Highly Cooperative Homodimerization Is a Conserved Property of Neural POU Proteins*

Jerry M. Rhee‡, Craig A. Gruber‡, Tammy B. Brodie‡, May Trieu‡, and Eric E. Turner‡§

From the ‡Department of Psychiatry and the §Program in Neuroscience, University of California, San Diego, La Jolla, California 92039-0603 and the ‡San Diego Veterans Affairs Medical Center, San Diego, California

POU-domain proteins have been shown to play important roles in the development of the nervous, endocrine, and immune systems. However, the distinctive DNA recognition properties of the six major POU subclasses have not been well defined. Here, we have used random oligonucleotide selection and competitive binding assays to determine the optimal DNA recognition elements for the POU-III and POU-VI protein classes, represented by Brn-2 and Brn-5, respectively. The optimal Brn-5 consensus binding sequence GCATAA(T/A)TTAT strongly resembles that previously determined for the POU-IV (Brn-3) class, whereas Brn-2 exhibits highest affinity for non-octamer sites of the form ATG(A/C)AT(ATA)\textsuperscript{o.o}2 ATTNAT and for octamer sites that contain a full associated heptamer sequence. Brn-2, Brn-3, and their invertebrate homologues all exhibit highly cooperative homodimerization on the Brn-2 consensus sequence, demonstrating that cooperative dimerization is a general property of these neural POU proteins. However, modified sites to which Brn-2 binds only as a monomer mediate the transcriptional effects of Brn-2 better than the consensus sequence, demonstrating that dimerization on these sites diminishes the transactivation ability of the protein. Together with the findings of our prior studies these data greatly facilitate the identification of functional POU recognition elements in the regulatory regions of neural genes.

The POU-domain transcription factors are a class of homeodomain proteins that interact with DNA via a two-part binding domain, consisting of a POU homeodomain and a POU-specific domain (1). POU proteins have been identified as developmental regulators in diverse invertebrate species, and in mammals they have important roles in the development of the immune, endocrine, and nervous systems (2). Based entirely on structural homology, the POU-domain factors have been divided into six distinct subclasses. In vertebrates, proteins of the POU-III, POU-IV, and POU-VI classes are expressed predominantly in the nervous system.

The POU-III class proteins Brn-1, Brn-2, and Brn-4 are expressed in specific hypothalamic nuclei and also in the neocortex (3), whereas SCIP/Tst-1/Oct-6 has a unique role in differentiation of myelinating glia and is also expressed in the neocortex (4). Expression of the POU-IV class proteins Brn-3.0, Brn-3.1, and Brn-3.2 is confined almost entirely to the caudal CNS\textsuperscript{3} and sensory system (5). Brn-5 (also known as emb and CNS-1) is significantly diverged from neural POU proteins of the POU-III and POU-IV classes and has been assigned to a separate structural category (POU-VI). Brn-5 is expressed widely in the developing mammalian CNS and has a somewhat lamina-specific pattern in the mature cerebral cortex (6–8). It is not currently known whether these proteins play similar regulatory roles in their respective brain regions or instead have distinct transcriptional functions conferred by different DNA binding properties between POU classes or by other unique features of the individual factors. We have previously demonstrated that within the POU-IV class, DNA recognition is highly conserved, and the nearly identical DNA binding domains of the POU-III proteins suggest that DNA recognition will also be conserved within this class.

Crystallographic data for the non-neural POU proteins Oct-1 and Pit-1 have shown quite distinct conformations of these proteins on DNA (9, 10). However, the distinct structures obtained may be partly due to the very different DNA sequences used for co-crystallization in these studies. In both of these crystal structures and in several NMR studies of POU-DNA interaction (11–13), the amino acids that form the principal DNA contacts are highly conserved throughout the POU family. This suggests that differences in specificity between POU subtypes may result from distinct spacing or orientation of the two tethered POU subdomains, rather than from distinct amino acid-DNA contacts (14, 15).

Random oligonucleotide selection has been used to show that the Oct-1 POU domain recognizes a sequence with an octamer (ATGCAAT) core, but with strong preferences for certain flanking bases (16, 17). Some progress has been made in determining the DNA recognition properties of the other POU subclasses, but among the POU proteins expressed predominantly in the nervous system, only members of the Brn-3 family have been examined in detail (18, 19). Recently we have shown that the optimal recognition sequence for the POU-IV (Brn-3) class proteins is a highly conserved non-octamer GCATAATTAT motif (19). Here, we show that, like the POU-IV class, the neural POU proteins Brn-2 and Brn-5 show high affinity for distinctive non-octamer recognition sites. On the consensus non-octamer Brn-2 binding site, invertebrate and vertebrate proteins of the POU-III and POU-IV classes exhibit highly cooperative homodimerization, demonstrating that this property is conserved among the neural POU factors. These results suggest new models for DNA binding by neural POU proteins that are consistent with the known Oct-1 and

* This work was supported in part by the Pfizer New Faculty Award, Scottish Rite Schizophrenia Research Program, Department of Veterans Affairs MERIT funding, and National Institutes of Health Awards HD33442, MH01581, and MH58447. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† A NARSAD Young Investigator. To whom correspondence should be addressed: Dept. of Psychiatry, 0603, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92039-0603. Tel.: 619-534-1568; E-mail: eturner@ucsd.edu.

