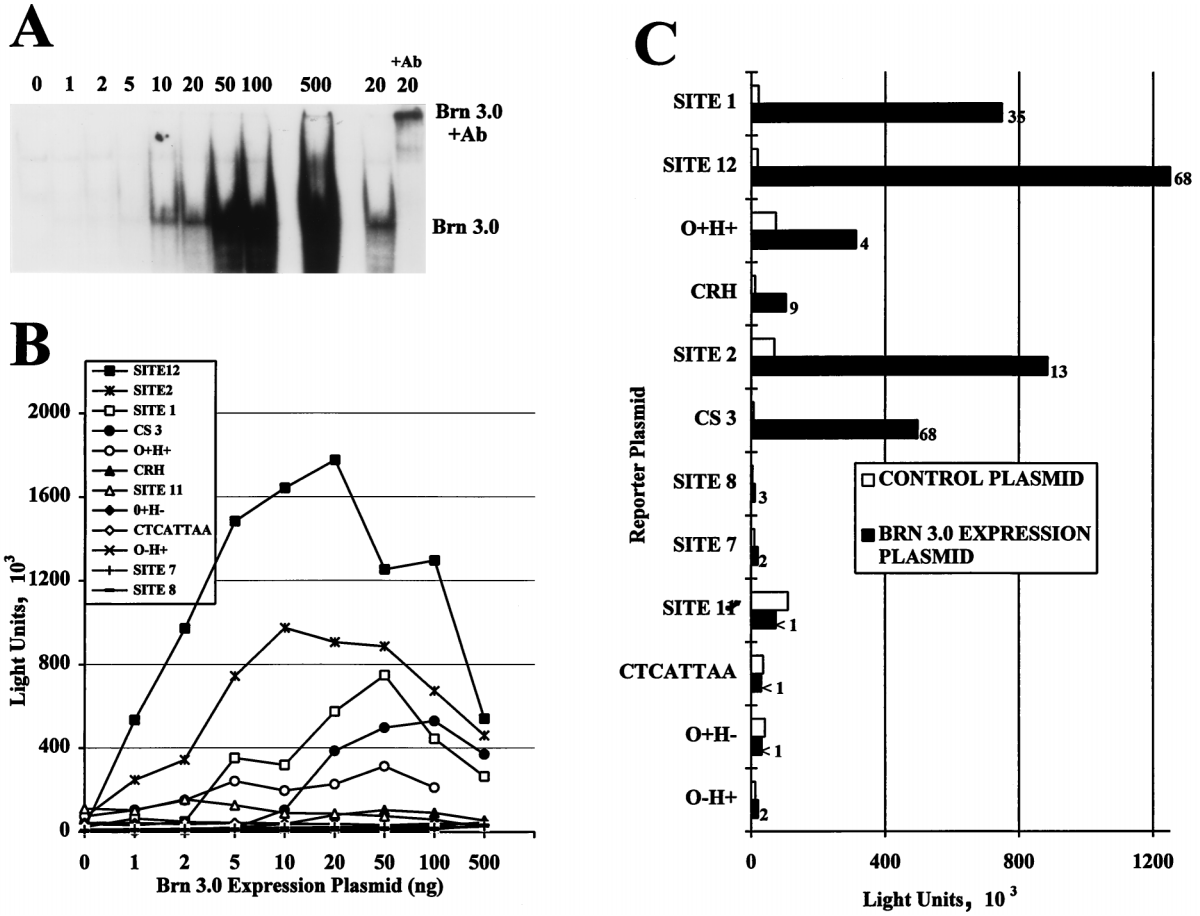


FIG. 4. High-affinity Brn-3.0 recognition sites mediate transcriptional activation. Transfection assays were performed in CV-1 cells as described in Materials and Methods. Full-coding Brn-3.0 protein was expressed in the vector pcDNA1/amp. The cotransfected reporter constructs contained three copies of the specified recognition sequence linked to a prolactin minimal promoter (20) in the luciferase reporter vector plasmid pGL-2 (Promega). (A) Expression of Brn-3.0 protein was assayed by EMSA for various amounts of transfected Brn-3.0 expression plasmid, and the amount (nanograms) of transfected plasmid per 3.5-cm-diameter culture dish appears above each lane. +Ab designates a Brn-3.0–site 1 complex supershifted with an affinity-purified rabbit anti-Brn-3.0 antibody (6). (B) Activity of expressed luciferase protein as a function of the amount of transfected plasmid. (C) Luciferase expression compared for each reporter construct in the presence of empty pcDNA1/amp control plasmid or 50 ng of Brn-3.0 expression plasmid. The sequences and derivations of the reporter sites appear in Fig. 2, and they are listed in descending order of in vitro binding affinity.

