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POU transcription factors control expression of CNS stem cell-specific genes

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SUMMARY

Multipotential stem cells throughout the developing central nervous system have common properties. Among these is expression of the intermediate filament protein nestin and the brain fatty acid binding protein (B-FABP). To determine if common mechanisms control transcription in CNS stem cells, the regulatory elements of these two genes were mapped in transgenic mice. A 257 basepair enhancer of the rat nestin gene is sufficient for expression throughout the embryonic neuroepithelium. This enhancer contains two sites bound by the class III POU proteins Brn-1, Brn-2, Brn-4, and Tst-1. Only one of the two POU sites is required for CNS expression. An adjacent hormone response element is necessary for expression in the dorsal midbrain and forebrain. The regulatory sites of the B-FABP gene are strikingly similar to those of the nestin gene. A hybrid POU/Pbx binding site is recognized in vitro by Pbx-1, Brn-1 and Brn-2. This site is essential for expression in most of the CNS. In addition, a hormone response element is necessary for forebrain expression. Both the nestin and B-FABP genes therefore depend on POU binding sites for general CNS expression, with hormone response elements additionally required for activity in the anterior CNS. These data indicate that regulation by POU proteins and hormone receptors is a general mechanism for CNS stem cell-specific transcription.

Key words: Neuroepithelium, Precursor cell, Nestin intermediate filament, Brain fatty acid binding protein, Mouse

INTRODUCTION

The diversity of cell types in the mammalian central nervous system (CNS) begins in the neuroectoderm. The neural tube is patterned from its inception: planar and vertical signals which induce neuroectoderm also impart an initial anteroposterior and dorsoventral polarity (for review see Ruiz i Altaba, 1994). These and subsequent signals within the neuroectoderm refine the regionalization and specify the highly localized expression of homeobox genes and other transcription factors (Marshall et al., 1992; Goulding et al., 1993; Papalopulu and Kintner, 1993).

However, recent studies on neuroepithelial precursor cells isolated from many regions of the embryonic or adult brain show that these cells have many common characteristics: (1) they can proliferate and self-renew in vitro in response to mitogens such as bFGF or EGF (Gensburger et al., 1987; Vicario-Abejón et al., 1995b; Ghosh and Greenberg, 1995; Reynolds and Weiss, 1992), (2) they are multipotent, able to produce differentiated progeny of several types including neurons and glia (Johe et al., 1996; Gritti et al., 1996; Shihabuddin et al., 1997), and (3) they can differentiate into neural subtypes appropriate to a new host region after transplantation (Vicario-Abejón et al., 1995a; Brüstle et al., 1995; Fishell, 1995; Campbell et al., 1995). These shared qualities define CNS stem cells.

The functional properties of stem cells correlate with expression of the intermediate filament protein nestin and the brain fatty acid binding protein (B-FABP). Both proteins are expressed in the ventricular zone during the peak of neurogenesis (Frederiksen and McKay, 1988; Kurtz et al., 1994). As cells migrate away from the ventricular zone the expression of nestin and B-FABP is shut down, but expression is maintained in radial glia (Hockfield and McKay, 1985; Feng et al., 1994; Kurtz et al., 1994). Both proteins mark the same CNS cells, except that nestin is present in cells of the floorplate and dorsal midline while B-FABP is not. Nestin and B-FABP are also expressed in stem cells which persist in the adult (Reynolds and Weiss, 1992; Johe et al., 1996; T.M., unpublished). B-FABP has been shown to have a high affinity for docosahexaenoic acid (DHA) (Xu et al., 1996), the major polyunsaturated long chain fatty acid in the brain phospholipid pool. Deficiencies in DHA cause severe and progressive neurological symptoms (Innis, 1991; Martinez, 1996). Meanwhile, nestin intermediate filaments may control neuroepithelial cell shape (Matsuda et al., 1996).

The shared gene expression and functional properties of CNS stem cells suggest that stem cells are defined by some common transcriptional mechanism in all brain regions. The nestin gene contains two tissue-specific transcriptional elements: a *cis*-element in the first intron drives nestin expression in somitic muscle precursors, while an enhancer in the second intron directs expression to CNS precursor cells (Zimmerman et al., 1994). Feng and Heintz (1995) have shown the importance of the proximal 0.8 kb of the B-FABP promoter for developmentally regulated expression in the neuroepithelium. We have defined a POU site and an HRE with similar roles in the nestin and B-FABP genes. The POU binding site is critical for general CNS expression, while the HRE is required for full expression in the anterior CNS. These parallel results in the two genes suggest that a common set of transcriptional controls establish fundamental features of the stem cell state.

MATERIALS AND METHODS

All standard molecular biology techniques were performed as described by Maniatis et al. (1989).

Preparation of transgenic mice

Transgenic mice were produced as described by Hogan et al. (1986). Mice used were C57Bl6×C3H F_1 embryo donors, C57Bl6 or C57Bl6×C3H F_1 stud males, and ICR foster mothers. Incorporation of transgene was assayed by yolk sac PCR as previously described (Zimmerman et al., 1994).

Whole-mount β-gal procedure

Nestin enhancer embryos were fixed in 0.2% glutaraldehyde, 2 mM $MgCl_2$, 0.1% Triton X-100 in phosphate-buffered saline (PBS) at room temperature for 60 minutes. They were washed in two changes of PBS plus 2 mM $MgCl_2$, soaked 20 minutes in PBS plus 2 mM $MgCl_2$, 0.1% Triton X-100, and stained with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.1% Triton X-100 in PBS at 37°C. Embryos were stained for 90 minutes to 3 hours, then washed with 70% ethanol to stop the reaction. B-FABP promoter embryos were fixed in 1% formaldehyde, 0.1% glutaraldehyde, processed as above, and stained overnight at 30°C. For frozen sections, dissected brains were soaked in 20% sucrose until loss of buoyancy, frozen and cut by cryostat into 40 µm sections. These were fixed for 40 minutes in 0.2% glutaraldehyde and processed for X-Gal histochemistry as described above.

Construction of plasmids

BSp-TKlacZ. The nestin second intron was cut with *Spe*I and *Bbs*I. The 587 bp fragment was isolated using Geneclean II (Bio101) and ligated into *Spe*I-*Hin*dIII digested pBluescriptKS+ (Stratagene). A 615 bp *Sal*I-*Not*I fragment of this plasmid was isolated and ligated into *Sal*I-*Not*I digested pTKlacZ (Zimmerman et al., 1994).

YSt-TKlacZ. The plasmid RgIItklacZ (Zimmerman et al., 1994) was cut with *Stu*I and *Sal*I. The overhangs were filled in with Klenow fragment and religated.

NY-TKlacZ. The plasmid gIITKlacZ (Zimmerman et al., 1994) was cut with *Nsi*I and *Sal*I. The ends were blunted with bacteriophage T4 polymerase or Klenow, and religated.

NY-pBt. An 857 bp *Hin*dIII-*Bst*YI fragment of NY-TKlacZ was isolated and ligated into *Hin*dIII-*Bam*HI digested pBluescriptKS+.

NYΔ257-TKlacZ. The 257 bp enhancer was deleted from the plasmid NY-pBt using an oligo-directed mutagenesis kit (Bio-Rad). The oligo used was 5′-GCTAACACTTTATATCA-GTCAAACGCC-AGTGG-3′. The resulting plasmid was digested with *Spe*I and *Xho*I, and the insert ligated into *Spe*I-*Sal*I digested pTKlacZ.

Cons-TKlacZ, 5′Cons-TKlacZ, 257-TKlacZ. Portions of the second intron were PCR amplified from a rat nestin genomic clone, using Vent polymerase (New England Biolabs) for 30 cycles as follows: 94°C 30 seconds, 55°C 30 seconds, 72°C 60 seconds, plus a final 8 minute extension at 72°C. PCR products were isolated using QIAEX (Qiagen) and cloned into the vector pCRII using the TA cloning kit (Invitrogen). The resulting plasmids were digested with *Spe*I and *Xho*I, and the inserts ligated into *Spe*I-*Sal*I digested pTKlacZ.

B-FABP promoter constructs. A *Sal*I site was introduced by PCR at position +82 just before the initiation codon of rat B-FABP. The *Xho*I-*Sal*I promoter fragment (−766 to +82) was ligated into the *Xho*I site of CMV-DF (Clontech), resulting in construct XS-Z. Deletions and point mutations in the XS-promoter fragment were generated by overlapping PCR.

Overlapping PCR mutagenesis

Nestin enhancer base substitutions and B-FABP promoter deletions were generated by overlapping PCR as described by Higuchi (1990). The nestin 1868 bp PCR product was subsequently digested with *Not*I and *Xho*I to generate 5′ overhanging ends, separated on 1% agarose and purified using GeneClean (Bio 101), then ligated into *Not*I-*Sal*I digested pTKlacZ. To mutate two binding sites (Δft2/3), a plasmid mutated at a single site (Δft2) was used as the template for a second PCR mutagenesis. All mutated plasmids were confirmed by DNA sequencing.

