Glucocorticoid and Androgen Activation of Monoamine Oxidase A Is Regulated Differently by R1 and Sp1*

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Monoamine oxidase (MAO) A is a key enzyme for the degradation of neurotransmitters serotonin, norepinephrine, and dopamine. There are three consensus glucocorticoid/androgen response elements and four Sp1-binding sites in the human monoamine oxidase A 2-kb promoter. A novel transcription factor R1 (RAM2/CDCA7L) interacts with Sp1-binding sites and represses MAO A gene expression. Luciferase assays show that glucocorticoid (dexamethasone) and androgen (R1881) increase MAO A promoter and catalytic activities in human neuroblastoma and glioblastoma cells. Gel-shift analysis demonstrates that glucocorticoid/androgen receptors interact directly with the third glucocorticoid/androgen response element. Glucocorticoid/androgen receptors also interact with Sp1-binding sites indirectly via transcription factor Sp1. In addition, dexamethasone induces R1 translocation from the cytosol to the nucleus in a time-dependent manner in both the neuroblastoma and wild-type UW228 cell lines but not in R1 knock-down UW228 cells. In summary, this study shows that glucocorticoid enhances monoamine oxidase A gene expression by 1) regulation of R1 translocation; 2) direct interaction of the glucocorticoid receptor with the third glucocorticoid/androgen response element; and 3) indirect interaction of glucocorticoid receptor with the Sp1 or R1 transcription factor on Sp1-binding sites of the MAO A promoter. Androgen also up-regulates MAO A gene expression by direct interaction of androgen receptor with the third glucocorticoid/androgen response element. Androgen receptor indirectly interacts with the Sp1, but not R1 transcription factor, on Sp1-binding sites. This study provides new insights on the differential regulation of MAO A by glucocorticoid and androgen.

Monoamine oxidase (MAO)[2] is located on outer membranes of mitochondria in neuronal, glial, and other cells. It catalyzes the oxidative deamination of monoamine neurotransmitters such as serotonin, norepinephrine, dopamine, and phenylethylamine (1). MAO exists in two isoforms, MAO A and MAO B. MAO A preferentially oxidizes serotonin (5-hydroxytryptamine), norepinephrine, and dopamine and is irreversibly inhibited by low concentration of clorgyline (2). MAO B preferentially oxidizes phenylethylamine and benzylamine and is irreversibly inactivated by low concentrations of pargyline and deprenyl (3). MAO A and B are coded by different genes (4) closely linked on the X chromosome, Xp11.2–11.4 (5). Both genes consist of 15 exons with identical exon-intron organization (6) and both are regulated by Sp1 factors; however, there are some differences in their cis-elements (7–9). The core promoter of MAO A consists of Sp1-binding sites that are activated by Sp1 (7) and repressed by a novel repressor R1 (RAM2/CDCA7L) (8). This core promoter has bi-directional activity (7). In addition, a functional polymorphism of a 30-bp repeat sequence has been identified and located ~1.2 kb upstream of the MAO A translation start site (10). The human MAO B promoter contains two clusters of overlapping Sp1 sites separated by a CACCC element (11). An Sp1-like transcription factor, transforming growth factor-β-inducible early gene, also activates the MAO B promoter via overlapping Sp1 sites (12). The expression of MAO B is regulated by protein kinase C and the MAPK signaling pathway (13).

A mutation in the human MAO A gene, which results in no enzymatic activity, is associated with aggressive behavior in males in a Dutch family (14). MAO A knock-out (KO) mice showed aggressive behavior in males (15), but MAO B KO mice did not show aggressive behavior (16). Interestingly, a spontaneous mutation of the MAO A gene in MAO B KO mice resulted in MAO A/B double KO mice, which showed hyperreactivity and anxiety-like behavior (12).

Steroid hormones are involved in the regulation of many functions in which MAO also plays an important roles such as response to stress, behavioral adaptation and mood (17). Significant hypersecretion of glucocorticoids has been shown to be associated with depression (18). The synthetic glucocorticoid, dexamethasone, increases MAO A activity in human fibroblasts (19) and rat brain frontal cortex (20). Both anti-glucocorticoid agents (21) and MAO A inhibitors (22, 23) have been used in the treatment of depression. Androgens have also been shown to increase MAO activity in the rat brain (24).

The biological action of glucocorticoids and androgens are mediated through their respective receptors, glucocorticoid receptor (GR) and androgen receptor (AR). GR and AR bind to derivatives of a common response element, i.e. the consensus glucocorticoid response element (GRE), 5′-GGTACAnnn-

TGTTCT-3′, where n is any nucleotide (25). There are three consensus glucocorticoid/androgen response elements (GRE/AREs) in the human MAO A promoter. However, how MAO A gene expression is regulated by glucocorticoid/androgen is unclear. In addition, R1 has recently been found to be a repressor for MAO A gene expression through Sp1-binding sites (8). This study describes the regulation of MAO A gene expression by GR, AR, R1, and Sp1. We show that R1 and Sp1 are glucocorticoid-responsive transcription factors and are involved in the regulation of glucocorticoid activation of MAO A, whereas R1 is not involved in androgen activation of MAO A.

MATERIALS AND METHODS

Cell Lines and Materials—The human cell line, neuroblastoma SK-N-BE(2)-C, was purchased from the American Type Culture Collection (ATCC), and glioblastoma 1242-MG was a gift obtained from Dr. B. Westermark (Dept. of Pathology, University Hospital, Uppsala, Sweden). SK-N-BE(2)-C cells were grown in medium containing a 1:1 mixture of Eagle's minimum essential medium with Earle's balanced salt solution and Ham's F-12 media with 2.5 mM L-glutamine, and 1242-MG cells were grown in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 10 g/ml streptomycin, and 10% fetal bovine serum (Invitrogen). Wild-type and siRNA-mediated R1 knockdown cell lines and the human medulloblastoma UW228 cells were gifts from Dr. Annie Huang (University of Toronto, Toronto, Canada) (26). UW228 cells were maintained in α-minimal essential medium with 10% fetal bovine serum. Goat polyclonal anti-Sp1 antibody, mouse monoclonal anti-GR or -AR antibody, and rabbit polyclonal anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against R1 was made in-house by Anaseptic Co. (San Jose, CA) (8). Dexamethasone (a synthetic glucocorticoid) and clorgyline (MAO A inhibitor) were purchased from Sigma Co. R1881 (a synthetic androgen) was purchased from PerkinElmer Life Sciences.

Human MAO A Promoter-Luciferase Reporter Gene Constructs—MAO A promoter fragments, $-2072/-14$ bp (2 kb), were cloned into the polylinker site (XhoI/HindIII) upstream of the luciferase gene in the pGL2-Basic vector (Promega, Madison, WI). Serial deletion constructs were generated from MAO A 2-kb promoter by PCR, and the PCR products were subcloned into Zero Blunt TOPO vector (PCR cloning kit, Invitrogen) for verifying DNA sequence. The PCR products were subcloned into pGL2-Basic luciferase reporter vector by restriction enzyme digestion (XhoI/HindIII) and self-ligation using T4 DNA ligase (Invitrogen). For construction of GRE/ARE-luciferase plasmids (GRE/ARE/SV40), the 24-bp-containing GRE/ARE1, GRE/ARE2, and GRE/ARE3 plasmids were subcloned into the MluI/XhoI sites, respectively, of the pGL3 promoter (Promega). Their sense DNA sequences were (consensus GREs are bold): GRE/ARE1: 5′-gtaccg**agaaca**gcc**tgaccg**tgg-3′; GRE/ARE2: 5′-aataca**agaacc**tcc**tgcacc**cag-3′; and GRE/ARE3: 5′-gacaca**aggaca**ttc**taaacc**taa-3′.

The plasmid DNA was extracted and purified by using a Miniprep kit (Qiagen) following the manufacturer's instructions. The identity and orientation of the subcloned fragments were confirmed by DNA sequencing.

Site-directed Mutagenesis of the Human MAO A Core Promoter—Site-directed mutagenesis was utilized to mutate each of three GRE/AREs in the human MAO A 2-kb promoter and to mutate each of four Sp1-binding sites in MAO A 2-kb or core promoter ($-270/-14$). GRE/AREs mutations were made by site-specific mutagenesis of the MAO A 2-kb promoter-luciferase reporter gene vector using a site-directed mutagenesis kit (QuikChange XL, Stratagene) following the manufacturer's instructions (27). Primers used for mutagenesis of three GRE sequences are indicated in the following (sense DNA sequence; GRE sequences are bold; the lowercase letters indicate base substitutions): GRE/ARE1: 5′-GAGTGGGTACCG**tttcca**gcc**gttccg**tggAGAAGGGCTGCG-3′; GRE/ARE2: 5′-GGGGTTCAATACA**tttccc**tcc**gttacc**cagTAATCCTTTCC-3′; and GRE/ARE3: CCAGCTGCCGACACA**tttcca**ttc**gttacc**taaTAACTCTC-GCC-3′.

Mutants of the first, second, third, or fourth Sp1-binding sites were generated using the human MAO A 2-kb promoter or core promoter-luciferase reporter gene construct as a template. To generate the mutation of all four Sp1-binding sites, the first Sp1-binding site was mutated using the human MAO A promoter-luciferase reporter gene construct as a template. The second Sp1-binding site was mutated using the first Sp1-binding site mutated construct. The third Sp1-binding site was mutated using the first and the second Sp1-binding site-mutated construct as templates, which were then used as the template for mutating the fourth Sp1-binding site. Four primers used for mutagenesis of four Sp1 sites are the following (Sp1-binding sites are bold; the lowercase letters indicate base substitutions: CG is replaced by tt in all four Sp1 sites): the first Sp1-binding site: 5′-CAAGGGTCCGC**CCttCTC**TCAGTGC-CCAGC-3′ (-249 to -220 bp); the second Sp1-binding site: 5′-GAAACATCAGC**TCttCCC**CTGGGCGCTCCC-3′ (-201 to -172 bp); the third Sp1-binding site: 5′-GCAAAAGGGTT-CGC**CCttCCC**ACAGTGCCC-3′ (-162 to -133 bp); and the forth Sp1-binding site: 5′-GAAGGATCGGC**TCttCCC**CCG-GGCTCCCCG-3′ (-111 to -82 bp). The mutated nucleotide sequences of all mutant constructs were confirmed by DNA sequencing.

Transient Transfection and Luciferase Assay—Transfections in cells were performed using SuperFect transfection reagent (Qiagen). Cells were plated at a density of 5×10^5 cells/well in 6-well plates (Costar, Cambridge, MA) with 2 ml of medium and 10% fetal bovine serum and grown until 50% confluency (24–36 h). Wild-type or deleted or mutated MAO A promoter-luciferase construct (0.5 μg for one well) was co-transfected into cells with 20 ng of plasmid pRL-TK (the herpes simplex virus thymidine kinase promoter fused upstream to the *Renilla* luciferase gene, which is used as an internal control, Promega) as described previously (28). For co-transfection of the human GR or AR expression vectors (hGR-pcDNA3 or hAR-pcDNA3), the total amount of DNA for each transfection was kept at ~1.0 μg. The plasmids were mixed with 100 μl of serum- and antibiotic-free medium and ~7 μl of SuperFect reagent (for one well) for ~40 min following the manufacturer's instructions. Cells were incubated with DNA precipitates for ~2 h and then replaced with fresh medium. For co-transfection of human R1 or Sp1 expression vectors, the total amount of

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DNA for each control group was kept constant by the addition of an empty expression vector pCMV3.1.

Following 12-h incubation, cells were supplemented with charcoal-stripped, steroid-free fetal calf serum for 8 h to remove endogenous hormones, then treated with 100 nM dexamethasone or 10 nM R1881 in the culture medium with charcoal-stripped serum for another 12 or 24 h. Controls were the same DNA constructs co-transfected with GR or AR but treated with vehicle (ethanol) without agonists. Cells were harvested with luciferase assay lysis buffer (Promega). Cell lysates were then assayed for luciferase activity using the Promega Dual Luciferase Assay system (Promega).

