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Identification of a Glucocorticoid Response Element in the Rat β_2-Adrenergic Receptor Gene

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ABSTRACT

Regulation of β_2-adrenergic receptor (β_2AR) levels by glucocorticoids is a physiologically important mechanism for altering β_2AR responsiveness. Glucocorticoids increase β_2AR density by increasing the rate of β_2AR gene transcription, but the *cis*-elements involved have not been well characterized. We now show that one of six potential glucocorticoid response elements (GREs) in the 5′-flanking region of the rat β_2AR gene is necessary for glucocorticoid-dependent stimulation of receptor gene expression. Using a nested set of deletion fragments of the rat β_2AR gene 5′-flanking region fused to a luciferase reporter gene, glucocorticoid-dependent induction of reporter gene expression in HepG2 cells was localized to a region between positions −643 and −152, relative to the transcription initiation site. In electrophoretic mobility shift assays, a double-stranded oligonucleotide incorporating a near-consensus GRE from this region (positions −379 to −365) formed complexes with the human recombinant glucocorticoid receptor, as well as with nuclear protein from dexamethasone-treated HepG2 cells. Mutation of a single base within this GRE sequence greatly diminished interaction of the mutated oligonucleotide with the human recombinant glucocorticoid receptor. The functional activity of the GRE was characterized using a luciferase reporter construct driven by a minimal thymidine kinase promoter. In HepG2 cells transfected with constructs containing the GRE, dexamethasone increased reporter gene expression approximately 3-fold, whereas a dexamethasone effect was not observed with constructs lacking the GRE. Taken together, these findings show that a GRE located at positions −379 to −365 in the 5′-flanking region of the rat β_2AR gene mediates glucocorticoid stimulation of β_2AR gene transcription.

The β_2AR is a member of a large superfamily of membrane-associated receptors that are coupled to G proteins and produce their effects by activating intracellular signal transduction pathways (Strader *et al.*, 1995). For many G protein-coupled receptors, modulation of receptor number is an established mechanism controlling responsiveness to hormones and neurotransmitters. Heterologous regulation of β_2AR levels by glucocorticoids is a physiologically important example of such control (Collins *et al.*, 1988). Numerous *in vitro* and *in vivo* studies have shown that β_2AR levels and β-agonist-stimulated adenylyl cyclase activity are increased by glucocorticoids (Cheng *et al.*, 1980; Norris *et al.*, 1987; Collins *et al.*, 1988; Takahashi and Iizuka, 1991; Dangel *et al.*, 1996). The increase in β_2AR number results from an increase in the rate of synthesis of new receptors (Norris *et al.*, 1987), which in turn is preceded by increased steady state levels of β_2AR mRNA (Collins *et al.*, 1988; Hadcock and Malbon, 1988; Zhong and Minneman, 1993; Mak *et al.*, 1995; Dangel *et al.*, 1996) and increased rates of transcription of the β_2AR gene (Collins *et al.*, 1988; Mak *et al.*, 1995). Taken together, these findings suggest that enhanced β_2AR gene transcription is a principal mechanism underlying glucocorticoid-mediated increases in β_2AR levels.

GREs mediate transcriptional activation of numerous eukaryotic genes by glucocorticoid receptors (Beato *et al.*, 1989). Comparison of these response elements has revealed a consensus, 15-nucleotide, nearly palindromic sequence that binds the glucocorticoid receptor (Nordeen *et al.*, 1990). The β_2AR genes from several mammalian species contain GRE-like sequences in both coding and noncoding regions (Emorine *et al.*, 1987; Kobilka *et al.*, 1987; Buckland *et al.*, 1990; Jiang and Kunos, 1995; McGraw *et al.*, 1996). Although there is indirect evidence to suggest that regions in the 5′-noncoding region of the β_2AR gene are involved (Malbon and Hadcock, 1988), the specific genetic elements responsible for the functional effect of glucocorticoids on β_2AR gene transcription have yet to be identified.

Recently, we extended the sequence of 5′-flanking DNA of the rat β_2AR gene to approximately 3.7 kilobases upstream

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ABBREVIATIONS: AR, adrenergic receptor; GRE, glucocorticoid response element; EMSA, electrophoretic mobility shift assay; bp, base pair(s); MMTV, mouse mammary tumor virus; TK, thymidine kinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

from the start of translation (McGraw *et al.*, 1996). Sequence analysis indicated that this segment of the gene contains six putative GREs that could potentially mediate the effect of glucocorticoids on β_2AR gene expression. Using a combination of transient transfection assays with β_2AR-luciferase fusion genes and EMSAs, we now show that a GRE-like element located at positions −379 to −365 (relative to the transcription start site) confers glucocorticoid inducibility to the rat β_2AR gene. Our data also suggest that, within the context of the β_2AR promoter, the activity of this GRE may be regulated by additional unidentified genetic elements.

Experimental Procedures

Materials. Dexamethasone was purchased from Sigma Chemical (St. Louis, MO) and was dissolved in ethanol to a stock concentration of 200 μM. The restriction enzymes *Avr*II and *Nhe*I were purchased from Boehringer Mannheim (Indianapolis, IN). All other restriction enzymes were purchased from Promega Corp. (Madison, WI). Plasmids pRShGRα and N-600 prATLUC were kindly provided by Robert McGehee (Arkansas Children's Hospital, Little Rock, AR).

Cell culture. HepG2 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY) that had been depleted of steroid hormones. Serum was stripped of steroids by the addition of 1 g of activated charcoal (Sigma)/100 ml of serum and incubation at 55° for 1 hr. The charcoal-treated serum was centrifuged at 23,000 × g for 10 min at 4°. The supernatant was filter-sterilized and stored at −20° before use.

