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Negative Regulation of the Rat Glutathione *S*-Transferase A2 Gene by Glucocorticoids Involves a Canonical Glucocorticoid Consensus Sequence

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ABSTRACT

Glucocorticoids (GCs) repress both basal and polyaromatic hydrocarbon-induced expression of the glutathione *S*-transferase Ya$_1$ gene (*gstA2*) in isolated rat hepatocytes and rat liver *in vivo*. Transient transfection experiments with HepG2 cells were used to identify GC-responsive elements (GREs). With cotransfected GC receptor, chloramphenicol acetyltransferase (CAT) constructs containing a palindromic GRE (pGRE) and three GRE hexanucleotide half-sites between −1.6 and −1.1 kb of the 5′-flanking region of *gstA2* were repressed >50% by GC when induced with polyaromatic hydrocarbon. This pGRE, if either mutated or deleted, significantly reduces GC responsiveness of the gene to 20–30%; no effect of GC was observed with CAT constructs containing −1.15 kb of the 5′-flanking region. The dexamethasone concentration dependence of the repression was consistent with involvement of the GC receptor and was antagonized by RU38486. Electrophoretic mobility shift assays demonstrated that pGRE formed a specific DNA/protein complex, which was prevented by the addition of excess unlabeled or mouse mammary tumor virus GRE but not by unrelated or mutated *gstA2* GRE double-stranded oligonucleotides. This complex was supershifted by incubation of nuclear extracts containing GC receptor with anti-GC receptor globulins. Constructs containing multiple copies of pGRE sequence were either nonresponsive or positively responsive (three copies) to GC. Luciferase constructs containing −1.62 to −1.03 kb of the 5′-flanking region also were regulated positively by GC. Chimeric GC-peroxisome proliferator activated receptor activated the constructs that were positively responsive to GC but did not mediate the negative effect in constructs containing 1.6 kb of 5′-flanking region. We conclude that pGRE and half-site GREs of *gstA2* participate in regulation of this gene; however, a second unidentified responsive element must exist between −1.03 and −0.164 kb, resulting in repression of *gstA2* expression.

The cytosolic glutathione *S*-transferases are a superfamily of dimeric enzymes of subunit molecular mass of 20–30 kD (Rushmore and Pickett, 1993; Hayes and Pulford, 1995). These proteins are classified as families based on their degree of sequence identity and on their ability to form heterodimeric proteins whose catalytic functions are intermediate relative to their respective homodimers (Mannervik *et al.*, 1985). These proteins play a major role in the detoxification of xenobiotic chemicals by catalyzing the conjugation of glutathione with their electrophilic centers, thus preventing protein or nucleic acid alkylation reactions. In addition, some glutathione *S*-transferases seem to serve as intracellular binding proteins for nonsubstrate ligands (Listowsky, 1993).

The molecular events involved with the transcriptional activation of rat *gstA2* subunit gene have been well characterized by Pickett and coworkers (Paulson *et al.*, 1990; Rushmore *et al.*, 1991). The *gstA2* subunit named using the nomenclature proposed by J. D. Hayes (Hayes and Pulford, 1995) is the rat gene whose cDNA was isolated as clone pGTB45-15 (Telekowski-Hopkins *et al.*, 1988). Single copies of two *cis*-acting responsive elements, the AHRRE and ARE (Rushmore *et al.*, 1990), have been characterized for their role in the induction of this protein subunit by xenobiotic compounds. The AHRRE core sequence (5′-TNGCGTG-3′) also is found in multiple copies in the 5′-flanking region of the *CYP1A1* gene, for which we have characterized the potentiation of the PAH-dependent induction by GC (Mathis *et al.*, 1989; Xiao *et al.*, 1995; Prough *et al.*, 1996). This responsive element is activated on binding the heterodimeric complex of the AHR and

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ABBREVIATIONS: *gstA2*, glutathione *S*-transferase 1 (Ya$_1$) subunit gene; AHRRE, aryl hydrocarbon receptor response element; AHR, aryl hydrocarbon receptor; ARE, antioxidant response element; BA, 1,2-benzanthracene; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer binding protein; bp, base pair(s); CMV, cytomegalovirus; DEX, dexamethasone; GC, glucocorticoid; GRE, hexonucleotide glucocorticoid response element [TGT(T/C)CT]; PCR, polymerase chain reaction; pGRE, palindromic glucocorticoid response element; HNF, hepatic nuclear factor; PAH, polyaromatic hydrocarbon; PPAR, peroxisome proliferator activated receptor; MMTV, mouse mammary tumor virus.

ARNT protein (AHR nuclear translocater). The ligands for the cytosolic AHR are planar chlorinated compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or PAHs such as BA and β-naphthoflavone. The ARE core sequence (GTGACNNNGC) is required for induction of *gstA2* message and protein by metabolites of aromatic compounds, phenolic antioxidants, phorbol esters, and hydrogen peroxide, suggesting a mechanism for activation involving reactive oxygen species (Rushmore *et al*., 1991; Nguyen *et al*., 1994). Similar ARE regulatory sequences are found in the human (Jaiswal *et al*., 1988) and rat (Favreau and Pickett, 1993) NAD(P)H:quinone acceptor oxidoreductase genes.

Several other responsive elements have been implicated in the constitutive expression of this protein (Paulson *et al*., 1990; Mendel and Crabtree, 1991; Pimental *et al*., 1993), namely for the hepatic nuclear factors HNF-1, HNF-4, and C/EBP. Genes regulated by these families of transcription factors use characteristic consensus sequences and may display developmental control. For example, genes regulated by HNF-1 in rats (Mendel and Crabtree, 1991) are expressed during neonatal life, whereas those regulated by C/EBPβ may not be fully expressed until adolescence (Lee *et al*., 1994).

We have shown that GCs modulate the PAH-dependent induction of the *gstA2* subunit protein (Sherratt *et al*., 1990; Linder and Prough, 1993; Xiao *et al*., 1995; Prough *et al*., 1996). In neonatal rats, GCs potentiate the PAH-dependent induction of the gene, whereas in adolescent rats, which display higher basal levels of expression, GCs suppress PAH induction (Linder and Prough, 1993). In both cases, these effects were shown to be regulated at the transcriptional level. Cell culture experiments conducted with both fetal (Sherratt *et al*., 1990) and adult (Xiao *et al*., 1995; Prough *et al*., 1996) rat hepatocytes have suggested two possible mechanisms of GC regulation. The first occurs at concentrations of the synthetic GC, DEX, of $<1 \times 10^{-7}$ M (i.e., in the concentration range expected for agonist/GC receptor binding) that potentiate the PAH-dependent induction of the subunit in fetal cells but suppress the effect in adult hepatocytes. A second mechanism is observed at higher concentrations of GC (1×10^{-6} to 1×10^{-4} M), resulting in increased expression of this protein. This second mechanism has been observed in both fetal (Sherratt *et al*., 1990) and adult (Xiao *et al*., 1995; Prough *et al*., 1996) rat hepatocytes.

In examining the 5′-flanking region of the *gstA2* subunit gene, we discovered several sequences between −1.65 and −1.15 kb that have homology to the canonical consensus hexanucleotides [5′-TGT(T/C)CT-3′] for the GC receptor. This report explores the hypothesis that the *gstA2* subunit gene is under regulatory control of GC due to binding of the ligand-activated GC receptor to its functional canonical consensus response element in the 5′-flanking region of the gene.

Experimental Procedures

Materials. Restriction endonucleases, pGL2-basic and T4 ligase, were purchased from Promega (Madison, WI) or New England Biolabs (Beverly, MA). pCR II cloning kits were obtained from InVitrogen (San Diego, CA). The 5′-flanking constructs of *gstA2* gene, p4.0YaCAT, p1.6YaCAT, p1.15YaCAT, and p0.164YaCAT, have been described previously (Telakowski-Hopkins *et al*., 1988; Paulson *et al*., 1990). pCMV-β was obtained from Clonetech (Palo Alto, CA). The reporter construct p2XDEX-LUC and pRSVGR, the expression vector for the human GC receptor, were a kind gift from Michael Mathis (LSU Medical Center, Shreveport, LA). pGR-PPAR has been described previously (Boie *et al*., 1993). pcDNA3 was purchased from InVitrogen.

