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Regulation of Human Monoamine Oxidase B Gene by Sp1 and Sp3

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ABSTRACT

The human monoamine oxidase (MAO) B plays a major role in the degradation of biogenic and dietary amines such as phenylethylamine, benzylamine, dopamine, and tyramine. We previously showed that the −246/−99 MAO B promoter region exhibited the highest activity and contained two clusters of overlapping Sp1 sites, a CACCC element and a TATA box. Here, using a series of 10 deletion constructs of the 2-kilobase pair 5′-flanking sequence, we identified additional potential regulatory elements, including activator proteins 1 and 4, CAAT, GATA, upstream stimulatory factor (USF), estrogen receptor (ER), and sex-determining region Y-box 5 (SOX5). Analysis of nine site-directed mutations of −246/−99 region reveals that both clusters of Sp1 sites contribute positively whereas the CACCC element contributes negatively to the transcriptional activity. Gel shift analysis demonstrates that in addition to Sp1, Sp3 can interact with both clusters of Sp1 sites. Cotransfection experiments show that Sp1 and its closely related family member Sp4 can *trans*-activate MAO B promoter activity through the proximal cluster of Sp1 sites and its activation can be repressed by the over-expression of Sp3 and a related family member BTEB2. These results suggest that the binding to the overlapping Sp1 sites by various members of Sp family is important for the regulation of the MAO B gene expression.

Monoamine oxidase (MAO) metabolizes biogenic and dietary amines in the central nervous system and peripheral tissues, and yields hydrogen peroxide (H_2O_2). Two distinct MAOs have been described: MAO A has higher affinity for serotonin, norepinephrine, and inhibitor clorgyline; MAO B exhibits higher affinity for phenylethylamine, benzylamine, and inhibitor deprenyl [for review, see Shih et al., (1999)]. In addition, MAO B converts 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine to its toxic metabolite 1-methyl-4-phenylpyridine, which selectively destroys nigrostriatal neurons (Gerlach et al., 1996). The neurodegeneration induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine is similar to the neuronal damage in Parkinson's disease and can be prevented by the MAO B inhibitor deprenyl (Heikkila et al., 1984).

Although both forms of MAO are expressed throughout the body, they differ in cell- and tissue-specific and developmental expressions. Placenta and fibroblasts express predominantly MAO A (Egashira 1976; Edelstein and Breakefield, 1981) and platelets and lymphocytes express only MAO B (Bond and Cundall, 1977; Donnelly and Murphy, 1977). Interestingly, in the brain, even though MAO A prefers serotonin and MAO B prefers phenylethylamine as substrates, MAO A is predominantly found in catecholaminergic neurons and MAO B is most abundant in serotonergic neurons and astrocytes (Westlund et al., 1985). Moreover, MAO B, but not MAO A, activity increases progressively in the brain throughout adult life (Fowler et al., 1980). Aberrant increase of MAO B activity in the elderly has been implicated in neurodegenerative diseases such as Parkinson's disease (Schneider et al., 1981), Alzheimer's disease (Saura et al., 1994), and Huntington's disease (Mann et al., 1986). On the other hand, low platelet MAO B activity has been associated with several psychiatric disorders and behavioral traits such as bipolar disorder, suicidal behavior, alcoholism (Oreland 1993), poor impulse control [for review, see Holschneider and Shih (1998)] and smoking (Fowler et al., 1996).

MAO A and MAO B genes are located on Xp11.2–11.4 (Lan et al., 1989) and consist of 15 exons with identical exon-intron organization (Grimsby et al., 1991). The promoter organization of human MAO A and B are different (Zhu et al., 1992). The MAO A promoter shows bidirectional activity (Zhu et al., 1994) and contains a functional polymorphism in the number of tandem repeat sequence (Sabol et al., 1998). However, the regulation of MAO B gene has not been addressed. In the present study, we show that several potential

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ABBREVIATIONS: MAO, monoamine oxidase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; WT, wild-type; DTT, dithiothreitol; bp, base pair(s); AP-1, activator protein 1.

cis-acting regulatory elements are present within the 2 kilobases of 5′-flanking sequence and the promoter region between −246 and −99 bp is critical for the transcription of MAO B gene. The transcription factors Sp1 or its closely related family member Sp4 can *trans*-activate the promoter activity and its activation can be repressed by the overexpression of Sp3 or related family member BTEB2.

Materials and Methods

Cell Lines and Reagents. The 1242-MG (human astrocytoma) cell line was obtained from Dr. B. Westermark, and HeLa (human cervical adenocarcinoma) and HepG2 (human hepatocytoma) cell lines were purchased from the American Type Culture Collection (Manassas, VA). All three cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 10 μg/ml streptomycin, and 10% FBS (Life Technologies, Gaithersburg, MD). Polyclonal antisera against Sp1 and Sp3 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Human MAO B Promoter-Luciferase Reporter Constructs. The *Bam*HI/*Bam*HI MAO B promoter fragment (−2099/−99 bp) was cloned into the polylinker site (*Bgl*II) upstream of the luciferase gene (LUC) in the pGL2-Basic vector (Promega, Madison, WI). The −2099/−99LUC was referred to wild-type (WT) promoter construct in the deletion study. Serial deletion mutants (D1–D10) were generated by restriction enzyme digestion using the −2099/−99LUC as a template followed by Klenow fill-in and self-ligation. The following restriction enzymes were used to generate the deletion mutant constructs: *Xho*I/AspI (D1); *Xho*I/*Bgl*II (D2); *Xho*I/*Spe*I (D3); *Xho*I/*Apa*I (D4); *Xho*I/*Pst*I (D5); *Spe*I/*Hin*dIII (D6); *Bgl*II/*Hin*dIII (D7); *AspI*/*Hin*dIII (D8); *Bgl*II/*Spe*I (D9), and *Nco*I/*Asp*I (D10). The restriction enzymes *Pst*I and *Hin*dIII were used to select positive clones and to verify the correct orientation. One recombinant clone for each of the constructs was chosen, and the plasmid DNA was extracted and purified using Qiagen Miniprep kit (Qiagen, Inc., Chatsworth, CA) following the manufacturer's instructions.

