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Transcription Factor E2F-Associated Phosphoprotein (EAPP), RAM2/CDCA7L/JPO2 (R1), and Simian Virus 40 Promoter Factor 1 (Sp1) Cooperatively Regulate Glucocorticoid Activation of Monoamine Oxidase B

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ABSTRACT

Glucocorticoid steroid hormones play important roles in many neurophysiological processes such as responses to stress, behavioral adaption, and mood. One mechanism by which glucocorticoids exert functions in the brain is via the modulation of neurotransmission systems. Glucocorticoids are capable of inducing the activities of monoamine oxidases (MAOs), which degrade monoamine neurotransmitters including serotonin, norepinephrine, phenylethylamine, and dopamine. However, the molecular mechanisms for such induction are not yet fully understood. Here, we report that dexamethasone, a synthetic glucocorticoid hormone, stimulates *MAO B* (an isoform of *MAO*s) promoter and catalytic activities via both the fourth glucocorticoid response element (GRE) and simian virus 40 promoter factor 1 (Sp1) binding sites in *MAO B* promoter. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation analysis demonstrated that glucocorticoid receptor binds to the fourth GRE in vitro and in vivo. Using Sp1-binding motifs as bait in a yeast one-hybrid system, we identified two novel transcriptional repressors of MAO B, E2F-associated phosphoprotein (EAPP) and R1 (RAM2/CDCA7L/JPO2), that down-regulate *MAO B* via *MAO B* core promoter, which contains Sp1 sites. EMSA suggested that EAPP and R1 competed with Sp1 for binding to the Sp1 site in vitro. Moreover, EAPP and R1 reduced Sp1-activated glucocorticoid activation of *MAO B* promoter. In response to dexamethasone, lower occupancy by EAPP and R1 and higher occupancy by Sp1 were shown at the natural *MAO B* core promoter. Together, this study uncovers for the first time the molecular mechanisms for glucocorticoid activation of *MAO B* gene and provides new insights into the hormonal regulation of *MAO*.

Introduction

Monoamine oxidase (MAO) degrades a number of monoamine neurotransmitters. MAO exists in two forms, A and B (Bach et al., 1988), which have different substrate preference and inhibitor specificities (Shih et al., 1999). MAO A preferentially oxidizes serotonin [5-hydroxytryptamine (5-HT)], norepinephrine (NE), and dopamine (DA), whereas MAO B oxidizes phenylethylamine (PEA) (Shih et al., 1999). In the absence of MAO A, MAO B also oxidizes 5-HT, NE, and DA because the brain levels of 5-HT, NE, DA, and PEA are higher in *Mao a*/*b* double knockout (KO) mice than *Mao a* or *b* single KO mice (Chen et al., 2004). MAO B also converts the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into 1-methyl-4-phenylpyridine, which selectively destroys nigrostriatal neurons and induces Parkinson's disease-like symptoms in rodents and humans (Nicotra and Parvez, 2000). The neurodegeneration induced by MPTP can be prevented by the MAO B inhibitor deprenyl, and *Mao b* KO mice do not suffer neurodegenerative

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ABBREVIATIONS: MAO, monoamine oxidase; ChIP, chromatin immunoprecipitation; EAPP, E2F-associated phosphoprotein; GR, glucocorticoid receptor; GRE, glucocorticoid response element; *luc*, luciferase; WT, wild type; EMSA, electrophoretic mobility shift assay; 5-HT, 5-hydroxytryptamine; NE, norepinephrine; DA, dopamine; PEA, phenylethylamine; KO, knockout; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; bp, base pair(s); PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; IP, immunoprecipitation; HA, hemagglutinin; Sp1, simian virus 40 promoter factor 1.

effects after MPTP administration (Heikkila et al., 1984; Grimsby et al., 1997). In addition, MAO B activity significantly increases in the brain with age in rats (Arai and Kinemuchi, 1988) and humans (Fowler et al., 1997). These studies suggest that MAO B may play an important role in aging and neurodegenerative processes.

MAO B gene expression can be activated by phorbol 12-myristate 13-acetate/12-*O*-tetradecanoylphorbol-13-acetate, which activates protein kinase C and mitogen-activated protein kinase signaling pathways such as extracellular signal-regulated kinase 2, mitogen-activated protein kinase kinase 1, and c-Jun NH_2-terminal kinase 1. Transcription factor c-Jun and Egr-1 are responsible for phorbol 12-myristate 13-acetate-induced *MAO B* gene up-regulation via the overlapping Sp1/Egr-1/Sp1-binding sites in *MAO B* core promoter, which is the sequences exhibiting the maximum *MAO B* promoter activity (Wong et al., 2002). Sp family and Sp family-like transcription factors are also involved in *MAO B* gene regulation. Sp1 and Sp4 activate the *MAO B* core promoter via two clusters of overlapping Sp1 sites, which is repressed by Sp3 (Wong et al., 2001). Transforming growth factor-β inducible early response gene 2, an Sp1-like transcription factor, also activates the *MAO B* promoter via Sp1 sites (Ou et al., 2004).

Glucocorticoid steroid hormones are involved in the regulation of many neurophysiological processes in which MAO also plays a critical role, such as responses to stress, behavioral adaption, and mood (de Kloet et al., 1990). Significant hypersecretion of glucocorticoids has been associated with depression (Duval et al., 2006), and antiglucocorticoid agents have been used in the treatment of depression (Wolkowitz and Reus, 1999). Dexamethasone, a potent synthetic glucocorticoid hormone, has been reported to increase both MAO A and MAO B activity in vitro (Edelstein and Breakefield, 1986) and in vivo (Slotkin et al., 1998). Glucocorticoids exert their effects by activating glucocorticoid receptor (GR) to bind to glucocorticoid response element (GRE) 5′-AGAACANNNTG-TACC-3′ ("N" is any nucleotide) and to regulate the transcription of target genes (Wang and Hodgetts, 1998; Adcock et al., 2004). Cross-talk between GR and other transcription factors also modulates the expression of glucocorticoid-responsive genes (Karin, 1998; Adcock et al., 2004). There are four consensus GREs in human *MAO B* 2-kb promoter. We have shown previously how MAO A is regulated by glucocorticoids (Ou et al., 2006a); however, the molecular mechanisms for glucocorticoid activation of MAO B still remains unclear.

In this study, we explored the canonical molecular pathways for glucocorticoid activation of the *MAO B* gene. Moreover, using the Sp1 binding motifs derived from *MAO B* core promoter as bait in a yeast one-hybrid system, we identified E2F-associated phosphoprotein (EAPP) and R1 (RAM2/CDCA7L/JPO2) as novel transcriptional repressors of *MAO B*, and further demonstrated the cooperative regulatory effects of EAPP, R1 and Sp1 in mediating glucocorticoid activation of *MAO B* gene.

Materials and Methods

Cell Lines and Reagents. The human glioblastoma 1242-MG cell line was a gift from Dr. B. Westermark (Department of Pathology, University Hospital, Uppsala, Sweden). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Monoclonal anti-Sp1, anti-β-actin, and polyclonal anti-Sp1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-GR antibody was purchased from Abcam Inc. (Cambridge, MA). Monoclonal anti-HA and anti-FLAG antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-EAPP antibody was made in house by Anaseptic Co. (San Jose, CA). Polyclonal anti-R1 antibody was generated as described previously (Ou et al., 2006a). Pure actin protein was purchased from GenWay Biotech, Inc. (San Diego, CA). Dexamethasone was purchased from Sigma-Aldrich and dissolved in ethanol.

