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Regulation of striatal D_{1A} dopamine receptor gene transcription by Brn-4

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ABSTRACT **Brn-4 is a member of the POU transcription factor family and is expressed in the central nervous system. In this study, we addressed whether Brn-4 regulates expression of the D_{1A} dopamine receptor gene. We found a functional Brn-4 responsive element in the intron of this gene by means of cotransfection chloramphenical acetyltransferase assays. This region contains two consensus sequences for binding of POU factors. Gel mobility-shift assays using glutathione *S*-transferase–Brn-4 fusion protein indicated that Brn-4 binds to these sequences. Both these sites are essential for transactivation by Brn-4 because deletion of either significantly reduced this enhancer activity. *In situ* hybridization revealed colocalization of Brn-4 and D_{1A} mRNAs at the level of a single neuron in the rat striatum where this dopamine receptor is most abundantly expressed. Gel mobility-supershift assay using rat striatal nuclear extract and Brn-4 antibody confirmed the presence of Brn-4 in this brain region and its ability to bind to its consensus sequences in the D_{1A} gene. These data suggest a functional role for Brn-4 in the expression of the D_{1A} dopamine receptor gene both *in vitro* and *in vivo*.**

POU transcription factors share a common bipartite DNA-binding domain that comprises an N-terminal POU-specific domain and a C-terminal homeodomain (1). Although the homeodomain is sufficient for recognizing specific DNA sequences, the POU-specific domain increases the binding affinity to the elements (2–4). POU members are expressed from the early embryo to adulthood in chronologically and topologically specific manners. Oct-3/4 is expressed in germ line cells and quickly disappears at the early embryo stage (5–7). On the other hand, POU-III subclass genes that include Brn-1, Brn-2 (8, 9), Brn-4/RHS2 (10, 11) and Tst-1/transcriptional repressor of myelin-specific genes/Oct-6 (9, 12–14) as well as POU-IV subclass genes that include Brn3.0/Brn-3 (9, 15) and Brn-3.2 (16) display specific and restricted expression patterns in the central nervous system (reviewed in refs. 17 and 18). These findings suggest that various POU family members play important roles in the central nervous system.

POU factors regulate specific molecules in the nervous system. In nonvertebrates, *Cf1a* regulates the expression of *Drosophila* dopa decarboxylase (19). In vertebrates, Pit-1 regulates prolactin and growth hormone gene expression in neuroendocrine cells (20–22), and a mutation in the human *PIT-1* gene results in combined deficiency of prolactin and growth hormone leading to dwarfism (23–25). Another POU factor, SCIP/Tst-1/Oct-6, modulates expression of the $\alpha 3$ acetylcholine receptor subunit gene and the P0 myelin gene (12, 13, 26–29). Despite the large number of POU transcription factors expressed in the central nervous system, information about their functions in neurons is limited. Additional knowledge about the relationship between POU factors and their neuronal target molecules is essential for a better understanding of the roles that these transcription factors play during brain development and in the adult organism. Since gene disruption studies evaluate the net outcome in the presence of other genes acting alternatively on the same biological system, we took classical approaches in this investigation to assess one of the functions of a specific POU transcription factor.

We previously reported that the human D_{1A} dopamine receptor gene is transcribed from a G+C-rich promoter (30) and is modulated by upstream activators to which as yet unknown transcription factors bind (31). It is noteworthy that the transcriptional activity of this gene is relatively strong even when the sequence 5′ to the core promoter is very short (30). Clusters of A+T-rich sequences are located among G+C-rich regions around the core promoter. We, therefore, tested whether POU transcription factors, which preferentially bind to A+T-rich sequence motifs (18), can modulate expression of the human D_{1A} gene. Brn-4 was selected as a candidate for this analysis because it is the most abundantly expressed POU factor in the striatum (9, 10). We found evidence for two functional Brn-4 responsive elements in the intron of the D_{1A} gene in both cultured neuroblastoma cells and in the rat striatum.

MATERIALS AND METHODS

Construction of Plasmids. Effector plasmids were made by subcloning the full-length cDNA of Brn-4 and Brn-2 into a eukaryotic expression vector directed by the cytomegalovirus (CMV) promoter (32). DD series of chloramphenicol acetyltransferase (CAT) reporter plasmids were constructed with D_{1A} promoter fragments that were PCR-amplified from plasmid pCAT-HD1G-D (30) by using the following sense primers: F, 5′-AAAAAGCTTGTGCCCGCGGGAA-3′ (−1108 to −1096); F1, 5′-AAAAAGCTTGTGCCAGCTCGGCTC-3′ (−1071 to −1057); F2, 5′-AAAAAGCTTCGAGCGCCCAGGAGC-3′ (−871 to −857); F3, 5′-AAAAAGCTTCGGAGCCGCTGCCCC-3′ (−671 to −657); F4, 5′-AAAAAGCTTCTGCTGAGGAGCCAG-3′ (−471 to −457); and antisense primers R, 5′-AAACTGCAGGTCACTGTCTTG-3′ (−236 to −253); R1, 5′-AAACTGCAGGAGCCGAGCTGGCAC-3′ (−1057 to −1071); R2, 5′AAACTGCAGGCTCCTGGGCGCTCG-3′ (−857 to −871); R3, 5′-AAACTGCAGGGGGCAGCGGCTCCG-3′ (−657 to −671); and R4, 5′-AAACTGCAGCTGGCTCCTCAGCAG-3′ (−457 to −471). Different portions of the D_{1A} gene were amplified using the following pairs of primers: F with R for DD1, F with R1 for DD2,

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Abbreviations: CMV, cytomegalovirus; PRE, PRE responsive elements; GST, glutathione *S*-transferase; CAT, chloramphenicol acetyltransferase.

