The rat D2 dopamine receptor gene is transcribed from a TATA-less promoter that has an initiator-like sequence and several putative Sp1 binding sites. The main activator of this gene is between nucleotides −75 and −29, and a strong negative modulator is located between bases −217 and −76 (Minowa, T., Minowa, M. T., and Mouradian, M. M. (1992) Biochemistry 31, 8389–8396). In the present investigation, a small deletion series within this negative modulator fused with the reporter gene for chloramphenicol acetyltransferase was used to transfect the D2-expressing cells, NB41A3. Two cis-acting functional DNA sequences were identified: a 41-base pair segment between nucleotides −116 and −76 (D2Neg-B), which decreased transcription from the D2 promoter by about 45%, and a 26-base pair segment between nucleotides −160 and −135 (D2Neg-A), which, in the presence of the downstream negative modulator, reduced transcription down to the level of a promoterless vector. DNase I footprinting, gel mobility shift, and competitive cotransfection experiments suggested that D2Neg-A functions without trans-acting factors, whereas D2Neg-B interacts with nuclear factors at its Sp1 binding sequences. Gel supershift with anti-Sp1 antibody and UV cross-linking experiments revealed that a novel 130-kDa factor as well as Sp1 interact with D2Neg-B in NB41A3 cells. This novel protein recognizing Sp1 binding sequences in the D2 gene negative modulator is also found in nucleolar extracts from the rat striatum.

Central dopaminergic neurotransmission has a key role in several brain processes including the control of movement, hypothalamic-pituitary axis regulation, and probably affect and cognition (Creese and Fraser, 1987). To date, five different dopamine receptor genes have been identified, each with distinct regional distribution in the brain. Based on their pharmacological profile and sequence homology, these receptors are classified into two main subfamilies: D1, which includes the D1a and D1b (D1) receptors; and D2, which includes the D2, D3, and D4 receptors (Bunzow et al., 1988; Derry et al., 1990; Monsma et al., 1990; Sokoloff et al., 1990; Sunahara et al., 1990, 1991; Van Tol et al., 1991; Zhou et al., 1990). Among these, the D2 dopamine receptor has been traditionally recognized to be the primary mediator of the motor, endocrine, and behavioral effects of dopaminergic transmission (Schacher et al., 1980; Seeman, 1981). Alterations in the functional state of dopamine receptors are thought to contribute to the clinical manifestations of a number of neuropsychiatric disorders as well as to the complications of their long term therapy (Lee et al., 1978; Mouradian et al., 1988). Thus, clarifying the molecular phenomena regulating the expression of the D2 dopamine receptor gene is essential for our efforts to modulate the dopaminergic system for therapeutic purposes.

We recently reported the primary structure of the promoter region of the rat D2 dopamine receptor gene and showed that it lacks a TATA box but has an initiator-like sequence where transcription begins preferentially, is rich in GC content, and has several putative Sp1 binding sites (Minowa et al., 1992). The main activator of this gene is between nucleotides −75 and −29, and a strong negative modulator is located between bases −217 and −76. Nuclear extract from the D2-expressing cells NB41A3 and purified human Sp1 bind to the Sp1 consensus sequence in the activator as well as to a TGGG repeat and Sp1 consensus sequences in the negative modulator. However, the DNase I protection pattern at this negative modulator is different between Sp1 itself and NB41A3 nuclear extract, suggesting that this DNA-protein interaction in these cells does not simply represent Sp1.

In the present study, we analyzed the negative modulator of the rat D2 gene in detail, examined its interaction with nuclear factors, and began to characterize the nature of these regulatory proteins.

MATERIALS AND METHODS

Plasmid Constructions—Serial 5′ deletion mutants of the rat D2 negative modulator region were constructed by replacing the 142-bp1 AccIII-Smal fragment of pCATD2-217 (Minowa et al., 1992) with DNA fragments extending between bases −160 and −76 (pCATD2-160, −136 and −76 (pCATD2-136), −116 and −76 (pCATD2-116), or between −95 and −76 (pCATD2-95) (see Figs. 1 and 5). All of these DNA fragments were either synthesized chemically or generated by PCR. A double-stranded oligonucleotide extending between bases −116 and −76 with four base substitutions at the Sp1 consensus sequence (putative Sp1 site A, 5′-GGCGCGG-3′ to 5′-GAACAAG-3′ at nucleotides −86 to −82) was used to construct pCATD2-116 mutA (see Fig. 5). The effectiveness of this mutation in eliminating Sp1 binding was tested by gel shift assay and DNase I footprinting (data not shown). All CAT constructs were verified by restriction analysis and partial sequencing.

