Brn-5 Is a Divergent POU Domain Factor Highly Expressed in Layer IV of the Neocortex*

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We have identified rat cDNA clones that encode a POU domain protein, referred to as brain-5 (Brn-5). During embryogenesis in the rat, Brn-5 is widely expressed, with highest levels in the developing brain and spinal cord from embryonic day 12.5. In the adult, Brn-5 mRNA is most abundant in the brain, where it is diffusely expressed with the exception of an enrichment in layer IV of the neocortex. However, Brn-5 is also found in multiple adult tissues outside the central nervous system, including kidney, lung, heart, adrenal, skin, testis, and anterior pituitary. This expression pattern contrasts with that of most other POU domain genes that are expressed predominantly in the developing nervous system and are progressively restricted to discrete regions of the brain. The predicted amino acid sequence of Brn-5 is considerably divergent from previously described POU domains and thus defines a new POU domain subclass (class VI). Consistent with its divergent sequence, the DNA-binding characteristics of Brn-5 overlap with, but are clearly distinct from, that of Oct-2. Although Brn-5 can bind to non-octamer sites, a random site selection indicates that its preferred binding site contains a variant octamer core motif. Finally, we show that the amino terminus of Brn-5 contains a transactivation domain.

Mammalian development is regulated by the temporal- and spatial-specific activity of factors that stimulate or suppress the transcription of developmentally important genes. Among the best defined developmental regulators are the homeotic genes which encode transcription factors characterized by a 60-amino acid long structural motif referred to as the homeobox (Scott and Carroll, 1987; Levine and Hoey, 1988). This domain resembles the helix-turn-helix motif of bacterial DNA-binding proteins and is responsible for the DNA-binding activity of these regulators (Gehring, 1987). The importance of homeodomain-containing proteins in development was initially revealed by homeotic mutations in Drosophila (McGinnis et al., 1984; Scott and Weiner, 1984). Subsequently, homologues with important developmental functions were described in several other species, including mammals (for review see Holland and Hogan (1988) and Kessel and Gruss (1990)).

A specific subclass of homeodomain proteins was identified by the cloning of factors that activate specific transcription units; the octamer-binding proteins Oct-1 (Sturm et al., 1988) and Oct-2 (Clerc et al., 1988; Ko et al., 1988; Müller et al., 1988; Scheidereit et al., 1988) and the pituitary transcription factor Pit-1 (Bodner et al., 1988; Ingraham et al., 1988). Comparison of these factors with the nematode developmental regulatory gene unc-86 (Finney et al., 1988) revealed a conserved 69- to 72-amino acid long domain on the NH2-terminal side of their homeodomain. This novel domain, referred to as the POU-specific domain, is required in conjunction with the POU homeodomain for high affinity interactions with most DNA-binding sites. The POU-specific domain is joined to the POU homeodomain by a 14- to 25-amino acid variable linker sequence, and together they are referred to as the POU domain (Herr et al., 1988). The four initially described members of the POU domain family are thought to exert critical roles in gene regulation and metazoan development (Fletcher et al., 1987; Bodner et al., 1988; Ingraham et al., 1988; Müller et al., 1988; Scheidereit et al., 1988; Tanaka et al., 1988; Finney and Ruvkun, 1990; Li et al., 1990; Corcoran et al., 1993). In addition, Oct-1 and several other POU domain factors are capable of regulating viral DNA replication in vitro (Verrijzer et al., 1990a, 1992a), and Pit-1 appears to regulate pituitary cell proliferation (Li et al., 1990).