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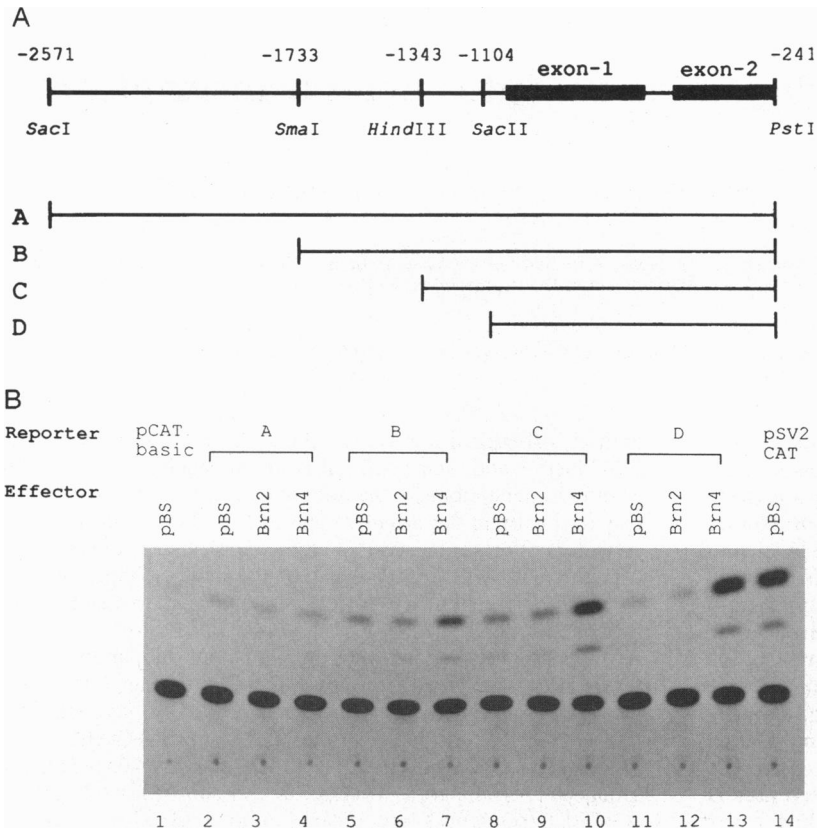

FIG. 1. (*A*) Structures of reporter CAT constructs (A to D) used for cotransfection CAT assays. Various lengths of the D_{1A} gene 5′ upstream region were subcloned into pCAT-Basic (Promega). Exon 1 and part of exon 2 are indicated by black boxes in the restriction map. Nucleotide numbers are relative to the first ATG codon (30). (*B*) Cotransfection CAT assays performed to evaluate the responsiveness of the D_{1A} gene 5′ upstream region to POU transcription factors. pCAT-Basic and pSV2CAT were negative and positive control reporter vectors, respectively. Effector plasmids were pBluescript SK (pBS) as control (lanes 1, 2, 5, 8, 11, and 14), pCMVBrn-2 (lanes 3, 6, 9, and 12) and pCMVBrn-4 (lanes 4, 7, 10, and 13). Neuro2A cells (1×10^6) were cotransfected with 15 μg of a reporter plasmid and 15 μg of an effector plasmid per 10-cm tissue grade dishes (Corning) using the calcium-phosphate coprecipitation method (5, 33). Three micrograms of pCH110 (Pharmacia) was included in each plate to control for transfection efficiencies. Transactivation by Brn-4 but not by Brn-2 was observed with reporter plasmids B, C, and D. Transactivation was gradually attenuated with reporter plasmids having longer D_{1A} gene 5′ regions (reporter plasmids A and B).

F with R2 for DD3, F with R3 for DD4, F with R4 for DD5, F1 with R for DD6, F2 with R for DD7, F3 with R for DD8, F4 with R for DD9, F1 with R4 for DD10, F2 with R3 for DD11, F1 with R3 for DD12, and F2 with R4 for DD13. The amplified fragments were digested with *Hin*dIII and *Pst*I, recovered with Geneclean II (Bio 101), and subcloned into the corresponding sites of pCAT-Basic (Promega).

