Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 10841–10845, November 1993
Biochemistry

Brn-3.0: A POU-domain protein expressed in the sensory, immune, and endocrine systems that functions on elements distinct from known octamer motifs

(c-*myc*/pituitary/corticotroph)

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Communicated by Daniel Steinberg, July 8, 1993

ABSTRACT **Characterization of Brn-3.0 and identification of a highly related member (Brn-3.1) of the class IV POU-domain family suggest potential roles of Brn-3.0 in the development of retinal ganglion cells and sensory neurons, as well as potential roles in the pituitary gland and the immune system. Brn-3.0 is expressed in the pituitary gland and in a corticotroph cell line. A functional DNA response element has been identified in the proopiomelanocortin promoter. In contrast to previously described mammalian POU-domain proteins, Brn-3.0 binds relatively ineffectively to known octamer DNA motifs, but instead binds with high affinity to a distinct set of DNA elements, functioning as a transcriptional activator. Brn-3.0, Brn-3.1, and the *Drosophila* tI-POU share an N-terminal region of homology, referred to as the "POU-IV box," which is similar to a conserved functional domain in the c-myc gene family.**

Comparison of three mammalian transcription factors, Pit-1, Oct-1, and Oct-2, as well as the *Caenorhabditis elegans unc-86* gene product, led to the identification of a gene family distinguished by a DNA-binding motif referred to as the POU domain (1–8). Numerous additional POU-domain genes have subsequently been identified (9–11), including Brn-1, Brn-2, Brn-3, Tst-1/SCIP/Oct-6, Brn-4, Oct-3/4, and Skn1-a/i in mammals (9–18). While all contain the bipartite DNA-binding domain composed of the POU-specific domain and the POU homeodomain, POU domain factors can be separated into six classes (POU-I to POU-VI) (9). Two members of the POU-domain gene family (unc-86 and Pit-1) have been demonstrated to serve as regulators of cellular differentiation. Pit-1 exerts critical roles in the development of three cell types in the mature anterior pituitary gland (19), but it is not required for the appearance of the proopiomelanocortin (POMC)-producing corticotroph cell type. unc-86 is required in *C. elegans* for the proper development of several sensory neuronal lineages (8).

By analogy to the actions of unc-86 and Pit-1, it is likely that the Brn-3 gene family will prove to exert determining roles during neurogenesis. In this manuscript, we characterize Brn-3.0 and identify a distinct highly related member of the POU-IV class, referred to as Brn-3.1, and identify the Brn-3.0 and Brn-3.1 response elements.

MATERIALS AND METHODS

Isolation and Analysis of Brn-3- and Brn-3.1-Specific cDNA and Genomic Clones. Nine genomic and seven cDNA clones of the Brn-3.0 family members (Brn-3.0, -3.1, and -3.2) were isolated from a mouse genomic library (λFix II) and a λgt11 trigeminal ganglion cDNA library (1), and the coding region was sequenced by the Sanger dideoxynucleotide (20) or chemical cleavage (21) method.

RNase Protection, *in Situ* Hybridization, DNA Binding, and Transfection Analyses. RNase protection assays were performed using *Dra* II-linearized pBKSII-Brn-3.0 (306-bp Brn-3.0 *Xho* I fragment) as template for T7-directed synthesis of a radiolabeled antisense RNA probe (18–22), using L32 as a control for cell line RNAs. *In situ* hybridization was performed using RNA probes corresponding to either the 400-bp 3′ untranslated Brn-3.0 information or to the first 270 bp of the Brn-3.1 cDNA (23). Binding assays used *in vitro*-translated Brn-3.0 and mutant Brn-3.0 proteins or a purified bacterially expressed glutathione *S*-transferase fusion protein (23) with DNA binding sites previously described (15) or CE-1 (24), 5′-GATCCGGGTCTGTGCTAACGC-CAGCCTCCGCACTTTCA-3′; CE-2, GATCCGAGTG-GAGATCCAACAGCATCCTTAATTAAGTTCCT-3′; DE-2 (24), 5′-GATCCTCCTCATTAGTGATATTTACCT-CAAATGC-3′; −113 to −134 bp of the rat corticotrophin-releasing factor (CRH) promoter (25), 5′-AGGGCCCTAT-TATTTATGCAGGAGCA-3′; or interleukin 2 proximal octamer binding site (26), 5′-GGAAGGATCCTTTGAA-AATATGTGTAATATGTAAAACATTTTG-3′. For relative affinity determinations, bound protein was challenged with excess unlabeled oligonucleotide, and equivalent aliquots were immediately loaded onto a 6.5% polyacrylamide gel. Retarded complexes were quantitated on a Molecular Dynamics PhosphorImager system. GraphPad data were fitted to a four-parameter logistic equation with the slope factor set to −1. CV-1 cells were cotransfected with 0.3 μg of expression plasmid and 1.7 μg of reporter plasmid as described (1).