Plasmids. The human *MAO B* 2-kb promoter fragment (−2099/−99 bp, BamHI/BamHI) was subcloned into the polylinker site (BglII) upstream of the luciferase (*Firefly*) reporter gene (*luc*) in pGL2-Basic vector (Promega, Madison, WI). Serial deletion constructs of *MAO B* promoter-*luc* (Ou et al., 2004), *R1* expression construct (Chen et al., 2005), *Sp1* expression construct (Wong et al., 2001), and *GR* expression construct (Ou et al., 2006a) were obtained as described previously. The human *EAPP* and *R1* genes were identified by Yeast One-Hybrid Screening (Matchmaker One-Hybrid System; Clontech, Mountain View, CA) using three copies of Sp1-binding motif 5′-ccggacgcgcagccccgcccgcccgcctacgcgcag-3′ derived from the human *MAO B* core promoter as bait. HA-*EAPP* expression construct was generated by inserting human *EAPP* coding region at KpnI/EcoRI sites of pcDNA3.1 vector (Invitrogen, Carlsbad, CA) engineered with N-terminal HA tag. HA-*R1* expression construct was generated in a similar way. FLAG-*R1* expression construct was generated by inserting human *R1* coding region at NotI/BglII sites of p3xFLAG-CMV vector (Sigma-Aldrich). HA-*Sp1* expression construct was a gift from Dr. Hans Rotheneder (Department of Medical Biochemistry and Molecular Biology, Medical University of Vienna, Vienna, Austria). All plasmids were extracted and purified using QIAGEN Miniprep kit (QIAGEN, Valencia, CA), and verified by DNA sequencing.

MAO B Catalytic Activity Assay. 1242-MG cells (10^6) were plated in 10-cm dishes. After 12 h, the medium was replaced to the medium supplemented with charcoal-stripped and steroid-free serum for 8 h. Cells were treated with 100 nM dexamethasone (ethanol was used as a vehicle) for another 48 h and harvested in cold phosphate-buffered saline (PBS), pH 7.4, by scraping. One hundred micrograms of total protein were incubated with 100 μM ^{14}C-labeled PEA in the assay buffer (50 mM sodium phosphate buffer, pH 7.4) at 37°C for 20 min, and the reaction was terminated by the addition of 100 μl of ice-cold 6 N HCl. The reaction products were extracted with toluene and centrifuged at 4°C for 7 min. The organic phase containing the reaction products was extracted, and the radioactivity was determined by liquid scintillation spectroscopy.

Transient Transfection and Luciferase Reporter Assay. Transfections in 1242-MG cells were performed using Superfect transfection reagent (QIAGEN) following the manufacturer's instructions. Cells were plated in the density of 5×10^5 cells/well in six-well plates (Costar; Corning Life Sciences, Lowell, MA) with 2 ml of medium/well and grown to 70% confluence. For determining the effect of EAPP, R1, or Sp1 on *MAO B* promoter activity, 0.5 μg of *MAO B* promoter-*luc* construct was cotransfected with 0.5 μg of *EAPP*, *R1,* or *Sp1* expression construct into cells per well, together with 20 ng of pRL-thymidine kinase (the herpes simplex virus thymidine kinase promoter fused upstream to the *Renilla reniformis* luciferase gene, which is used as internal control; Promega) as described previously (Wong et al., 2001). For determining the effect of dexamethasone on *MAO B* promoter activity, 0.5 μg of *MAO B* 2-kb *luc* construct or various serial deletion MAO B promoter-*luc* construct was cotransfected with 0.5 μg of *GR* expression construct into cells per well, together with 20 ng of pRL-thymidine kinase. For determining the effect of EAPP, R1, or Sp1 on glucocorticoid activation of *MAO B* promoter, additional 0.3 μg of *EAPP, R1,* or *Sp1* expression construct was cotransfected into cells per well. The total amount of DNA for each transfection was kept constant by the addition of parental empty vectors such as pcDNA3.1. For dexameth-

asone treatment, cells were grown in the medium supplemented with charcoal-stripped and steroid-free serum for 8 h and treated with 100 nM dexamethasone (ethanol used as a vehicle) in the medium supplemented with charcoal-stripped and steroid-free serum for another 12 or 24 h. Cells were then harvested and assayed for luciferase activity using the Dual-Luciferase Reporter 1000 Assay System (Promega).

Nuclear Protein Extraction and Electrophoretic Mobility Shift Analysis. 1242-MG cells treated with or without dexamethasone (100 nM, 48 h, ethanol used as a vehicle) were washed with cold PBS and harvested by scraping. The cell pellets were resuspended in five pellet-volume of buffer A [10 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)], incubated on ice for 10 min, and centrifuged at 4°C for 10 min. The pellet was next resuspended in three pellet-volume of buffer A plus 0.1% NP-40, incubated on ice for 10 min, and centrifuged at 4°C for 10 min. The pellet was 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 PMSF, and 15% glycerol) and incubated on ice for 30 min with gentle shaking. Nuclear proteins were centrifuged at 4°C for 30 min and dialyzed at 4°C for 4 h 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 PMSF, and 15% glycerol). Nuclear extracts were cleared by centrifugation at 4°C for 15 min. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA).

MAO B promoter-derived oligonucleotide harboring the GRE4 (5′-GGCTTCCACCA**AGGACA**CAC**TGCATT**GCTTAA-3′, GRE is in boldface type) was used as probe for electrophoretic mobility shift analysis (EMSA) and radioactively labeled by Klenow fill-in reaction. ^{32}P-labeled probe was purified using Nucleotide Removal kit (QIAGEN). For determining the DNA-protein binding, 15 μg of nuclear extracts was diluted in binding buffer [40 mM HEPES, pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol, and 10 μg/ml poly(dI-dC)] in a total volume of 20 μl and incubated in the absence or presence of anti-GR antibody at room temperature for 20 min. ^{32}P-labeled probe (~600,000 cpm) was added and incubated at room temperature for another 20 min. Samples were analyzed on 5% nondenaturing polyacrylamide gel in 1× Tris borate/EDTA buffer at 150 V for 3 h. Gel was dried and visualized by autoradiography.

For determining Sp1 binding to Sp1 sites in *MAO B* promoter, *MAO B* promoter-derived oligonucleotide harboring wild-type Sp1 sites were used as probe. In brief, 5 to 15 μg of purified Sp1 protein was diluted in binding buffer in a total volume of 20 μl. Nonradioactively labeled probes in 100- or 500-fold excess or anti-Sp1 antibody (3 μl) was added (when required), and the reaction was incubated for 20 min at room temperature. ^{32}P-labeled probe (~600,000 cpm) was added, and the reaction was incubated for another 20 min at room temperature. Samples were then analyzed as described above.

For determining the competitive binding among Sp1, R1, and EAPP to the Sp1 sites in *MAO B* promoter, *MAO B* promoter-derived oligonucleotide harboring wild-type Sp1 sites was used as probe. In brief, 2 μg of purified Sp1 protein was mixed with 1 or 2 μg of purified R1, EAPP, or actin proteins in binding buffer in a total volume of 20 μl. Actin was used as a negative control. ^{32}P-labeled probe (~600,000 cpm) was added, and the reaction was incubated for 20 min at room temperature. Samples were then analyzed as described above.

