Retinoic Acid Activates Monoamine Oxidase B Promoter in Human Neuronal Cells

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Monoamine oxidase (MAO) B deaminates a number of biogenic and dietary amines and plays an important role in many biological processes. Among hormonal regulations of MAO B, we have recently found that retinoic acid (RA) significantly activates both MAO B promoter activity and mRNA expression in a human neuroblastoma BE(2)C cell line. RA activates MAO B promoter in both concentration- and time-dependent manners, which is mediated through retinoic acid receptor α (RARα) and retinoid X receptor α (RXRα). There are four retinoic acid response elements (RAREs) as identified in the MAO B 2-kb promoter, and mutation of the third RARE reduced RA-induced MAO B promoter activation by 50%, suggesting this element is important. Electrophoretic mobility shift analysis and chromatin immunoprecipitation assay demonstrated that RARα specifically binds to the third RARE both in vitro and in vivo. Moreover, transient transfection and luciferase assays revealed that Sp1 enhances but not essentially required for the RA activation of MAO B through two clusters of Sp1-binding sites in the MAO B promoter. RARα physically interacts with Sp1 via zinc finger domains in Sp1 as determined by co-immunoprecipitation assay. Further, RARα was shown to be recruited by Sp1 and to form a transcriptional regulation complex with Sp1 in the Sp1-binding sites of natural MAO B promoter. Taken together, this study provides evidence for the first time showing the stimulating effect of RA on MAO B and new insight into the molecular mechanisms of MAO B regulation by hormones.

Monoamine oxidase (MAO) B deaminates a number of biogenic and dietary amines (1), and exists in two forms, MAO A and MAO B (2). Both enzymes catalyze the oxidative deamination of monoamine neurotransmitters such as serotonin, norepinephrine, phenylethylamine, and dopamine with different affinity (3). MAO B preferentially oxidizes phenylethylamine and benzylamine, as well as dopamine, and is irreversibly inhibited by low concentration of pargyline and deprenyl (4). Abnormal neurotransmitter levels are implicated in several neurological and psychiatric disorders. For example, low platelet MAO B activity with elevated level of phenylethylamine is linked with alcoholism and stress-related disorders (1, 5–8). Moreover, MAO B is predominantly found in serotoninergic and histaminergic neurons and glial cells (9–14). MAO B activity progressively increases in brain throughout adult life (15, 16). aberrant increase of dopamine oxidation by MAO B in the elderly is associated with loss of dopaminergic neurons in the substantia nigra, which underlies Parkinson disease (17, 18).

The transcriptional regulation of MAO B has been extensively studied in recent years. There are two clusters of overlapping Sp1-binding sites in the human MAO B promoter (19). Sp1 and Sp1-like family transcription factors are involved in the regulation of MAO B (20–22). Sp1, Sp4, and TIEG2 activate the MAO B core promoter via Sp1 sites, and this activation is repressed by Sp3 (20–22). DNA methylation of CpG sites in the MAO B promoter epigenetically inhibits MAO B gene expression (22). Moreover, MAO B can be activated by extracellular stress inducer, phorbol 12-myristate 13-acetate, which activates protein kinase C and mitogen-activated protein kinase signaling pathways, including Ras, MEK1, MEK3, MEK7, ERK2, JNK1, and p38/RK, with transcription factors c-Jun and Egr1 as the ultimate targets (23). Emerging evidence has recently suggested the importance of MAO gene regulation by hormones, because hormones such as androgen and glucocorticoid influence neurotransmitter levels by modulating MAO, which further affects mood and behaviors in humans (24, 25). Among hormones associated with the regulation of MAO B is estrogen, and estrogen-related receptors up-regulate MAO B transcription through the fourth estrogen response element (−291/−286) in the MAO B promoter (26), which has been recently found to overlap with a consensus retinoic acid (RA) response element (RARE) (−303/−287), suggesting potential regulation of MAO B by RA. Further, previous studies showed that RA stimulates MAO B activity in embryonic chick hepatocytes with little known about the molecular mechanisms (27).
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RA, as a non-steroid hormone, plays a critical role in mammalian development and homeostasis through their regulatory effects on cell differentiation, cell proliferation, and apoptosis (28–30). RA directly transactivates downstream target genes through retinoic acid receptors (RARs) and retinoid X receptors (RXRs) that bind to RAREs in the regulatory sequences of target genes (29). A canonical RARE can be a 7 bp-spaced inverted repeat (referred to as IR7), 5’-GGAANNNNNNNT-GACC-3’ (N is any nucleotide), as identified in the MAO B promoter (31). Several recent studies have also reported RARE-independent regulatory mechanisms via interactions of RAR/RXR with other transcription factors (32–34). In the present study, we demonstrate that RA activates the MAO B transcription via the third RARE and Sp1 sites in the MAO B promoter with RARα/RXRα and Sp1 involved.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—The human neuroblastoma BE(2)C cell line was purchased from the American Type Culture Collection (ATCC). BE(2)C cells were grown in a medium containing a 1:1 mixture of Eagle’s minimum essential medium with Earle’s balanced salt solution and Ham’s F-12 medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.05 mM non-essential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin. All culture materials were purchased from Mediatech Inc. All chemicals, including all-trans retinoic acid and mithramycin, were purchased from Sigma. Monoclonal anti-RXRα (sc-46659), anti-Sp1 (sc-17824), anti-β-actin (sc-47778), anti-GST (sc-138), polyclonal anti-RARα (sc-773), anti-RARβ (sc-551), anti-RXRβ (sc-774), and anti-GFP (sc-8334) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti-HA (H3663) antibody was purchased from Sigma. Polyclonal anti-Sp1 antibody (A300-133A) was purchased from Bethyl Laboratories, Inc. Mouse TrueBlot™ horseradish peroxidase (HRP) anti-mouse IgG (13-8817) was purchased from eBioscience, Inc. Human Sp1 recombinant protein was purchased from Promega (Madison, WI).