Since the molecular cloning of the original POU domain genes, several additional members of this family have been isolated (for review see Rosenfeld, 1991; Ruvkun and Finney, 1991; Scholer, 1991; Wegner et al., 1993). These additional POU domain proteins have permitted a classification of existing POU genes into five subclasses based on similarities in the sequence of the POU domain, including the linker region, and weaker homologies of the NH2-terminal domains (He et al., 1989; Hara et al., 1992). Although no unifying rule for expression pattern has emerged, examination of the developmental expression of POU domain genes in the mammalian nervous system has revealed that all known family members, except Oct-3/4 and Skn-1a/i, are expressed during development of the nervous system (He et al., 1989; for review see Rosenfeld (1991) and Treacy and Rosenfeld (1992)). Thus, transcripts of the Oct-1, Oct-2, Pit-1, Brn-1, Brn-2, Brn-3.0, Brn-4, and Tst-1 genes are detected in the developing neural tube of the rat at embryonic days 9 to 12. During early stages, expression is diffuse, and, in contrast to most classic homeodomain genes, the region of expression includes the developing midbrain and forebrain. During later embryological stages, and in the adult, expression of most of these genes becomes completely restricted to subregions of the brain (He et al., 1989, 1991; Hatzopoulos et al., 1990; Monuki et al., 1990; Stoykova et al., 1992; Le Moine...
and Young, 1992; Mathis et al., 1992; Gerrero et al., 1993).

Because many POU domain genes were initially identified using a polymerase chain reaction approach with a common set of oligonucleotides, the divergence of this family may have been underestimated. Using a modified PCR-based approach, we have cloned cDNAs encoding a new POU domain protein, Brn-5, which has a prominent neuronal expression and is characterized by a highly divergent POU domain.

**EXPERIMENTAL PROCEDURES**

PCR Cloning of POU Domains—Poly(A) RNA from rat anterior pituitary glands was used as a template for cDNA synthesis with random hexamer primers and reverse transcriptase (Superscript, Life Technologies Inc.) according to instructions from the vendor. This cDNA was used as a template in PCR reactions with degenerate primers to confirm the open reading frame. The POU-specific and the POU homeodomains are bored. The two direct repeats of 7 amino acids in the NH2 terminus are underlined. The numbers on the right refer to amino acids with the initial in-frame methionine assigned number 1.

FIG. 1. Nucleotide sequence and predicted amino acid sequence of Brn-5. The sequence shown includes in-frame stop codons 5' and 3' (designated ****) to the open reading frame. The POU-specific and the POU homeodomains are boxed. The two direct repeats of 7 amino acids in the NH2 terminus are underlined. The numbers on the right refer to amino acids with the initial in-frame methionine assigned number 1.

The abbreviations used are: PCR, polymerase chain reaction; 5' or 3', embryonic day; CMV, cytomegalovirus; SAAB assay, Selected And Amplified Binding site assay; bp, base pairs; kb, kilobase(s).

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**FIG. 2. Amino acid sequence comparison of mammalian POU domains.** Known mammalian POU domains were assigned to classes I to V and compared as previously described (Rosenfeld, 1991). Brn-5 was placed in a new class (VI). The sequences are organized from left to right, and the lower half is continuous from the top. The numbering systems for the POU-specific domain and the POU homeodomain are according to Assa-Munt et al. (1995) and Laughon (1991), respectively. Highly conserved residues are inside black and white boxes. Residues that are different in Brn-5 compared to most other POU domains are indicated with an asterisk. The four α helices in the POU-specific domain and the three predicted α helices in the POU homeodomain are indicated below. r indicates rat; h, human; and m, mouse. Other names that have been used for these mammalian POU domain factors are (for references see Wegner et al. (1993)): GHF-1 (for Pit-1); OTF-1, OBP100, NFII, and NF-A1 (for Oct-1); OTF-2 and NF-A2 (for Oct-2); Oct-11 (for Skn-la); N-Oct-3 (for Bm-2); RHS2 (for Bm-4); Oct-6 and SCIP (for Tst-1); Bm-3 (for Bm-3.0); Oct-3 and Oct-4 (for Oct-3/4); Emb (for Bm-5).

