



Published in final edited form as:

Drug Metab Dispos. 2008 February ; 36(2): 409–417.

Regulation of the Rat UGT1A6 by Glucocorticoids Involves a Cryptic Glucocorticoid Response Element

K. C. Falkner, J. K. Ritter, and R. A. Prough

Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, Kentucky (K.C.F., R.A.P.); and the Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia (J.K.R.)

Abstract

Glucocorticoids precociously induce fetal rat UGT1A6 and potentiate polycyclic aromatic hydrocarbon (PAH)-dependent induction of this enzyme in vivo and in isolated rat hepatocytes. To establish whether induction was due to glucocorticoid receptor (GR), luciferase reporter vectors were tested in transfection assays with HepG2 cells. Using a reporter construct containing approximately 2.26 kilobases of the 5'-flanking region of the UGT1A6-noncoding leader exon (A1*), dexamethasone increased basal activity 3- to 7-fold in cells cotransfected with an expression plasmid for GR. PAH increased gene expression 23-fold, but the presence of dexamethasone only induced PAH-dependent expression by 1.5-fold, suggesting interaction between GR and the aryl hydrocarbon (Ah) receptor. Furthermore, the GR antagonist RU 38486 [17 β -hydroxy-11 β -(4-dimethylamino-phenyl)-17 α -(prop-1-ynyl)-estra-4,9-dien-3-one] was a partial agonist that increased, rather than inhibited, basal activity 3-fold. 5'-deletion analysis defined the 5'-boundary for a functional glucocorticoid-responsive unit between base pairs -141 and -118 relative to the transcription start site. This region contains the Ah receptor response element (AhRE), and both PAH and glucocorticoid-dependent gene activation were lost when this area was deleted. Mutation of a single base pair located in the AhRE region simultaneously reduced induction by PAH and increased glucocorticoid induction. Thus, the sequences of both the AhRE and glucocorticoid response elements seem to overlap, suggesting that Ah receptor binding may decrease glucocorticoid-dependent induction due to interactions of these two *cis*-acting elements. Mutation of a putative GRE located between base pair -81 and -95 reduced, but did not completely eliminate, glucocorticoid-dependent induction of the reporter, suggesting that a non-classic mechanism of induction is involved in this response.

The UDP-glycosyltransferases (UGTs) are a superfamily of enzymes with a molecular mass between 50 to 60 kDa (Mackenzie et al., 1997) that are located in the endoplasmic reticulum and nuclear envelope. These enzymes apparently evolved to catalyze the glucuronidation of either endogenous compounds such as bile acids, bilirubin, and steroids or xenobiotic compounds, such as metabolites of drugs and foreign chemicals (Wells et al., 2004). Many of the substrates for UGTs are oxidized metabolites formed by the cytochrome P450 system. Conjugation of the electrophilic centers of these molecules with UDP-glucuronic acids prevents protein or nucleic acid adduction and facilitates excretion by making the molecule more hydrophilic and a substrate for the anion transporter systems.

UGT1A6 is of special interest because its expression can be induced by polycyclic aromatic hydrocarbons (PAHs), such as benzo-[*a*]pyrene, or chlorinated compounds, such as 2,3,7,8-tetrachloro-*p*-benzodioxin (Emi et al., 1995, 1996). These compounds constitute a major class

of environmental pollutants and carcinogens and are ligands for the Ah receptor. The Ah receptor (Whitlock et al., 1996) is a ligand-activated cytosolic receptor of the periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded domain class that dimerizes with Ah receptor nuclear translocator protein and subsequently binds to the consensus sequence TNGCGTG found in the 5'-flanking region of responsive genes, such as CYP1A1, 1A2, 1B1, aldehyde dehydrogenase 3C, glutathione S-transferase A2, and NAD-(P)H:quinone oxidoreductase. For *UGT1A6* (Emi et al., 1996), the AhRE is located in the region that flanks the noncoding leader exon between base pairs -134 and -129 relative to the transcription start site in the 5'-flanking region of the rat gene.