Chromatin Immunoprecipitation Assay. 1242-MG cells were grown in 10-cm dishes. Formaldehyde was directly added to the medium at a final concentration of 1% at 37°C for 10 to 15 min to cross-link nuclear proteins with genomic DNA. Cells were washed once in ice-cold PBS before being scraped into PBS containing protease inhibitors (1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF). Cells were centrifuged for 3 min at 2000 rpm and resuspended in 350 μl of lysis buffer (50 mM Tris-HCl, pH 8.0; 1% SDS, and 10 mM EDTA) containing protease inhibitors. After brief sonication, the resulting supernatant contains DNA fragments ranging from ~200 to 1000 bp, with the majority centered at ~300 to 500 bp. Ten percent of the supernatant was saved as input, and the remainder was diluted (1:10) in chromatin immunoprecipitation (ChIP) dilution buffer (16.7 mM Tris-HCl, pH 8.0, 0.01% SDS, 1.1% Triton X-100, and 1.2 mM EDTA) containing protease inhibitors. Samples were precleared with 2 μl of salmon sperm DNA at 4°C for 30 min before overnight incubation at 4°C with mouse anti-GR or anti-Sp1 antibody (with goat anti-mouse BioMag beads; QIAGEN) or rabbit anti-EAPP or anti-R1 antibody (with goat anti-rabbit BioMag beads; QIAGEN). DNA-protein complexes were recovered from beads with elution buffer (1% SDS and 0.1 M sodium bicarbonate), reversely cross-linked by incubating at 65°C for 4 h, and analyzed by quantitative real-time PCR.

Quantitative Real-Time PCR. PCR was performed with SYBR Green reagent (Bio-Rad) using the iCycler optical system (Bio-Rad) following the manufacturer's instructions. The primers for the GRE4 (−901/−703, 199 bp), Sp1 sites in *MAO B* core promoter (−287/−134, 154 bp), and 5′-irrelevant locus (negative control, −1940/−1780, 161 bp) are as follows: GRE4 forward, 5′-CAATGCCGATCCACCAC-CTCT-3′ (−901/−881); and reverse, 5′-TATTTGGAAGGAGGGT-GAGGTGG-3′ (−725/−703); Sp1 sites in *MAO B* core promoter forward 5′-TCTCCGCCCAGGCACCCGCCCTCC-3′ (−287/−263) and reverse 5′-TCGGCGAGCCGCTATATTAGCCCC-3′ (−157/−134), 5′-irrelevant locus forward 5′-TTTGCTGTCTCAGGCCCTTTATA-3′ (−1940/−1917) and reverse 5′-ATGAATGGAGAGGATCTGCTACG-3′ (−1802/−1780). PCR was performed in triplicate under the following conditions: an initial denaturation step of 3 min at 95°C followed by 35 cycles of PCR consisting of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C. The data were analyzed by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) and presented as the percentages of input (input set as 100%).

Stable Cell Line Establishment. *EAPP*, *R1*, and *Sp1* expression construct or the parental pcDNA3.1 empty vector was transfected into 1242-MG cells separately. All constructs carry neomycin-resistant gene. After 24 h, cells were replated into 5-cm dishes and treated with G418 (Geneticin, 600 μg/ml). Resistant clones were isolated into separate dishes after 6 days and cultured under G418 selection continuously.

Protein Expression and Purification. 1242-MG cells were transfected with HA-*Sp1*, HA-*R1*, or HA-*EAPP* for 48 h. Cells were lysed in the buffer containing 25 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT, 5% glycerol, and protease inhibitors (500 μl/10-cm dish). Anti-HA agarose slurry (6 μl; ProFound HA Tag IP Kit; Pierce Chemical, Rockford, IL) was incubated with 500 μl of cell lysates. Immunoprecipitation (IP) reactions were performed at 4°C overnight following the manufacturer's instructions. IP products were eluted with 25 μl of nonreducing sample buffer provided in the kit and stored at −80°C. The high affinity of anti-HA antibody-coupled agarose ensured the IP specificity of HA-tagged protein.

Western Blotting Analysis. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical). Fifty micrograms of total protein from cells lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitors were separated by 8 to 12% SDS-polyacrylamide gel electrophoresis and transferred to the 0.45-μM pore size polyvinylidene difluoride membrane. After the transfer, the membrane was blocked at room temperature for 1 h with 2% bovine serum albumin in 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, and 0.05% Tween 20. The membrane was incubated with anti-HA (1:1000), anti-FLAG (1:1000), anti-Sp1 (1:1000), anti R1 (1:2000), anti-GR (1:500) or anti-β-actin (1:1000) antibody in 1% bovine serum albumin in 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, and 0.05% Tween 20 at 4°C overnight. After incubating the membrane with horseradish peroxidase-conjugated secondary antibody against appropriate species at room temperature for 1 h, bands were visualized with the ECL Western Blotting Substrates (Pierce Chemical) (Wu et al., 2009a).

Statistical Analysis. Data were presented as the mean ± S.D. and analyzed with unpaired t test. A p value of less than 0.05 was considered significant.

Results

Glucocorticoid Activates MAO B Catalytic and Promoter Activities through both the Fourth Glucocorticoid Response Element and Sp1-Binding Sites. The ability of glial cells to metabolize monoamine neurotransmitters has been firmly established; in fact, more than 90% of brain MAO is found extraneuronally (Oreland et al., 1983). MAO B is abundant in glial cells (Shih et al., 1999), and the interaction between glucocorticoids and MAO B has also been well defined in the glial culture system (Carlo et al., 1996). Hence, in this study, we used a human glioblastoma 1242-MG cell line as a model system to investigate the molecular mechanisms responsible for glucocorticoid activation of *MAO B*. 1242-MG cells express reasonable levels of MAO B and show responses to glucocorticoids in terms of the activation of GR (Tazik et al., 2009). First, we determined the effect of glucocorticoids on MAO B catalytic activity. As shown in Fig. 1, MAO B catalytic activity increased by ~2.8-fold in response to dexamethasone (a potent synthetic glucocorticoid hormone, 100 nM, 48 h) in 1242-MG cells. This is consistent with the observations as reported by other groups using different neuronal/glial culture systems (Carlo et al., 1996; Tazik et al., 2009). The concentrations of dexamethasone as used under current experimental conditions did not produce cytotoxicity, which was ensured in our previous study (Ou et al., 2006a).

Next, we examined whether this induction is reflected at the transcriptional level as well. The human *MAO B* promoter has been cloned and extensively studied in recent years. A core promoter region (−246/−99, 0.15 kb) in 2-kb (−2099/−99) promoter exhibits the maximum promoter activity to drive *MAO B* gene transcription. There are two clusters of overlapping Sp1-binding sites consisting of totally five Sp1-binding motifs in *MAO B* core promoter (Fig. 2A). Mutation or deletion of these Sp1 sites abolished the basal *MAO B* promoter activity (Wong et al., 2001). By examining

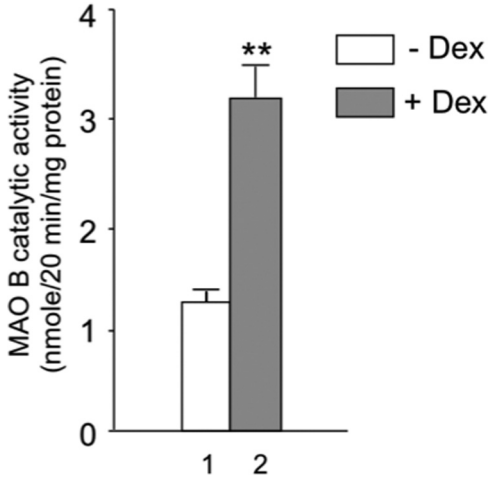

Fig. 1. Glucocorticoid increases MAO B catalytic activity. 1242-MG cells were treated with 100 nM dexamethasone for 48 h and then harvested for MAO B catalytic activity determination. Ethanol was used as a vehicle. The data were presented as the mean ± S.D. from at least three independent experiments. **, $p < 0.01$.

MAO B 2-kb promoter sequence, we identified four consensus GREs. The distal cluster of Sp1 sites is located 577-bp downstream of the fourth GRE (GRE4) (Fig. 2A). The location and sequence of each GRE are summarized in Fig. 2A.