‡ The abbreviations used are: CNS, central nervous system; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase.
Pit-1 structures and provide the first clear basis for the recognition of POU recognition elements in the regulatory sequences of neural genes.

EXPERIMENTAL PROCEDURES

Expression of POU-domain Proteins—DNA fragments encoding the POU-domain of murine Brn-2, Brn-3.0, and Brn-5 and -5 were generated by polymerase chain reaction from cDNA templates and ligated into a modified pGEX vector containing a FLAG epitope, as described previously (19). The oligonucleotides for Brn-2, incorporating the FLAG epitope, were GCCGCAGAATTCAGTACTACAGGACGAGCAGAGTGACAAGTGGCACAGGAGGACCCGGACCTCA and GGCAGATTTACAGTACTACAGGACGAGCAGAGTGACAAGTGGCACAGGAGGACCCGGACCTCA and the oligonucleotides for Brn-5 were GGCAGGCTGGACAGGAGGACCCGGACCTCA and GGCAGATTTACAGTACTACAGGACGAGCAGAGTGACAAGTGGCACAGGAGGACCCGGACCTCA and for Brn-3.0, the oligonucleotides were GGCAGATTTACAGTACTACAGGACGAGCAGAGTGACAAGTGGCACAGGAGGACCCGGACCTCA and GGCAGATTTACAGTACTACAGGACGAGCAGAGTGACAAGTGGCACAGGAGGACCCGGACCTCA. The resulting constructs were cloned into the expression vector pCDNA-1/amp. Cell extracts of CV-1 epithelial cells (20) after calcium phosphate-mediated transformation with cDNA constructs contained in the vector pGEX-1-amp.

Random Oligonucleotide Selection and Screening—Random oligonucleotide selection with GST-POU-domain fusion protein was performed as described previously (19), except that an oligonucleotide containing an internal 24-base pair random sequence CCAGGCTCGAGGT- was used, and eight rounds of selection were employed. The polymerase chain reaction primers for amplification of the selected sequences were CCAGGCTCGAGGTCCCTC and GGAAGCTCTCAGAGGAAGAGCTC and GGAAGCTCTCAGAGGAAGAGCTC. Following eight rounds of selection and amplification, the selected oligonucleotide pool was ligated into the vector pGEX-1-amp and transformed into bacteria, and individual colonies were selected for analysis. Inserted plasmid clones were amplified by polymerase chain reaction and assayed for their ability to compete for POU protein binding in electrophoretic mobility shift assays that were performed as described previously (19). Each screening competition assay contained 1.25 \times 10^{-9}M of the appropriate competitor DNA consisting of the polymerase chain reaction product from one of the selected clones. Consensus binding sequences were derived only from the competitor sequences, which at least half-competed POU protein binding to the octamer-octamer sites.

Determination of the Relative Affinity of DNA Recognition Sites—The relative affinity of oligonucleotides containing POU recognition sites was determined in EMSAs containing 5 \times 10^{-11} M of the appropriate POU protein, 1.25 \times 10^{-9} M of P32-labeled IgG octamer-heptamer oligonucleotide (21), sufficient POU protein to gel-shift 10–20% of the labeled DNA, and 6.25 \times 10^{-9} M of competitor DNA consisting of the polymerase chain reaction product from one of the selected clones. Consensus binding sequences were derived only from the competitor sequences, which at least half-competed POU protein binding to the octamer-octamer sites.

RESULTS

Brn-2 and Brn-5 Recognize Distinctive Non-octamer Sequences—To determine the optimal DNA recognition sequence for Brn-2, we employed random oligonucleotide selection with a GST-POU domain fusion protein, followed by affinity screening of individual selected sites. The random selection method and the importance of determining the relative affinity of the sites obtained from the random pool have been described in previous work (19). The majority of the selected sites exhibited a very similar, novel core sequence that did not contain an octamer motif but instead conformed to the consensus ATG(A/C)ATA/TG

T0,2-ATTNAT (Fig. 1). The preferred base at the N position is C; A and T were also observed at this location, but not G. If the site is considered as two nearly symmetrical half-sites (Fig. 2, box), the usual number of A-T base pairs separating the half-sites was one, but zero and two were also observed. This highly conserved 12–14-base pair motif was frequently followed by GAG, although in several cases this was contributed by the invariant flanking sequence. A minority of Brn-2 selected sites instead contained the variant octamer motif ATG(C/T)ATA/TG within an extended recognition site that was distinct from the non-octamer sites described above. Unlike the octamer element selected by Oct-1 (16), T and C were found interchangeably in the third position of the Brn-2 octamer, and T, not A, was found exclusively in the fifth position. Also, the octamer element alone was not sufficient for optimal binding. In the octamer-containing sites selected by Brn-2, the octamer motif was always preceded by AT or TT, and the sequence ATGC or GCAT was found within 2–5 base pairs of the extended octamer motif in every Brn-2 selected site.