Early biochemical studies (Wishart and Dutton, 1977a,b) demonstrated that administration of glucocorticoids to pregnant rats induced microsomal UGT activity with *o*-aminophenol, *p*-nitrophenol, 1-naphthol, and serotonin as substrates in the fetus/neonate and that the developmental pattern of expression of the activities paralleled the levels of circulating glucocorticoids. Other UGT activities were refractory to glucocorticoid treatment, most notably bilirubin glucuronidation. Another characteristic of these activities is that their expression is delayed until the neonatal stage of development. This observation led to the suggestion that there are two clusters of activities: a late-fetal cluster that is glucocorticoid-sensitive and a neonatal cluster that is glucocorticoid-insensitive.

The UDP-glycosyltransferase 1 gene (Emi et al., 1995, 1996) is unusual because enzymes encoded by this gene are generated from unique first-exons that encode regions of the protein that dictate substrate specificity and share exons 2 through 5, which encode catalytic function. A rare inherited disease, Crigler-Najjar syndrome, which results in a fatal jaundice, is caused by mutations in these shared exons (Ritter et al., 1993). Historically (Ritter et al., 1992; Emi et al., 1995, 1996), the first exons were initially named in clusters with the most distal 5'-exons being named the A cluster based in sequence homology. The first exon in the A cluster (A1, A2, and A3) encode enzymes whose substrate specificities are consistent with being members of the late fetal cluster described by Wishart and Dutton (1977a). The second cluster containing exons B1 through B5 encodes enzymes with substrate specificities consistent with being members of the neonatal cluster. *UGT1A6* is the cluster A member (A1) that encodes an enzyme characterized by having relatively high specific activity with *p*-nitrophenol as substrate (Falany et al., 1986).

Our work (Xiao et al., 1995; Prough et al., 1996) has focused on understanding the effects of glucocorticoids at the molecular level on drug-metabolizing enzymes whose expression is regulated by the Ah receptor. In studies with isolated rat hepatocytes (Xiao et al., 1995), the PAH-dependent induction of mRNA for *UGT1A6* was potentiated 2- to 3-fold by inclusion of the synthetic glucocorticoid dexamethasone (DEX). In these studies, CYP1A1 expression was also potentiated, whereas other genes, namely NAD(P)H:quinone oxidoreductase, glutathione S-transferase A2, and aldehyde dehydrogenase 3A1, were repressed.

The glucocorticoid transcriptional response is mediated by a cytosolic member of the nuclear receptor superfamily, namely the glucocorticoid receptor (Beato, 1989). This ligand-activated receptor of the zinc finger class binds to sequences, TGTYCT, that are often found as an imperfect palindromic sequence separated by three base pairs. The binding of the receptor to these sequences either activates or represses gene transcription depending upon the interaction of surrounding *cis*-acting elements for other transcription factors. This report explores the hypothesis that glucocorticoids induce *UGT1A6* gene expression via receptor binding to a responsive element located in the 5'-flanking region of the 1A6 leader exon. To facilitate this study, we have prepared luciferase reporter constructs that contain the 5'-flanking sequence of the *UGT1A6* gene and tested them for inducibility with PAH and glucocorticoids.

Materials and Methods

Materials

Restriction endonucleases, T4 ligase, and pGI₃-basic were obtained from Promega (Madison, WI) or New England Biolabs (Beverly, MA). pCMV- β , an expression vector for β -galactosidase with a CMV promoter, was obtained from Stratagene (La Jolla, CA). The cDNA used to prepare the expression vector for the human glucocorticoid receptor, pCMVGR (Falkner et al., 1998), and a glucocorticoid responsive reporter plasmid, p2XDEX-Luc, which contains two copies of the MMTV-GRE and a proliferin gene promoter, were provided by Michael Mathis (Louisiana State University Medical Center, Shreveport, LA). The expression plasmid for the chimeric glucocorticoid receptor (GR)-peroxisome proliferator-activated receptor (PPAR) plasmid was provided by T. H. Rushmore (Merck Research Laboratories, West Point, PA) as has been described previously (Boie et al., 1993).