To determine the importance of each consensus GRE, we cotransfected either wild-type (WT, 2 kb) or one of various deleted *MAO B* promoter luciferase (*luc*) reporter constructs with the *GR* expression construct into 1242-MG cells. After 24-h dexamethasone (100 nM) treatment, cells were assayed for luciferase activity. WT *MAO B* 2-kb promoter activity increased by 3-fold upon dexamethasone treatment (Fig. 2B, construct 2). Deletion of GRE1 (Fig. 2B, construct 3) or GRE1, 2, and 3 (construct 4) resulted in a similar extent of dexamethasone activation of *MAO B* promoter, whereas deletion of GRE1, 2, 3, and 4 (construct 5) or Sp1 sites (construct 6) relatively reduced this activation by 37 and 30% ($p < 0.05$), respectively. Deletion of both GRE4 and Sp1 sites (construct 7) further completely abolished this activation. No induction was observed for promoterless luciferase reporter construct (pGL2 only, construct 1). These data suggest that both GRE4 and Sp1 sites are important and have similar potency for glucocorticoid activation of *MAO B* promoter.

Glucocorticoid Receptor Binds to GRE4 Both In Vitro and In Vivo. Glucocorticoids exert their effects on target genes through GR. In response to ligand binding, activated GR translocates into the nucleus and binds to specific GRE to activate gene transcription (Adcock et al., 2004). To study whether GR binds to GRE4 in *MAO B* promoter, we conducted EMSA with radioactively labeled oligonucleotide harboring GRE4 as probe. The result showed two radioactive bands on the gel after incubating 1242-MG nuclear extracts with the probe (Fig. 2C, lane 2). The intensity of the top band increased when the same amount of nuclear extracts treated with dexamethasone (100 nM, 48 h) was incubated in DNA-protein binding reaction (lane 3); because more GR translocate into the nucleus upon dexamethasone treatment, this band thus represents the GR-DNA complex. Furthermore, this band was shifted when anti-GR antibody was incubated in the reaction (lane 4), suggesting that this band is also specific. Thus, GR is capable of directly binding to GRE4.

To examine whether GR is recruited to GRE4 in the natural *MAO B* promoter, we conducted ChIP assay coupled with PCR using primers specific for the region harboring GRE4 (−832/−817) in 1242-MG cells. The 5′-irrelevant locus (−1940/−1780) at the distal end of 2-kb promoter served as a negative control. 1242-MG cells treated with either vehicle (ethanol) or dexamethasone (100 nM, 24 h) were subjected to ChIP assay using anti-GR antibody, and the levels of GR association with specific promoter regions were presented as the percentage of input. As summarized in Fig. 2D, compared with negligible inclusions of GR at irrelevant locus (columns 1 and 3), GR indeed interacted with GRE4 in the natural *MAO B* promoter (column 2, 1.57%), and this association increased by 240% upon dexamethasone treatment (column 4, 3.77%). In line with these observations, we demonstrate the functional GRE4 under both in vitro and in vivo conditions.

EAPP and R1 Are Novel Transcriptional Repressors of MAO B. It is worth noting that the deletion of Sp1 sites in *MAO B* promoter significantly diminished dexamethasone activation of *MAO B* (Fig. 2B, compare construct 6 with construct 2). This suggests a critical involvement of Sp1 sites

as well as other potential Sp1 site-interacting transcriptional regulators in mediating the glucocorticoid effect. We have demonstrated previously that Sp family and Sp family-like transcription factors, such as Sp1, directly interact with these sites (Wong et al., 2001; Ou et al., 2004). To search for additional novel transcription factors, which may interact with Sp1 sites and contribute to the glucocorticoid activation of MAO B, we used three copies of Sp1-binding motifs derived from *MAO B* core promoter as bait to screen a human cDNA library in the yeast one-hybrid system. Two novel transcription factors, EAPP and R1, were identified. EAPP is an E2F-binding protein that modulates E2F-regulated transcription and influences cell proliferation (Novy et al., 2005). R1 was characterized previously as a transcriptional repressor of *MAO A* (Chen et al., 2005) and is involved in apoptotic signaling pathways (Ou et al., 2006b).

To study the role of EAPP and R1 in regulating *MAO B* promoter, WT *MAO B* 2-kb *luc* (Fig. 3A, construct 1) was cotransfected with *EAPP* or *R1* expression construct into 1242-MG cells. After 24-h incubation, cells were assayed for luciferase activity. The results show that both EAPP and R1 (Fig. 3A, construct 1, lanes 2 and 3) repressed *MAO B* promoter activity by ~40% in comparison with the control cotransfected with the parental empty vector (Fig. 3A, construct 1, lane 1). However, EAPP and R1 had no effect on *MAO B* promoter when deleted *MAO B* 2-kb *luc* without the core promoter (Fig. 3A, construct 2) was used. These data indicate that EAPP and R1 are capable of repressing *MAO B* promoter activity via the core promoter region.

To examine the presence of EAPP and R1 in *MAO B* core promoter in vivo, we conducted ChIP assay coupled with PCR using primers specific for the core promoter (−287/−153) in 1242-MG cells. Anti-EAPP and anti-R1 antibodies were used to immunoprecipitate endogenous EAPP and R1, respectively. The 5′-irrelevant locus (−1940/−1780) at the distal end of 2-kb promoter served as a negative control (Fig. 3B). The levels of occupancy by EAPP and R1 at specific locus were presented as the percentage of input. As demonstrated in Fig. 3C, compared with negligible inclusions of EAPP and R1 at irrelevant locus, both EAPP (column 4, 1.38%) and R1 (column 5, 1.27%) were associated with Sp1 sites in the core promoter in vivo. The association of Sp1 (Fig. 3C, column 6, 1.51%) with this region was also examined as a positive control.

MAO B catalytic activity assay was subsequently used to examine the repressing effects of EAPP and R1 on MAO B

