Heterodimerization of Mineralocorticoid and Glucocorticoid Receptors at a Novel Negative Response Element of the 5-HT1A Receptor Gene*

Received for publication, June 20, 2000, and in revised form, December 20, 2000

Published, JBC Papers in Press, February 2, 2001, DOI 10.1074/jbc.M00593200

Xiao-Ming Ou‡§, John M. Storring‡¶, Neena Kushwaha‡, and Paul R. Albert‡**

From the ‡Neuroscience Research Institute, University of Ottawa, Ottawa, Ontario K1H-8M5, Canada and the §Department of Pharmacology and Therapeutics, McGill University, Montreal, Canada, H3G-1Y6

Negative regulation of neuronal serotonin (5-HT1A) receptor levels by glucocorticoids in vivo may contribute to depression. Both types I (mineralocorticoid) and II (glucocorticoid) receptors (MR and GR, respectively) participate in corticosteroid-induced transcriptional repression of the 5-HT1A gene; however, the precise mechanism is unclear. A direct repeat 6-base pair glucocorticoid response element (GRE) half-site 5′-TGTTCT-3′ separated by 6 nucleotides was conserved in human, mouse, and rat 5-HT1A receptor promoters. In SN-48 neuronal cells that express MR, GR, and 5-HT1A receptors, deletion or inactivation of the nGRE (negative GRE) eliminated negative regulation of the rat 5-HT1A or heterologous promoters by corticosteroids, whereas its inclusion conferred corticosteroid-induced inhibition to a heterologous promoter. Bacterially expressed recombinant MR and GR preferentially bound to the nGRE as a heterodimer, as identified in nuclear extracts of MR/GR-transfected COS-7 cells, and with higher affinity than MR or GR homodimers. In SN48 and COS-7 cells, concentration-dependent coactivation of MR and GR was required for maximal inhibitory action by corticosteroids and was abrogated in the L501P-GR mutant lacking DNA binding activity. Corticosteroid-mediated transcriptional inhibition was greater for MR/GR in combination than for MR or GR alone. These data represent the first identification of an nMRE/GRE and indicate that heterodimerization of MR and GR mediates direct corticosteroid-induced transrepression of the 5-HT1A receptor promoter.

Adrenal corticosteroids readily enter the central nervous system to regulate a diversity of processes that involve the serotonergic system, including mood and emotion. For example, gene knockout of neuronal glucocorticoid type II (GR)3 or corticotrophin-releasing hormone receptor genes results in increased anxiety behaviors in mice (1, 2). In contrast, knockout of the 5-HT1A receptor gene results in increased anxiety (3–5).

The 5-HT1A receptor is strongly expressed in the hippocampus, septum, and other limbic areas (17, 18) and functions as a key regulator of the limbic system. Transcription of the 5-HT1A receptor gene is negatively regulated by corticosteroids especially in the limbic system. In the rat, adrenalectomy is followed by a rapid increase (within hours) in de novo 5-HT1A RNA synthesis (as measured by nuclear run-on assay (19)), total 5-HT1A mRNA, and binding sites. These changes are completely suppressed by low concentrations of corticosterone that preferentially activate MR (20–23). The extent of increase in 5-HT1A receptor gene transcription upon adrenalectomy was most robust in the hippocampus and septum, with smaller changes in the raphe nuclei. Using MR- and GR-selective ligands (in rat) and gene knockout approaches (in mouse) (24–26), MR (primarily) and GR have been implicated in negative regulation of 5-HT1A gene expression, but the precise mechanism remains unclear.

Positive gene regulation by glucocorticoids results in an 8–12-fold induction of transcription and is mediated by binding of GR/MR homo- or heterodimers to a canonical GRE composed of an inverted repeat of GRE half-sites (TGTTCT) separated by 3 nucleotides (27, 28). However, negative regulation of gene transcription by glucocorticoids results in 40–70% inhibition of transcription and involves diverse mechanisms (29). Direct steroid-induced repression of target gene transcription occurs at an nGRE (e.g. proopiomelanocortin (30)). Indirect mechanisms are mediated via interactions of glucocorticoid receptors with a variety of other transcription factors such as Pbx and...
Negative Regulation by Glucocorticoid Receptor Heterodimerization

Oct-1 (31, 32), XTF (33), or c-Jun and c-Fos (34). The mechanism of glucocorticoid action on the 5-HT1A gene was examined using deletion constructs of the promoter to localize the site of gene repression by corticosteroids in SN-48 cells, a sepal cell line that expresses 5-HT1A receptors. Because interactions between MR and GR appear to play an important role in glucocorticoid-mediated regulation of 5-HT1A receptor transcription in vivo, gel shift and cotransfection experiments were designed to assess functional interactions at the 5-HT1A promoter. Our findings indicate that a novel nGRE confers direct negative regulation by corticosteroids to the rat 5-HT1A receptor or heterologous promoters and suggest that MR and GR form a novel head-to-tail heterodimeric complex with the nGRE to mediate glucocorticoid-induced gene repression.