Plasmid construction. We have extended the known sequence of the rat β_2AR gene to position −3491 (McGraw *et al.*, 1996). The nucleotide numbering system that we used in this study is based on the assignment of +1 to the transcription start site (nucleotide −220 relative to the start of translation, as previously determined) (Jiang *et al.*, 1996). Chimeric gene constructs were made by fusing β_2AR gene fragments with pGL3-Basic (Promega), a promoterless luciferase expression vector. Initially, pβ_2AR(−3129/+126), pβ_2AR(−1115/+126), and pβ_2AR(−152/+126) were prepared by subcloning restriction fragments from the 5′-flanking region of the rat β_2AR gene into the available sites of pGL3-Basic, as previously reported (McGraw *et al.*, 1996). pβ_2AR(−2552/+126), pβ_2AR(−643/+126), and pβ_2AR(−62/+126) were prepared by linearization of β_2AR(−3129/+126) with *Mlu*I and *Nhe*I and unidirectional digestion of the linearized plasmid with exonuclease III/mung bean nuclease (Stratagene, La Jolla, CA). Short segments of β_2AR DNA containing putative GREs were subcloned into pT81LUC (Nordeen, 1988), a luciferase expression vector driven by a minimal TK promoter, to test their ability to enhance expression of a heterologous promoter. For these constructs, double-stranded oligonucleotides containing either GRE_1 or GRE_5 and the polymerase chain reaction-generated β_2AR gene fragment containing GRE_2, GRE_3, and GRE_4 were cloned into the *Xma*I site of pT81LUC. The sequence and orientation of all constructs were confirmed by dideoxy sequencing (Sanger *et al.*, 1977) using Sequenase version II (United States Biochemical Corp., Cleveland, OH). All plasmids used in transfections were prepared using Bigger Prep plasmid preparation kits (5 Prime-3 Prime, Boulder, CO).

Mutagenesis. GRE_5 was mutated in plasmid pβ_2AR(−3129/+126) using the oligonucleotide gaaagaataagctcacccggacacgc and the GeneEditor *in vitro* site-directed mutagenesis system (Promega). The resulting mutant, pβ_2ARm1(−3129/+126), replaced the guanine at position +6 of GRE_5 with an adenine. The mutation was confirmed by dideoxy sequencing (Sanger *et al.*, 1977). The identity between the remainder of pβ_2AR(−3129/+126) and pβ_2ARm1(−3129/+126) was confirmed by restriction site analysis.

Polymerase chain reaction. The relatively short segment (−831 to −708) in the β_2AR gene containing three putative GREs was amplified from rat genomic DNA using a Perkin-Elmer model 480 thermal cycler (Norwalk, CT) and β_2AR gene-specific primers. The primers were 5′-catatacccgggcgaagttactgccttggtgcggttg-3′ (sense) and 5′-catatacccgggggcaagaacacaggaggtgactc-3′ (antisense). Each primer was synthesized with a *Xma*I site to facilitate cloning of the amplified product into pT81LUC. The reaction mixture included 0.5 μg of rat genomic DNA, 0.5 pmol of each primer, 0.2 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 2.5 units of *Thermus aquaticus* DNA polymerase (Promega). A 5-min hot start at 94° was used, followed by 30 cycles of 94° for 1 min and 66° for 1 min and then a final single cycle of 72° for 7 min.

Transient transfections. HepG2 cells were transfected by calcium phosphate coprecipitation in 60-mm dishes, as described previously (Chen and Okayama, 1987). For experiments in which dexamethasone-stimulated promoter activity in different 5′-deletion constructs was tested, subconfluent cells in Dulbecco's modified Eagle medium with 10% fetal bovine serum that had been stripped of steroids were transfected with 0.38 pmol of the β_2AR-luciferase fusion genes, 2 μg of pRSVβgal, 1 μg of pRShGRα, and pGEM-7Zf(−) to adjust the amount of total DNA in each dish to 8.33 μg. HepG2 cells were co-transfected with pRShGRα, a human glucocorticoid receptor expression vector (Brasier *et al.*, 1990), because replicating cells can be deficient in *trans*-acting factors such as the glucocorticoid receptor (Johnson, 1990). Cells were exposed to precipitates overnight and the medium was changed. Either vehicle or dexamethasone (final concentration, 0.1 μM) was added 24 hr after transfection, and cells were harvested after an 8-hr incubation. After harvesting, cell lysates were prepared and assayed for luciferase using the Promega luciferase assay system or for β-galactosidase using the Galacto-Light system (TROPIX, Bedford, MA), with a Monolight model 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). For experiments in which the ability of putative β_2AR GREs to enhance the activity of a heterologous promoter in the presence of dexamethasone was assessed, the dual-luciferase reporter assay system (Promega) was used as described above, except that pRSVβgal was omitted from the transfection mixture. In its place, pRL-SV40 encoding *Renilla* luciferase was used as a measure of transfection efficiency.

Human recombinant glucocorticoid receptor and nuclear extract preparation. Human recombinant glucocorticoid receptor was obtained from Affinity Bioreagents (Golden, CO). Nuclear extracts were prepared essentially as previously described (Andrews and Faller, 1991). Except where otherwise noted, centrifugations were performed in an Eppendorf model 5415C microfuge at maximum speed, at room temperature. Approximately 10^6 to 10^7 HepG2 cells that had been transiently transfected with pRShGRα were scraped into 1.5 ml of cold phosphate-buffered saline, pH 7.4, and pelleted. Cells were resuspended in 400 μl of buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) at 4°. Cells were allowed to swell for 10 min, they were vortex-mixed for 10 sec and then centrifuged for 10 sec, and the supernatant was discarded. The pellet was resuspended in 20–50 μl of buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) at 4° and incubated for 20 min. Cellular debris was removed by centrifugation for 2 min at 4°, and the supernatant containing DNA-binding proteins was stored at −70°. Nuclear extract protein concentrations were determined (Bradford, 1976) using bovine serum albumin as the standard.