Reagents for culturing *Escherichia coli* were obtained from Difco Laboratories (Detroit, MI). Penicillin, streptomycin, nonessential amino acids, and Dulbecco's modified Eagle's medium (DME/high modified) were purchased from JRH BioSciences (Lexena, KS). Fetal bovine serum, Fungizone, and Geneticin were obtained from Life Technologies (Gaithersburg, MD). Magic or Wizard Minipreps kits purchased from Promega were used for initial screening, whereas Mega preps kits from Qiagen (Chatworth, CA) were used to produce transfection-quality plasmid DNA. PCR reagents were purchased from Perkin-Elmer Cetus (Norwalk, CT).

1,2-Benzanthracene, DEX, and chloramphenicol were obtained from Sigma Chemical (St. Louis, MO). Nafenopin was obtained from Ciba-Geigy Chemical (Ardsley, NY). RU 38486 [17β-hydroxy-11β(4-dimethylamino-phenyl)-17α-(prop-1-ynyl)-estra-4,9-dien-3-one] was obtained from Roussel Uclaf (Romainville, Cedex, France). Chlorophenol red-β-D-galactopyranoside was purchased from Boehringer-Mannheim (Indianapolis, IN). *n*-Butyryl CoA and poly(dI-dC) were obtained from Pharmacia (Piscataway, NJ). Radiolabeled compounds [^{3}H]chloramphenicol and [^{32}P]dCTP were obtained from Du Pont New England Nuclear (Boston, MA.). Oligonucleotides were synthesized using a Applied Biosystems model 308B DNA synthesizer (Applied Biosystems Division, Perkin-Elmer, Foster City, CA). All other reagents were purchased from commercial suppliers and were either American Chemical Society or molecular biology grade.

Cells and culture conditions. *E. coli DH5α* and HB 101 cells were transformed routinely with plasmids of interest. pCRII-derived plasmids were grown in *E. coli* of the ONE-SHOT strain (InVitrogen). The human hepatoblastoma cell line HepG2 (HB8065, American Type Culture Collection, Rockville, MD) was maintained in Dulbecco's modified Eagle's medium supplemented with 250 μg/ml Fungizone, 10 units/ml penicillin, 10 units/ml streptomycin, and 10% fetal bovine serum. The hepatoma cells were incubated at 37° in a 5% carbon dioxide atmosphere and were subcultured every 2–3 days.

PCR products. A 1651-bp PCR product used to construct p1.62YaLUC was synthesized using an upstream primer that contains bp complementary to position −1620 to −1578 of p1.6YaCAT and a 5′ *Nde*I site with extra bp (YaGRETOP, 5′-GGAATTCCAT ATGTGGGAGC ATTCCAGAACA AGCTGTACCA CCAAGGGTCA CT-3′) and a 33-mer downstream primer with a 5′ *Hin*dIII extension and extra bp (BMYaUNI, 5′-AGACTAAGCT TGGGTTGTAA AAGAGAGTAC TGA-3′). A 371-bp PCR product used to construct 0.164YaLUC was synthesized from 0.164YaCAT using the upstream primer PRIMNDE1 (5′-GTGAGCGAGGAAGCGGAAGA-3′), which is complementary to bp 2523–2503C of pRSVoCAT and BMYaUNI. PCR was performed in a Thermolyne Amplitron II thermal cycler (Barnstead/Thermolyne, Dubuque, IA) with 2 mM Mg^{2+}. The products were generated through 20 cycles of the following steps: denaturing temperature at 94° for 0.5 min, annealing temperature 50° for 1 min, and elongation temperature 72° for 1 min. A 604-bp product was generated from p1.6YaCAT using YaGRETOP and a downstream primer complementary to bp −1052 to −1032 of the 5′-flanking region of the *gstA2* gene and a restriction site for *Nde*I with extra bp (YaHALFBOT, 5′-GGAATTCCAT ATGGCCATTT GCCTGTGGTC ACG-3′). These were designed for cloning DNA products into the unique *Nde*I restriction site of p0.164YaLUC vector.

To introduce a mutated pGRE at position −1606 bp of the 5′-flanking region of *gstA2*, a mutant pGRE PCR product was produced by initially making two PCR products. The first was prepared using p1.6YaCAT as a template, a universal top primer (5′-TGATGCGGTA TTTTCTCCTT AC-3′) for pSVoCAT and a mutated GRE-containing bottom primer (5′-TGGTACAGCT CGTGCTGGAA TGCT-3′) to yield a 160-bp product, whereas the second was prepared by using a

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mutated GRE-containing top primer (5′-AGCATTCCAG CACGAGCTGT ACCA-3′) and a universal bottom primer (5′-GGTTCATGGG TCTTGGTTAT TAA-3′) for p1.6YaCAT to yield a 930-bp product. Both PCR products were mixed in the absence of primers, and subsequently two cycles of PCR at 94° for 30 sec, 57° for 30 sec, and 72° for 60 sec were performed using standard PCR reagents to produce a full-length template. At this point, the universal top primer and bottom primers were added and the PCR procedure was continued for an additional 29 cycles to form a pGRE-mutated 1060-bp fragment. The mutation of the GRE was confirmed by sequencing and found to contain only the two changes specified above.

Plasmid constructs. pCMV-GR was produced by subcloning the *Xho*I/*Kpn*I fragment from pRSVGR containing the coding region for the human GC receptor into the unique *Xho*I/*Kpn*I sites of pcDNA3. p1.52YaCAT was synthesized by digesting p1.6YaCAT with *Xba*I and *Nde*I, followed by treatment with Klenow fragment and religation of the resultant fragment. pCR-GREMUT was generated by subcloning the mutated 1060-bp PCR product described previously into a pCRII vector. pKCF28, a construct nearly identical to p1.6YaCAT but containing a mutated pGRE at bp −1609 to −1594, was made by digesting both p1.6YaCAT and pCR-GREMUT with *Xba*I and *Nde*I and by religating the 90-bp fragment containing the mutated GRE into the *Xba*I/*Nde*I-restricted p1.6YaCAT parent vector.

p1.62YaLUC was synthesized by subcloning the 1651-bp PCR product made from YaGRETOP and BMYaUNI initially into pCRII. Orientation of the insert was determined by restriction analysis using *Hin*dIII. The insert in reverse orientation was removed by digestion with *Kpn*I and *Hin*dIII, and the resultant fragment was subcloned into the unique *Kpn*I and *Hin*dIII sites of pGL2-basic. p0.164YaLUC was synthesized by cloning the 371-bp PCR product made using PRIMNDE1 and BMYaUNI initially into pCRII and subcloning the insert by digestion with *Kpn*I and *Hin*dIII. The resultant fragment was subcloned into the unique restriction sites of pGL2-basic to form 0.164YaLUC.

pKCF68 and 74 were generated by subcloning the 604-bp PCR product that contains bp −1620 to −1032 of the 5′-flanking region of *gstA2*, produced using YaHalfBOT and YaGRETOP from p1.6YaCAT into the pCRII vector. Digestion with *Nde*I allowed subcloning of the fragment into the *Nde*I restriction site of the minimal promoter construct p0.164YaLUC, yielding pKCF68 and pKCF74, with the fragment in the reverse and forward orientation, respectively. The pGRE series of plasmids was generated by subcloning annealed oligonucleotides of either the 25 mers complementary to bp −1614 to −1594 (5′-ATTCCAGAAC AAGCTGTACC AAGGG-3′ and 5′-ATCCCTTGGT ACAGCTTGTT CTGGA-3′) or the 47 mers complementary to bp −1620 to −1580 and an *Nde*I site (5′-TATGTGGGAG CATTCCAGAA CAAGCTGTAC CACCAAGGGT CACTCCA-3′ and 5′-TATGGAGTGA CCCTTGGTGG TACAGCTTGT TCTGGAATGC TCCCACA-3′) into the *Nde*I cloning site of p0.164YaCAT. After annealing the 25- or 47-mer oligonucleotides, the double-stranded oligonucleotides with overhanging AT sequences were mixed with the *Nde*I-cleaved p0.164YaCAT construct to allow incorporation of one or more copies of the pGRE double-stranded oligonucleotides. The stoichiometry of vector to insert was optimized to favor insertion of multiple copies of the pGRE into the vector (i.e., 1:50–100 ratio, respectively). After ligation with T4 ligase, *E. coli* DH5α cells were transformed with the newly ligated plasmids, and plasmid DNA was isolated for analysis with *Ava*I/*Hae*II restriction enzymes. The plasmid constructs were sequenced and noted to contain the expected sequence identical to the original oligonucleotide in the orientations noted below. pKCF29 was synthesized by subcloning the annealed 47 mers into the unique *Nde*I site of p1.15YaCAT.