MATERIALS AND METHODS

Plasmid Construction—Previously reported DNA fragments or constructs of the rat 5-HT1A receptor promoter (35) (GenBank™ accession number AF217200) were cloned into the pG3-32 Basic vector (Promega, Madison, WI) to generate luciferase reporter constructs (1519-bp, 1186-bp, 922-bp) in the rat GR (p6RGR), MR (p6RMR), or heterodimers, which contain full-length rat GR, L501P-GR, and MR, respectively, of the pGL3 promoter (Promega). DNA sequencing was used to confirm the identity and orientation of the subcloned fragments. For construction of normal or mutant nGRE-luciferase plasmids three tandem 39-bp-containing nGREs (3xGRE/SV40), one 39-bp-containing nGRE (1xGRE/SV40), or the mutated nGRE were subcloned into the Smal or MluI/Xhol sites, respectively, of the pG3-32 vector (Promega). DNA sequencing was used to confirm the identity and orientation of the subcloned fragments. The 1519-, 1186-, 922-bp p6RMR, and 1148 bp p6RGR expression plasmids, which contain full-length rat GR, L501P-GR, and MR, respectively, were gifts from Dr. Robert Hache, Ottawa Hospital Research Institute, Ottawa, Canada.

Cell Culture and Transfections—SN-48 and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum at 37 °C in 5% CO2. SN-48 and COS-7 cells were grown to 50–60% confluence, and the medium in each plate were replaced 12 h before transfection. Calcium phosphate coprecipitation was used for both cell lines (35). Cells were transfected with the following amounts of normal or mutant constructs: 0.25 μg reporter or heterologous promoters and suggest that MR and GR negative regulation by corticosteroids to the rat 5-HT1A receptor or heterologous promoters and suggest that MR and GR form a novel head-to-tail heterodimeric complex with the nGRE to mediate glucocorticoid-induced gene repression.

Electrophoretic Mobility Shift Assay (EMSA)—Complementary 39-bp oligonucleotides (−1180 to −1141 bp) containing the dexamethasone-responsive element were end-labeled with [γ-32P]dATP and used in EMSA (40). Nuclear extracts or recombinant GR and MR were preincubated at room temperature for 10 min with or without competitor DNA or antibodies in a 20-μl reaction containing EMSA buffer (20 μg Hepes, 0.2 μM EDTA, 0.2 mM EGTA, 100 mM KCl, 5 mM MgCl2, 5% glycerol, and 2 mM dithiothreitol, pH 7.9). Following incubation samples were digested with DNase I (Amersham Pharmacia Biotech) for 5 min at room temperature with the addition of about 10 μg of yeast tRNA as carrier. After phenol/chloroform extraction, samples were resuspended in formamide dye solution and electrophoresed through 8% polyacrylamide/urea gel. The total nuclear protein for all extracts was quantitated according to the Bradford method with bovine albumin as standard.

Western Blot Analysis—Nuclear protein was extracted from SN48 cells as described above. Western blot was performed as described previously (42). Proteins were electrophoresed on sodium lauryl sulfate containing 10% polyacrylamide gel. The MR or GR antibody (1/1000) was rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.). The secondary antibody (1/3000) and chemiluminescence procedures were conducted according to the manufacturer (Amersham Pharmacia Biotech).

Statistical Analysis—The statistical significance was evaluated using a Student’s t test for two-group comparisons or by one-way analysis of variance with the Tukey test for multiple comparisons among more than two groups.

RESULTS

Identification of nGRE-like Sequence in the Rat 5-HT1A Receptor Promoter—We have previously identified a major TATA-driven transcriptional initiation site at −967 bp from the translatable translational initiation ATG of the rat 5-HT1A receptor gene, which is regulated by proximal GC-rich enhancer elements and by upstream dual repressor elements that confer neuron-specific expression (35, 40). To identify DNA elements that confer corticosteroid-mediated transcriptional repression, a series of six 5′-deletion fragments fused to a luciferase reporter gene was transfected in sepa
tal SN-48 cells differentiated to a neuronal phenotype, and luciferase activity in the absence or presence of 1 μM dexamethasone was measured (Fig. 1). Dexamethasone induced a 40% reduction in luciferase activity for the 1519- or 1186-luciferase constructs, but no significant effect was observed for any of the shorter luciferase constructs (Fig. 1). Dexamethasone induced a 40% reduction in luciferase activity for the 1519- or 1186-luciferase constructs, but no significant effect was observed for any of the shorter luciferase constructs (Fig. 1A), including 955-, 800-, and 618-luciferase (not shown). Within the dexamethasone-responsive region between positions 1186 and −1145 bp, a GRE-like sequence (−1169 to −1148 bp) was identified, and the 6-bp spacing between repeated 6-bp GRE half-sites (TGTCCT) was conserved in mouse and rat 5-HT1A promoters. Mutations were incorporated to disrupt the GRE half-sites (1519m and 1186m-luciferase, see “Materials and Methods”), resulting in a complete loss of corticosteroid transrepression for both constructs (Fig. 1B). Similarly, in rat raphe RN46A cells (35), another 5-HT1A-expressing neuronal cell line, 1 μM dexamethasone induced a 32% reduction in transcriptional activity of...
the 1186-luciferase; and this was absent in the mutant 1186m construct (data not shown). Thus, the nGRE was critical for dexamethasone-mediated negative regulation of 5-HT1A receptor gene transcription in neuronal cells.

**MR and GR Associate with the 5-HT1A nGRE**—To determine whether nuclear proteins interact with the putative nGRE, the DNase I protection assay was conducted using SN-48 nuclear extracts or purified recombinant glucocorticoid receptors (Fig. 2). DNase I digestion using nuclear extracts revealed a pair of strongly protected segments between −1173 and −1145 bp and corresponding to the two GRE-like half-sites (Fig. 2B, lanes 2 and 3). The interaction between GR and MR at the nGRE was examined directly using recombinant GR and MR (Fig. 2, A and B, lanes 4–9). Purified GR or MR or GR/MR heterodimerization protected the same nGRE sequence observed using SN-48 nuclear extracts (Fig. 2B, lanes 2 and 3), but GST protein did not protect this region (Fig. 2A, lanes 2 and 3), suggesting that the DNA-protein complex observed involves a direct interaction of GR or MR at two half-sites on the nGRE sequence.