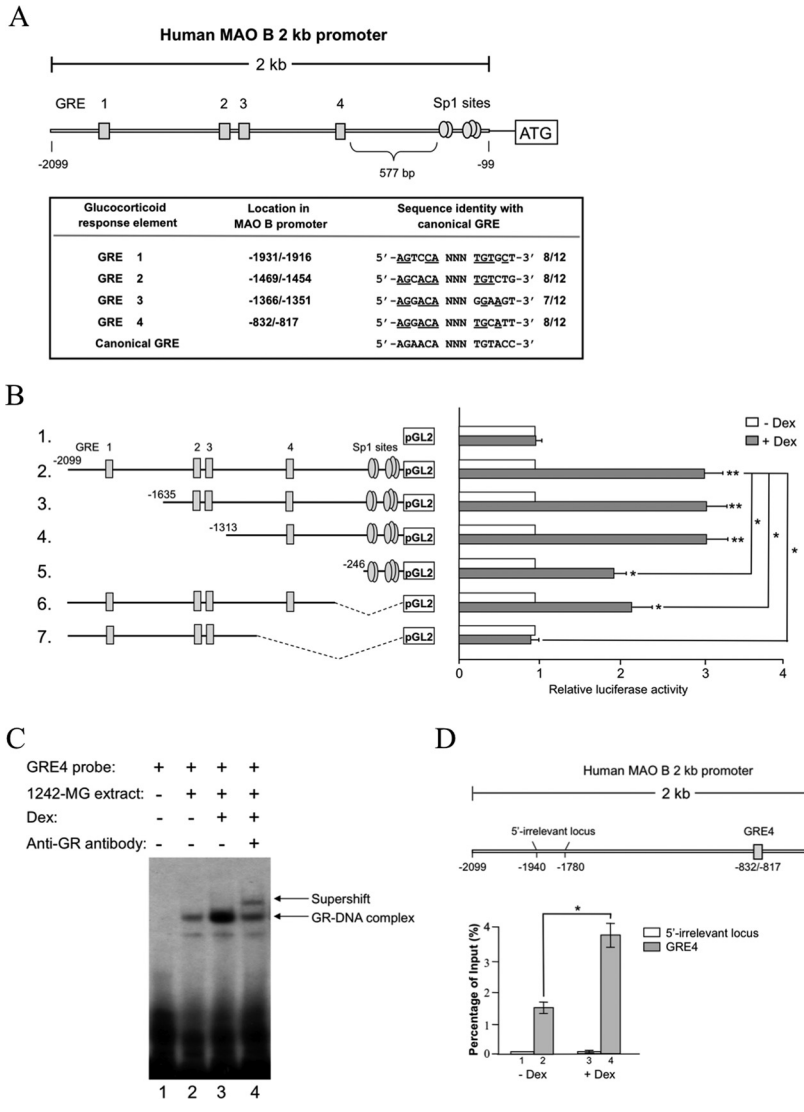

Fig. 2. Glucocorticoid activates *MAO B* 2-kb (−2099/−99) promoter via GRE4. A, a schematic diagram of *MAO B* 2-kb promoter showing four consensus GREs and two clusters of overlapping Sp1 sites. The GRE4 is 577 bp upstream of the distal cluster of Sp1 sites. The sequence and location of each GRE is shown. The nucleotide identity shared with canonical GRE of each consensus GRE is underlined. B, wild-type (2 kb) or deleted *MAO B* promoter-*luc* was cotransfected with *GR* expression construct into 1242-MG cells. Cells were then treated with 100 nM dexamethasone for 24 h followed by luciferase activity determination. Ethanol was used as a vehicle. Activity of *MAO B* promoter-*luc* cotransfected with *GR* expression construct under no treatment was set as 1. C, the radioactive probe harboring GRE4 derived from *MAO B* promoter was incubated with nuclear extracts of 1242-MG cells treated either with or without dexamethasone (100 nM, 48 h). Ethanol was used as a vehicle. Anti-GR antibody was added into DNA-protein binding reaction when required. Arrows indicate GR-DNA complex and shifted GR-DNA complex conjugated with anti-GR antibody. A representative gel is shown. D, a schematic diagram of *MAO B* 2-kb promoter showing the location of GRE4 (−832/−817) and 5′-irrelevant locus (−1940/−1780) used in ChIP assay. 1242-MG cells treated with or without dexamethasone (100 nM, 24 h, ethanol used as a vehicle) were subjected to ChIP assay using anti-GR antibody followed by real-time PCR with primers specific for the region encompassing GRE4. PCR with primers targeting 5′-irrelevant locus was used as a negative control. GR occupancy at specific locus was quantitated using the $2^{-\Delta\Delta C_T}$ method and presented as the percentage of input. All data were presented as the mean ± S.D. from at least three independent experiments.*, $p < 0.05$; **, $p < 0.01$.

catalytic activity in 1242-MG stable cells. As shown in Fig. 3D, stable overexpression of EAPP or R1 decreased MAO B catalytic activity by ~30% compared with the control stably transfected with the parental empty vector, suggesting that these repressions have physiological effects. Moreover, consistent with our previous finding that Sp1 serves as an activator of MAO B, there was a ~2-fold increase of MAO B catalytic activity observed in 1242-MG cells that stably overexpress Sp1 (Fig. 3D).

EAPP and R1 Repress *MAO B* Promoter by Competing with Sp1. We have previously demonstrated that transcriptional repressor Sp3, a member of Sp family proteins, represses *MAO B* promoter by competing with transcriptional activator Sp1 for binding to Sp1 sites (Wong et al., 2001). This led us to hypothesize that similar mechanisms may be used by EAPP and R1 to exert their repressing effects on MAO B. WT (Fig. 4A, construct 1) or deleted (without Sp1 sites, construct 2) *MAO B* 2-kb *luc* construct was cotransfected with *Sp1* expression construct, a key transcriptional activator of *MAO B*, together with *EAPP* or *R1* expression construct into 1242-MG cells. As shown in Fig. 4A, cotransfection of *Sp1* increased WT *MAO B* 2-kb promoter activity by ~300% (construct 1, compare lane 2 with lane 1), whereas this activation was significantly reduced to ~200% ($p < 0.05$) in the presence of EAPP or R1 (construct 1, compare lanes 3 and 4 with lane 2). However, Sp1, EAPP, and R1 had no effect on the deleted *MAO B* 2-kb promoter without Sp1 sites (Fig. 4A, construct 2). The transfection efficiency of *EAPP* (with HA tag), *R1* (with FLAG tag), and *Sp1* as used in promoter assays was confirmed by Western blotting (Fig. 4B). These data suggest competitive regulatory effects of EAPP/R1 and Sp1 on *MAO B* promoter via the core promoter.

To examine whether EAPP and R1 directly compete with Sp1 for binding to Sp1 sites in *MAO B* core promoter, we conducted EMSA with purified Sp1, EAPP, and R1 protein and radioactively labeled oligonucleotide harboring Sp1 sites derived from *MAO B* core promoter as probe. One radioactive band indicating Sp1-DNA complex was shown when 15, 10, or 5 μg of Sp1 protein were incubated with the probe, and the intensity of this band decreased along with lower amounts of Sp1 protein used (Fig. 4C, lanes 2–4). The intensity of this band also decreased in the presence of 100- or 500-fold excess of nonradioactively labeled probes (Fig. 4C, lanes 5 and 6), suggesting that it is specific. Furthermore, this band was shifted when anti-Sp1 antibody was added into the DNA-protein binding reaction (Fig. 4C, lane 7). We also conducted EMSA in the presence of EAPP or R1, as revealed in Fig. 4D, the intensity of Sp1-DNA complex decreased along with increasing amounts of R1 (lanes 2 and 3) or EAPP (lanes 4 and 5) protein incubated in the DNA protein binding reaction. As a negative control, Actin did not recognize the probe and showed no interference with the binding of Sp1 to probe (Fig. 4D, lanes 6 and 7). Taken together, these data suggest that EAPP and R1 may repress *MAO B* promoter by a mechanism of direct competition with Sp1 for binding to Sp1 sites.

EAPP, R1, and Sp1 Cooperatively Regulate Glucocorticoid Activation of *MAO B* Promoter. Because Sp1 sites mediate glucocorticoid activation of *MAO B* promoter (Fig. 2B), we postulate that transcription factors that bind to Sp1 sites, such as EAPP, R1, and Sp1, may also play a role in regulating this activation. To explore this possibility, we first determined the effect of each individual factor on glucocorticoid activation of *MAO B* promoter. WT or deleted (without core promoter) *MAO B* 2-kb *luc* was cotransfected with *GR* expression construct together with *EAPP*, *R1,* or *Sp1* expression construct into 1242-MG cells. After 24-h incubation with dexamethasone (100