**Protein-DNA-binding Assays—**The proteins used for gel-shift analyses were in vitro translated or expressed in bacteria using a T7 promoter, as previously described (Ingraham et al., 1990). Gel mobility shift assays were done as previously described (Andersen et al., 1993). All binding sites were 32P-labeled to similar specific activity. The Brn-5 protein was purified by glutathione agarose affinity chromatography (Smith and Johnson, 1988) followed by cleavage with factor X. We observed no difference in binding specificity between proteins prepared by the three different methods. Gel mobility shift assays were done as previously described (Andersen et al., 1993). All binding sites were 32P-labeled to similar specific activity. The Brn-5 protein was purified by glutathione agarose affinity chromatography (Smith and Johnson, 1988) followed by cleavage with factor X. We observed no difference in binding specificity between proteins prepared by the three different methods. Gel mobility shift assays were done as previously described (Andersen et al., 1993). All binding sites were 32P-labeled to similar specific activity. The Brn-5 protein was purified by glutathione agarose affinity chromatography (Smith and Johnson, 1988) followed by cleavage with factor X. We observed no difference in binding specificity between proteins prepared by the three different methods. Gel mobility shift assays were done as previously described (Andersen et al., 1993). All binding sites were 32P-labeled to similar specific activity. The Brn-5 protein was purified by glutathione agarose affinity chromatography (Smith and Johnson, 1988) followed by cleavage with factor X.

**Cell Culture and Transfections—**CV-1 cells were grown and transfected with the calcium phosphate method as previously described (Andersen et al., 1993). CMV Lex A Brn-5 contains the coding region for amino acids 1 to 146 fused in-frame with the DNA-binding domain of LexA and placed downstream of the CMV enhancer/promoter as previously described (Ingraham et al., 1990). CMV LexA Pit-1, 2X LexA–36 luciferase and –36 luciferase plasmids have been described before (Ingraham et al., 1990).

**RESULTS**

**Cloning and Characterization of Brn-5 cDNAs—**Degenerate oligonucleotides representing all possible codons in two different 9-amino acid conserved regions in Oct-1, Oct-2, Pit-1, and Unc-86 were initially utilized as primers for PCR to identify novel POU domain genes (He et al., 1989). However, with the subsequent cloning of the Oct-3/4 gene (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990a), it became evident that the 5′ region on which one of the degenerate oligonucleotide primers was based, located in the POU-specific domain, was not fully conserved. This observation raised the possibility that additional POU domain genes might have escaped detection by the initial PCR approach. Therefore, we used an oligonucleotide to another highly conserved region in the middle of the POU-specific domain (5′-oligonucleotide) in combination with an oligonucleotide to the highly conserved third helix of the POU homeodomain (3′-oligonucleotide). The cDNA was generated from rat anterior pituitary mRNA using random hexanucleotides to increase the chance of detecting POU
domain transcripts with long 3'-untranslated sequences. Because Pit-1 is a highly abundant POU domain transcript in the pituitary, we excluded Pit-1 transcripts by treating the amplified material with either restriction endonuclease XhoI or BstXI, both of which cleave in the Pit-1 POU domain. The amplified material was then size-fractionated by agarose gel electrophoresis, and DNA corresponding to a full-length PCR fragment was isolated and reamplified. These products were subsequently cloned into a plasmid vector and analyzed by dideoxynucleotide sequencing.

We used this method to isolate several copies of a novel POU domain, referred to as Brn-5, which was selected for further analysis. The Brn-5 PCR fragment was used to screen a rat pituitary cDNA library, yielding three overlapping clones of 4.6 kb, 4.7 kb, and 2.1 kb denoted λZAP Brn-5A, -B, and -C, respectively. All three clones contained an identical open reading frame of 900 bp (Fig. 1). The translation start site was assigned based on a in-frame stop codon to the putative initiating ATG, as well as homology to a consensus Kozak sequence (Kozak, 1984). All three clones contained poly(A) tracks at their 3' end. Clones A and B were similar in that both contained 2.9 kb of 3'-untranslated sequences, whereas clone C contained only 750 bp of 3'-untranslated sequences, suggesting the use of an alternative poly(A) signal site. Although heterogeneity was detected in the 5'-untranslated regions, this did not alter the open reading frame. Brn-5 is a single copy gene (data not shown), and we have localized the gene to mouse chromosome 15 and human chromosome 12 (Xia et al., 1993).