Plasmids—Human MAO B 2-κb or 0.15-κb promoter-luciferase reporter constructs (MAO B 2-κb- or 0.15-κb-luc) and their mutant forms with specific Sp1-binding sites mutated were generated as described previously (20, 21, 23). Human Sp1 expression construct was obtained as described previously (21). Human RARα and RXRα expression constructs were a gift from Dr. Henry M. Sucov (Dept. of Biochemistry and Molecular Biology, Keck School of Medicine, University of Southern California, Los Angeles, CA). GST-RARα was generated by inserting human RARα coding region at BamHI/EcoRI sites of pGEX-2T vector, a gift from Dr. Ron T. Hay (Center for Biomolecular Sciences, University of St. Andrews, St. Andrews, UK). GST-RXRα was generated by inserting human RXRα coding region at EcoRI/NotI sites of pGEX-4T1 vector (Amersham Biosciences). EGFP-RARα was generated by inserting human RARα coding region at BamHI/BglII sites of pEGFP-C1 vector (Clontech). The 3′ Sp1-binding sites luciferase reporter construct was a gift from Dr. Harry P. Elsholtz (Dept. of Laboratory Medicine and Pathobiology, Banting and Best Diabetes Center, University of Toronto, Toronto, Ontario, Canada) (35). Wild-type and truncated forms of HA-tagged Sp1 expression constructs were a gift from Dr. Hans Rotheneder (Dept. of Medical Biochemistry and Molecular Biology, Medical University of Vienna, Vienna, Austria) (36). All plasmids generated were verified by DNA sequencing.

Transient Transfection and Luciferase Assay—MAO B promoter luciferase reporter constructs with various site-directed mutation/deletion were cotransfected into cells with pRL-TK, which was used as internal control (Promega). Transfections were performed with Lipofectamine® 2000 (Invitrogen) following the manufacturer’s instructions using 12-well plates. Prior to 24-h RA treatment at different concentrations (DMSO used as a vehicle), cells were grown in the medium containing charcoal-treated serum for 5–8 h. Cells were harvested, and cell lysates were assayed for luciferase activity using the Dual-Luciferase® Reporter 1000 Assay System (Promega).

RNA Isolation and Quantitative Real-time RT-PCR—Total RNA was isolated with genomic DNA contamination efficiently eliminated by RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. Two microgram of total RNA was used for reverse transcription by Moloney murine leukemia virus reverse transcriptase (Promega) following the manufacturer’s instructions. The RT products were used as the template for quantitative real-time PCR. PCR products were determined by SYBR Green reagent (Maxima™ SYBR Green qPCR Master Mix 2×, Fermentas) using the iCycler optical system (Bio-Rad) following the manufacturer’s instructions. The primers for MAO B were forward (5′-GCTCTCTGTGTCTCTGTGATGTTG-3′) and reverse (5′-TCCGCTCATCTGACCATGATT-3′) and the fragment length, 118 bp (37). The primers for glyceraldehyde-3-phosphate dehydrogenase were forward 5′-GACACCGCTCAATGACTCGA-3′ and reverse 5′-ATGGCATGACTGTGGCTATGAG-3′ (fragment length, 122 bp) (37). PCR condition included an initial denaturation step of 3 min at 94 °C followed by 40 cycles of PCR consisting of 30 s at 94 °C, 30 s at 60 °C, and 40 s at 72 °C. The PCR data were analyzed by 2−ΔΔCT method (38).

Western Blot Analysis—100 µg of total proteins from cells lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) were separated by 8–12% SDS-PAGE and transferred to nitrocellulose membranes. After the transfer, membranes were blocked at room temperature for 1 h with 2% bovine serum albumin in PBST (10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride, and 0.05% Tween 20). The membranes were incubated with primary antibody in 1% bovine serum albumin in PBST at room temperature for 2 h or at 4 °C overnight. After incubating the membranes with HRP-conjugated secondary antibody against appropriate species at room temperature for 1 h, bands were visualized with the enhanced chemiluminescent (ECL) Western blotting detection reagents (Amersham Biosciences).

Site-directed Mutagenesis of the Human MAO B 2-κb Promoter—Site-directed mutagenesis was used to mutate each putative retinoic acid response element as identified in the MAO B 2-κb promoter. MAO B 2-κb-luc mutants (m1–4) were generated using wild-type MAO B 2-κb-luc as the template. Mutagenesis was carried out using QuickChange® XL site-directed
buffer D (20 mM HEPES, 200 mM KCl, 1 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 15% glycerol), and cleared by centrifugation at 4 °C for 15 min. Protein concentration was determined by BCA™ Protein Assay Kit (Pierce).

The RARE3 oligonucleotide (5'-GGTTTTGAAGTCCTAGGTGACCTCTCT-3', RARE3 in bold) was used as the probe and radiolabeled by Klenow fill-in reaction. A 32P-labeled probe was purified using the Nucleotide Removal Kit (Qiagen). For determining the DNA-protein binding, 15 µg of nuclear extract were diluted in 1× binding buffer (40 mM HEPES, pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol, and 10 µg/ml of poly(dI-dC)) in a total volume of 20 µl. Excess unlabeled probes (competitor) and 2 µg of anti-RARα/anti-RXRα antibody were added when required, and the mixture was incubated at room temperature for 20 min. The 32P-labeled probe (~600,000 cpm) was then added, and the mixture was incubated at room temperature for another 20 min. Samples were analyzed on 5% non-denaturing polyacrylamide gel in 1× Tris borate/EDTA buffer at 150 V at room temperature for 3 h. Gel was dried and visualized by autoradiography as described previously (20).

**siRNA Interference**—Small interfering RNA (siRNA) was transfected into cells with Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s instructions. The sequences to silence the translation of Sp1, RARα, and RXRα were 5’-GGUAGCUCUAAAGUUUGAUU-3’ (sense) (39), 5’-GGAUUAGUAAUUUCUCGCUUGGUU-3’ (sense) (34), and 5’-AAGCCAGUGAGAGUCAUU-3’ (sense) (41), respectively. A non-silencing RNA with sense strand as 5’-UUUCGGAACGUGCUCAGUUU-3’ was used as control (42).