Reagents for culturing *Escherichia coli* were purchased from Difco Laboratories (Detroit, MI). Antimycotic/antibiotic solution, nonessential amino acids, and Dulbecco's modified essential medium (high-modified) was obtained from Mediatech (Heron, VA). Fetal bovine serum was purchased from Harlan Bioproducts for Science (Indianapolis, IN). DNA purification kits were obtained from Qiagen (Chatworth, CA) to produce transfection-quality DNA. Oligonucleotides were purchased from Operon Technologies (Alameda, CA). PCR reagents were purchased from Fisher Scientific (Pittsburgh, PA).

BA and DEX were obtained from Sigma Chemical Co. (St. Louis, MO). Nafenopin was obtained from Ciba-Geigy Chemical (Ardsley, NY). RU 38486 was obtained from Roussel Uclaf (Romainville, Cedex, France). Chlorophenol red β -D-galactopyranoside was purchased from Boehringer-Mannheim (Piscataway, NJ). All other reagents used were purchased from commercial sources and were either American Chemical Society or Molecular Biology grade.

Cells and Culture Conditions

E. coli DH5 α cells were purchased from Invitrogen (Carlsbad, CA) and were routinely transformed with plasmids of interest. HepG2 cells (ATCC HB8065), a human hepatoblastoma-derived cell line, were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (high-modified) supplemented with 10% fetal bovine serum, antimycotic/antibiotic, and nonessential amino acids. The cells were incubated at 37°C in a 5% carbon dioxide atmosphere and subcultured every 2 days.

PCR Products

To create reporter constructs that contain various portions of the 5'-flanking region of the UGT1A6 gene, PCR reactions were performed using the primers shown in Table 1 using OKF44 as the common downstream primer and either primers OKF45, OKF 46, OKF47, and OKF49. To mutate the AhRE, a mutated version of the primer OKF47, named OKF48, was synthesized that contains a single base change of C to G causing a mutation of the AhRE, TGCGTGA to TGCCTGA. To mutate the putative GRE located between base pairs -95 to -81 in the 5'-flanking region, we used a four-primer strategy similar to that previously described (Ripp et al., 2003). Initially, we produced two PCR products using primers OKF44 and OKF154 and OKF46 and OKF153 to generate two PCR products (Table 1). After gel purification and annealing, the final PCR product was obtained using OKF44 and OKF47.

PCR was performed in a Thermolyne Amplitron II thermal cycler (Barnstead Thermolyne, Dulbuque, IA) with a Mg²⁺ concentration of 2 mM. The products were produced through 20 cycles of the following: annealing temperature 55°C for 1 min, elongation 70°C for 1 min, and

denaturation 90°C for 30 s. The PCR products were then subcloned into pCR2 or pCR2.1 (Invitrogen), and the insert was sequenced at the University of Louisville Center for Genetics and Molecular Medicine core facility (Louisville, KY). The sequences in all but the mutant constructs agreed with that previously described (Emi et al., 1996) with the exception of a deletion of T at position -41.

Reporter Constructs

The reporters used in this study were constructed from p2.6BX that was subcloned from λ RPT6 (Metz and Ritter, 1998). p1.106UGT1A6 was synthesized by initially subcloning a 1.1-kb HindIII/EcoRI fragment from pRPT6 2.6 BX into the unique restriction sites of pBSIKS+ (Stratagene). A KpnI/EcoRI fragment was subcloned into the KpnI/EcoRI sites of a modified version of the pGL₃-basic vector (Promega). p2.26UGT1A6 was produced by subcloning a 2.7-kb XhoI/SacI fragment from pRPT6 2.6 BX into the unique XhoI/SacI sites of pGL₃-basic.

The plasmids p0.434UGT1A6 (OKF44,45), p0.191UGT1A6 (OKF44,46), p0.141UGT1A6 (OKF44,47), p0.141UGT1A6M1 (OKF44,48), and p0.141UGT1A6M2 (OKF44,47) were generated by excising EcoRI fragments from the pCR2.1 recovery vector and subcloning this fragment into the EcoRI site in the modified version of pGL₃-basic. Orientation of the fragment was determined by digestion with TthIII/HindIII. p0.118UGT1A6 (OKF44,49) was generated by subcloning a KpnI/EcoRI fragment into those unique sites in the modified pGL₃-basic vector. The flanking sequences included in these luciferase reporter constructs are shown in Table 2.