EMSAs. Oligonucleotides were either commercially synthesized (Bio-Synthesis, Inc., Lewisville, TX) or, in the case of oligonucleotides containing the human tyrosine aminotransferase gene GRE, were obtained from Affinity Bioreagents. The sequences of the sense oligonucleotides only are shown in Table 1. Previously, glucocorticoid receptor bound to a functional GRE in MMTV was shown to occupy no more than 30 bp of DNA (Nordeen *et al.*, 1990). Therefore, the oligonucleotides that we used in EMSAs included 35 nucleotides of

β_2AR gene sequence plus an additional five nucleotides comprising a restriction site for subcloning of the fragment. Complementary oligonucleotides in equimolar amounts were heated to 100°, cooled overnight to 25°, divided into aliquots, and stored at −20° before use. Double-stranded oligonucleotide probes were end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Binding reactions were performed in a 20-μl volume containing approximately 20,000 cpm of labeled probe, 6–12 μg of nuclear extract or human recombinant glucocorticoid receptor (Affinity Bioreagents), 20 mM HEPES, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 10% glycerol, 200 ng of poly(dI·dC), 1 μg of bovine serum albumin, and unlabeled competitor oligonucleotides. The instructions provided by the manufacturer were followed when the human recombinant glucocorticoid receptor was used in EMSAs. The binding reaction mixtures were incubated at 25° for 30 min and then loaded onto 6% nondenaturing polyacrylamide gels in 25 mM Tris, pH 8.3, 25 mM boric acid, 0.5 mM EDTA. For supershift experiments, incubations with polyclonal anti-human glucocorticoid receptor antibody (Affinity Bioreagents) or preimmune antiserum (diluted 1:500) were performed for 30 min at room temperature after addition of radiolabeled GRE_5 and nuclear extract. Gels were dried and autoradiographed overnight at −70°, using Fujifilm RX-Fuji medical X-ray film and intensifying screens.

Results

The β_2AR gene. In an earlier study, in addition to correcting an error in the previously reported sequence of the rat β_2AR gene (Buckland *et al.*, 1990), we cloned an additional 1400 bp of 5′-flanking DNA (McGraw *et al.*, 1996). Analysis of the known sequence of the rat β_2AR gene yielded seven potential glucocorticoid regulatory elements (Fig. 1A). Six of the potential GREs are located upstream from the receptor open reading frame, whereas the seventh GRE is located in the 3′-flanking region of the gene. Sequence comparisons were made between the putative GREs in the β_2AR gene and the consensus sequence for previously demonstrated, positively modulated GREs. This consensus GRE sequence arose from analysis of GRE-like elements in the following genes: MMTV, Moloney murine sarcoma provirus, metallothionein IIa, lysozyme, vitellogenin, growth hormone, uteroglobin, tyrosine aminotransferase, tryptophan oxygenase, and acidic glycoprotein (Nordeen *et al.*, 1990) (Fig. 1B). In preliminary studies, the putative GRE downstream from the receptor open reading frame was found to be nonfunctional (data not shown); therefore, we focused our attention on the six GRE-like elements in the 5′-flanking region of the gene. We have numbered these GRE sequences 1 through 6, with GRE_6 being the most proximal.

Transient transfections using β_2AR promoter truncations. To first determine which regions of the β_2AR 5′-flanking region are necessary for glucocorticoid-mediated transcriptional activation, a series of six 5′-deletion fragments were fused to a luciferase reporter gene. Among these fragments, pβ_2AR(−3129/+126) and pβ_2AR(−2552/+126) contain all six putative GREs, pβ_2AR(−1115/+126) contains the proximal five putative GREs, pβ_2AR(−643/+126) contains GRE_5 and GRE_6, and pβ_2AR(−152/+126) and pβ_2AR(−62/+126) contain only the most proximal GRE. To test the effect of dexamethasone on the expression of the β_2AR-luciferase fusion genes, luciferase activity was determined in transfected HepG2 cells after incubation with either vehicle or 0.1 μM dexamethasone for 8 hr. Results from preliminary experiments indicated that 8 hr was the optimal time to observe dexamethasone responsiveness, because cell viability decreased with longer exposures to dexamethasone (data not shown). Fig. 2 depicts the results of experiments in which progressively truncated β_2AR-luciferase fusion genes transiently transfected into HepG2 cells were tested for dexamethasone responsiveness. Approximately 2-fold induction with dexamethasone (compared with levels in the absence of added glucocorticoid) was observed with pβ_2AR(−3129/+126), pβ_2AR(−2552/+126), and pβ_2AR(−1115/+126). This level of induction of luciferase activity is similar to the 2–4-fold induction in β_2AR levels that has been observed in the lung after injection of rats with dexamethasone (McGraw *et al.*, 1995) or after addition of glucocorticoids to cultured cells (Collins *et al.*, 1988; Takahashi and Iizuka, 1991; Dangel *et al.*, 1996). With pβ_2AR(−643/+126), the dexamethasone induction was approximately 1.3-fold. In contrast, no dexamethasone effect was observed with pβ_2AR(−152/+126) or pβ_2AR(−62/+126). As a positive control, we used N-600 prATLUC, a fusion gene containing a segment of the rat angiotensinogen gene with two functional GREs coupled to a luciferase-encoding gene. In the presence of dexamethasone, expression of N-600 prATLUC was increased approximately 8–10-fold, consistent with the level of glucocorticoid induction previously demonstrated with this fusion gene in HepG2 cells (Brasier *et al.*, 1989). These results indicated that the region between positions −643 and −152 was necessary for dexamethasone induction of β_2AR gene expression. This region contains a single GRE (GRE_5), which then became the focus of additional experiments.

EMSAs using nuclear extracts. To determine whether nuclear transcription factors could indeed bind to GRE_5, we performed EMSAs using a 35-bp double-stranded oligonucleotide that includes GRE_5 and nuclear extracts prepared from HepG2 cells that had been treated with 0.1 μM dexamethasone for 8 hr. In preliminary experiments in which increasing amounts of HepG2 cell nuclear extracts were added to radiolabeled GRE_5 probe, we determined that 6 μg of nuclear extract resulted in optimal levels of shifted product (data not shown). Incubation of radiolabeled GRE_5 with HepG2 nuclear extracts resulted in a prominent shifted band (Fig. 3). The specificity of binding was confirmed by observing that the addition of increasing concentrations of unlabeled GRE_5 displaced radiolabeled GRE_5 in a dose-dependent manner, whereas the unlabeled random oligonucleotide did not suppress radiolabeled GRE_5 binding (Fig. 3).