Plasmids used for competitive cotransfection experiments were prepared by modifying pCATD2 plasmids with restriction enzymes (see Fig. 2a). The control plasmid pZeo was prepared by cutting out the 2.5-kilobase EcoRI fragment of pCATD2-852 (Minowa et al., 1992), resulting in deletion of the CAT gene and all rat D2 gene sequences from the plasmid. Removal of the 2-kilobase Smal-EcoRI fragment from pCATD2-217 generated pNeg-1, whereas deletion of the same fragment from pCATD2-116 yielded pNeg-2. All of these plasmids were verified by sequencing.

Cell Culture and Transient Expression Assays: The murine neuroblastoma cell line NB41A3, obtained from ATCC, was cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (CELLect GOLD, ICN Flow, Irvine, CA) at 37 °C in a humidified atmosphere containing 10% CO2. Transfections were carried out using the CaPO4 coprecipitation method.
**Fig. 1. S′ Deletion mutants of the rat D2 gene negative modulator and their transcriptional activity in NB41A3 cells.** Transcriptional activity of various DNA fragments of the rat D2 gene 5′-flanking region was tested by transient expression assays in NB41A3 cells. The black bar on top shows organization of the D2 gene upstream region. Nucleotide numbers are relative to the main transcription start site (Minowa et al., 1992). Restriction sites AccI and SmaI are shown. D2Neg-A and D2Neg-B refer to the two negative modulatory regions in this gene. Ellipses represent the putative Sp1 binding sites designated as A and B, and the TGGG repeat. The AP2 consensus sequence in D2Neg-A is boxed. Shadowed bars on the left represent the 5′-deletion mutants of the rat D2 gene generated by synthetic oligonucleotides or PCR and subcloned upstream of the CAT gene in the 5′−3′ orientation. Hatched bars on the right show promoter activity of each pCAT construct in NB41A3 cells expressed as ratio to pCATD2-75. Data shown are means ± S.E. for four to six transfections. Transfection efficiencies were normalized according to β-galactosidase activity. Transfections were repeated at least four times yielding reproducible results. *, analysis of variance p < 0.05 compared with pCATD2-75.

**Results**

Transcriptional Activity of S′ Deletion Mutants of the Rat D2 Gene Negative Modulator—Previously we had found a strong negative modulator in the rat D2 gene between nucleotides −217 and −76 which decreases transcription level from the D2 promoter down to that of a promoterless vector (Minowa et al., 1992). To localize this negative modulator further, we created small 5′ deletion series in this region and measured its ability to decrease transcriptional activity of the D2 promoter in NB41A3 cells which express the D2 gene endogenously (Fig. 1). The region between bases −116 and −76 (D2Neg-B), where nuclear factors from NB41A3 cells as well as purified Sp1 interact at the three TGGG repeats and putative Sp1 site A (Minowa et al., 1992), significantly decreases transcriptional activity by about 45% compared with the most active construct pCATD2-75. Nucleotides −160 to −135 (D2Neg-A), which include a consensus sequence for AP2 binding site, were found necessary in addition to nucleotides −116 to −76 to fully achieve the negative modulatory function of the −217 to −76 region in repressing transcription from the D2 promoter. No significant changes in CAT activity were observed by deleting the nucleotides between −217 and −160 or between −135 and −116. These results indicate that the D2 negative modulator can be divided into two regions: D2Neg-B and D2Neg-A.