The following oligonucleotides, and their exact complements, were used to mutate nestin binding sites: gIIUPnot, 5′-ATTTGCGGCCGCAGATCCTGGAAGGTGG-3′; gIIDNxho, 5′-CCGCTCGAGTTCCAAGGAGAGCAG-3′; Δftpt1, 5′-GGTA-ACAGACAAAGATATCAGAATTCAGCTCC-3′; Δftpt2, 5′-GC-AGAGAGAGAGCCATCAGGCCTCAACAGCCTGAGAATTC-3′; Δftpt3, 5′-CTGAGAATTCCCACTTCGGATCCGCAGCCCTCCCT-TCTTA-3′; Δftpt4, 5′-GGGAGGCCGATTCTCAACTAGTTTAT-TGCCTTTTGTCC-3′; Δftpt5, 5′-CTCCCAGAGGAGTAGGCCTT-CGGCCTTGGC-3′ with phosphothioate linkage at 3′ end.

Nuclear extracts

Timed pregnant Sprague-Dawley rats (Taconic) or CD1 mice (Charles River) were killed by CO_2 asphyxiation. Embryos were dissected in Hank's Balanced Salt Solution (Gibco BRL) with 3.7 g/l $NaHCO_3$ and 3.9 g/l HEPES (pH 7.2). Nuclear extracts were prepared by the method of Dignam et al. (1983). Protein concentration was determined using a Pierce BCA kit and BSA standards.

DNAse I footprinting

The plasmid pCRII-5669, containing 257 bp of the nestin second intron, was digested with *Hin*dIII or *Eco*RV and labeled with [α-^{32}P]dCTP and Klenow fragment. Unincorporated label was removed via a sephadex G-50 spin column (5 Prime→3 Prime). A second digestion with *Eco*RV or *Hin*dIII released the single end-labeled probe, which was separated from the vector on low-melting agarose and purified on an Elutip column (Schleicher & Schuell) before being ethanol-precipitated and resuspended in TE (pH 8.0).

10000 disints/minute of probe was mixed with 14 µg of tissue nuclear extract or 15 µg of bovine serum albumin (BSA) plus 9 µg of poly(dI-dC) in footprint buffer (12 mM Tris-HCl [pH 8.0], 50 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM NaCl, 1 mM dithiothreitol (DTT), 5% glycerol (vol/vol)). This reaction was incubated for 30 minutes at room temperature. One volume (50 µl) of footprint buffer was added just before the addition of DNAse I (RQ1 DNAse; Promega). BSA controls received 0-0.5 µg DNAse I, while nuclear extract samples received 0.4-0.8 µg. Digestion proceeded at room temperature for exactly 2 minutes before being stopped with an equal volume of stop buffer (50 mM EDTA, 0.2% SDS, 100 mg/ml yeast tRNA). The reaction was extracted once in phenol, and ethanol precipitated. The DNA was resuspended in formamide loading dyes and heated 5 minutes at 90°C before analysis on a 6% acrylamide, 7 M urea sequencing gel.

Methylation interference

1-2×10^6 disints/minute of probe was methylated as described by Baldwin (1988).

100,000 disints/minute of methylated probe and 40 µg of tissue nuclear extract were combined in binding buffer (12% glycerol [vol/vol], 12 mM HEPES [pH 7.9], 4 mM Tris-HCl [pH 7.9], 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol [DTT]) for 1 hour at room temperature (23°C). Binding reactions were electrophoresed at 200 volts on a 4% 80:1 acrylamide/bis-acrylamide gel. The bound and free probes were located using a phosphor screen (Molecular Dynamics), cut out and electrophoresed into NA45 paper (Schleicher and Schuell). Probe was eluted at 65°C in 50 mM Tris-HCl (pH 8.0), 1.0 M NaCl, 10 mM EDTA, and cleaved in piperidine as described by Baldwin (1988). Equal activities of bound and free probes were analyzed by denaturing PAGE on a 12% acrylamide, 7 M urea sequencing gel. A Maxam and Gilbert G+A sequencing reaction was run alongside as a positional marker. The dried gel was visualized on X-OMAT AR film (Kodak).

Oligonucleotides used in methylation interference and electrophoretic mobility shift assays

Oligonucleotide probes were designed as complementary pairs with 4 bp 5′ overhangs at each end. The forward sequence of each pair is listed below. Nestin oligos (Ftpt1-5) were synthesized with a *Hin*dIII overhang (AGCT) at the 5′ end of each forward oligo and a *Sal*I overhang (TCGA) at the 5′ end of each reverse oligo. B-FABP oligos (bs2, bs8, bs9) were synthesized with a CATG overhang at the 5′ end of the forward oligo.

Ftpt1: 5′-*AGCT*GGAGAAGGGGAGCTGAATTCATTTGCTTTTGTCTGTTACCAG-3′;
Ftpt2: 5′-*AGCT*GCAGAGAGAGAGCCATCCCCTGGGAACAGCCTGAGAATTC-3′;
Ftpt3: 5′-*AGCT*CAGCCTGAGAATTCCCACTTCCCCTGAGGAGCCCTCCCTTCT-3′;
Ftpt4: 5′-*AGCT*GTGTGGACAAAAGGCAATAATTAGCATGAGAATCGGCCTC-3′;
Ftpt5: 5′-*AGCT*CCCTCCCAGAGGATGAGGTCATCGGCCTTGGCCTTGGGTGGG-3′;
bs2 5′-*CATG*TTTTTCTTATCCCCTGCTGAACCTGAAAAACCCTCTCTTT-3′; bs8 5′-*CATG*TTAAGGAAATCAATCTCAATGCCC-3′; bs9 5′-*CATG*TGCTGAACCTGAAAAACCCTCT-3′.

Complementary oligonucleotides were annealed at 10 pmoles/µl in 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA while slowly cooling from 80°C. The double stranded probes were labeled at a single end with [α-^{32}P]dCTP and Klenow fragment, adding 200 mM dATP and dGTP to fill in *Hin*dIII overhanging ends or 200 mM dTTP for *Sal*I overhangs. Unincorporated nucleotides were removed by spin column.

Electrophoretic mobility shift assays

Protein binding reactions were performed by combining 10,000 disints/minute of double-stranded oligonucleotide probe and 4 µg of tissue nuclear extract with 2.2 µg poly(dI-dC) and 6.0 µg BSA in binding buffer (see above). For supershifts, 1 µl of antibody was added to the protein-DNA binding reactions after 30 minutes at room temperature and incubated for a further 30 minutes at room temperature or 180 minutes on ice before gel loading. Binding reactions were electrophoresed at 200 volts on a 4% 80:1 acrylamide/bis-acrylamide/2.5% glycerol gel. Gels were dried and visualized on X-OMAT AR film (Kodak).

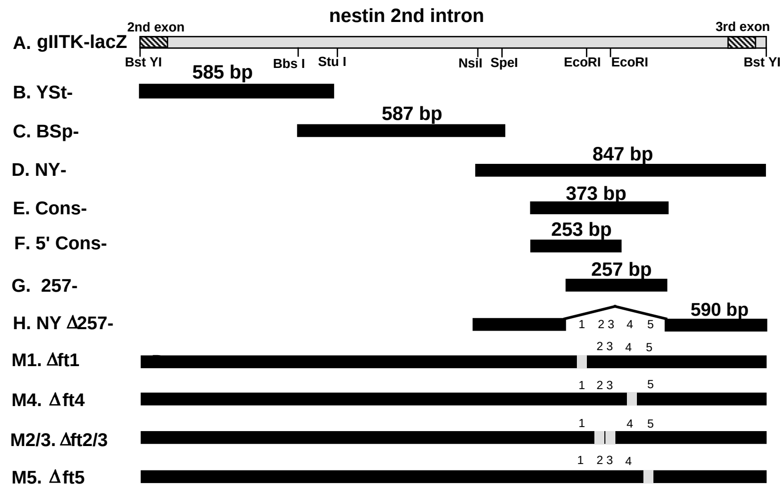
The following antibodies were used undiluted for supershift assays. Guinea pig anti-Brn-1, guinea pig anti-Brn-2, rabbit anti-Tst-1, rabbit anti-Brn-4, rabbit anti-Pit-1, and guinea pig anti-Brn-3a were kind gifts from Dr M. G. Rosenfeld. Rabbit anti-Cns-1 (Brn-5) was generously provided by Dr R. Bulleit. Rabbit anti-Pbx1 was a considerate gift from Dr M. Kamps. Rabbit anti-9-*cis*-retinoic acid receptor beta (RXRβ) was kindly provided by Dr K. Ozato. Rabbit anti-chicken ovalbumin upstream promoter transcription factor (COUP-TF) was a generous gift from Dr M-J. Tsai. Mouse anti-AP-2 was a kind gift from Dr T. Williams. Rabbit anti-9-*cis*-retinoic acid receptor alpha (RXRα) and rabbit anti-thyroid receptor alpha (TRα1) were purchased from Santa Cruz Biotechnical. Anti-CREB and anti-phosphoCREB (New England Biolabs), anti-phosphoCREB (Upstate Biotechnical), and anti-c-fos (Oncogene Science) were used as a 1:10 dilution in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

The following double-stranded oligonucleotides were used as competitors (complementary strand not shown): MBP-TRE 5′-CAGAACAATGGGACCTCGGCTGAGGACACGGGG-3′ (Huo et al., 1997), TR (DR-4) 5′-CTGAGGTGGAGGGCATCTGAGGACATTGAC-3′ (Thompson and Bottcher, 1997), octamer 5′-CTAGGACAAAAGGCAATAATTTGCATGAGAATC-3′, tailless 5′-TCGGCAATTAAGAAGTCAAATTTCT-3′ (Yu et al., 1994), bs8mut(c→g) 5′-CCTTAAGGAAATGAATCTCAATGCCCTATTATC-3′. The following competitors were obtained from Santa Cruz Biotechnical: Pbx-RE 5′-CGAATTGATTGATGCACTAATTGCAG-3′, Oct1-RE 5′-TGTCGAATGCAAATCACTAGAA-3′, TR (IR-0) 5′-AAGATTCAGGTCATGACCTGAGGAGA-3′, RAR (DR-5) 5′-AGGGTAGGGTTCACCGAAAGTTCACTC-3′, RXR (DR-1) 5′-AGCTTCAGGTCAGAGGTCAGAGAGCT-3′, Pit1-RE 5′-TGTCTTCCTGAATATGAATAAGAAATA-3′, SP1-RE 5′-ATTCGATCGGGGCGGGGCGAGC-3′, AP1-RE 5′-CGCTTGATGACTCAGCCGGAA-3′.