Transfection

HepG2 cells were plated in 12-well plates and transfected at 40% confluence by a calcium phosphate-based transfection technique described previously (Falkner et al., 2001). The cells were treated 24 h following transfection with various agents made up as 500× stock solutions in DMSO (controls received DMSO alone). After 24 h, the cells were harvested with 100 μ l of cell lysis buffer (Promega) according to the manufacturer's instructions and subjected to a single-freeze thaw event. All cells were cotransfected with 500 ng of pCMV- β as a transfection control. Routinely, 125 ng of receptor expression plasmid, pCMVGR or pGR-PPAR, and 1 μ g of the various UGT1A6 expression plasmids were added to each well.

Assays for β -Galactosidase and Luciferase Activity

Luciferase activity was determined using the Promega Luciferase Assay System in a PerkinElmer Victor³ 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). For β -galactosidase activity, cell extracts were incubated with chlorophenol red β -galactopyranoside at 37°C for 15 to 60 min. The activity was determined spectrophotometrically at 595 nm using a Bio-Tek μ Quant Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT).

Statistical Analysis

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

Results

Effects of BA, DEX, and RU 38486 on Luciferase Expression of UGT1A6 Expression Vector p2.26UGT1A6Luc

We sought to examine whether the effects of PAH and DEX treatment on UGT1A6 gene expression in isolated hepatocytes could be reproduced in transient transfection experiments in HepG2 cells. As a positive control, BA induced expression of a reporter vector containing 2360 bp of the 5'-flanking region of UGT1A6 (Metz and Ritter, 1998) by 22-fold (Fig. 1). This result is similar to previous studies (Emi et al., 1996) that demonstrated that a chloramphenicol acetyl transferase UGT1A6 reporter was induced 9-fold following treatment with the PAH 3-methylcholanthrene through action of AhR. Treatment with the synthetic glucocorticoid DEX alone caused a 6.8 ± 0.6 -fold increase in luciferase activity but did not significantly induce luciferase activity in BA-treated cells (1.3 ± 0.3 -fold; not statistically different from BA-treated). This is in contrast to our previous results in isolated hepatocytes in which this concentration of DEX ($1 \mu\text{M}$) significantly potentiated the PAH-dependent induction by 2.3-fold. The DEX-dependent induction required cotransfection of an expression vector for the glucocorticoid receptor (results not shown). These results are consistent with observations in vivo by Wishart and Dutton (1977a,b), in which administration of glucocorticoids induces glucuronidation rates of substrates for UGT1A6.

Treatment with the glucocorticoid and progesterone receptor antagonist RU 38486 induced the basal level of expression of p2.26UGT1A6Luc by 3.5 ± 0.4 -fold, suggesting it is an agonist for this gene. RU 38486 was also an effective antagonist of the DEX-dependent induction of this reporter system, as the expression of luciferase activity with DEX treatment in the presence of RU 38486 was not different from RU 38486 alone.

PAHs Interfere with the Glucocorticoid-Dependent Induction of Luciferase Activity in HepG2 Cells Transfected with p2.26UGT1A6Luc

In Fig. 1, we noted that DEX-potentiated PAH induction of the UGT luciferase reporter is significantly less than the changes observed in primary rat hepatocytes or rat liver in vivo. To test whether activation of AhR interferes with the DEX-dependent induction of these UGT reporters, we determined the effect DEX had on the concentration dependence of induction by PAH (Fig. 2A) compared with a classic GRE-driven reporter (Fig. 2B). As shown in Fig. 2A, $50 \mu\text{M}$ BA induced the UGT reporter 7.8 ± 1.1 -fold, and $5 \mu\text{M}$ BA treatment induced this construct 4.89 ± 1.12 -fold, whereas the lowest concentration of BA tested ($0.5 \mu\text{M}$) did not significantly induce luciferase activity. In control cells, the DEX-dependent induction was 3.44 ± 0.72 -fold, whereas in cells treated with $50 \mu\text{M}$ BA the -fold induction (1.32 ± 0.186) was reduced by approximately 87%. In the $5\text{-}\mu\text{M}$ BA treatment group, intermediate results were obtained, with the DEX-dependent induction being 1.83 ± 0.24 -fold. Thus, we observed a simple monotonic induction of this gene with BA that coincides with the loss of ability to induce these cells with glucocorticoids. This suggests that either AhR-dependent induction of this gene interferes with the glucocorticoid receptor-dependent induction of this gene or vice versa.