Site-Directed Mutagenesis of the Human MAO B Proximal Promoter (−246/−99 bp). Site-directed mutagenesis was used to mutate potential transcription elements (Sp1, CACCC, and TATA elements) in the proximal promoter region (−246 to −99). Mutant promoter constructs (m1–m10) were generated using deletion construct D5 as a template. Mutagenesis was carried out using the Amersham Mutagenesis kit (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's instructions. The primers used for mutagenesis (mutations underlined) were the following:

5′ ACCGCCCCCGAACGCAGCTCTG 3′ (m1); 5′ AGGGCCACCGAACCCGCCCGCA 3′ (m2); 5′ GGCAGGCCGGAAGCAGCGCAGG 3′ (m3); 5′ CGCAGCCCCGAACGCCCGCCTACGCGC 3′ (m4); 5′ CGCAGCCCCGCCCGCAAGCCTACGCGC 3′ (m5); 5′ CGCAGCCCCGAACGCAAGCCTACGCGC 3′ (m6); 5′ CGCCCGCCCGAATACGCGCAGG 3′ (m7); 5′ ACGCGCAGCCAAGCCCGCCCGC 3′ (m8); 5′ CGCAGCCAAGCCCGCCCGAATACGCGC 3′ (m9); 5′ GGCGAGCCGCTAGGTTACCAGCCCCCG 3′ (m10). The mutated nucleotide sequences of all mutant constructs were confirmed by DNA sequencing.

Transient Transfection and Luciferase Assay. Transfections in 1242-MG, HeLa, and HepG2 cells were performed using Superfect transfection reagent (Qiagen, Inc.) following the manufacturer's instructions. Exponentially growing cells were plated at a density of 5×10^5 cells/well in six-well plates (Costar, Cambridge, MA) with 2 ml of DMEM and 10% FBS, and grown until 80% confluent (24–36 h). For promoter deletion and mutagenesis studies, 2 μg of MAO B promoter constructs were cotransfected into the 1242-MG, HeLa, and HepG2 cells with 20 ng of plasmid pRL-TK (the herpes simplex virus thymidine kinase promoter fused upstream to the *Renilla* luciferase gene, which is used as an internal control; Promega). The plasmids were mixed with 100 μl of serum- and antibiotic-free medium and 10 μl of Superfect reagent. Following a 15-min incubation at room temperature, 600 μl of DMEM (with 10% FBS and antibiotics) were added to the DNA-Superfect complexes. The cells were washed once with phosphate-buffered saline and then incubated with DNA-Superfect complexes. After 2-h incubation, the cells were washed with phosphate-buffered saline and incubated with fresh DMEM (with 10% FBS and antibiotics). Cells were harvested 48 h later with Luciferase Assay lysis buffer (Promega). The cell lysates were then assayed for luciferase activity using the Promega Dual Luciferase Assay system (Promega). The expression plasmid pCMV-Sp1 was kindly provided by Dr. Robert Tjian, and the pCMV-Sp3 and pCMV-Sp4 were generous gifts from Dr. Guntram Suske. The cDNA sequence for BTEB2 has been described in GenBank (Accession no. AB030824). The expression plasmid pCMV-BTEB2 was generated by cloning BTEB2 cDNA into the polylinker sites of pCMV3.1 (Invitrogen, La Jolla, CA). For cotransfection experiments, the total amount of DNA for each transfection was kept constant by the addition of empty expression vector pCMV3.1.

Nuclear Protein Extraction and Gel Shift Assay. Cells were washed with cold phosphate-buffered saline, harvested by scraping, and pelleted. The cell pellets were then resuspended in 5 pellet-volumes of buffer A (10 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride), incubated on ice for 10 min, and centrifuged for 10 min. The pellets were resuspended in 3 pellet-volumes of buffer A plus 0.1% Nonidet P-40, incubated on ice for 10 min, and centrifuged for 10 min. The pellets were then resuspended in buffer B (10 mM HEPES, 400 mM NaCl, 0.1 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 15% glycerol) and incubated on ice for 30 min with gentle shaking. Nuclear proteins were then centrifuged for 30 min and dialyzed for 4 h at 4°C against 1 liter of buffer D (20 mM HEPES, 200 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 15% glycerol). Protein extracts were cleared by centrifugation at 4°C for 15 min. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA).

The DNA fragment for gel shift assay was radiolabeled by Klenow fill-in. Labeled probes were purified by gel electrophoresis (5% polyacrylamide) and eluted in Tris/EDTA buffer. For DNA-protein binding, 5-μg nuclear extracts were diluted in binding buffer [40 mM HEPES, pH 8.0, 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 10 μg/ml of poly (dI-dC) (Sigma, St. Louis, MO)] with total volume of 20 μl. Antibodies against Sp1 or Sp3 were added (when required) and the mixture was incubated for 20 min at room temperature. Labeled probe (0.2 ng) was added to the mixture and incubated for additional 20 min at room temperature. The samples were then run on a 5% nondenaturing polyacrylamide gel in 1× Tris/borate/EDTA at 150 V for 3 h. Gels were dried and visualized by autoradiography.

Statistical Analysis. All values were presented as means ± SEM. Student's *t* test was used for statistical analysis, and differences were considered significant when $p < 0.05$.