The non-octamer Brn-2 consensus recognition site has several notable features. First, the site exhibits imperfect palindromic symmetry. Among the selected sites, the presence of a central A-T base pair usually gives the core recognition site an odd number of residues and thus prevents perfect symmetry, but some selected sites contain a complete 12-base pair palindrome (e.g. site 78). Second, the terminal part of the consensus sequence significantly resembles the heptamer motif, CT-
CATGA or TCATGAG found in the Oct-1/2 recognition sequences of IgH promoters. Third, the central core of the most frequent length variant of the Brn-2 consensus sequence, AT(T/A)ATTNAT, resembles the optimal binding site for the Brn-3 (POU-IV) protein class, which contains the core motif ATAAATTAAT (19).

To determine the optimal DNA recognition site for Brn-5, we performed oligonucleotide selection and affinity screening with Brn-5 POU-domain protein as described for Brn-2 above. The selected oligonucleotides yielded the consensus sequence GCATAA(T/A)TTAT (Fig. 2). This sequence is very similar to the consensus recognition sequence previously derived for proteins of the Brn-3 class (GCATAA(T/A)TAAT) (19), with the possible exception of a Brn-5 preference for T over A at position 9, where the Brn-3 proteins prefer A but tolerate sequence variability.

It has recently been suggested that GST fusions of DNA binding proteins may undergo GST-mediated dimerization, which for some Zn-finger transcription factors interferes with the analysis of DNA-binding specificity (22). In the present study, no evidence was found for GST-mediated dimerization of POU fusion proteins, and dimerization was only observed as a binding site-specific phenomenon. As shown in Fig. 3A, only single bands representing protein complexes with the monomeric binding site b3s1 were observed in EMSAs over a wide range of GST-Brn-2 concentrations, strongly suggesting exclusively monomeric binding. Monomeric binding is confirmed by the fact that GST-Brn-2 fusion protein (Mr = 46 × 10^3) DNA complexes migrate slightly faster than Brn-2 holoprotein (Mr = 44 × 10^3) or Brn-3 holoprotein (Mr = 43 × 10^3), and much more slowly than DNA complexes with the Brn-2 POU domain alone (Mr = 20 × 10^3). Although GST-mediated dimerization appears unlikely to be influencing DNA binding for these POU proteins, we have observed some effects of the GST moiety on site specificity. The most profound of these effects is impairment of DNA-dependent dimer formation, most likely due to conformational constraints. For these reasons, all subsequent DNA binding studies described here were performed with thrombin-cleaved GST fusion proteins, with in vitro translated POU domain proteins lacking the GST moiety, or with native POU proteins expressed by transient transfection of cultured mammalian cells.
To better understand the significance of the consensus Brn-2 recognition sequence (b2s1), we generated a series of specific variants of this site and determined the relative affinity of these variants based on the relative concentration of competitor oligonucleotide required to half-inhibit binding of Brn-2 POU domain protein to the consensus site (Joh, Fig. 3B). These results suggest that Brn-2 has considerable flexibility in its conformation on DNA. Despite the near symmetry of the consensus site, alteration of the initial AT sequence (positions 1 and 2, b2s3) reduced affinity by only slightly, whereas disruption of the terminal GAG sequence (b2s2) lowered binding 9-fold, and changing the terminal AT residues (positions 12 and 13, b2s6) reduced affinity by over 50-fold, together indicating that the two nearly symmetrical halves of the consensus sequence are not equivalent. Alteration of the core residues of the weaker half-site (b2s5) resulted in a 5-fold loss of affinity. This shows that Brn-2 may interact with a single “half-site,” but together with the oligonucleotide selection results, it also demonstrates that the full sequence is required for optimal affinity.

Among the selected random sites, we observed variation in the number of A/T pairs separating the highly conserved near-palindromic sequences. For this reason, we systematically tested placement of 0, 1, 2, and 3 T residues between the conserved hexanucleotide (b2s1, b2s7, b2s8, and b2s9). Changing site length in this way had only a modest effect on affinity.

To begin to assess differences in DNA site recognition by the POU-domain subclasses, we also examined the affinity of Brn-2 for variants of the octamer/heptamer element. The octamer/heptamer sequence used has been defined as a target of Oct-2 for variants of the octamer/heptamer element. The octamer/heptamer sequence are not equivalent. Alteration of the core residues of the weaker half-site (b2s5) resulted in a 5-fold loss of affinity. This demonstrates that the full sequence is required for optimal affinity.

As expected based on the resemblance between the central bases of the Brn-2 consensus site and the optimal Brn-3 binding site, Brn-2 had high but not optimal affinity for the Brn-3 consensus (b3s1, Fig. 3B). Brn-2 also showed suboptimal but significant affinity for a related site (b2s11), which was previously proposed as a consensus Brn-2/Brn-3 recognition element (23) but that was subsequently shown to bind Brn-3.0 poorly (19). A model for site discrimination by the POU subclasses incorporating the octamer and non-octamer binding data is presented in the discussion.

**Brn-2 Exhibits Highly Cooperative Homodimerization on Its Non-octamer Recognition Sites**—One of the most notable features of the optimal Brn-2 binding site is that it exhibits (imperfect) palindromic symmetry, consisting of an inverted repeat of the sequence ATG(A/C)AT. Repeated or symmetrical DNA elements in transcription factor binding sites are often associated with dimeric binding, and for this reason we wished to examine the stoichiometry of Brn-2 binding to its optimal site and variations of this site. Homodimerization has been reported for some POU proteins, including Pit-1 and Brn-4 on certain sites, but most known POU-DNA interactions are monomeric.