MAO A Catalytic Activity Assay—MAO A catalytic activity was determined to access effects of glucocorticoid and androgen in SK-N-BE(2)-C and MG-1242 cells. Cells were plated at a density of 10^6 cells in a 10-cm dish at 37 °C for 12 h, then supplemented with charcoal-stripped, steroid-free fetal calf serum for 8 h. Subsequently, cells were treated with 100 nM dexamethasone or 10 nM R1881 or vehicle (ethanol) for another 24 or 48 h and harvested. MAO A catalytic activity was determined.

Approximately 100 μg of total proteins from cells were incubated with 100 μM $[^{14}C]$5-hydroxytryptamine in 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 6 N HCl. Reaction products were then extracted with benzene/ethyl acetate and centrifuged at 4 °C for 10 min. The organic phase containing the reaction product was extracted, and its radioactivity was determined by liquid scintillation spectroscopy (29).

Nuclear Extracts and Gel-Shift Assay—Nuclear extracts were prepared by resuspending SK-N-BE(2)-C cells, which were treated with either 100 nM dexamethasone or 10 nM R1881 from a 10-cm dish in 20 μl of buffer A (10 mM HEPES (pH 7.9 at 4 °C), 1.5 mM MgCl$_2$, 10 mM KCl, and 0.5 mM DTT) and 0.1% Nonidet P-40 (freshly added), incubating 10 min on ice, mixing, and centrifuging at 6,000 rpm in a microcentrifuge for 10 min at 4 °C. The supernatant was removed, and the nuclear pellet was suspended in 15 μl of buffer C (20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM phenylmethysulfonyl fluoride (freshly added) and 0.5 mM DTT (freshly added)), incubated 15 min at 4 °C, mixed and centrifuged at 3,000 rpm in a microcentrifuge for 10 min at 4 °C. The supernatant containing nuclear proteins was diluted with 75 μl of buffer D (20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethysulfonyl fluoride (freshly added), and 0.5 mM DTT (freshly added)) and stored at −70 °C (30).

The following two DNA fragments: 1) the third GRE/ARE element (bold), sense 5′-cgacaca**aggaca**ttc**taaacc**tagg-3′ (we added 2 gg to the 3′-end to disrupt the "TAATAA" box) or 2) Sp1-binding sites in MAO A core promoter (the four Sp1-binding sites are bold), sense 5′-cg c**cccgctc**tc agtgcccagc tccccccggg tatcagctga aacatcagc**t ccgccc**ctgg gcgctccggg agtatcagca aaagggttcg c**cccgccc**ac agtgcccggc tccccccggg tatcaaaaga aggatcgg**ct ccgccc**ccgg gctccccggg gg-3′ were radiolabeled by Klenow fill-in. 20-μg nuclear extracts were diluted in binding buffer (20 mM Hepes (pH 7.9), 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% glycerol, and 2 mM dithiothreitol, 2 μg of poly(dI-dC)) (31) with a total volume of 20 μl. Antibody against

GR, AR, R1, or Sp1 was added when required, and the mixture was incubated for 20 min at room temperature. Labeled probe (∼60,000 cpm) was added, and the mixture was incubated for an additional 20 min at room temperature. Samples were then run on a 5% non-denaturing polyacrylamide gel in 250 mM Tris and 1.95 M glycine (pH 8.3) at 150 V for 3 h. The gel was dried and visualized by autoradiography.

Co-immunoprecipitation—Nuclear proteins were extracted from SK-N-BE(2)-C or 1242-MG cells (1×10^7) and treated with 100 nM dexamethasone or 10 nM R1881 or vehicle for 24 h as described previously (32). Briefly, the nuclear proteins were extracted by adding the immunoprecipitation buffer (50 mM Hepes buffer, pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.5% Nonidet P-40), supplemented with protease inhibitors (10 μM antipain, 1 g/ml aprotinin, 30 μM leupeptin, 30 μM chymostatin, 1 μM pepstatin A, and 1 mM phenylmethysulfonyl fluoride), and incubated on ice for 30 min with gentle shaking. Soluble nuclear proteins were collected as supernatant by centrifuging at 1,200 rpm in a microcentrifuge for 30 min. The supernatant was dialyzed for 4 h at 4 °C against 1 liter of immunoprecipitation buffer with protease inhibitors.

After dialysis, the nuclear proteins were cleared by centrifugation at 4 °C for 15 min. The concentration of proteins in the supernatant fraction of the lysates was determined by the Bio-Rad protein assay, using bovine serum albumin as a standard. Samples were adjusted to 4 mg/ml with ice-cold immunoprecipitation buffer. The nuclear protein was immunoprecipitated by incubating with anti-GR or -AR antibody (4 μl of antibody in 1 ml of immunoprecipitation buffer) with BioMag beads (Anti-Mouse, Qiagen) overnight at 4 °C with rotation. As a negative control, BioMag beads without antibody were used. Beads and bound proteins were pelleted by centrifugation and washed three times with 1 ml of immunoprecipitation buffer. Proteins were eluted from beads by boiling them in SDS sample buffer (60 mM Tris-HCl, pH 6.8, 100 mg/ml sucrose, 2% SDS, 0.05 mg/ml bromphenol blue, and 100 mM DTT) for 5 min. Proteins co-immunoprecipitated with GR or AR were analyzed by Western blot with anti-R1 or anti-Sp1 antibodies.

Western Blot Analysis of Proteins Co-immunoprecipitated with GR or AR—Twenty micrograms of total proteins that co-immunoprecipitated with anti-GR or -AR antibody were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After the transfer, membranes were blocked at room temperature for 2 h with 5% bovine serum albumin in TTBS (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). The membranes were then incubated with anti-R1 antibody (1:500), with anti-Sp1 antibody (1:250), or with anti-β-actin antibody (negative control) overnight at 4 °C. The secondary antibody (goat anti-rabbit antibody for R1 and β-actin, 1/10,000, or mouse anti-goat antibody for Sp1, 1/4000), and chemiluminescence procedures were conducted according to the manufacturer (Amersham Biosciences). ∼0.5% of nuclear proteins of each sample (input) before co-immunoprecipitation experiments were loaded on gel and blotted by anti-R1 or -Sp1 antibody as positive controls.

Immunofluorescence and Quantitative Image Analysis—SK-N-BE(2)-C and UW228 cells were plated on coverslips the day before experiment. After 16 h, cells were supplemented with

A. **Human MAO A promoter (2 kb region)**
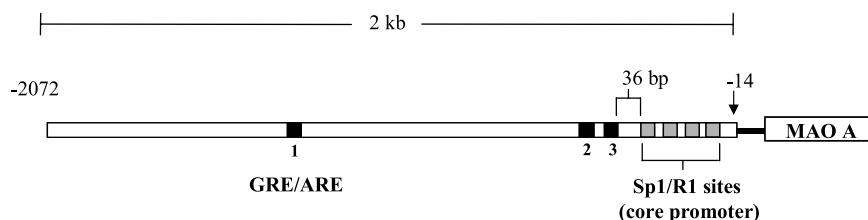
B. **Three consensus GR/AR response elements in MAO A promoter**

Glucocorticoid/ Androgen Response Element		Sequence Identity With Consensus GRE/ARE (antisense)		Location in MAO A promoter
GRE/ARE	1	5'-C<u>GGTCA</u> NNN <u>TGTTCT</u>-3'	9/12	**-1350/-1335**
GRE/ARE	2	5'-<u>GGTGCA</u> NNN G<u>GTTCT</u>-3'	10/12	**-331/-317**
GRE/ARE	3	5'-<u>GGTTTA</u> NNN <u>TGTCCT</u>-3'	9/12	**-289/-275**
GRE		5'-GGTACA NNN TGTTCT-3'		

FIGURE 1. *A*, a map of human MAO A 2-kb promoter showing three GRE/AREs (GRE/ARE1, -2, and -3) and four Sp1-binding sites or R1-binding sites. There are 36 nucleotides between the third GRE/ARE and Sp1-binding sites. *B*, the sequences (antisense) of the three GRE/AREs in the human MAO A promoter were compared with classic GRE sequence. The shared identity of nucleotides between GRE/AREs in MAO A promoter and classic GRE are *underlined*. The location of each GRE/ARE in the human MAO A 2-kb promoter is also indicated.

charcoal-stripped, steroid-free fetal calf serum for 8 h, then treated with 100 nM dexamethasone for another 12, 24, or 48 h. Controls were treated with vehicle for the same time. Cells were then fixed in 4% paraformaldehyde in phosphate-buffered saline for 30 min and permeabilized in 0.2% Triton X-100 and 5% goat serum in phosphate-buffered saline for 15 min. Cells were incubated overnight with rabbit anti-R1-antibody (1/1000) at 4 °C, followed with fluorescein-conjugated anti-rabbit secondary antibody (1/1000, Vector Laboratories) for 2 h. Stained slides were mounted by Vectashield (Vector Laboratories) in the presence of 4,6-diamino-2-phenylindole (DAPI, for nuclear staining) and examined under a fluorescence microscope.

Quantitative analysis of R1 expression in both the nucleus and cytosol was carried out by calculating the ratios of green color (R1 signal) intensity (nucleus/cytosol) using image analysis software (ComPix, Inc., Cranberry Township, PA). Images were acquired using a Q-Imaging Retiga EXI Cooled-CCD digital camera at 1392×1040 pixel resolution and 12-bit/channel color depth. The acquired images were then analyzed using the Compix imaging software. The total area of green color in the nuclei in an image field was determined. To measure the R1 signal in cytosol, total R1 signal in both nuclei and cytosol was segmented and counted. The R1 signal in cytosol was obtained by subtracting the intensity value of cytosol from the total intensity of nuclei and cytosol. This result was used to calculate the R1 expression ratio of nucleus/cytosol.

Statistical Analysis—The statistical significance was evaluated using a Student's *t* test for two-group comparisons when needed. A value of $p < 0.05$ was considered to be significant.

RESULTS

Dexamethasone and R1881 Increased Human MAO A Promoter Activity—Three consensus glucocorticoid/androgen response elements (GRE/ARE1, -2, and -3, from 5'- to 3'-ends, Fig. 1*A*) and a core promoter that contains four Sp1-binding

sites (or R1-binding sites, -238 to -14 bp, Fig. 1*A*) have been identified within the human MAO A 2-kb promoter region (Fig. 1*A*). Sp1-binding sites (-239 to -93) are located 36-bp downstream of the third GRE/ARE (GRE/ARE3, -289 to -275 bp, Fig. 1*B*). To study effects of dexamethasone (glucocorticoid) and R1881 (androgen) on MAO A gene expression, an MAO A 2-kb luciferase construct was co-transfected with or without a GR-expression vector (Fig. 2, *A* and *B*) or an AR-expression vector (Fig. 2, *C* and *D*) into SK-N-BE(2)-C (Fig. 2, *A* and *C*) or MG-1242 (Fig. 2, *B* and *D*). Cells were then treated with synthetic glucocorticoid, dexamethasone (100 nM), or synthetic androgen, R1881 (10 nM) for 24 h.

As shown in Fig. 2 (*A* and *B*), the MAO A promoter activity was 2.6-fold increased upon treatment with dexamethasone in both cell lines compared with their respective controls (treatment with vehicle, *column 4 versus 3*). When co-transfecting glucocorticoid receptor, MAO A promoter activity was further increased to 5.3-fold upon treatment with dexamethasone in both cell lines compared with their respective controls (*column 6 versus 5*).

Similarly, R1881 increased the MAO A promoter activities to 1.8-fold in both SK-N-BE(2)-C and MG-1242 cell lines as shown in Fig. 2 (*C* and *D*, *column 4 versus 3*). When co-transfecting with androgen receptor, MAO A promoter activity was further increased to 3-fold upon treatment with R1881 in both cell lines compared with controls (Fig. 2, *C* and *D*, *column 6 versus 5*). However, dexamethasone or R1881 alone did not increase the pGL2 basic luciferase reporter gene activity (Fig. 2, *A–D*, *column 2 versus 1*).