TABLE 1

Oligonucleotides used in EMSAs and luciferase assays with pT81LUC

With the exception of GRE_{TAT}, both sense and antisense oligonucleotides were synthesized by Bio-Synthesis. The sense and antisense oligonucleotides for GRE_{TAT} (the human tyrosine aminotransferase GRE) were obtained from Affinity Bioreagents. Only the sense oligonucleotides, of the complementary pairs, are shown. Bold nucleotides represent putative core GREs within each oligonucleotide. Underlined nucleotides represent restriction enzyme sites added to facilitate cloning into plasmid vectors. GRE_5, GRE_1, and MMTV GRE were prepared with both *Xma*I and *Hin*dIII ends. *Xma*I sites were used to clone double-stranded oligonucleotides into plasmid pT81LUC. Only GRE_5 with *Hin*dIII ends was used as a probe in EMSAs. The mutated nucleotide in the putative core GRE in GRE_5 is bold and underlined.

Oligonucleotides	Sequence
GRE_5	AGCTTCGTGTCC**GGGTGAGCTTGTTCT**TTCTGTTA
m1GRE_5	CCGGGCGTGTCC**GGGTGAGCTTATTCT**TTCTGTTC
GRE_1	AGCTTGAGGCAA**CGAGTAACGTGTTCT**AAATGAGA
MMTV GRE	AGCTTGTTTTTG**GTTACAAACTGTTCT**TAAAACGA
GRE_{CON}	CCGGGCGTGTCC**GGTACAGCTTGTTCT**TTCTGTTC
GRE_{TAT}	CTAGGC**TGTACAGGATGTTCT**GCCTAG
Random oligonucleotide	AGCTTGTGTGTGTTTTATTCCTGTTTGGCAAGA

To assess the immunological identity of the proteins in the band shift complex produced with HepG2 cell nuclear extracts and GRE_5, supershift experiments were carried out with polyclonal anti-human glucocorticoid receptor antibody. The anti-human glucocorticoid receptor antibody interacted with the GRE_5-protein complex, as evidenced by the appearance of a more slowly migrating band (Fig. 4). Nonspecific antiserum had no effect on the mobility of the GRE_5-protein complex, nor did the anti-human glucocorticoid receptor antibody interact directly with GRE_5 (Fig. 4).

EMSAs using the human recombinant glucocorticoid receptor. We further characterized the ability of GRE_5 to bind the glucocorticoid receptor *in vitro* using EMSAs with human recombinant glucocorticoid receptor and serial dilutions of competitor oligonucleotides. Radiolabeled GRE_5 incubated with human recombinant glucocorticoid receptor resulted in a single shifted band (Fig. 5). Addition of increasing concentrations of unlabeled GRE_5 displaced radiolabeled GRE_5 in a dose-dependent manner (Fig. 5). In contrast, increasing concentrations of either unlabeled GRE_1 or unlabeled mutant m1GRE_5 (with a single nucleotide change in the core sequence of GRE_5) showed decreased ability to compete with radiolabeled GRE_5 for binding to human recombinant glucocorticoid receptor (Fig. 5).

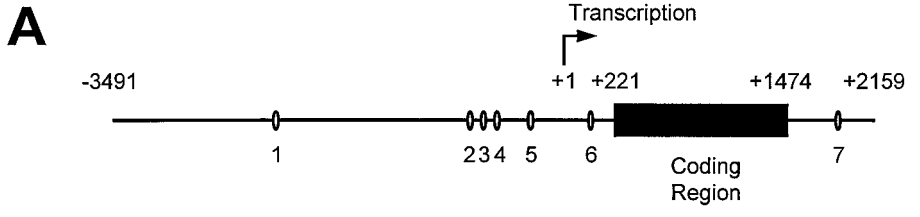
Incubation of radiolabeled GRE_{TAT} (the GRE from the human tyrosine aminotransferase gene) with the human recombinant glucocorticoid receptor resulted in a single shifted band (Fig. 6). Addition of increasing concentrations of unlabeled GRE_{TAT}, GRE_5, or GRE_{CON} (a 15-bp consensus GRE flanked by sequence surrounding GRE_5 in the β_2AR gene) displaced radiolabeled GRE_{TAT} from the human recombinant glucocorticoid receptor in a dose-dependent manner (Fig. 6).

Transient transfections using a β_2AR-luciferase fusion gene with mutated GRE_5. To further test the involvement of GRE_5 in glucocorticoid regulation of β_2AR expression, a plasmid [pβ_2ARm1(−3129/+126)] was constructed that had been mutated at position +6 of GRE_5 (gggtgagctgttct to gggtgagct*a*ttct). This mutation, the same base change as in oligonucleotide m1GRE_5 (Table 1), is essential for glucocorticoid inducibility of a MMTV GRE (Nordeen *et al.*, 1990). Our results demonstrated loss of glucocorticoid inducibility using pβ_2ARm1(−3129/+126) (Fig. 7). Interestingly, in the absence of added dexamethasone, the activity of pβ_2ARm1(−3129/+126) was markedly lower than that of pβ_2AR(−3129/+126) (Fig. 7). A possible explanation is that basal expression of pβ_2AR(−3129/+126) in HepG2 cells that overexpress the glucocorticoid receptor is relatively high, despite removal of glucocorticoids from the serum by charcoal stripping. Alternatively, GRE_5 contributes to the basal activity of the β_2AR gene promoter.

Transient transfections using putative GREs fused to a heterologous promoter. To further examine the functionality of the putative GREs, fragments that contained either GRE_1, GRE_5, or GRE_2 plus GRE_3 and GRE_4 together were fused to a luciferase expression plasmid driven by a minimal TK promoter. An MMTV fragment containing a functional GRE (Majors and Varmus, 1983) cloned into pT81LUC was used as a positive control. Approximately 4-fold induction was observed with dexamethasone using the MMTV-pT81LUC fusion gene (Fig. 8). Activity of GRE_5-pT81LUC was induced 3.2-fold in the presence of dexamethasone (Fig. 8), a value that was higher than that observed with any of the β_2AR-luciferase fusion genes that included GRE_5. Activity of GRE_1-pT81LUC was not induced by dexamethasone (Fig. 8). Transfection of HepG2 cells with the segment −831 to −708 (which contains GRE_2, GRE_3, and GRE_4) fused to pT81LUC resulted in luciferase activity in either vehicle- or dexamethasone-treated cells that was below the level of detection in the assay (Fig. 8). This result suggests the presence of an element in the segment −831 to −708 of the β_2AR gene that negatively affects the activity of the minimal TK promoter.