The Brn-2 POU domain dimerizes avidly on its optimal recognition site (b2s1, Fig. 4A), and examination of the stoichiometry of Brn-2 POU-domain binding to several key variants sites revealed the critical features of the consensus Brn-2 site with respect to dimerization. Alteration of the terminal GAG residues at position 14–16 of the consensus site did not impair dimer formation (b2s2, Fig. 4A). Alteration of the initial AT residues at position 1–2, however, completely eliminated dimer formation, although this change had little effect on binding affinity (Fig. 3B). The importance of the initial AT residues is confirmed by the failure of Brn-2 to dimerize on the consensus Brn-3.0 recognition site (b3s1), which lacks this initial AT sequence, although the Brn-2 POU-domain monomer also binds b3s1 with high affinity.

Alteration of site length also affected dimerization. Separation of the two near-palindromic ATG(A/C)AT elements by zero or one T residues allowed avid dimerization (b2s7 and b2s1, Fig. 4A). Separation by two T residues (b2s8) impaired dimerization, and three intervening T residues (b2s9) eliminated it. These results could be interpreted to mean that various “spacing” distances between the two nearly symmetrical halves of the consensus site, each occupied by a Brn-2 monomer, may allow or disallow dimerization. However, this model may not be correct, because GST-Brn-2 POU domain fusion protein, which binds only as a monomer, requires the extended ~16-base pair sequence in b2s1 for optimal affinity (data not shown). This strongly suggests that the two Brn-2 recognition sites in the dimeric complex must significantly overlap. Under “Discussion,” we incorporate these considerations into a model for Brn-2 dimerization.

Because the Brn-2 POU domain dimerizes avidly and the GST-POU domain fusion protein does not, it was important to assess the dimerization properties of the native Brn-2 protein, of which only about 40% consists of the POU domain. For these experiments, the full coding sequence of Brn-2 was introduced by transfection into CV-1 cells, and lysates of the transfected cells were used as a source of Brn-2 protein (see under “Experimental Procedures”). As shown in Fig. 4B, Brn-2 holoprotein exhibits avid dimerization on its consensus binding site, and its dimerization properties on several variants of this site corresponded well with the dimerization characteristics of the isolated POU domain.

The binding of Brn-2 to its non-octamer consensus site is suggestive of highly cooperative dimerization. This can be ob-
served in the titration of Brn-2 protein on its consensus site in Fig. 4, A and B, in which a significant amount of dimeric complex appears when overall site occupancy is still low. In the cooperative binding of two monomeric proteins to a DNA site, the affinity of the protein for the second site is increased by the occupancy of the first. If there is no cooperativity of binding, occupancy of the second site should occur without regard to whether the first site is occupied. When site occupancy is analyzed by EMSA, the data reveal either free DNA probe, or a monomeric or dimeric protein-DNA complex. In the monomeric complex, it is only possible to determine that exactly one site is occupied, and not which site, because monomeric occupancy of either the first or second site produces a complex with very similar migration properties. If the two monomeric complexes cannot be distinguished by electrophoresis, and the two sites are different in sequence and affinity, it is not possible to quantitate precisely the effect occupancy of one site has on the other. In some cases, variant oligonucleotides in which the first or second site has been specifically altered may allow determination of the individual affinities of the half-sites, and provide a solution to this problem. However, if the sites are overlapping in sequence, as appears to be the case in the dimeric binding of Brn-2 to its consensus site, the alteration of one site will affect the sequence of the other, and determination of the affinity of the protein for separate “half sites” cannot provide a solution.

To demonstrate conclusively the cooperativity of Brn-2 homodimerization, we designed a completely symmetrical (palindromic) DNA recognition element that was related as closely as possible to the consensus DNA sequence (b2s10, Fig. 5A). Analysis of DNA binding to this symmetrical site can be simplified by the assumption that the two monomer complexes are identical and do not need to be specifically identified in the EMSA. The overall fraction of site occupancy can be determined from the fraction of oligonucleotide in the dimeric complex, plus one-half of the fraction of the oligonucleotide in the monomeric complex. If there is no positive or negative cooperativity of

![Diagram of Brn-2 POU-domain and Brn-2 holoprotein binding sites](image-url)
binding, the ratio of the oligonucleotide that appears as free site, monomeric complex, and dimeric complex can be predicted from the overall fraction of site occupancy ($s$) according to the following equations: fraction of dimer complex $= s^2$; fraction of free site $= (1 - s)^2$; fraction of monomer complex $= 1 - $ dimer free $= 1 - s^2 - (1 - s)^2 = 2s - 2s^2$. For example, if the overall site occupancy is 10% ($s = 0.1$), and there is no cooperativity of binding, 1% of the oligonucleotide should appear in the dimeric complex, 18% in the monomeric complex, and 81% as free probe.