We have also examined the effect of dihydrotestosterone (DHT) on the human MAO A 2-kb promoter activity. DHT increases MAO A promoter activity similarly to R1881 (data not shown). DHT is produced from testosterone with different effects in certain physiological conditions (33). R1881 is a synthetic androgen and works as effectively as either DHT or testosterone in most cell model systems and has similar affinity as DHT for androgen receptor (34). Thus we have used R1881 in this study.

Dexamethasone/R1881 Increases the Human MAO A Promoter Activity through the Third GRE/ARE and Sp1-binding Sites—Next, the roles of each GRE/ARE and Sp1-binding site on MAO A promoter activity were studied using deleted and mutated MAO A promoter luciferase constructs. To determine the effect of dexamethasone, each mutant was co-transfected with GR into SK-N-BE(2)-C and treated with dexamethasone or vehicle (ethanol) for 24 h (Fig. 3*A*). Deleting the first GRE/ARE (Fig. 3*A*, *construct 2*) or both the first and second GRE/AREs (Fig. 3*A*, *construct 3*) showed the same -fold activation by
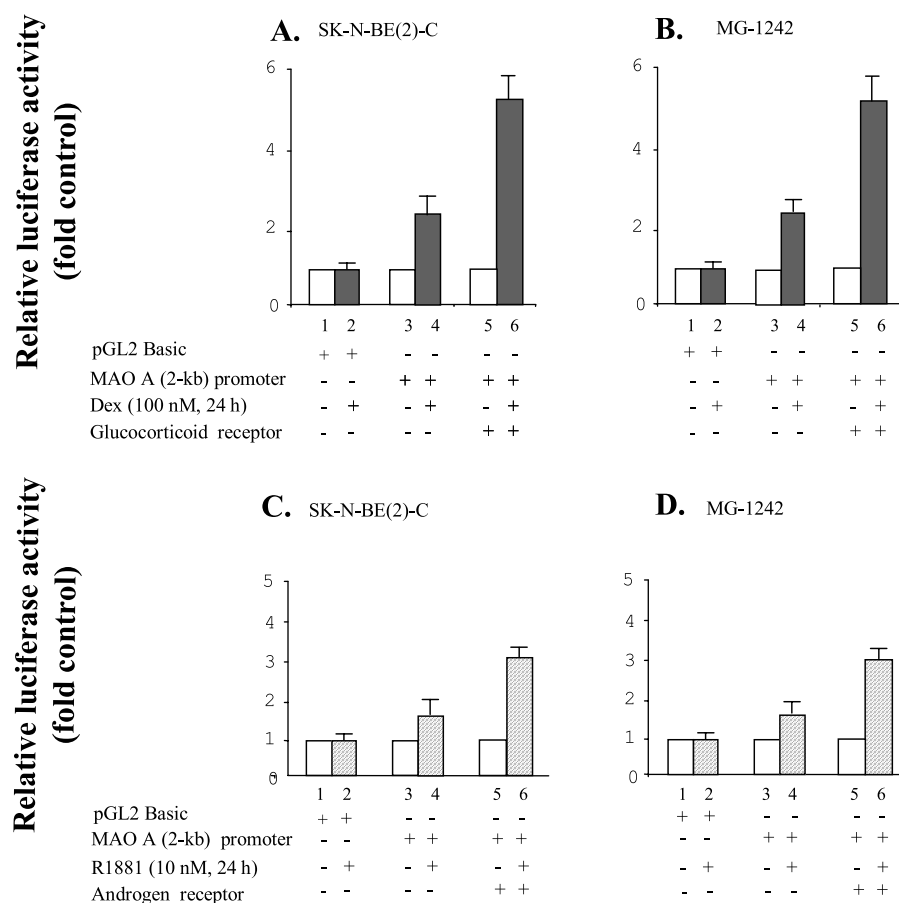
FIGURE 2. **The effect of dexamethasone or R1881 on MAO A promoter activity in SK-N-BE(2)-C and 1242-MG cell lines.** *A*, MAO A 2-kb-luciferase construct alone (*lanes 3* and *4*) and with GR expression vector (*lanes 5* and *6*) were co-transfected into SK-N-BE(2)-C cells for 16 h. The culture medium was then changed to charcoal-treated serum to remove endogenous hormones. 8 h later, a charcoal-treated serum containing a synthetic glucocorticoid, dexamethasone (100 nM), was added. After another 24 h, cells were collected and luciferase activity was determined. The pGL2-basic without MAO A promoter is the negative control (*lanes 1* and *2*). The same experiments were performed in *B* with 1242-MG cells. The effect of a synthetic androgen, R1881 (10 nM), was also studied in a similar way in SK-N-BE(2)-C (*C*) and 1242-MG cell lines (*D*). Controls were respective luciferase constructs with vehicle (ethanol) treatment (without agonists), which were taken as 1. Data are the mean ± S.D. from three independent experiments with triplicates for each experiment.

struct 8) showed the same activity as *construct 6*, *i.e.*, presence of all three GRE/ARE without Sp1-binding sites. These results suggested again that the third GRE/ARE was involved in dexamethasone activation. This finding was further supported by the result shown with *construct 9*, in which both the third GRE/ARE and Sp1-binding sites were deleted and there was no effect of dexamethasone.

To ensure that the Sp1-binding sequence, but no other sequence in the MAO A core promoter, was involved in androgen activation, Sp1-binding sites were mutated (Fig. 3*A*, *construct 10*; CG was mutated to tt in four Sp1 sites, see "Materials and Methods") and co-transfected with GR and treated with dexamethasone. The result displayed a similar -fold increase to the finding of MAO A promoter without Sp1-binding sites (Fig. 3*A*, *construct 10 versus 6*, 2.8-fold *versus* 2.7-fold increase).

To study the effect of R1881 on MAO A promoter, AR was co-transfected with various mutants into SK-N-BE(2)-C and treated with R1881 or vehicle (control) for 24 h (Fig. 3*B*). Similar results were obtained as with GR agonist-treated samples, except that the -fold increase of the effect of dexamethasone was significantly higher in the constructs containing

dexamethasone compared with that of wild-type MAO A 2 kb (Fig. 3*A*, *constructs 2* and *3 versus 1*), suggesting that the first and second GRE/AREs were not involved in the dexamethasone activation of the MAO A promoter. Deleting the third GRE/ARE, the glucocorticoid effect was lower than that of wild type, however, it still showed the increase of promoter activity as compared with cells not treated with dexamethasone (Fig. 3*A*, *construct 4*, 3.6-fold *versus construct 1*, 5.7-fold). These results suggested that the third GRE/ARE element was important for the GR activation. We also examined the possibility of Sp1-binding sites regulating MAO A promoter activity induced by dexamethasone.

To test this latter possibility, Sp1-binding sites alone were fused with the luciferase reporter gene (Fig. 3*A*, *construct 5*). The result showed that dexamethasone indeed activated MAO A promoter activity through Sp1-binding sites.

In the absence of Sp1-binding sites, but in the presence of all three GRE/ARE (Fig. 3*A*, *construct 6*), the promoter activity was reduced slightly compared with the WT (Fig. 3*A*, *construct 6 versus 1*). However, deleting the first (Fig. 3*A*, *construct 7*) or both first and second GRE/ARE (Fig. 3*A*, *con-*

Sp1-binding sites than that of the effect of androgen R1881 (Fig. 3*A*, *constructs 1–5 versus* Fig. 3*B*, *constructs 1–5*). In the absence of Sp1-binding sites, effects of glucocorticoid/androgen were similar (Fig. 3, *A* and *B*, *constructs 6–10*).

The basal luciferase values of different deleted MAO A promoter-luciferase reporter genes co-transfected with either GR- or AR-expression vector, without dexamethasone or R1881, are shown in Fig. 3*C*. The results show that MAO A promoter containing Sp1-binding sites (*upper panel*, Fig. 3*C*) had much higher luciferase activity than that of promoter constructs without Sp1-binding sites, which is consistent with our previous finding (35).

Similar results were obtained in 1242-MG or COS-7 cells (data not shown). COS-7 cells were chosen because they lack endogenous GR (32) and AR (25) and, therefore, provide a null background for expression of transfected receptors.

We have also systematically mutated each GRE/ARE in the human MAO A 2-kb promoter (mutated sequences were shown under "Materials and Methods"), and the effects upon treatment with GR/AR agonist are shown in Fig. 4*A*. Results showed that the mutation of GRE/ARE3 reduced the
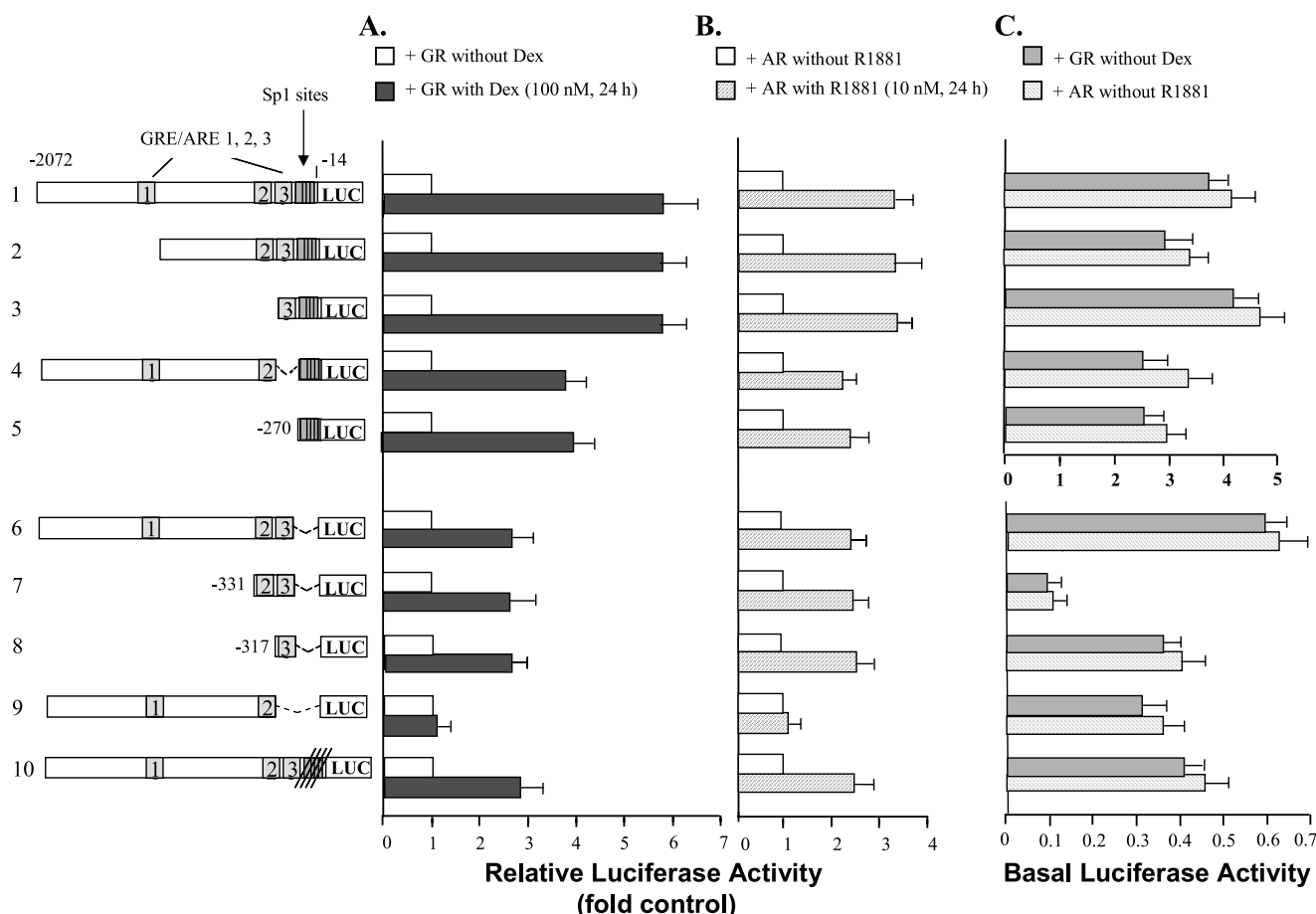
FIGURE 3. **Dexamethasone/R1881 activates MAO A promoter activity through the third GRE/ARE and Sp1-binding sites in the MAO A promoter.** The wild-type 2 kb or deleted or mutated luciferase constructs of MAO A promoter were co-transfected with GR (*A*) or AR (*B*) into human neuroblastoma SK-N-BE(2)-C for 16 h. The culture medium was then changed to charcoal-treated serum to remove endogenous hormones. 8 h later, the charcoal-treated serum containing dexamethasone (100 nM) (*A*) or R1881 (10 nM) (*B*) was added. After another 24 h, cells were harvested, and luciferase activity was determined. Controls were respective DNA constructs co-transfected with GR (*A*) or AR (*B*) and treated with vehicle (ethanol), which were taken as 1. *C*, the absolutely basal luciferase values of different deleted MAO A promoter-luciferase reporter gene, co-transfected with either GR or AR without dexamethasone or R1881 (with ethanol), are shown. Please note that MAO A promoter-luciferase constructs in the *upper panel* contains Sp1-binding sites. Data are the mean ± S.D. from three independent experiments with triplicates for each experiment.