RESULTS

Characterization of Brn-3.0 and Brn-3.1 and Identification of the "POU-IV Box". The open reading frame of Brn-3.0 cDNA predicted a 43-kDa protein containing 421 amino acid residues (Fig. 1*A*). Analysis of isolated mouse Brn-3.0 genomic clones revealed a 730-bp intron between codons 41 and 42 in the mature transcripts and a 1.3-kb intron in the 3′ untranslated region, located 48 nt 3′ of the translation termination codon. We have also identified two additional distinct highly related gene transcripts [Brn-3.1 (Fig. 1*B*) and Brn-3.2]. Both exhibit a distinct ontogeny and both genes contain an intron in the N-terminal coding region (data not

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Abbreviations: CRH, corticotrophin-releasing factor; POMC, proopiomelanocortin; e, embryonic day.

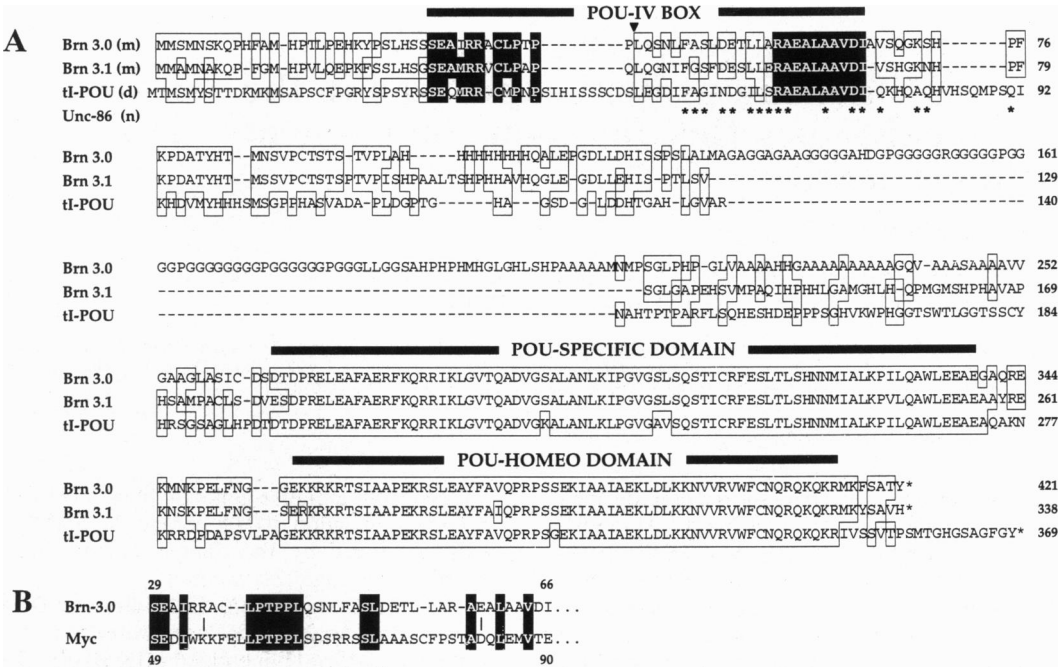

FIG. 1. POU-IV class homologies. (*A*) Comparison of Brn-3.0 amino acid sequence with Brn-3.1 and tI-POU. The POU-IV box is overlined; the residues with the highest homology are set in white with a black background. The splice junction between exons 1 and 2 of Brn-3.0 is shown as a solid triangle. m, Mouse; d, *Drosophila*; n, nematode. (*B*) Region of similarity between Brn-3.0 and Myc (amino acids 49–90 of chicken c-myc).

shown). While the N terminus of Brn-3.0 and Brn-3.1 are relatively divergent from those of unc-86, we note a 40-amino-acid region of similarity, referred to as the "POU-IV box," including a 10 amino acid sequence entirely conserved between Brn-3.0, Brn-3.1, and the presumptive *Drosophila* homolog, tI-POU (Fig. 1*B*). The N-terminal portion of the box is similar to a highly conserved domain found in the N terminus of all c-myc family members (27) (Fig. 1*B*).