Results

Deletion Analysis of the Human MAO B Promoter. To identify the transcriptional elements regulating the transcription of MAO B gene, the 5′-flanking sequence between −2099 and −99 bp (+1 was assigned to the start of translation) was inserted into the promoter-less luciferase reporter gene construct (pGL2-Basic). This clone was isolated from the X chromosome-specific library (Zhu et al., 1992). The potential *cis*-acting regulatory elements are shown in Fig. 1A. A series of deletion constructs were generated from the 5′ or 3′ end of the MAO B promoter as described under *Materials and Methods*. Each of these deletion constructs was

transiently transfected into 1242-MG, HeLa, and HepG2 cells along with the pRL-TK plasmid to monitor transfection efficiency. Both 1242-MG and HeLa cells have MAO A (0.35 and 0.44 nmol/20 min/mg of protein, respectively) and MAO B (0.64 and 0.53 nmol/20 min/mg of protein, respectively) activity but HepG2 cells show higher MAO A (6.46 nmol/20 min/mg of protein) and MAO B (8.2 nmol/20 min/mg of protein) activity. The WT MAO B promoter was constitutively active (Fig. 1B legend) in all three cell lines studied, as shown in Fig. 1B. Deletion of the region that contained GATA, AP-1, upstream stimulatory factor, and estrogen receptor binding sites (D1) increased promoter activity by ~ 2-fold. Further deletion of two GATA binding sites (D2) increased MAO B promoter activity to ~ 3 folds, indicating that this region (−2099 to −1180 bp) contained silencer(s) that negatively regulate the transcription activity. However, further deletion of the AP-1, CAAT, and activator protein-4 sites (D3) decreased the promoter activity, suggesting that the region between −1180 and −868 bp contained enhancer element(s) that positively regulated the promoter activity. Deletion of the region that contains three AP-1 and one sex-determining region Y-box 5 binding sites (D4) increased promoter activity over 3-fold (compared with WT), indicating the presence of silencers in this region (−868 to −425 bp). Further deletion of the GATA and Sp-1 binding sites (D5, −425 to −246 bp) decreased the promoter activity.

On the other hand, the promoter activity was almost abolished (~2–5% of the WT) when the proximal regions of the promoter were deleted (D6, D7, and D8). Therefore, sequences within 246 bp upstream of the transcription start site seemed to be critical for the transcription of MAO B gene in transient transfections. Deletions D9 and D10 (−868 to −425 bp and −1703 to −1313 bp) increased MAO B promoter activity to ~2-fold, which was consistent with the results

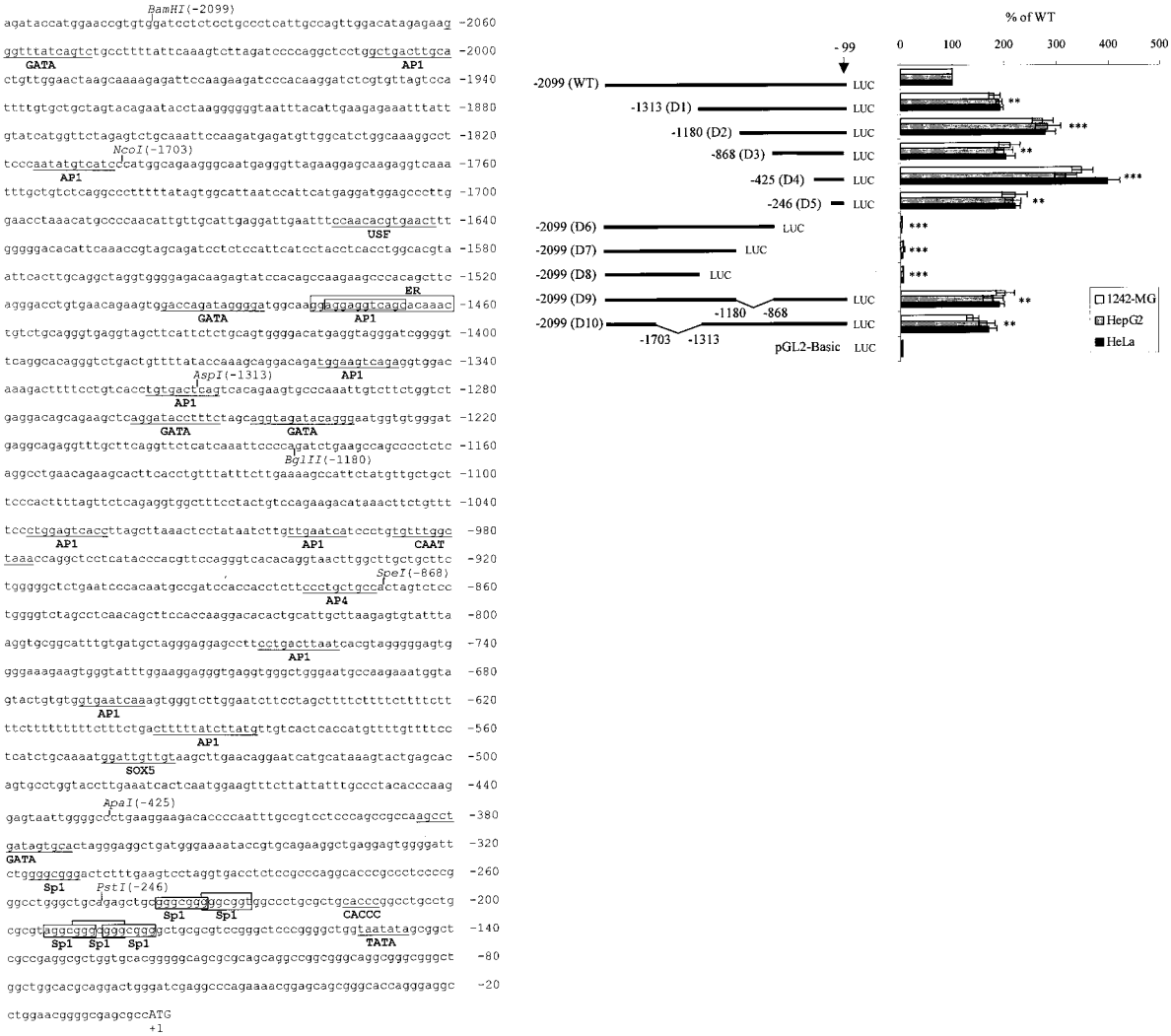

Fig. 1. Effect of deletion mutations on human MAO B promoter activity. A, 5′-flanking sequence of human MAO B gene. The A of the ATG codon of the translational start site is labeled as +1. Potential regulatory elements within the human MAO B promoter are underlined and labeled in bold. The restriction enzymes used to generate deletion constructs are indicated with their corresponding locations. Overlapping sites are shown in open boxes. B, the promoter activity of the WT and the 10 deletion constructs (D1–D10) are shown. Two micrograms of WT or deletion constructs and 20 ng of the pRL-TK vector (herpes simplex virus thymidine kinase promoter upstream of Renilla luciferase reporter gene, as an internal control) were cotransfected into HeLa, 1242-MG, and HepG2 cells. Luciferase (LUC) activity was determined in duplicates and was normalized with activity of internal control pRL-TK. The normalized luciferase activity of the WT constructs (1242-MG, 5.34; HepG2, 7.23; HeLa, 4.45) is taken as 100% and the activities of the deletion constructs are shown as percentage of the WT. The pGL2-Basic is the promoterless luciferase vector used as a negative control. Data are the mean ± S.D. from three independent experiments with duplicates for each experiment. Statistical analysis was performed comparing the activity of the deletion contructs to that of WT. $^{**}p < 0.01$, $^{***}p < 0.001$.