activation of MAO A significantly upon treatment with dexamethasone and R1881, respectively (Fig. 4*A*, *panels a* and *b*, *construct 4 versus 1*) in SK-N-BE(2)-C and 1242-MG (data not shown). Furthermore, each GRE/ARE has been inserted into pGL3 promoter-luciferase reporter gene (driven by SV40 promoter), and effects upon treatment with GR/AR agonist are shown in Fig. 4*B*. The third GRE/ARE-pGL3 exhibited higher promoter activity than that of first and second GRE/ARE (Fig. 4*B*, *construct 4 versus 3* or *2*). In addition, the effect of each Sp1-binding site on the MAO A core promoter (−270/−14 bp) has been examined by mutation of each Sp1-binding site (Fig. 5). The mutated constructs were co-transfected with GR (Fig. 5*A*) or AR (Fig. 5*B*) and treated with agonists. Results showed that the third Sp1-binding site contributes the most to the activation of MAO A by GR/AR agonist (Fig. 5, *construct 4 versus 1*). The fourth Sp1-binding site is less important than that of third Sp1-binding site (Fig. 5, *construct 5 versus 4*), whereas the first and second Sp1-binding sites have little effect on activation (Fig. 5, *construct 2 and 3 versus 1*). Taken together, these results further suggest that the third GRE/ARE and Sp1-binding sites are important in the activation of MAO A by GR/AR agonist.

GR and AR Interact with the Third GRE/ARE, whereas R1 and Sp1 Interact with Sp1-binding Sites as Shown by Gel-Shift Assay—The third GRE/ARE (GRE/ARE3) was radiolabeled and incubated with nuclear proteins isolated from SK-N-BE(2)-C cells that were treated with either dexamethasone (Fig. 6*A*, *lanes 2–5*) or R1881 (Fig. 6*A*, *lanes 6–9*). Two DNA-protein complexes were observed (Fig. 6*A*, *lanes 2* and *6*). A 500-fold excess of cold wild-type oligonucleotides reduced all binding (Fig. 6*A*, *lanes 3* and *7*), suggesting that these DNA-protein complexes were specific. However, a 500-fold excess of cold mutated probe (Fig. 6*A*, *lanes 4* and *8*) did not affect the DNA-protein binding. The upper complex was supershifted by incubation with anti-GR and anti-AR antibody (Fig. 6*A*, *lanes 5* and *9*), respectively, suggesting that the GR homodimer or AR homodimer has occurred in the upper DNA-protein complex in dexamethasone (*lane 5*)- or R1881 (*lane 9*)-treated samples. Based on our data, it does not suggest the formation of GR/AR heterodimer. Similar results were observed in 1242-MG cells (data not shown).

The first and second GRE/ARE (GRE/ARE1 or GRE/ARE2) were also used as probes to perform gel-shift assay. But the anti-GR or anti-AR antibody did not supershift or block the
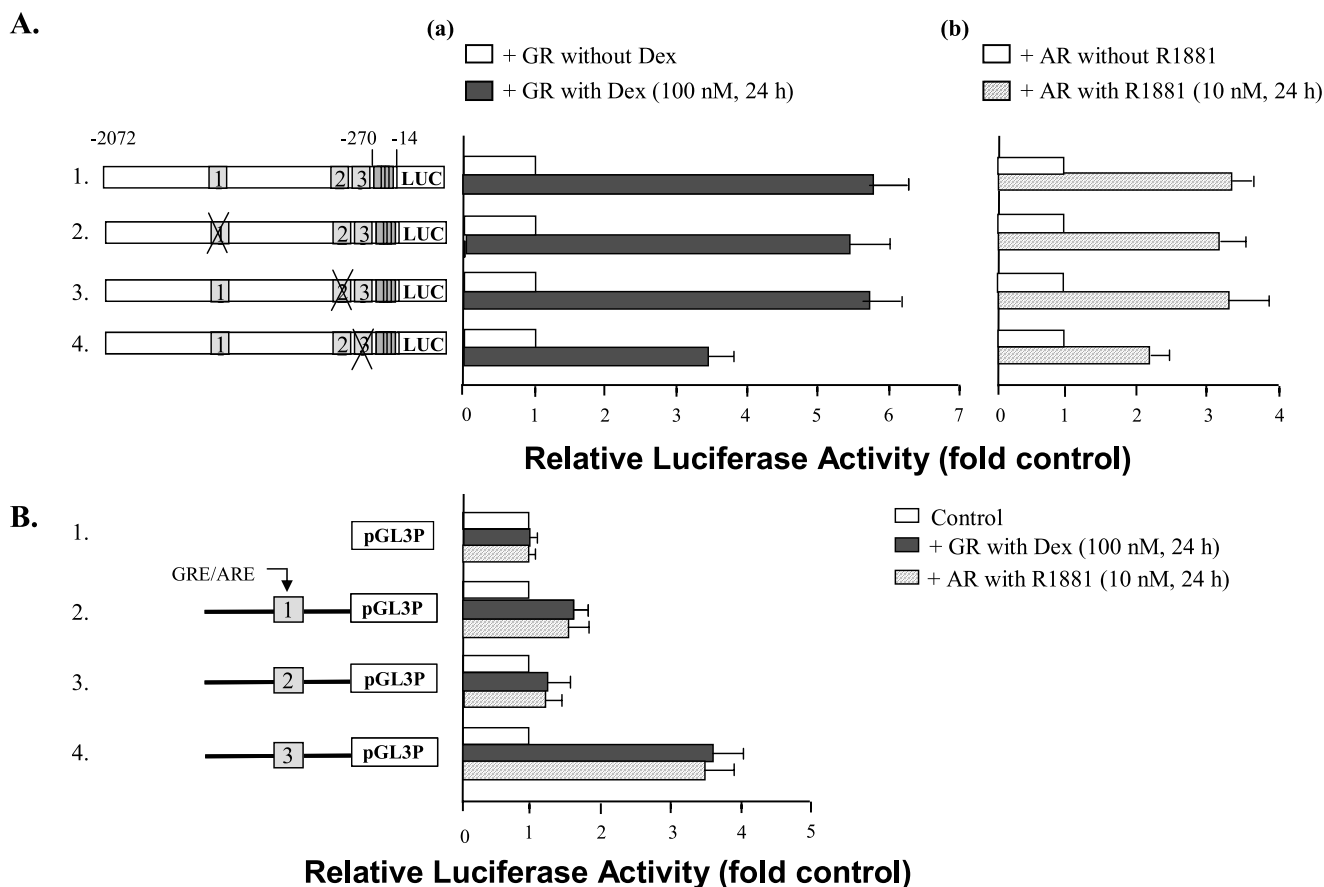
FIGURE 4. **Effects of each GRE/ARE in GR and AR activation.** *A*, effects of each GRE/ARE in MAO A 2-kb promoter. Each GRE/ARE in the human MAO A 2-kb promoter was mutated separately. Mutated sequences are shown under "Materials and Methods." The wild-type MAO A 2-kb promoter or mutated luciferase construct was co-transfected with GR (*a*) or AR (*b*) into SK-N-BE(2)-C for 16 h. The culture medium was then changed to charcoal-treated serum. 8 h later, the charcoal-treated serum containing dexamethasone (100 nM) (*a*) or R1881 (10 nM) (*b*) was added. After another 24 h, cells were harvested and luciferase activity was determined. Controls were respective DNA constructs co-transfected with GR (*a*) or AR (*b*) and treated with vehicle (ethanol), which were taken as 1. *B*, effects of each GRE/ARE in pGL3 promoter-luciferase reporter gene. Each GRE/ARE has been inserted into pGL3 promoter-luciferase reporter gene (driven by SV40 promoter) and co-transfected with GR or AR into SK-N-BE(2)-C as indicated. After treatment with dexamethasone or R1881, cells were harvested and luciferase activity was determined. Controls were respective pGL3 promoter-luciferase reporter constructs, which were taken as 1. Data are the mean ± S.D. from four independent experiments with triplicates for each experiment.

DNA-protein complex (data not shown) suggesting that GR/AR did not bind to the first or second GRE/ARE. There are four nucleotides that differ between the third GRE/ARE sequence and the first or second GRE/ARE sequence (Fig. 1*B*), which may be the reason why GR/AR could not bind to the first or second GRE/ARE of the MAO A promoter.

It was interesting that dexamethasone/androgen agonists activated the luciferase activity when there were only Sp1-binding sites present on MAO A promoter (Fig. 3, *construct 5*). Gel-shift assay was performed to determine whether GR/AR interacted with Sp1-binding sites. The Sp1-binding sites were radiolabeled and incubated with nuclear proteins (Fig. 6*B*). One DNA-protein complex was observed in SK-N-BE(2)-C (Fig. 6*B*, *lane 2*) and 1242-MG cells (data not shown). A 100-fold excess of cold identical oligonucleotide reduced this binding significantly (Fig. 6*B*, *lane 3*), suggesting that this DNA-protein complex was specific. Further, this DNA-protein complex could be supershifted by incubation with anti-Sp1 or anti-R1 antibodies (Fig. 6*B*, *lanes 6* and *7*) but not by anti-AR or anti-GR antibody (Fig. 6*B*, *lanes 4* and *5*), which suggested that R1 or Sp1 but not GR or AR, bind to Sp1-binding sites. Further, an irrelevant antibody (anti-β-actin antibody) was used as a negative control and

did not affect the protein-DNA complex (Fig. 6*B*, *lane 8*), similar to anti-GR and anti-AR antibodies.

R1 Interacts with GR, Not AR, and Represses the Dexamethasone Activation of MAO A, Whereas Sp1 Interacts with Both GR and AR and Enhances the Dexamethasone Activation of MAO A—Next, we studied whether GR interacted with R1 and Sp1 transcription factors, respectively, and regulated the MAO A gene expression (Fig. 7*A*). Nuclear proteins were extracted from SK-N-BE(2)-C treated with or without dexamethasone for 24 h and immunoprecipitated by incubating with anti-GR antibody (Fig. 7*A*, *panel a*, *lanes 1–4*). The immunoprecipitated proteins were analyzed by Western blot using anti-R1 or anti-Sp1 antibodies. The results showed that R1 interacted with GR (Fig. 7*A*, *panel a*, *lanes 1* and *2*), and the intensity of R1 protein band was decreased slightly in cells treated with dexamethasone for 24 h compared with the untreated cells (Fig. 7*A*, *panel a*, *lanes 2 versus 1*). The intensity of Sp1 protein band increased significantly after 24 h of dexamethasone treatment as compared with the untreated cells (Fig. 7*A*, *panel a*, *lanes 4 versus 3*).