**Purification of Human RARα and RXRα—GST-RARα or GST-RXRα expression constructs were transformed into Escherichia coli BL21-competent cells (Sigma) separately. Bacterial culture was harvested in LB supplemented with 100 µg/ml ampicillin at 37 °C followed by isopropyl β-D-thiogalactopyranoside induction (0.4 mM) when A₆₀₀ was above 0.6. Induction was performed at room temperature overnight with gentle shaking, and bacteria were collected when the A₆₀₀ was above 2.0. Induction results were verified by Coomassie Blue staining and Western blot with anti-GST, anti-RARα, and anti-RXRα antibodies. GST-RARα and GST-RXRα fusion proteins were purified using B-PER GST Fusion Protein Purification Kit (Pierce) following the manufacturer’s instructions.
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**FIGURE 2. RARα and RXRα activate MAO B promoter activity in BE(2)C cells.** A, BE(2)C cells were transiently transfected with MAO B 2-kb-luc and human RARα/RXRα expression constructs, and treated with RA (10 μM) for 24 h followed by luciferase activity determination. B, Western blot analysis of endogenous and transfected RARα and RXRα protein expression levels in BE(2)C cells. β-Actin was used as loading control. C, MAO B 2-kb-luc was cotransfected with RARα siRNA or/and RXRα siRNA into BE(2)C cells followed by 24 h RA (10 μM) treatment and luciferase activity determination. Nonsense (NS) siRNA was used as control for siRNA transfections. D, Western blot analysis of siRNA-mediated knockdown of endogenous RARα and RXRα in BE(2)C cells. β-Actin was used as loading control. Activity of MAO B 2-kb-luc transfected alone without RA treatment was set as 1% (A) or 100% (C). All data are presented as the mean ± S.D. from at least three independent experiments with triplicates for each experiment. **, p < 0.01.

**In Vitro Translation**—In vitro translation was conducted with TNT Coupled Reticulocyte Lysate System (Promega) following the manufacturer’s instructions. All plasmids used as DNA templates carry T7 promoter. All in vitro translated products were verified by Western blot.

**Co-immunoprecipitation (Co-IP) Assay**—Two hundred nanograms of human recombinant Sp1 protein was incubated with either 200 ng of pure GST-RARα protein or 200 ng of pure GST-RXRα protein in 50 μl of PBS on ice for 2 h. Samples were diluted in 950 μl of PBS with 1× protease inhibitor, and immunoprecipitated with rabbit polyclonal anti-RARα antibody (sc-773, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit polyclonal anti-RXRα antibody (sc-774, Santa Cruz Biotechnology) at the final concentration of 10 μg/ml at 4 °C overnight on a rotating mixer. After 50 μl of Protein A-Sepharose beads (Amersham Biosciences) was added into each IP reaction, samples were incubated at 4 °C for another 2 h on a rotating mixer. Beads were washed three times with cold PBS and boiled in 2× SDS sample buffer for 5 min followed by Western blot with mouse monoclonal anti-Sp1 antibody (sc-17824, Santa Cruz Biotechnology). Incubations of 200 ng of Sp1 protein with either anti-RARα antibody or anti-RXRα antibody only were used as a negative control for IP. 200 ng of Sp1, GST-RARα, and GST-RXRα was used as 100% input and analyzed by Western blot with anti-Sp1, anti-RARα, and anti-RXRα antibodies, respectively.

For determining the specific RARα-binding region in Sp1, both in vitro translated RARα (10 μl) and wt/truncated HA-Sp1 (10 μl) proteins were incubated in TNE buffer (250 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4, and 1× protease inhibitor) in a total volume of 250 μl on ice for 3 h, and immunoprecipitated with rabbit polyclonal anti-RARα antibody (sc-551, Santa Cruz Biotechnology) at the final concentration of 10 μg/ml at 4 °C for 4 h on a rotating mixer. After 40 μl of Protein A beads was added into each IP reaction, samples were incubated at 4 °C for another 1 h on a rotating mixer. Beads were then washed once with cold TNE buffer and boiled in 2× SDS sample buffer for 5 min followed by Western blot with mouse monoclonal anti-HA antibody and TrueBlot HRP-conjugated anti-mouse IgG (secondary antibody). The incubation of in vitro translated wt HA-Sp1 (10 μl) with anti-RARα antibody only was used as a negative control for IP. 2 μl of in vitro translated wt/truncated HA-Sp1 and RARα was used as 20% input and analyzed by Western blot with anti-HA and anti-RARα antibodies, respectively.

**Chromatin Immunoprecipitation Assay and PCR**—BE(2)C cells cultured in 10-cm dishes upon confluence were treated with formaldehyde at the final concentration of 1% at room temperature with gentle shaking for 10 min to cross-link nuclear proteins with genomic DNA, which was quenched by incubating with glycine at the final concentration of 2.5 M at room temperature with gentle shaking for another 5 min. Cells were quickly washed by cold PBS twice, harvested by scraping, and centrifuged at 2000 rpm at 4 °C for 5 min. Cell pellets were lysed in 350 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, and 2× protease inhibitor) on ice for 10 min followed by sonication using the Branson 450 sonifier to shear genomic DNA into 500- to 1000-bp fragments. Ten percent of supernatant was saved as input. Appropriate volume of supernatant was diluted (1:10) in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, and 167 mM NaCl), and blocked with 60 μl of sheared salmon sperm DNA/Protein A/G-agarose at 4 °C for 2–4 h. The supernatant obtained by brief centrifugation after blocking was immunoprecipitated with 2–5 μg of specific anti-
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A canonical retinoic acid response element (RARE)