Seven additional oligonucleotides were synthesized to construct reporter plasmids having internal deletions of the POU responsive elements PRE1 and PRE2 and of the AP2 consensus sequence. These primers were mDX1, 5′-AATCTAGAGCCCGTCCGAGCCACCTA-3′ (−581 to −598); mDX2, 5′-AATCTAGAGAATTATTAGGTTGGGCT-3′ (−571 to −554); mDX3, 5′-AATCTAGACGTCAAGCCCAACCTAAT-3′ (−549 to −566); mDX4, 5′-AATCTAGATGATGCCATCTGCTTAAG-3′ (−541 to −524); mDX5, 5′-AATCTAGATTCCCAGGGCGAGCCCGT-3′ (−569 to −586); mDX6, 5′-AATCTAGAGGTTGGGCTTGACGTGAG-3′ (−562 to −545); and mDX7, 5′-AATCTAGAGGGCTTGACGTGAGGATGCCATCTGCTTAA-3′ (−558 to −545/−540 to −525). DNA fragments were amplified from plasmid pCAT-HD1G-D (30) with F1/mDX1 and R4/mDX4 for Δcent, with F1/mDX1 and R4/mDX2 for ΔAP-2, with F1/mDX5 and R4/mDX6 for ΔPRE1, with F1/mDX3 and R4/mDX4 for ΔPRE2, and with F1/mDX5 and R4/mDX7 for ΔPRE3. The amplified fragments were digested with *Hin*dIII/*Xba*I and with *Xba*I/*Pst*I and then subcloned into pCAT-Basic (Promega) that had been digested with *Hin*dIII/*Pst*I. Δcent lacked the region from −580 to −542, ΔAP-2 lacked the sequence TGCCCCTGG from −580 to −572, ΔPRE1 lacked TTATTA from −568 to −563, ΔPRE2 lacked TGAGTAA from −548 to −542, and ΔPRE3 lacked TTATTAGGTT from −568 to −559 and TAAT from −544 to −541. The integrity of all constructs was verified by restriction analysis and sequencing.

To construct a bacterial expression vector for glutathione *S*-transferase (GST)–Brn-4 fusion protein, the 2.3-kb *Bam*HI–*Eco*RI fragment of the rat Brn-4 gene (11) was subcloned in pGEX2T (Pharmacia). The fusion protein contains the entire Brn-4 amino acid sequence plus three extra residues at the junction. The bacterial extract containing the fusion protein was prepared according to the supplier's recommendations.

CAT Assay. CAT assays were conducted essentially as described (5, 33). Plasmids were prepared using Qiagen (Chatsworth, CA) plasmid kits, and their respective purities and concentrations were checked by photodensitometry. Transfections were done by the calcium phosphate coprecipitation method using mammalian transfection kits (Stratagene). Transfection efficiency was verified by pCH110 (Pharmacia), a eukaryotic expression vector containing the simian virus early promoter and the *Escherichia coli*-β-galactosidase (*LacZ*) structural gene. Each experiment was repeated at least four times and variations in transfection efficiencies were <20%.

Gel Mobility-Shift Assay. Gel mobility-shift assays were performed as described (5, 33). GST–Brn-4 fusion protein was induced from the bacterial expression vector by isopropyl β-D-thiogalactoside in *E. coli* JM109. Two sets of complementary oligonucleotides, 5′-AGCTCCCTGGGAATTATTAGGTTGGGCT-3′/5′-AGCTAGCCCAACCTAATAATTCCCAGGG-3′ and 5′-AGCTCTTGACGTGAGTAATGATGCCATC-3′/5′-AGCTGATGGCATCATTACTCACGTCAAG-3′, were synthesized for PRE1 and PRE2 probes, respectively. The sequences of mutated oligonucleotides are shown in Fig. 3*B*. These oligonucleotides were annealed and radiolabeled with [α-^{32}P]dCTP using the Klenow fragment (Takara Shuzo, Kyoto, Japan). The probes (1000 cpm) were incubated with 400 ng of GST–Brn-4 fusion protein in 40 μl of the reaction mixture (20 mM Hepes, pH 7.9/80 mM NaCl/0.3 mM EDTA, pH 8.0/0.2 mM EGTA/1 mM DTT/0.2 mM phenylmethylsulfonyl fluoride/10% glycerol) at 20°C for 20 min and loaded on 4% polyacrylamide gel containing 2.5% glycerol. For gel mobility-supershift assay, an antiserum (4 μl) raised against GST–Brn-4 was also added to the reaction mixture. This antiserum was prepared by immunizing rabbits with 0.1 mg of