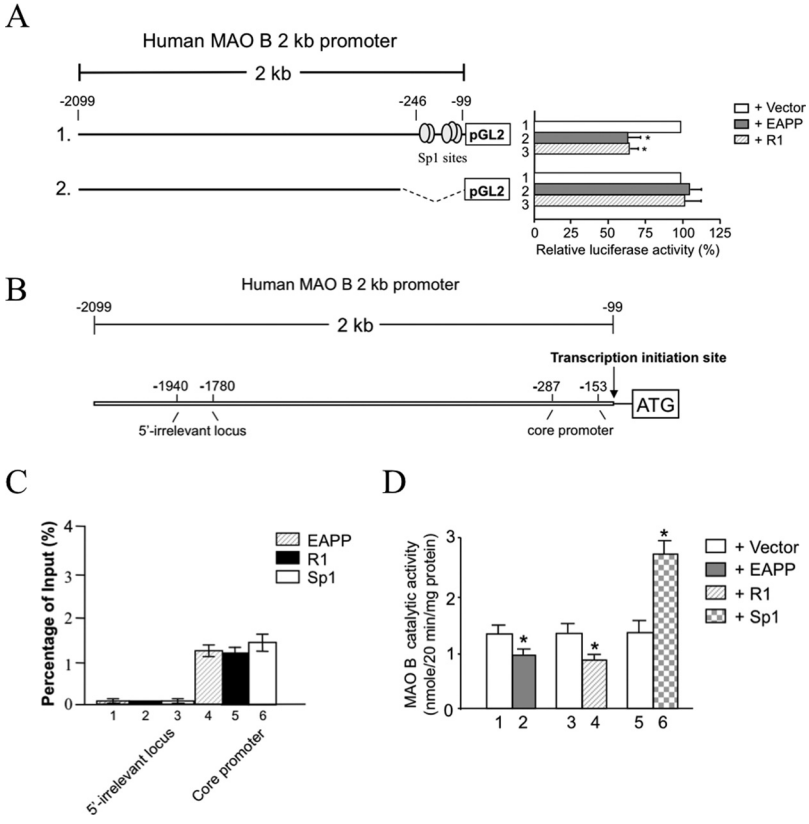

Fig. 3. EAPP and R1 repress *MAO B* promoter and catalytic activities. A, Wild-type (2 kb, construct 1) or deleted (without core promoter, construct 2) *MAO B* 2-kb *luc* was cotransfected with *EAPP* or *R1* expression construct into 1242-MG cells. After 24-h incubation, cells were harvested and assayed for luciferase activity. Activity of *MAO B* promoter-*luc* cotransfected with the parental empty vector was set as 100%. B, a schematic diagram of *MAO B* 2-kb promoter showing the location of core promoter region that encompasses Sp1 sites (−287/−153) and 5′-irrelevant locus (−1940/−1780) used in ChIP assay. C, 1242-MG cells were subjected to ChIP assay using anti-EAPP, anti-R1, or anti-Sp1 antibody followed by real-time PCR with primers specific for *MAO B* core promoter. PCR with primers targeting 5′-irrelevant locus was used as a negative control. The occupancy by EAPP, R1, and Sp1 at the core promoter was quantitated using the $2^{-\Delta\Delta C_T}$ method and presented as the percentage of input. D, MAO B catalytic activity was determined in 1242-MG cells that stably overexpress EAPP, R1, or Sp1. 1242-MG cells stably transfected with the parental empty vector was used as a control. All constructs carry neomycin-resistant genes. All data were presented as the mean ± S.D. from at least three independent experiments. *, $p < 0.05$.

nM), cells were assayed for luciferase activity. As demonstrated in Fig. 5A (left), cotransfection of *EAPP* or *R1* relatively reduced dexamethasone activation of WT *MAO B* 2-kb promoter (EAPP, compare lane 4 with lane 2; R1, compare lane 6 with lane 2, $p < 0.05$), whereas Sp1 enhanced this activation (compare lane 8 with lane 2, $p < 0.05$). However, these factors had no effect on dexamethasone induction of *MAO B* promoter when Sp1 sites were deleted (Fig. 5A, right). The transfection efficiency of *EAPP* (with HA tag), *R1*, *Sp1,* and *GR* as used in promoter assays was confirmed by Western blotting (Fig. 5B).

We next studied whether these factors exert competitive regulatory effects on glucocorticoid activation of *MAO B*. WT *MAO B* 2-kb *luc* was cotransfected with *Sp1* expression construct along with increasing amounts of *EAPP* or *R1* expression construct into 1242-MG cells followed by dexamethasone treatment (100 nM, 24 h). The results demonstrate that both EAPP (Fig. 5C, left) and R1 (Fig. 5C, right) relatively repressed Sp1-enhanced dexamethasone activation of *MAO B* promoter ($p < 0.05$) in an *EAPP* or *R1* concentration-dependent manner.

To study whether these regulatory effects of EAPP, R1, and Sp1 on glucocorticoid activation of *MAO B* promoter are also reflected in vivo, we conducted ChIP assay coupled with PCR using primers specific for the core promoter (−287/−153) in 1242-MG cells. The 5′-irrelevant locus (−1940/−1780) at the distal end of 2-kb promoter served as a negative control. 1242-MG cells treated with either vehicle (ethanol) or dexamethasone (100 nM, 24 h) were subjected to ChIP assay using anti-EAPP, anti-R1, or anti-Sp1 antibody, and the levels of association of each factor at specific locus were presented as the percentage of input. As revealed in Fig. 5D, consistent with previous findings, EAPP (column 4, 1.38%), R1 (column 5, 1.27%), and Sp1 (column 6, 1.51%) were all recruited to the natural *MAO B* core promoter. It is noteworthy that, in response to dexamethasone, a ~30% decrease of occupancy by EAPP (column 10, 0.92%) and R1 (column 11, 0.85%) and a ~230% increase of occupancy by Sp1 (column 12, 3.46%) in the natural core promoter were observed (Fig. 5D). These glucocorticoid-responsive changes of occupancy by EAPP, R1, and Sp1 in *MAO B* promoter show a positive correlation with glucocorticoid activation of *MAO B* gene. Taken together, these data demonstrate that EAPP, R1, and Sp1 cooperatively regulate glucocorticoid activation of *MAO B* promoter via *MAO B* core promoter.

Discussion

Emerging evidence from recent studies has brought into focus the physiological significance of hormonal regulations