GTTAA NNNNNN TGACC

Potential RAREs in MAO B 2 kb promoter

1. GTAA NNNNNN TGAG (-1907/ -1891)
2. GTAA NNNNNN TGGCTG (-940/ -924)
3. TGAG NNNNNN TGACC (-303/ -287)
4. GTAA NNNNNN GCTCG (-154/ -138)

Mutated RAREs in MAO B 2 kb promoter

1. TTTA NNNNNN TGAG (-1907/ -1891)
2. TTTA NNNNNN TGGCTG (-940/ -924)
3. TGAG NNNNNN TGAAA (-303/ -287)
4. TTTA NNNNNN GCTCG (-154/ -138)

B Relative luciferase activity (wt %) of RA activation

C

Probe
Nuclear extract
Competitor
Anti-RAR antibody
Anti-RXR antibody

D

FIGURE 3. RA activates MAO B promoter activity through the third RARE in the MAO B promoter. A, the canonical RARE, four potential RAREs as identified in the MAO B 2-kb promoter with their specific location upstream from the first transcription-initiation site, and the introduced point mutations (in italic) used to inactivate each RARE. B, BE(2)C cells were transfected with wild-type (wt) or mutant (with each RARE mutated separately, m1–4) MAO B 2-kb-luc, and treated with RA (10 μM) for 24 h followed by luciferase activity determination. C, electrophoretic mobility shift analysis demonstrating the RARα binding to RARE3 in vitro. Nuclear extract from BE(2)C cells treated with RA (10 μM) for 48 h was incubated with radiolabeled RARE3 probe (lanes 2–5). An excess of unlabeled probes as competitor (lane 3), anti-RARα antibody (lane 4), or anti-RXRα antibody (lane 5) was added into the DNA-protein-binding reaction when required. Arrows showed free probes, RARα-DNA complex, and supershifted RARα-DNA complex with anti-RARα antibody. D, ChIP assay demonstrating RARα binding to the RARE3 in vivo. BE(2)C cells were treated with RA (10 μM) for 24 h followed by ChIP assay with anti-RARα antibody targeting the endogenous RARα (lower panel). A separate ChIP assay was performed with BE(2)C cells transiently transfected with EGFP-RARα followed by RA (10 μM) treatment for 24 h, and anti-GFP antibody was used to target the transfected RARα (lower panel). PCR was conducted with primers targeting the RARE3-containing region. IgG was used as a negative control for IP. Genomic DNA isolated (gDNA) from BE(2)C cells was used as the template as a positive control for PCR. Distilled H2O was used as the template as a negative control for PCR. PCR products were analyzed by agarose gel electrophoresis, and the intensity of DNA bands was quantified by Labworks analysis software (UVP).

Retinoic acid activates MAO B promoter activity through the third RARE in the MAO B promoter at 4 °C overnight. IgG was used as a negative control for IP. After incubating 40 μl of salmon sperm DNA/Protein A/G-agarose with IP samples at 4 °C for another 2 h, beads were sequentially washed by low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 0.5 M NaCl), LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl pH 8.0), and TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The DNA-protein complex was eluted by elution buffer (1% SDS and 0.1 M sodium bicarbonate) with gentle rotation at room temperature for 15 min twice, reversely cross-linked by incubating at 65 °C for 5–8 h, and purified using QIAquick PCR Purification Kit (Qiagen). Purified DNA was used as the template for the following PCR analysis.

For ChIP/re-ChIP assay to determine the simultaneous presence of RARα and Sp1 in the MAO B promoter, BE(2)C cells were performed with ChIP assay using anti-Sp1 antibody as described above. The remaining proteins bound within the beads in anti-Sp1 immunoprecipitates were recovered in 50 μl of DTT buffer (2% SDS, 10 mM DTT, and 2X protease inhibitor in 1X TE buffer) at 37 °C for 30 min twice, and subjected to the second ChIP assay (re-ChIP) using anti-RARα antibody as described above.

The primers used for the RARE3 were forward (5'-ATTGGCCCTA- CACCCAGGAG-3') and reverse (5'-GGAGAGTCCTAGGAC- TTC-3') (fragment length, 176 bp). The primers used for the Sp1 sites were forward (5'-TGAAGTCCTAGGCTACACCAT- TTC-3') and reverse (5'-GACCAAGACAGTCGAG- TTTG-3') (fragment length, 333 bp). PCR condition included an initial denaturation step of 3 min at 94 °C followed by 30–40 cycles of PCR consisting of 30 s at 94 °C, 30 s at 60 °C, and 40 s at 72 °C, with a further extension step of 7 min at 72 °C. PCR mix included 5% DMSO or/and 1 M betaine when needed. Distilled H2O was used as the template as a negative control for PCR.

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A

MAO B 2 kb promoter

Retinoic acid response element (RARE)
Sp1-binding site

0.15 kb core promoter

wt: GGGCCGGGAGCCG....AGGCCGGCAGCCGGG
mut: GttCGGGtCGAG....AGGCCttCGGtGtG

B

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C

Western blot analysis of endogenous and transfected Sp1 protein expression levels in BE(2)C cells. β-Actin was used as loading control. D, BE(2)C cells were transfected with wild-type (wt) or mutant (mut) MAO B 2-kb-luc together with various amounts of Sp1 expression construct, and treated with RA (10 μM) for 24 h followed by luciferase activity determination. Activity of the wt MAO B 2-kb-luc and 0.15 kb-luc was measured through RARα and RXRα expression constructs into BE(2)C cells. After transfection, cells were treated with RA (10 μM) for 24 h followed by luciferase activity determination. As shown in Fig. 2A, introductions of RARα alone, RXRα alone, and both receptors increased MAO B promoter activity by 60, 50, and 370%, respectively (Fig. 2A, compare lanes 3, 5, and 7 with lane 1). Further, additional increases up to 60-fold (lane 8) in the presence of RARα/RXRα were observed upon RA treatment, which suggests that this activation is mediated through RARα and RXRα. In addition, both RARα and RXRα are well expressed in BE(2)C cells (Fig. 2B, lane 1), with higher expression levels when transfected into cells (Fig. 2B, compare lane 3 with lane 1).