Discussion

Glucocorticoids increase β_2AR expression and β_2-agonist-stimulated adenylyl cyclase activity in a variety of tissues and cell types (Cheng *et al.*, 1980; Norris *et al.*, 1987; Collins *et al.*, 1988; Takahashi and Iizuka, 1991; Zhong and Minneman, 1993; Dangel *et al.*, 1996). For most steroid hormone-responsive genes, including those regulated by glucocorticoids, changes in the level of expression induced by hormone



B

Glucocorticoid Response Element	Location in β_2AR	Sequence	Identity With Consensus GRE
GRE_1	-2235/-2221	CGAGTAACGTGTTCT	11/15
GRE_2	-809/-795	TTGCCTGCATGTTCT	10/15
GRE_3	-765/-751	TTCACCAAGTGTGCT	10/15
GRE_4	-726/-712	CACCTCCTGTGTTCT	9/15
GRE_5	-379/-365	GGGTGAGCTTGTTCT	12/15
GRE_6	+60/+74	GCACCCACGAGCTCA	8/15
GRE_7	+1909/+1923	AATATCCTGTGGTCT	11/15
Consensus		GGTACANNNTGTTCT	

Fig. 1. Schematic diagram of the rat β_2AR gene and location of putative GREs. A, The coding region is indicated by a *solid rectangle*. The first nucleotide in the ATG codon that codes for the initiator methionine is designated +1. The approximate locations of putative GREs are indicated by *open ellipses* and are numbered 1 through 7. B, For each putative GRE, the exact location, sequence, and identity with the consensus GRE sequence (Nordeen *et al.*, 1990) are shown. *Underlined nucleotides*, identity with the consensus GRE.

are principally controlled by changes in the rate of transcription of the target gene (Beato *et al.*, 1989). These *trans*-activation effects are a result of the glucocorticoid hormone-receptor complex interacting with GREs within the target gene and thus acting as a transcription factor (Beato *et al.*, 1989). In the case of the β_2AR gene, the results of numerous studies suggest transcriptional regulation by glucocorticoids. Steady state levels of β_2AR mRNA in several cell lines and tissues have been shown to be increased in the presence of glucocorticoids (Collins *et al.*, 1988; Hadcock and Malbon, 1988; Takahashi and Iizuka, 1991; Zhong and Minneman, 1993; Mak *et al.*, 1995; Dangel *et al.*, 1996). The rate of transcription of the β_2AR gene has been shown, by nuclear run-off transcription assays, to be increased by glucocorticoids in both DDT_1 MF-2 cells (Collins *et al.*, 1988) and rat lung (Mak *et al.*, 1995).

In this study, the 5′-flanking region of the rat β_2AR gene was isolated and used to prepare β_2AR-luciferase fusion genes, as a means to identify *cis*-acting elements that mediate the stimulatory effects of glucocorticoids on β_2AR gene expression. The HepG2 human liver cell line was chosen for these studies because human hepatocytes are known to express the β_2AR (Bevilacqua *et al.*, 1987) and HepG2 cells were previously used to study glucocorticoid regulation of angiotensinogen gene expression (Brasier *et al.*, 1989, 1990). HepG2 cells are deficient in functional glucocorticoid receptors (Brasier *et al.*, 1990); however, this deficiency enabled us to investigate the role of glucocorticoids in the regulation of β_2AR gene expression by performing co-transfection with a glucocorticoid receptor-encoding expression plasmid.

Early reports demonstrated that, in rats, glucocorticoids decrease β_2AR number and β-agonist-stimulated adenylyl cyclase activity (Wolfe *et al.*, 1976; Chan *et al.*, 1979). More

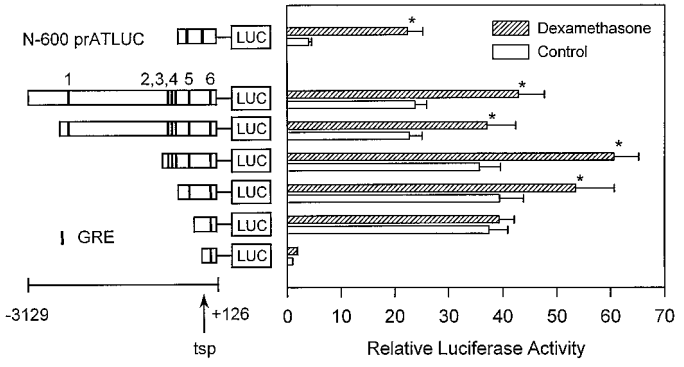


Fig. 2. Expression of β_2AR-luciferase fusion genes in HepG2 cells incubated in the absence or presence of dexamethasone. Human HepG2 cells were transiently transfected with the human glucocorticoid receptor expression vector pRShGRα (Brasier *et al.*, 1990), the β-galactosidase expression vector pRSVβgal, and β_2AR-luciferase fusion genes containing 3254, 2677, 1240, 768, 277, or 187 bp of 5′-flanking DNA from the β_2AR gene linked to a firefly luciferase-encoding gene (*LUC*) in pGL3-Basic (Promega), as described in the text. After transfection, the cells were incubated for 8 hr in either the absence or presence of 0.1 μM dexamethasone and were harvested. Values are means ± standard errors of data from nine independent experiments, each performed in triplicate. *Arrow*, primary transcription start site in the β_2AR gene. *, Significant ($p < 0.05$) difference in luciferase activities in lysates from untreated and dexamethasone-treated cells, as determined by Student's *t* test.