Expression of Brn-3.0 and Brn-3.1 in the Immune, Endocrine, and Nervous System. The pattern of Brn-3.0 and Brn-3.1

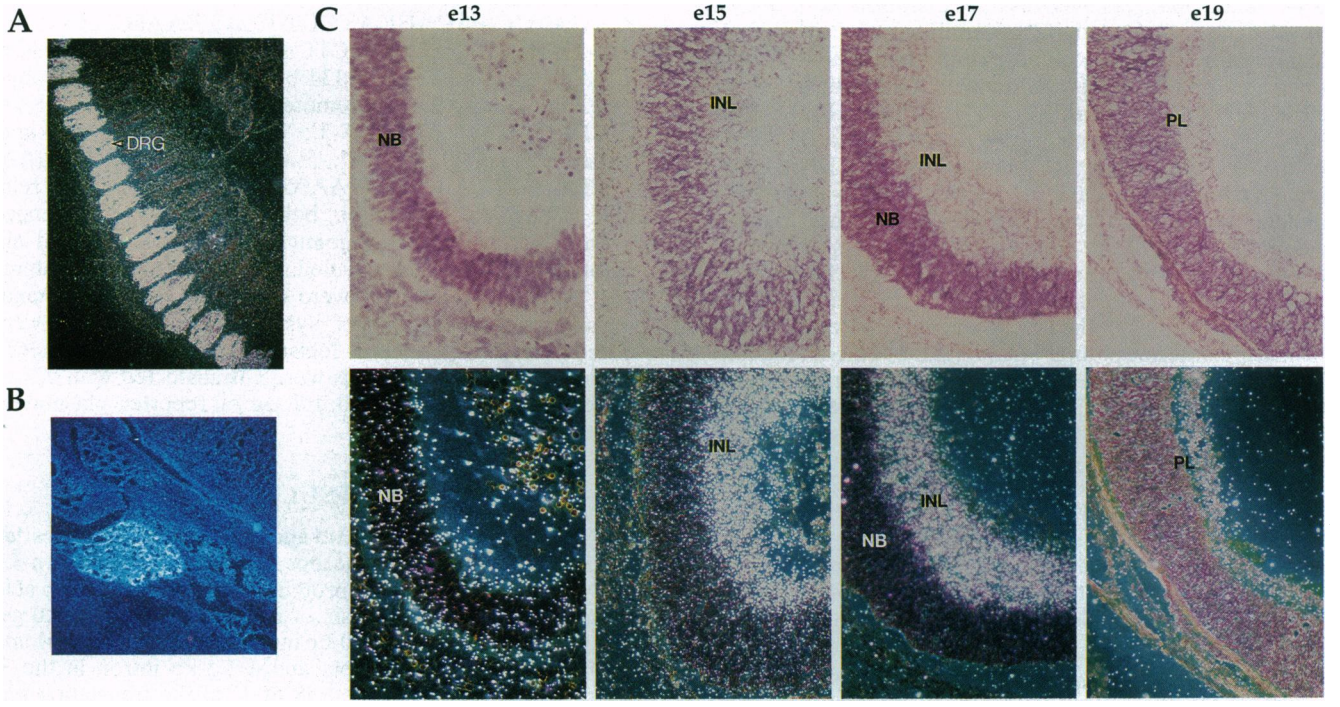

FIG. 2. Brn-3.0 mRNA expression in the developing embryo. Parasagittal sections through an embryonic rat were hybridized with an ^{35}S-labeled antisense Brn-3.0 RNA probe. (*A*) Hybridizing regions include e13 dorsal root ganglion (DRG). The section was counterstained with hematoxylin/eosin (23). (×5.) (*B*) e17 trigeminal ganglion. The section was counterstained with bisbenzimide. (×20.) (*C Upper*) Brn-3.0 mRNA expression in the developing retina. Transmission photomicrographs were counterstained with hematoxylin/eosin. (e13, ×70; e15–e19, ×35.) (*C Lower*) Autoradiographic analysis of *in situ* hybridization analyses. Increased silver grain density is indicated in the inner edge of the developing neuroblastic layer (INL) on e15 and e17, with no signal in the outer neuroblastic layer (NB). On e19, Brn-3.0 transcripts are present in the inner plexiform layer (PL) but at a reduced level of signal.