Transfection of hepatoma cells. HepG2 cells were transfected at 40% confluence, treated with various agents, and harvested after 24 hr using methods described previously (Rushmore *et al.*, 1990). All cells were cotransfected with pCMV-β as a transfection control and pRSVGR, the expression plasmid for human GR. Routinely, 2 μg of plasmids with viral promoters (pCMV-β or pRSVGR) or 4 μg of the respective *gstA2* promoter CAT or luciferase construct was added per flask. The inducing agents, BA, DEX and nafenopin, were added as 500× concentrated stocks in dimethylsulfoxide; controls received dimethylsulfoxide alone. Cells transfected with luciferase reporter plasmids were harvested with 0.5 ml of cell lysis buffer (Promega, Madison, WI) according to manufacturer's instructions.

Assays of CAT, β-galactosidase, and luciferase activity. The CAT assay used in this study was a variation of the method of Gorman *et al.* (1982), which includes xylene extraction of the products and liquid scintillation quantification. Reactions were performed in 100-μl reactions of cell extract (120 μg of protein) in 0.25 M Tris·HCl, pH 7.5, containing 3.7 mM chloramphenicol (25 nCi) and 5 μg of *n*-butyryl CoA for 1 hr at 37°. Samples initially were extracted with 300 μl of xylene, and after reextraction of 250 μl of the xylene phase with 100 μl of reaction buffer, the enzyme activity was calculated as the volume-adjusted ratio of radioactivity in 200 μl of organic and 50 μl of aqueous phase. This method gave identical results (not shown) to the thin layer chromatography method described by Gorman *et al.* (1982). Luciferase activity was determined using the luciferase assay system from Promega. Luciferase activity was measured with 20 μl of cell extract over a 10-sec time period in a Berthold model LB9501 Lumat luminometer (Wallac, Gaithersburg, MD). For the β-galactosidase assays, cell extracts (30 μg of protein) were incubated with chlorophenol red β-galactopyranoside at 37° for 1 hr. Activity was determined spectrophotometrically at 595 nm on a Titretek Uniskan II plate reader (Flow Laboratories, McLean, VA).

Electrophoretic mobility shift assays. Nuclear extracts were prepared from rat liver as described previously (Gorski *et al.*, 1986), placed into aliquots, and stored at −70°. Polyclonal anti-human GC receptor antibodies (PA1–511) were obtained from Affinity Bioreagents (Golden, CO). Nuclear extracts were incubated with radiolabeled probe at 30° for 30 min before resolution on a polyacrylamide gels using low ionic strength buffers (Chodosh, 1995). The gels were dried and analyzed by exposure to a Molecular Dynamics Phosphor Screen in a Molecular Dynamics PhosphorImager (Sunnyvale, CA). HepG2-GR4 cells were produced by selection of cells transfected with pCMV-GR, which formed clonal colonies in the presence of 1.8 mg/ml (~2 weeks). Individual colonies thereafter were maintained on 0.9 mg/ml Geneticin. Both concentrations of geneticin were toxic to untransfected cells. The colony HepG2-GR4 was selected for its GC responsiveness in transfection assays with p2XDEX-LUC, a reporter containing two copies of the MMTV-GRE, relative to HepG2 cells cotransfected with pRSV-GR.

Statistical analysis. Student's *t* tests were used to discriminate significance between groups. Fold induction and the degree of repression were analyzed by fitting to theoretical equations with the least-squares regression program Kineti77 (Clark and Carrol, 1986).

Results

Deletion and mutational analysis. In preliminary experiments, we tested the ability of DEX to effect expression of CAT constructs containing various segments of the 5′-flanking region of the rat *gstA2* gene. Although DEX did down-regulate *gstA2* gene expression slightly in HepG2 cells, the results were not consistent when the expression vector for the GC receptor was omitted. In our hands, consistent repression of 1.6YaCAT expression occurred only when the GC receptor expression plasmid was cotransfected with the CAT construct (data not shown). We determined that 2 μg of pRSVGR, the expression plasmid for the human GC receptor, gave consistent responsiveness to DEX, suggesting that HepG2 cells express the GC receptor (Lui *et al.*, 1993) at levels much lower than those found in hepatic tissues *in vivo*.

The effects of treatment of various 5′-deletion constructs

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containing the *gstA2* promoter with PAH and GCs are shown in Fig. 1. BA caused an ~8-fold increase in CAT activity with all constructs tested (data not shown). DEX had no consistent effect on the low basal level of activity of these CAT constructs but suppressed the BA-dependent induction in p4.0YaCAT and p1.6YaCAT by ~40–50%. With the construct, p1.56YaCAT, the suppression was ~20–30%, and no significant repression was observed with p1.15YaCAT. These results are consistent with at least one functional GC-responsive *cis*-acting element existing between −1630 bp and −1560 bp in the 5′-flanking region upstream of the translation start site. Sequence analysis indicates that there is an imperfect pGRE located in this region whose sequence is identical to that described by Beato (1989) for the GC consensus palindrome. Our results are consistent with the involvement of this pGRE in the full GC-mediated decrease in expression.

To test further the involvement of the pGRE in GC regulation, we constructed a plasmid (pKCF28) in which two bases had been mutated in the perfect half (AGAACA to AGCACG) of the palindrome. Relative affinity determinations made using electrophoretic mobility shifts (La Baer and Yamamoto, 1994) with the MMTV GRE showed that replacement of A to C and A to G in the perfect consensus half of its palindrome caused 3.6- and 6.4-fold reductions in binding affinity for the GC receptor, respectively. The ability of DEX to suppress PAH-dependent activation of this plasmid was nearly identical to that of p1.56YaCAT, the construct in which the pGRE had been deleted. This implicates the palindromic GRE sequence as a critical element in the maximal negative regulation of this gene.

Although much of the GC sensitivity of the *gstA2* constructs was lost when the pGRE was either mutated or deleted, we wanted to test the hypothesis that the GRE half-sites, located between −1562 and −1150 bp, also might play an important role in this process, acting cooperatively with the pGRE. To test this hypothesis, we constructed a plasmid (pKCF29) that contained the pGRE, but not the half-sites, ligated to a −1150-bp 5′-flanking CAT construct. This plasmid was regulated negatively by GC by ~20–30%. These results are consistent with the half-sites also being involved and acting cooperatively with the pGRE to effect maximal repression. However, we cannot eliminate the possibility that other response elements or the spacing of the GREs also may play important roles in this negative regulatory effect.

Effects of RU38486. To document the involvement of the GC receptor in this regulation, we used the GC antagonist RU38486 to inhibit receptor function (Fig. 2). Administration of 10 μM RU38486 alone had little or no significant effect on either basal or BA-induced expression of p1.6YaCAT. RU38486 antagonized the DEX-dependent repression of PAH induction because expression levels of p1.6YaCAT were identical in samples treated with either BA or BA plus DEX when RU38486 was present. Because RU38486 is a type II antagonist of the GC receptor (i.e., it is translocated to the nucleus but does not form a transcriptionally-active DNA/protein complex), our results support the involvement of both the GC receptor and DNA binding of the receptor in the repressive effect of GCs on *gstA2* gene expression.