Statistical Analysis—Statistical analysis was performed with unpaired t test. A p value of less than 0.05 was considered as significant.

RESULTS

RA Activates MAO B Promoter Activity and mRNA Expression in BE(2)C Cells—To examine the effect of RA on the MAO B promoter, MAO B 2-kb promoter-luciferase reporter construct (MAO B 2-kb-luc) was transfected into human neuroblastoma BE(2)C cells, the luciferase activity was determined after 24-h RA treatment. RA activated the MAO B promoter in a concentration-dependent manner with increases of 1.4, 5.3, and 13.2-fold at 10⁻⁷ M, 10⁻⁶ M, and 10⁻⁵ M, respectively (Fig. 1A). It was also time-dependent with the highest induction achieved after 24-h treatment with RA (10 μM) (Fig. 1B).

Next, we performed quantitative real-time RT-PCR to examine the stimulating effect of RA on the MAO B transcript. As revealed in Fig. 1C, MAO B mRNA increased by 2-fold (Fig. 1C) after 48-h RA treatment (10 μM) in BE(2)C cells, which is consistent with the promoter activation.

RA Activates MAO B Promoter Activity through RARα and RXRα—To study whether nuclear retinoid receptors (RARs and RXRs) are involved in the RA activation of MAO B promoter, MAO B 2-kb-luc was cotransfected with human RARα/RXRα expression constructs into BE(2)C cells. After transfection, cells were treated with RA (10 μM) for 24 h followed by luciferase activity determination. As shown in Fig. 2A, introductions of RARα alone, RXRα alone, and both receptors increased MAO B promoter activity by 60, 50, and 370%, respectively (Fig. 2A, compare lanes 3, 5, and 7 with lane 1). Further, additional increases up to 60-fold (lane 8) in the presence of RARα/RXRα were observed upon RA treatment, which suggests that this activation is mediated through RARα and RXRα. In addition, both RARα and RXRα are well expressed in BE(2)C cells (Fig. 2B, lane 1), with higher expression levels when transfected into cells (Fig. 2B, compare lane 3 with lane 1).
down of RARα, RXRα, and both reduced the RA activation of MAO B promoter by 74%, 48%, and 69%, respectively (Fig. 1C, compare lanes 2–4 with lane 1). Moreover, the basal MAO B promoter activity was not affected by the knockdown of these RA receptors (data not shown). Taken together, RA-induced MAO B promoter activation depends on RARα and RXRα.

RA Activates MAO B Promoter Activity through the Third RA Response Element in the MAO B Promoter—There are four potential RAREs as identified in the MAO B 2-kb promoter, which all consist of consensus sequence of a 7-bp-spaced inverted repeat 5’-GGTAANNNNNNTGACC-3’ (N is any nucleotide) (Fig. 3A, top and middle boxes). To determine which RARE is important for the RA activation of MAO B promoter, site-directed mutagenesis was carried out to specifically mutate each RARE (Fig. 3A, bottom box). Our results showed that only mutation of the third RARE repressed RA-induced MAO B promoter activation (by 50%), whereas mutations of RARE1, -2, and -4 had no effect (Fig. 3B). To study whether RARα and RXRα directly bind to the third RARE, electrophoretic mobility shift analysis was conducted using radiolabeled RARE3 as the probe. Our results showed one radioactive band indicating a DNA-protein complex on the gel after BE(2)C cell nuclear extract was incubated with the probe (Fig. 3C, lane 2). This band was not observed in the presence of 500-fold excess of unlabeled probes as competitor, suggesting it is specific (Fig. 3C, lane 3). Further, this band was supershifted when anti-RARα but not anti-RXRα antibody was incubated in the DNA-protein binding reaction (Fig. 3C, lanes 4 and 5). To study whether RARα binds to this element in vivo, chromatin immunoprecipitation (ChIP) assay coupled with PCR using primers specifically targeting this region was performed with BE(2)C cells. The binding of endogenous RARα to the RARE3 was demonstrated by using specific anti-RARα antibody (Fig. 3D, upper panel), suggesting that RARα indeed interacts with the RARE3-containing region in native chromatin of the MAO B promoter. Likewise, the ectopically expressed RARα (with EGFP tag) also bound to the functional RARE3 both in vitro and in vivo.

Sp1 Enhances but Is Not Essentially Required for the RA Induction of MAO B Promoter Activity by RA—MAO B 2-kb promoter consists of two clusters of Sp1-binding sites in the 0.15-kb core promoter region independent of RAREs: two and three sites in the distal and proximal clusters, respectively (Fig. 4A). To study the role of Sp1 in the RA activation of MAO B promoter, all five Sp1 sites were mutated (Fig. 4A), which led to a lower basal MAO B promoter activity as expected (Fig. 4B, compare mut with wt without RA treatment). Wild-type (wt) or mutant (mut) MAO B 2-kb-luc was cotransfected with various amounts of Sp1 expression construct into BE(2)C cells, and the luciferase activity was determined after 24 h RA (10 μM) treatment. As shown in Fig. 4B, RA increased wt MAO B promoter activity up to 50-fold in a Sp1 concentration-dependent manner (lanes 1–3); however, only marginal activation was observed when all five Sp1 sites were mutated (lanes 4–6). To determine the importance of each cluster of Sp1 sites, three mutant MAO B 0.15-kb-luc constructs containing Sp1 sites only were generated: m1 (with distal Sp1 sites mutated), m2 (with proximal Sp1 sites mutated), and m3 (with both sites mutated) (Fig. 4D). Mutation of distal Sp1 sites repressed RA-induced MAO B promoter activation by 50% (Fig. 4D, compare the -fold activation of m1 with wt), whereas this activation was reduced to 30% when proximal Sp1 sites were mutated (Fig. 4D, compare m2 with wt). Muta-
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A

|                      | RARα/RXRα RA |
|----------------------|-------------|
| wt MAO B promoter    | - - 1       |
| + - 2                |
| + + 4                |
| with Sp1 sites and TATA box only |
| - - 5                |
| + - 6                |
| + + 8                |
| without Sp1 sites    |
| - - 9                |
| + - 10               |
| + + 12               |