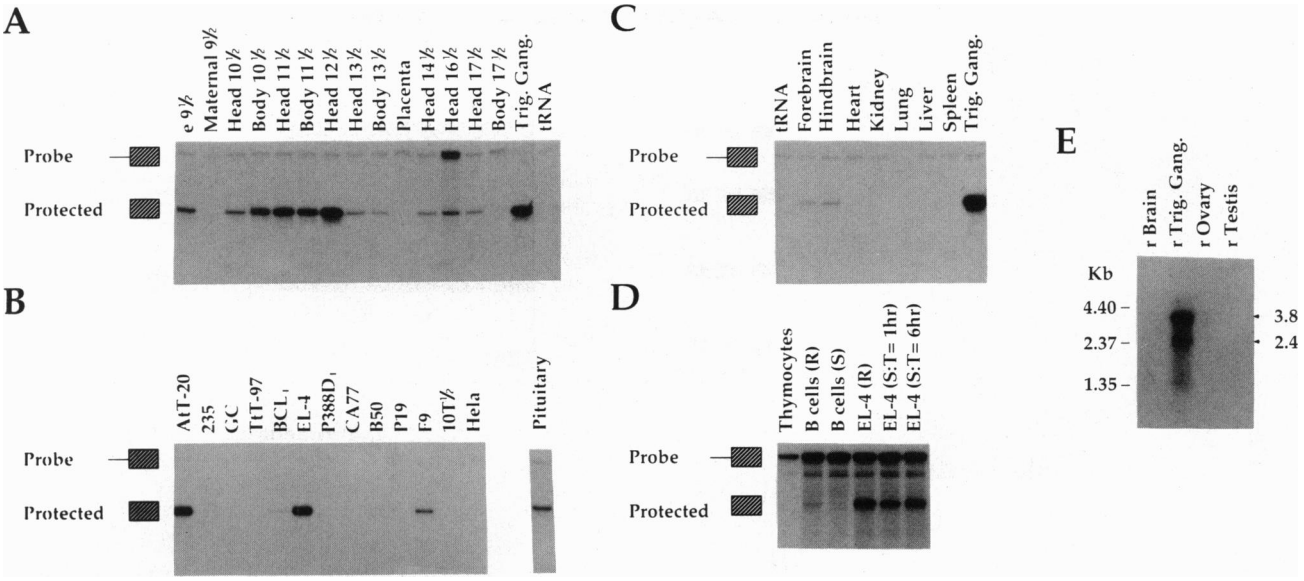

FIG. 3. Analysis of Brn-3.0 transcripts and gene expression. (*A*) Ontogeny of Brn-3.0 expression in the mouse embryo was examined by hybridizing total RNA (20 mg) prepared from whole embryo (e9.5) or embryonic head and/or body (e10.5–e17.5). Maternal e9½ refers to adjacent endometrium plus myometrium. Trig. Gang., trigeminal ganglia. (*B*) Expression of Brn-3.0 in the mouse corticotroph (AtT-20), mouse T (EL-4), mouse pre-B (BCL_1), and mouse embryonic carcinoma (F9) cell lines. Analyses used 5 μg of total RNA except for the mouse pituitary (≈200 mg of total RNA) and EL-4 and BCL_1 (total RNA from 5×10^5 cells). (*C*) Tissue distribution of Brn-3.0 transcripts: trigeminal ganglion (Trig. Gang.), 4 μg of total RNA; mouse forebrain and hindbrain, 20 μg total of RNA; other samples, 20 μg total RNA. tRNA, yeast tRNA. (*D*) For thymocytes and B cells, total RNA from 3×10^6 cells was hybridized; for EL-4, RNA from 2.5×10^5 cells was used. Phorbol 12-myristate 13-acetate stimulation of EL-4 cells (10 ng/ml). R, resting; S, stimulated; T, time. (*E*) Analyses used 5 μg of poly(A)-selected RNA. r, rat.

expression was reassessed by *in situ* hybridization using specific probes. Brn-3.0 was highly expressed in dorsal root ganglia (Figs. 2*A* and 3*C*) and also in the fifth cranial nerve (Fig. 2*B*). While Brn-3.1-specific probes hybridized weakly to sensory ganglia and dorsal horn cells (data not shown), Brn-3.0 transcripts were detected by RNase protection from all stages of murine development tested [embryonic day (e) 9.5–e19.5], with a progressive increase from e9.5 to e12.5, followed by an apparent decrease between e12.5 and e13.5 (Fig. 3*A*). High levels of Brn-3.0 transcripts (2.4 and 3.8 kb) were present in mature mouse trigeminal ganglia (Fig. 3*E*).

Brn-3.0 gene expression was also developmentally regulated in the retina; Brn-3.0 transcripts were not detectable in undifferentiated neuroblasts (e13) but initially appeared at high levels at the inner edge of the retinal neuroblastic layer at e15 (Fig. 2*C*), when ganglion cells begin differentiating and axonal outgrowth was initiated (28, 29). High levels of expression, limited to the ganglion cell layer, were maintained through e17 and e19 (Fig. 2*C*), but retinal Brn-3.0 hybridization signals decreased after birth (data not shown). In addition to expression in neural tissue, Brn-3.0 was expressed at low levels in the pituitary gland (Fig. 3*B*), below detection by *in situ* hybridization. Brn-3.0 was expressed in the corticotroph cell line (AtT-20) but not in somatotroph (GC), lactotroph (235, MMQ), or thyrotroph (TtT-97) cells (Fig. 3*B*). The BCL_1 B-cell line and the EL-4 T-cell line contained measurable levels of Brn-3.0 transcripts, whereas the macrophage-like $P388D_1$ line scored as negative (Fig. 3*B*). A transient 2- to 3-fold reduction of Brn-3.0 expression was observed at 1 h in EL-4 cells treated with phorbol 12-myristate 13-acetate (Fig. 3*D*). No apparent expression of Brn-3.0 could be detected in adult thymocytes, whereas splenic B cells clearly exhibited Brn-3.0 expression (Fig. 3*D*).