The POU domain of Brn-5 is unusual because it has significant alterations in several predicted amino acids that are invariant or exhibit only conservative changes in previously described mammalian POU domains (Fig. 2). In the POU-specific domain, 11 of the highly conserved amino acids are different, and 6 of these changes are nonconservative. In the POU homeodomain, seven near-invariant amino acids are altered in Brn-5. Five of the changes in the POU homeodomain are nonconservative. The 20-amino acid linker region of Brn-5 bears no homology to any of the previously described POU proteins. Because of this high degree of divergence, we assigned Brn-5 to a new subclass (class VI) of the POU domain family (Fig. 2).

Indeed, two highly similar sequences from zebrafish and mouse, referred to as pou(c) (Johansen et al., 1993) and Emb (Okamoto et al., 1993), respectively, were recently reported. The predicted amino acid sequences of pou(c) and Emb are 70% and 97% homologous to that of rat Brn-5, respectively. The pou(c) coding region contains a 302-amino acid NH2-terminal extension that is not in Emb or Brn-5. Other structural features of Brn-5 include an unusually short COOH terminus of only 10 amino acids. The NH2 terminus, which is 144 amino acids long, bears no obvious homology to previously described POU domain genes. However, it features an over-representation of prolines (17%), a characteristic which has been associated with trans-activation domains in several other transcription factors (Mitchell and Tjian, 1989), including Oct-3/4 (Imagawa et al., 1991). In addition, the NH2 terminus contains a 7-amino acid sequence, NAQQGQVI, that is repeated twice (Fig. 1).

Expression of Brn-5 mRNA during Development and in the Adult—RNA blot analysis using RNA from multiple tissues and cell lines was used to determine the size and distribution of Brn-5 transcripts (Fig. 3). A major transcript of 5.5 kb was observed in several somatomammotroph pituitary cell lines (GC, MMQ, 235-1) and in a thyrotroph tumor (Tt97). Upon longer exposure, low level expression was found in embryonic cell lines F9 and P19 and in the corticotroph cell line AtT-20, but no expression was detected in HeLa, HL-60, or CV-1 cells (data not shown). In addition, minor bands of 2.8 kb and 2.1 kb were found in pituitary cell lines and embryonic cell lines, respectively. Although the nature of these minor transcripts is unclear, the 2.8-kb mRNA is consistent with the use of the alternative poly(A) signal site found in λZAP Brn-5C.

To further determine the pattern of Brn-5 expression, we performed RNase protection assays on RNAs from several different rat adult and embryonic tissues in addition to cell lines (Fig. 4, A and B). In the adult, Brn-5 is most highly expressed in the brain but transcripts are also readily detected in kidney, lung, heart, skin, adrenal, and placenta. Low level expression was observed in spleen, muscle, liver, anterior pituitary, testis, and ovary. During development, expression was readily detected in the head at embryonic day 15, with levels decreasing gradually until postnatal day 10. On embryonic day 17, expression was detected in brainstem, cortex, and hypothalamus, with highest levels in the cortex. The low level of expression in testes appears to be developmentally controlled, being highest during the prepubertal stage (Fig. 4B). Together, these data suggest that Brn-5 is expressed at highest levels in the central nervous system, but at lower levels in many different organs.