RESULTS

A CNS-specific element is contained within 257 bp of the nestin second intron

The second intron of the rat nestin gene promotes *lacZ* reporter expression throughout the CNS of embryonic mice, even when the native promoter is replaced by a herpesvirus thymidine kinase (TK) minimal promoter (Zimmerman et al., 1994). The current study further localizes the nestin CNS-specific enhancer in transgenic mice. In these ‘transient’ transgenic animals, each embryo analyzed carries the transgene at a unique integration site. Embryos were initially analyzed at embryonic day (E) 12.5 or 13.5; at these ages the second intron drives reporter expression in all regions of the neural tube. Overlapping fragments of the rat nestin second intron were generated and placed upstream of the TK minimal promoter and *lacZ* (Fig. 1). CNS enhancer activity is located in the 3′ end of the second intron; this region is highly conserved between the rat and human nestin genes (Lothian and Lendahl, 1997). The nestin genes are 52% identical through the second intron, but the 3′ 847 bp are 64% identical. This conserved region produces strong CNS-specific activity in mice (Fig. 1D). A PCR fragment encompassing the most highly (75%) conserved 373 bp also has strong CNS activity (Fig. 1E). A 257 bp subregion of this fragment (Fig. 1G) is 78% conserved and is sufficient to drive expression throughout the CNS at E13.5 (Fig. 2A). Overall, the expression pattern obtained with 257-TKlacZ is very similar to that given by the entire second intron. Portions of the CNS which are marked poorly by this construct, such as the small gap between the cerebral cortices and the midbrain, are also stained weakly by the entire second intron at the same age (Zimmerman et al., 1994).



Construct	TRANSGENIC EMBRYOS	EXPRESSION: CNS FULL	EXPRESSION: CNS PARTIAL	EXPRESSION: ECTOPIC
A. gIITK-lacZ	12	8	0	1
B. YSt-	3	0	0	0
C. BSp-	5	0	1	3[6]
D. NY-	5	3	0	1
E. Cons-	10	6	0	1
F. 5' Cons-	10	0	3	4[7]
G. 257-	8	6	0	4
H. NY Δ257-	13	0	4[8]	2
M1. Δft1	24	16	1	3
M4. Δft4	12	0	5	3
M2/3. Δft2/3	16	8	0	1
M5. Δft5	19	15[9]	0	0

Fig. 1. Map of nestin second intron fragments assayed for CNS-specific activity in transgenic embryos. A 1847 bp rat genomic fragment containing the second intron (construct A) drives strong CNS-specific expression when inserted upstream of the herpesvirus thymidine kinase (TK) basal promoter and the *E. coli lacZ* gene (Zimmerman et al., 1994). On the right is shown the number of transgenic embryos analyzed for each construct, and the number of embryos having β-galactosidase staining in all regions of the CNS, some regions of the CNS, or outside of the CNS. **M1.** The upstream octamer site was substituted at 6 of 8 bases by overlapping PCR. **M4.** The downstream octamer site was substituted at 6 of 8 bases. **M2/3.** Both AP2-like sites were substituted at 7 of 11, and 6 of 11, bases. **M5.** The nuclear hormone response element was substituted at 4 of 8 bases. [1-5]Locations of the five footprinted sites. [6]Various patterns of ectopic staining were seen in three of five transient transgenic embryos. [7]Various ectopic expression patterns, plus some CNS activity, were seen in four of ten transgenic embryos. None of the embryos had expression selectively in the CNS. [8]Four of 13 transgenics had expression in small portions of the CNS. [9]In no embryo did staining fully penetrate the telencephalon.

The 257 bp element is sufficient for proper onset and shutoff of expression

Additional mice injected with the plasmid 257-TKlacZ were analyzed at E9.5 or E16.5. At E9.5, expression of 257-TKlacZ is mostly limited to the ventral neural tube (Fig. 2B). The dorsal neural tube is stained only in the metencephalon and rostral spinal cord. Early expression is discontinuous; the telencephalon and anterior hindbrain are relatively unstained. The same pattern of early ventral expression of *lacZ* has been observed in transgenic mice carrying 5.8 kb of nestin upstream sequences and 5.3 kb of intragenic sequences including the entire second intron (Zimmerman et al., 1994).

Transgenic embryos at E16.5 have strong β-galactosidase (β-Gal) staining in the subventricular zone (SVZ) surrounding the lateral ventricles of the telencephalon (Fig. 2D). Nestin protein in the telencephalon at this age is predominantly in the ventricular and subventricular zones (Hockfield and McKay, 1985). At higher magnification (Fig. 2E), strong reporter expression is seen in the SVZ, with blue radial processes extending across the intermediate zone and a blue layer at the pial surface in the endfeet of the radial cells. This matches the distribution of nestin protein (Hockfield and McKay, 1985). Very little β-Gal staining is seen in the outer layers of the cortex where the maturing neurons are located. These data show that differentiating neurons down-regulate expression via the 257 bp element.

Deletion of the 257 bp element abolishes reporter expression in most of the CNS

To test whether all of the nestin CNS-specific elements are contained within 257 bp, this region was deleted by PCR from the 847 bp conserved 3′ end of the second intron. Only 4 of 13 transgenic embryos made with this construct (NYΔ257-TKlacZ; Fig. 1H) showed any β-Gal staining. Three of these four had staining in three limited regions of the CNS: the most caudal portion of the spinal cord, the rhombic lip, and the ventral portion of the midbrain (Fig. 2C). The finding that three embryos have the same discontinuous CNS staining suggests that elements which lie outside of the 257 bp element can specifically direct expression to these three portions of the CNS. However, this staining is relatively weak and these three regions are also strongly marked by the 257 bp enhancer, so perhaps ordinarily these elements do not function independently.

DNAse I footprinting reveals five sites of protein interaction with the 257 bp nestin neural enhancer region

The 257 bp nestin enhancer defined in vivo was mapped in vitro for binding sites recognized by nuclear proteins of the brain. Fig. 3A shows the results of DNAse I footprinting assays on a double-stranded DNA probe containing the 257 bp neural element. Nuclear extracts (Dignam et al., 1983) were prepared from midbrain, cerebellum, spinal cord and cerebral cortex or telencephalon of rats at E13, E16, E17 and E20 or postnatal day 5 (P5). A total of five regions are protected from DNAse I digestion. Footprinting occurs at the same five locations on the opposite strand (not shown). DNAse I hypersensitive sites are found adjacent to footprints 2, 3 and 5. These results are summarized in Fig. 3C.

None of the five footprints is detectable in every age and tissue from which nuclear extracts were prepared. In these and other experiments not shown, footprints 1 and 4 were protected by proteins from most brain regions taken at ages from E13 to P5. Footprint number 5 was almost as widespread, but was not protected by some midbrain and spinal cord extracts. By contrast, footprint 2 is bound only by extracts of midbrain, cerebellum and spinal cord from E16 to P5, but never by cortex. Footprint 3 is the most restricted of the five. It is protected only by proteins from E16 cerebellum and spinal

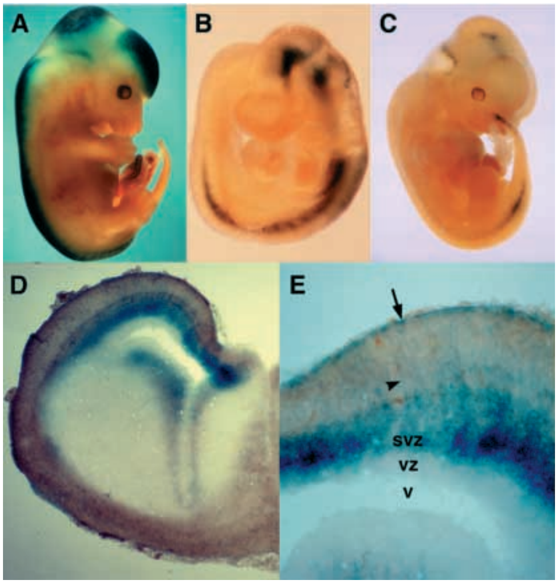


Fig. 2. The nestin 257 bp CNS-specific element is necessary and sufficient to drive correct expression in the neural tube. (A) Whole-mount E13.5 transgenic embryo with 257-TKlacZ (construct G). Strong staining is seen in all regions of the CNS. (B) Whole-mount E9.5 transgenic embryo with 257-TKlacZ. CNS staining is seen only from the optic vesicle to just posterior of the forelimb bud. CNS expression appears first in the ventral neural tube and discrete dorsal regions of the hindbrain. Expression from the 257 bp element is noticeably weaker in the ventral myelencephalon and telencephalon than in other regions. (C) Whole-mount E12.5 transgenic embryo with NYΔ257-TKlacZ (construct H). Weak CNS staining remains only in ventral midbrain, rhombic lip and posterior spinal cord. The embryo shown here also has ectopic staining in the mesoderm of the tail. (D,E) Coronal sections through forebrain of an E16.5 transgenic embryo with the plasmid 257-TKlacZ. Strong βGal staining is seen surrounding the telencephalic ventricles in D. An adjacent section at higher magnification (E) shows that the strongest expression is in the subventricular zone, the proliferating region of cortex at this age. The arrowhead points to fine radial processes which stain for β-galactosidase, and the arrow indicates a band of staining along the pial surface. These are the elongated cell bodies and pial endfeet, respectively, of radial glia. v, telencephalic vesicle; vz, ventricular zone; svz, subventricular zone.

cord (Fig. 3A) and P5 cerebellum (data not shown). Thus the 257 bp enhancer is active in all regions of the CNS, but some proteins that bind it may be regionally restricted.