To examine further the interaction of GR or MR with the nGRE, an EMSA was conducted using double-stranded 39-bp oligonucleotides incorporating the nGRE sequence incubated with nuclear protein extracts from SN-48 cells (Fig. 3) or with recombinant GR and MR proteins (Fig. 4). A specific protein complex from SN-48 nuclei associated with the nGRE and was competed by excess unlabeled nGRE oligonucleotide but not by the unrelated E2F sequence (Fig. 3A, lane 4) or by the mutated nGRE sequence (data not shown). The inclusion of either anti-GR or anti-MR antibody displaced this complex (Fig. 3A, lanes 5 and 6). As observed by others (32, 43), the interaction of antibody with either rat GR or MR at the nGRE prevented high affinity binding to labeled nMRE/GRE (39-oligo), displacing the DNA-protein complex rather than inducing a supershift of the complex. By contrast, inclusion of either nonspecific antibody or preimmune serum did not displace the complex (Fig. 3A, lanes 5 and 6), suggesting that the DNA-protein complex observed involves a direct interaction of GR or MR at two half-sites on the nGRE sequence.

To assess GR/MR receptor interactions in vivo, COS-7 cells were transfected with expression plasmids for Flag-GR alone, GST-MR alone, or both, and receptor expression was assessed by EMSA performed on nuclear extracts using the nGRE oligonucleotide (Fig. 4D). The COS-7 cells are derived from CV-1 cells, which lack endogenous MR and GR and therefore provide a null background for expression of transfected receptors (38). Consistent with an absence of detectable MR/GR, no gel retarded bands were present in extracts from COS-7 cells not transfected with MR or GR plasmids (lane 9). Nuclear extracts

**Fig. 1**. Activity of the 5-HT1A receptor promoter expressed in SN-48 cells is suppressed by dexamethasone. A, SN-48 cells treated with dexamethasone (1 μM, 48 h) showed inhibition of transcriptional activity (plotted as percent of vehicle control) of the rat 5-HT1A gene 5'-flanking reporter constructs. A schematic diagram of the rat 5-HT1A receptor gene is shown on the left. The percent inhibition by dexamethasone is shown on the right. B, loss of glucocorticoid transrepression by mutation of the nGRE. Mutated nGRE constructs were generated in the context of the 1519- and 1186-luciferase gene by site-specific mutagenesis (1519m and 1186m; see “Materials and Methods”). The pGL3-Basic vector was measured to determine background activity. All measurements were performed in triplicate in four independent experiments. Shown is the average ± S.E. expressed as percent inhibition compared with vehicle control. *, p < 0.02 compared with control.
from COS-7 cells transfected with 2 μM GST-MR, 2 μM Flag-GR, or 1 μM each GST-MR and Flag-GR plasmids together resulted in specific protein-DNA complexes that were supershifted with the appropriate antibodies (anti-GST or anti-Flag). The predicted molecular masses of these full-length recombinant receptor constructs were 140 kDa for GST-MR and 95 kDa for Flag-GR; hence the electrophoretic mobilities of homodimers or heterodimers were distinguishable (MR, MR/GR, and GR were indicated by arrows 1, 2, and 3, respectively, Fig. 4D) in 4% non-denatured gel. These results indicate that the transfected MR and GR in COS-7 cells were functional and present at a similar density as detected by Western blot (data not shown) and that both associated with the nGRE of the 5-HT1A receptor gene. Furthermore, anti-GST or -Flag antibody independently supershifted binding of the nGRE-protein complex in cells transfected with GR+MR (lanes 8, 9), indicating that the GR/MR heterodimer forms the major species of the complex.

The binding affinity of recombinant MR, GR, or MR/GR heterodimer to the nGRE/GRE was evaluated by EMSA using increasing concentrations of radiolabeled 39-oligo followed by quantification of bound and free oligonucleotide (Fig. 5). Scatchard analysis revealed that the dissociation constant ($K_d$) value was $0.13 \times 10^{-9}$ M for GR, $0.12 \times 10^{-9}$ M for MR, and $0.08 \times 10^{-9}$ M for GR + MR (Fig. 5B). These $K_d$ values are in the same range as for GR binding to the nGRE ($0.25 \times 10^{-9}$ M) in the pro-opiomelanocortin gene (30). The $K_d$ value of GR + MR was significantly lower than that of MR or GR alone ($p < 0.05$). These results indicate that the binding affinity of MR/GR heterodimer to nGRE/GRE is significantly enhanced compared with homodimers, suggesting that MR/GR heterodimerization may play an important role in down-regulation of the 5-HT1A receptor gene.

**MR and GR Coactivation for Glucocorticoid Transrepression at the 5-HT1A nGRE**—The relative importance of MR, GR, or both receptors in the transcriptional repression was assessed by cotransfection of MR, GR, or both expression plasmids with the 1186-luciferase 5-HT1A construct in COS-7 cells (Fig. 6A). A MR/GR molar ratio of 1:1 was found to be optimal for glucocorticoid transrepression (data not shown) and was used in these experiments. Dexamethasone and aldosterone had no effect on luciferase activity of the 1186-luc construct in the absence of MR or GR. Transrepression was greatest upon coactivation of both MR and GR (53% inhibition), less for MR alone (33% inhibition), and least for GR alone (26% inhibition). The mutated nGRE (1186 m-luc) was not responsive to corticosteroids in the presence of both MR and GR, confirming the results obtained in SN-48 cells (Fig. 1). The greater activity (53 versus 41% inhibition) of the GR/MR combination in COS-7 (Fig. 6A) versus SN-48 cells (Fig. 1A) may be due to higher levels of receptor expression in transfected COS-7 cells.