We utilized in situ hybridization to localize the expression of Brn-5 more precisely both during development and in the adult brain. A series of rat embryos ranging from stage e9.5 through e15.5 were probed with sense and antisense Brn-5 RNA probes. No expression of Brn-5 was detected before e12.5 (Fig. 5, left panel, and data not shown). On e13.5 and e15.5, expression of Brn-5 was detected diffusely throughout the developing brain and spinal cord (Fig. 5, middle and right panels). Because Brn-5 signal could be detected in whole adult brain by RNase protection, it was likely that Brn-5 was either expressed diffusely or, alternatively, restricted with very high levels in expressing cells. To distinguish between these possibilities, we carried out in situ hybridization studies on regularly spaced coronal brain sections. Expression was diffuse throughout the brain, and, in general, the signal correlated with cell density, including regions such as the hippocampus and cerebellum.
**Fig. 4. Expression of Brn-5 in development and in the adult rat analyzed by RNase protection assays.** A, 20 µg of total RNA from the indicated tissues, cell lines, and yeast tRNA were hybridized to a 32P-labeled antisense riboprobe from part of the POU domain of Brn-5. After treatment with RNase, the products were size-fractionated on a 5% denaturing polyacrylamide gel and analyzed by autoradiography. The numbers on the right show the size of the protected fragment in rat tissues (290 bp) as judged by the migration of a labeled 1-kb DNA ladder (Life Technologies Inc.). Hybridization to RNA of mouse origin gives rise to two fragments, 160 and 100 bp long, apparently due to sequence divergence between mouse and rat in this region of the Brn-5 gene. B50 and B109 are neuroblastoma cell lines. CA 77 and GC are thyroid C cell and somatotroph cell lines, respectively. TtT 97 is a mouse thyrotroph tumor. Head refers to a brain of a 10-day-old rat. B, 20 µg of total RNA from the indicated sources were hybridized to a Brn-5 (top panel) and β-actin probes (lower panel) and analyzed as described above.

**Fig. 5. Localization of Brn-5 mRNA during development by in situ hybridization.** Rat embryos from the embryonic stages indicated at the top were hybridized with a 35S-labeled cRNA probe corresponding to the NH2 terminus of Brn-5. Basal telencephalic plate (B), cerebellum (C), diencephalon (D), lateral ventricle (L), mesencephalon (M), rhombencephalon (R), spinal cord (S), and telencephalon (T) are indicated.

The one clear exception was a higher expression level found in neocortex, and, within the cortex, the signal was most intense in the inner granular layer (layer IV, Fig. 6A). Observation under high magnification light field showed that silver grains were localized over neurons (data not shown). No signal was observed when we used a sense Brn-5 riboprobe (data not shown). An RNase protection assay using RNA from various brain regions was used to independently confirm the expression pattern showed by the in situ hybridization studies. This experiment revealed that Brn-5 was expressed in all brain regions, with highest expression in the neocortex (Fig. 6B). Taken together, the in situ hybridization and RNase protection data indicate that Brn-5 mRNA has widespread distribution in adult brain, and that the expression is enriched in the neocortex.

**DNA-binding Properties of Brn-5.** To test whether the divergent sequence of the Brn-5 POU domain led to different DNA-binding properties, we expressed Brn-5 both in bacteria and in vitro using rabbit reticulocyte lysate. Binding of the expressed protein was tested in the gel-mobility shift assay using a series of AT-rich candidate binding sites for POU proteins or classic homeodomain proteins (Fig. 7). Brn-5 binding to the following elements was easily detected in order of decreasing affinity: HSV Oct, CRH Oct, POMC DE2, Ftz, pOct, and H+O+. Unexpectedly, Brn-5 bound poorly to an element, H-O+, where the heptamer binding site was mutated. This suggests that, in contrast to classic octamer-binding proteins, the Brn-5 binding site overlaps with both the octamer and heptamer sequences. We could not detect binding to unrelated sequences, such as a thyroid hormone response element and a site that binds helix-loop-helix proteins (E box; data not shown). This binding pattern is different from that exhibited by Oct-2, which prefers all sites that contain a core octamer element: H+O', H-O', pOct, and H12 Oct (data not shown). Thus, consistent with the divergent sequence of the Brn-5 POU domain, its binding preference appears to be distinct from that of Oct-2.
Alignment of the high affinity binding sites for Brn-5 revealed a potential consensus sequence: 5'-GCATNN(N)TAAT-3'.

The POU-specific domain contacting the -ATGC- on the 5' side (Kristie and Sharp, 1990; Verrijzer et al., 1990b, 1992b; Laughon, 1991). The site described here for Brn-5 could be considered different because of a presumed separation of the two binding regions and the relaxed sequence specificity between the two halves. To test whether Brn-5 binds as a dimer to this site, we took advantage of the approach described by Hope and Struhl (1987). The binding of Brn-5 holoprotein and Brn-5 glutathione S-transferase fusion protein to the CRH site was tested using the gel-mobility shift assay. Brn-5 holoprotein (Fig. 8C, lane 2) and Brn-5 glutathione S-transferase fusion protein (Fig. 8C, lane 4) form complexes with distinct mobility patterns. When the two proteins were mixed together, no complexes with intermediate migration were observed (Fig. 8C, lane 3). Similar results were obtained using the HSV octamer site and when we used co-translated products from templates encoding Brn-5 holoprotein and POU domain (data not shown). These data suggest that Brn-5 binds as a monomer to both the HSV Oct and CRH sites.