The same approach was used to study the interaction of AR with R1 and Sp1. SK-N-BE(2)-C cells were treated with or with-

out R1881 for 24 h. Nuclear proteins were then extracted and immunoprecipitated by incubating with anti-AR antibodies (Fig. 7*B*, *panel a*, *lanes 1–4*). The result showed that R1 protein is not present in the AR immunoprecipitated protein (Fig. 7*B*,

panel a, *lanes 1* and *2*), but Sp1 protein is found in AR immunoprecipitated protein (Fig. 7*B*, *panel a*, *lane 3*). We have exposed the filter long enough to produce equivalent Sp1 signal with R1 in the AR immunoprecipitation but did not observe any R1 signal in the AR immunoprecipitation. The intensity of the Sp1 protein band was increased in R1881-treated cells compared with untreated cells (Fig. 7*B*, *panel a*, compare *lanes 4* to *3*). These data provided evidence that AR interacted with Sp1 but not R1.

Input samples treated with or without agonist were directly blotted by anti-R1 or anti-Sp1 antibody as positive controls (Fig. 7, *A* (*panel b*) and *B* (*panel b*)). Nuclear extracts alone without anti-GR or anti-AR antibody during immunoprecipitation, and nuclear extracts with an irrelevant anti-β-actin antibody in immunoprecipitation assay, did not show any band, which were used as negative controls (Fig. 7, *A* (*panel c*) and *B* (*panel c*)).

The relative intensity of each GR-bound R1 or Sp1 was quantified by a PhosphorImager system (Fig. 7*A* (*panel d*)) and expressed as -fold
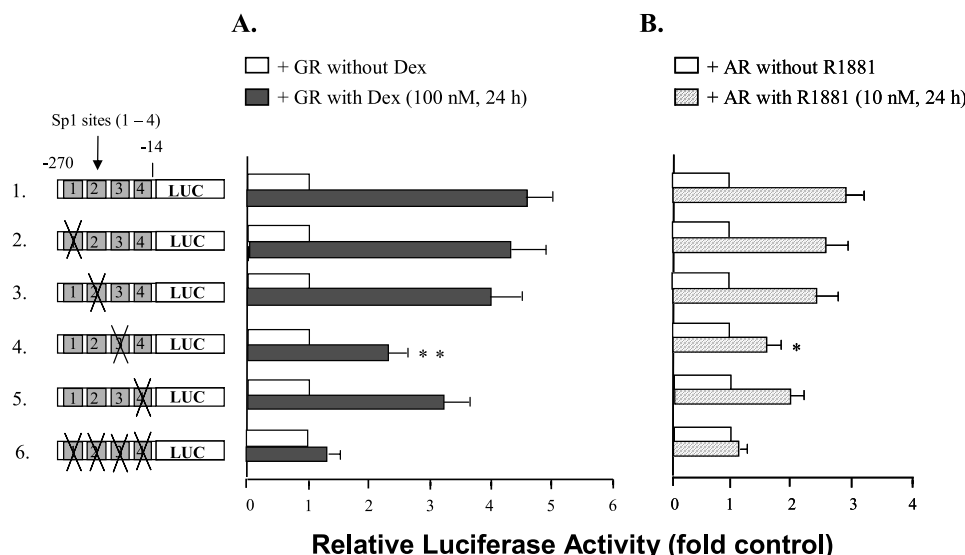

FIGURE 5. Effects of each Sp1-binding site in the GR and AR activation. The wild-type or Sp1-binding site mutated luciferase constructs of MAO A core promoter (−270/−14) was co-transfected with GR (*A*) or AR (*B*) into SK-N-BE(2)-C for 16 h. The culture medium was then changed to charcoal-treated serum. 8 h later, the charcoal-treated serum containing dexamethasone (100 nM) (*A*) or R1881 (10 nM) (*B*) was added. After another 24 h, cells were harvested and luciferase activity was determined. Controls were respective DNA constructs co-transfected with GR or AR and treated with vehicle (ethanol), which were taken as 1. Data are the mean ± S.D. from three independent experiments with triplicates for each experiment. *, $p < 0.05$ and **, $p < 0.02$ compared with their respective wild-type control (*construct 1*).
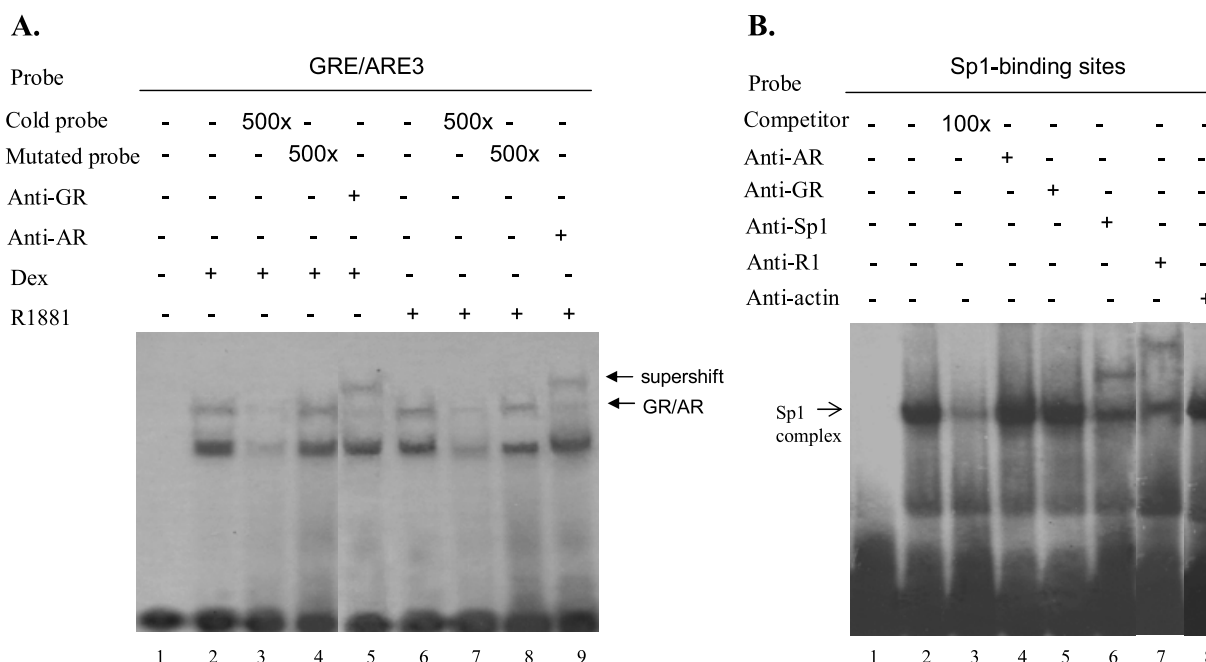

FIGURE 6. Glucocorticoid/androgen receptors interact with the third GRE/ARE (*A*) but not with Sp1-binding sites (*B*). *A*, nuclear proteins from SK-N-BE(2)-C cells treated with either dexamethasone (*lanes 2–5*) or R1881 (*lanes 6–9*) for 24 h were incubated with radiolabeled GRE/ARE3 (sense DNA sequence: 5′-cgacaca**aggaca**ttc**taaacc**tagg-3′, GRE/ARE is in *boldface*) was incubated with (*lanes 2–9*). Unlabeled GRE/ARE3 probe (*lanes 3* and *7*) or mutated GRE/ARE3 (5′-cgacaca***tttcca***ttc***ttgacc***tagg-3′, mutated nucleotides are in *italic* and *underlined*; *lanes 4* and *8*) or anti-GR antibody (*lane 5*) or anti-AR antibody (*lane 9*) were added as indicated. *Arrows* show the GR- or AR-DNA complex and the supershifted GR- or AR-DNA complexes. *B*, nuclear proteins isolated from SK-N-BE(2)-C cells (*lanes 2–6*) were incubated with Sp1-binding sites (5′-cg **ccccgctc**tc agtgcccagc tcccccgggg tatcagctga aacatcag**t ccgccc**ctgg gcgctcccgg agtatcagca aaagggttcg **cccgccc**ac agtgcccggc tcccccgggg tatcaaaaga aggatcggc**t ccgccc**ccgg gctccccggg gg-3′; four Sp1-binding sites are in *boldface*), which was end-labeled. In addition, excess cold Sp1-binding sites probe (*lane 3*), anti-AR (*lane 4*), anti-GR (*lane 5*), anti-Sp1 (*lane 6*), anti-R1 (*lane 7*), or anti-β-actin antibodies (*lane 8*) were added. *Arrows* indicate Sp1- or R1-DNA complexes or supershifted protein-DNA complexes.

A.

(a)

Co-IP anti-GR antibody: +   +   +   +
Dexamethasone 24 h: –   +   –   +

R1–   Sp1–

(b) Input (before co-IP):   +   +   +   +
Dexamethasone 24 h: –   +   –   +

R1–   Sp1–

Co-IP anti-Actin antibody: +   +   +   +
(c) Dexamethasone 24 h: –   +   –   +

R1–   Sp1–
   1   2   3   4

(d)

B.

(a)

Co-IP anti-AR antibody: +   +   +   +
R1881 24 h :   –   +   –   +

R1–   Sp1–

(b) Input (before co-IP)):   +   +   +   +
R1881 24 h :   –   +   –   +

R1–   Sp1–

Co-IP anti-Actin antibody: +   +   +   +
(c) R1881 24 h :   –   +   –   +

R1–   Sp1–
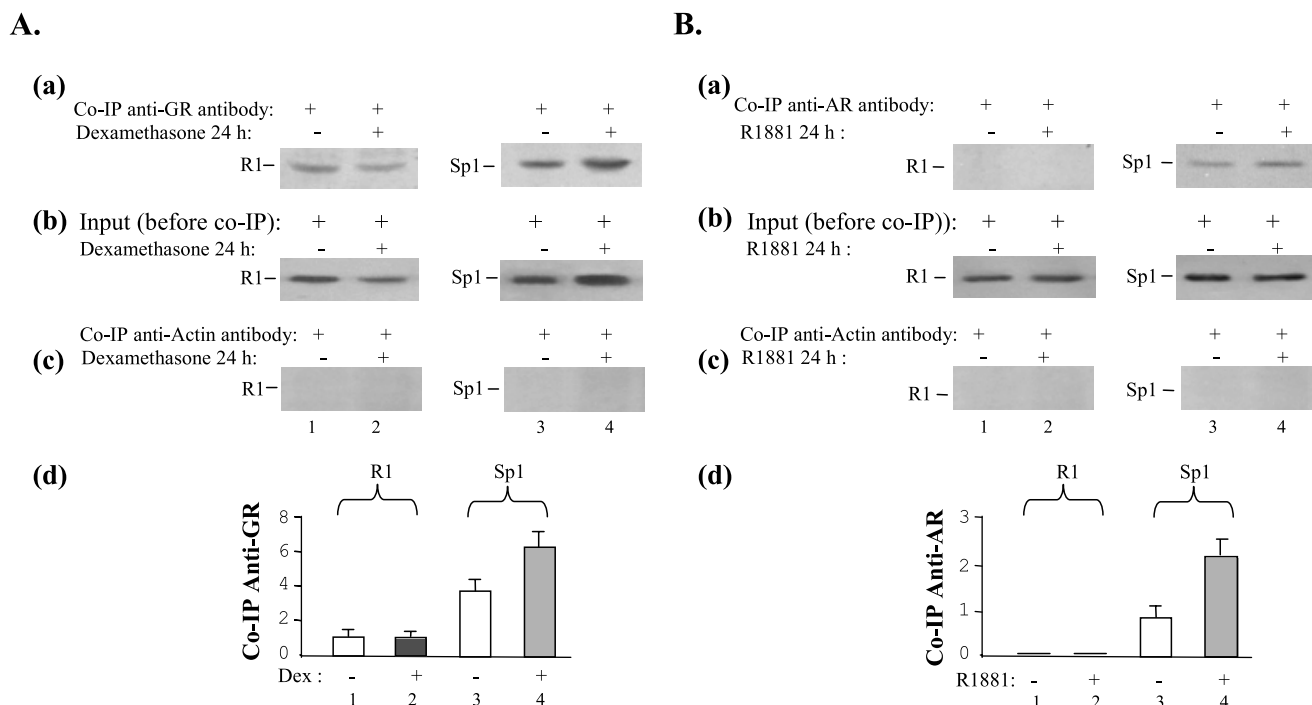   1   2   3   4

(d)