The activity of the nGRE to mediate glucocorticoid transrepression at a heterologous promoter was examined by placing one to three copies of the nGRE sequence upstream of the SV40 promoter in the pGL3 luciferase vector (Fig. 6, B and C). The SV40 promoter was unresponsive to corticosteroids, even in the presence of cotransfected MR and GR (data not shown). For each construct, no effect of corticosteroids was observed in the absence of cotransfected MR or GR, and the mutated nGRE was unresponsive to corticosteroids in the presence of both MR and GR, as observed for the 5-HT1A promoter. In each case, cotransfection with both MR/GR conferred significantly more corticosteroid responsiveness on the nGRE-containing constructs than that of transfection with MR or GR alone as seen for the 5-HT1A promoter (Fig. 6A). The presence of three copies of the nGRE did not enhance the corticosteroid responsiveness of the SV40 promoter (Fig. 6C), which is consistent with previous findings (30). Interestingly, the corticosteroid responsiveness of the 5-HT1A promoter (53% inhibition in COS-7 cells) was greater than for the SV40 promoter (41% inhibition). These results indicate that the nGRE independently confers glucocorticoid transrepression on a heterologous promoter, with preferential repression activity for coactivation of MR and GR compared with independent activation of either receptor.

To assess the importance of DNA binding in glucocorticoid action, the mutant L501P-GR, which incorporates a mutation in the zinc finger domain to inactivate DNA binding (37), was cotransfected with each of the reporter constructs (Fig. 6, A–C). This mutant receptor was completely inactive in all cases, indicating that direct receptor-DNA interaction is required for glucocorticoid-induced repression at the nGRE of the 5-HT1A receptor gene.

**Pharmacology and Receptor Dependence of the nGRE Response**—The concentration dependence of Dex- or Ald-mediated dexamethasone- or aldosterone-mediated repression of the 3xnGRE/SV40 construct in COS-7 cells cotransfected with GR (Fig. 7A) or MR (Fig. 7B) alone, respectively. Both Dex (EC$_{50}$ = 2.5 nM) or Ald (EC$_{50}$ = 0.08 nM) mediated saturable responses
at concentrations similar to those required for ligand binding to their respective receptors. The functional importance of coactivation of MR and GR in SN-48 and COS-7 cells was addressed further using a pharmacological approach (Fig. 7, C and D). Three treatment conditions were used to selectively activate, respectively, GR alone, MR alone, or both: dexamethasone + MR antagonist spironolactone (Dex + Sp); aldosterone (MR agonist) + GR antagonist RU38486 (Ald + RU); or Dex + Ald. In SN-48 cells cotransfected with 1186-luc (Fig. 7C), treatment with Dex and Ald resulted in the greatest level of transrepression (40% of control), whereas the other treatments produced a smaller but significant repression. COS-7 cells cotransfected with MR and GR plasmids in an equal molar ratio, and 3xnGRE/SV40 displayed glucocorticoid receptor selectivity similar to that in SN-48 cells (Fig. 7D). Corticosteroid-induced repression was greatest (40% of control) upon activation of both MR and GR, whereas activation of either receptor alone displayed significant less repression activity. Thus, coactivation of GR and MR is required for optimal transrepression at the nGRE in both neuronal SN-48 cells and COS-7 cells.

**DISCUSSION**

A Novel nGRE Regulates the 5-HT1A Receptor Gene—The level of expression of the 5-HT1A receptor has been implicated in mental illnesses such as major depression, anxiety, and related disorders. For example, gene knockout of the 5-HT1A receptor gene results in mice with increased anxiety-related behaviors (3–5). On the other hand, depressed suicides show elevated levels of 5-HT1A autoreceptors compared with nondepressed suicides (46). Dysregulation of glucocorticoids is observed in a large proportion of depressed patients, suggesting a link between regulation of the 5-HT1A receptor by glucocorticoids and the etiology or maintenance of depression.