palindromic because they contain an odd number of bases, but they exhibit symmetry around a central A-T base pair at position 6. Affinity of the Brn-3.0 POU domain for the sites containing extended symmetry was similar to the affinity for the consensus site. The near symmetry of the Brn-3.0 recognition element suggests that the protein may bind many high-affinity sites with reversible orientation and that for many sites, identification of a POU-specific domain and POU homeodomain half-site may be somewhat arbitrary.

Transcriptional activation correlates with in vitro Brn-3.0 binding. To test the relationship between binding affinity and activation of transcription, three copies of the Brn-3.0 consensus oligonucleotide, selected mutant sites, and several naturally occurring POU recognition elements were ligated into the luciferase reporter pGL-2, adjacent to a minimal promoter derived from the prolactin gene (20). To determine the optimal conditions for transcriptional activation by Brn-3.0, cotransfection assays were performed with a wide range of expression plasmid concentrations. The amount of transfected plasmid correlated well with the amount of Brn-3.0 protein detected by EMSA in lysates of the transfected cells (Fig. 4A). As shown in Fig. 4B, maximal enhancement of luciferase expression from reporters containing the Brn-3.0 recognition elements occurred at 20 to 100 ng of transfected plasmid per 3.5-cm-diameter cell culture dish. As expected, the highest-affinity recognition elements exhibited peak luciferase expression at the lowest amount of transfected expression plasmid (10 to 20 ng). Reporters containing recognition sites with 4- to 10-fold lower affinity than site 1 showed peak luciferase activity at 50 to 100 ng of transfected plasmid, and sites with still lower affinity did not mediate detectable activation of transcription at any plasmid concentration.

Several factors, including variable rates of cell transfection and the inability to isolate neural tissues in which all neurons express Brn-3.0, make it difficult to precisely correlate the amount of functional Brn-3.0 protein in transfected cells with levels expressed in neurons which express Brn-3.0 endogenously. However, EMSAs using cellular extracts from chick embryonic retina and tectum (not shown) suggest that transfection of 10 to 20 ng expression plasmid per 3.5-cm-diameter culture dish approximates physiological Brn-3.0 levels, and transfection of greater than 100 ng of plasmid results in significant overexpression of Brn-3.0 protein.

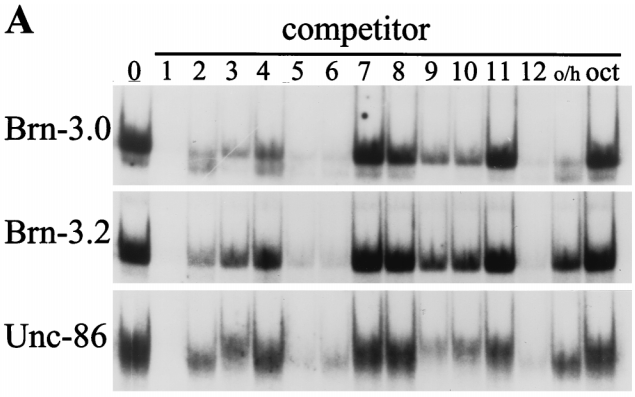
For most of the Brn-3.0 recognition sites studied, the in vitro

affinity for Brn-3.0 was highly correlated with transcriptional activation. The high-affinity elements site 1 (consensus) and site 12 mediated 35- and 68-fold increases in reporter gene expression, respectively. Several other sites which bound Brn-3.0 with slightly lower affinity also mediated significant transcriptional activation, as did the naturally occurring POU recognition elements CRH and CS3.

Three mutations of the Brn-3.0 consensus recognition sequence (sites 7, 8, and 11) which bound Brn-3.0 poorly also failed to significantly activate reporter gene expression, as did the previously reported Brn-3.2 consensus site (27) and the octamer (O^+H^-) and heptamer (O^-H^+) elements (18). The O^+H^+ site mediated a slight increase in luciferase expression but was a poorer mediator of transcriptional activation by Brn-3.0 than expected based on its in vitro affinity. This may be due in part to the much higher background levels of luciferase activity observed with the O^+H^+ reporter and to other atypical characteristics of this site, discussed below.