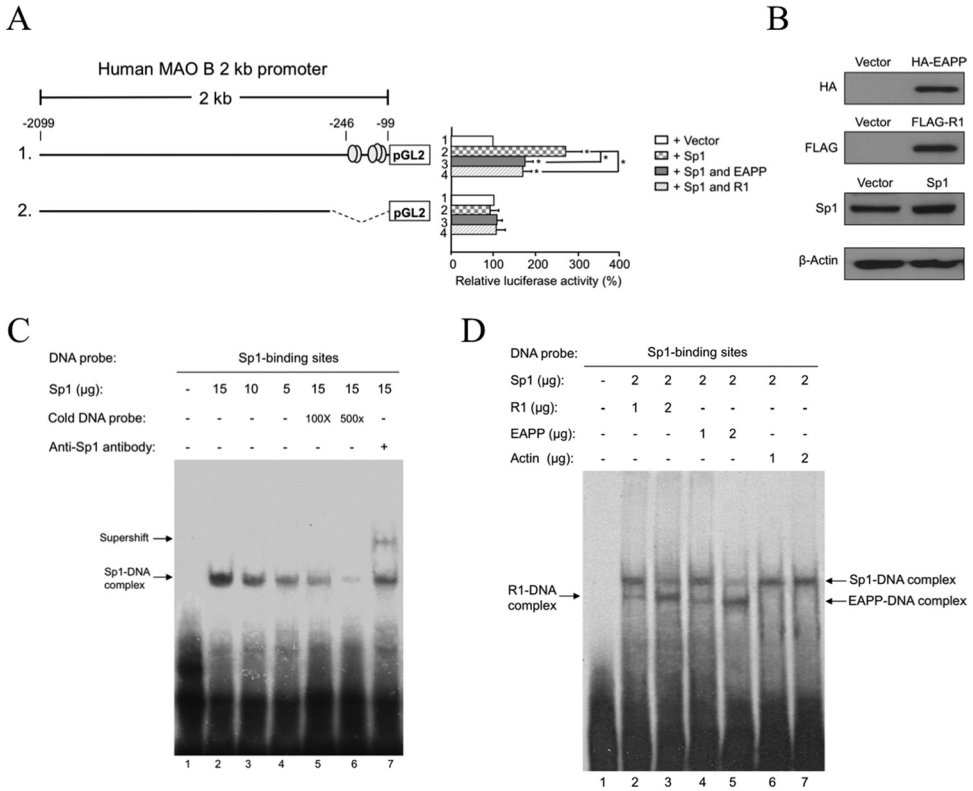

Fig. 4. EAPP and R1 repress *MAO B* promoter by competing with Sp1. A, wild-type (2 kb, construct 1) or deleted (without core promoter, construct 2) *MAO B* 2-kb *luc* was cotransfected with *Sp1* expression construct together with *EAPP* (with HA tag) or *R1* (with FLAG tag) expression construct into 1242-MG cells. After 24-h incubation, cells were harvested and assayed for luciferase activity. Activity of *MAO B* promoter-*luc* cotransfected with the parental empty vector only was set as 100%. B, Western blotting analysis of transfection efficiency of HA-*EAPP*, FLAG-*R1,* and *Sp1* as used in A. β-Actin was used as a loading control. Representative gels are shown. C, electrophoretic mobility shift analysis showed that Sp1 directly binds to *MAO B* core promoter-derived Sp1 sites in vitro. The radioactively labeled probe harboring Sp1 sites derived from *MAO B* core promoter was incubated with various amounts (15, 10, and 5 μg) of purified Sp1 protein as indicated. Nonradioactively labeled probes (lanes 5 and 6) or anti-Sp1 antibody (lane 7) was added into DNA-protein binding reaction when required. Arrows indicate Sp1-DNA complex and shifted Sp1-DNA complex conjugated with anti-Sp1 antibody. A representative gel is shown. D, EAPP and R1 compete with Sp1 for binding to Sp1 sites. The radioactively labeled probe harboring Sp1 sites derived from *MAO B* core promoter was incubated with purified Sp1 protein in the presence of purified R1 (lanes 1 and 2) or EAPP (lanes 3 and 4) protein. Actin protein was used as a negative control. Arrows indicate R1-DNA, EAPP-DNA, and Sp1-DNA complexes. A representative gel is shown. All data were presented as the mean ± S.D. from at least three independent experiments. *, $p < 0.05$.

of *MAO* gene. Steroid and nonsteroid hormones have been shown to influence brain neurotransmission and further to affect mood and behavior, which could in part be gained by modulating MAO activity (de Kloet et al., 1990). Indeed, the roles of a number of hormones, such as androgen, glucocorticoid (Ou et al., 2006a), estrogen (Zhang et al., 2006), and retinoic acid (Wu et al., 2009b), have been demonstrated in *MAO* gene regulation. In this study, we explored the molecular mechanisms responsible for glucocorticoid activation of *MAO B* gene. We have identified four consensus GREs in *MAO B* 2-kb promoter and demonstrated that GR directly binds to GRE4 under both in vitro and in vivo conditions (Fig. 2). Glucocorticoids exert their effects though GR that translocates into the nucleus upon ligand binding and binds to specific DNA response elements to regulate gene transcription (Adcock et al., 2004). Many hormone-response elements share sequence identity such as androgen and glucocorticoid response elements (Ou et al., 2006a). Hence, it would be interesting to determine whether GRE4 serves as a common site to mediate the regulation of *MAO B* by other hormones, which also suggests a structure-function correlation. Indeed, as shown in Fig. 2C, multiple radioactive bands were observed on the gel by the EMSA experiment, which reinforces the possibility of association of other nuclear hormone receptors with this element.

Besides GRE4, we showed that Sp1 sites in *MAO B* core promoter are also necessary for glucocorticoid activation of *MAO B* promoter. Deletion of Sp1 sites downstream of GRE4 reduced the responses of *MAO B* promoter to glucocorticoid (Fig. 2B, construct 6). In contrast, the *MAO B* core promoter, encompassing Sp1 sites but without any GRE, was inducible by glucocorticoid as well (Fig. 2B, construct 5). These observations suggest that the Sp1 site by itself, in addition to maintaining the basal *MAO B* promoter activity, may also mediate the glucocorticoid activation of *MAO B* promoter. Given the largely distinct sequence identity shared between GRE and Sp1 (GC-rich) sites (Li et al., 2004), GR is unlikely to directly bind to Sp1 sites, which led us to speculate that other transcriptional regulators capable of recognizing Sp1 sites may play a potential contributing role(s) in glucocorti-