One of the most powerful regulators of 5-HT1A receptor expression in the rat brain is corticosterone, which rapidly and completely inhibits adrenalectomy-induced expression of the 5-HT1A receptor gene. Transcriptional repression of the 5-HT1A receptor gene by glucocorticoids has been demonstrated in the rat hippocampus, septum, and frontal cortex (20, 21, 23). However, the precise site of glucocorticoid regulation at the 5-HT1A receptor gene has yet to be clarified. We have identified a novel nGRE that mediates direct transcriptional repression of the rat 5-HT1A receptor gene by corticosteroids in 5-HT1A-expressing septal SN-48 or raphe RN46A neuronal cells. The 5-HT1A promoter activity was reduced by 40–60%, consistent with the extent of inhibition observed at other nGRE elements (29, 31). Importantly, we demonstrate that transrepression of the 5-HT1A promoter or heterologous promoters is mediated by the nGRE and is transduced preferentially by MR over GR, but transrepression is strongest upon coactivation of both receptors. Thus the nGRE of the 5-HT1A receptor gene represents the first nMRE/GRE to be identified. The ligand- and receptor-dependent properties of this novel nGRE correspond well with the known properties of regulation of the 5-HT1A receptor transcription in limbic areas of the brain in vivo. In particular, both pharmacologic and gene knockout studies have provided evidence that MR is involved in corticosteroid-induced repression of 5-HT1A transcription. For example, low concentrations of corticosteroids or synthetic ligands that selectively activate MR are sufficient to reverse the adrenalectomy-induced increase in 5-HT1A receptor level in the
respectively, were supershifted by the addition of 0.4 pmol, anti-MR antibody (B, lane 5), or anti-NF1 antibody or normal rabbit serum (lanes 6 and 7). C, heterodimerization of recombinant MR and GR at the 5-HT1A nGRE. Labeled 39-oligo (-1180 to -1141 bp) was incubated with equimolar GR (2 pmol, lane 2), MR (4 pmol, lane 3), or both (1 pmol each of GR and MR plasmids, lanes 4 and 5), or both (1 pmol each of MR and GR plasmids, lanes 6–8). Nuclear proteins were extracted, incubated with labeled 39-bp nGRE oligonucleotide, and analyzed on 4% polyacrylamide gel. No bands were detected in the absence of nuclear extract (lane 1) or in nuclear extracts from cells not transfected with MR or GR (lane 9). The nGRE-protein complexes (MR, MR/GR, and GR), indicated by arrows labeled 1, 2, and 3, respectively, were supershifted by the addition of 0.4 μg of anti-GST or anti-Flag antibody (lanes 2, 4, 7, and 8) as indicated.

Fig. 4. Heterodimerization of recombinant MR and GR with the 5-HT1A nMRE/GRE. Labeled 39-oligo (-1180 to -1141 bp) was incubated (50,000 cpm/lane) with 2 μg of recombinant GST-GR (A) or 4 μg of recombinant GST-MR (B) as indicated. No bands were detected in the absence of nuclear extract (lane 1). A single specific complex (indicated by an arrow) was observed with either GR or MR (lane 2) that was competed by unlabeled 39-oligo (Cold 39, lanes 3 and 4). Recombinant proteins were preincubated with 0.4 μg of anti-GR antibody (A, lane 5), anti-MR antibody (B, lane 5), or anti-NF1 antibody or normal rabbit serum (lanes 6 and 7). C, heterodimerization of recombinant MR and GR at the 5-HT1A nGRE. Labeled 39-oligo (-1180 to -1141 bp) was incubated with equimolar GR (2 pmol, lane 2), MR (4 pmol, lane 3), or both (1 pmol each of GR and MR plasmids, lanes 4 and 5) as indicated. D, heterodimerization of MR and GR from nuclear extracts at the 5-HT1A nGRE. COS-7 cells, which lack endogenous GR and MR, were transfected with 8 pmol, expression plasmids Flag-GR for GR alone (2 pmol, lanes 2 and 3), 12 μg of GST-MR for MR alone (2 pmol, lanes 4 and 5), or both (1 pmol each of MR and GR plasmids, lanes 6–8). Nuclear proteins were extracted, incubated with labeled 39-bp nGRE oligonucleotide, and analyzed on 4% polyacrylamide gel. No bands were detected in the absence of nuclear extract (lane 1) or in nuclear extracts from cells not transfected with MR or GR (lane 9). The nGRE-protein complexes (MR, MR/GR, and GR), indicated by arrows labeled 1, 2, and 3, respectively, were supershifted by the addition of 0.4 μg of anti-GST or anti-Flag antibody (lanes 2, 4, 7, and 8) as indicated.

Hippocampal (24, 25, 47). Similarly, knockout of the murine GR gene did not greatly impair corticosterone-mediated repression of hippocampal 5-HT1A RNA, suggesting that MR alone is sufficient for transrepression of the 5-HT1A gene by glucocorticoids (26).

Glucocorticoid-induced repression of the rat 5-HT1A receptor gene at the nGRE proceeded via direct protein-DNA interaction as opposed to indirect mechanisms involving protein-protein interactions (29). We demonstrate that both GR and MR bind to the nGRE and that mutations in the nGRE or the receptor that disrupt this interaction block repression. In particular, the point mutant L501P-GR, which lacks DNA binding capability, failed to mediate or alter GR/MR-induced transrepression. This is in contrast to an indirect mechanism proposed for corticosterone action of an adjacent nGRE-like sequence of the 5-HT1A promoter (at -1149 to -1134 bp). Furthermore, inclusion of the nGRE element upstream of heterologous promoters conferred corticosteroid-induced inhibition by both GR and MR receptors, the same receptor preference observed for 5-HT1A gene regulation in hippocampal neurons. Thus, direct glucocorticoid-induced transrepression at the nGRE is likely to represent the dominant mechanism of basal regulation of the 5-HT1A gene.

The NFκB-dependent indirect pathway of glucocorticoid regulation may provide a complementary mechanism to regulate 5-HT1A receptor expression, especially in fibroblast or immune cells where NFκB is implicated in the stimulation of 5-HT1A receptor expression (49). For example, in non-neuronal Chinese hamster ovary cells, NFκB mediates 5-HT1A agonist-induced expression of the 5-HT1A receptor (50), regulation that is opposite to the negative regulation of the 5-HT1A receptor gene by agonists in hippocampal neurons (51). In B and T lymphocytes, where mitogens induce NFκB-dependent transcriptional activation of the 5-HT1A promoter (49, 52, 53), antagonism of NFκB action by glucocorticoids could inhibit mitogen action.