Relative luciferase activity

B

|                      | RARα/RXRα RA |
|----------------------|-------------|
| 3X Sp1-binding sites | - - 1       |
| + + 2                |
| + - 3                |
| + + 4                |
| Promoterless         |
| - - 5                |
| + - 6                |
| + - 7                |
| + + 8                |

Relative luciferase activity

C

|                      | RA - | RA + |
|----------------------|------|------|
| MAO B promoter       |      |      |
| + - 1                |
| - - 2                |
| + - 3                |
| - - 4                |

Relative luciferase activity
the importance of Sp1 sites for driving the MAO B promoter.

Next, we further studied the role of endogenous Sp1 in MAO B promoter activation by RA using RNA interference technology. Fig. 5A showed that the introduction of siRNA successfully knocked down endogenous Sp1 in BE(2)C cells. Transient transfection and luciferase assays revealed that Sp1 knockdown significantly reduced the basal MAO B 2-kb promoter activity by 37% (Fig. 5B, compare lane 3 with lane 1) in BE(2)C cells. However, the extent of RA-induced MAO B promoter activation remained the same after Sp1 was knocked down (Fig. 5B, 5.6-fold increase with nonsense siRNA in lanes 1 and 2, and 5.7-fold increase with Sp1 siRNA in lanes 3 and 4), suggesting that Sp1 is not essentially required for this activation. In addition, the effect of mithramycin, a well known specific inhibitor that interferes with Sp1 binding to Sp1 sites, was studied to further examine the role of Sp1 in the RA activation of MAO B promoter. Fig. 5C showed that RA increased MAO B promoter activity in a RA concentration-dependent manner in BE(2)C cells (upper curve), whereas mithramycin treatment attenuated this activation (lower curve). The concentration of mithramycin used under current experimental condition is not toxic to cells (data not shown), and the specificity of mithramycin was ensured in the previous report (32). In line with these observations, we suggest that Sp1 enhances but is not essentially required for MAO B promoter activation by RA.

RARs and RXRs Mediate the RA Activation of MAO B Promoter through the Sp1-binding Sites in the Core Promoter Region—As shown in Fig. 4D, RA activated MAO B promoter activity when MAO B 0.15-kb-luc containing Sp1 sites only was used, suggesting that RAR and RXR might also transactivate the MAO B promoter through a RARE-independent mechanism. To explore the idea that RAR and RXR activate the MAO B promoter through Sp1 sites, we used wild-type (2 kb) and deletion (0.15 kb with Sp1 sites and TATA box only, and 2 kb without Sp1 sites) MAO B promoter-luc in transfections (Fig. 6A). Fig. 6A showed that basal activities of deletion MAO B promoters with and without Sp1 sites were 230 and 37% of wt, respectively (Fig. 6A, compare lanes 5 and 9 with lane 1), indicating the importance of Sp1 sites for driving the MAO B promoter. Transfections of RARα and RXRα uniformly enhanced basal promoter induction with all three constructs (Fig. 6A, compare lanes 3, 7, and 11 with lanes 1, 5, and 9, respectively), suggesting the existence of RARα/RXRα-responsive elements both inside and outside Sp1 sites in the MAO B 2-kb promoter. As expected, these promoter inductions were augmented under RA treatment (Fig. 6A, lanes 4, 8, and 12). The strongest induction of the MAO B promoter by RARα and RXRα was obtained with the MAO B 0.15-kb promoter construct containing Sp1 sites only (Fig. 6A, lanes 7 without RA, and lane 8 with RA), which implies possible direct or indirect interaction of RARα and RXRα with the Sp1 sites. Moreover, when a promoter reporter construct containing three tandem Sp1 sites only was used, RARα and RXRα significantly activated this promoter as well with higher induction upon RA treatment (Fig. 6B), which further solidifies the possibility that RARα and RXRα might directly or indirectly interact with the Sp1 sites in the MAO B promoter.

In addition, the combined effect of the RARE3 and Sp1 sites on the RA activation of MAO B promoter was examined. Various MAO B 2-kb-luc constructs, including wild-type and mutants (with RARE3 mutated, with Sp1 sites mutated, and with both sites mutated) were used in transfections. Fig. 6C showed that mutation of either site significantly attenuated RA-induced MAO B promoter activation with more repression observed when the Sp1 sites were mutated (compare lanes 3 and 2 with lane 1), which suggests that both the RARE3 and Sp1 sites are involved in this activation with Sp1 sites more potent.

RARα Interacts with Sp1 via Zinc Finger Domains in Sp1—Sp1-binding sites are GC-rich and thus completely different from all types of RAREs as identified so far (29, 43). Although RARα does not bind to Sp1 sites by itself (32, 44), we hypothesized that RARα and RXRα might indirectly interact with Sp1 sites via the association with Sp1. To study whether RARα and RXRα interacts with Sp1, we incubated equal amounts of Sp1 protein with RARα or RXRα protein in vitro (Fig. 7A, lanes 3 and 4) followed by co-IP assay (with anti-RARα or anti-RXRα antibody) and Western blot (with anti-Sp1 antibody). Incubations of Sp1 protein with either anti-RARα or anti-RXRα antibody served as negative control for IP (Fig. 7A, lanes 6 and 7). As revealed in Fig. 7A, RARα physically interacted with Sp1 (compare lane 4 with lane 6); however, there was no association between RXRα and Sp1 observed (compare lane 5 with lane 7). Pure RARα and RXRα proteins as input used in co-IP assay was also analyzed by Western blot with anti-RARα and anti-RXRα antibodies respectively (Fig. 7A, lanes 8 and 9). This interaction might contribute to the transactivation of MAO B promoter by RARα and Sp1 via Sp1 sites (32).