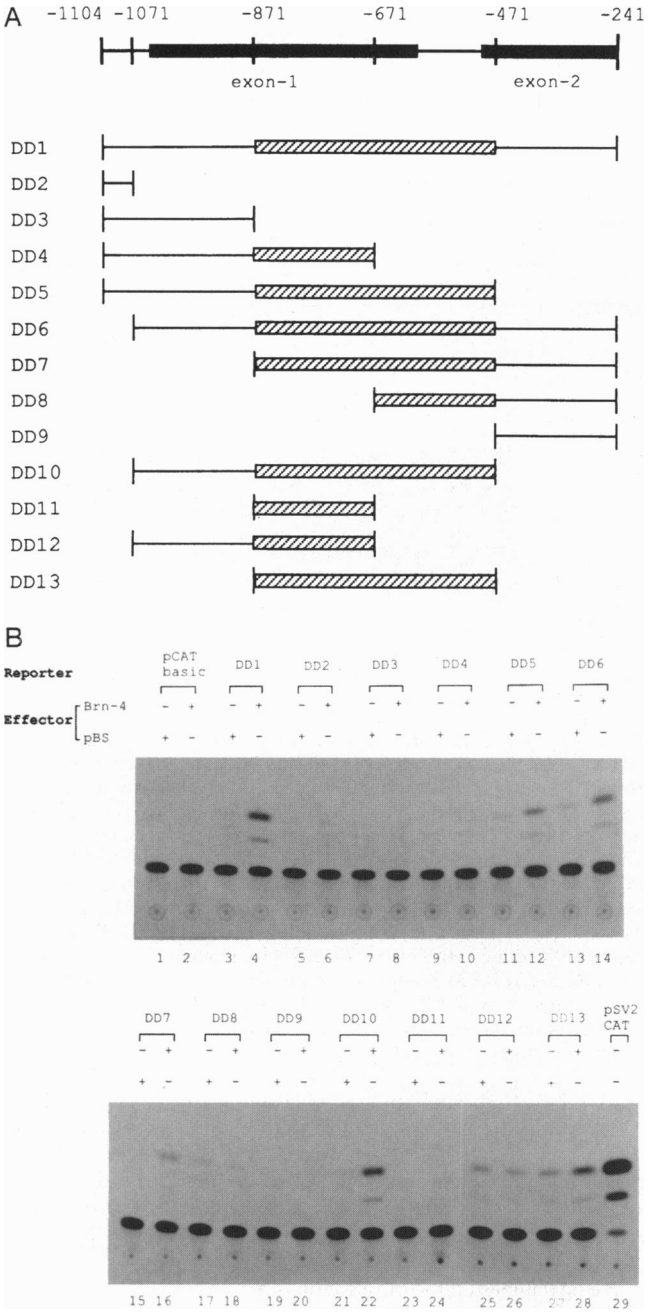

FIG. 2. (*A*) Localization of the Brn-4 responsive element in the human D_{1A} gene. Structures of 5′ and 3′ deletions of the D_{1A} gene upstream region subcloned in pCAT-Basic. Exons 1 and 2 are indicated by black boxes in the restriction map at the top. The Brn-4 responsive element determined by CAT assays is shown by shaded boxes. (*B*) Cotransfection CAT assays to localize the Brn-4 responsive element using pCMVBrn-4 as the effector plasmid. pBS is the negative control effector plasmid. Transactivation was observed with reporter plasmids containing the putative Brn-4 responsive elements (the region from −871 to −471 indicated with shaded boxes in *A*): DD 1, 5, 6, 7, 10, and 13.

recombinant GST–Brn-4 purified with glutathione Sepharose 4B (Pharmacia) according to the supplier's protocol. The rabbits were initially immunized with the recombinant protein and Freund's complete adjuvant followed 21 days later by a booster immunization by Freund's incomplete adjuvant. Rabbits were bled at day 28.

***In Situ* Hybridization.** A rat brain was fixed with 2% paraformaldehyde by perfusion. Frozen 10- or 15-μm-thick serial sections were cut using a cryostat. For nonradioisotope *in situ* hybridization, oligonucleotides were 5′-biotinated with biotin amidite (Applied Biosystems) using a DNA synthesizer (Applied Biosystems; model 392). The D_{1A} oligonucleotide probe sequence was 5′-TAAAGGAACTTTCAGACTGGGCGCATTCGACGGGGTTCCCGTTACCTG-3′, which is located in the third cytoplasmic loop (34). The Brn-4 antisense oligonucleotide used was 5′-AGCGGGCCACCTCCTTGCTTCCTCCAGTCAGAGATCGTGGCAGGACGC-3′, located between bases 1117–1070 of the rat Brn-4 cDNA (8). Hybridization and washing steps were performed as described (35). The oligonucleotide probes were bound to ExtrAvidin-peroxidase (Sigma) and visualized with DAB tablets (Sigma). For *in situ* hybridization using radioisotope, a Brn-4 riboprobe was transcribed *in vitro* (CLONTECH kit) from pBluescript containing the *Bam*HI fragment of Brn-4 genomic DNA (11). For a D_{1A} probe, the *Pst*I–*Hin*dIII genomic fragment (30) containing 608 bp from the first ATG was subcloned in the 3′–5′ orientation between the *Hin*dIII and *Pst*I sites of pBluescript SK, digested with *Nco*I to excise the nontranslated sequence, and transcribed from the T_7 promoter. The hybridization solution had 50% formamide/0.6 M NaCl/10 mM Tris, pH 7.4/1 mM EDTA/1× Denhardt's solution/100 μg/ml *E. coli* DNA/1 mg/ml yeast tRNA/0.1% SDS. Hybridized slides were washed in 2× SSC − 20 μg/ml RNase A at 40°C for 60 min followed by 0.1× SSC at 55°C for 40 min.

RESULTS

Localization of the Brn-4 Responsive Element in the D_{1A} Gene. Initially, cotransfection CAT assays employed a cell line that does not express the D_{1A} gene to rule out the effects of endogenous factors on the reporter plasmids flanked by D_{1A} promoter fragments. The neuroblastoma cell line, Neuro2A, was used since it has D_2 but no D_1 dopamine receptor binding sites (36). Lack of D_{1A} expression in this cell line was confirmed by Northern blot analysis (data not shown). Two effector plasmids expressing either Brn-4 or Brn-2 were used. The former POU factor is expressed in the striatum (10), where the D_{1A} receptor is abundant, whereas the latter is not (9). Neuro2A cells were further considered suitable for functional analysis of Brn-4 in this experimental paradigm because they do not express this transcription factor as assessed by Northern blot analysis (data not shown).