obtained from D1 and D3, suggesting that these two regions may contain silencer(s) negatively regulating MAO B promoter activity. Results from the deletion study demonstrated that the MAO B promoter contained multiple positive and negative regulatory elements, and the transcription of MAO B gene depended on the *cis*-elements within the proximal region (−246/−99 bp) of the promoter. This proximal promoter contained two clusters (distal and proximal) of overlapping Sp1 binding sites, a CACCC element and a TATA box. Our previous results from primer extension experiments had shown that multiple transcription initiation sites were present upstream of the MAO B coding sequences (Zhu et al., 1992). These initiation sites were located at −150, −142, −139, −132, −128, −123, −119, and −117 (Zhu et al., 1992). All initiation sites except −150 were downstream of the TATA box, suggesting that a TATA-dependent transcription was present for the MAO B gene.

Distinct Functions of Sp1 Clusters, CACCC Element, and TATA Box in the MAO B Promoter. To define the *cis*-elements in the proximal promoter (−246/−99 bp) that may contribute to MAO B gene transcription, a series of mutant promoter constructs were generated using the construct D5 (from the deletion experiment, Fig. 1B) as a template. Mutations were introduced into each of the five Sp1 elements, CACCC element, and TATA box (Fig. 2A). Each of these mutant promoter constructs was transiently transfected into HeLa, 1242-MG, and HepG2 cells along with the plasmid pRL-TK (internal control to monitor transfection efficiency). The normalized luciferase activity of the construct D5 (the WT promoter) was set to 1 and the fold induction of the mutant constructs were determined. As shown in Fig. 2B, mutation of the first Sp1 element in the distal Sp1 cluster (m1) had no effect on MAO B promoter activity in HeLa and HepG2 cells but reduced ~50% promoter activity in 1242-MG cells. Mutation of the second Sp1 element (m2) resulted in ~50% reduction in promoter activity in HeLa and 1242-MG cells but ~40% increase in promoter activity in HepG2 cells. When the distal Sp1 cluster was left intact, single mutations in the proximal Sp1 cluster (m7 and m8) resulted in a 40 to 80% reduction in promoter activity in all of these cell lines. The promoter activity was also reduced when double mutations were introduced into proximal Sp1 cluster (m4, m5, and m9). Finally, when all three Sp1 elements in the proximal cluster were mutated (m6), the MAO B promoter activity was reduced by ~80%. These results indicated that the Sp1 elements in the proximal Sp1 cluster were critical for the transcription of MAO B gene. In contrast, the promoter activity was increased ~2.5 to 13-fold when the CACCC element was mutated (m3, Fig. 2B), suggesting that the CACCC element functioned as a negative regulatory element for MAO B promoter. A significant reduction (~90%) in promoter activity was observed when the TATA box was mutated (m10), indicating its functional role for the transcription of MAO B gene. Results from the mutagenesis analysis of the MAO B proximal promoter demonstrated that the Sp1 elements, particularly in the proximal Sp1 cluster, contributed positively whereas the CACCC element negatively to the MAO B promoter activity, and the TATA box was critical for the basal transcription of the gene.

The Transcription Factors Sp1 and Sp3 Bind to the MAO B Proximal Promoter (−246/−99 bp). To determine whether nuclear proteins may bind to the MAO B proximal promoter (−246/−99 bp; D5), nuclear extracts from HepG2 cells were analyzed by gel shift assay using the radiolabeled