To identify the specific regions in Sp1 protein that are necessary for RARα binding, we incubated in vitro translated RARα with various in vitro translated wt/truncated HA-tagged Sp1 in equal amounts under in vitro condition followed by co-IP assay (with RARα antibody) and Western blot (with anti-HA antibody) (Fig. 7B). Besides the full-length wt HA-Sp1-1(1–788), RARα retained HA-Sp1-1(1–668) and HA-Sp1-2(622–788) but not HA-Sp1-1(1–293), HA-Sp1-1(1–621), and HA-Sp1-1(648). Prior to co-IP assay, 20% input fraction was analyzed by Western blot (with anti-HA and anti-RARα antibodies) (Fig.

**FIGURE 6.** RA activates MAO B promoter activity via both the Sp1-binding sites and RARE3. A, transient transfection and luciferase assays demonstrating the effect of RARα and RXRα on MAO B promoter activity with various deletion promoter reporter constructs used in BE(2)C cells. Wild-type (wt, 2 kb) or deletion (0.15 kb with Sp1 sites and TATA box only, or 2 kb without Sp1 sites) MAO B promoter-luc was cotransfected with RARα/RXRα expression constructs into BE(2)C cells. Cells were treated with RA (10 μM) for 24 h followed by luciferase activity determination. B, luciferase reporter construct containing three tandem Sp1 sites only was cotransfected with RARα/RXRα into BE(2)C cells followed by 24 h RA (10 μM) treatment and luciferase activity determination. Promoterless pGL2-Basic luciferase reporter construct was used as a negative control. C, BE(2)C cells were transfected with wt (lane 1) or mutant MAO B 2-kb-luc (lane 2 with RARE3 mutated, lane 3 with Sp1 sites mutated or lane 4 with both sites mutated), and treated with RA (10 μM) for 24 h followed by luciferase activity determination. Activity of the wt MAO B 2-kb-luc without cotransfection of RARα/RXRα and RA treatment was set as 1. All data were presented as the mean ± S.D. from at least three independent experiments with triplicates for each experiment.
As shown in a schematic diagram of Sp1 protein structure in Fig. 7D, Sp1 comprises three zinc finger DNA-binding domains and domain D that mediates Sp1 self-association (45, 46). Taken together, we conclude that amino acids 622–668 in Sp1 are sufficient for RARα binding, which contains the first and partial of the second zinc finger domains.

RARα Is Recruited by Sp1 and Forms a Transcriptional Regulation Complex with Sp1 in the Sp1-binding Region of Natural
Retinoic Acid Activates MAO B Promoter

To determine the possible simultaneous presence of RAR and Sp1 in this region, we conducted ChIP assay with BE(2)C cells using anti-Sp1 antibody, and anti-Sp1 immunoprecipitates were subjected to re-ChIP assay using anti-RAR antibody. As revealed in Fig. 8B, RAR was detected in anti-Sp1 immunoprecipitates, indicating that RAR does indeed form a complex with Sp1 in the Sp1 sites of natural MAO B promoter. Also, higher occupancy of RAR/Sp1 complex in Sp1 sites upon 48 h RA (10 μM) treatment was observed (Fig. 8B, 1.86-fold and 2.54-fold increases for Sp1 and RAR, respectively).

In addition, the recruitment of RAR to Sp1 sites in the MAO B promoter by Sp1 was further studied by ChIP/re-ChIP assays coupled with RNA interference approach in BE(2)C cells. We introduced siRNA to knock down the endogenous Sp1 in BE(2)C cells followed by 24-h treatment with or without RA (10 μM). As expected, this Sp1 knockdown significantly attenuated Sp1 binding to Sp1 sites by 79 and 68% in the absence and presence of RA, respectively (Fig. 8C, top panel). Re-ChIP assay was subsequently performed with anti-Sp1 immunoprecipitates using anti-RAR antibody. As a consequence of Sp1 knockdown, less RAR in anti-Sp1 immunoprecipitates was shown to associate with Sp1 sites, with reductions of 95 and 87% in the absence and presence of RA, respectively (Fig. 8C, middle panel). As determined by ChIP assay using anti-RAR antibody directly, lower RAR occupancy in Sp1 sites was also observed after the endogenous Sp1 was knocked down, with decreases of 90 and 77% in control and treated cells, respectively (Fig. 8C, bottom panel). Taken together, these in vivo studies demonstrate that RAR is recruited by Sp1 and forms a complex with Sp1 in the Sp1-binding region to modulate MAO B transcription in response to RA signaling.

DISCUSSION

We provide evidence for the first time showing that RA activates MAO B promoter activity and mRNA expression in human neuroblastoma cells. RA activates MAO B transcription through RAR/RXR and the third retinoic acid response element (RARE3) in the MAO B promoter (Figs. 2 and 3). RAR but not RXR specifically binds to the RARE3 under both in vitro and in vivo conditions (Fig. 3). In the absence of RXR, RAR might work with other RXR isotypes and function as heterodimers in the RARE3. The RARE3 has been found to overlap with the fourth estrogen response element in the MAO B 2-kb promoter (26). Previous report showed that estrogen receptors (ERs) compete with estrogen-related receptors in estrogen response element sites, although ERs alone have no impact on the MAO B promoter (26). Retinoids and estrogens play similar roles in many biological processes such as vertebrate early development and reproduction, which are mediated through their respective nuclear receptors (29, 47). In light of sequence identities shared by ER and RAR response elements, although ERs alone have no role in mammalian models, the transcriptional crosstalk between ER and RAR pathways is implicated in similarities of their biological functions.