Fig. 5A shows titration of Brn-2 protein binding to the palindromic site b2s10 over a wide concentration range, from zero site occupancy to nearly 100%. Even at the lowest protein concentrations, which gave a detectable complex, most of the POU protein was incorporated into dimeric complexes, and the monomeric complex could only be observed on very long autoradiographic exposure times. Because the monomeric complex was too faint to be measured accurately, it was not possible to calculate precisely the “cooperativity coefficient” or the contribution of occupancy of the first site to the affinity of the second (24). However, these results conclusively demonstrate a very high degree of cooperativity and imply that occupancy of one of the two identical binding sites in the symmetrical oligonucleotide greatly increases the affinity of Brn-2 for the second otherwise identical site. In Fig. 5B, the fraction of this symmetrical oligonucleotide observed in the dimeric complex is plotted as a function of total site occupancy, and these results are compared with the predicted fractions of monomeric and dimeric site occupancy for a hypothetical noncooperative site. Similar cooperative dimerization on near-palindromic sites has previously been observed for homeodomain proteins of the paired class (24).

Cooperative Homodimerization Is a Conserved Property of Neural POU Proteins—Proteins of the Brn-3 (POU-IV) class have to date been described as interacting with DNA as monomers. However, cooperative Brn-2 dimerization requires an extended recognition site, longer than that previously used for studies of Brn-3 DNA binding. Thus, it seemed reasonable to assess the ability of Brn-3 proteins to form dimeric complexes on Brn-2 class sites, if Brn-3 factors exhibited high enough affinity for these sites to allow this test. As a preliminary to these dimerization studies, we assessed the affinity of the Brn-3.0 POU domain for various sites derived from the consensus Oct-2 and Brn-2 recognition sequences (Fig. 6A). In general, the site specificity of Brn-3.0 was significantly more restricted than Brn-2. The Brn-3.0 POU domain showed fair affinity for the consensus Brn-2 site, as expected given the resemblance of the central region of the Brn-2 site to the optimal Brn-3.0 site previously determined (19). Alteration of the initial AT sequence in the consensus Brn-2 site (b2s3) resulted in only a slight loss of binding, but other variants of the Brn-2 consensus bound Brn-3 very poorly.

Next, we assessed the stoichiometry of binding of the Brn-3.0 POU domain on the Brn-2 consensus site (b2s1). These results (Fig. 6C) demonstrate that Brn-3.0 dimerizes cooperatively on this site, with an even greater degree of dimer formation at any level of overall site occupancy than observed for Brn-2. Brn-3.0 complex formation was then examined on the completely palindromic site used to quantitate cooperative dimerization of Brn-2 (Fig. 6B), with similar results: dimerization was highly cooperative, and the presence of the monomer complex was minimal even at low levels of site occupancy.

The Brn-2 (POU-III) protein class is represented by four mammalian genes with a single Drosophila counterpart, named drifter (dfr, formerly termed cfa) (25, 18). The Brn-3 (POU-IV) protein class consists of three known vertebrate proteins, again with a single Drosophila homologue, I-POU and its splicing variant twin-of-I-POU (18). Within the POU domain, these Drosophila factors strongly resemble all of the mammalian genes of the same subclass, and the invertebrate POU proteins thus provide an opportunity to determine whether the dimerization properties of the neural POU factors are conserved. Fig. 6C demonstrates that the POU domains of dfr and tI-POU exhibit a high degree of dimerization on the consensus Brn-2 recognition site and exhibit dimerization properties that are remarkably similar to their mammalian counterparts. Thus, it is very likely that cooperative homodimerization on the appropriate class of DNA sites will be a universally conserved property of these classes of neural POU proteins.

Dimerization May Alter the Transcriptional Properties of Brn-2—To test the relationship between DNA-binding and transcriptional activation for the neural POU proteins we examined the effect of Brn-2 and Brn-3.0 on luciferase expression
from reporter constructs containing three copies of the appropriate recognition sites (Fig. 7). In these co-transfection assays, Brn-2 significantly activated luciferase expression from reporters containing the consensus Brn-3 recognition site (b3s1), as previously reported for Brn-3.0 (19). However, Brn-2 did not activate transcription from reporters containing its own consensus site (b2s1), although Brn-2 binds both sites with similar affinity.

One reason for poor activation of transcription from reporters containing the Brn-2 consensus site is that inclusion of b2s1 in the reporter construct greatly increased luciferase expression in the absence of co-transfected Brn-2 expression plasmid, presumably due to activation by endogenous transcription factors. In contrast, reporters containing the optimal Brn-3 recognition site were silent in the absence of a POU-activator, giving background transcription levels similar to the minimal prolactin promoter (prl) alone. Because Brn-2 has similar affinity for b2s1 and b3s1, it is quite possible that Brn-2 activates transcription on both of these sites, but that activation of transcription from the b2s1 reporter construct is masked by the high basal level of transcription in these cells. The endogenous activating factors are unknown, but known proteins of the POU-III and POU-IV classes are not expressed in the CV-1 epithelial cells used in the transfection assays.

The poor transcriptional activation produced by Brn-2 on its consensus site also suggests that dimerization on Brn-2 recognition sites may impair transcription. To further test the effect of dimerization on transcription we assayed the activation of luciferase expression from a reporter containing oligonucleotide b2s3, a variant of the Brn-2 site with similar affinity to b2s1, but to which Brn-2 binds as a monomer (Fig. 4B). Brn-2 produced a modest but significant increase in luciferase expression from this construct. Brn-3.0 failed to stimulate transcription on either of the Brn-2 class sites tested, but this may be due to its significantly lower affinity for these sites (Fig. 6).