FIGURE 7. **The interaction between glucocorticoid/androgen receptors and R1 or Sp1.** *A*, GR co-immunoprecipitation with R1 and Sp1, respectively. *a*, the interaction of GR with R1 (*lanes 1* and *2*) or Sp1 (*lanes 3* and *4*). Nuclear proteins isolated from SK-N-BE(2)-C cells, which were treated with dexamethasone (*lanes 2* and *4*) or vehicle (*lanes 1* and *3*), were immunoprecipitated by incubating with anti-GR antibody. Protein co-immunoprecipitation with GR was analyzed by Western blot with anti-R1 (*lanes 1* and *2*) or anti-Sp1 (*lanes 3* and *4*) antibodies. *b*, ~0.5% of nuclear protein of each sample before co-immunoprecipitation experiments was included on gel and blotted by anti-R1 (*lanes 1* and *2*) or -Sp1 (*lanes 3* and *4*) antibody as positive controls. *c*, nuclear extracts, which were immunoprecipitated by incubating with an irrelevant anti-β-actin antibody were used as negative controls. *d*, quantitative analysis. The relative intensity of each GR-bound R1 or Sp1 band was quantified by PhosphorImager. Values are expressed as -fold of control, in which the basal GR-bound R1 (*column 1*) is taken as 1. *B*, AR co-immunoprecipitation with R1 and Sp1, respectively. *a*, the interaction of AR with Sp1 (*lanes 3* and *4*) but not with R1 (*lanes 1* and *2*). A similar approach was performed using SK-N-BE(2)-C cells treated with R1881 (*lanes 2* and *4*) or vehicle (*lanes 1* and *3*). Nuclear proteins were immunoprecipitated by incubating with anti-AR antibody, and then analyzed by Western blot with anti-R1 (*lanes 1* and *2*) or anti-Sp1 (*lanes 3* and *4*) antibodies. *b*, ~0.5% of input of each sample before co-immunoprecipitation experiments was included on gel and blotted by anti-R1 (*lanes 1* and *2*) or -Sp1 (*lanes 3* and *4*) antibody. *c*, nuclear extracts, which were immunoprecipitated by incubating with an irrelevant anti-β-actin antibody, were used as negative controls. *d*, quantitative analysis. The relative intensity of each AR-bound R1 or Sp1 band was quantified by PhosphorImager. Values were expressed as -fold of control, in which the basal GR-bound R1 (*A* (*panel d*), *column 1*) was taken as 1. Data represent the mean ± S.D. of three independent experiments. Note that the amount of GR-bound Sp1 is more than AR-bound Sp1.

control, in which the GR-bound R1 (Fig. 7*A* (*panel d*), *lane 1*) was taken as 1. These quantitative data showed that GR interacts with both R1 and Sp1. The 24-h treatment of dexamethasone did not increase the interaction of R1 with GR (0.8-fold *versus* 1.0-fold; Fig. 7*A* (*panel d*), compare *column 2 versus 1*). But the GR-bound Sp1 was further increased (~6-fold) in cells treated with dexamethasone than Sp1 in untreated cells (~4-fold, Fig. 7*A* (*panel d*), compare *column 4 versus 3*).

The AR-bound Sp1 was also quantified (Fig. 7*B*, *panel d*) and expressed as -fold control, in which the GR-bound R1 (Fig. 7*A*, *panel d*, *lane 1*) was taken as 1. The AR-bound Sp1 was further increased (~2-fold) in cells treated with R1881 than AR-bound Sp1 in untreated cells (~1-fold, Fig. 7*B*, *panels d* and *c*), compare *column 4 versus 3*).

The result also showed that more Sp1 protein interacted with GR (~4- to 6-fold) than AR (~1- to 2-fold; Fig. 7, compare *A* (*panel d*), *columns 3* and *4*, to *B* (*panel d*), *columns 3* and *4*). Similar results were obtained when using nuclear proteins from 1242-MG cells (data not shown). These results suggested that GR/AR regulate MAO A gene expression also by interacting with R1 and Sp1.

To determine whether the novel transcriptional repressor R1 has an effect on steroid hormone involved regulation of MAO

A, R1 was co-transfected with MAO A 2-kb promoter and GR- or AR-expression vector and then treated with dexamethasone or R1881 for 24 h (Fig. 8). The transcriptional activator Sp1 has previously been shown to be involved in dexamethasone/androgen activation but has not yet shown involvement in MAO A gene regulation. Therefore, the effect of Sp1 was also studied together with R1 by transient transfection and luciferase assay (Fig. 8).

As shown in Fig. 8*A*, R1 inhibited the activation induced by dexamethasone on MAO A 2-kb promoter (Fig. 8*A*, *lanes 4 versus 2*) but did not inhibit the activation induced by R1881 (Fig. 8*B*, *lanes 4 versus 2*), whereas Sp1 enhanced the activation of MAO A 2-kb promoter by either dexamethasone (Fig. 8*A*, *lanes 6 versus 2*) or R1881 (Fig. 8*B*, *lanes 6 versus 2*).

The absolute basal luciferase values, of MAO A 2-kb promoter-luciferase reporter gene (together with GR-expression vector (Fig. 8*C*), or with AR-expression vector (Fig. 8*D*)) co-transfected with either R1 or Sp1, without agonist treatment, are shown in Fig. 8*C*. Results show that R1 inhibits, but Sp1 increases, MAO A promoter activity. These results are consistent with our previous publications for R1 (8) and Sp1 (35) in the absence of hormone.
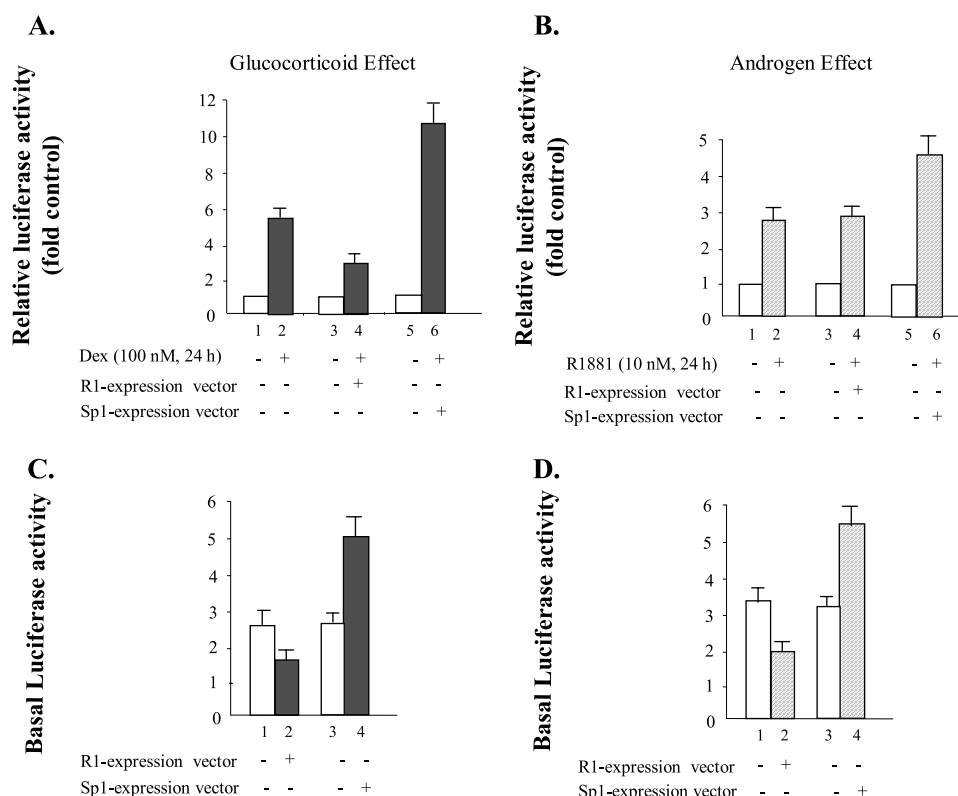
A.

Glucocorticoid Effect

Relative luciferase activity (fold control)

1	2	3	4	5	6

	1	2	3	4	5	6
Dex (100 nM, 24 h)	-	+	-	+	-	+
R1-expression vector	-	-	-	+	-	-
Sp1-expression vector	-	-	-	-	-	+

B.

Androgen Effect

Relative luciferase activity (fold control)

	1	2	3	4	5	6
R1881 (10 nM, 24 h)	-	+	-	+	-	+
R1-expression vector	-	-	-	+	-	-
Sp1-expression vector	-	-	-	-	-	+

C.

Basal Luciferase activity

	1	2	3	4
R1-expression vector	-	+	-	-
Sp1-expression vector	-	-	-	+

D.

Basal Luciferase activity

	1	2	3	4
R1-expression vector	-	+	-	-
Sp1-expression vector	-	-	-	+

FIGURE 8. **Effects of R1 and Sp1 on dexamethasone/R1881 activation of MAO A promoter activity.** The MAO A 2-kb promoter was co-transfected with GR (*A*) or AR (*B*) and together with R1-expression vector (*lane 4*) or Sp1-expression vector (*lane 6*) as indicated into human neuroblastoma SK-N-BE(2)-C. Then the culture medium was changed to charcoal-treated serum containing 100 nM dexamethasone (*A*) or 10 nM R1881 (*B*) and incubated for another 24 h. The luciferase activity was determined. Controls were MAO A 2-kb promoter co-transfected with GR (*A*) or AR (*B*) or pcDNA3.1 (instead of R1- or Sp1-expression vector, *lanes 1*, *3*, and *5*) and treated with vehicle (ethanol), which are taken as 1. *C*, the absolutely basal values of MAO A 2-kb promoter-luciferase reporter gene (together with GR) co-transfected with either R1 or Sp1 without dexamethasone or R1881 are shown. *D*, the absolutely basal values of MAO A 2-kb promoter-luciferase reporter gene (together with AR) co-transfected with either R1 or Sp1 without dexamethasone or R1881 are shown. Controls for *C* and *D* were MAO A 2-kb promoter co-transfected with GR (*C*) or AR (*D*) or pcDNA3.1 (instead of R1- or Sp1-expression vector, *lanes 1* and *3*). Data are the mean ± S.D. from three independent experiments with triplicates for each experiment.

out dexamethasone treatment), R1 staining was found in both of nucleus and cytosol at a ratio of nucleus/cytosol 1/2.74 (Fig. 10, *A* and *B* (*panel a*)). However, nuclear R1 signal was increased upon treatment with dexamethasone for 12 h (nucleus/cytosol: 1/1.64; Fig. 10, *A* and *B* (*panel b*)), then decreased at 24 h of treatment (nucleus/cytosol: 1/3.73; Fig. 10, *A* and *B* (*panel c*)), and relocated into cytosol at 48 h of treatment (nucleus/cytosol: 1/10.4; Fig. 10, *A* and *B* (*panel d*)). When cells were incubated with either first antibody or secondary antibody alone, it displayed only background immunoreactivity (data not shown). In addition, the nucleus was stained by DAPI (*blue color*), and a merge of nucleus with R1 staining is also shown in Fig. 10. Similar results were found for 1242-MG cells (data not shown).

Activation of MAO A Promoter Activity by 12 h of Dexamethasone/ R1881 Treatment Was Increased after 24 h of Dexamethasone Treatment, Which Is Similar to MAO A Catalytic Activity, but Was Not Increased by R1881 Treatment—To test whether the movement of endogenous R1 induced by dexamethasone influences MAO A gene expression, the MAO A promoter (Fig. 9, *B* and *C*) and catalytic (Fig. 9,

Endogenous R1 Is Translocated into the Nucleus upon 12 h of Dexamethasone Treatment and Relocated into Cytosol after 24 or 48 h of Treatment—Because R1 was found to inhibit the dexamethasone activation of MAO A, we speculated that R1 might have different expression levels in the nucleus upon dexamethasone treatment. To test this possibility, SK-N-BE(2)-C (Fig. 9A) or 1242-MG (data not shown) cells were treated with or without dexamethasone for 12, 24, and 48 h. Then nuclear proteins were extracted and subjected to Western blot analysis. The result showed that nuclear levels of R1 were increased at 12 h and then decreased at 24 h and further decreased at 48 h of dexamethasone treatment (Fig. 9A). In contrast, nuclear levels of Sp1 were increased at 12 h, and then did not change significantly at 24 or 48 h of dexamethasone treatment (Fig. 9A).