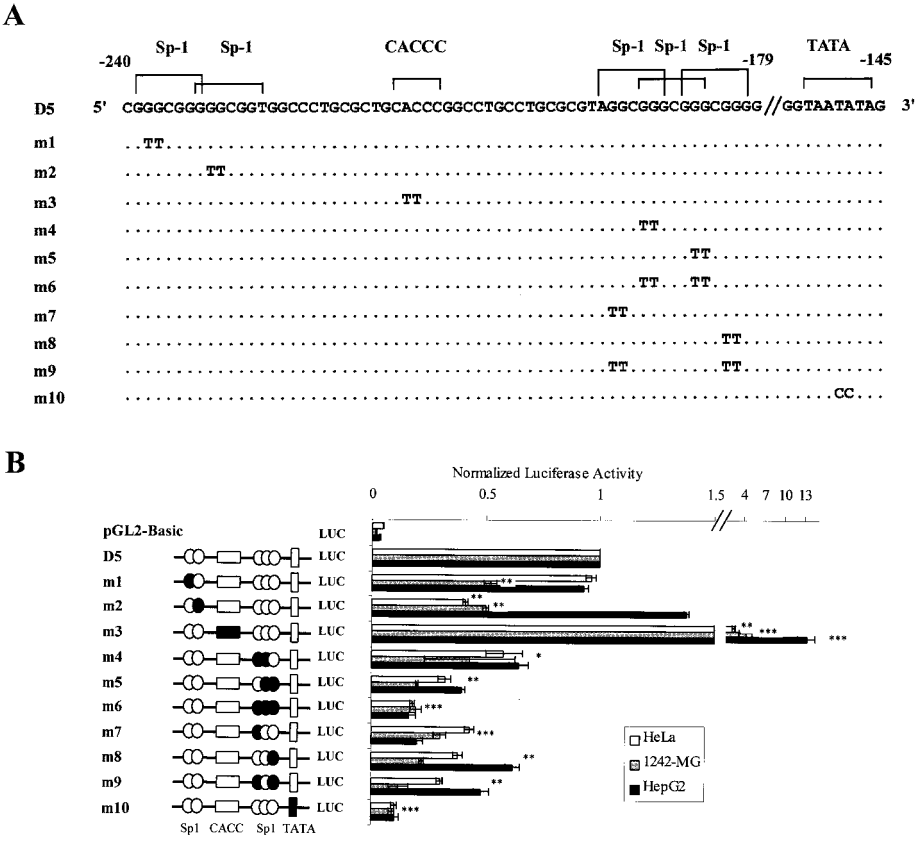

Fig. 2. Functional analysis of the MAO B proximal promoter. A, site-specific mutations were introduced into CACCC element, TATA box, and each of the overlapping Sp1 sites with various combinations (−246/−99 bp, D5). The mutated nucleotide sequences were indicated. B, the proximal promoter (D5) and its mutant constructs were cotransfected with pRL-TK into HeLa, 1242-MG and HepG2 cells. Luciferase (LUC) activity was determined in duplicates and was normalized with activity of internal control pRL-TK. The effect of site-specific mutations on MAO B proximal promoter activity is shown. The activity of the wild-type proximal promoter construct (D5) (HeLa, 7.67; 1242-MG, 6.24; HepG2, 8.65) is set to 1 to determine the fold of induction of the mutant constructs. Statistical analysis was performed comparing the activity of the deletion constructs to that of D5. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

promoter fragment D5 as probe. As shown in Fig. 3A, five DNA-protein complexes (I, II, III, IV, and V) were detected. To determine the specificity of the five complexes, an excess of unlabeled promoter fragment was used as competitor. The formation of all five complexes was abolished in the presence of cold competitors, indicating that the complexes were specific to the MAO B promoter (lane 2). Because the D5 promoter region contains multiple Sp1 binding sites, these protein complexes may belong to other members of Sp1 family. To further investigate the identities of the proteins in the band shift complexes, supershift experiments were performed using Sp1 and Sp3 antibodies. The complex II was supershifted in the presence of Sp1 antibodies (lane 3), and complexes I and III were supershifted by Sp3 antibodies (lane 4). The complexes I, II, and III were all supershifted when both Sp1 and Sp3 antibodies were present (lane 5). These results showed that the transcription factors Sp1 and Sp3 could specifically interact with the proximal MAO B promoters. The two differently migrating bands of Sp3 may correspond to the two Sp3 isoforms that arise from Sp3 mRNA via translational initiation at two internal sites located within the *trans*-activation domain (Kennett et al., 1997). Although the internally initiated Sp3 could bind to Sp1 binding sites, it was shown to be unable to stimulate the transcription of Sp-regulated genes (Kennett et al., 1997). The identities of complexes IV and V remained to be determined, although their intensities were decreased by Sp1 antibodies, but not by Sp3 antibodies, suggesting that they may be partially degraded Sp1 or Sp1-related protein.

To define which elements in the MAO B promoter are involved in the binding of Sp1 and Sp3, the mutant promoter constructs (m1-m9) were radiolabeled and analyzed with nuclear extracts from HeLa and 1242 MG cells in gel shift assays. As shown in Fig. 3, B and C, mutation of the first Sp1 element in the distal Sp1 cluster (m1) had no effect on all complexes compared with the wild-type (D5), whereas mutation of the second Sp1 element (m2) decreased the binding of Sp1 and Sp3. Mutation of the CACCC element (m3) and the first and second Sp1 elements in the proximal Sp1 cluster (m4) had virtually no effect on all complexes, suggesting that these elements were not the major Sp1 and Sp3 binding sites. However, when the second and third Sp1 elements in the proximal Sp1 cluster were mutated (m5), the formation of all complexes was decreased, indicating that the second and third proximal Sp1 elements were important for the binding of Sp1 and Sp3 to MAO B promoter. Similar gel shift pattern was observed when the first Sp1 element in the proximal Sp1 cluster was further mutated (m6). In addition, mutation of the first Sp1 element in the proximal Sp1 cluster alone (m7) had no effect on all complexes comparing with the wild-type. The findings from mutant m6 and m7 were consistent with that from m4 that the first Sp1 element in the proximal Sp1 cluster was not crucial for the binding of Sp1 and Sp3. When the third Sp1 element in the proximal Sp1 cluster was mutated alone (m8) or together with the first Sp1 element (m9), the formation of all complexes was abolished, indicating that the third Sp1 element was critical for the binding of Sp1 and Sp3.

Sp1 and Sp4, but not Sp3 and BTEB2, Are *trans*-Activators of the Human MAO B Promoter. To determine whether members of the Sp family could modulate MAO B promoter activity, the D5 promoter construct (−246/−99 bp) was transiently transfected into 1242-MG cells with increasing amount (10–1000 ng) of expression plasmids for Sp1, Sp3, Sp4, or BTEB2. As shown in Fig. 4A, both Sp1 and Sp4 stimulated MAO B promoter activity in a dose-dependent manner, with Sp4 being the most potent *trans*-activator. In contrast, the promoter activity was not affected by the over-expression of Sp3 or BTEB2. These results indicated that although Sp1, Sp3, Sp4, and BTEB2 contain similar