Common transcription factor binding motifs lie at the core binding sequences

Sites on DNA that closely interact with proteins can be mapped by the methylation interference assay. This was done with five 44- to 46-mer oligonucleotide probes carrying each footprinted region of the nestin enhancer individually (Ftpt1-5). Protein interactions with the bottom strand of the footprint 4 region are shown in Fig. 3B. Nuclear proteins from the E17 rat cortex produce several complexes with this probe, of which four were isolated. Three adenines are relatively uncleaved in all four complexes. The location of the methylation interference sites relative to the footprinted area is summarized in Fig. 3C. The adenine/thymine-rich sequence, which includes the three blocked adenines, overlaps an eight basepair sequence (5′-ATTAGCAT-3′) nearly identical to the well known octamer sequence (5′-ATTTGCAT-3′). This octamer is the consensus binding site of the Pit-1/Oct/Unc-86 (POU) transcription factor family (Wirth et al., 1987; Wegner et al., 1993). The six blocked adenines on the footprint 1 probe are part of an A/T-rich sequence overlapping a seven-of-eight basepair homology (5′-ATTTGCTT-3′) to the octamer sequence (not shown). The presence of octamer-like motifs indicates that footprints 1 and 4 are likely to be occupied by POU family members.

Methylation interference also demonstrates strong similarities between footprints 2 and 3 (Fig. 3C). Binding to the Ftpt2 probe is blocked by methylation of two adjacent guanines on the top strand, or two guanines on the bottom strand, within the sequence 5′-TCCCCTGGGAA-3′. When methylation interference was performed on the bottom strand of the Ftpt3 probe, two clusters of guanines were involved in binding. One cluster of four guanines was complementary to the sequence 5′-TCCCCTGAGGA-3′. The second cluster of three guanines is complementary to an upstream sequence 5′-TTCCCACTTCC-3′. These sequences and the footprint 2 site are related to the binding site for AP-2 proteins (Imagawa et al., 1987). The comparable methylation interference sites on the two probes suggest that footprints 2 and 3 are bound by similar proteins.

Finally, methylation interference of the Ftpt5 probe reveals a third type of binding site. The three critical guanines on Ftpt5 are part of an eight basepair sequence, 5′-TGAGGTCA-3′, with homologies to multiple transcription factor binding sites. It is identical to an eight basepair site in the MHC class I promoter which binds a tri-iodothyronine receptor/9-*cis*-retinoic acid receptor beta heterodimer (TR/RXRβ) (Marks et al., 1992). The same eight bases are also similar to the consensus cAMP response element (CRE) (Habener, 1990). In a gel shift competition experiment, binding to this site was eliminated by a five-hundred-fold excess of 9-*cis* retinoic acid response elements (RXRE; direct repeat-1) or thyroid hormone response elements (TRE; inverted repeat), but not by CREs (data not shown). Thus footprint 5 of the *nestin* enhancer is likely to be a hormone response element.

Members of the POU transcription factor family bind to the *nestin* neural enhancer

In footprint assays, the two octamer sites in the *nestin* enhancer were protected by proteins from all tested regions of the brain, which suggests that POU domain proteins may be involved in transcription of nestin throughout the CNS. Supershift assays were performed to determine whether the two octamer-containing probes are recognized by class III POU proteins, which are widely expressed in the developing nervous system.

Fig. 4 shows supershift assays in which anti-POU antibodies were added to binding reactions on the octamer sites. In the absence of antibody, E13 cortical extract produces six strong complexes on Ftpt4 (Fig. 4A). Addition of antibody against Brn-1 completely supershifts one of these complexes. Brn-2 antibody supershifts two different complexes; the two forms of Brn-2 could arise from alternative translational forms (Schreiber et al., 1993). The same complexes are also seen with E13 extract on a Ftpt1 probe (not shown). Extract from E17 cortex forms five similar complexes on the Ftpt1 probe (Fig. 2B). Again, antibodies show that Brn-1 is part of one complex and Brn-2 is part of two. In addition, antibody against Brn-4 detects binding to Ftpt1 (Fig. 4C).

In P5 cortex, two strong complexes remain (Fig. 4D). Antibody supershifts reveal that these two bands are formed by Brn-1 and Brn-2. The faster-migrating form of Brn-2 is no longer seen. However, a less abundant complex which is seen just below the Brn-2 band is supershifted by antibody against Tst-1 (Fig. 4D). Binding of other CNS-specific POU proteins, including Pit-1, Brn-3a, and Brn-5, was not detected in extracts of any age tested. Both octamer sites of the nestin enhancer are thus specifically bound in vitro by all four class III POU proteins.