The first set of CAT assays was performed with four reporter constructs containing various stretches of the D_{1A} 5′-flanking region (Fig. 1*A*). Cotransfection with Brn-4, but not with Brn-2, resulted in up-regulation of the transcriptional activity of all four CAT constructs. This suggested that Brn-4 responsive cis-elements are located within the genomic fragment contained in the shortest construct D (Fig. 1*B*). Interestingly, transactivation by Brn-4 was gradually attenuated with extension of the D_{1A} gene fragment in the reporter construct, perhaps suggesting that other endogenous factor(s) acting at upstream site(s) repress the enhancing effect of Brn-4. We have confirmed that Brn-4 itself does not act as this repressor by cotransfection CAT assays using plasmids containing D_{1A} gene fragments and a constitutive promoter (data not shown).

Additional deletion constructs were made from plasmid D to further localize the Brn-4 responsive element (Fig. 2*A*). Deletions from the 3′ end revealed a discrepancy between constructs DD5 and DD4, whereas deletions from the 5′ end demonstrated the difference between DD7 and DD8 (Fig. 2*B*). Thus, the entire region between nucleotides −871 and −471 relative to the adenosine of the initial methionine codon (30) was judged necessary and sufficient for transactivation by Brn-4. Furthermore, this region must contain a second promoter because DD7 and DD13, which lack the D_{1A} promoter located around −1060 (30), could still be transactivated by Brn-4.

Structure of the Brn-4 Responsive Element. The 400-bp Brn-4 responsive region contained several sequences with the core ATTA motif that has been proposed as the minimum consensus sequence for homeobox and POU transcription factors (17, 18, 37). The tandemly aligned motif ATTATTA is

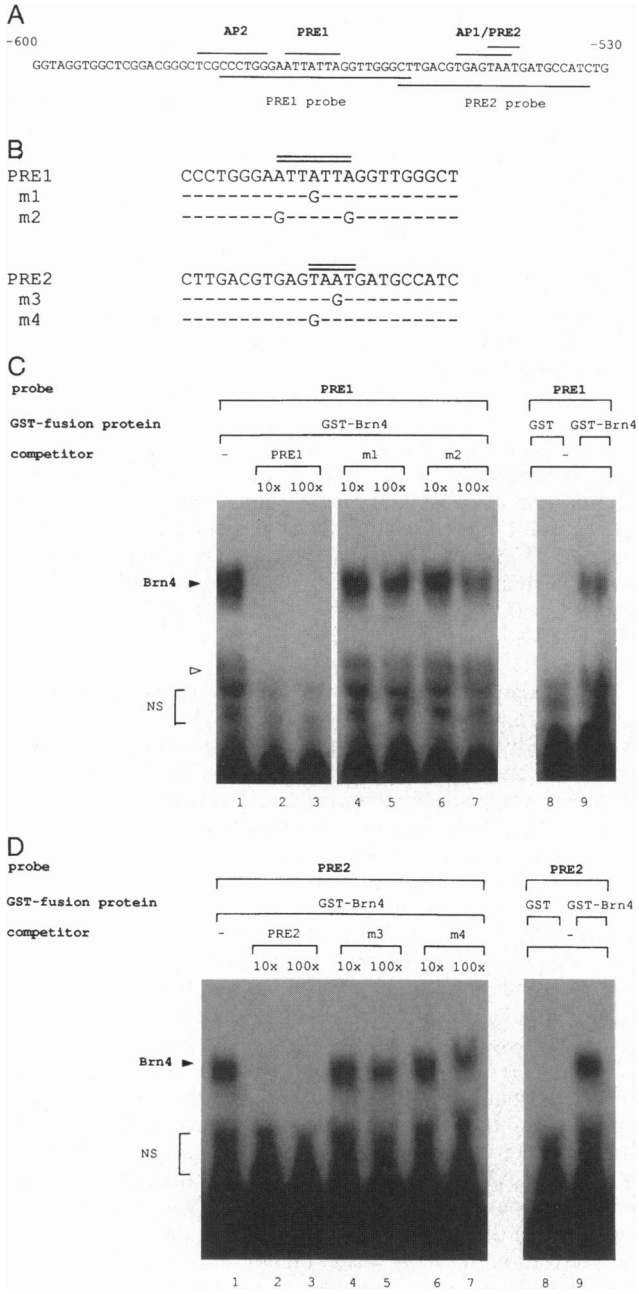

FIG. 3. (*A*) Sequence of the core Brn-4 responsive elements in the human D_{1A} gene. Two tandemly aligned ATTA sequences were collectively named PRE1. Another ATTA motif (PRE2) overlaps with an AP-1 consensus sequence. The oligonucleotides used for gel mobility-shift assays are underlined. (*B*) Mutated oligonucleotides used for competition in gel mobility-shift assays. m1 and m2 were mutations of the PRE1 probe sequence; m3 and m4 were mutations of PRE2. Specific mutations in PRE1 and PRE2 are indicated below the respective sequences. (*C*) Gel mobility-shift assay showing Brn-4 binding to PRE1 that was inhibited by wild-type but not mutated oligonucleotides. GST–Brn-4 fusion protein was incubated with PRE1 probe in the absence (lane 1) or presence of 10- or 100-fold excess competitor (lanes 2–7). The retarded complex was efficiently inhibited by 10-fold excess wild-type competitor (lanes 2 and 3) whereas 100-fold excess mutated oligonucleotides, m1 through m4, could not affect the shifted complex significantly (lanes 4–7). This complex was not formed by the negative control GST protein (lane 8). Nonspecific bands (NS) were observed both with non-fused GST protein (lane 8) and with GST–Brn-4 fusion protein (lane 9). A weak band (white arrow head) seen with GST–Brn-4 could possibly represent a degradation product of Brn-4. (*D*) Gel mobility-shift assay showing Brn-4 binding to PRE2. These results are similar to those obtained with the PRE1 probe (*C*).