Brn-3.0 Functions on a Distinct Type of DNA Response Element. Although all POU-domain proteins thus far studied bound effectively to octamer DNA elements (9–11), Brn-3.0 was surprisingly ineffective in binding either the herpes simplex virus or the proximal interleukin 2 promoter octamer elements (26) and bound only weakly to the immunoglobulin octamer/heptamer site (Fig. 4*A*). However, a high-affinity DNA-binding site for Brn-3.0 (Fig. 4*A*) was identified as a high-affinity Brn-2 recognition sequence in the CRH promoter (34). Competition analyses reveal that octamer sites have a 20- to 30-fold lower affinity than the CRH site (Fig. 4*B*).

Brn-3.0 produced a 20- to 30-fold stimulation of a minimal promoter containing CRH DNA-binding sites but failed to transactivate promoters under control of a series of octamer elements, including the octamer/heptamer IgH site (Fig. 4*C*), indicating that octamer sites tested were not Brn-3.0 response elements. Systematic mutation of the GCATAAATAAT CRH site revealed that binding was selectively abolished or severely compromised only with mutations of the CAT or TAAT sequences, respectively (Fig. 4*D*). Intriguingly, I-POU, a potent transcriptional inhibitor that was ineffective at binding previously known DNA sites, is capable of binding to the CRH site (data not shown).

Elements involved in POMC gene activation (24, 30) include a CE2 site similar to the Brn-3.0 CRH binding sequence, except that the central nucleotides were CCT instead of AAA (Fig. 5*A*). DNase I footprint analysis and electrophoretic mobility-shift assay demonstrated that Brn-3.0 bound effectively to the CE-2 site (Fig. 5 *B* and *C*) and that Brn-3.0 was a major protein binding to the CE-2 site in AtT-20 cells (Fig. 5*C*) but not to the CE-1 or the DE-2 sites. Brn-3.0 was capable of transactivating the POMC promoter (−480 to +18) or a heterologous promoter containing multiple CE-2 sites (Fig. 5*D*).

DISCUSSION

Brn-3.0 and Brn-3.1 are expressed in the sensory nervous system, with initial appearance closely linked to the appearance of mature sensory neurons. The mammalian POU-IV family contains an N-terminal region conserved from *Drosophila* to mammals, referred to as the POU-IV homology box, similar to a domain that is conserved within all members of the c-*myc* gene family and transfers microtubule-