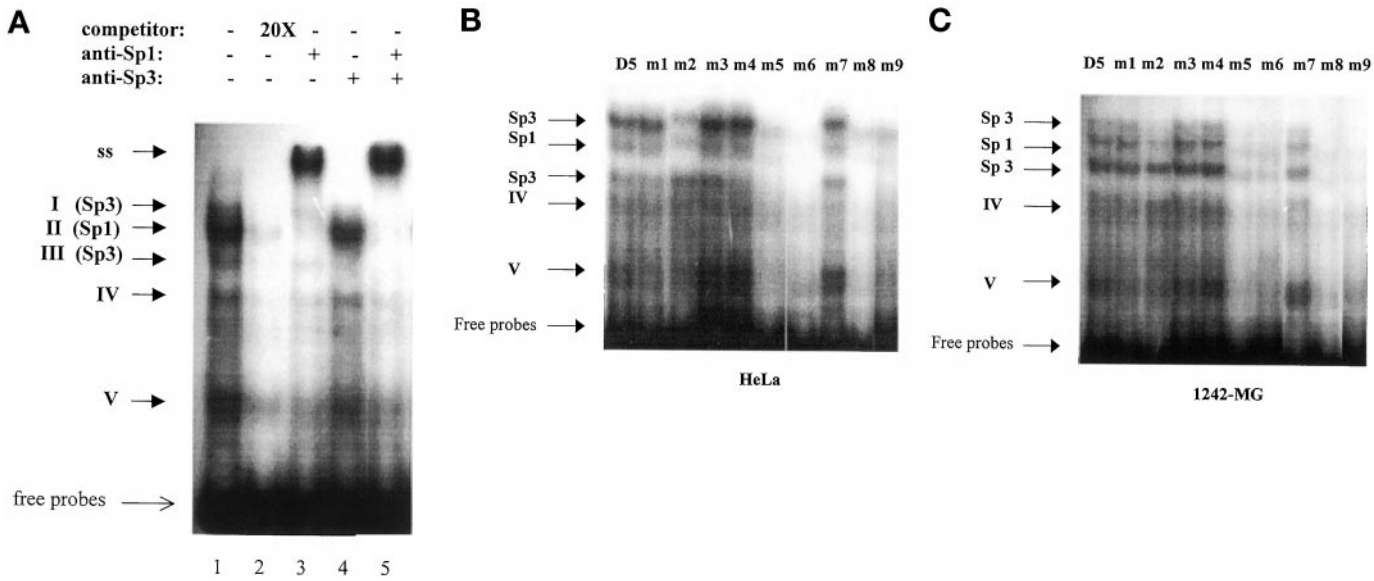

Fig. 3. Gel shift assay of complex formation over the MAO B proximal promoter. A, gel shift assay was performed with 5 μg of nuclear extracts from HepG2 cells and ^{32}P-labeled (20,000 cpm) wild-type (D5, −246/−99 bp) probe (lane 1). Arrows indicate protein-DNA complexes and free probes. Competition analysis was performed in the presence of a 20 M excess of unlabeled wild-type (D5) (lane 2). Supershift analysis was performed with 5 μg of nuclear extracts from HeLa cells in the presence of anti-Sp1 (lane 3), anti-Sp3 (lane 4), and both anti-Sp1 and anti-Sp3 antibodies (lane 5). "ss" denotes supershifted band. B, the effect of site-specific mutations on proteins binding to the *cis*-elements of proximal promoter region (−246/−99) is shown. Gel shift assay was performed with 5 μg of nuclear extracts from HeLa (Fig. 3B) or 1242 MG (Fig. 3C) cells and radiolabeled wild-type (D5) or mutant constructs (m1–9). The shifted bands for Sp1 and Sp3 are indicated by arrows and Sp1-related-DNA complexes are indicated by "IV" and "V".

functional domains and can recognize the GC boxes, only Sp1 and Sp4 could *trans*-activate the MAO B promoter.

Even though Sp3 and BTEB2 had no effect itself on the MAO B promoter activity in transient transfection assays, a dynamic interaction between Sp1/Sp4 and Sp3/BTEB2 in the regulation of MAO B promoter cannot be ruled out because all of these factors contain conserved DNA binding domains that can recognize the overlapping Sp1 elements in the promoter. Moreover, the proximal MAO B promoter contains overlapping Sp1 elements and the binding of the overlapping Sp1 elements by one of these factors may exclude the binding by the other because of spatial hindrance (Gidoni et al., 1985). To determine whether competition between members of the Sp family may play a role in MAO B transcription, cotransfection experiments were performed using various combinations of these factors. As shown in Fig. 4B, Sp1- or Sp4-mediated activation was reduced by cotransfection of Sp3 or BTEB2. These results indicated that Sp3 and BTEB2 could repress the MAO B promoter activity by inhibiting Sp1 or Sp4-mediated activation. No synergistic or additive effect on promoter activity was observed when Sp1 and Sp4 were both over-expressed, which may be because of saturation of Sp1 in the cells.

Identification of Transcriptional Elements Responsible for Sp1- and Sp4-Mediated MAO B Promoter Activation. The MAO B proximal promoter contains five Sp1 binding sites and one CACCC element that both Sp1 and Sp4 can recognize. To define the elements that were responsible for the promoter activation mediated by Sp1 and Sp4, each of the mutant MAO B promoter constructs (m2, m3, m6, and m7) was transiently cotransfected into 1242-MG cells with expression plasmid for Sp1 or Sp4. As shown in Fig. 5, no significant change of Sp1- or Sp4-mediated activation was observed (compared with WT) when the distal Sp1 cluster was mutated (m2). Mutations of the proximal Sp1 (m6 and m7) resulted in ~80 to 90% reduction in Sp1- or Sp4-mediated promoter activation. In contrast, mutation of the CACCC element led to more than 3-fold enhancement of Sp1 and Sp4-mediated promoter activations. These results indicated that the proximal Sp1 cluster was largely responsible