FIG. 6. Highly cooperative homodimerization is a conserved property of neural POU proteins. In A, oligonucleotides related to the Brn-2 and Oct-2 optimal sites were tested for their affinity for the Brn-3.0 POU domain relative to the Brn-3.0 complex with the POU-IV class consensus binding site b3s1. The relative affinity of Brn-3.0 for o2s1 in the present study is lower than that previously reported (19). This difference is attributable to the use of the isolated POU domain in the present work and a GST-Brn-3.0 POU-domain fusion in the prior study, which somewhat reduced site selectivity. In B, titration of thrombin cleaved Brn-3.0 (expressed as 10^{-9} g of total Brn-3.0 protein) on site b2s10 demonstrates highly cooperative homodimerization of Brn-3.0 on this site. C demonstrates cooperative homodimerization of Brn-3.0 (10^{-9} g), the Drosophila POU-IV class protein t-POU (expressed as µl of programmed reticulocyte lysate), and the Drosophila POU-III class protein drifter (µl of lysate) on the Brn-2 consensus oligonucleotide b2s1.

FIG. 7. Brn-2 and Brn-3.0 activate transcription from monomeric but not dimeric recognition elements. CV-1 epithelial cells were transfected with luciferase reporter plasmids containing three copies of the stated POU-recognition element adjacent to a minimal prolactin promoter (prl) or the minimal prolactin promoter alone. In A, cells were co-transfected with a Brn-2 expression plasmid, and in B, they were co-transfected with a Brn-3.0 expression plasmid, or in both cases with pBKS control DNA. Data shown represent mean values of three assays in a single experiment. Three separate experiments gave differing total light units due to variable transfection efficiency but similar fold activation of luciferase expression with each transfected construct. For co-transfection assays, three copies of the appropriate oligonucleotide were inserted into the luciferase expression vector pGL-2 containing a minimal prolactin promoter, and POU proteins were expressed in the vector pCDNA-1/amp, both as described previously (19).
combined with the high background expression levels from these reporters. In summary, these results suggest that the cooperative dimerization seen on b2s1 impairs transactivation by Brn-2 and that the enhancement of transcription seen will also depend on the selectivity of the recognition site for the POU-III/POU-IV class proteins.

**DISCUSSION**

In this study and a related one (19), we have determined the optimal DNA recognition sequences of the three major groups of POU-domain proteins expressed primarily in the nervous system, including the POU-III (Brn-2), POU-IV (Brn-3.0, Brn-3.2, Unc-86), and POU-VI (Brn-5) classes. These studies have employed methods that represent a significant technical improvement over many prior reports, primarily because only the highest affinity sites derived from random sequence selection have been used to determine a consensus binding sequence. Our results show that the DNA binding preferences of the major POU classes can be clearly distinguished, yet exhibit similar affinity for some sequences. In some cases the results reported here differ significantly from previous assumptions about the DNA recognition properties of these factors. Our results could be clearly distinguished on several sites. Particularly, a number of characteristic variant octamer sequences were obtained from Brn-2 oligonucleotide selection that were never among Brn-3 selected sites (19) and Brn-2 retains high affinity for synthetic sites containing extended octamer or decamer sequences (ATATGCAAAT, o2s4), which Brn-3.0 binds very poorly. The relatively high affinity of POU-III class proteins for extended octamer sites accounts for the initial discovery of some of these factors, but not the Brn-3 proteins, in octamer binding assays of neural tissue extracts (26).

In contrast to Brn-2, the binding site selected by the Brn-5 POU domain was quite similar to the optimal recognition sequence of the Brn-3 class. The Brn-3 genes are expressed in the habenula, midbrain, hindbrain, and spinal cord but are excluded from the neocortex, where Brn-5 is significantly expressed (25, 27). These results suggest that Brn-5 and the Brn-3 proteins may regulate the same set of target genes in different brain regions.

Prior studies of the DNA binding properties of POU factors have shown results that are consistent with various aspects of the present study. Random oligonucleotide selection has been used to derive a binding site for Brn-4 (28). Although the authors of that study did not publish the sequences of the individual selected oligonucleotides or affinity data for the sites, the consensus sequence derived, CAATATGCTAAT, is in good agreement with the octamer-containing sites selected by Brn-2 in this study, including a preference for T instead of A in position 5 of the octamer motif and an extended AT-rich sequence preceding the octamer element. However, these authors (28) did not obtain the preponderance of non-octamer sites...
observed here. High Brn-2 affinity for the non-octamer Brn-3 consensus binding site has also been identified for a single sequence (GCATAATGAATAGT, OA25) originally identified by Oct-1 selection, but subsequently shown to have higher affinity for neural POU proteins than for Oct-1 (29, 30).

Detailed analysis of sequences in the 5'-flanking region of the Drosophila POU-III class gene dfr has revealed two autoregulatory sites with marked similarity to some of the Brn-2 selected sequences in this study (31). The regulatory site DFRE1 contains an extended sequence GCATATGATGC, which is not typical of the Brn-2 non-octamer consensus but is identical to a sequence contained in the Brn-2 selected oligonucleotide number 69 (Fig. 1). A second site, DFRE2, contains an octamer element preceded by a GCAT element in a very similar arrangement to the other Brn-2 selected octamer sites.