The DNA recognition site of POU-IV class transcription factors is highly conserved. The significant binding of Brn-3.0 to the Unc-86 recognition sites CS2 and CS3 from the *mec-3* gene regulatory region suggests that DNA recognition by the POU-IV class proteins is highly conserved. However, prior studies have attributed functional differences between POU-IV proteins to diverged amino acid residues within the POU domain (16, 27). To test the extent of the conservation of DNA binding function between POU-IV proteins from distantly related sources, and between different mammalian members of the POU-IV class, we used competition EMSAs to compare the affinities of the Brn-3.0, Brn-3.2, and Unc-86 POU domains for the Brn-3.0 consensus and mutant oligonucleotides (Fig. 5A). These competition assays demonstrate that these proteins have very similar affinities for this defined set of recognition elements. In addition, results published elsewhere (22) demonstrate that the *Drosophila* POU-IV proteins I-POU and twin-of-I-POU also share similar affinity for these sites.

To further define the recognition sites of Brn-3.2 and Unc-86, we performed random oligonucleotide selection with Unc-86 and Brn-3.2 POU domain proteins by the method used to derive the Brn-3.0 consensus sequence (Fig. 5B and C). The oligonucleotides selected by these proteins strongly resemble those selected by Brn-3.0 and converge on a nearly identical consensus sequence, including a highly symmetrical A T(A/T) A(T/A)T(A/T)AT core, and a preference for flanking GC residues. We also observed that the Unc-86 oligonucleotides exhibited a preference for a flanking GAG sequence on the end opposite the initial GC pair, which had also been observed as a trend but not as a clear consensus with Brn-3.0. These results demonstrate that the DNA binding properties of the POU-IV proteins are highly conserved and strongly suggest that any functional differences between these proteins will result from changes in properties other than DNA site recognition.



B

BRN 3.2

```
2        gtgcATAATTAATGGCTTTAcgag
3        gtgcATAATTAATGGCTTCCggag
7        gtgcATAATTAATGCCTGCCcgag
8        gtgcATAAATAATTAATGGTcgag
11       gtgcATAATTAATGGGCCCTcgag
13 gtgcATATAAATTATTAATGGCCcgag
15   gtgcTCGAATAATTAATcgag
16       gtgcATAATTAATGGTTGCCcgag
19   ctcgGGCCATCAATAATTTAgcac
20       gtgcATAATTAATTCTGCCCcgag
23       gtgcATATTTAATCGTTTCCcgag
31      ctcgCATAAATAATTAATGTgcac
32       gtgcATAATTAATGGACTGTcgag
36    ctcgGGCATGATTAATTTATgcac
Consensus  gcATAATTAAT
                  A
```

C

UNC-86

```
1         gtgcATAATTAATGAGTTCGcgag
3         gtgcATAATTAATGAGCTGCCgag
4         gtgcATACTTAATGAGTGctcg
5         gtgcATAATTAATGAGCCCCCgagac
10        gtgcATAATTAATTAGGCCcgag
11     ctcgTGAATAATTAATGAGCTCgctc
14     ctcgTACATAATTAATGAGTgcac
21        gtgcATATTTAATTAGGGCCcgag
27        gtgcATAGTTAATTAGCTTGcgag
29        gtgcATAATTAATGAGTGCCcgag
31        gtgcATAATTAATCAGCTGTcgag
Consensus  gcATAATTAATNAG
```

FIG. 5. Conservation of DNA recognition by the POU-IV class proteins. (A) Competition EMSAs of Brn-3.0, Brn-3.2, and Unc-86 binding to specific recognition sites. Site sequences appear in Fig. 2. Each assay was performed with 1.25×10^{-9} M ^{32}P-labeled site 1, 5.0×10^{-8} M each unlabeled competitor oligonucleotide, and equal amounts of the appropriate bacterially expressed POU protein. Loss of signal indicates effective competition and thus high-affinity binding by the competitor. For example, site 1 competitor abolishes binding to the radiolabeled site 1 by all three proteins, but the Oct site does not compete effectively. o/h, octamer-heptamer site; oct, octamer-mutant heptamer site. (B and C) Results for selection of an oligonucleotide containing an internal random 16-bp sequence with Brn-3.2 and Unc-86 POU domain proteins, as described for Brn-3.0. The initial G and C residues of the consensus sequences are shown in lowercase to indicate that they were frequently contributed by the invariant flanking sequence.