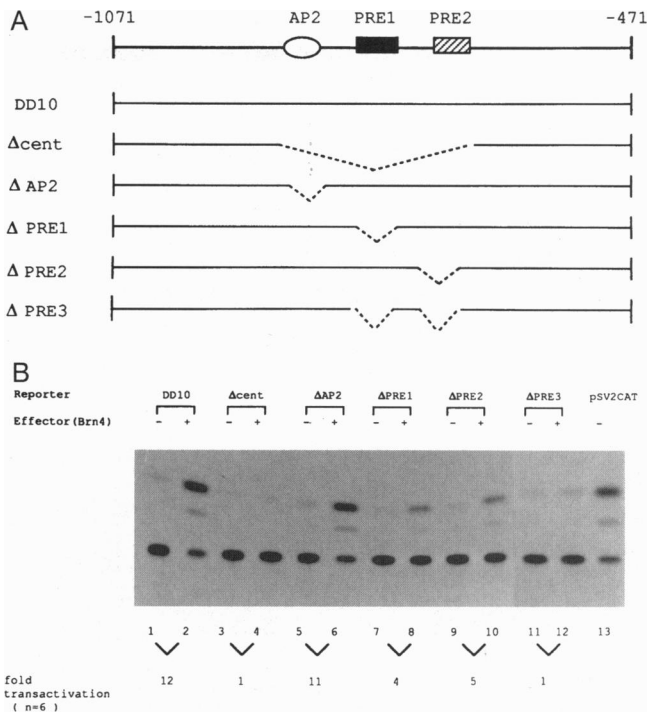

FIG. 4. (*A*) Structures of reporter plasmids lacking the AP-2, PRE1, or PRE2 sequences. These plasmids were used for cotransfection CAT assays to define Brn-4-induced enhancer activity. Δcent is deletion of all AP-2, PRE1, and PRE2 sites. Plasmids ΔAP-2, ΔPRE1, and ΔPRE2 lack the corresponding consensus sequences, respectively. ΔPRE3 lacks both PRE1 and PRE2. (*B*) Cotransfection CAT assays localizing the core enhancer activity. Deletion of all three consensus sequences combined (Δcent) eliminated Brn-4 induced transactivation (lanes 3 and 4) and individual deletions of PRE1 or PRE2 also significantly reduced this effect (lanes 7–10). Double deletion of PRE1 and PRE2 (ΔPRE3) completely eliminated the transactivation by Brn-4 (lanes 11 and 12). On the other hand, deletion of the AP-2 element alone did not change the transactivation by Brn-4 (lanes 5, 6). Mean fold increase in CAT activities ($n = 6$) are indicated below.

located between −569 and −563 (PRE1), and a single ATTA is located in the reverse orientation between −544 and −541 (PRE2) (Fig. 3*A*). We tentatively designated them as PREs (*P*OU *r*esponsive *e*lements) because we have found that another POU factor, Oct-2, also transactivates a reporter gene through these elements (data not shown). PRE2 overlaps with the AP-1 consensus sequence.

We next analyzed the putative binding sites of Brn-4 in the D_{1A} gene. Gel mobility-shift assays using GST–Brn-4 fusion protein revealed that Brn-4 can bind to both candidate sequences (Fig. 3 *C*, lane 1, and *D*, lane 1, respectively) but not to the AP-2 binding site or other non-POU consensus sequences (data not shown). The highest complex with GST–Brn-4 was judged specific because no similarly shifted band was observed when GST protein itself was used as negative control (Fig. 3 *C*, lane 8, and *D*, lane 8) and because it was specifically inhibited by PRE1 or PRE2 oligonucleotides but not by the mutated oligonucleotides (m1 to m4) lacking the ATTA motif (Fig. 3 *B–D*).

Based on the GST–Brn-4 binding sequences in the D_{1A} gene, the enhancer activity could be localized more precisely. Reporter plasmids lacking PRE1, PRE2, or AP-2 (designated as ΔPRE1, ΔPRE2, and ΔAP-2, respectively) and another lacking both PRE1 and PRE2 (designated ΔPRE3) were constructed from plasmid pCAT-HD1G-D (30) and used for cotransfection assays with the expression vector pCMV-Brn-4. The enhancement by Brn-4 was clearly reduced by deleting either PRE1 or PRE2 but not the AP-2 consensus sequence and was completely abolished by deleting both PRE1 and PRE2 (Fig. 4*B*). These functional analyses are in accordance with the data from DNA-protein

binding assays and further support the notion that PRE1 and PRE2 are the core enhancer sequences in the D_{1A} gene activated by POU transcription factors.