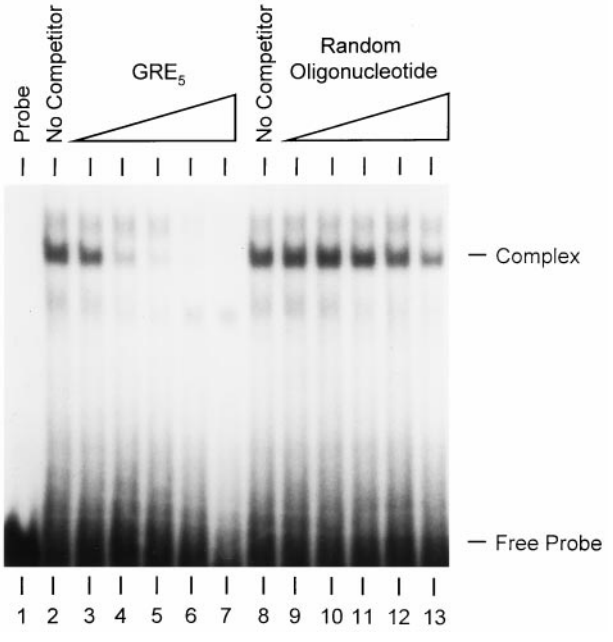


Fig. 3. EMSA characterization of HepG2 cell nuclear proteins that interact with GRE_5. Nuclear extracts (6 μg) prepared from HepG2 cells transfected with pRShGRα were incubated with radiolabeled GRE_5 in the presence of increasing concentrations of the indicated double-stranded oligonucleotide, as described in the text. *Lane 1*, radiolabeled GRE_5 alone; *lanes 2* and *8*, radiolabeled GRE_5 incubated with nuclear extract; *lanes 3–7*, radiolabeled GRE_5 incubated with nuclear extract and a 10-, 50-, 100-, 250-, or 500-fold molar excess of GRE_5, respectively; *lanes 9–13*, radiolabeled GRE_5 incubated with nuclear extract and a 10-, 50-, 100-, 250-, or 500-fold molar excess of the random oligonucleotide, respectively. The sequences of the oligonucleotides are shown in Table 1. Labeled complexes were separated on polyacrylamide gels and visualized by autoradiography.

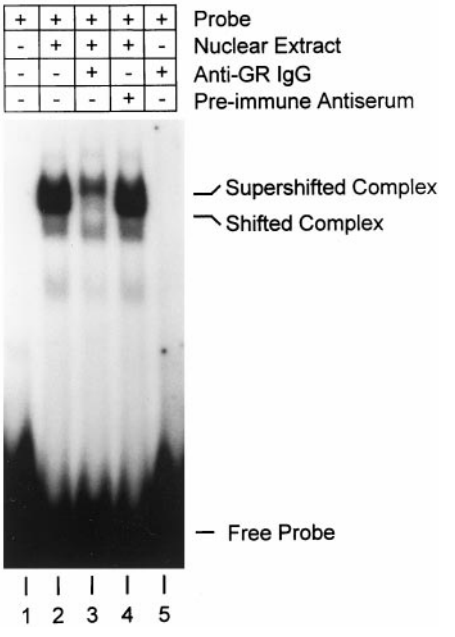


Fig. 4. Immunological characterization of the protein-GRE_5 complexes. Supershift assays were performed as described for EMSAs except that, after the addition of either anti-human glucocorticoid receptor antibody or preimmune antiserum to the mixture of radiolabeled GRE_5 and nuclear extract, the incubation was continued for 30 min at room temperature. *Lane 1*, radiolabeled GRE_5 alone; *lane 2*, radiolabeled GRE_5 incubated with nuclear extract; *lane 3*, radiolabeled GRE_5 incubated with nuclear extract and anti-human glucocorticoid receptor (*Anti-GR*) antibody; *lane 4*, radiolabeled GRE_5 incubated with nuclear extract and preimmune antiserum (diluted 1:500); *lane 5*, radiolabeled GRE_5 incubated with anti-human glucocorticoid receptor antibody. Labeled complexes were separated on polyacrylamide gels and visualized by autoradiography.

recent evidence suggests that glucocorticoid regulation of rat liver β_2AR expression is age dependent. Hepatic β_2AR number, although decreased in young rats, is actually increased in aged rats by glucocorticoids (Slotkin *et al.*, 1996). To the best of our knowledge, glucocorticoid regulation of the β_2AR has not been studied in human liver. However, our data clearly demonstrate that the HepG2 cell line is a valid model system in which to study glucocorticoid regulation of β_2AR gene expression.

Cloning of the rat β_2AR gene (Buckland *et al.*, 1990; Jiang and Kunos, 1995; McGraw *et al.*, 1996) has allowed investigation of the genetic elements involved in glucocorticoid regulation of β_2AR gene expression. Indirect evidence obtained in an early study suggested that the putative *cis*-acting elements were located in the 5′-flanking region of the β_2AR gene (Malbon and Hadcock, 1988). Examination of the rat β_2AR gene sequence for known transcription factor binding sites indicated that six potential GREs are located within the proximal 3.7 kilobases of 5′-flanking DNA. In the transfection analyses using various β_2AR-luciferase fusion genes, we were able to demonstrate that dexamethasone responsiveness was mediated by a region spanning nucleotides −643 to −152. Within this segment is a single putative GRE, with the sequence gggtgagcttgttct (Fig. 1). This GRE, designated GRE_5, displays reasonable similarity to the consensus GRE sequence ggtacannntgttct (Beato *et al.*, 1989). Approximately 2-fold induction with dexamethasone was observed with the longer β_2AR-luciferase fusion gene constructs that contained GRE_5. This level of induction was less than that observed with N-600 prATLUC. However, it should be noted that numerous investigators have obtained similar levels of induction (2–4-fold) with glucocorticoid administration, in measurements of either receptor numbers, steady state mRNA levels, or β_2AR gene transcription rates, in a variety of cells and tissues (Norris *et al.*, 1987; Collins *et al.*, 1988; Hadcock and Malbon, 1988; Takahashi and Iizuka, 1991; Zhong and Minneman, 1993; Mak *et al.*, 1995; Dangel *et al.*, 1996). Therefore, the GRE that we have identified as lending glucocorticoid responsiveness to β_2AR gene expression seems to be physiologically relevant.