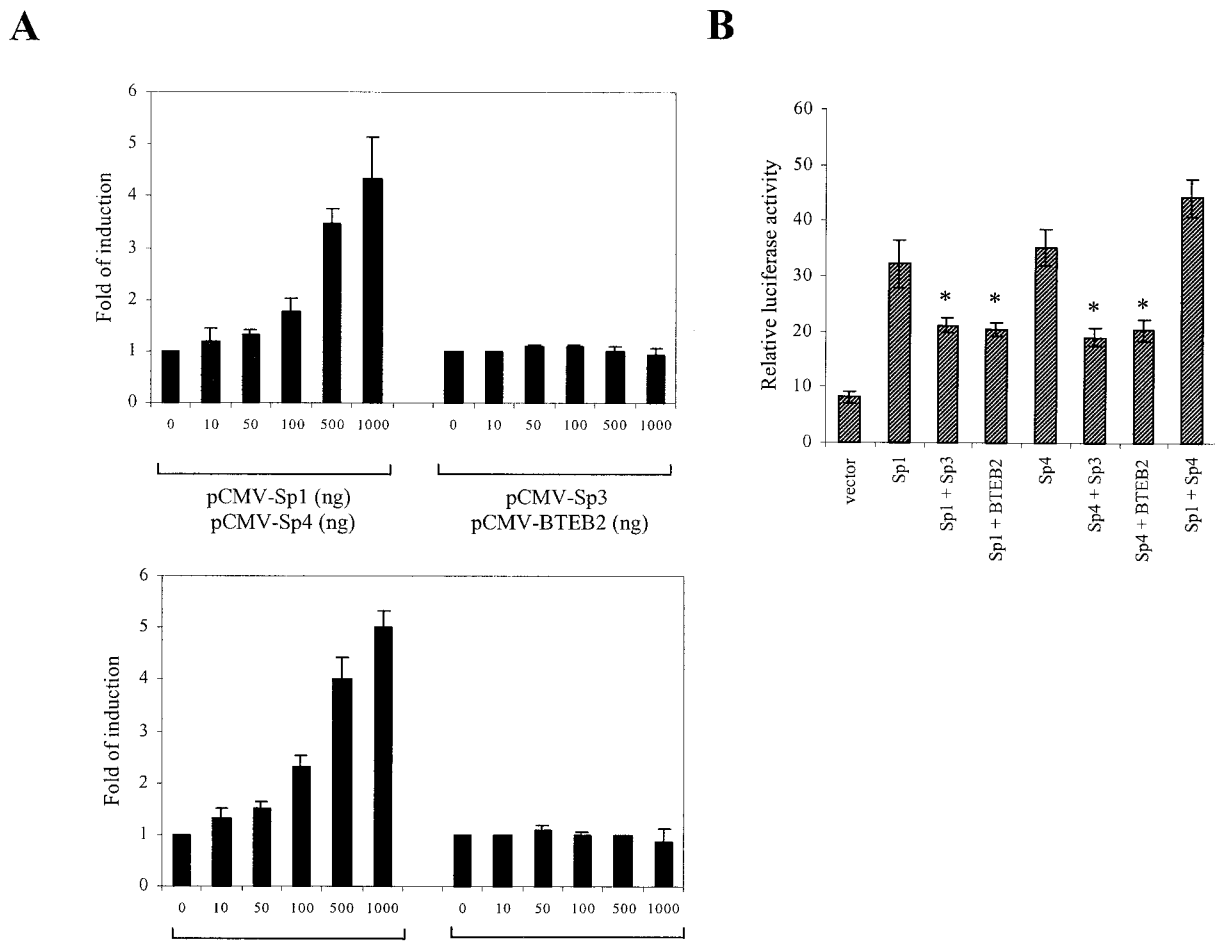

Fig. 4. The transcription factors Sp1 and Sp4, but not Sp3 and BTEB2, activate human MAO B proximal promoter activity. A, effect of each transcription factor Sp1, Sp3, Sp4 and BTEB2 on MAO B promoter activity. One microgram of the proximal promoter construct (D5) was cotransfected with variable amounts (10–1000 ng) of the expression plasmids for Sp1, Sp3, Sp4, and BTEB2, respectively, into 1242-MG cells along with 20 ng of internal control plasmid pRL-TK. The "Fold of Induction" represents the fold increase in luciferase activity relative to that obtained from cotransfection of expression vector (pCMV3.1) alone. Luciferase activity was determined in duplicates and was normalized with activity of internal control pRL-TK. B, effect of coexpression of Sp1, Sp3, Sp4, and BTEB2 on promoter activity is shown. Five hundred nanograms of the expression plasmid for each transcription factor was cotransfected with 1 μg of the proximal promoter construct (D5) into 1242-MG cells. The amount of transfected plasmid was kept constant (2 μg) using vector construct (pCMV3.1). Data are the mean ± S.D. from three independent experiments with duplicates for each experiment. Statistical analysis was performed comparing the activity obtained from cotransfection with that from transfection of Sp1 or Sp4 alone. *$p < 0.05$.

for Sp1- and Sp4-mediated *trans*-activation of MAO B gene promoter. Furthermore, the CACCC element seemed to involve in both Sp1 and Sp4-mediated activation because its mutation enhanced the MAO B promoter activation by Sp1 and Sp4.

Discussion

MAO B has been associated with a number of psychiatric disorders and neurodegenerative diseases; therefore, it is important to understand the molecular basis of its gene expression. In the present study, we have identified several potential *cis*-elements in the 2 kilobases of 5′-flanking sequence of the MAO B gene. Moreover, we have shown that the promoter region between nucleotides −246 and −99 was sufficient to provide the basal promoter activity in the 1242 MG, HepG2, and HeLa cells. This proximal promoter region (−246/−99) contains multiple Sp1 consensus elements arranged into two clusters, a negatively regulating element CACCC and a functional TATA box for the transcription of MAO B gene. Although both Sp1 and Sp3 can interact with both clusters of Sp1 elements, the proximal cluster was critical for the binding of these factors because the mutation of this cluster significantly affects their binding. Functional studies have demonstrated that Sp1 and Sp4, but not Sp3 and BTEB2, can *trans*-activate MAO B promoter activity. Furthermore, over-expression of Sp3 or BTEB2 can repress the Sp1- or Sp4-mediated promoter activation, possibly by competing with Sp1 and Sp4 for binding to the overlapping Sp1 elements.