***In Vivo* Interaction of Brn-4 With the D_{1A} Gene.** To verify the *in vivo* validity of a potential role for Brn-4 in D_{1A} gene transactivation, we looked for coexpression of Brn-4 and D_{1A} genes in the same neurons by *in situ* hybridization. First, both Brn-4- and D_{1A}-positive neurons, which were primarily medium-sized, were found predominantly in the striosomes of the caudate-putamen with a small number in the matrix (Fig. 5 *A* and *B*). This finding is in agreement with previous data derived from binding (38) and *in situ* hybridization studies (35). Second, *in situ* hybridization using Brn-4 and D_{1A} probes on adjacent tissue sections clearly demonstrated colocalization of these two gene transcripts at the cellular level within the rat striatum (Fig. 5 *C–H*). After excluding neurons whose cell bodies were found only in one section, we calculated the relative abundance of single and double positive neurons (Fig. 5*I*). Statistical analyses using these percentages confirmed the significant coexpression of Brn-4 and D_{1A} in individual striatal neurons. Since the percentages of Brn-4 $+/D_{1A}$ $-$ or Brn-4 $-/D_{1A}$ $+$ cells are not negligible, additional factors might influence D_{1A} gene expression.

We also tested whether Brn-4 in the striatum could bind to the PREs in the D_{1A} gene. We performed gel mobility-supershift assays using nuclear extract from the rat striatum, PRE oligonucleotide probes, and Brn-4-antiserum. This ex-