Heterodimerization of MR and GR at the nGRE—Both MR and GR bound directly to the 5-HT1A nGRE, and when both were present formation of a heterodimeric complex was fa-
vored. Consistent with this finding, the affinity for the nMRE/GRE of the MR+GR combination was significant higher than that of GR or MR alone. Corticosteroid repression of transcriptional activity of the rat 5-HT1A receptor gene proceeded through a mechanism involving preferential coactivation of both MR and GR. Cooperative interactions between MR and GR at a positive GRE have been reported in the nervous system (54), in which MR and GR activate transcription synergistically through heterodimer formation. It has been proposed that glucocorticoid receptor heterodimerization may play a crucial role in glucocorticoid action in the brain, particularly in tissues (e.g. hippocampus) that express MR and GR (45). The high affinity of MR for corticosterone would allow for high sensitivity to the actions of glucocorticoids in regions that express this receptor (55). The strong expression of 5-HT1A receptors in MR-enriched hippocampal CA1 pyramidal cells and the cooperative interactions between MR and GR at the 5-HT1A nGRE strongly suggest that heterodimerization is the key mechanism for inhibitory regulation by glucocorticoids of the 5-HT1A receptor gene in the brain.

Structure and Conservation of the nGRE—Negative regulation of gene transcription by glucocorticoids involves repression via an nGRE composed of one GRE half-site (56, 57) or three half-sites (30, 58). An nGRE-like element has been found in several genes including the following: the pro-opiomelanocortin gene (30, 59, 60), the corticotrophin-releasing hormone gene (58), the gonadotropin-releasing hormone gene (32), the human interleukin-1β gene (61), and the bovine prolactin gene (33, 56). In the present study, the GRE-like sequence has a repeated 6-bp GRE half-site (TgTGCCCT) separated by 6 nucleotides, which is conserved (uppercase) in both human (−1249 TgTGCCCT-TTgnnn-TgTGCCCTTA) and mouse (−1158 TgTGCCCT-ccAnnn-TgTGCCCTTcc) 5-HT1A receptor genes. Note that in all cases the direct repeat GRE half-site (TgTGCCCT) and 6-bp gap are absolutely conserved. Although the nGRE contains two GRE-like half-sites, suggesting the formation of receptor dimers, the exact number of receptors involved in the present interaction needs to be elucidated by x-ray crystallography. Nevertheless, a theoretical consideration of the topology of GR homodimer binding to the nGRE, based on the crystal structure of GR homodimer binding to a consensus GRE (62), suggests that two receptors would bind in a head-to-tail conformation on the same face of the double helix. The critical parameter for this arrangement is the 6-nucleotide spacing between the two conserved GRE half-sites, which is sufficient for a complete 360° rotation of the two sites into alignment. The absolute conservation among 5-HT1A gene homologues of the 6-bp spacing between two GRE half-sites suggests that this is a crucial element of the nGRE. Indeed, elimination of 3 bp of the 6-bp gap in the 5-HT1A nGRE to rotate the receptor dimer 180° converts the negative GRE into a positive GRE that mediates glucocorticoid-induced gene transcription of 3–4-fold the

Fig. 5. Saturation binding of nMRE/GRE to recombinant GR, MR, or GR + MR combination. A, increasing amounts of 32P-labeled 39-oligo (1–25 × 10⁶ cpm/lane) were incubated with equimolar amounts of recombinant GST-GR (2 μg), GST-MR (4 μg), or GST-GR + GST-MR (1 μg/2 μg) and analyzed by EMSA. The arrows indicate the migration of the protein-DNA complexes. B, bound and free 39-oligo (nMRE/GRE) in panel A were quantitated by Microcomputer Imaging Device (Imaging Research Inc.), and the Kd of nMRE/GRE binding to GR + MR, GR, or MR was determined by Scatchard analysis. Scatchard plot is displayed together with the Kd values calculated from four independent experiments.

Fig. 6. Coactivation of MR and GR for maximal transrepression at the nGRE in the 5-HT1A promoter or a heterologous promoter. The indicated reporter constructs were cotransfected with expression plasmids MR or GR alone or MR/GR together as indicated. The reporter constructs used were: A, 1186-luc or 1186 m-luc, constructs of the 5-HT1A gene, as indicated; B, 1 × nGRE/SV40, containing the 5-HT1A nGRE or mutant nGRE located upstream of the SV40 promoter; and C, 3 × nGRE/SV40, containing three tandem copies of the 5-HT1A nGRE or mutant nGRE located upstream of the SV40 promoter (see “Materials and Methods”). Cells were transfected with equimolar concentrations of MR, GR, both (or neither), or DNA binding mutant L501P-GR and treated for 48 h with 20 nm Ald, 100 nm Dex, or both Ald and Dex, respectively. The activity of SV40 vectors cotransfected with MR/GR was not altered by treatment with Ald and Dex (not shown). Note that mutated nGRE or receptor (L501P-GR) failed to mediate corticosteroid repression. Data represent percent inhibition compared with vehicle control and are plotted as mean ± S.E. of at least six independent transfections done with triplicate samples. *, p < 0.05 and **, p < 0.02 compared with control.
samples and represent the average and both MR- and GR-expression plasmids in equal ratio. Cells were treated for 48 h as described in C. 6-Glucocorticoid inhibition is expressed as a percentage of the maximal inhibition. Each point is the mean (± S.E. of three independent experiments. 6-Dexamethasone (1186-luc), 100 nM dexamethasone and 100 nM spiroxatrine (Dex + Spi), 10 nM aldosterone and 100 nM RU-486 (Ald + RU), or Dex + Ald. D, pharmacology of corticosteroid-induced repression in COS-7 cells. COS-7 cells were transfected with 3 × nGRE/SV40 and both MR- and GR-expression plasmids in equal ratio. Cells were treated for 48 h as described in C. All measurements were done in triplicate samples and represent the average ± S.E. of three independent experiments. Luciferase activity was normalized to β-galactosidase activity and is expressed as relative light units. *, p < 0.05 and **, p < 0.02, in comparison with control.

basal level in SN48 or GR-transfected COS-7 cells (data not shown).