Effects of BA and DEX on luciferase expression of p1.62YaLUC. Because DEX had no apparent effect on the very low basal level expression of *gstA2* CAT constructs, we constructed a plasmid similar to p1.6YaCAT that contains the luciferase structural gene. In comparison to CAT constructs, luciferase reporter gene systems have low backgrounds and high assay sensitivity (Alam and Cook, 1990). The effects of BA and DEX on expression of p1.62YaLUC are presented in Fig. 3. As anticipated, BA caused a 15-fold induction, whereas *t*-butylhydroquinone caused a significant induction (~5-fold) in reporter expression (results not shown). Administration of DEX caused a 75 ± 7% and 82 ± 6% (average ± standard deviation) suppression in both basal and PAH-induced expression of this reporter system, respectively. The levels of suppression are similar to those observed

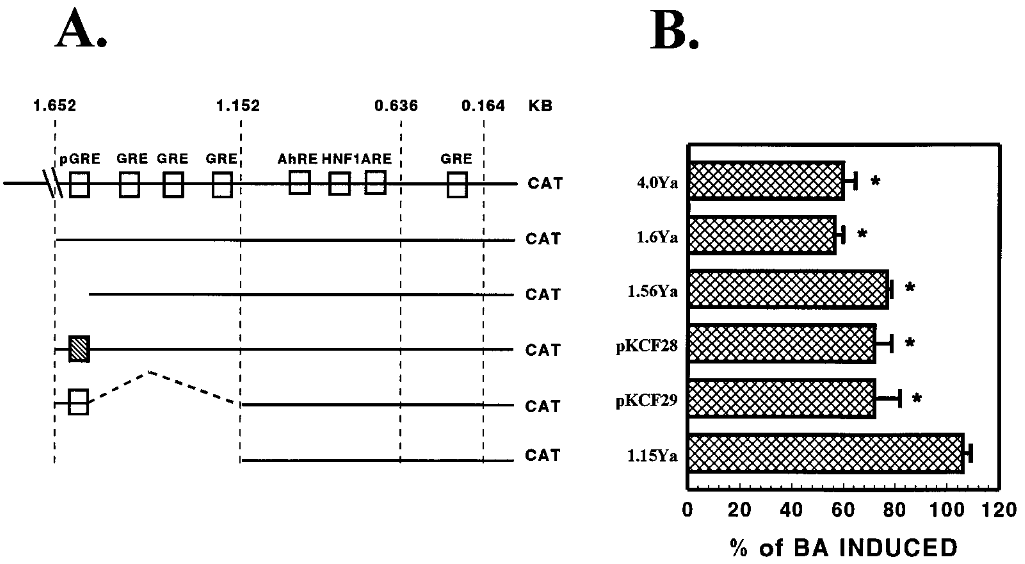

Fig. 1. Induction of CAT activity in HepG2 cells transiently transfected with 5′-deletion constructs of *gstA2*. A, Diagrammatic representation of the 5′-constructs used. *pGRE*, palindromic GRE. B, Activity of various deletion CAT constructs of *gstA2*. CAT and β-galactosidase assays were performed on lysed HepG2 cell transiently transfected with YaCAT constructs and the expression plasmids for pCMVβ and pRSVGR and subsequently treated with either 50 μM benzanthracene, 1 μM DEX ,or a combination for 24 hr as described in Experimental Procedures. CAT activity is expressed as the percent conversion of chloramphenicol to its acetyl derivative relative to β-galactosidase activity and is the mean ± standard deviation of three flasks. Activities were normalized for BA-induced transcription rate. *, Statistically different from PAH-induced CAT activity ($p < 0.05$).

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in animals (Linder and Prough, 1993) or isolated primary rat hepatocytes (Xiao *et al.*, 1995) and clearly demonstrates that GCs inhibit basal and PAH-induced gene expression. Basal expression of this gene is regulated transcriptionally by HNF1 and ARE responsive elements (Paulson *et al.*, 1990; Rushmore *et al.*, 1990). Suppression of both basal and induced expression is consistent with DEX negative regulation being independent of the action of the AHR (Xiao *et al.*, 1995).

Concentration-dependent repression of basal expression of p1.62YaLUC by DEX. To determine whether the response may be due to interaction with the GC receptor or to "nonclassic" mechanisms, the concentration dependence of the effects of DEX on the basal expression of p1.62YaLUC was tested (Fig. 4). DEX also suppressed the PAH-dependent induction at all concentrations tested except at $\leq 1 \times 10^{-11}$ M (data not shown). The repression was greatest at GC concentrations of 1×10^{-7} M, with significant reductions being observed with doses as low as 1×10^{-9} M. The concentration dependence of DEX suppression was the same as that observed with BA-induced CAT activity from p1.6YaCAT (results not shown). This concentration-dependent, monotonic decline is consistent with the process being mediated by the GC receptor. The concentration-dependence curve was extended to 1×10^{-5} M to examine whether any nonclassic mechanism of GC induction occurred as observed in both the fetal (Sherratt *et al.*, 1990) and adult (Xiao *et al.*, 1995) hepatocytes. No evidence of a biphasic concentration-response relationship was observed in the expression of p1.62YaLUC or p1.6YaCAT in HepG2 cells. Therefore, nonclassic induction mechanisms apparently do not influence this transient transfection system or affect the results with the concentration of DEX ($\leq 1 \times 10^{-6}$ M) routinely used in this study. Our work is similar to that observed by others in which nonclassic mechanism of GC action could be observed only in whole animals or primary cultures of hepatocytes (Schuetz *et al.*, 1984). The concentration dependence is similar to that observed in adult hepatocytes at low DEX concentrations (Prough *et al.*, 1996).

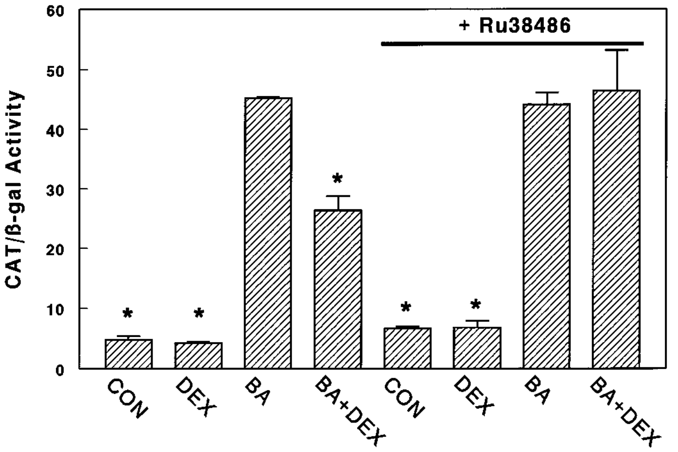

Fig. 2. Effects of RU38486 on CAT activity of HepG2 cells transiently transfected with p1.6YaCAT and treated with BA, DEX, or a combination. CAT and β-galactosidase assays were performed on lysates from HepG2 cells that had been transiently transfected with p1.6YaCAT and the expression plasmids for pCMVβ and pRSVGR and then treated with either 50 μM benzanthracene, 1 μM DEX, 10 μM RU38486, or combinations of these compounds for 24 hr as described in Experimental Procedures. *CON*, control. The normalized CAT activity is the percent conversion of chloramphenicol to its acetyl derivative relative to β-galactosidase activity and is the mean ± standard deviation of three flasks. *, Statistically different from CAT activity of BA-treated cells ($p < 0.01$).

Electrophoretic mobility shift assays. To test whether the palindromic GRE is capable of binding the GC receptor, we performed electrophoretic mobility shift experiments using the ^{32}P-labeled double-stranded oligonucleotides (Fig. 5A) whose sequence is identical to the pGRE of *gstA2* and nuclear extracts from rat liver, HepG2 cells, or HepG2-GR4 cells. HepG2-GR4 cells are stably transfected with a expression vector for the human GC receptor as described in Experimental Procedures. As can be seen in Fig. 5B, a specific DNA/protein complex was observed when the pGRE oligonucleotide of *gstA2* was mixed with rat nuclear extract and