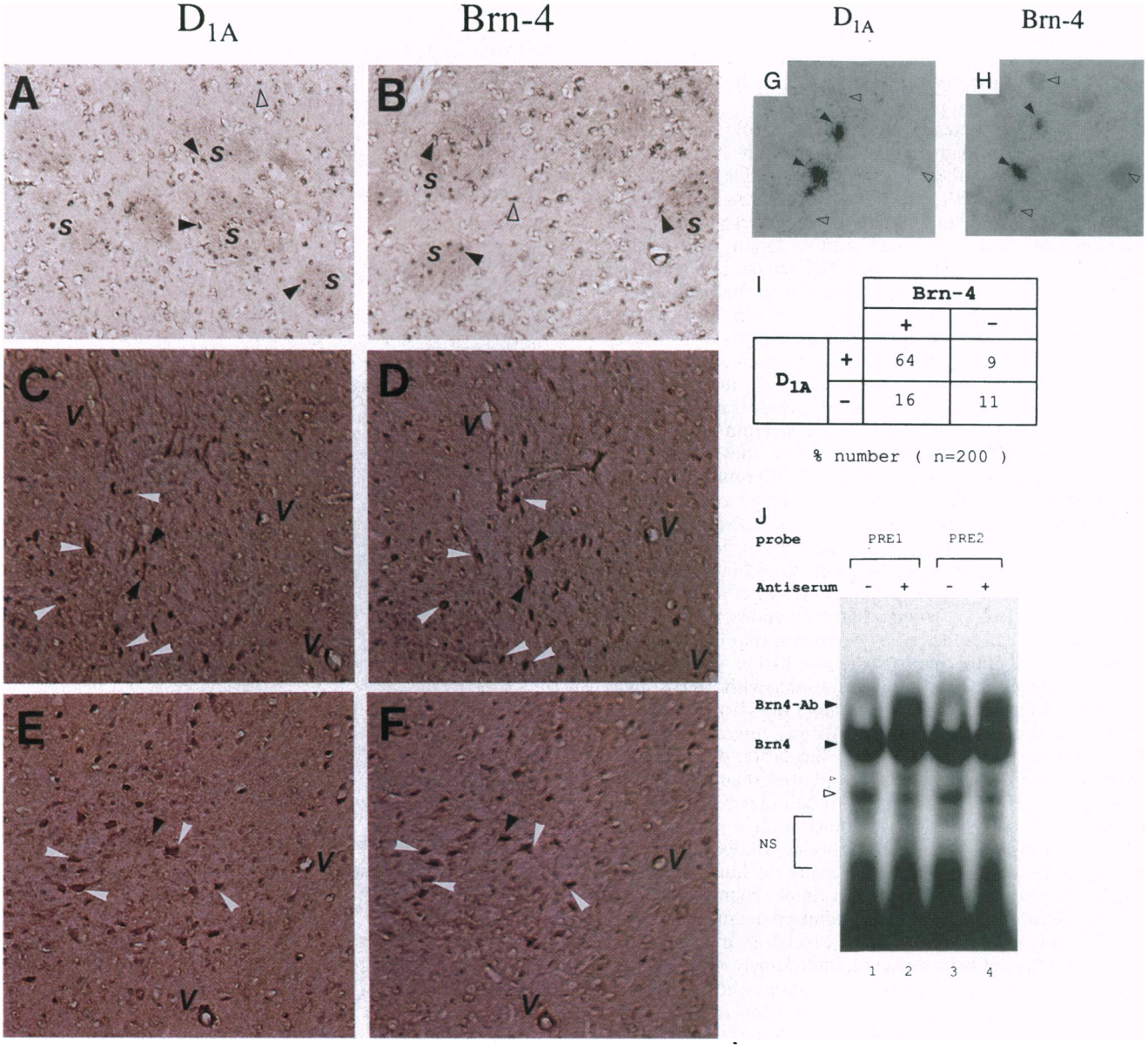

FIG. 5. *In situ* hybridization by biotinated oligonucleotide probes (*A–F*) or radioisotope-labeled riboprobes (*G* and *H*) of the rat striatum. Representative neurons positive for D_{1A} and Brn-4 in the striosomes(s) are indicated with black arrowheads (*A* and *B*). Positive neurons were seen predominantly in striosomes (black arrowheads) with a small proportion (white arrowheads) located in the matrix (*A* and *B*). Adjacent sections showed coexpression of Brn-4 and D_{1A} mRNAs in striatal neurons (*C* and *D*, *E* and *F*, *G* and *H*). Each slide was hybridized with a Brn-4 or D_{1A} oligonucleotide probe (*C–F*) or with a riboprobe (*G* and *H*). Representative neurons positive for both signals are indicated by white arrowheads (*C–F*). Some neurons were divided unequally between adjacent sections and thus hybridized differentially to each probe (*C* and *D*: black arrowheads). A few neurons hybridized strongly to Brn-4 but scarcely to D_{1A} (*E* and *F*: black arrowheads). *In situ* hybridization using radiolabeled riboprobes (*G* and *H*) similarly detected double positive (black arrowheads) and double negative neurons (white arrowheads). Blood vessels are indicated by the letter "V." Magnifications are ×100 in *A* and *B* ×200 in *C–F*, and ×600 in *G* and *H*. (*I*) Percentage of neurons in the four groups showing different hybridization patterns. (*J*) Gel mobility-supershift assay showing the binding of striatal Brn-4 to PREs. Nuclear extract from rat striatum was incubated with PRE1 (lanes 1 and 2) or PRE2 (lanes 3 and 4) probes, respectively. A specific band was supershifted by Brn-4 antiserum (lanes 2 and 4). A weak band (white arrowhead) was shifted by the antiserum (small white arrowhead), which could correspond to a degraded Brn-4 protein. Non-specific bands are indicated by NS.

periment showed supershifted complexes of striatal Brn-4 with PRE1 or PRE2 (Fig. 5*J*), indicating that Brn-4 in this brain region could bind to these regulatory elements in the D_{1A} gene. These findings support the conclusion that Brn-4 modulates expression of the D_{1A} gene in the striatum.

DISCUSSION

POU transcription factors appear to have important functions in the central nervous system based on their wide distribution in the brain. Anatomic perturbations due to disruptions of POU family genes (24, 25, 39) are far less detrimental than those caused by disruptions of homeobox genes (40–42). Thus, POU and homeobox proteins seem to differ in their roles in morphogenesis. On the other hand, mutation of the Pit-1 gene disturbs the expression of specific molecules that determine the phenotypes of select cell populations. Considering the complex expression pattern of POU transcription factors, we postulated that POU family proteins could regulate the expression of specific molecules in terminally differentiated neurons.

In the present study, we addressed the role of Brn-4 in regulating the D_{1A} dopamine receptor gene. The Neuro2A cell line was selected for these reconstitution experiments since these cells express neither D_{1A} nor Brn-4 allowing interpretation of the functional interaction between these two genes in the absence of other native transcription factors. Our results indicated the presence of functional PREs in the D_{1A} gene that could be activated by Brn-4 in cultured neuronal cells. Deletions of these PREs abrogated the *in vitro* binding to Brn-4 and resulted in loss of Brn-4 induced transactivation in Neuro2A cells. Furthermore, we observed significant colocalization of Brn-4 and D_{1A} mRNAs in the rat striatal neurons. These two findings strongly suggest that Brn-4 promotes transcription of the D_{1A} dopamine receptor gene in the striatum.

Brn-4 but not Brn-2 could transactivate the D_{1A} gene in Neuro2A cells, thus, suggesting differential regulation of this gene by different members of the POU family factors (Fig. 1). This discrepancy was observed despite equal expression levels of Brn-4 and Brn-2 transcripts in these cells by Northern blot analysis (data not shown). Furthermore, their binding abilities to the PREs were similar when assessed by gel mobility-shift assay using GST fusion proteins (data not shown). These data might suggest the presence of a coactivator that discriminates Brn-4 from Brn-2 in this neuroblastoma cell line. The presence of an AP-1 consensus sequence overlapping with PRE2 might suggest *in vivo* interaction of AP-1 factors with Brn-4, although we have not detected *in vitro* Brn-4/*c-jun* interaction at the PRE2 sequence (data not shown). Elucidation of the molecular mechanism underlying this differential regulation of the D_{1A} gene by POU factors remains to be determined.

Confirmation of the net *in vivo* function of a gene requires analysis of experimental animals harboring a knock-out of the gene, naturally occurring mutant strains or human patients carrying the gene mutation. Mutations in or around the human Brn-4 gene is associated with X-linked mixed deafness (43). The expression level or function of the D_{1A} receptor in the brains of these patients has yet to be reported. Interestingly, genes responsible for some types of mental retardation with or without deafness are positioned in the same locus as Brn-4 (44, 45). Thus, careful neurologic and psychologic evaluations of those patients are important, since knock-out of the D_{1A} dopamine receptor gene causes only functional changes rather than neuronal death or gross morphological anomalies in the nervous system (46, 47).

The D_{1A} dopamine receptor has been implicated in a number of disorders such as Parkinson disease, Huntington disease, drug addiction, and psychiatric disorders, based on its brain distribution and pharmacological observations. Furthermore, the D_{1A} dopamine receptor may be essential for the working memory function of the brain (48). Therefore, information about regulation of the D_{1A} gene could be useful for developing novel therapeutic strategies by modulating its expression.

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