In conclusion, we have identified a novel nMRE/GRE that demonstrates the crucial role of MR/GR heterodimers in mediating responses to corticosteroids, not only for transcriptional activation but also for transcriptional repression. This is consistent with the general importance of heterodimerization of the steroid receptor family of nuclear receptors (44, 63). Furthermore, our results provide a plausible mechanism for the sensitivity of the rat 5-HT1A receptor gene to corticosteroid-induced transrepression, particularly in the hippocampus where MR and GR are both expressed.

Acknowledgments—We thank Dr. Robert Haché, University of Ottawa, for providing plasmids and experimental compounds, and Dr. M. H. Ghaehremani University of Ottawa for assistance.

Note Added in Proof—Recent evidence that MR and GR interact to form heterodimers in solution (Savory, J. G., Préfontaine, G. G., Lamprecht, C., Liao, M., Walther, R. F., Lefebvre, Y. A., and Haché, R. J. (2001) Mol. Cell Biol. 21, 781–793) may account in part for the preferred formation of heterodimers at the nGRE of the 5-HT1A receptor gene.

REFERENCES
1. Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P. C., Bock, R., Klein, R., and Schutz, G. (1998) Nat. Genet. 23, 99–103
2. Timpl, P., Spanagel, R., Silhaker, I., Kreese, A., Reul, J. M., Stallia, G. K., Blankvet, V., Steckler, T., Holsteiner, F., and Wurst, W. (1998) Nat. Genet. 19, 162–166
3. Heisser, L. K., Cho, H. M., Brennan, T. J., Danan, J. A., Bajwa, P., Parsons, L. H., and Tocchi, L. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15049–15054
4. Ramboz, S., Oetting, R., Amara, D. A., Kung, H. F., Blier, P., Mendelsonh, M., Mann, J. J., Brunser, D., and Hen, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14476–14481
5. Parks, C. L., Robinson, P. S., Sibille, E., Shenk, T., and Toth, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10734–10739
6. Diaz, R., Brown, R. W., and Seckl, J. R. (1998) J. Neurosci. 18, 2570–2580
7. McAllister-Williams, R. H., Ferrier, I. N., and Young, A. H. (1998) Psychol. Med. 28, 573–584
8. Azmitia, E. C., Jr., and McEwen, B. S. (1989) Science 245, 1274–1276
9. Laaris, N., Haj, D. S., Hamon, M., and Landuyt, L. (1999) Neuropharmacology 38, 1201–1210
10. Joels, M., and Vreugdenhil, E. (1998) Mol. Neurobiol. 7, 87–108
11. Lopes, J. P., Chalmers, D. T., Little, K. Y., and Watson, S. J. (1998) Biol. Psychiatry 43, 547–573
12. De Kloet, E. R., Vreugdenhil, E., Otzi, M. S., and Joels, M. (1998) Endocrin. Res. 19, 289–301
13. Verge, D., Daval, G., Patay, A., Goulan, H., e Mestikawi, S., and Hamon, M. (1985) Eur. J. Pharmacol. 113, 463–464
14. Hoyer, D., Clarke, D. E., Fozard, J. R., Hartig, P. R., Martin, G. R., Mylecharane, E. J., Szaenza, P. R., and Humphrey, P. (1994) Pharmacol. Rev. 46, 157–203
15. Pineyro, G., and Blier, P. (1999) Pharmacol. Rev. 51, 533–591
16. Meijer, O. C., and de Kloet, E. R. (1998) Crit. Rev. Neurobiol. 12, 1–20
17. Albert, P. R., Zhou, Q. Y., Van Tole, H. H., Buzov, J. R., and Civelli, O. (1990) J. Biol. Chem. 265, 5825–5832
18. Pompeiano, M., Palacios, J., and Mengod, G. (1992) J. Neurosci. 12, 440–453
19. Zhong, P., and Ciaramello, R. D. (1995) Mol. Brain Res. 29, 23–34
20. Mendelson, S. D., and McLennan, B. S. (1992) Neuroendocrinology 55, 444–450
21. Chalmers, D. T., Kwak, S. F., Mansour, A., Akil, H., and Watson, S. J. (1993) J. Neurosci. 13, 914–923
22. Liao, B., Miesak, B., and Amtizia, E. C. (1993) Mol. Brain Res. 19, 328–332
23. Meijer, O. C., and de Kloet, E. R. (1994) Eur. J. Pharmacol. 265, 255–261
24. Meijer, O. C., and de Kloet, E. R. (1995) J. Neuroendocrinology 7, 653–657
25. Nishi, M., and Amtizia, E. C. (1996) Brain Res. 722, 190–194
26. Meijer, O. C., Cole, T. J., Schmid, W., Schutz, G., Joels, M., and De Kloet, E. R. (1997) Mol. Brain Res. 46, 290–296