DISCUSSION

In this study, we have identified a consensus binding site for the POU-IV class of proteins by selection from a pool of random oligonucleotides and have demonstrated the transcriptional function and evolutionary conservation of this recognition element. The consensus site derived here strongly resembles the naturally occurring DNA recognition elements identified for this POU subclass which have been previously demonstrated to mediate transcriptional activation. These include the CRH-II site from the rat CRH gene promoter for Brn-3.0 and Brn-3.2 (10, 15, 20) and the closely related CS2 and CS3 binding sites for Unc-86 identified in the *mec-3* gene promoter (24, 25). In contrast, the consensus site derived in this study differs markedly from a random oligonucleotide-derived site previously reported for BRN-3.2, CTCATTAA

(27). However, we note that a significant number of the individual selected oligonucleotide sequences reported in the previous study and used to derive the CTCATTAA consensus are closely related to the core consensus derived here, suggesting that the primary data obtained in both studies have considerable overlap. The derivation of a higher-affinity consensus sequence in the present study probably results from screening the selected sites individually against a known standard (the CRH site) by competition EMSAs, and using only the highest-affinity class of sites to derive a consensus. In general, because the stringency of random oligonucleotide selection cannot be strictly controlled, it is not sufficient in studies of this type to perform selection for an arbitrary number of cycles and conclude that the resulting sites represent an optimal recognition sequence without determining the affinities of the individual sites.

Li et al. (15) have previously analyzed the binding of the Brn-3.0 POU domain and Brn-3.0–Brn-2 chimeras for a series of synthetic recognition sites of the form GCATnTAAT, where n is a variable unit consisting of G, GC, or GCG. These results show that among these sites, Brn-3.0 has highest affinity for n = GCG (site 11 of this study). However, even the best site used in that study has very low affinity for Brn-3.0 relative to the optimal Brn-3.0 recognition site determined here (site 1), or in comparison to the naturally occurring CS3 and CRH-II sites, and the n = GCG site cannot mediate transcriptional activation by Brn-3.0 (Fig. 4C). Thus, the consensus site derived in the present study is likely to be a better model than the previously described sites for functional Brn-3.0 recognition elements which can be expected to occur in the regulatory regions of neural genes.

Affinity of Brn-3.0 for its recognition sites in vitro is highly correlated with the ability of these sites to mediate transcriptional activation by Brn-3.0 in cotransfection assays. A partial exception to this relationship was observed for the octamer-heptamer (O^+H^+) site derived from the IgG heavy-chain promoter, which exhibited moderately high affinity for Brn-3.0 but only weak activation of transactivation. Because the O^+H^+ site deviates significantly from other high-affinity Brn-3.0 sites, it is possible that Brn-3.0 assumes a different conformation on this site, as discussed below, and that Brn-3.0 is less likely to activate transcription in this alternate conformation. However, a much simpler and more likely explanation is that the O^+H^+ site is less selective for Brn-3.0 and is occupied by other factors, including Oct-1, in vivo. This possibility is supported by the much higher background transcriptional activity of the O^+H^+ reporter constructs than of the selective Brn-3.0 reporters (e.g., site 1, site 12, and CS3) when transfected in the absence of Brn-3.0 and by the presence of abundant factors expressed in all cell lines that we have tested that bind to the O^+H^+ site in EMSAs. This finding demonstrates that both the affinity and the selectivity of a POU factor recognition site will be important to its in vivo activity.

Determination of the optimal recognition site for the Brn-3 family of POU domain factors has important implications for understanding the interaction of the POU domain with DNA. Two key features distinguish the core Brn-3.0 recognition element, ATAATTAAT, from the optimal Oct-1 site ATGC (A/T)AAT (23). First, the Brn-3.0 site lacks a central GC base pair, and second, it is extended over 9 key positions, or 11 positions if the relative preference for initial G and C residues is included. The structure of the Oct-1–DNA complex has been determined by nuclear magnetic resonance (NMR) spectroscopy and crystallography (1, 4, 13). In these structures, most of the critical contacts between Oct-1 and the major groove and phosphate backbone of DNA are made by key amino acid residues which are conserved throughout the POU domain family (Fig. 6A), suggesting that all classes of POU proteins contact DNA in similar manners. However, the clearly distinct recognition sites for Brn-3.0 and Oct-1 demonstrate that significant flexibility must be available within a generally conserved DNA binding strategy.