To determine whether this effect is specific to the UGT1A6 gene or is a more general effect, we tested the ability of BA to inhibit the glucocorticoid-dependent action of a classic glucocorticoid-responsive reporter. The reporter, p2XDEX-Luc, has two copies of the palindromic glucocorticoid response element found in the MMTV LTR. The results, shown in Fig. 2B, unexpectedly indicated that BA induced this reporter construct 2.47 ± 0.42 -fold and 1.54 ± 0.23 -fold at $50 \mu\text{M}$ and $5 \mu\text{M}$ BA, respectively. This induction by BA was unexpected because this reporter was not known to contain an AhRE. As anticipated, DEX robustly induced expression of this reporter construct in control cells (81 ± 13 -fold), and only at the highest

concentration of BA treatment (50 μ M) was a reduction in the -fold induction by DEX observed (54- \pm 15-fold). This 33% reduction in -fold activation is significantly smaller than the 87% reduction observed with the UGT reporter. This suggests that BA treatment and therefore AhR translocation has only a slight effect on classic glucocorticoid responses, possibly due to the AhR-squelching GR function in a non-DNA-dependent manner. Although we cannot discount the possibility that the glucocorticoid receptor affects AhR function, these results are consistent, with most of the effect being DNA-specific with the *UGT1A6* gene and most likely being due to cross-talk between the GRE and AhRE.

The Concentration Dependence of the Glucocorticoid-Dependent Induction of the *UGT1A6* Reporter Is Consistent with Involvement of the Glucocorticoid Receptor

To further document that the DEX-dependent induction of the *UGT1A6* is a glucocorticoid receptor-mediated event, we determined the concentration dependence of this process relative to a classic glucocorticoid receptor-dependent reporter. As shown in Fig. 3, a simple monotonic increase was observed for both the *UGT1A6* reporter p2.26*UGT1A6*Luc (Fig. 3A) and the “classic” glucocorticoid-responsive reporter p2XDEX-Luc (Fig. 3B). The IC₅₀ for both processes was approximately 8.3 \times

10⁻⁸ M. This agrees well with previously published results using DEX as a model ligand for glucocorticoid receptor-mediated processes (Szapary et al., 1996). The magnitude of the glucocorticoid-dependent induction with the *UGT1A6* reporter (~4-fold) was considerably lower than that observed with the model MMTV-GRE-containing reporter p2XDEX-Luc (80-fold). Thus, the concentration dependence of the response obtained in this study is consistent with the process being mediated through the glucocorticoid receptor, and it is sensitive to RU 38486 (Fig. 1). It is unlikely that this response involves receptors such as the pregnane X receptor.

5'-Deletion Analysis of the *UGT1A6* Indicates That a Glucocorticoid-Responsive Element Is Located Close to the AhRE

To verify the presence of a functional GRE in the 5'-flanking region of the *UGT1A6* gene, we tested the ability of glucocorticoids to induce basal level activities with a series of 5'-deletion constructs shown in Fig. 4A. As shown in Fig. 4B, there was a major difference in the basal level of expression between p2.26*UGT1A6*Luc and p1.1*UGT1A6*Luc, whose basal activity is 98-fold higher than p2.26*UGT1A6*Luc, suggesting that either intron/exon (-2.26 to +400 bp) boundaries affected luciferase expression or that negative regulatory regions are lost. In addition, significant basal level activity is lost with the deletion of base pairs between -191 and -141. This area contains a CCAAT box and an AATGTG repeat sequence that may be important in basal level transcription (Emi et al., 1996).