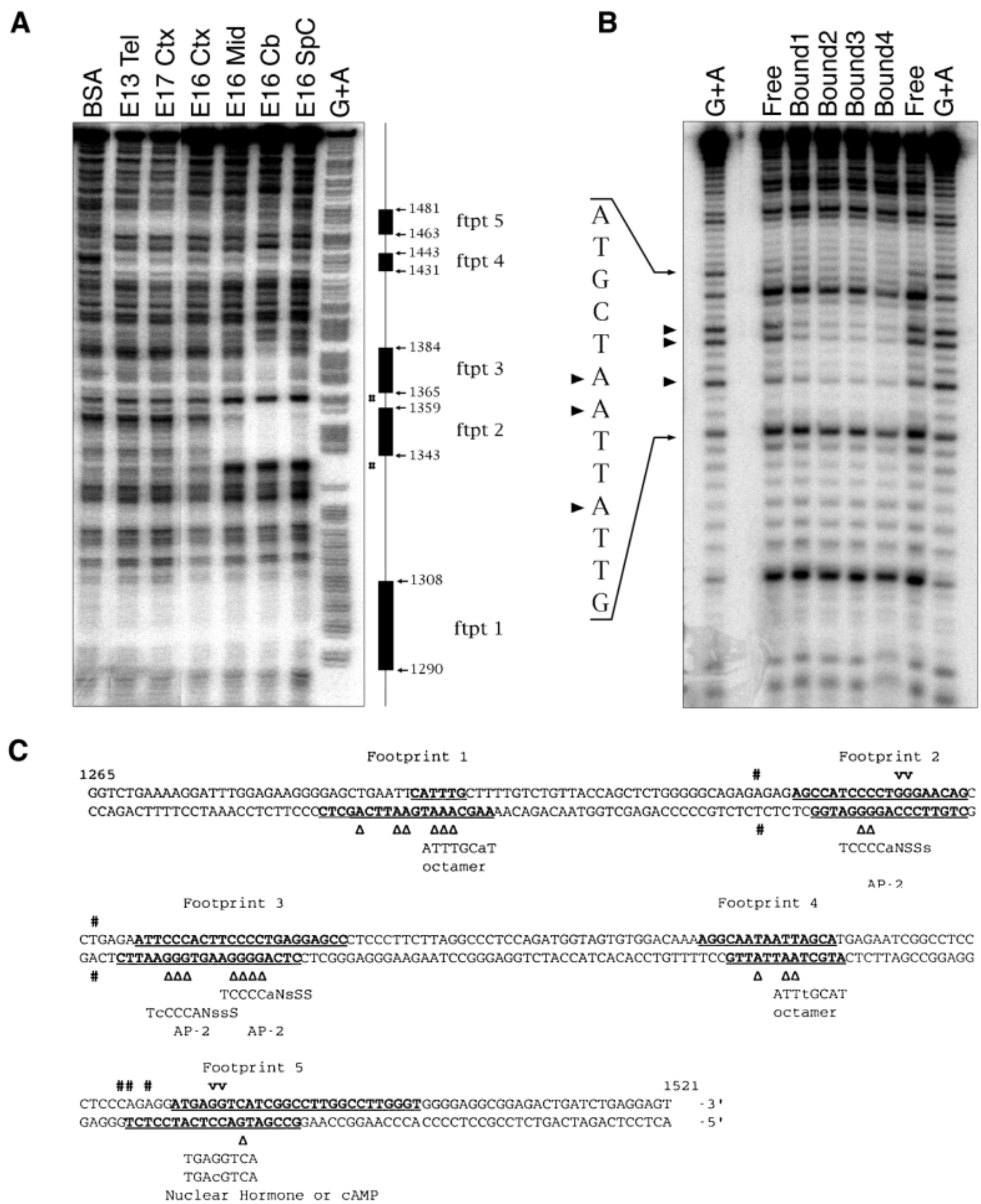


Fig. 3. In vitro mapping of the nestin 257 bp CNS-specific element. (A) DNAse I footprinting on the 257 bp CNS-specific element of the rat nestin second intron. The reverse strand of the DNA probe is shown. Five regions of protection are marked by black boxes at the right of the figure. Sites of greater digestion in the presence of some tissue extracts (hypersensitive sites) are marked with #. BSA, bovine serum albumin control; Tel, telencephalon; Ctx, cortex; Mid, midbrain; Cb, cerebellum; SpC, spinal cord; G+A, Maxam-Gilbert guanine+adenine sequencing reaction performed on the same end-labeled probe as the footprinting assay. (B) Methylation interference assay on nestin footprint 4. Cleavage on the reverse strand of a double-stranded oligonucleotide probe containing the nestin second intron sequence from 1417 to 1456 bp, which includes the footprint 4 region. The cleavage pattern of unbound DNA (lanes marked Free) is compared with cleavage of DNA bound by nuclear extract (lanes marked Bound). Maxam and Gilbert purine-specific sequencing of the probes is shown for reference (lanes marked G+A). The bases at which methylation interferes with protein binding are indicated by arrowheads. Methylation interference occurs at the same three adenines in each of four different complexes (Bound1-4) isolated by EMSA. (C) Summary of binding sites on the nestin enhancer detected by DNAse I footprinting and methylation interference. Sequence of both strands of the 257 basepair CNS-specific element of the rat nestin second intron is shown. Five footprinted regions are detected on each strand (underlined bold text) and footprints 2, 3, and 5 are flanked by DNAse I hypersensitive sites (#). Within each footprint are several bases at which methylation interferes with protein binding (arrowheads). These core binding sites are homologous to known transcription factor motifs: there are two octamer motifs (footprints 1 and 4), two AP-2 sites (footprints 2 and 3), and one site similar to the cyclic AMP response element or nuclear hormone response elements (footprint 5). The bases are numbered relative to the upstream end of the 1847 bp second intron region shown in Fig. 1A.

Activities which recognize other binding sites of the nestin enhancer are not identified by supershift assays

The oligonucleotide probes Ftpt2 and Ftpt3 contain sequences similar to the recognition motif for AP-2 proteins. In EMSA assays, both probes are strongly bound by proteins present in E16 rat spinal cord, but these DNA/protein complexes are not recognized by antibodies against AP-2α, β, or γ (data not shown).

The probe Ftpt5 contains a binding site similar to the cAMP response element or the half-site for non-steroid nuclear receptors. E17 rat cortex nuclear extract produces two strong complexes on this probe. Antibodies against possible binding proteins RXRα, RXRβ, TRα, COUP-TF, c-fos, CREB, and two antibodies against serine133-phosphorylated CREB were used in efforts to supershift the Ftpt5 probe, without success (data not shown).

A POU protein binding site and a hormone response element have different roles in CNS-specific expression

In order to test whether the transcription factor binding sites identified in vitro are required for nestin enhancer function in vivo, transgenic mice were generated carrying binding sites that were altered by PCR mutagenesis. These mutations were made in the 1.8 kb *nestin* second intron and inserted upstream of TKlacZ for injection into transgenic mice. In all cases, sites were mutated by base substitution to preserve the exact spacing between the remaining sites.

Each of the two octamer sites was altered at 6 of 8 basepairs, removing the octamer homology (constructs M1 and M4; see Fig. 1). The hormone response element was substituted at 4 of 8 basepairs within the half-site (construct M5). Similarly, the two AP-2 sites were altered at 6 or 7 of 11 basepairs; in this case, a single second intron-TKlacZ construct was tested bearing mutations at both AP-2 loci (construct M2/3).

Fig. 5 shows β-Gal staining of transgenic embryos made with mutated enhancers. The wild-type second intron produces reporter expression along the full length of the CNS (Zimmerman et al., 1994). On E11.5, the wild-type second intron produced strong expression throughout the CNS except for dorsal regions of the telencephalon and mesencephalon (Fig. 5A). Mutation of the upstream octamer (construct M1) had no effect on the pattern of reporter expression. M1 embryos at E11.5 had strong CNS-specific staining except in the dorsal forebrain and midbrain (Fig. 5D), and complete CNS staining at E12.5 and E13.5 (Fig. 5E,F). This gradual spread of nestin reporter expression from the ventral to dorsal CNS has been reported for the intact nestin enhancer (Zimmerman et al., 1994). Similarly, mutation of both AP-2 sites (construct M2/3) had no effect on CNS-specific β-Gal activity. Complete staining of the CNS is observed at both E12.5 and E13.5 (Fig. 5B,C). Thus three of the transcription factor binding sites identified in vitro appear to be dispensable for CNS-specific activation in vivo.

Mutation of the hormone response element (construct M5) indicates that this element is critical for a portion of the nestin CNS expression pattern. Reporter expression in M5 embryos is normal at E11.5 (Fig. 5G). However, by E12.5 it is apparent that reporter expression is much weaker in the telencephalon than in the rest of the CNS at an age when the entire CNS should be positive (compare Fig. 5H and 5B). This poor expression in the telencephalon is still apparent at E13.5 (Fig. 5I) and E14.5 (data not shown). It appears that the hormone response element is necessary for reporter expression only in telencephalon and dorsal mesencephalon.

Mutation of the downstream octamer (construct M4) has a

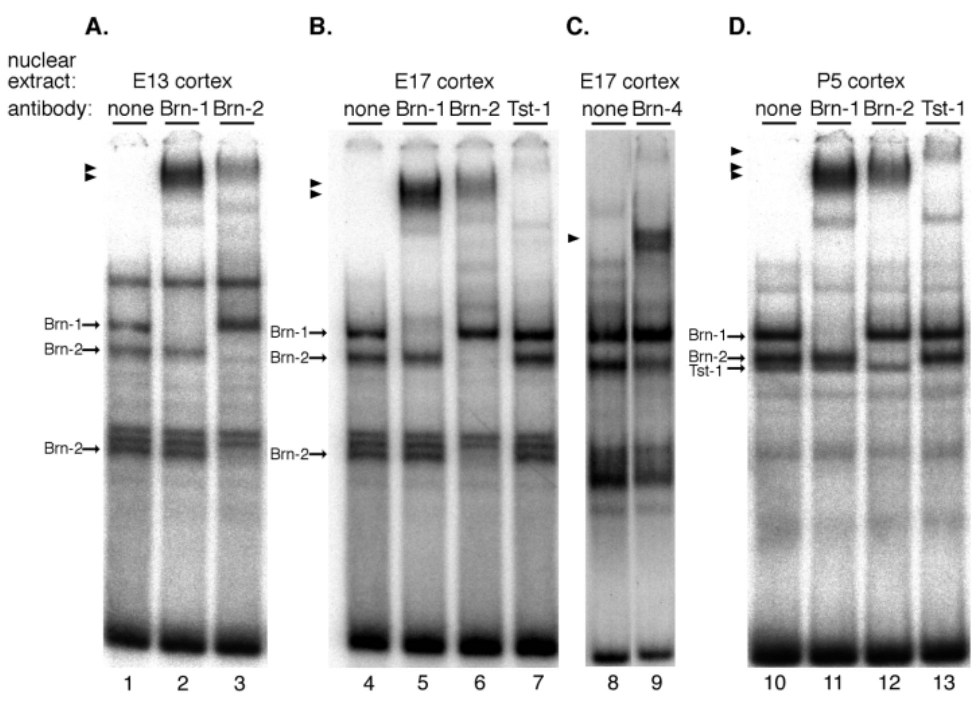


Fig. 4. The two octamer sites in the nestin enhancer are recognized by the class III POU transcription factors. (A) Electrophoretic mobility shift assay (EMSA) of Ftpt4 probe with E13 rat cortex nuclear extract shows six protein-DNA complexes (lane 1). Addition of antibody against Brn-1 disrupts one complex (Brn-1, lane 2) and produces a supershift (arrowhead). Brn-2 antibody supershifts two other bands (Brn-2, lane 3). (B) EMSA of Ftpt1 probe with nuclear extract from E17 rat cortex. Five complexes are produced (lane 4). Antibodies against Brn-1 (lane 5) or Brn-2 (lane 6) supershift bands migrating similarly to those seen in E13 extract. Antibody to Tst-1 has little effect (lane 7). (C) EMSA of Ftpt1 probe with nuclear extract from E17 rat cortex. Antibody against Brn-4 (lane 9) produces a supershift (arrowhead), but only slightly disrupts the two Brn-2 complexes. (D) EMSA of Ftpt4 probe with nuclear extract from P5 rat cortex. Two strong protein-DNA complexes are seen (lane 10), and these are supershifted by antibodies against Brn-1 and Brn-2 (lanes 11 and 12). A weak band just below the Brn-2 complex is supershifted by antibody against Tst-1 (lane 13).