The reason for the diminished responsiveness of the β_2AR gene to glucocorticoid induction, compared with that of other glucocorticoid-inducible genes such as N-600 prATLUC (Brasier *et al.*, 1989), is unclear at this time. The limited similarity of GRE_5 to the consensus GRE (Fig. 1B) suggests that the biological response of the β_2AR gene to dexamethasone stimulation may be related to the extent of homology to the consensus GRE. Of interest was the observation that GRE_5, when fused to a luciferase expression vector driven by

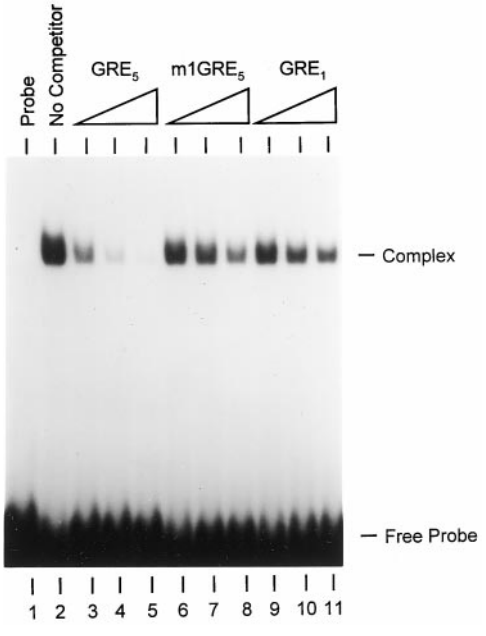


Fig. 5. Specificity of the interaction between GRE_5 and the human recombinant glucocorticoid receptor, as determined in EMSAs. Human recombinant glucocorticoid receptor (6 μg), in the absence of added glucocorticoids, was incubated with radiolabeled GRE_5 in the presence of increasing concentrations of the indicated double-stranded oligonucleotide, as described in the text. *Lane 1*, radiolabeled GRE_5 alone; *lane 2*, radiolabeled GRE_5 incubated with human recombinant glucocorticoid receptor; *lanes 3–5*, radiolabeled GRE_5 incubated with human recombinant glucocorticoid receptor and a 25-, 100-, or 250-fold molar excess of GRE_5, respectively; *lanes 6–8*, radiolabeled GRE_5 incubated with human recombinant receptor and a 25-, 100-, or 250-fold molar excess of $m1GRE_5$, respectively; *lanes 9–11*, radiolabeled GRE_5 incubated with human recombinant receptor and a 25-, 100-, or 250-fold molar excess of GRE_1, respectively. The sequences of the oligonucleotides are shown in Table 1. Labeled complexes were separated on polyacrylamide gels and visualized by autoradiography.

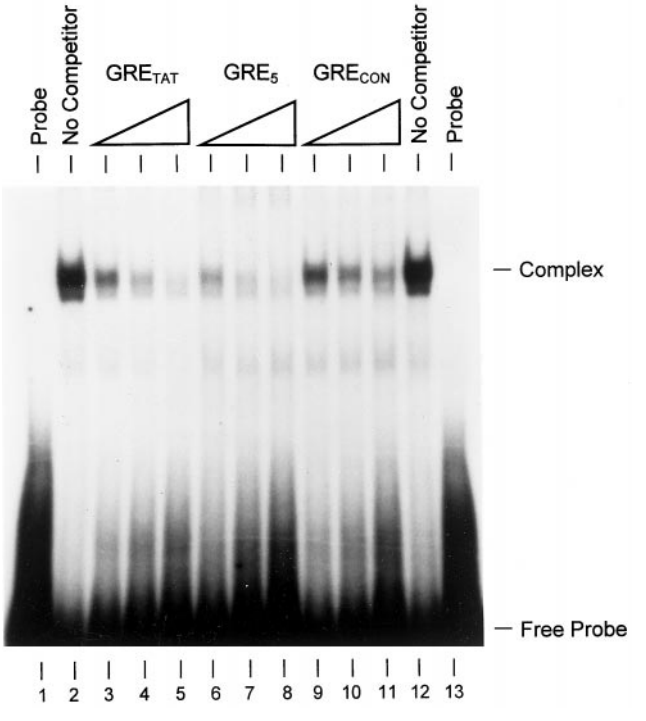


Fig. 6. Specificity of the interaction between GRE_5 and the human recombinant glucocorticoid receptor, as determined in EMSAs. The human recombinant glucocorticoid receptor (6 μg), in the absence of added glucocorticoid, was incubated with radiolabeled GRE_{TAT} in the presence of increasing concentrations of the indicated double-stranded oligonucleotide, as described in the text. *Lanes 1 and 13*, radiolabeled GRE_{TAT} alone; *lanes 2 and 12*, radiolabeled GRE_{TAT} incubated with human recombinant glucocorticoid receptor; *lanes 3–5*, radiolabeled GRE_{TAT} incubated with human recombinant glucocorticoid receptor and a 25-, 100-, or 250-fold molar excess of GRE_{TAT}, respectively; *lanes 6–8*, radiolabeled GRE_{TAT} incubated with human recombinant glucocorticoid receptor and a 25-, 100-, or 250-fold molar excess of GRE_5, respectively; *lanes 9–11*, radiolabeled GRE_{TAT} incubated with human recombinant glucocorticoid receptor and a 25-, 100-, or 250-fold molar excess of GRE_{CON}, respectively. The sequences of the oligonucleotides are shown in Table 1. Labeled complexes were separated on polyacrylamide gels and visualized by autoradiography.

the basal TK promoter and transiently transfected into HepG2 cells, resulted in 3.2-fold induction of activity. This induction was similar to that obtained with MMTV sequences cloned into pT81LUC. These results suggest either that the β_2AR promoter is a relatively weak substrate for GRE enhancer activity, that the activity of GRE_5 is constrained by sequences in the 5′-flanking region of the β_2AR gene, or that other segments of the β_2AR gene that are missing from our reporter gene constructs are necessary for greater induction by glucocorticoids. In many genes, GREs have been demonstrated to synergize with other transcription factor-binding elements to increase gene activity. For example, a direct interaction between interleukin-1-inducible NFκB binding to an acute-phase response element and two flanking GREs has been demonstrated to underlie the acute-phase activation of the angiotensinogen gene in rat liver (Ron *et al.*, 1990). Through recruitment of hepatic nuclear factor 3, protein kinase A has been shown to modulate the activity of a GRE in the rat tyrosine aminotransferase gene (Espinas *et al.*, 1995) and phosphoenolpyruvate carboxykinase genes (Wang *et al.*, 1996). Finally, widely spaced GREs have been shown to be additive in stimulating glucocorticoid induction of the tryptophan oxygenase gene in rat liver (Danesch *et al.*, 1987).