The transcription factors Sp1, Sp3, Sp4, and BTEB2 are members of the Sp family encoding proteins with similar structural features and highly conserved zinc finger DNA binding domain that can recognize the GC box (GGGGCGGGC) and/or the GT motif (GGGTGTGGC) with similar affinities. The Sp family was further divided into three subgroups based on the structural relationships between the protein members: 1) the Sp transcription factors; 2) the close relatives such as TIEG1 and TIEG2; and 3) the kruppel-like factors such as BTEB2 and AP-2rep. Sp1, Sp3, and Sp4 contain glutamine and serine/threonine-rich *trans*-activation domains, whereas BTEB2 contains a proline-rich *trans*-activation domain [for review, see Philipsen and Suske (1999)]. The existence of proteins with DNA-binding specificity similar to Sp1 indicates that gene regulation by Sp1 is complex. Although Sp1 and Sp3 are expressed ubiquitously, Sp4 is predominantly expressed in the brain and BTEB2 is only expressed in placenta and testis [for review, see Philipsen and Suske (1999)]. Interestingly, the human MAO B is also highly expressed in the brain (Westlund et al., 1985) but not detected in the placenta (Egashira, 1976). In the present study, we have demonstrated that the Sp1 and Sp4 are potent *trans*-activators of the MAO B promoter whereas the Sp3 and BTEB2 repress the Sp1- or Sp4-mediated activation of the promoter. Thus, it is tempting to speculate that the similar structural but distinct functional and expression features of these Sp family members may play an important role in the tissue-specific expression of the human MAO B gene.

The human MAO B core promoter (−246/−99 bp) contains overlapping Sp1 sites that can be recognized by Sp1 and Sp3, and presumably by Sp4 and BTEB2, based on their conserved DNA binding domains. Previous studies have demonstrated that the binding of two adjacent Sp1 molecules to a DNA sequence requires at least 10 bp between the central C of the two Sp1 elements (GGGCGGG) (Gidoni et al., 1985). Because the Sp1 elements in the MAO B core promoter are overlapped, only one Sp1 can bind to each cluster of Sp1 binding sites at a time. On the other hand, the activity and cellular content of Sp1 have been shown to be regulated during development (Ammendola et al., 1992), cellular proliferation (Shin et al., 1992), apoptosis (Piedrafita and Pfahl 1997), and other cellular processes by post-translational modification including phosphorylation (Jackson et al., 1990) and glycosylation through the *O*-linkage of the monosaccharide, *N*-acetylglucosamine (*O*-GlcNAc) (Jackson and Tjian 1988). Sp3 has been shown to act as a bifunctional regulator whose activating or repressing activity is dependent upon both the promoter and the cellular context (Majello et al., 1997). Because only one of these factors can bind to each cluster at one time, alterations of the these factors and/or their DNA binding activities, and the competition between these factors for binding to the same cluster may account in part for the differential regulation of MAO B gene expression.

Mutation of the CACCC element (m3) activated the MAO B promoter activity by ~2- to 13-fold (Fig. 2B) and enhanced Sp1- and Sp4-mediated promoter activations (Fig. 5), suggesting that the CACCC element is a negative regulatory element. It is unclear at this time whether other proteins might interact with the CACCC sequence motif. Future experiments will be needed to determine the mechanism for this negative regulation. The −246/−99 region contains two clusters of overlapping Sp1 elements that contribute positively to the MAO B transcription. The transcription factors Sp1 and Sp3 can interact with both clusters of overlapping Sp1 sites. Furthermore, Sp1 and Sp4 can *trans*-activate MAO B promoter activity via the overlapping Sp1 sites and the promoter activations can be repressed by the over-expression of Sp3 or BTEB2. The arrangement of overlapping Sp1 sites in the promoter suggests a complexity of transcriptional regulation of MAO B gene. A number of promoters have been shown to contain overlapping Sp1 sites in their proximal

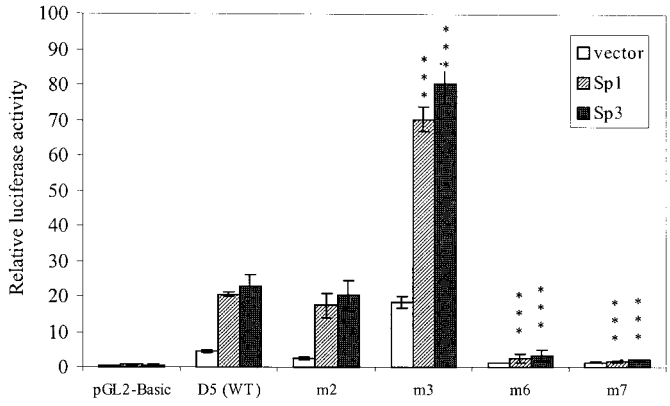

Fig. 5. Effect of site-specific mutations of MAO B proximal promoter on Sp1 and Sp4 activations. One microgram of wild-type (D5) or mutant promoter constructs (m2, m3, m6, and m7) was cotransfected with 1 μg of expression plasmid for Sp1, Sp4, or vector alone into 1242-MG cells. Luciferase activity was determined in duplicates and was normalized with activity of internal control pRL-TK. Data are the mean ± S.D. from three independent experiments with duplicates for each experiment. Statistical analysis was performed comparing the activity of the deletion contructs to that of WT. ***$p < 0.001$.

regions including platelet-derived growth factor A-chain (Khachigian et al., 1995) and acetylcholinesterase (Mutero et al., 1995). Redundant Sp1 binding site had been shown to block epigenetic gene inactivation in the mouse aprt gene promoter (Mummaneni et al., 1998). It is unclear at this point why the MAO B core promoter contains such arrangement of overlapping Sp1 sites. Future experiments will be needed to elucidate the function of overlapping Sp1 elements in the MAO B core promoter.

In summary, we have identified both positive and negative regulatory elements in the proximal promoter of human MAO B gene. We have also demonstrated the importance of the *cis*-elements within the proximal promoter region in the regulation of MAO B gene. Moreover, we have shown that Sp1 and Sp3 could bind to either proximal or distal clusters of Sp1 sites in the proximal region of MAO B promoter. Overexpression of Sp1 or Sp4 activated MAO B promoter activity and its activation could be repressed by over-expression of Sp3 or BTEB2. This study provides novel information on how the MAO B gene is regulated. Given the importance of the members of Sp1 family in the MAO B gene regulation, it will be of interest to determine whether any of these factors is aberrantly expressed in the MAO B-related psychiatric disorders and behavioral traits as well as neurodegenerative diseases. A further understanding of the molecular basis of MAO B regulation will provide insights into the pathophysiology of these disorders and diseases and may ultimately lead to design of new therapeutics.

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