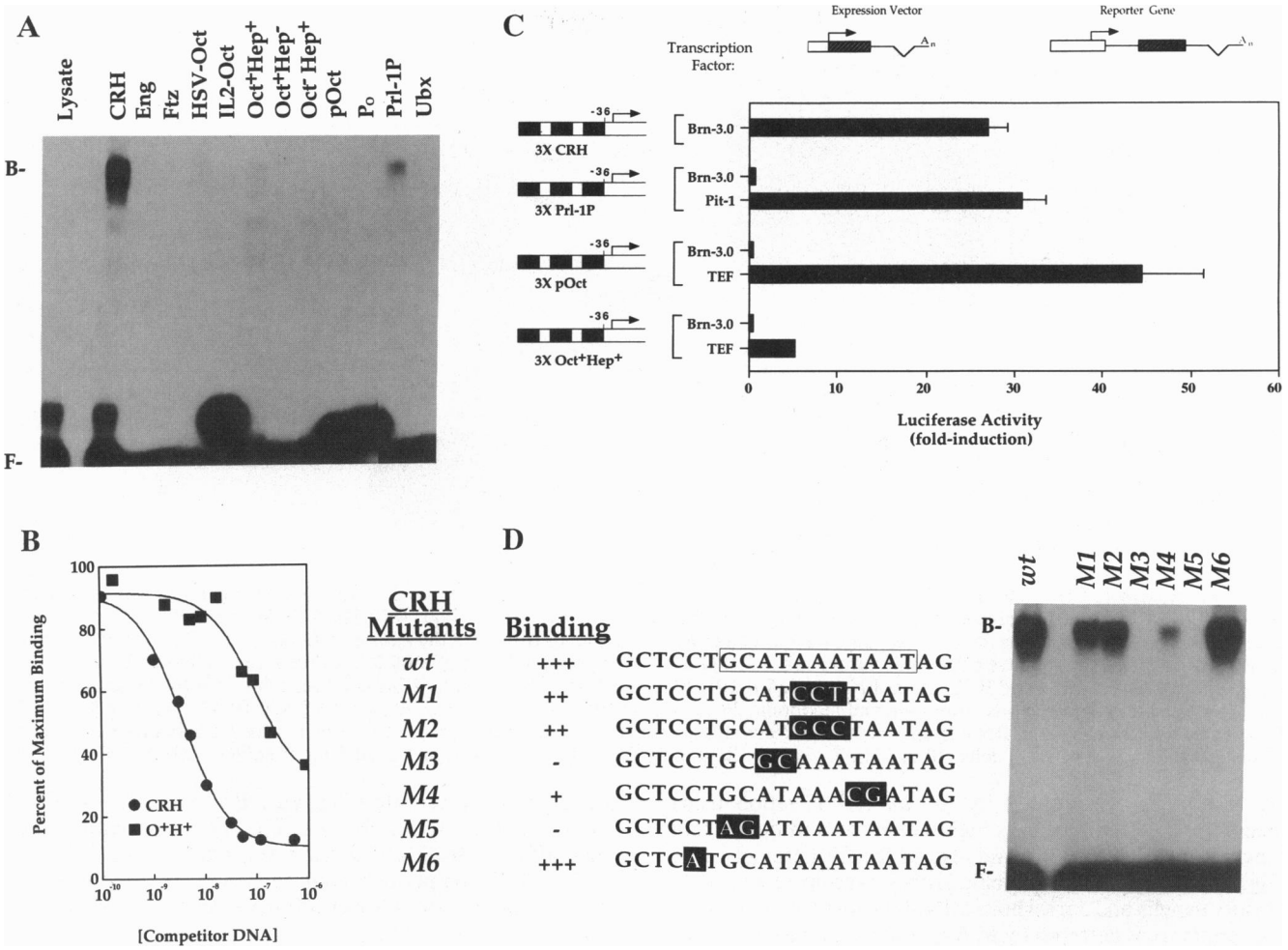

FIG. 4. DNA binding and transcriptional activity of Brn-3.0. (*A*) Electrophoretic mobility-shift assays employing the CRH II Brn-2 binding site (CRH), herpes simplex virus (HSV-Oct) and interleukin 2 (IL2-Oct) promoter octamer-binding sites, IgG octamer/heptamer (Oct^+Hep^+) promoter octamer-binding sites, Oct^+Hep^- and Oct^-Hep^+ mutations, the P_o promoter Tst-1 site (P_o), the pOct mutation of the PRL-1P binding site (pOct), the rat prolactin Pit-1 binding site (PRL-1P), and Eng, Ftz, and Ubx sites. (*B*) Oct^+Hep^- (O^+H^-) and CRH competition curves of Brn-3.0 binding to the CRH II site. Molar concentrations are as indicated. CRH $EC_{50} = 3.83 \times 10^{-9}$ M; O^+H^- $EC_{50} = 9.44 \times 10^{-8}$ M. (*C*) A Brn-3.0 eukaryotic expression vector was cotransfected into CV-1 cells with a reporter plasmid consisting of three CRH Brn-3.0 (3× CRH), PRL-1P Pit-1 (3× Prl-1P), Oct-1 (3× pOct), or Oct^+Hep^+ (3× Oct^+Hep^+) binding sites, fused to the rat prolactin minimal promoter (−36 to +33) upstream of a firefly luciferase reporter gene (1). pCMV/Pit-1 and pCMV/TEF cotransfecting provided positive controls and fold inductions relative to pCMV1 cotransfection (1). Average basal activities in light units per 10 sec per 100 μg of total protein were 372 for 3× CRH, 163 for 3× Prl-1P, 218 for 3× pOct, and 80 for Oct^+Hep^+. Results of triplicate determinations ± SEM are shown. (*D*) Binding activity of Brn-3.0 on the wild-type and mutant CRH sites determined by electrophoretic mobility-shift assay is denoted as follows: +++, wild-type (wt) binding; ++ and +, reduced binding; −, no detectable DNA binding.

associated protein 2 (MAP-2) kinase-dependent transactivation (31). Brn-3.0 is also expressed in a highly restricted fashion that links its expression to critical differentiation events in retinal ganglion cell development. In differentiating sensory neurons, the role of Brn-3.0 is potentially analogous to the role of unc-86 in several sensory neuronal lineages in *C. elegans* (32). We have presented evidence that suggests that Brn-3.0 is expressed, and may function, in pituitary and cells of the immune system. Brn-3.0 transcripts were present in cell lines representative of both the B- and T-lymphocyte lineages but absent in a macrophage-like cell line. We have found that Brn-3.0 is also expressed in normal splenic B cells and in nontransformed, cloned $CD4^+$ T cells derived from mice (M.V.H., unpublished observation). Because Brn-3.0 is expressed in a corticotroph cell line, in the pituitary gland, and can bind to and function on one element that can combinatorially target POMC transgene expression in transgenic mice (30), Brn-3.0 might exert functions in the complex molecular events that regulate expression of the POMC gene. Because Brn-3.0 binds with high affinity to a restricted set of high-affinity DNA sites that are structurally similar to putative regulatory elements in the *mec-3* gene promoter (33), but is relatively ineffective in binding to known octamer elements, Brn-3.0 and Brn-3.1 appear to exhibit unique properties among POU-domain proteins.