Another previous report examined the binding of the Brn-3.0 and Brn-2 POU domains to a variety of synthetic sites and proposed a non-octamer “space of three” site GCATGCGTAAT as a consensus recognition element for these proteins (23). We have previously shown that Brn-3.0 binds this site poorly (19). However, Brn-2 shows suboptimal but significant affinity for this sequence (b3s11, Fig. 3B), and our data agree with the overall conclusion from this previous work that Brn-2 exhibits much more flexible site recognition properties than Brn-3.0.

The Neural POU Proteins Share DNA Recognition Properties with Both Oct-1 and Pit-1—Crystallographic data for an Oct-1 monomer complex with the octamer-containing oligonucleotide GTATGCCAATAA (9) have revealed that the major DNA contacts of the POU homeodomain are centered on the AT residues at positions 7 and 8 of the octamer element and that the POU-specific DNA contacts are principally the ATGC residues at octamer positions 1–4 (Fig. 8A). In this structure, the POU homeodomain and POU-specific domain lie in the major groove on opposite sides of the DNA helix. More recently, a co-crystal structure for Pit-1 on a symmetrical non-octamer recognition element has also been published (10). In this model, the Pit-1 POU domain interacts with DNA as a dimer in an extended conformation in which the POU homeodomain and POU-specific domain reside on the same side of the DNA helix, and the recognition sites of the two Pit-1 molecules significantly overlap (Fig. 8B). In contrast, no structural data are available for the POU-III, POU-IV, or POU-VI protein classes.

The results presented here demonstrate clearly that Brn-2 and the related POU-III class proteins exhibit more flexibility in DNA recognition than either the Oct-1/2 or Brn-3 protein classes. Brn-2, like Oct-1/2, exhibits fairly high affinity for extended octamer (ATATGCCAAT) sites, and its conformation on this site can be expected to mimic Oct-1. However, Brn-2 retains high affinity for an octamer/heptamer site in which octamer positions 7–8 have been altered (Fig. 8C, box), suggesting that Brn-2 may also recognize a non-octamer sequence that overlaps the octamer and heptamer domains of this complex site and strongly resembles the Brn-2 non-octamer consensus. Optimal affinity is only obtained with the full octamer/heptamer, which can accommodate Brn-2 in both conformations, and the increment in affinity obtained with this composite site is about 10-fold.

Brn-2 also exhibits high affinity for the Brn-3 class consensus binding site. No structural data have been published for the Brn-3 class proteins, but the similarity of this site to the non-octamer site used in crystallographic studies of Pit-1 suggests that the Brn-3 proteins will assume similar extended conformations on this site (Fig. 8D).

The structural data for Oct-1 and Pit-1 suggest two possible models for the interaction of Brn-2 with its non-octamer consensus site (Fig. 8E). In one model, each Brn-2 DNA binding domain occupies a separate half-site, and the POU-specific and POU homeodomain reside on opposite sides of the DNA helix. In a second possible conformation, the Brn-2 monomers occupy overlapping sites on opposite sides of the DNA strand. A definitive answer to this question will require crystallographic studies. However, the effects of the specific alterations of the Brn-2 site shown in Fig. 3, the remarkable similarity of the Brn-2 consensus to the site used in co-crystallization studies of Pit-1 (10), and the avid dimerization of Pit-1 and Brn-2 on these sites all favor the second model.

The neural POU proteins have been shown, by analysis of naturally occurring mutations in humans and targeted deletions in mice, to have critical roles in development of the hypothalamus, retina, sensory ganglia, auditory system, and the myelination of peripheral axons (2). However, the specific regulatory targets that account for these profound effects are not known. In this study and in our prior studies of the DNA recognition properties of POU proteins (18, 19), several consistent principles have emerged: 1) the major classes of POU proteins, including the POU-II (Oct-1/2), POU-III (Brn-1, Brn-2, Brn-4, SCIP/Tst-1/Oct-6, and driffer), POU-IV (Brn-3.x, L-POU, and Unc-66), and POU-VI (Brn-5) factors have clearly distinguishable functional DNA binding properties, although they may exhibit similar affinity for certain complex sites. 2) Within each subclass, the DNA binding properties of the POU domains of the invertebrate and the multiple vertebrate genes are indistinguishable. 3) The proteins exhibit optimal affinity for DNA sites that are surprisingly long and highly specific and that can accommodate POU binding in multiple conformations. 4) The POU-III and POU-IV class proteins, as well as Pit-1, exhibit highly cooperative homodimerization on appropriate symmetrical sites but may also bind and function as monomers. 5) There is a strong correlation between binding affinity and transcriptional activation, at least for monomeric binding sites. Together, these findings form the necessary basis for recognizing functional targets of the POU-domain factors in the regulatory regions of neural genes.

Acknowledgments—We thank B. Andersen, S. Rhodes, and M. Wegner for useful DNA constructs.