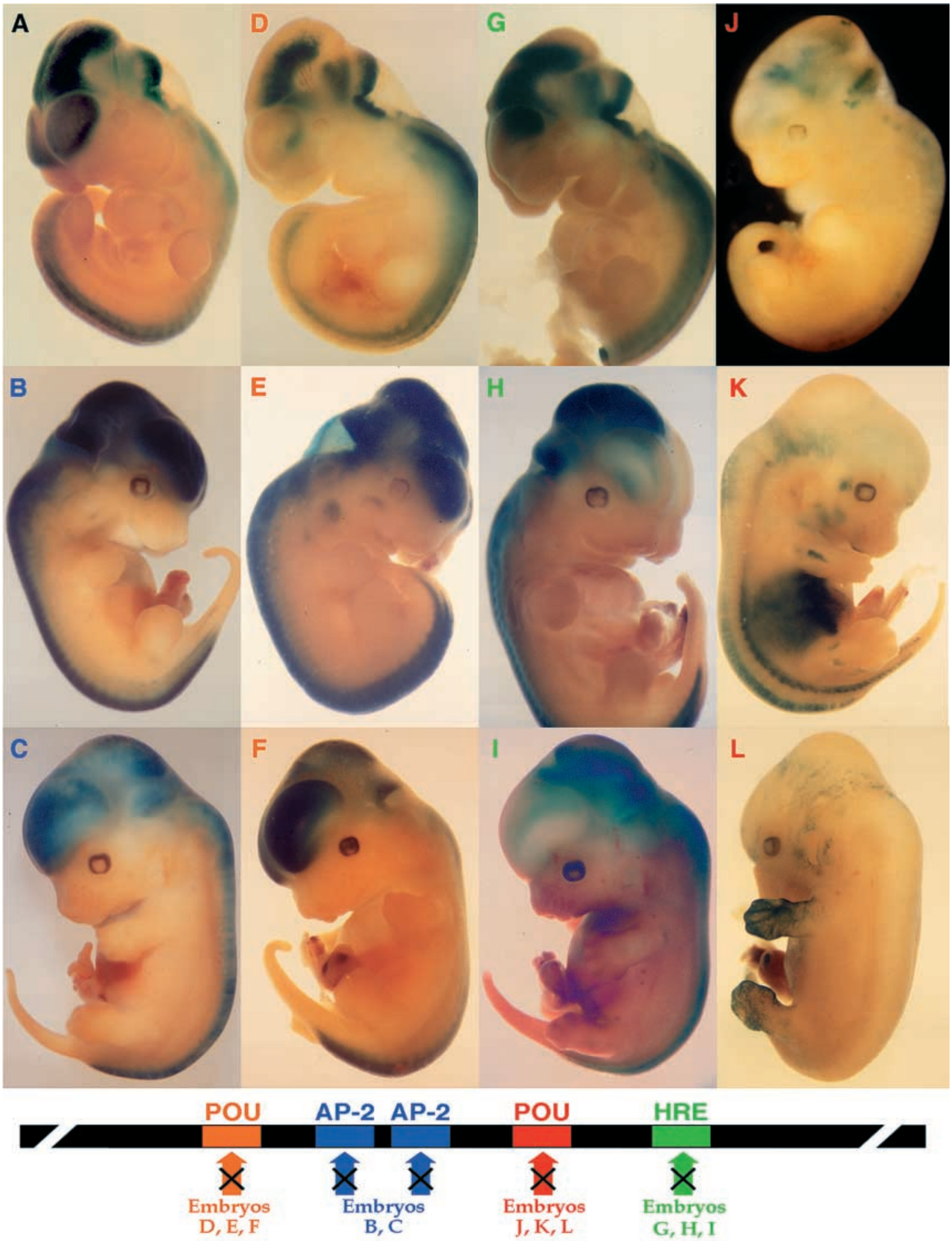


Fig. 5. Mutations of single binding sites within the nestin second intron disrupt CNS expression. (A) Transgenic embryo made with the wild-type nestin second intron (construct A), whole-mounted at E11.5 and stained for β-Gal. At this age, wild-type reporter expression is weak in the dorsal telencephalon and mesencephalon. (B,C) Embryos at E12.5 and E13.5 made with a mutation of both AP2-like sites at footprints 2 and 3 (construct M2/3). These embryos show the wild-type pattern of reporter expression throughout the CNS. (D,E,F) Mice at E11.5, E12.5 and E13.5 made with a mutated octamer site at footprint 1 (construct M1). This mutation does not disturb the normal reporter expression at each age. (G,H,I) Mice at E11.5, E12.5 and E13.5 made with a mutated hormone response element at footprint 5 (construct M5). Reporter expression at E11.5 is strong in all regions of the CNS except the anterior telencephalon and dorsal mesencephalon, similar to wild type (compare A and G). At E12.5 and E13.5 (H and I), expression remains weak in the telencephalon and dorsal midbrain. (J,K,L) Mice at E12.5, E13.5 and E13.5, made with a mutated octamer site at footprint 4 (construct M4). Weak expression in varying portions of the CNS is seen in a few embryos, but there is no strong, consistent CNS expression.

significant effect on the CNS-specific activity of the enhancer. M4 embryos had at most partial staining of the CNS (5 of 12 transgenics), and the pattern varied in each embryo (Fig. 5J,K,L). This is a striking contrast to the upstream octamer site, which was unnecessary for CNS-specific staining. Significant but variable non-neural staining was also observed in some of the M4 embryos. This result indicates that the downstream octamer site is required for full CNS-specific expression.

A CNS-specific element is located in the B-FABP proximal promoter

The B-FABP gene has a pattern of expression similar to that

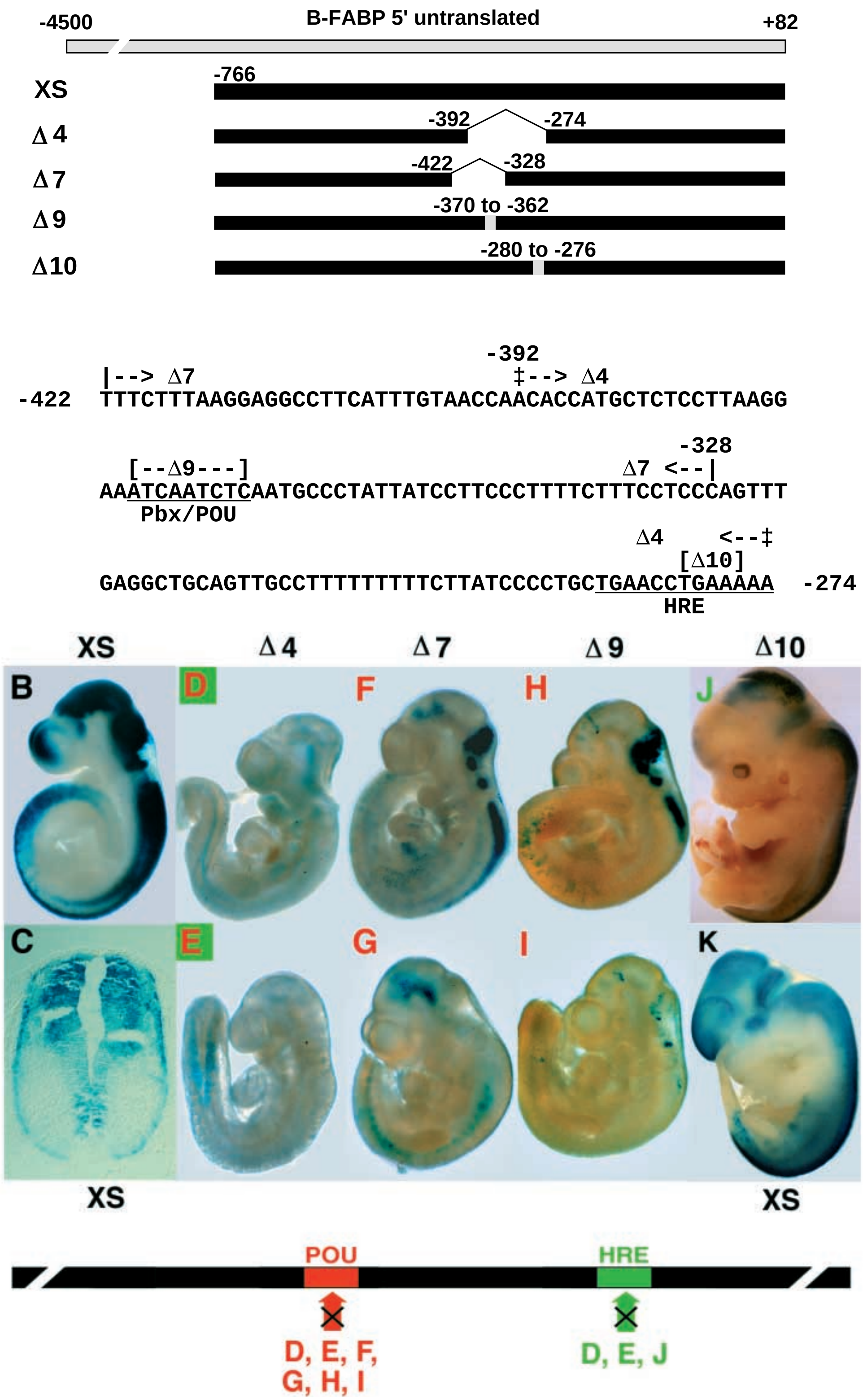


Fig. 6. Deletion analysis of the B-FABP upstream promoter. Segments of the B-FABP 5′ flanking region, including the endogenous promoter, were linked to the *lacZ* reporter gene. (A) A schematic map of the constructs shows the limits of the deleted regions and their nucleotide sequences. The **XS** fragment is sufficient for strong expression throughout the developing CNS of E10.5 and E12.5 transgenic embryos (B and K). XS drives expression in the ventricular zone of E11.5 spinal cord (C). Several deletions were made within the XS construct, of which the 118 bp deletion **Δ4** had the greatest effect: there was little expression anywhere in E10.5 embryos (D,E). An overlapping deletion, **Δ7**, allowed CNS expression from the rhombic lip through the anterior cord (F). Other Δ7 transgenic embryos showed less CNS expression (G). The 64 bp removed by both Δ4 and Δ7 contain a putative Pbx/POU binding site, which was deleted to make the **Δ9** transgene. Δ9 mice matched the Δ7 animals in pattern and frequency of expression, indicating that this site was critical for expression outside of the hindbrain and anterior spinal cord (H,I). A putative hormone response element resides within the Δ4 deletion. A five nucleotide deletion within the HRE, **Δ10**, produces a transgene which gave poor expression in the forebrain of E12.5 embryos (J) but in all other CNS regions performed similarly to the complete XS fragment (compare to K).