Competitive Cotransfection Assays—The functional importance of the interaction between trans-acting nuclear factor(s) and cis-acting negative modulatory sequences in the rat D2 gene was examined next by competitive cotransfections of NB41A3 cells (Fig. 2). The plasmid pCATD2-116, which contains the TGGG repeat and Sp1 site A (D2Neg-B), increased its promoter activity by about 40% when cotransfected with 4 × molar excess of the competitor plasmid pNeg-2 which includes the −116 to −76 fragment (D2Neg-B). In contrast, plasmid pCATD2-75, which lacks the negative modulatory regions, predictably did not change its promoter activity when cotransfected with pNeg-2. These results strongly suggest that a trans-acting factor(s) interacts in vivo with D2Neg-B but not with the positive modulator sequence, Sp1 site B. Interestingly, the plasmid pCATD2-217, which harnesses both D2Neg-A and D2Neg-B sequences, increased its CAT activity signifi-

**D2 Gene Negative Modulator**

11657
**DNA-Protein Interactions in the Negative Modulator of the Rat D_2 Gene**—The relationship between in vivo negative modulatory activity and in vitro DNA-protein interaction(s) was initially assessed by DNase I footprinting using a probe extending between nucleotides -225 and -67 (Fig. 3). Consistent with our earlier observations (Minowa et al., 1992), both nuclear extract from NB41A3 cells and purified human Spl protected the Spl consensus sequence (Spl site A) and the three consecutive 5'-TGGG-3' repeats that collectively constitute the functional D,Neg-B region. Also in agreement with our previous data, the protection of the TGGG repeat and Spl site A from DNase I had a different pattern and was notably weaker when using nuclear extract from NB41A3 cells compared with purified human Spl (Fig. 3b). On the labeled (+)-strand, the footprint of NB41A3 nuclear extract extended further upstream compared with that of human Spl, and at least two DNase I hypersensitive sites were observed with Spl but not with NB41A3 nuclear extract or no protein. These differences might suggest that the nuclear factor(s) interacting with D,Neg-B in NB41A3 cells has a different character than Spl.

The AP2 consensus sequence at nucleotides -150 to -143 in D,Neg-A was not protected from DNase I by any nuclear protein tested including purified human AP2 despite the potent negative modulatory activity of this region. This observation supported the data of cotransfection experiments that there are no trans-acting factors interacting with D,Neg-A. We have previously confirmed that this AP2 preparation can bind to its consensus sequence in the human D_3 dopamine receptor gene using this assay (Minowa et al., 1993).

To study further the DNA-protein interaction(s) within the negative modulator region of the rat D_2 gene, gel mobility shift experiments were employed with the same probe used in footprinting (Fig. 4). In the absence of competitors, two major retarded bands (b and c) having very close electrophoretic mobility and a faster moving much weaker band (a) were observed. All three bands represented specific binding since they disappeared by adding excess cold probe. These bands were also effectively competed off by oligonucleotides having either the Spl consensus sequence or the TGGG repeat, suggesting that the factors interacting with the D_2 gene negative modulator...
recognize both these sequences. On the other hand, none of the shifted bands was affected by adding 200 x molar excess of an oligonucleotide having the D2Neg-A sequence (data not shown). These observations further confirmed that the only DNA-protein interaction in the negative modulator of the D2 gene occurs within the D2Neg-B region but not in the D2Neg-A region.

**Functional Analysis of the D2Neg-B Region**—To determine the relative functional contribution of the two nuclear factor binding sites within D2Neg-B, two additional constructs derived from pCATD2-116 were tested: plasmid pCATD2-98 with 5' deletion of the TGGG repeat, and pCATD2-116 mutA, in which 4 bases in the Spl site A sequence were mutated (Fig. 5). Deletion of the TGGG repeat (pCATD2-95) did not impact on the promoter activity of pCATD2-116 whereas deletion (pCATD2-75) as well as substitution mutation of Spl site A (pCATD2-116 mutA) significantly increased transcriptional activity. These data suggest that between the two nuclear factor binding sites within D2Neg-B, putative Spl site A is a stronger negative modulator than the TGGG repeat.