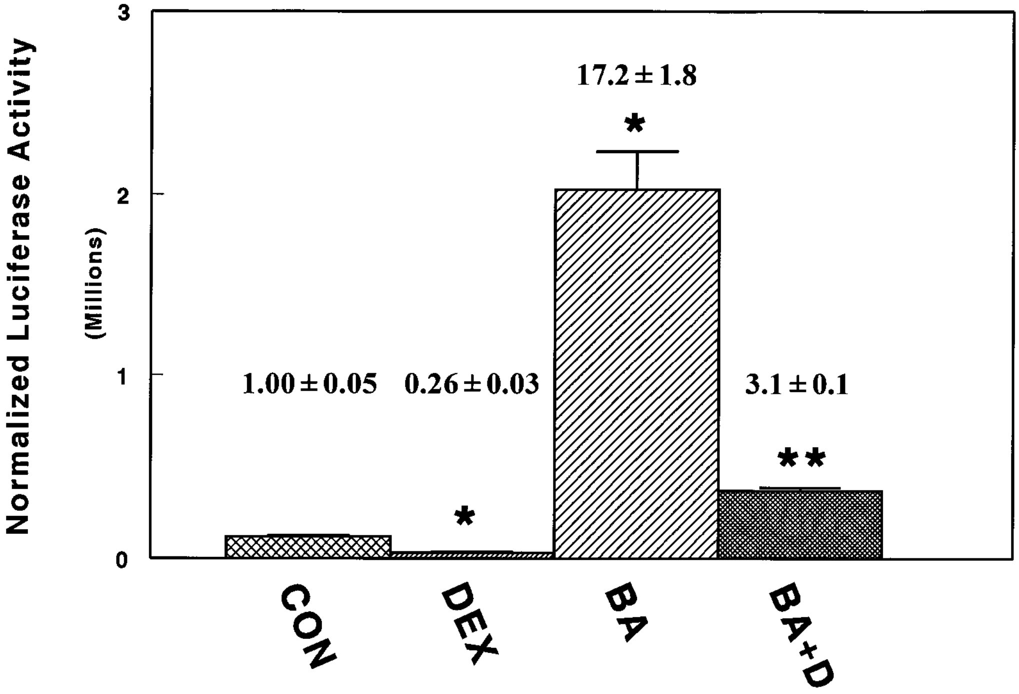

Fig. 3. Effects of DEX on the basal and PAH-induced luciferase activity in HepG2 cells transiently transfected with the plasmid p1.62YaLUC. Luciferase and β-galactosidase assays were performed on lysed HepG2 cells that had been transiently transfected with p1.62YaLUC and the expression plasmids for pCMVβ and pRSVGR subsequently treated with either 50 μM benzanthracene, 1 μM DEX, or in combination for 24 hr as described in Experimental Procedures. *CON*, control. The normalized luciferase activity is expressed as relative light units divided by β-galactosidase activity and is the mean ± standard deviation of three flasks. *, Statistically different from control cells ($p < 0.05$). **, Statistically different from cells treated with BA alone ($p < 0.05$).

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resolved by gel electrophoresis. The DNA/protein complex formed could be competed for effectively by double-stranded oligonucleotides whose sequence was identical to either the pGRE from the MMTV long terminal repeat or the pGRE from *gstA2* itself but not by an unrelated oligonucleotide, such as an oligonucleotide identical to the AHRRE from *CYP1A1*. When the MMTV pGRE was used as radiolabeled probe, 100- and 200-fold molar ratios of *gstA2* pGRE to MMTV pGRE double-stranded-oligonucleotide blunted DNA/protein complex formation by >80%; cold MMTV pGRE double-stranded oligonucleotide completely reversed complex formation, demonstrating that both pGREs compete for GR binding but that MMTV pGRE has a slightly higher affinity for the receptor than *gstA2* pGRE (data not shown). Using the same mutation strategy used in the transient transfection experiments, competition for DNA/protein complex formation by an oligonucleotide containing a mutated pGRE from *gstA2* was diminished significantly. A reduction in the ability of the mutated pGRE oligonucleotide to compete for complex formation is consistent with a loss of binding affinity for the GC receptor. Thus, complex formation could be prevented by inclusion of unlabeled oligonucleotides with sequence identity to the GC hexanucleotide consensus sequence (Fig. 5A), and mutation of the *gstA2* pGRE core sequence greatly reduced the ability of the oligonucleotide to bind protein.

To characterize further the identity of the specific DNA/protein complex formed, we performed antibody supershift experiments (Fig. 5C) with a polyclonal antibody raised against the GC receptor. Interestingly, control HepG2 extract and extracts form HepG2-GR4 cells both formed specific DNA/protein complexes of similar mobility. However, the antibody caused a significantly larger supershift in the extract derived from the GC receptor expressing cell line (HepG2-GR4). This result suggests that other proteins in the HepG2 extract may be capable of binding this element; however, when activated GC receptor is present, it may prevent other proteins from binding. In concert, these results strongly support the postulate that this response element binds the GC receptor, resulting in regulation of the *gstA2* gene.

Palindromic GRE CAT constructs. Because the pGRE of the *gstA2* gene apparently binds the GC receptor, we sought to establish whether introduction of these sequences into reporter constructs possibly accounts for the negative regulation of this gene by GC. To facilitate this, we synthesized oligonucleotides containing the pGRE, either 25 or 47 mers, and ligated them as double-stranded oligonucleotides into the *Nde*I site of p0.164Ya CAT. Of the plasmids tested (Fig. 6), only one was GC responsive; this plasmid pGRE5CAT contained three copies of the pGRE. All other plasmids tested contained either one or two copies of the GRE in several orientations and displayed levels of basal expression similar to the minimal promoter construct p0.164YaCAT; none were GC responsive. What is striking is the fact that the CAT activity of this plasmid is induced 15-fold by GC, whereas the PAH-induced CAT activity of the reporter gene containing the 1.6-kb 5′-flanking region of the native gene is repressed >60% by GCs. The 47-mer constructs were made to establish whether spacing between pGREs or the immediate flanking sequences were critical, as suggested by Schule *et al.* (1988); at least two palindromic sequences or a palindrome and several half-sites apparently are required for GC responsiveness, and adequate spacing must exist between the palindromes, half-sites, or both to ensure the optimal geometry for cooperativity of receptor binding and function. Our results (Fig. 6) suggest that unlike other strong pGREs (Lanz *et al.*, 1994) that confer GC responsiveness when present in two copies, at least three copies of the *gstA2* palindromic GRE are required for CAT-reporter constructs to be GC responsive. This response may be related in part to the strength of binding of GR to this pGRE. Schule *et al.* (1988) have shown that binding to weak GREs have greater synergistic effects than those to strong GREs, such as those found in MMTV, which forms a hormone-responsive element when present in only two copies.

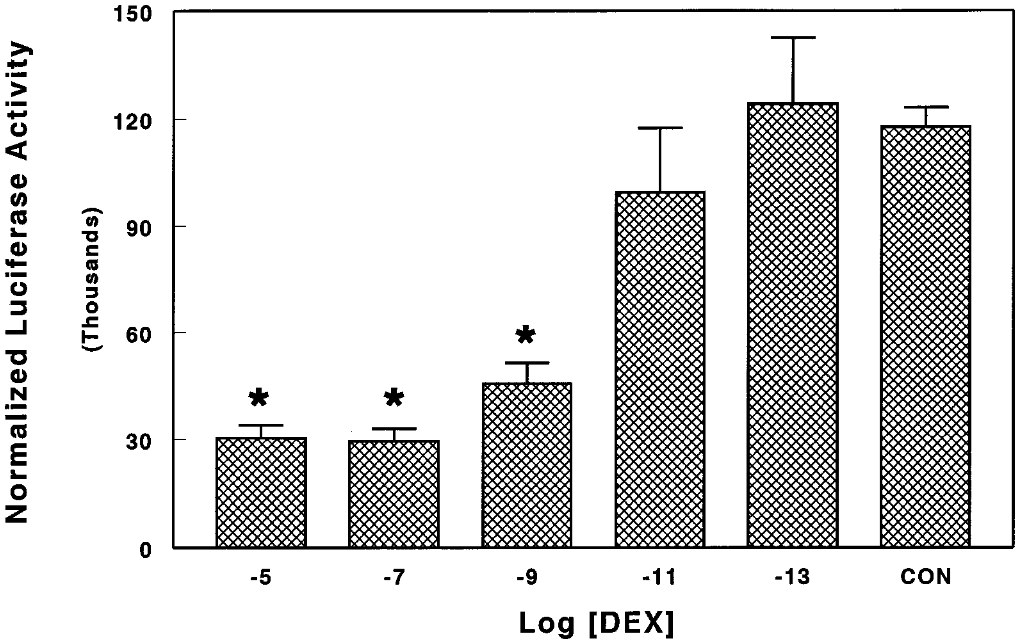

Fig. 4. Concentration dependence of DEX repression of luciferase activity in HepG2 cells transiently transfected with the plasmid p1.62YaLUC. Luciferase and β-galactosidase assays were performed on lysed HepG2 cells that had been transiently transfected with p1.62YaLUC and the expression plasmids for pCMVβ and pRSVGR and subsequently treated with varying doses of DEX for 24 hr as described in Experimental Procedures. The normalized luciferase activity is expressed as the relative light units divided by β-galactosidase activity and is the mean ± standard deviation of three flasks. *, Statistically different from untreated cells ($p < 0.05$).