27. Beato, M., Truse, M., and Chavez, S. (1996) Annu. N. Y. Acad. Sci. 784, 93–123
28. Evans, R. M. (1988) Science 240, 889–895
29. Webster, J. C., and Cidlowski, J. A. (1999) Trends Endocrinol. Metab. 10, 396–402
30. Drouin, J., Sun, Y., L., Chamberland, M., Gauthier, Y., De Lean, A., Nemer, M., and Schmidt, T. J. (1993) EMBO J. 12, 145–156
31. Subramanian, N., Cairns, W., and Okret, S. (1998) J. Biol. Chem. 273, 25967–25974
32. Chandran, U. R., Warren, B. S., Baumann, C. T., Hager, G. L., and DeFranco, D. B. (1999) J. Biol. Chem. 274, 25723–25728
33. Subramanian, N., Cairns, W., and Okret, S. (1997) DNA Cell Biol. 16, 153–163
34. Diamond, M. I., Miner, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990) Science 249, 1266–1272
35. Storring, J. M., Charest, A., Cheng, P., and Albert, P. R. (1999) J. Neurochem. 72, 2238–2247
36. Godowski, P. J., Rusconi, S., Miesfeld, R., and Yamamoto, K. R. (1987) Nature 325, 365–368
37. Schena, M., Freedman, L. P., and Yamamoto, K. R. (1989) Genes Dev. 3, 1590–1601
38. Pearce, D., and Yamamoto, K. R. (1993) Science 259, 1161–1165
39. Charest, A., Wainer, B. H., and Albert, P. R. (1993) J. Neurosci. 13, 5161–5171
40. Ou, X. M., Jafar-Nejad, H., Storring, J. M., Meng, J. H., Lemonde, S., and Albert, P. R. (2000) J. Biol. Chem. 275, 8161–8168
41. Giffin, W., Kwast-Welfeld, J., Rodda, D. J., Prefontaine, G. G., Traykova-Andonova, M., Zhang, Y., Weigel, N. L., Leebvre, Y. A., and Hache, R. J. (1997) J. Biol. Chem. 272, 5647–5658
42. Grahnenani, M. H., Cheng, P., Lembo, P. M., and Albert, P. R. (1999) J. Biol. Chem. 274, 9238–9245
43. Radoja, N., Komine, M., Jho, S. H., Blumenberg, M., and Tomic-Canic, M. (2000) Mol. Cell. Biol. 20, 4328–4339
44. Liu, W., Wang, J., Sauter, N. K., and Pearce, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12480–12484
45. Trapp, T., and Holsboer, F. (1996) Trends Pharmacol. Sci. 17, 145–149
46. Stockmeier, C. A., Shapiro, L. A., Diley, G. E., Kolli, T. N., Friedman, L., and Rajkowska, G. (1998) J. Neurosci. 18, 7394–7401
47. Neumaier, J. F., Sexton, T. J., Hamblin, M. W., and Beck, S. G. (2000) Brain Res. Mol. Brain Res. 82, 65–73
48. Wissink, S., Meijer, O., Pearce, D., van Der, B.urg, B., and van Der, S.aag, P. T. (2000) J. Biol. Chem. 275, 1321–1326
49. Abdouh, M., Storring, J. M., Risal, M., Paquette, Y., Albert, P. R., Drebetasky, E., and Kouassi, E. (2001) J. Biol. Chem. 276, 4383–4388
50. Cowen, D. S., Molinoff, P. B., and Manning, D. R. (1997) Mol. Pharmacol. 52, 221–226
51. Huang, J., and Armitia, E. C. (1999) Neurosci. Lett. 270, 5–8
52. Aune, T. M., Golden, H. W., and McGrath, K. M. (1994) J. Immunol. 153, 489–486
53. Iken, K., Chheng, S., Farina, G., Goulet, A. C., and Kouassi, E. (1995) Cell Immunol. 163, 1–9
54. Trapp, T., Ruprecht, R., Castren, M., Reul, J. M., and Holsboer, F. (1994) Neurosci. Lett. 147–1482
55. Funder, J. W. (1996) J. Steroid Biochem. Mol. Biol. 56, 179–183
56. Sakai, D. D., Helms, S., Carlestedt-Duke, J., Gustafsson, J. A., Rottman, F. M., and Yamamoto, K. R. (1988) Genes Dev. 2, 1144–1154
57. Bahouth, S. W., Park, E. A., Beauchamp, M., Cui, X., and Malbon, C. C. (1996) Recept. Signal Transduct. 6, 141–149
58. Malkoski, S. P., and Dorin, R. I. (1999) Mol. Endocrinol. 13, 1629–1644
59. Drouin, J., Trifiro, M. A., Plante, R. K., Nemar, M., Eriksson, P., and Wrage, O. (1989) Mol. Cell. Biol. 9, 5305–5314
60. Nakai, Y., Usui, T., Tsukada, T., Takahashi, H., Fukata, J., Fukushima, M., Senoue, K., and Imura, H. (1991) J. Steroid Biochem. Mol. Biol. 40, 301–306
61. Zhang, G., Zhang, L., and Duff, G. W. (1997) DNA Cell Biol. 16, 145–152
62. Luisi, B. F., Xu, X. X., Otwinski, Z., Freedman, L. P., Yamamoto, K. R., and Sigler, P. B. (1991) Nature 352, 497–505
63. Lee, Y. F., Shyr, C. R., Thin, T. H., Lin, W. J., and Chang, C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14724–14729