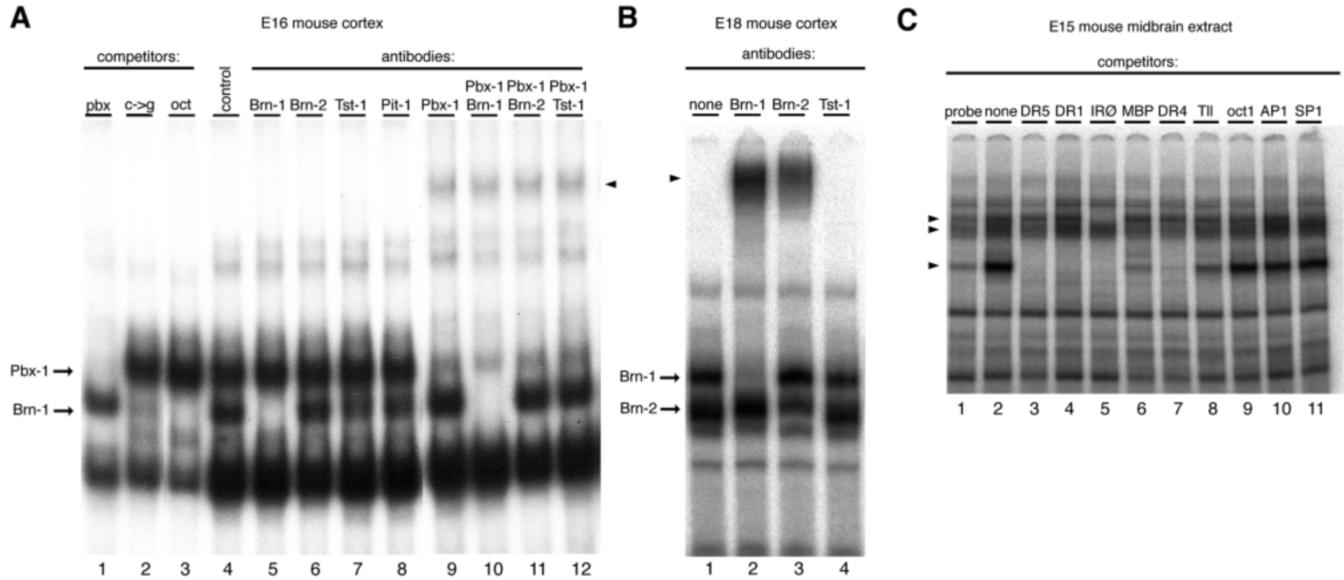


Fig. 7. Supershift assays show that the B-FABP promoter contains a Pbx/POU binding site and a hormone response element. (A) EMSA on an oligonucleotide probe containing the Pbx site of the B-FABP promoter. Nuclear extract from E16 mouse cortex forms three protein-DNA complexes on the labeled probe (lane 4). One hundred-fold excess unlabeled oligonucleotide containing the Pbx consensus site competes away the uppermost band (lane 1). An oligonucleotide identical to the probe except for a one basepair mutation competes away only the middle band (lane 2), as does an oligonucleotide containing the octamer consensus site for POU proteins (lane 3). The lowest band is not sequence-specific (not shown). Addition of antibody against Brn-1 protein disrupts the middle band (lane 5), while antibody against Pbx1 produces a supershift (arrowhead) and partially disrupts the uppermost band (lane 9). (B) EMSA on the same Pbx site probe, using nuclear extract from E18 mouse cortex. Multiple specific complexes are formed, but two bands are strongest (lane 1). Antibody against Brn-1 disrupts the upper of these (lane 2) and produces a supershift (arrowhead). Antibody against Brn-2 partially disrupts the lower complex and produces a supershift (lane 3). (C) EMSA on an oligonucleotide probe containing the B-FABP hormone response element. Nuclear extract from E15 midbrain forms three sequence-specific complexes (arrowheads). These complexes are competed to varying degrees by 50-fold excess oligonucleotides bearing consensus binding sites for nuclear hormone receptors, including all-trans-retinoic acid receptors (lane 3), 9-cis-retinoic acid receptors (lane 4), thyroid hormone receptors (lanes 5-7), and the orphan receptor tailless (100-fold excess; lane 8). No competition of any complex is given by POU, AP-1, or SP-1 consensus sites (lanes 9-11).

of nestin and correlates with the distribution of stem cells. Feng and Heintz (1995) showed that a region between 0.3 kb and 0.8 kb upstream of the B-FABP gene is sufficient for developmentally regulated reporter expression throughout the fetal CNS. We have used the same proximal promoter fragment (−766 to +82 bp) to drive *lacZ* expression in transient transgenic mice. This fragment shows high levels of expression on E10.5 (Fig. 6B), at an age when B-FABP mRNA is detected throughout the neuroepithelium in most of the CNS (Kurtz et al., 1994). Reporter expression is continuous throughout the CNS except for the dorsal telencephalon and a small gap in the hindbrain at the level of rhombomere five. In a section through the spinal cord at E11.5 (Fig. 6C) reporter expression is seen throughout the ventricular zone and at the pial surface, where it marks the endfeet of radial glia. Unlike nestin, expression is not seen in the floorplate or dorsal midline.

To further localize the transcriptional control elements of the B-FABP promoter, a series of seven overlapping deletions in the 0.8 kb fragment were generated by PCR (Fig. 6A). Of these, a deletion of 118 bp (Δ4) removed promoter sequences from −392 to −274. This deletion completely eliminated CNS expression in transgenic mice (Fig. 6D,E). An overlapping deletion, Δ7, was made distally from −422 to −328. This 94 bp deletion allows limited CNS expression in some transgenic mice. Individual E10.5 embryos had various patterns of reporter expression, probably due to varying transgene insertion sites. The most extensive CNS expression was in hindbrain and anterior spinal cord, plus slight expression in ventral midbrain (Fig. 6F). All other embryos had less extensive or no CNS expression (Fig. 6G). While the 94 bp deletion does not completely abolish CNS reporter expression, it is not possible with this deletion for expression to extend throughout the CNS.

The crucial portion of the B-FABP promoter contains a Pbx/POU binding site which is recognized by Pbx-1 and Brn-1

By inspecting the 64 basepairs deleted in both the Δ4 and Δ7 constructs, a 7 of 9 bp consensus binding site for Pbx proteins (ATCAATCtc) was found. Gel supershift assays were performed on this sequence in vitro, using protein from E16 mouse cortex. An oligonucleotide probe corresponding to the B-FABP promoter from −378 to −355 forms three complexes with E16 cortex (Fig. 7A). The fastest-migrating complex is not a sequence-specific activity, as demonstrated by competition assay (not shown). Antibody against Pbx-1 supershifts the upper specific complex but not the lower, while antibody against Brn-1 disrupts the lower complex. A second POU protein also binds to this site in extracts of E18 cortex (Fig. 7B). Antibodies against Brn-1 and Brn-2 supershift distinct complexes. Thus the B-FABP promoter contains a hybrid Pbx/POU binding site at −370 to −362, recognized by Pbx-1, Brn-1 and Brn-2.

The Pbx/POU site is necessary for full CNS expression

In the 0.8 kb B-FABP promoter, the 9 basepairs of the Pbx/POU binding site were deleted by PCR. Transgenic mice with this deletion had very limited reporter expression in the CNS; the *lacZ* product was seen predominantly in the hindbrain and anterior spinal cord (Fig. 6H,I). Overall, the expression pattern of the Pbx/POU mutation closely resembles that in the 94 bp deletion Δ7 (compare Fig. 6H with 6F). In both cases, expression is variable in each embryo but the greatest observed extent of CNS expression is limited to midbrain, hindbrain and anterior spinal cord. This shows that the Pbx/POU site is the critical element within the 94 bp deletion.

The B-FABP promoter Pbx/POU site is bound by both Pbx-1 and POUs in vitro. To determine whether both activities are required for promoter function, the Pbx/POU site (ATCAATCTC) was altered at the first cytosine residue to produce the sequence ATGAATCTC. This sequence is not bound by Pbx according to published data (Chang et al., 1996), however it resembles more closely the typical octamer site. The activity of this mutation was tested in vitro and in vivo. For in vitro tests, the mutant Pbx/POU site was synthesized as a double-stranded oligonucleotide competitor for EMSA. The oligo competes away the identified Brn-1 complex on the wild-type probe, but does not affect the Pbx-1 complex (Fig. 7A, lane 2). Thus a single basepair change in the Pbx/POU competitor creates exclusive recognition of the POU protein, Brn-1. Mice bearing the 0.8 kb proximal promoter with the same Pbx/POU site mutation which prevented binding of Pbx-1 nevertheless had strong and full CNS expression (not shown). These data indicate that Pbx-1 binding is dispensable while POU binding to the promoter is required for expression throughout the CNS.