The unusual binding site determined in these experiments suggested the possibility that Brn-5 might bind preferentially to non-octamer sites. To test this possibility more rigorously, we used the “selected and amplified binding site” (SAAB) assay (Blackwell and Weintraub, 1990) to identify a preferred site for Brn-5. The site in this study contained the 5'-GCAT-3' sequence followed by 8 random nucleotides. By the means of this assay we identified a preferred site: 5'-GCATATGATAAT-3' (Fig. 9A). Surprisingly, this site is very similar to the sites for Oct-1: 5'-GATGATATTGACT-3' (Fig. 9A). This analysis revealed that mutation of a -5' to the consensus had no effect on binding (Fig. 8B, lane 2), whereas double mutations through the -GAT- sequence on the left (Fig. 8B, lanes 3 and 4) and the -TA- on the right (Fig. 8B, lane 7) completely obliterated binding. Although two separate mutations of the 3-bp sequence between the two half-sites decreased binding affinity, the effect of these mutations was less dramatic (Fig. 8B, lanes 5 and 6). The relaxed sequence requirements in this region of the binding site are consistent with two critical regions of Brn-5 binding sites separated by 3 base pairs.

The classic octamer site, 5'-ATGCAAAT-3' has been previously described as a bipartite site with the homeodomain contacting the -AAAT- sequence on the 3' portion of the site, and
identified in Fig. cated on the protein and Bm-5 glutathione S-transferase fusion protein together and the indicated mutant sites were radioactively labeled, incubated with Bm-5 protein, and analyzed by the gel-mobility shift assay. Bound well as to that of CMV LexA Brn-4 (Mathis glutathione S-transferase fusion protein contains a domain capable of transcriptional activation, a CMV monomer. A radioactively labeled CRH site was incubated in the absence of protein (lune 3). Bound (B1, Bm-5 glutathione S-transferase-DNA complex, and B2, Bm-5 holoprotein-DNA complex) and free (F) probes are indicated on the right.

(data not shown). To independently test whether Brn-5 contains a domain capable of transcriptional activation, a CMV LexA Brn-5 fusion plasmid containing the NH2 terminus of Brn-5 linked to a LexA DNA-binding domain, was co-transfected with a reporter plasmid where 2 Brn-5 linked to a minimal promoter and a luciferase reporter. Under activation was specific because a Lex fusion containing the NH2 terminus of Skn-li, which is incapable of DNA binding, did not activate in these same experiments (data not shown). The ac-

Fig. 8. Identification of a consensus high affinity DNA-binding site for Brn-5. A, alignment of highest affinity binding sites for Brn-5 identified in Fig. 7. In some instances, two possible alignments are shown. Sense (S) and antisense (AS) strands are indicated. B, mutational analyses of the CRH site. The wild type CRH oligonucleotide or the indicated mutant sites were radioactively labeled, incubated with Brn-5 protein, and analyzed by the gel-mobility shift assay. Bound (B) and free (F) probes are indicated on the right. C, Brn-5 binds as a monomer. A radioactively labeled CRH site was incubated in the absence of protein (lane 1), with Brn-5 holoprotein (lane 2), with Brn-5 glutathione S-transferase fusion protein (lane 4), or with Brn-5 holoprotein and Brn-5 glutathione S-transferase fusion protein together (lane 3). Bound (B1, Brn-5 glutathione S-transferase-DNA complex, and B2, Brn-5 holoprotein-DNA complex) and free (F) probes are indicated on the right.