We gratefully acknowledge the contributions of B. Anderson, P. Li, and C. Nelson, D. Drolet, L. Erkman, and M. Wegner and the Preuss Foundation. E.T. is a Howard Hughes Physician Fellow and a National Association for Research on Schizophrenia and Depression Young Investigator. M.G.R. is an Investigator with Howard Hughes Medical Institute. These studies were supported by National Institutes of Health Grant MH47137. M.V.H. is supported by U.S. Public Health Service Grant AG09822 and the American Federation for Aging Research.

1. Ingraham, H. A., Chen, R., Mangalam, H. J., Elsholtz, H. P., Lin, C. R., Swanson, L. & Rosenfeld, M. G. (1988) *Cell* **55**, 519–529.
2. Bodner, M., Castrillo, J. L., Theill, L. E., Deerinck, T., Ellisman, M. & Karin, M. (1988) *Cell* **50**, 267–275.

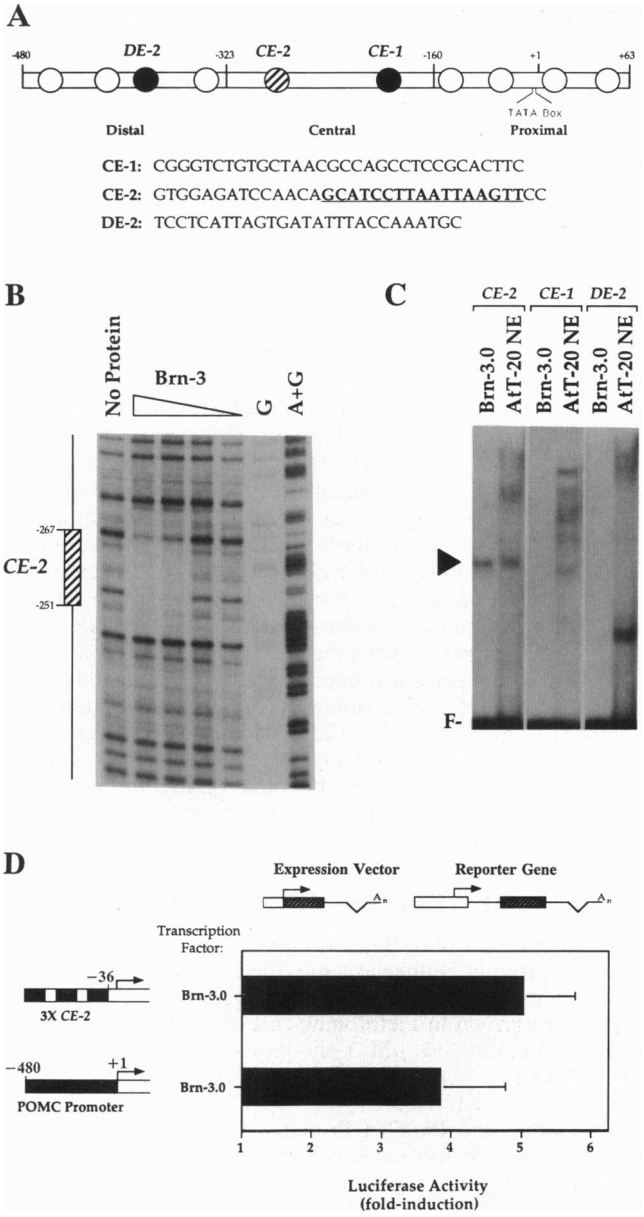

FIG. 5. Brn-3.0 binds and transactivates a regulatory cis-active element in the POMC gene promoter. (*A*) POMC promoter sequences necessary for tissue-specific expression (24, 30). (*B*) Localization of a Brn-3.0 binding site on the rat POMC promoter (−182 to −480) by DNAse I protection assay. The amount of Brn-3.0 bacterially expressed POU-domain protein added was, from left to right, 0 μg (No Protein), 8.75 μg, 4.38 μg, 0.875 μg, and 0.088 μg. G and G+A sequencing reactions are shown. (*C*) Mobility-shift analysis of *in vitro* Brn-3.0-translated protein or AtT-20 nuclear extracts (NE) on CE-2, CE-1, and DE-2 sites. (*D*) Cotransfection assays in CV-1 cells were used to determine the ability of Brn-3.0 to transactivate luciferase reported in the control of the prolactin minimal promoter (−36 to +33) with three CE-2 elements (3× CE-2) or −480 to +18 bp of the POMC promoter. Basal activity in light units per 10 sec per 100 μg of total protein was 478 for 3× CE2 and 8329 for the POMC promoter. Values given are the mean ± SEM (n = 3).