As can be seen in Fig. 4C, all of the constructs tested except p0.118*UGT1A6*Luc were responsive to glucocorticoids. This was a surprising result because this plasmid contains a putative GRE located between base pairs -81 and -95. All of the responsive constructs showed at least a 3-fold induction of luciferase activity when administered DEX. Glucocorticoid sensitivity is greatly enhanced when base pairs between -191 and -141 are deleted, 2.8-fold and 12.2-fold for p0.195*UGT1A6*Luc and p0.141*UGT1A6*Luc, respectively. This suggests that the response elements involved with basal level transcriptional activity of this gene may negatively regulate glucocorticoid receptor function. When base pairs -141 to -118 were deleted, GC sensitivity was almost completely lost, suggesting that this sequence is essential for glucocorticoid-mediated induction of luciferase activity expression. The AhRE that is responsible for the PAH-dependent induction of this gene is located in this region between base pairs -134 and -129. Interestingly, the region does not contain any sequences with high sequence homology with the canonical GRE sequence of TGTYCT. These results suggest that

the same DNA regions are responsible for both the AhR-dependent and glucocorticoid-dependent induction of this gene.

Mutation of the AhRE Reduces Induction of UGT1A6 Reporters by PAHs but Increases the Ability of Glucocorticoids to Induce Gene Expression

To test whether AhR binding to the AhRE is directly involved in the suppression of the glucocorticoid-dependent induction, we mutated a single base pair in the AhRE found in the 5'-flanking region of the *UGT1A6* gene. Previous studies (Emi et al., 1996) have shown that either mutation of base pairs in the AhRE TGCGT to TGCCT or deletion of this sequence essentially eliminates the AhR-dependent induction of this gene. Using this same mutation strategy, we compared the ability of these reporter constructs to be induced with either BA, DEX, or a combination of both compounds as shown in Fig. 5.

As anticipated, reporter constructs containing an intact AhRE, namely p2.26UGT1A6Luc and p0.141UGT1A6Luc, were induced 13.5- and 16.6-fold by BA treatment, respectively. In reporter constructs that had either a deleted AhRE (p0.118UGT1A6) or a mutated AhRE (p0.141UGT1A6LucM1), BA-dependent induction was either abolished or in the case of the mutated construct, p0.141UGT1A6LucM1, greatly diminished (2.6 ± 0.7 -fold). The mutation used was identical to that used previously (Emi et al., 1996) to produce the M1 mutant and confirms that this substitution greatly reduces the ability of PAHs to induce these reporter constructs. As anticipated, from the deletion analysis, glucocorticoids induced our -2.26-kb construct, p2.26UGT1A6Luc, 4.3 ± 0.3 -fold, the -141-bp construct, p0.141UGT1A6Luc, 8.9 ± 2.6 -fold, but did not induce the -118-bp construct, p0.118UGT1A6Luc (Fig. 5). Interestingly, the AhRE-mutated construct, p0.141UGT1A6LucM1, which has a single base pair mutation difference from p0.141UGT1A6Luc, was induced 20.2 ± 2.4 -fold, which was significantly greater than the 8.9-fold induction seen with the wild-type sequence. These results clearly indicate that this region of DNA is critical for both PAH and glucocorticoid-dependent inductions of this gene. What is more surprising is that this occurs in the absence of PAH, a situation in which we would anticipate that AhR is located in the cytosol. This suggests that there is a DNA-dependent effect of the AhRE on glucocorticoid-dependent induction of this gene that is independent of the occupancy of AhRE by the AhR. However, BA treatment reduced the -fold-induction by glucocorticoids in all PAH-responsive plasmids, suggesting that binding of the AhR also affects the glucocorticoid-dependent induction of this gene. In the -141-bp construct, p0.141UGT1A6Luc, the -fold induction by BA was reduced from 8.9-fold to 1.2-fold by the AhRE mutation. Whereas the presence of DEX and BA causes a trend toward induction in the 2260- and 141-bp luciferase constructs, there was no difference between BA-treated and BA + DEX-treated cells using the mutated construct. These results demonstrate that GR regulation is significantly affected by occupancy of the AhRE by AhR, as seen by the suppressed DEX response and the striking results of the M1 mutation on DEX induction. Although it is unlikely that the GR binds to the AhRE, it is possible that this mutation either affects the binding of another transcription factor to DNA or its ability to recruit coactivators to the *UGT1A6* glucocorticoid-responsive unit.