**Gel Mobility Supershift Assays with Anti-Spl Antibody**—The identity of the nuclear factor(s) binding to D2Neg-B was addressed next. In supershift assays using anti-Spl antibody, the middle shifted band (b) with probe -225/-67 was shifted further to a higher position (band b*), whereas the mobility of the other two bands (a and c) was unaffected (Fig. 6a). This supershift was graded with increasing concentrations of antibody, reaching completion at 40 ng/reaction. Since this polyclonal antibody is raised against the partial amino acid sequence of Spl, our observations suggest that NB41A3 cells have at least three types of factors interacting with Spl binding sequences in the D2 gene, all bind to these sequences, but only one (band b) is recognized by anti-Spl antibody. Similarly, when the TGGG repeat or Spl consensus sequence oligonucleotides were used as probes in supershift experiments (Fig. 6b), only part of the shifted band (S) could be supershifted (SS) with 30 ng of anti-Spl antibody or even with 100 ng of antibody when the Spl consensus probe was used (data not shown). Identical results were obtained with an oligonucleotide probe having the Spl site B sequence (data not shown).

**UV Cross-linking**—The molecular masses of the proteins binding to the TGGG repeat sequence were estimated by UV cross-linking experiments (Fig. 7a). Purified human Spl was used to compare the gel migration patterns between Spl and the nuclear factors in NB41A3 cells. Spl is known to yield two
different apparent molecular masses, 95 and 105 kDa, due to phosphorylation (Jackson et al., 1985). Thus, UV cross-linking with the TGGG probe, human Sp1 formed two complexes, about 108 and 115 kDa (determined in an 8% gel, data not shown), although the slower migrating band was much weaker than the faster moving one. Since this method detects proteins as complexes with oligonucleotides, the apparent molecular masses could be slightly higher than those of the proteins alone. UV cross-linking with NB41A3 nuclear extract yielded three types of factors, two of which had gel migrations similar to that of purified human Sp1, about 105 and 110 kDa as complexes with the probe (determined in an 8% gel, data not shown), whereas the third had a greater apparent molecular size, about 130 kDa. Electrophoresis in the higher concentration gel did not show any additional specific bands other than those seen in Fig. 7 (data not shown).

The combination of gel supershift and UV cross-linking provided additional information about these factors in NB41A3 cells interacting with the Sp1 binding sequences in the D2 gene (Fig. 7b). First, gel supershift with anti-Sp1 antibody was done using the TGGG repeat, Sp1 consensus sequence, and Sp1 site B oligonucleotides as probes followed by UV cross-linking in the gel. Subsequently, both the supershifted band and the retarded but not supershifted band (see Fig. 6b) with each probe were cut out from the gel and subjected to SDS-PAGE (Fig. 7b). As expected, the molecules that were recognized by anti-Sp1 antibody (SS) had apparent molecular sizes (105 and 110 kDa) similar to human Sp1, whereas the factor that did not cross-react with this antibody (S) had a higher apparent molecular mass (130 kDa). These results strongly suggest that the 130-kDa factor in NB41A3 cells is distinct from Sp1. We also conclude that band b in the gel shift assays (Fig. 4) is composed of the 105- and 110-kDa proteins visualized in UV cross-linking and that band c corresponds to the 130-kDa protein, based on the cross-reactivity of these bands with anti-Sp1 antibody. The protein forming band a in Fig. 4, which is much weaker in intensity than bands b and c, apparently could not be detected by UV cross-linking.

Gel Mobility Shift with Rat Striatal Nuclear Extract—To ascertain if the nuclear proteins interacting with the D2 gene negative modulator in NB41A3 cells are also present in the rat brain, gel shift assays were performed using nuclear extract from rat striata where the D2 dopamine receptor gene is expressed (Fig. 8). Among the four major specific complexes shifted with striatal nuclear extract (bands a–d), bands a, b, and c were found to be common with the complexes formed by NB41A3 nuclear extract. Furthermore, band d shifted by both extracts was supershifted by anti-Sp1 antibody. These observations strongly suggest that the striatum has the same three proteins (105, 110, and 130 kDa) which recognize the Sp1 binding sites in the D2 gene as in NB41A3 cells. The slower migrating complex, band d seen in the striatum but not in NB41A3 cells, likely originates from non-D2-expressing cells in striatal tissue.