3. Clerc, R. G., Corcoran, L. M., LeBowitz, J. H., Baltimore, D. & Sharp, P. A. (1988) *Genes Dev.* **2,** 1570–1581.
4. Ko, H.-S., Fast, P., McBride, W. & Staudt, L. M. (1988) *Cell* **55,** 135–144.
5. Müller, M. M., Rupert, S., Schafner, W. & Matthias, P. (1988) *Nature (London)* **336,** 544–551.
6. Scheidereit, C., Cromlish, J. A., Gerster, T., Kawakami, K., Balmaceda, C.-G., Currie, R. A. & Roeder, R. G. (1988) *Nature (London)* **336,** 552–557.
7. Sturm, R. A., Das, G. & Herr, W. (1988) *Genes Dev.* **2,** 1582–1599.
8. Finney, M., Ruvkun, G. & Horvitz, H. R. (1988) *Cell* **55,** 757–769.
9. He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. W. & Rosenfeld, M. G. (1989) *Nature (London)* **340,** 35–42.
10. Suzuki, N., Rohdewohld, H., Neuman, T., Gruss, P. & Schöler, H. R. (1990) *EMBO J.* **9,** 3723–3732.
11. Rosner, M. H., Vigano, M. A., Ozato, K., Timmons, P. M., Piorier, F., Rigby, P. W. J. & Staudt, L. M. (1990) *Nature (London)* **345,** 686–691.
12. Schöler, H. R., Ruppert, S., Suzuki, N., Chowdhury, K. & Gruss, P. (1990) *Nature (London)* **344,** 435–439.
13. Monuki, E. S., Kuhn, R., Weinmaster, G., Trapp, B. D. & Lemke, G. (1990) *Science* **249,** 1300–1303.
14. Okazawa, H., Okamoto, K., Ishino, F., Ishino-Kaneko, T., Takeda, S., Toyoda, Y., Muramatsu, M. & Hamada, H. (1991) *EMBO J.* **10,** 2997–3005.
15. Mathis, M. J., He, X., Simmons, D. M., Swanson, L. W. & Rosenfeld, M. G. (1992) *EMBO J.* **11,** 2551–2561.
16. Hara, Y., Rovescalli, A. C., Kim, Y. & Nirenberg, M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3280–3284.
17. Le Moine, C. & Young, W. S., III (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3285–3289.
18. Andersen, B., Schonemann, M., Flynn, S., Pearse, R., Singh, H. & Rosenfeld, M. G. (1993) *Science* **260,** 78–81.
19. Li, S., Crenshaw, E. B., Rawson, E. J., Simmons, D. M., Swanson, L. W. & Rosenfeld, M. G. (1990) *Nature (London)* **347,** 528–533.
20. Sanger, F., Nicklen, S. & Coulson, A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
21. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
22. Hobbs, M. V., Weigle, W. O., Noonan, D. J., Torbett, B. E., McEvilly, R. J., Koch, R. J., Cardenas, G. J. & Ernst, D. N. (1993) *J. Immunol.* **150,** 3602–3614.
23. Simmons, D. M., Voss, J. W., Ingraham, H. A., Holloway, J. M., Broide, R. S., Rosenfeld, M. G. & Swanson, L. W. (1990) *Genes Dev.* **4,** 695–711.
24. Therrien, M. & Drouin, J. (1991) *Mol. Cell. Biol.* **11,** 3492–3503.
25. Thompson, R. C., Seasholtz, A. F. & Herbert, E. (1987) *Mol. Endocrinol.* **1,** 363–367.
26. Kamps, M. P., Corcoran, L., LeBowitz, J. H. & Baltimore, D. (1990) *Mol. Cell. Biol.* **10,** 5464–5472.
27. van Beneden, R. J., Watson, D. K., Chen, T. T., Lautenberger, J. A. & Papas, T. S. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3698–3702.
28. Raedler, A. & Sievers, J. (1975) *Adv. Anat. Embryol. Cell. Biol.* **50,** 3–88.
29. Kuwabara, T. & Weidman, T. A. (1974) *Invest. Opthalmol.* **13,** 725–739.
30. Liu, B., Hammer, G. D., Rubinstein, M., Mortrud, M. & Low, M. J. (1992) *Mol. Cell. Biol.* **12,** 3978–3990.
31. Xue, D., Finney, M., Ruvkun, G. & Chalfie, M. (1992) *EMBO J.* **11,** 4969–4979.
32. Finney, M. & Ruvkun, G. (1990) *Cell* **63,** 895–905.
33. Seth, A.; Alvarez, E., Gupta, S. & Davis, R. J. (1991) *J. Biol. Chem.* **266,** 23521–23524.
34. Li, P., He, X., Gerrero, M. R., Mok, M., Aggarwal, A. & Rosenfeld, M. G. (1993) *Genes Dev.*, in press.