DISCUSSION

Defining additional POU domain proteins is of interest because at least three of these factors, Pit-1 (Li et al., 1990), Oct-2 (Corcoran et al., 1993), and one-86 (Finney and Ruvkun, 1990) have been genetically shown to determine cell fate or differentiated function, suggesting that there may be important roles for each of the diverse members of this family. We have identified a CDNA encoding a novel POU domain factor referred to as Brn-5, whose predicted amino acid sequence is highly diverged from other POU domain proteins, thus defining a new class of POU domain factors (He et al., 1988; Johansen, 1993; Okamoto et al., 1993).

The POU domain is a bipartite DNA-binding domain composed of a POU-specific domain joined by a short linker sequence to the POU homeodomain. The POU-specific domain contains four α helices, homologous to the helix-turn-helix motif in the λ repressor (Assa-Munt et al., 1993; Dekker et al., 1993). The structure of the POU homeodomain is thought to be similar to that of the classic homeodomains with a cluster of basic amino acids at the NH2 terminus and three α helices (Laughon, 1991; Rosenfeld, 1991; Schöler, 1991). In fact, POU domains are highly conserved, even across species including Drosophila (Johnson and Hirsh, 1990; Billin et al., 1991; Dick et al., 1991; Lloyd and Sakonju, 1991; Treacy et al., 1991, 1992; Prakash et al., 1992), Caenorhabditis elegans (Finney et al., 1988), Xenopus (Agarwal and Sato, 1991; Frank and Harland, 1992; Hinkley et al., 1992; Whitfield et al., 1993); zebrafish (Matsuzaki et al., 1992; Johansen et al., 1993), chicken (Petryniak et al., 1990), and mammals (Rosenfeld, 1991; Ruvkun and Finney, 1991; Schöler, 1991; Hara et al., 1992; Wegner et al., 1993). Although the predicted structure of the Brn-5 POU domain probably conforms to these general rules, it has alterations of many amino acids that are conserved across all previously described mammalian POU domains. For instance, the residue that replaces a lysine at position 22 in the basic region of helix 1 of the POU-specific domain of Brn-5 creates a potential phosphorylation site for protein kinase A (Kennelly
and Krebs, 1991). Similarly, Oct-3/4 contains a threonine at the same position. This is reminiscent of the basic region in the NH₂ terminus of the POU homeodomain in which a phosphorylation site regulates the binding activity of Pit-1 (Kapiloff et al., 1991) and Oct-1 (Segil et al., 1991), raising the possibility that activity of the POU-specific domain of Brn-5 and Oct-3/4 might also be regulated by phosphorylation. Interestingly, Brn-5 has an alteration in the DNA recognition helix (alanine instead of threonine at position 46) of the POU-specific domain. However, this residue may not be directly involved in DNA recognition (Dekker et al., 1993). Two of the altered amino acids in the POU homeodomain, at position 25 (leucine instead of a basic residue) and 55 (threonine instead of a basic residue), are involved in phosphate backbone contacts in binding of classic homeodomains to their cognate site (Laughon, 1991). The NH₂ terminus of Brn-5 contains a 7-amino acid sequence, NAQGQVI, that is repeated twice. Although its function is unknown, it is likely to be important because it is completely conserved in the mouse. Furthermore, in zebrafish, one of the repeats is unchanged while the other has two conservative changes. Because the NH₂ terminus is a transactivation domain, it is tempting to speculate that this domain might be involved in protein-protein interactions that are required for transcriptional activation.

Brn-5 exhibits a pattern of expression that is qualitatively distinct from most other POU domain factors. The most striking feature of Brn-5 gene expression is the widespread distribution of Brn-5 transcripts, somewhat similar to that of Oct-1 (Sturm et al., 1988; Kambe et al., 1993). Whereas, Oct-3/4 and Tst-1 are expressed early in embryogenesis and in embryonic cell lines (Meijer et al., 1990; Okamoto et al., 1990; Rosner et al., 1990; Schöler et al., 1990a, 1990b; Suzuki et al., 1990), Brn-5 is expressed at low levels in these cell lines and is not detected by in situ hybridization in the rat embryo until embryonic day 12.5. Therefore, high levels of Brn-5 expression appear to be associated with the appearance of more differentiated cell phenotypes.