A comparison of the primary structures of Oct-1 and the POU-IV class factors suggests some possible ways in which these different DNA recognition properties may arise. As shown in Fig. 6A, the POU-IV class proteins are distinguished from Oct-1 by a three-amino-acid insertion at the amino terminus of helix 3 of the Brn-3.0 and Unc-86 (7) POU-specific domains. Helix 3 contributes the POU-specific domain major groove DNA contacts, and it is possible that these three additional amino acids serve to extend helix 3 and permit additional major groove contacts which lengthen the POU-IV class DNA recognition site by one base pair. The DNA recognition helices of the Oct-1 and Brn-3.0 POU-specific domains also differ by a Thr-Ser change at position 45. In the Oct-1–DNA complex, this threonine residue contacts the second and third positions of the ATGCAAAT core sequence (13). The hydroxy group of serine would be expected to allow similar hydrogen bonds, and this conservative substitution is unlikely to provide a basis for the diverged recognition sites.

A second potential explanation for the difference in DNA binding specificity between Oct-1 and the Brn-3 family proteins is a possible difference in the relative orientations of the POU homeodomain and POU-specific domain. In the Oct-1–DNA complex, the amino-to-carboxy-terminal direction of the DNA binding helices of the POU-specific domain and POU-homeodomain run in opposite orientations to one another as they lie in the major groove of the target DNA (Fig. 6B). Li et al. (15) have proposed a model for binding of the POU-III class protein Brn-2 in which the relative orientation of the POU-specific domain is reversed. Although the optimal DNA binding site for the POU-III class has not been determined, Brn-2 binds to sites of the form GCAT(N_X)TAAT (X = 0, 2, or 3 [Fig. 6B]), providing support for this model. A model in which the relative orientation of the POU-specific domain and POU-homeodomain are different than in the known Oct-1 structure is plausible because the linker region provides a flexible tether connecting these domains. Reversal of the orientation of the Brn-3.0 POU-specific domain with respect to Oct-1 is also an attractive model for the interaction of Brn-3.0 with its optimum site, given the similarity between the ideal Brn-3 site and the site proposed for Brn-2.

The concept of the reversible orientation of the POU-specific domain between POU subclasses may also be extended to provide a possible explanation for flexible site recognition by the Brn-3 class proteins. Brn-3.0 binds the O^+H^+ site with unexpectedly high affinity given the low affinity of Brn-3.0 for the heptamer and octamer elements alone and the significant divergence of the O^+H^+ sequence from the optimal Brn-3.0 site. As shown in Fig. 6B, the top strand of the preferred Brn-3.0 site read in the 5′-to-3′ direction (GCATAAT) resembles closely the lower strand of the alternate octamer-heptamer site read in the same direction (GCATATT), suggesting similar recognition sequences for the POU-specific domain in these two sites, but with reversed orientation. However, this model and any other discussion of the conformation of Brn-3.0 on its recognition sites must be considered hypothetical until data from crystallography or NMR spectroscopy are available.

In summary, the results reported here significantly advance the understanding of DNA recognition by the POU-IV class of transcription factors, which have important neurodevelopmen-