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Because the magnitude of induction of pGRE5CAT is much smaller (15-fold) than that we have observed with plasmids containing two copies of the MMTV-GRE (40–100-fold; results not shown), the functional interaction of the GR with this specific sequence seems weaker than that seen with the MMTV-GRE, and therefore greater synergistic effects might be expected (Lanz *et al*., 1994).

Using PCR, we incorporated the putative GC responsive elements residing between −1620 and −1032 bp 5′ from the translation start site, which consists of the pGRE and three half-sites [TGT(T/C)CT], into a luciferase construct containing the *gstA2* promoter. We wanted to examine whether this section of 5′-flanking region contains *cis*-acting elements that could serve as a "classic" response element (i.e., capable of operating in a position- and an orientation-independent manner). DEX had a small positive effect (<30%) on the expression of the plasmid p0.164YaLUC containing just the basal promoter of the *gstA2* gene linked to the *LUC* gene (Fig. 7). Basal expression of the 1620-bp 5′-flanking construct, p1.62YaLUC, was significantly higher than either of the constructs containing the 588-bp region encompassing the four GREs or the basal plasmid (p0.164YaLUC). This is consistent with the results of Paulson *et al*. (1990), who demonstrated that the ARE and HNF-1 *cis*-acting elements were critical for the basal expression of this gene. The basal expression of pKCF68 and pKCF 74 was not greater than that of the minimal promoter (p0.164YaLUC), suggesting there are no functional, positive basally active *cis*-acting elements

A Oligonucleotides Used for Electrophoretic Mobility Shift Assays

gstA2 pGRE 5'-ATGGGGAGCATTCC**AGAACAAGCTGT**ACCACCAAGGGTC-3'

☺ ☺

Mutated pGRE 5'-ATGGGGAGCATTCC**AGCACGAGCTGT**ACCACCAAGGGTC-3'

MMTV LTR GRE 5'-AGCTTGGTCGACATGGTT**ACAAACTGTTCT**TAAAACAAGG-3'

AhRE1A1 5'-AGCTTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3'

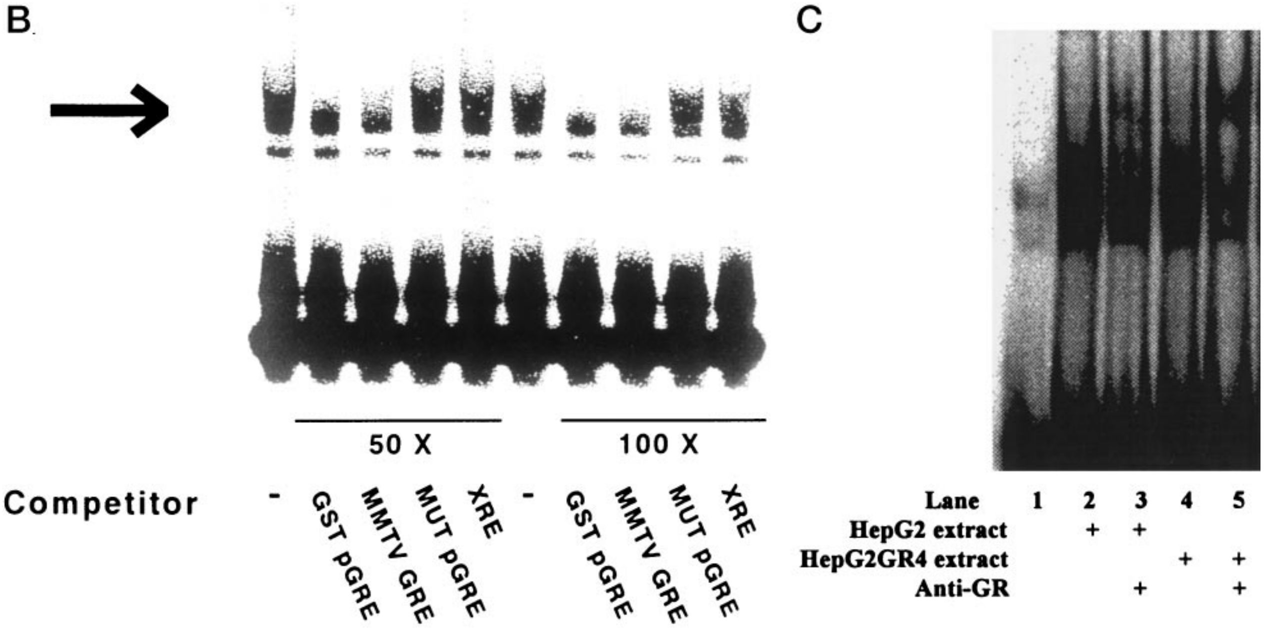

Fig. 5. Interaction between the pGRE located between bp −1611 and −1594 in the 5′-flanking region of *gstA2* and rat nuclear extract using electrophoretic mobility shift assay. A, Double-stranded oligonucleotides used for the ^{32}P-labeled probe and for competition are shown (coding strand only). *Boldface*, consensus nucleotides to the GRE palindrome (Schule *et al*., 1988). *Smiley faces*, aligned mutated sequences. B, Nuclear extract from livers of untreated male rat was incubated with the radiolabeled probe and resolved using a low ionic strength polyacrylamide gel electrophoresis. *Lanes 1–10*, probe and nuclear extract. *Lanes 2 and 7*, 50- and 100-fold excess of MMTV GRE, respectively. *Lanes 3 and 8*, 50- and 100-fold excess of *gstA2* pGRE, respectively. *Lanes 4 and 9*, 50- and 100-fold excess of mutant pGRE, respectively. *Lanes 5 and 10*, 50- and 100-fold excess of the unrelated oligonucleotide AHRRE, respectively. C, Nuclear extract from either HepG2 or HepG2-GR4 cells was incubated with probe in the absence or presence of a polyclonal antibody elicited against human GR. *Lanes 1*, probe alone. *Lanes 2*, probe and HepG2 nuclear extract. *Lane 3*, probe and HepG2 nuclear extract plus antibody. *Lanes 4*, probe and HepG2-GR4 nuclear extract. *Lanes 5*, probe and HepG2-GR4 nuclear extract plus antibody. The DNA/protein responses were measured using a PhosphorImager.

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in the 604-bp (588 bp of 5′-flanking region) GRE-containing region. Neither pKCF68 nor pKCF74 was sensitive to BA, which is consistent with the lack of a functional AHRRE in these constructs (results not shown). When GC responsiveness of the GRE-containing plasmids was tested (Fig. 7), both constructs, with the 604-bp fragment in forward (pKCF74) and reverse (pKCF68) orientation, displayed a positive response to GC (7–12-fold). These results suggest that this GC-responsive sequence can be described as classic because the effect of the response element is positive as well as orientation and position independent. However, the 1.65-kb construct including the 588-bp region functions negatively. In comparing the GC-repressed expression of p1.62YaLUC with p0.164YaLuc, the inhibited expression always is greater than that seen with the basal promoter, suggesting that the mode of regulation affects both constitutive and inducible enhancers interaction with the basal promoter in a DNA-dependent manner.