We demonstrate that Sp1 enhances but is not essentially required for the RA activation of MAO B promoter (Figs. 4 and 5). Sp1 is a ubiquitous transcription factor in mammals, in particular a key activator of MAO B that directly binds to GC-rich sites in the promoter for maintaining the basal MAO B expres-

MAO B Promoter—To study whether RAR associates with Sp1 sites in the MAO B promoter in vivo, we performed ChIP assay with BE(2)C cells followed by PCR with primers specifically targeting the Sp1-binding region, and Sp1 occupancy in this region was also examined as a positive control. As shown in Fig. 8A, the region encompassing Sp1 sites was prominently amplified from both anti-RAR (upper panel) and anti-Sp1 (lower panel) immunoprecipitates, suggesting that both RAR and Sp1 target Sp1 sites in the MAO B promoter. Moreover, stronger RAR and Sp1 binding to Sp1 sites were observed upon 48 h RA (10 μM) treatment (Fig. 8A, 3.48-fold and 2.47-fold increases for RAR and Sp1, respectively).
Retinoic Acid Activates MAO B Promoter

Retinoic acid (RA) has been shown to activate MAO B transcription in various cell lines (1, 2, 3). Recent studies have suggested that RA receptors (RARs) physically interact with Sp1 sites in the MAO B promoter to activate transcription (4, 5). However, the mechanism by which RA regulates MAO B transcription remains unclear.

Previous studies have shown that RA receptors interact with other transcription factors to mediate gene transcription, and this crosstalk between nuclear receptors and transcription factors is an important gene regulatory mechanism (6–9). For example, RARα functionally interacts with Sp1 to corporately activate the transcription of interleukin-1β (33) and 17β-hydroxysteroid dehydrogenase type 2 (34). Consistent with these findings, we demonstrate that RARα physically interacts with Sp1 via a zinc finger domain in Sp1 (Fig. 7). This interaction, to some extent, enhances the Sp1-binding ability as suggested in previous studies (32, 52).

Recent studies have shown that RA receptors interact with other transcription factors to mediate gene transcription, and this crosstalk between nuclear receptors and transcription factors is an important gene regulatory mechanism (49–51). For example, RARα functionally interacts with Sp1 to corporately activate the transcription of interleukin-1β (33) and 17β-hydroxysteroid dehydrogenase type 2 (34). Consistent with these findings, we demonstrate that RARα physically interacts with Sp1 via a zinc finger domain in Sp1 (Fig. 7). This interaction, to some extent, enhances the Sp1-binding ability as suggested in previous studies (32, 52).

Mouse RXRα was reported to interact with human Sp1 as well (32), whereas there is no obvious interaction observed between human RXRα and Sp1 in the present study (Fig. 7A), despite highly conserved sequences shared between human and mouse RXRα. Moreover, RARα/RXRα are implicated in the interaction with Sp1 sites in the MAO B promoter, because they significantly activate MAO B promoter activity when a promoter deletion construct containing GC-rich sites only was used (Fig. 6A). In addition, RARα/RXRα also activate 3×Sp1-luc solely via Sp1 sites out of the MAO B promoter context (Fig. 6B), which further implies a possible interaction of RA receptors with Sp1 sites. Previous studies have shown that RARα could not directly bind to Sp1 sites by itself in both the absence (44) and presence (32) of Sp1 in vitro, to a large degree, due to contrasting nucleotide composition of RAREs and Sp1 sites (GC-rich) (29, 43). However, here we show that RARα interacts with the Sp1-binding region in vivo (Fig. 8A), which was also observed in the regulation of the folate receptor type β promoter (53). Further, we raise and demonstrate the possibility that RARα indirectly associates with Sp1 sites in which RARα recruited by Sp1 interacts and forms a complex with Sp1 in Sp1 sites of native chromatin to modulate MAO B transcription (Figs. 7A, 8B, and 8C).

In addition to BE(2)C cells, we also examined the stimulating effect of RA on the MAO B promoter in another two human neuronal cell lines, the human glioblastoma 1242-MG and neuroblastoma SH-SY5Y cells. Both cell lines support the RA activation of MAO B promoter by 50 and 70% with significance in 1242-MG and SH-SY5Y cells, respectively, although not as robust as in BE(2)C cells (supplemen tal Fig. S1A). With a further dissection of the expression levels of RA receptors in all three neuronal cell lines, we reveal that both RARα and RXRα are present in these cells with different expression levels (supplemental Fig. S1B). Both 1242-MG and SH-SY5Y cells express much lower levels of RXRα than BE(2)C cells, and SHSY-5Y cells express more RXRα than another two cell lines. These differences might partially explain the discrepancy of MAO B promoter activation by RA among three cell lines, in which RARα is suggested as an important mediator.

One possible mechanism by which RA activates MAO B transcription is through epigenetic regulation of the MAO B promoter. There is a CpG island containing multiple CpG methylation sites in the MAO B core promoter region, and MAO B gene expression is up-regulated by DNA methylation inhibitors such as 5-aza-2′-deoxycytidine (22). Emerging evidences have recently suggested that RA treatment induces epigenetic modifications at its target loci and restores epigenetically silent genes to a transcriptionally active state by triggering DNA demethylation and histone acetylation at the promoter level (40, 54, 55). Because the CpG island in the MAO B promoter encompasses several Sp1 sites (22), we speculate that the RA activation of MAO B promoter also correlate with RA-induced epigenetic alterations. As a consequence of reducing DNA methylation and decompacted chromatin structure, transcription factors are recruited to this region and activate the MAO B promoter. This is consistent with the observation of higher Sp1 occupancy in the Sp1 sites of native MAO B promoter in response to RA (Fig. 8A).

In summary, we demonstrate that RA activates MAO B transcription at both promoter and mRNA levels. RA activates MAO B through 1) RARα/RXRα in the functional RARE3 in the MAO B promoter, 2) Sp1 and Sp1-binding sites, and 3) interactions of RARα/RXRα with Sp1/Sp1-binding sites. Taken together, this study has explored for the first time the novel regulatory mechanism of RA-induced MAO B activation in human neuronal cells and provided new insight into hormonal regulation of MAO B.

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