Similar to most POU domain genes, Brn-5 is initially diffusely expressed in the developing central nervous system. However, in contrast to neuronally expressed POU domain genes of class III and IV, Brn-5 mRNA expression does not subsequently become restricted to limited regions of the nervous system. Instead, Brn-5 is expressed diffusely throughout the adult brain, and signal strength correlates with cell density in structures such as the hippocampal formation (Okamoto et al., 1993) and cerebellum. A clear exception to this pattern is focally enriched expression in the neocortex, especially layer IV. In this regard, Brn-5 expression is distinct from Brn-2 and Tst-1, which are expressed in isocortex layers 2–5 and 5–6, respectively. In layer IV, sensory inputs from the thalamus or adjacent cortical regions terminate on neurons that connect to adjacent cortical laminae (Schmitt et al., 1981). Interestingly, we also observed enriched expression in the piriform cortex, which receives olfactory input. Therefore, Brn-5 joins the growing list of POU domain genes that are expressed in neurons involved in sensory pathways. In C. elegans, unc-86 specifies several sensory neuronal pathways (Finney and Ruvkun, 1990) and in mammals the Brn-5 gene is highly expressed in sensory ganglia (He et al., 1989; Gérrero et al., 1993). Furthermore, various products of the Oct-2 gene (Stoykova et al., 1992) and Brn-2 (He et al., 1989) are expressed in the olfactory pathway.

Therefore, it appears that several POU domain genes may have important roles in the development of neurons involved in different aspects of processing sensory information in the mammalian nervous system. Brn-5 is also expressed in several organs outside the nervous system. Expression of Brn-5 in testis becomes lower after puberty, suggesting that Brn-5 is expressed in non-germ cells or in immature spermatogonia.

Although POU homeodomain DNA recognition is thought to be similar to that of the classic homeodomain, high affinity DNA binding to cognate sites also requires the POU specific domain (Sturm and Herr, 1988; Ingraham et al., 1990; Kristie and Sharp, 1990; Verrijzer et al., 1990b; Imagawa et al., 1991), which makes direct DNA contacts (Aurora and Herr, 1992; Verrijzer et al., 1992b). Like the POU domain, the DNA recognition sequences for POU homeodomain proteins also appear to be bipartite. Oct-1 binds with highest affinity to sites containing an octamer motif: 5'-ATGCAAAT-3' (for review see Laughon (1991)). The 5' half of this site (-AAAT-, or a variant thereof) is recognized by the homeodomain whereas the 3' half (-ATGC-) is recognized by the POU-specific domain (Kristie and Sharp, 1990; Verrijzer et al., 1990b, 1992b). Oct-1 can also bind to a different type of sequence, the so-called TAATGARAT motif (Sturm et al., 1987); however, it binds to this site with lower affinity.
affinity and may not require the POU-specific domain for this interaction (Verrijzer et al., 1990b). Because of the divergent sequence of the Brn-5 POU domain, it was of interest to determine whether Brn-5 preferred octamer DNA-binding sites. These experiments are especially relevant because the zebrafish homolog of Brn-5 was reported to represent the initial example of a non-octamer-binding POU domain protein (Johansen et al., 1993). Although our results indicate that Brn-5 is capable of binding to non-octamer sites containing the consensus sequence: 5'-GCATNN(Y)ATT3', a random binding site selection demonstrates that Brn-5 binds preferentially to a site containing a variant octamer element, surprisingly similar to that preferred by both Oct-1 (Verrijzer et al., 1992b) and a new class V POU domain protein. Therefore, our results are consistent with previous studies demonstrating that, although different POU homeodomain proteins may recognize distinct sequences, they generally have the ability to bind to certain sites containing the octamer motif (Singh et al., 1986; Sturm et al., 1987; Baumruker et al., 1988; LeBowitz et al., 1989; Poellinger and Roeder, 1989; Scholer et al., 1989; Meijer et al., 1990; Okamoto et al., 1990; Suzuki et al., 1990; Hinkley et al., 1992; Mathis et al., 1992).

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