Chimeric PPAR-GR receptor specificity. To test further the hypothesis that the GC receptor acts differently in regulating the expression of p1.6YaCAT construct than for a CAT construct containing three copies of the pGRE (pGRE5CAT), we determined the ability of a chimeric receptor GR-PPAR to activate either pGRE5CAT or p1.6YaCAT. This receptor contains the GC receptor DNA binding domain fused to the PPARα ligand binding domain (Fig. 8A). Previous work (Boie *et al.*, 1993) has shown that this chimeric PPARα receptor is capable of activating genes with functional positively acting GREs and is inducible by peroxisome proliferators. When pGRE5 was cotransfected with GR-PPAR (Fig. 8B), a significant (2.0-fold) increase in basal level transcription was observed, and this activity was induced

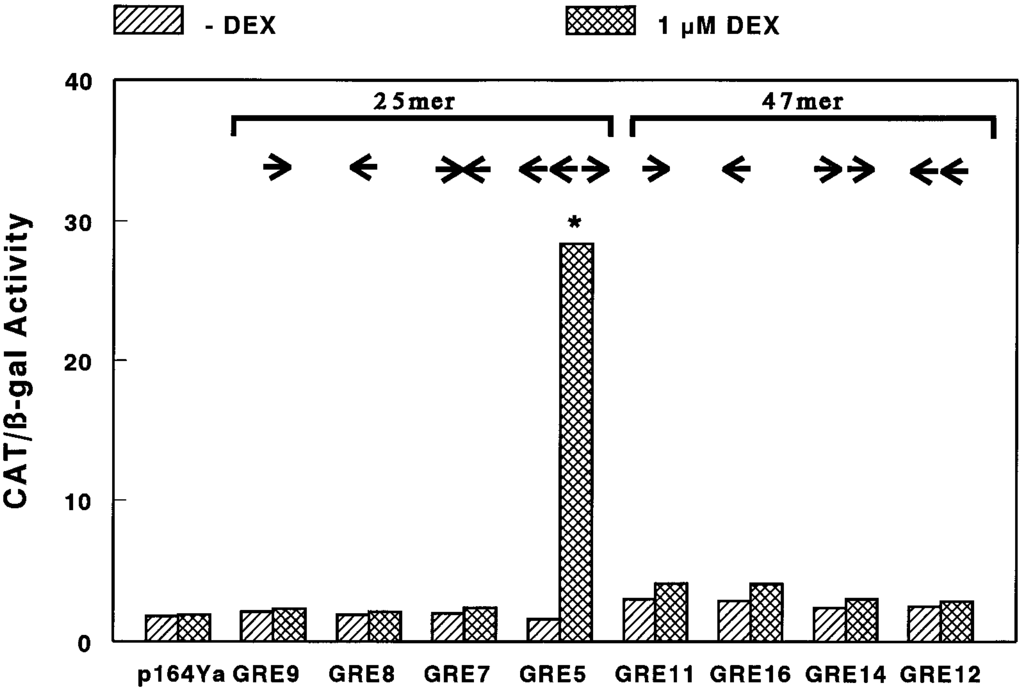

Fig. 6. Induction of CAT activity by DEX in HepG2 cells transiently transfected with CAT constructs containing the palindromic GRE minimal sequences of *gstA2*. CAT and β-galactosidase assays were performed on lysates of HepG2 cells that had been transiently transfected with GRE-containing CAT constructs containing either one or more copies of a 25- or 47-mer oligonucleotide spanning the palindromic GRE and the expression plasmids for pCMVβ and pRSVGR. *Arrows*, orientation and number of oligonucleotide insert. The HepG2 cells were treated with 1 μM DEX for 24 hr as described in Experimental Procedures. The normalized CAT activity is the percent conversion of chloramphenicol to its acetyl derivative relative to β-galactosidase activity and is the mean ± standard deviation of three flasks. *, Statistically different from untreated cells ($p < 0.05$).

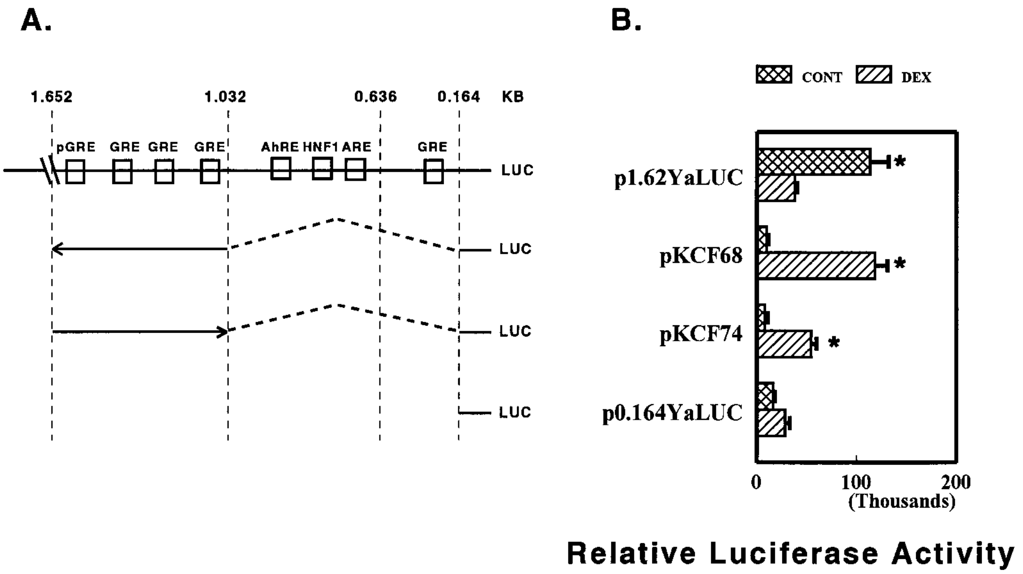

Fig. 7. Induction of luciferase activity by DEX in HepG2 cell transiently transfected with homologous promotor constructs containing bp −1620 to −1032 of *gstA2*. Luciferase and β-galactosidase assays were performed on lysed HepG2 cells that had been transiently transfected with various LUC constructs that contained a 588-bp PCR product cloned into p0.164YaLUC and the expression plasmids pCMVβ and pRSVGR. *Arrows*, orientation of the insert. The HepG2 cells were treated with 1 μM DEX for 24 hr as described in Experimental Procedures. The normalized luciferase activity is expressed as relative light units divided by β-galactosidase activity and is the mean ± standard deviation of three flasks. *, Statistical difference from cells treated with BA alone ($p < 0.5$).

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17-fold on the addition of 50 μM nafenopin, a potent peroxisome proliferator. Nafenopin had no effect on these reporter genes when administered in the absence of the chimeric receptor or after cotransfection of the GC receptor (results not shown). With pGRE5CAT, similar effects were observed when other peroxisome proliferators, ciprofibrate and clofibrate, were administered (results not shown). Interestingly, cotransfection of GR-PPAR in either the presence or absence of nafenopin had no significant effect on the fold induction of CAT activity of p1.6YaCAT by PAH (Fig. 8C). There was a modest increase in basal expression, suggesting that although the chimeric receptor does contain the domains required for *trans*-activation of some genes regulated by the GC receptor, it does not have the domains required to mediate the negative regulation of the native rat *gstA2* gene.

Discussion

Our results demonstrate that the negative regulation of the *gstA2* gene by GCs occurs via a GC receptor-dependent process and is similar to the responses observed in intact animal models (Linder and Prough, 1993) and in adult hepatocytes (Prough *et al.*, 1996). The expression of the rat *gstA2* gene is known to be under multiple regulatory processes and differs during the various stages of development from the fetal to adult state (Abramovitz *et al.*, 1989; Sherratt *et al.*, 1990; Linder and Prough, 1993; Xiao *et al.*, 1995; Prough *et al.*, 1996). In addition, Paulson *et al.* (1990) and Rushmore *et al.* (1990) have demonstrated the presence of two functional xenobiotic responsive elements that allow regulation by the AHR and a novel responsive element that allows transcriptional regulation by a variety of antioxidants/electrophilic chemicals termed the ARE. These two elements, controlled by exogenous chemicals, seem to function independently of each other. Other liver-specific transcription factors also may regulate expression of this gene, as was shown for C/EBP by Pimental *et al.* (1993). Because GCs apparently play a role in regulating the expression of *gstA2* in adrenalectomized animals or animals deficient in normal circulating levels of this steroid hormone (Linder and Prough, 1993), we identified putative GREs in the 5′-flanking region of the *gstA2* subunit gene: one palindromic consensus GRE (−1609 to −1694 bp) and four consensus GRE half-sites (−1637, −1361, −1063,