To provide further evidence, immunocytochemistry was performed to examine cellular localization of R1 protein upon treatment with dexamethasone for 12, 24, and 48 h. The immunofluorescent staining of SK-N-BE(2)-C cells in culture was done (Fig. 10) using rabbit anti-R1 antibody (*green color*). R1 expression in both nucleus and cytosol was quantified in ~110 individual cells in each group. In the control group (with-

D and *E*) activities were determined upon treatment with dexamethasone or R1881 for 12 and 24 h (for promoter activity assay, in Fig. 9, *B* and *C*) and 24 and 48 h (for catalytic activity assay, in Fig. 9, *D* and *E*), respectively.

For promoter activity, upon 12 h of treatment with dexamethasone or R1881, the MAO A 2-kb promoter activity was increased by ~3-fold compared with their respective controls (Fig. 9B, *lanes 2 versus 1* and *lanes 4 versus 3*). In contrast, upon 24 h of treatment, dexamethasone further increased the MAO A promoter activity from ~3-fold to ~6-fold (Fig. 9B, *lanes 4 versus 2*) compared with vehicle (ethanol)-treated groups, but R1881 did not (Fig. 9C, *lanes 4 versus 2*).

In addition, the nuclear expression level of GR and AR was examined in cells treated with dexamethasone (Fig. 9B) or R1881 (Fig. 9C) for 12 and 24 h, respectively, by Western blot, similar protein levels between GR and AR in both 12 and 24 h of treatments were found (Fig. 9, *B* and *C*).

Results for MAO A catalytic activities in SK-N-BE(2)-C (Fig. 9, *D* and *E*) or 1242-MG (data not shown) showed that dexamethasone or R1881 increased MAO A catalytic activity by ~2-fold upon 24 h of treatment (Fig. 9, *D* and *E*, compare *lanes 4 to 3*). In contrast, upon 48 h of treatment, dexametha-
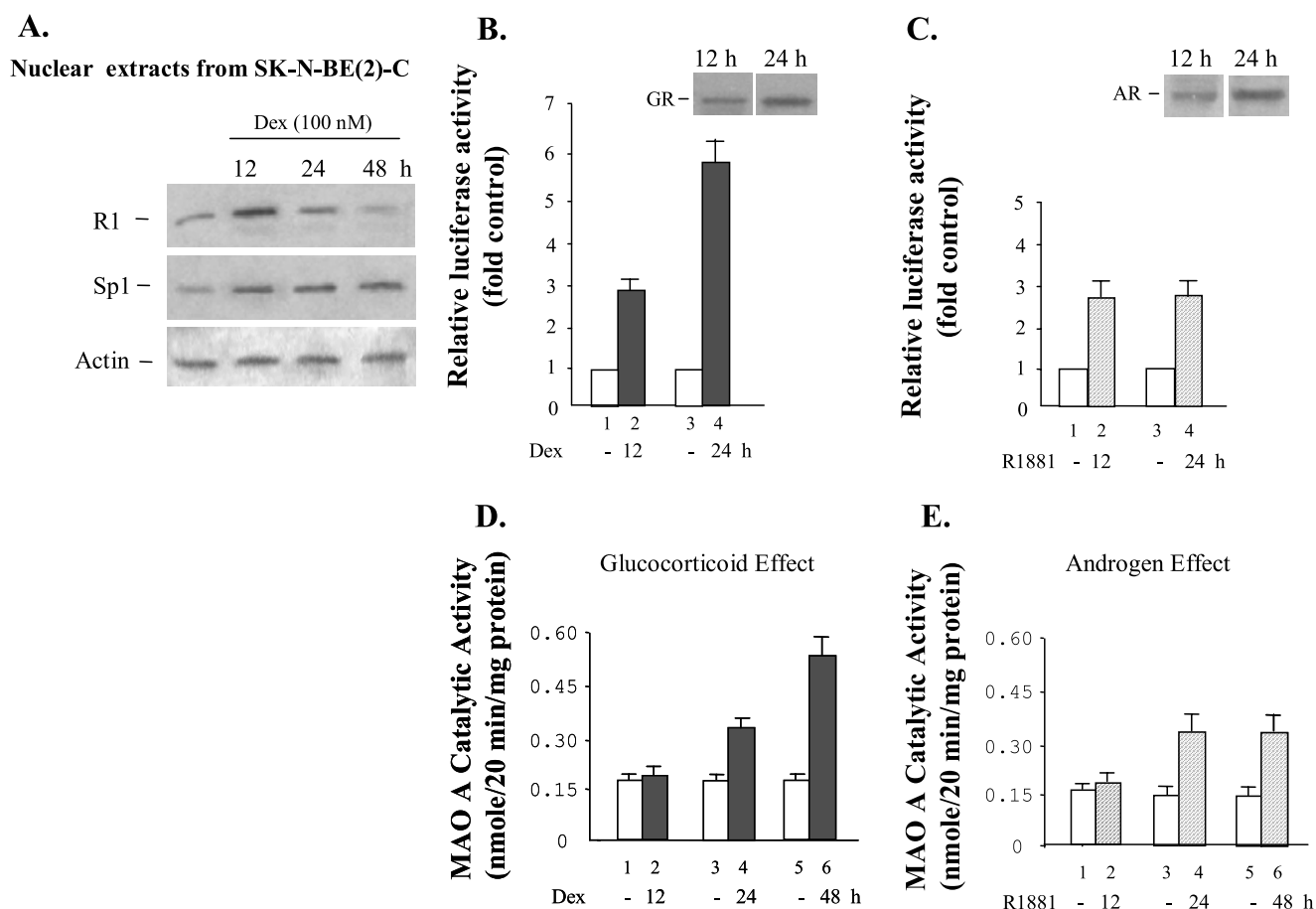
FIGURE 9. **Effects of dexamethasone on nuclear R1/Sp1 expression and MAO A promoter/catalytic activities.** *A*, Western blot analysis showing that endogenous levels of R1 in nucleus are increased after dexamethasone treatment for 12 h but decreased afterward. SK-N-BE(2)-C cells were treated with or without dexamethasone for 12, 24, and 48 h. Then nuclear proteins were extracted and subjected to Western blot analysis using anti-R1 or anti-Sp1 antibody. Note that nuclear levels of Sp1 were increased at 12 h and then not changed at 24 or 48 h of dexamethasone treatment. *B* and *C*, transient transfection and luciferase assays showing that the activation of MAO A promoter activity is increased upon 24 h of dexamethasone treatment compared with those of 12 h of treatment but is not increased by 24 h of R1881 treatment. The MAO A 2-kb promoter was co-transfected with GR (*B*) or AR (*C*) into SK-N-BE(2)-C cells. Then dexamethasone (*lane 2*) (*B*) or R1881 (*lane 4*) (*C*) was added for another 12 and 24 h, respectively, as indicated. The luciferase activity was then determined. Controls were MAO A 2-kb promoter co-transfected with GR and AR and treated with vehicle (ethanol; *lanes 1* and *3*), which are taken as 1. The expression of GR and AR protein levels in cells treated with dexamethasone and R1881, respectively, in each group were also determined by Western blot and shown in *B* and *C*. *D* and *E*, effects of dexamethasone/R1881 on MAO A catalytic activity. SK-N-BE(2)-C cells were plated at a density of 10^6 cells in a 10-cm dish and treated with 100 nM dexamethasone (*D*) or 10 nM R1881 (*E*) for another 12, 24, and 48 h, respectively. Then cells were harvested, and MAO A catalytic activity was determined. Cells treated with vehicle (ethanol; *lanes 1*, *3*, and *5*) were used as controls. Data are the mean ± S.D. from three independent experiments.

sone further increased the MAO A catalytic activity from ~2-fold to ~3-fold (Fig. 9D, *lanes 6 versus 4*), but R1881 did not (Fig. 9E, *lanes 6 versus 4*), compared with vehicle (ethanol)-treated cells.

Thus, these results suggested that the translocation of R1 induced by dexamethasone influences the MAO A gene expression at both transcriptional and enzymatic levels. It also suggests that the stronger effect of dexamethasone than androgen is mainly regulated by R1.

*The Increase in Glucocorticoid Activation on MAO A Was Not Observed, the Same as Androgen, in R1-knockdown Cells—*An siRNA-mediated R1 knockdown cell line, the human medulloblastoma UW228 (a gift from Dr. Annie Huang, University of Toronto, Toronto, Canada), was used to determine whether differences between GR and AR are eliminated. First, immunofluorescence results showed that the expression of R1 was found in both nucleus and cytosol in wild-type UW228 cells (Fig. 11A, *panel b*), which was similar to SK-N-

BE(2)-C cells but much less in R1-knockdown UW228 cells (Fig. 11A, *panel a*) and nothing in cells without anti-R1 antibody staining (data not shown). This provides direct evidence for the specificity of our anti-R1 antibody.

In addition, whether dexamethasone can induce R1 translocation in wild-type UW228 cells was determined in the same way. In the control group (without dexamethasone treatment), R1 staining was found in both the nucleus and cytosol at a ratio of nucleus/cytosol of 1/3.24 (Fig. 11, *A* and *B* (*panel b*)). However, nuclear R1 signal is increased upon treatment with dexamethasone for 12 h (nucleus/cytosol: 1/1.98, Fig. 11, *A* and *B* (*panel c*)), then decreased at 24 h of treatment (nucleus/cytosol: 1/4.37, Fig. 11, *A* and *B* (*panel d*)), and relocated into cytosol at 48 h of treatment (nucleus/cytosol: 1/12.2, Fig. 11, *A* and *B* (*panel e*)). These results indicate that wild-type UW228 cells have a similar property to that of SK-N-BE(2)-C cells. R1-knockdown UW228 cells, which were treated with dexamethasone, did not display this
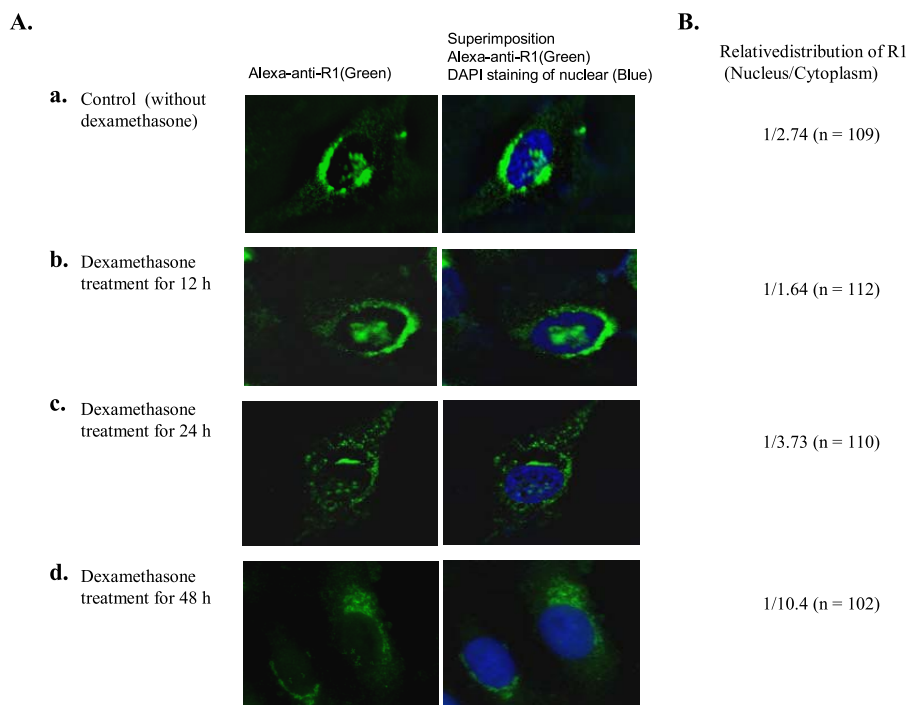
A.