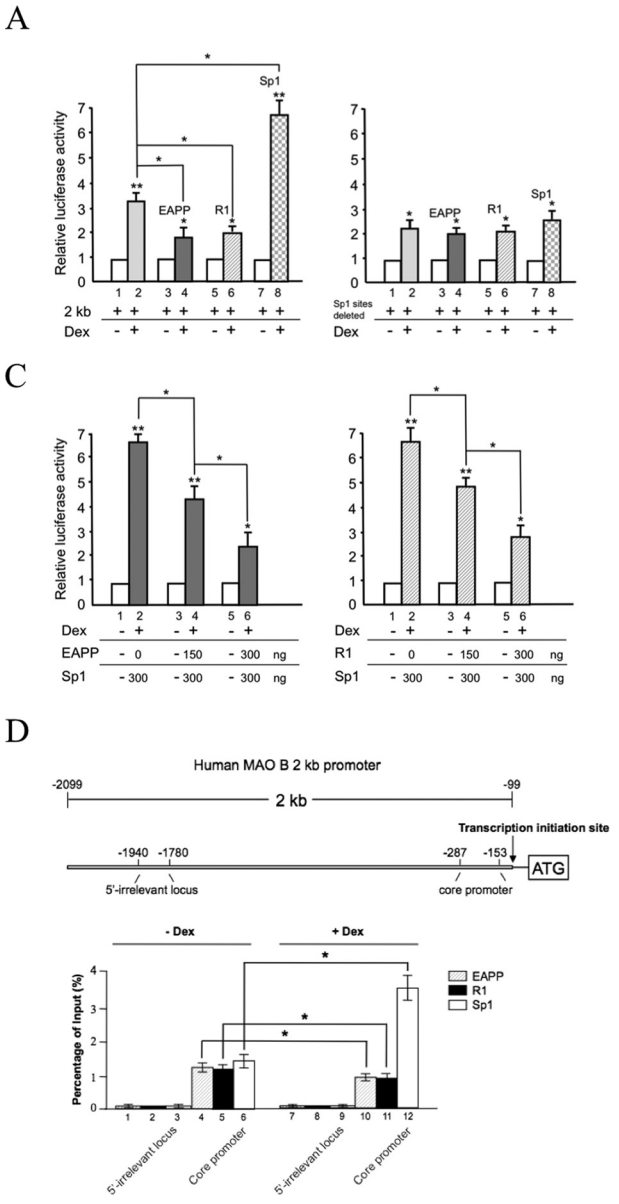

Fig. 5. EAPP, R1, and Sp1 cooperatively regulate glucocorticoid activation of *MAO B* promoter. A, wild-type (2 kb, left) or deleted (without core promoter, right) *MAO B* 2-kb *luc* was cotransfected with *GR* expression construct together with *EAPP*, *R1,* or *Sp1* expression construct into 1242-MG cells. Cells were then treated with 100 nM dexamethasone for 24 h followed by luciferase activity determination. Ethanol was used as a vehicle. Activity of *MAO B* promoter-*luc* cotransfected with *GR* expression construct only under no treatment was set as 1. B, Western blotting analysis of transfection efficiency of HA-*EAPP*, *R1*, *Sp1,* and *GR* as used in A. β-Actin was used as a loading control. Representative gels are shown. C, *MAO B* 2-kb *luc* was cotransfected with *Sp1* expression construct together with various amounts of *EAPP* (left) or *R1* (right) expression construct into 1242-MG cells. Cells were then treated with 100 nM dexamethasone for 24 h followed by luciferase activity determination. Ethanol was used as a vehicle. Activity of *MAO B* 2 kb-*luc* transfected with the parental empty vector only under no treatment was set as 1. D, a schematic diagram of *MAO B* 2-kb promoter showing the location of core promoter region that encompasses Sp1 sites (−287/−153) and 5′-irrelevant locus (−1940/−1780) as used in ChIP assay. 1242-MG cells treated with or without dexamethasone (100 nM, 24 h, ethanol used as a vehicle) were subjected to ChIP assay using anti-EAPP, anti-R1, or anti-Sp1 antibody followed by real-time PCR with primers specific for the core promoter region. The occupancy by EAPP, R1, and Sp1 at specific locus was quantitated using the $2^{-\Delta\Delta C_T}$ method and presented as the percentage of input. All data were presented as the mean ± S.D. from at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

coid activation of *MAO B*. However, it is also possible that other glucocorticoid/GR-derived transcription factors, such as Egr-1 (Sarrazin et al., 2009), up-regulate the *MAO B* promoter by interacting with specific binding elements, neighboring the Sp1 sites (Wong et al., 2002), in the core promoter. Moreover, GR has shown the capability to recruit the general transcription machinery by directly contacting the basal transcription factors, such as TATA-binding protein and TFIIB, and other coactivators via its transactivation domain to stimulate transcription (McEwan et al., 1997). Both GRE4 and Sp1 sites are located at the proximal end of *MAO B* promoter, in particular, closely upstream of the initiator sequences (e.g., TATA) and the ATG transcription start site (Zhu et al., 1992), which provides bases for GR to exert its ability to facilitate the basal transcription of *MAO B* gene. This is also consistent with the observation that *MAO B* promoter was more efficiently transcribed with the presence of GRE4 (Fig. 2B, compare construct 5 with construct 4).

In addition to the known transcriptional activator Sp1, we identified for the first time EAPP and R1 as novel transcriptional repressors of *MAO B*, both of which recognize and use Sp1 sites to down-regulate *MAO B* gene (Fig. 3). The EMSA experiment, albeit in vitro, further demonstrated that EAPP and R1 might compete with Sp1 for binding to Sp1 sites in *MAO B* core promoter, thereby exerting the repressing effects on *MAO B*. However, it would be interesting to explicitly address the way these three factors function at the endogenous *MAO B* promoter in more defined in vivo systems. Moreover, overexpression of both factors significantly repressed MAO B catalytic activity (Fig. 3D), suggesting their physiological effects. EAPP is a ubiquitous nuclear protein that interacts with the activating members of E2F-family transcription factors and increases the activity of several cell cycle-regulated promoters in an E2F-dependent manner (Novy et al., 2005; Schwarzmayr et al., 2008). Hence, it is possible that EAPP also indirectly regulates *MAO B* by modulating the expression of E2F-responsive *MAO* regulators. In addition to *MAO B*, R1 shows the capability to repress *MAO A* gene as well via a similar mechanism involving competitive binding to Sp1 sites with Sp1 (Chen et al., 2005). *MAO A* and *B* genes display similar patterns of transcriptional regulation with regard to specific regulators such as Sp family transcription factors (Chen, 2004). This may arise from the fact that *MAO A* and *B* core promoters share an approximately 60% sequence identity, and, in particular, both consist of Sp1 sites (Zhu et al., 1992).

We further showed the effect of each individual factor on glucocorticoid activation of *MAO B*. Sp1 enhanced this activation (Fig. 5A); in response to glucocorticoid, higher occupancy by Sp1 at *MAO B* core promoter was observed (Fig. 5D). However, the expression levels of Sp1 protein were not changed upon dexamethasone treatment (Fig. 5B), which suggests that Sp1 enhances glucocorticoid activation of *MAO B* by increasing its DNA-binding ability. Similar responses of Sp1 to other hormones, such as retinoic acid, have also been observed previously (Wu et al., 2009b). In contrast, overexpression of EAPP and R1 reduced both the basal (Fig. 5A) and Sp1-enhanced (Fig. 5C) glucocorticoid activation of *MAO B* promoter. Moreover, a partial release of EAPP and R1 from *MAO B* core promoter upon dexamethasone treatment was observed (Fig. 5D), which could be a direct consequence of their competitive binding at Sp1 sites with Sp1.

It is also possible that the glucocorticoid-induced dynamic equilibrium of occupancy by these factors is coupled with specific cellular events. Recent studies have indicated that glucocorticoids increase the susceptibility of certain cell types to apoptosis (Reagan and McEwen, 1997). Long-term stress-induced hypersecretion of glucocorticoids leads to neuronal atrophy and eventually cell death (Sapolsky, 1996). Specifically, dexamethasone, as used in the present study, has been reported to exert an antiproliferative effect in certain tumor cells such as glioma cells (Liu et al., 2009). EAPP and R1 both have shown a positive correlation with cell proliferation. Overexpression of EAPP results in a significant increase of cells in S phase, whereas RNA interference-mediated knockdown of EAPP reduces the fraction of cells in S phase (Novy et al., 2005). R1 potentiates and complements c-Myc transforming activity (Huang et al., 2005) and shows antiapoptotic activity (Ou et al., 2006b). Furthermore, R1 expression increases along with induction of cell cycle progression (unpublished data). Given these findings, the glucocorticoid-induced decrease of transcriptional activities of EAPP and R1 could also be indirectly driven by dexamethasone-mediated antiproliferative effects.

Glucocorticoids are the major form of hormones to respond to stress and are secreted from the adrenal gland during stress. Abnormally elevated levels of glucocorticoids, which could be a consequence of long-term exposure to stress, are associated with major depression (Duval et al., 2006). Induction of MAO activity by glucocorticoids down-regulates neurotransmitter levels such as serotonin, which, to some extent, could further deteriorate depressive situations. MAO inhibitors have shown their effectiveness in the treatment of depression by increasing the neurotransmitter levels to date (Bortolato et al., 2008). However, in this study, the identification of EAPP and R1 as novel transcriptional repressor of *MAO* provides new targets for treating MAO-associated neuropsychiatric diseases with a neurotransmitter imbalance. Modulation of endogenous levels of EAPP and R1 expression could be an alternative approach to maintain normal MAO activity, which avoids unwanted side effects of drugs taken exogenously.

Together, we demonstrated the molecular mechanisms of glucocorticoid activation of *MAO B* gene, which involves a canonical pathway of direct GR binding to the fourth GRE as well as cooperative regulatory effects exerted by EAPP, R1, and Sp1 via *MAO B* core promoter that contains Sp1 sites. Moreover, we identified for the first time EAPP and R1 as novel transcriptional repressors of *MAO B* and further showed that these factors may compete with Sp1 for binding to Sp1 sites in *MAO B* core promoter, which expands the current knowledge on *MAO B* gene regulation. This study also provides new insights into the molecular mechanisms of glucocorticoid action.

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Authorship Contributions

Participated in research design: Chen, Ou, Wu, and Shih.
Conducted experiments: Ou and Wu.
Performed data analysis: Ou, Wu, and Shih.
Wrote or contributed to the writing of the manuscript: Ou, Wu, and Shih.

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