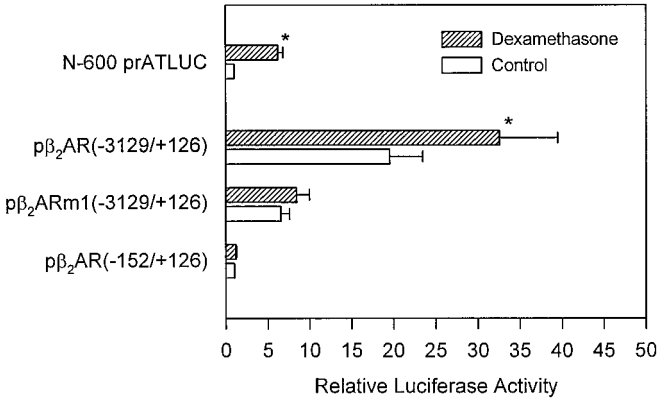


Fig. 7. Effect of a single point mutation in GRE_5 on the dexamethasone inducibility of a β_2AR-luciferase fusion gene. Human HepG2 cells were transiently transfected with pRShGRα, pRSVβgal, and either pβ_2AR(−3129/+126) or pβ_2ARm1(−3129/+126), as described in the text. After transfection, the cells were incubated for 8 hr in either the absence or presence of 0.1 μM dexamethasone and were harvested. Values are means ± standard errors of data from five independent experiments, each performed in triplicate. *, Significant ($p < 0.05$) difference in luciferase activity, compared with untreated and dexamethasone-treated cells, as determined by Student's *t* test.

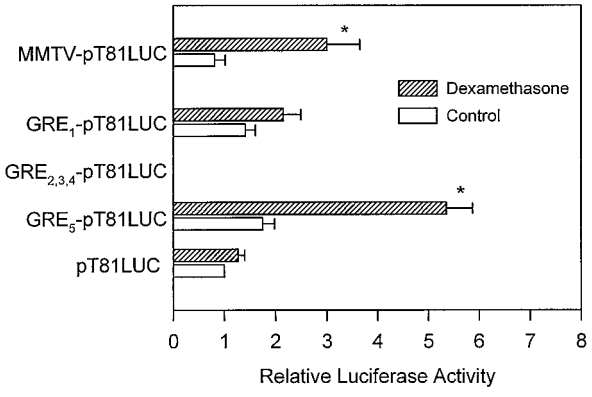


Fig. 8. Expression of GRE-luciferase fusion genes in HepG2 cells incubated in the absence or presence of dexamethasone. HepG2 cells were transiently transfected with the human glucocorticoid receptor expression vector pRShGRα (Brasier *et al.*, 1990), pRL-SV40, and luciferase fusion genes consisting of either GRE_5, GRE_1, or MMTV double-stranded oligonucleotides linked to a firefly luciferase-encoding gene in pT81LUC (Nordeen, 1988), as described in the text. The sequences of the double-stranded oligonucleotides are shown in Table 1. After transfection, the cells were incubated for 8 hr in either the absence or presence of 0.1 μM dexamethasone and were harvested. Values are means ± standard errors of data from five independent experiments, each performed in triplicate. *, Significant ($p < 0.05$) difference in luciferase activities in lysates from untreated and dexamethasone-treated cells, as determined by Student's *t* test.

The results from the EMSAs confirmed that GRE_5 is capable of binding nuclear proteins isolated from glucocorticoid-treated HepG2 cells, as well as the human recombinant glucocorticoid receptor. A double-stranded oligonucleotide containing GRE_5 and surrounding sequence bound a protein in nuclear extracts prepared from glucocorticoid-treated HepG2 cells that was immunologically identified as a glucocorticoid receptor. Moreover, the same double-stranded oligonucleotide bound the human recombinant glucocorticoid receptor. Interestingly, a single base change in GRE_5 greatly reduced the ability of the mutant GRE ($m1GRE_5$) to compete with radiolabeled GRE_5 for binding to the human recombinant glucocorticoid receptor. This change (guanine to adenine in position +6 of the GRE) was previously shown to result in the complete loss of glucocorticoid inducibility of a MMTV GRE fused to a luciferase reporter gene (Nordeen *et al.*, 1990).

Glucocorticoids are important therapeutic agents used in the treatment of asthma, but a small proportion of asthmatic patients are resistant to the beneficial effects of glucocorticoids (Cypcar and Busse, 1993). The molecular mechanisms underlying this form of steroid resistance remain unclear, although one of the beneficial effects of using glucocorticoids to treat asthma is increased β_2AR expression (Barnes, 1996). Our EMSA results demonstrated that a single nucleotide change in GRE_5 of the rat β_2AR gene resulted in a greatly diminished ability to bind the human recombinant glucocorticoid receptor. This same nucleotide change in pβ_2AR(−3129/+126) resulted in a fusion gene, pβ_2ARm1(−3123/+126), that was no longer inducible by dexamethasone, as determined in transient transfection assays. Interestingly, mutations in the regulatory DNA of the human apolipoprotein (Suzuki *et al.*, 1997) and 5-lipoxygenase (In *et al.*, 1997) genes have been shown to alter their expression, although the functional significance is currently not clear. The *cis*-acting elements in the human β_2AR gene that mediate glucocorticoid responsiveness have not yet been identified. Nevertheless, the demonstration of steroid resistance in human patients, together with our present findings regarding glucocorticoid regulation of β_2AR gene expression, suggests that the molecular basis for steroid resistance in a subset of asthmatic patients could involve mutations in GREs in the β_2AR gene.

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