Alexa-anti-R1(Green)

Superimposition
Alexa-anti-R1(Green)
DAPI staining of nuclear (Blue)

a. Control (without dexamethasone)

b. Dexamethasone treatment for 12 h

c. Dexamethasone treatment for 24 h

d. Dexamethasone treatment for 48 h

B.

Relativedistribution of R1
(Nucleus/Cytoplasm)

1/2.74 (n = 109)

1/1.64 (n = 112)

1/3.73 (n = 110)

1/10.4 (n = 102)

FIGURE 10. **The translocation of R1 in SK-N-BE(2)-C cells upon dexamethasone treatment.** Immunofluorescence microscopy was performed with anti-R1 antibody. SK-N-BE(2)-C cells were plated on coverslip the day before experiment. Then, 100 nM dexamethasone (*A, panels b–d*) or vehicle (*A, panel a*) was added into the medium for another (*b*) 12 h, (*c*), 24 h or (*d*) 48 h. Cells were fixed and incubated with rabbit anti-R1-antibody, followed with fluorescein-conjugated anti-rabbit secondary antibody (*green*). Stained slides were mounted in the presence of DAPI for nuclear staining (*blue*). The Alexa-anti-R1 staining and the merge of R1 (*green*) and nucleus (*blue*) are indicated at the *top*. One (*a–c*) or two (*d*) single cells are represented for each group. *B*, the relative distribution of R1 in nucleus and cytosol was quantified by image analysis software (ComPix) and expressed as the ratio of nucleus/cytosol as indicated with each group on the *right*. The cell number (*n*) is shown in each group.

genes at other DNA sites (such as Sp1-binding sites) (39–43), which is a DNA-independent mechanism (38).

Our results indicate a positive interaction between GR/AR and transcription factor Sp1, whereas, a negative regulation between a novel repressor R1 and GR is observed for the first time in this study. Crosstalk between transcription factors and nuclear hormone receptors have previously been found to produce either positive (39, 40, 44) or negative (41, 45) regulation.

Our findings also demonstrate that dexamethasone was more effective in activating MAO A gene expression than R1881 upon 24 h or longer time treatment (Figs. 2–5, 8, 9, and 11). This observation may be in part explained by the stronger interaction between Sp1 transcription factor with GR than with AR as demonstrated by co-immunoprecipitation experiments (Fig. 7). But in the beginning (12 h) of the treatment, both glucocorticoid and androgen exhibit the same -fold activation in MAO A gene expression. This could be explained by a specific interaction of R1 with GR, but not with AR (Fig. 7*B*). Because R1 interacts with GR and inhibits glucocorticoid activation, but does not inhibit androgen activation, in the beginning of the treatment, R1 is translocated into nucleus and competes with Sp1 to interact with GR so that both dexamethasone and R1881 exhibited the same -fold activation. However, with the longer dexamethasone treatment (24 h), nuclear R1 is reduced due to R1 returning to cytosol and Sp1 remains at the same level. Thus MAO A gene expression is further increased. This explanation can be supported by the use of R1-knockdown cells. In this cell line, dexamethasone was more effective in activating MAO A gene expression than R1881 upon 12- and 24-h treatment, respectively (Fig. 11*D*).

Previously we found that R1 repressed MAO A promoter activity in the human 1242-MG, SH-SY5Y, HepG2, and LNCaP cell lines. Our co-immunoprecipitation experiments showed interactions between GR and R1 in the absence of GR stimulation (Fig. 7*A, panel a*). It raised a question whether this GR-R1 interaction is required for the function of R1 on transcription. Therefore, we have examined the R1/GR effect by comparing the inhibitory effect of R1 on MAO A promoter in the presence or absence of GR-expression vector in SK-N-BE(2)-C cells by transient transfection and luciferase assay. Results showed that there is no difference between the presence and absence of GR-expression vector (data not shown), which suggested that the
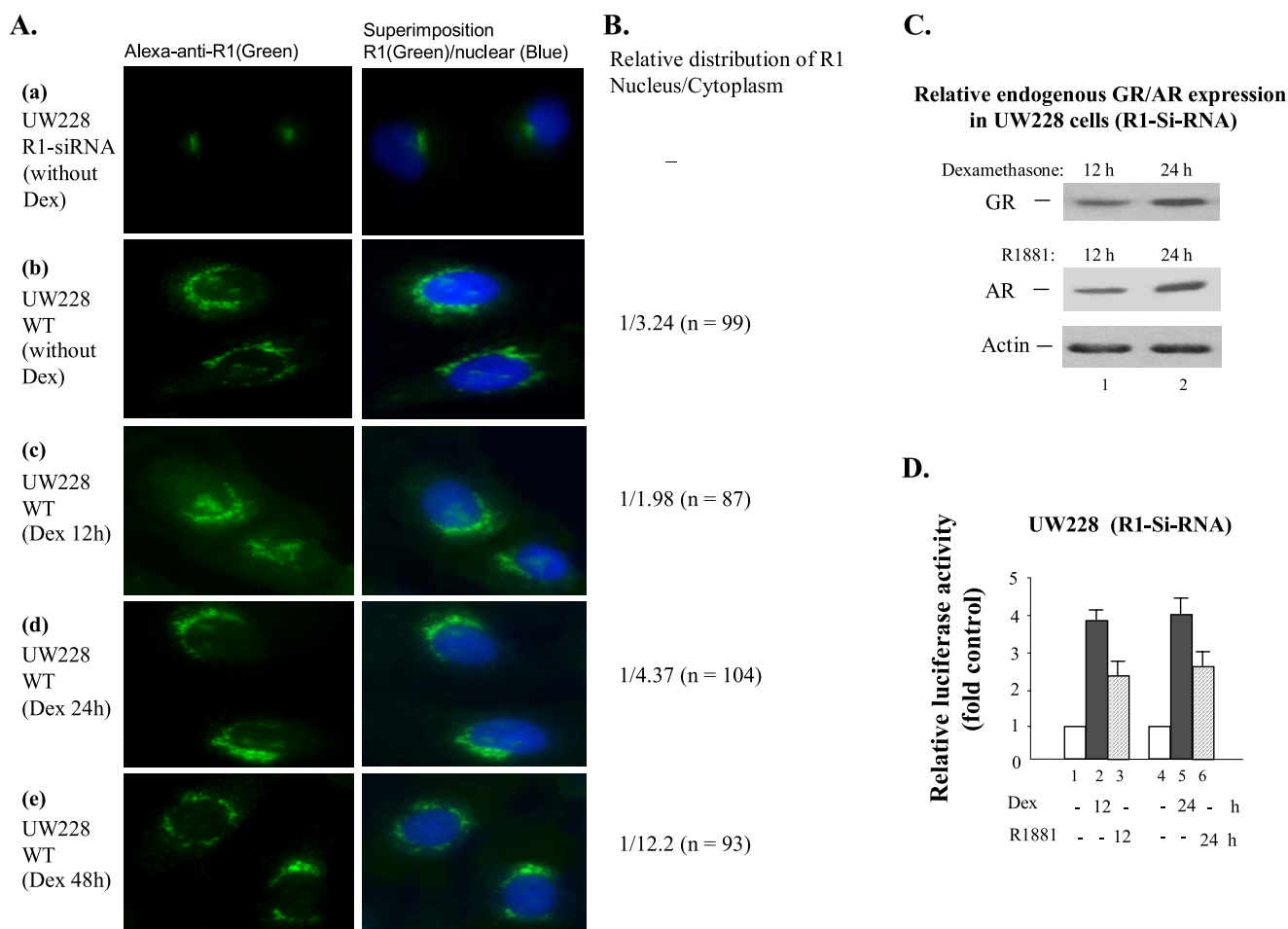
phenomena due to the lack of R1 gene expression (data not shown).

Next, Western blot analysis showed that the protein level between GR and AR was similar in both 12- and 24-h treatment with dexamethasone or R1881 in R1-knockdown cells (Fig. 11*C*). Then transient transfection and luciferase assay were performed in R1-knockdown cells, and the results showed that the -fold activation of MAO A by dexamethasone and R1881 was similar upon 12- and 24-h treatment (Fig. 11*D*), respectively. Therefore, the increase in glucocorticoid activation on MAO A was not observed in R1-knockdown cells.

DISCUSSION

This study provides the first evidence that glucocorticoid/androgen agonist activates the human MAO A promoter and increases catalytic activities. The transcriptional activation is mediated through the third GRE/ARE and Sp1-binding sites. We have also shown that both DNA-dependent and DNA-independent mechanisms are present in the regulation of MAO A by GR/AR activation. Steroid hormone receptors, such as glucocorticoid/androgen receptors (GR/AR), can be activated by their respective agonists and translocated into nucleus binding to consensus GRE/ARE as homodimers or heterodimers to regulate their target genes transcription (36, 37). This is a DNA-dependent mechanism (38). On the other hand, GR/AR can interact with other transcription factors to regulate their target

FIGURE 11. **The effect of R1 on GR/AR activation of MAO A in wild-type and R1-knockdown cells.** *A*, R1 expression was examined in siRNA-mediated R1 knockdown (*a*) and wild-type (*b–e*) cells, the human medulloblastoma UW228, by immunofluorescence microscopy. *c– e*, 100 nM dexamethasone was added into the medium for 12 (*c*), 24 (*d*), and 48 h (*e*), respectively. Cells were fixed and incubated with rabbit anti-R1-antibody, followed with fluorescein-conjugated anti-rabbit secondary antibody (*green*). Stained slides were mounted in the presence of DAPI for nuclear staining (*blue*). The Alexa-anti-R1 staining and the merge of R1 (*green*) and nucleus (*blue*) are indicated at the *top*. *B*, the relative distribution of R1 in nucleus and cytosol was quantified by image analysis software (ComPix) and expressed as the ratio of nucleus/cytosol as indicated. The cell number (*n*) is shown in each group. *C*, expression of GR and AR in R1-knockdown UW228 cells treated with dexamethasone or R1881 for 12 or 24 h was determined by Western blot as indicated. *D*, the activation of MAO A promoter by dexamethasone or R1881 in R1-knockdown cells. The MAO A 2-kb promoter was co-transfected with GR (*lanes 2* and *5*) or AR (*lanes 3* and *6*) into SK-N-BE(2)-C cells. Then dexamethasone (*lanes 2* and *5*) or R1881 (*lanes 3* and *6*) was added for another 12 and 24 h, respectively. The luciferase activity was then determined. Controls were MAO A 2-kb promoter co-transfected with GR or AR and treated with vehicle (ethanol; *lanes 1* and *4*), which are taken as 1. Data are the mean ± S.D. from three independent experiments with triplicates for each experiment.

interaction between GR and R1 is not required for the effect of repressor R1.

Our results also show that R1 is translocated between nucleus and cytosol upon treatment with glucocorticoid, suggesting that glucocorticoid translocates R1 between nucleus and cytosol. The level of glucocorticoid is increased during long term stress (46), which increases MAO A expression. MAO A is a pro-apoptotic gene (47) that is involved in cell death induced by nerve growth factor withdrawal through the p38 MAPK pathway in PC12 cells (47). Therefore, long term stress-induced neuronal cell death (18) may result partially from a glucocorticoid-induced increase in MAO A. R1 is a repressor for MAO A and thus may have anti-apoptotic function.[3]

In summary, dexamethasone/R1881 agonists activate human MAO A gene expression via both a DNA-dependent mechanism (GR/AR directly binding to androgen/glucocorticoid

response element) and a DNA-independent mechanism (GR/AR indirectly cross-talking with transcription factors at Sp1-binding sites). A novel transcriptional repressor R1 specifically regulates glucocorticoid activation of MAO A. Therefore, the differential activation of MAO A by glucocorticoid and androgen is regulated by R1.

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