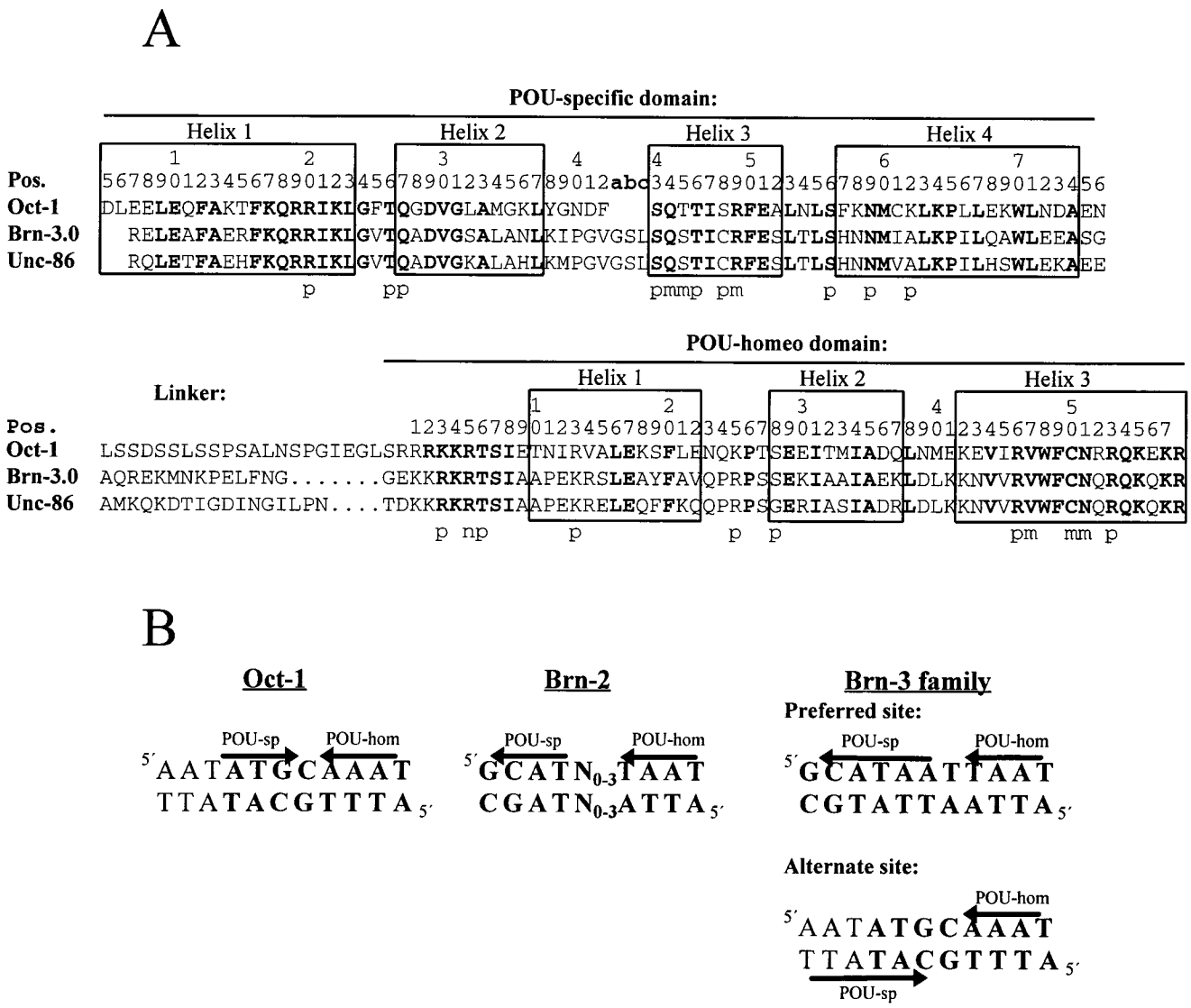


FIG. 6. Comparison of divergent POU domain structures in relationship to binding site specificity. (A) The primary structure of Oct-1 and two members of the POU-IV class. The helical domains of Oct-1 are derived from NMR and crystallographic data, while the Brn-3.0 and Unc-86 domains are proposed based on their homology to Oct-1. The **abc** between residues 42 and 43 of the POU-specific domain denotes a three-amino-acid insertion unique to the POU-IV class, adjacent to helix 3 of the POU-specific domain. Oct-1 protein–DNA contacts are designated as follows: p, phosphate backbone contact; m, major groove contact; n, minor groove contact. (B) A possible scheme for the relative orientation of the POU-specific domain (POU-SP) and POU homeodomain (POU-hom) on DNA. The Oct-1 conformation on the octamer site has been demonstrated crystallographically. The Brn-2 binding site and reversed orientation of the Brn-2 POU-specific domain have been proposed by Li et al. (15). In the proposed mechanism of Brn-3.0 DNA binding, Brn-3.0 exhibits a conformation similar to that proposed for Brn-2 on its preferred site and a conformation similar to that of Oct-1 on the alternate octamer-heptamer site. There is significant sequence similarity between the top strand of the preferred Brn-3.0 site (GCATAAT) and the bottom strand of the octamer-heptamer site (GCATATT). The arrows are intended to demonstrate amino-to-carboxy-terminal orientation of the DNA binding domains only and are not intended to show specific DNA contacts, which are not known for Brn-2 or Brn-3.0.

tal functions in organisms ranging from nematodes to mammals. Especially significant are the findings that the highly conserved DNA binding structures of the POU-IV class genes correspond to extremely similar DNA binding functions and that there are clear functional differences between the recognition elements for the different classes of POU proteins. In addition, these data show that there is a close relationship between the affinity of the Brn-3 family proteins for their recognition elements and their ability to activate transcription on these sites, thus providing a foundation for understanding the transcriptional signals encoded in the regulatory elements of neural genes.

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