REFERENCES

1. Herr, W., and Cleary, M. A. (1995) Genes Dev. 9, 1679–1693
2. Ryan, A. K., and Rosenfeld, M. G. (1993) Genes and Dev. 11, 1207–1225
3. Alvarez-Bolado, G., Rosenfeld, M. G., and Swanson, L. W. (1995) J. Comp. Neurol. 355, 237–295
4. Franz, G. D., Bohner, A. P., Aker, R. M., and McConnell, S. K. (1994) J. Neurosci. 14, 472–485
5. Fedtsova, N., and Turner, E. E. (1995) Mech. Dev. 53, 291–304
6. Andersen, B., Schmemann, M. D., Pearse, R. V., II, Jenne, K., Sugarman, J., Rosenfeld, M. G. (1993) J. Biol. Chem. 268, 23390–23398
7. Okamoto, K., Waka, M., Noji, S., Koyama, E., Taniguchi, S., Takeuchi, R., Copeland, N. L., Gilbert, D. J., Jenne, K. A., Muramatsu, M., and Hamada, H. (1993) J. Biol. Chem. 268, 7449–7457
8. Bullet, R. F., Cui, H., Wang, J., and Lin, X. (1994) J. Neurosci. 14, 1584–95
9. Klemm, J. D., Roud, M. A., Aurora, R., Herr, W., and Pabo, C. O. (1994) Cell 77, 21–32
10. Jacobsen, E. M., Li, P., Leon-del-Sol, A., Rosenfeld, M. G., and Aggarwal, A. (1997) Genes Dev. 11, 198–212
11. Dekker, N., Cox, M., Boelsens, R., Verrijzer, C. P., van der Vliet, P. C., and Kaptein, R. (1993) EMBO J. 12, 3057–3065
12. Assa-Munt, N., Mortishire-Smith, R. J., Aurora, R., Herr, W., and Pabo, C. O. (1994) Cell 77, 21–32
13. Fedtsova, N., and Turner, E. E. (1995) Mech. Dev. 53, 291–304
14. Andersen, B., Schmemann, M. D., Pearse, R. V., II, Jenne, K., Sugarman, J., Rosenfeld, M. G. (1993) J. Biol. Chem. 268, 23390–23398
15. Okamoto, K., Waka, M., Noji, S., Koyama, E., Taniguchi, S., Takeuchi, R., Copeland, N. L., Gilbert, D. J., Jenne, K. A., Muramatsu, M., and Hamada, H. (1993) J. Biol. Chem. 268, 7449–7457
16. Bullet, R. F., Cui, H., Wang, J., and Lin, X. (1994) J. Neurosci. 14, 1584–95
17. Klemm, J. D., Roud, M. A., Aurora, R., Herr, W., and Pabo, C. O. (1994) Cell 77, 21–32
18. Turner, E. E. (1996) Proc. Nat. Acad. Sci. U. S. A. 93, 15997–15101
19. Gruber, C. A., Klee, J. M., Gleiberman, A., and Turner, E. E. (1997) Mol. Cell Biol. 17, 2391–2400
20. Scholer, H. R., Hatzopoulos, A. K., Balling, R., Suzuki, N., and Gruss, P. (1989) EMBO J. 8, 2543–2550
21. Poellinger, L., and Roeder, R. G. (1989) Mol. Cell Biol. 9, 747–756
22. Lencz, D., Nikolaev, L., and Felsenhok, B. (1997) J. Biol. Chem. 272.
23. Li, P., He, X., Guerrero, M. R., Mok, M., Aggarwal, A., and Rosenfeld, M. G. (1993) Genes Dev. 7, 2483–2496
24. Wilson, D., Sheng, G., Lecuit, T., Dostatni, N., and Desplan, C. (1993) Genes Dev. 7, 2120–2134
25. Anderson, M. G., Perkins, G. L., Chittick, P., Shrigley, R. J., and Johnson, W. A. (1995) Genes Dev. 9, 123–137
26. Suzuki, N., Rohdeowld, H., Neuman, T., Gruss, P., and Scholer, H. (1990) EMBO J. 9, 3723–3732
27. Turner, E. E., Jenne, K., and Rosenfeld, M. G. (1994) Neuron 12, 205–218
28. Malik, K. F., Kim, J., Hartman, A. L., Kim, P., and Young, W. S. (1996) Brain Res. Mol. Brain Res. 38, 209–221
29. Bendall, A. J., Sturm, R. A., Danay, P. A. C., and Molloy, P. L. (1993) Eur. J. Biochem. 217, 799–811
30. Thomson, A. F., Murphy, K., Baker, E., Sutherland, G. R., Parsons, P. G., and Sturm, R. A. (1995) Oncogene 11, 691–700
31. Certel, K., Anderson, M. G., Shrigley, R. J., Johnson, W. A. (1996) Mol. Cell. Bio. 16, 1813–1823
Highly Cooperative Homodimerization Is a Conserved Property of Neural POU Proteins

Jerry M. Rhee, Craig A. Gruber, Tammy B. Brodie, May Trieu and Eric E. Turner

J. Biol. Chem. 1998, 273:34196-34205.
doi: 10.1074/jbc.273.51.34196

Access the most updated version of this article at http://www.jbc.org/content/273/51/34196

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 17 of which can be accessed free at http://www.jbc.org/content/273/51/34196.full.html#ref-list-1