**DISCUSSION**

The rat D2 dopamine receptor gene has a strong negative modulator between nucleotides –217 and –76 in which the Sp1 consensus (Sp1 site A) and TGGG repeat sequences are bound by Sp1 and by nuclear extract from the D2-expressing cells NB41A3 (Minowa et al., 1992). In the present investigation, we further localized this negative modulator by testing the transcriptional activity of small deletion series of the rat D2 gene promoter. We found that repression of D2 gene expression is contributed by two segments: the first comprises the two Sp1 binding sites between nucleotides –116 and –76 (D2Neg-B), which reduces promoter activity by about 45%, and the second extends between bases –160 and –135 (D2Neg-A), which in the presence of the first segment decreases transcription rate by an additional 36%. Both these regions are necessary to achieve the full extent of the negative modulatory activity of the –217/-76 fragment in the D2 gene (Fig. 1).

Within the D2Neg-A region, no in vitro DNA-protein interactions could be detected using NB41A3 nuclear extract or even recombinant AP2 protein, despite the presence of an AP2 consensus sequence at –143/-150. Lack of nuclear factor binding in this region was concluded not only based on DNase I footprinting experiments (Fig. 3) but also gel shift assays with a synthetic oligonucleotide harboring this sequence as a probe or as a competitor (data not shown). Furthermore, in competitive cotransfection experiments using pCATD2-217 as the expression plasmid, the –217/-76 region (pNeg-1) had no additional competitive capacity compared with the smaller –116/-76 region (pNeg-2) (Fig. 2). Although changes in the molar ratios of these competitor plasmids to the CAT plasmids after transfection cannot be totally excluded, these observations suggest that there is no trans-acting factor binding to D2Neg-A in NB41A3 cells despite its strong negative modulatory activity. One hypothesis to explain this phenomenon is that the DNA strand in D2Neg-A might form a structural control element, which has been suggested to reduce transcription from the mammalian dihydrofolate reductase gene promoter due to its rigid structure rather than by interacting with trans-acting factors (Pierce et al., 1992), although no homology exists between these two sequences. Additional investigation is required to reveal the intriguing mechanism by which the D2Neg-A region affects D2 promoter activity.
D, Gene Negative Modulator

Various amounts of anti-Spl antibody were added to the same binding reaction as in Fig. 4. The total amount of gelatin which is included in the antibody preparation was adjusted to 200 ng/reaction. Panel a, the probe is the rat D, gene fragment extending from -225 to -67 generated by PCR as in Fig. 4. The shifted bands are designated as a, b, and c as in Fig. 4. The supershifted band is indicated as b*. Panel b, the probes are the TGGG repeat or Spl consensus oligonucleotides. The supershifted band is designated as SS and the nonsupershifted band as S. A similar result was obtained with the Spl site B oligonucleotide probe.

The mechanism of the negative modulatory function of D, Neg-B also appears interesting because this region is composed of two Spl binding sites (TGGG repeat and Spl site A), and yet Spl is generally recognized to function as a positive modulator (Gidoni et al., 1985). Competitive cotransfection experiments using CATD2-116 or CATD2-75 as the CAT plasmid and pNeg-2 harboring D,Neg-B as the competitor (Fig. 2) suggested that a negative trans-acting factor(s) indeed interacts in vivo with D,Neg-B but not with the positive modulator sequence, Spl site B, since pNeg-2 increased the promoter activity of CATD2-116 but did not affect that of CATD2-75. Thus, there must be at least two kinds of nuclear factors in NB41A3 cells which recognize Spl binding sites in the D, gene. One of these factors might have greater affinity for Spl site B and positively regulates the D, promoter, whereas the other might prefer the Spl binding sites in D,Neg-B and negatively modulates this gene.

The functional role of the TGGG repeat and Spl site A was assessed by testing the promoter activity of various deletion or substitution mutants in D,Neg-B (Fig. 5). Between the TGGG repeat and Spl site A, the latter appears to be a more potent negative modulator of the D, promoter since deletion or alteration of its sequence recovers some of the transcriptional activity, whereas deletion of the TGGG repeat does not result in significant change.