The crucial region of the B-FABP promoter also contains a nuclear hormone response element

The 118 bp deletion Δ4 had a more severe effect on CNS expression than the 94 bp deletion Δ7 or Pbx/POU site deletion Δ9. Therefore it is likely that the 118 bp region contains additional sequences which contribute toward CNS expression. By inspection of the 54 bp of this sequence which do not overlap Δ7, a possible hormone response element was found at −286 to −275. The reverse strand (5′-TTTTCAGGTTCA-3′) resembles a direct repeat of the half-site (AGKTCA) for non-steroid nuclear hormone receptors. Gel shifts show that this sequence is recognized by multiple activities in E16 cortex nuclear extract (Fig. 7C). Competition with canonical binding sites for various non-steroid nuclear hormone receptors reinforces the conclusion that this sequence is a hormone response element. Binding sites typically recognized by thyroid hormone receptors and/or retinoid receptors were able to compete away the binding activity of the B-FABP hormone response element.

Five basepairs of the hormone response element were deleted from the 0.8 kb B-FABP promoter to make the construct Δ10. In E12.5 transgenic mice generated with the Δ10 reporter construct, expression was normal in most of the CNS. However, the level of expression in the telencephalon was low except for a patch of dorsal expression at the anterior extreme of the hemispheres (Fig. 6J) while expression from the wild-type promoter fills the telencephalon at a similar age (Fig. 6K). This expression pattern from the HRE deletion is remarkably similar to that produced by mutation of the nestin enhancer at its HRE (see Fig. 5I). The only notable difference between the two embryos is in the remaining expression in the telencephalon. In nestin enhancer mutants the ventrolateral portion of the hemispheres, just above the eyes, expresses the reporter; in B-FABP HRE mutants, expression is maintained only in the most anterior region.

DISCUSSION

Neuroepithelial precursor cells express the intermediate filament gene nestin and the brain fatty acid binding protein. In both genes a single regulatory region is sufficient for expression throughout the early CNS. In vitro assays show that the control regions of both genes are bound by class III POU proteins and unidentified nuclear hormone receptors. The POU sites are critical for establishing the CNS expression domain of each gene, and the hormone response elements are required for full expression in the rostral CNS.

Parallel transcriptional regulation

In the nestin gene a conserved 257 bp enhancer located in the second intron is sufficient to drive reporter transcription in all regions of the CNS, allowing proper onset and shutoff of expression. These results agree closely with those of Lothian and Lendahl (1997), who localized the CNS-specific element of the human nestin gene to 714 bp at the conserved 3′ end of the intron. They showed 78% sequence identity between the rat and human genes in the 257 bp sequence we have identified. The five footprinted sites identified in the rat enhancer are especially conserved: these sequences are identical in the two species at 19 of 19 bp, 15 of 19 bp, 21 of 25 bp, 13 of 16 bp, and 29 of 32 bp.

Of these five sites in the nestin enhancer, one is a hormone response element which is required for reporter activity in the dorsal portions of the forebrain and midbrain. Two are octamer sites recognized by the class III POU proteins Brn-1, Brn-2, Brn-4 and Tst-1. Deletion of the downstream octamer from the complete second intron allows only weak and inconsistent CNS activity. Therefore all additional transcriptional elements in the intron, including the hormone response element and the elements outside the 257 bp enhancer which drive weak midbrain, rhombic lip and spinal cord expression, are dependent upon the activity of this POU binding site.

In B-FABP, expression is controlled by a 1.6 kb upstream sequence that has been identified by Feng and Heintz (1995). Only the proximal 0.8 kb was required for early CNS expression. In this region, putative transcriptional control elements have been identified. One is a Pbx/POU site which is bound by Pbx-1, Brn-1 and Brn-2 in CNS nuclear extracts. Deletion of the Pbx/POU site abolishes expression in most of the neuroepithelium. Alteration of a single nucleotide eliminates Pbx-1 binding and retains both POU binding and expression in the early CNS. Thus we have identified a POU binding site in the B-FABP promoter which drives full CNS expression. Mutation of a nuclear hormone response element had an effect on reporter expression in the forebrain, which resembles a similar deletion in the nestin enhancer but does not affect dorsal midbrain expression. While the control derived from POU factors is widespread, both hormone response elements control expression only in the anterior regions of the neural tube.

These data suggest that POU proteins and nuclear hormone receptors cooperate to drive gene expression in anterior CNS. A number of studies have shown synergy between nuclear hormone receptors and the POU gene Pit-1 in the forebrain. Pit-1 sites synergize with estrogen, thyroid hormone, and retinoic acid response elements in the enhancers of the prolactin, growth hormone, and Pit-1 genes, respectively (see Rhodes et al., 1994 for review). Also, the POU domains of Brn-3a and Brn-3b bind the estrogen receptor (Budhram-Mahadeo et al., 1998). Cooperation between POU proteins and nuclear hormone receptors may be a general phenomenon.

A switch in POU expression

The class III POU proteins are candidate regulators of CNS-specific genes because they are widely expressed in embryonic and adult CNS (Alvarez-Bolado et al., 1995; He et al., 1989). Like nestin and B-FABP, Brn-1, Brn-2, and Brn-4 are broadly expressed in the embryonic neuroepithelium. Brn-2 is transcribed in the forebrain by E8.5 (Schonemann et al., 1995), which is close to the first appearance of nestin transcript at E7.75 (Dahlstrand et al., 1995) and well ahead of B-FABP emergence at E10. The other POU-III genes (Brn-1, Brn-4 and Tst-1) are all detected in the neuroepithelium by E10 (Alvarez-Bolado et al., 1995).

In the cortex, Brn-1 and Brn-2 are strongly expressed in the ventricular zone, and repressed when stem cells first differentiate into neurons (Alvarez-Bolado et al., 1995). They are often re-expressed in mature neurons. In contrast, Tst-1 in cortex is first expressed during migration and differentiation of layer 5 and layer 2/3 neurons (Frantz et al., 1994). POUs of two other classes, Brn-3a and Brn-5, are not expressed in proliferating precursors of the CNS but appear almost immediately upon neuronal differentiation (Andersen et al., 1993; Okamoto et al., 1993; Cui and Bulleit, 1997; Fedtsova and Turner, 1995). These results emphasize that the expression of POU genes is abruptly altered when neurons differentiate from their precursors.

Another POU protein, Oct-3, is only expressed in cells of the very early embryo and germ cells (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990). Oct-3 expression in headfold ectoderm is last seen at E8.5 (Rosner et al., 1990), close to the time of neural induction. Treatment of P19 embryonal carcinoma cells with retinoic acid produces neurons and other differentiated cell types while repressing Oct-3 (Okamoto et al., 1990). Antisense inhibition of Brn-2 expression in P19 cells specifically blocks the neuronal fate (Fujii and Hamada, 1993). Forced expression of Oct-3 in certain differentiated P19 cells extinguishes expression of Brn-2 and nestin (Shimazaki et al., 1993). These data suggest that a change in POU expression is required for the acquisition of neural potential. POU proteins could specify neural precursor identity in mammals as they do in *Drosophila* (Bhat et al., 1995; Yeo et al., 1995).

Additional functional information on POUs comes from homologous recombination experiments. Unfortunately, Brn-1 and Brn-4 deletions in mice have not been described. Disruption of Brn-2 results in the loss of the paraventricular nucleus (PVH) and supraoptic nucleus (SO) of the hypothalamus as well as the posterior pituitary (Nakai et al., 1995; Schonemann et al., 1995). In the absence of Brn-2, the neuroendocrine cells of the two hypothalamic nuclei die during migration or terminal differentiation. No other brain regions are grossly abnormal, and no disruption of development is seen prior to E12.5. Tst-1 is expressed in dividing glial cell precursors in the CNS and PNS (Collarini et al., 1991; Monuki et al., 1990; Monuki et al., 1993). Deletion of Tst-1 prevents Schwann cell differentiation (Bermingham et al., 1996; Jaegle et al., 1996). Thus deletions of individual POU genes affect only a few postmitotic cell types. However, POU functions in dividing CNS cells may be complemented by redundant family members.

In conclusion, we propose a novel role for POU proteins in mitotic neuroepithelium. Gene targeting shows that individual POUs are required for differentiation of select tissues. However, our data indicate that multiple class III POUs regulate the transcription of diverse genes in CNS stem cells. Thus, expression of a set of class III POU proteins could define the stem cell state. Exit from this state is accompanied by changes in POU expression, with Brn-1, Brn-2, and Brn-4 widely shut off while Brn-3a and Brn-5 are turned on in newly postmitotic cells. Creation of the stem cell state during neural induction may also involve a switch in POU expression. This is suggested by overexpression of the *Xenopus* Brn-4 homolog XlPOU-2, which induces neural markers in ectoderm (Witta et al., 1995). Ectodermal cells, like P19 cells, may be required to shut off Oct-3 and express class III POUs to become neural. Thus switches in POU expression may control both the acquisition and the loss of stem cell characteristics in the CNS.

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