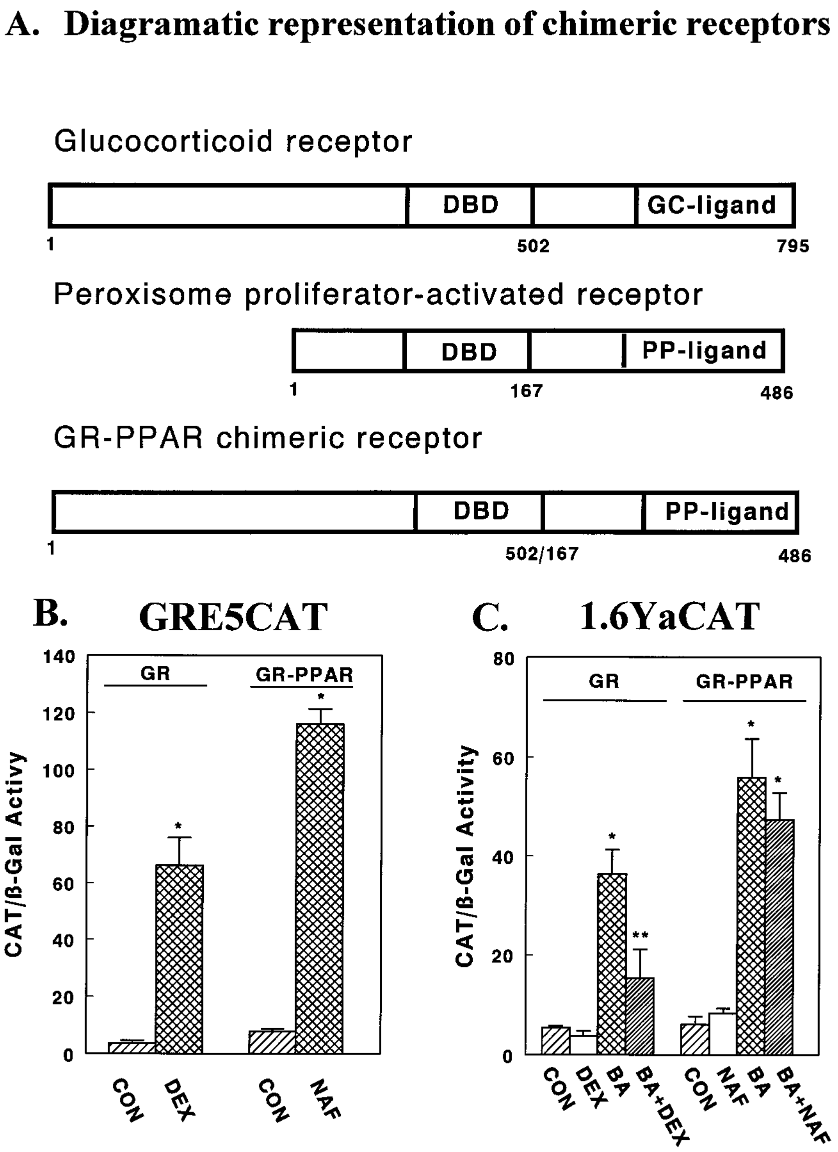

Fig. 8. The effects of nafenopin and BA on CAT expression in HepG2 cells transiently transfected with GSTYaCAT constructs and a chimeric receptor, GR-PPAR. A, Diagrammatic representation of the chimeric construct between the GC and peroxisome proliferator activated receptor. Transfected pGRE5CAT (B) and transfected p1.6YaCAT; CAT, and β-galactosidase assays (C) were performed on lysates from HepG2 cells that had been transiently transfected with the expression vector pCMVβ and either pRSVGR (GC receptor) or pGR-PPAR (chimeric GC receptor). pGRE5CAT or p1.6YaCAT also were cotransfected, and the HepG2 cells were subsequently treated with 50 μM benzanthracene, 1 μM DEX, 50 μM nafenopin, or in combination for 24 hr as described in Experimental Procedures. The normalized CAT activity is expressed as the percent conversion of chloramphenicol to its acetyl derivative relative to β-galactosidase activity and is the mean ± standard deviation of three flasks. *, Statistically different from control cells ($p < 0.05$). **, Statistically different from cell treated with BA ($p < 0.05$).

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and −646 bp). Therefore, we sought to demonstrate that these were functional GREs, accounting for some of the changes in expression shown in intact animals.

In both primary hepatocytes and immortalized cells, significant changes are observed in the levels of expression of constitutive transcription factors, such as HNF-1. These changes are thought to be important in the basal expression of the *gstA2* gene. In cultured primary hepatocytes, expression of the *gstA2* gene falls during the first 24 hr of culture (results not shown). In human renal tumors, there is a strong correlation between the expression of HNF-1 and the levels of GSTα expression (Clairmont *et al.*, 1994). Consequently, the basal expression of CAT constructs in HepG2 cells is likely to be low. In our studies, we measured CAT activities 24 hr after treatment. The inability to observe maximal negative regulation (only 40–60%) of the basal expression of *gstA2* promoter-driven CAT plasmids simply may be due to the relatively short dosing period in cultured HepG2 cells and the inherent stability of the CAT protein. However, the basal expression of our luciferase reporter constructs was more clearly repressed by GCs. Because the fold suppression of the basal expression of the LUC construct is similar to that observed with PAH-dependent induction and the basal rate is affected by the presence of the ARE, HNF-1, and a moderately strong promoter (Rushmore *et al.*, 1990), the DEX-dependent repression phenomenon seems to be independent of gene activation by the AHR (Prough *et al.*, 1996). However, our results do show that the structural reporter gene used in transient transfection assays may affect the magnitude of both the suppressive GC effect and the PAH-dependent induction that were observed. With the luciferase reporter construct, the degree of suppression was similar or slightly greater than that observed for the native gene either *in vivo* or in primary hepatocyte models over the same time period (Linder and Prough, 1993; Prough *et al.*, 1996).

Previously, we have shown that GC negatively regulates inducible activities of the *gstA2*, NAD(P)H:quinone oxidoreductase, and aldehyde dehydrogenase 3 but potentiated the AHR-dependent activation of CYP1A1 and UDP-glucuronosyl transferase 1A6 proteins in cultured adult hepatocytes (Xiao *et al.*, 1995; Prough *et al.*, 1996). In concert, these results suggest it is unlikely that the effects of GC on these genes involve the direct interaction of the liganded GC receptor with the AHR. Other interactions, possibly with constitutive transcription factor elements, also may be involved.

Several mechanisms of regulation have been described involving the GC receptor (Starr *et al.*, 1996). Negative mechanisms of regulation include removal of essential factors from the nucleus before receptor binding. This mode of inhibition (squelching) is seen with genes such as nuclear factor-κB (Mukaida *et al.*, 1994) and is distinguished from the mode of inhibition displayed in our current work in that the presence of a GRE is not required and RU38486 serves as an effective agonist of gene expression rather than an antagonist. Other mechanisms of negative regulation involve overlapping composite response elements, as seen in the proliferin gene (Miner and Yamamoto, 1992), or competition for transcription factor binding to the promoter element for the TATA box, as seen in the osteocalcin gene (Stromstedt *et al.*, 1991). The GREs found in *gstA2* are located upstream from all other known *cis*-acting elements, in an area that does not seem to significantly affect basal activity and acts as a positive hormone-responsive element when placed with the minimal promoter. These results collectively suggest that negative interaction is not simply due to competition of transcription factor binding to a composite pGRE response element. In the pro-opiomelanocortin gene (Drouin *et al.*, 1993), a negative regulatory sequence has been described that binds three GC receptor molecules. In this gene, the response element has a 2-bp difference compared with the GRE consensus palindrome found in the MMTV GRE. The negative GRE of the pro-opiomelanocortin gene is characterized by not forming GC-sensitive plasmid constructs when the pGRE is present in three copies. Because the palindromic sequence found in *gstA2* has identity with the consensus palindromic GRE described by Beato (1989) and is positively GC responsive when present in three copies or when a portion of the 5′-flanking region (−1032 to −164 bp) is omitted, its regulation clearly is different from that of the pro-opiomelanocortin gene.

Our results demonstrate that although the response does involve receptor binding to its canonical response element, the response is complex. Clearly, the normal function of the pGRE is not a classic response element because it is negatively regulated by GC. Furthermore, chimeric receptor studies suggest that the domains involved in the repressive effect are different from those involved with positive *trans*-activation. In concert, these results suggest that the GC receptor interacts with other elements of the 5′-regulatory region of *gstA2* gene (between bp −1032 and −164) through protein/protein interactions, which may involve DNA looping. Identification of these elements will be a focus for further study.

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