The nature of the trans-acting factor(s) in NB41A3 cells interacting with Spl binding sequences in the D, gene was addressed by gel mobility supershift and UV cross-linking experiments. Gel supershift assays with anti-Spl antibody suggested that there are at least two main factors in these cells which interact with D,Neg-B since only one (band b) of the two major retarded bands (b and c) using the negative modulator sequence as probe cross-reacted with anti-Spl antibody (Fig. 6). UV cross-linking experiments and the combination of gel supershift and UV cross-linking (Fig. 7) confirmed that there are indeed three kinds of nuclear proteins recognizing the Spl binding sequences. Two of these factors cross-react with anti-Spl antibody and have apparent molecular masses (105 and 115 kDa).
110 kDa complexes with the probe) very close to those of purified human Sp1 (108 and 115 kDa as complexes with the probe) and, therefore, are likely to be Sp1. The slight discrepancy in the apparent molecular masses between purified human Sp1 and the two factors in the murine NB41A3 cells might be due to species differences. The third nuclear factor in these cells is not recognized by the anti-Sp1 antibody and has an apparent molecular mass (130 kDa) greater than that of Sp1. Thus, this 130-kDa factor is likely to be different from Sp1.

In vitro transfection experiments suggested that a trans-acting factor(s) in NB41A3 cells recognizes the Sp1 binding sites of D2 Neg-B and negatively modulates the D2 promoter, whereas in vitro experiments revealed that these cells have a factor that recognizes these Sp1 binding sites but is distinct from Sp1 in size and cross-reactivity with anti-Sp1 antibody. Although there is no direct experimental evidence that these two observations are related, it is highly probable that the 130-kDa protein which we detect in vitro functions as a trans-acting negative modulator of the D2 gene in NB41A3 cells. Furthermore, the fact that this 130-kDa factor is likely present in both NB41A3 cells and the rat striatum (Fig. 8) suggests that this novel protein plays a role in controlling D2 gene expression within the brain. Purification and molecular characterization of this protein are needed to test these possibilities. Recently, at least five cDNAs encoding Sp1-related proteins have been cloned (Kingsley and Winoto, 1992; Hagen et al., 1992; Imataka et al., 1992; Sagowa et al., 1993). Similar to Sp1, these proteins bind to GC box (Sp1 consensus sequence) and/or GT box sequences such as GTGGG. Whether the 130-kDa protein found in the present investigation is one of these five proteins remains to be determined.

The D2 Neg-B sequence is highly homologous to at least two other reported silencers or negative modulators. The first is the silencer of the α T cell receptor gene which has the sequence GTGGGGTGGGTT (Winoto and Baltimore, 1989) homologous to the TGGG repeat of the D2 gene. However, the TGGG repeat in the D2 gene is not a strong silencer, and therefore the trans-acting factors recognizing these similar sequences in the two genes might be different. The second is the Sp1 binding sites in the simian virus 40 genome which function positively for the early promoter but negatively for the late promoter in the absence of T antigen (May et al., 1992). In this virus, formation of a transcription initiation complex including Sp1 protein at the early promoter prevents transcription from the late promoter. Thus, the regulatory mechanism at the D2 gene negative modulator is probably different from the SV40 promoter, since a negatively acting factor is suggested to interact with D2 Neg-B.

In summary, transcription from the D2 gene promoter is negatively modulated by two cis-acting regions: a 26-bp segment (D2 Neg-A) which appears to function without trans-acting factors, and a 41-bp segment (D2 Neg-B) composed of two Sp1 binding sites which functions by interacting with trans-acting factors. Besides Sp1 itself, a novel 130-kDa factor recognizing these Sp1 binding sequences is found in nuclear extract from the D2-expressing cells NB41A3 as well as the rat striatum.

Acknowledgments—We thank Dr. Kai-Xing Huang of NINDS for supplying nuclear extract from the rat striatum and Dr. Sadamitsu Asoh of NHLBI for critical reading of the manuscript.

REFERENCES


FIG. 8. Gel mobility shift assay with rat striatal nuclear extract. Gel mobility shift assay was done using 15 μg of rat striatal nuclear extract (the four left lanes) or 4 μg of NB41A3 cell nuclear extract (the two right lanes) as in Fig. 4. The probe was the D2 gene fragment extending from nucleotide −225 to −67 generated by PCR as in Fig. 4. One hundred ng of anti-Sp1 antibody was added to the lanes indicated as Sp1 antibody. Competitors were added in about 200-fold molar excess relative to the radiolabeled probe. The bands designated as a, b, and c are the same as in Fig. 4. The supershifted band is indicated as b*.