Mutation of a Putative GRE Located between Base Pairs -81 and -95 in the 5'-Flanking Region Reduces Glucocorticoid Sensitivity

An examination of the 5'-flanking region of the *UGT1A6* gene revealed a potential palindromic GRE located between base pairs -81 and -95 in the 5'-flanking region (Fig. 6A). To test the hypothesis that the pivotal event conferring glucocorticoid responsiveness to this gene is due to the glucocorticoid receptor binding to this element, we mutated two base pairs in the consensus half-site of this element (AGAACA to ACAGCA) as shown in Fig. 6A and tested the responsiveness of reporter constructs in transient transfection assays. The mutation strategy was based on the studies with the GRE from the MMTV LTR that indicate that these sequences

are critical for receptor binding (La Baer and Yamamoto, 1994) and in our study (Falkner et al., 1999), in which mutation of these base pairs in the GRE from the rat aldehyde dehydrogenase 3C gene prevented the glucocorticoid-dependent suppression of this gene in transient transfection and abolished binding to the GRE in electrophoretic mobility shift assay studies. We used our most glucocorticoid-sensitive wild-type UGT1A6 reporter construct, p0.141UGT1A6Luc, as a positive control. As anticipated, this construct was induced 7.3 ± 1.7 -fold by $0.1 \mu\text{M}$ DEX treatment and 13.1 ± 3.18 -fold by BA treatment, as shown in Fig. 6B. In the GRE-mutant construct p0.141UGT1A6LucM2, the basal level of the reporter activity ($44,729 \pm 10,804$ relative light units) was relatively unchanged from the wild-type construct ($35,044 \pm 11,803$ relative light units), indicating that this mutation strategy did not interfere with any transcription factors involved with the proximal promoter. Expression from this reporter was also induced by treatment with $0.1 \mu\text{M}$ DEX (4.0 ± 0.7 -fold), albeit to a lesser extent than the wild-type construct. Similar results were observed for the condition of BA + DEX. This suggests that although this GRE is likely to be involved in the glucocorticoid response, it is not solely responsible for the glucocorticoid-dependent activation of this gene and that gene activation occurs through sequences other than a canonical glucocorticoid response element alone. In contrast to the glucocorticoid response, the AhR-mediated BA-dependent induction (11.2 ± 1.9 -fold) of this reporter was unchanged. This demonstrates that the AhR function seems to be independent of mutation of GRE and, therefore, GRE occupancy. However, as seen in Fig. 5, this is not the case for GR binding, which probably involves a multiprotein complex, such as the glucocorticoid-responsive unit in phosphoenol pyruvate carboxykinase (Wang et al., 1999).

The Glucocorticoid Response of UGT1A6 Reporters Cannot Be Robustly Supported Using a Chimeric GR-PPAR Receptor Using Nafenopin as a Ligand

To prove a direct role for the glucocorticoid receptor in this response, we tested the ability of this response to be supported by a chimeric receptor that contains the GR ligand-binding domain and the PPAR ligand-binding domain. This receptor can robustly induce a response through a classic GRE as shown in Fig. 7. As anticipated, cells transfected with the p2XDex-Luc reporter and an expression vector for GR, DEX induced luciferase activity 30 ± 12 -fold. Likewise, in cells transfected with p2XDex-luc and an expression vector for the GR-PPAR chimeric receptor, nafenopin induced luciferase activity 35 ± 8 -fold. This demonstrates that either GR or GR-PPAR, the chimeric receptor, can robustly induce a classic GRE in a ligand-dependent manner. The UGT1A6 reporters were all induced by DEX when an expression vector for the glucocorticoid receptor was cotransfected. However, when the chimeric GR-PPAR receptor was cotransfected with the UGT1A6 reporters and the cells were treated with nafenopin, the -fold-induction of luciferase activity of these reporters was much lower than the DEX-induced luciferase activity. For example, with the -2.26 -kb reporter p2.26UGT1A6Luc, DEX induced luciferase activity 4.5 ± 0.6 -fold, whereas nafenopin induced luciferase activity 1.3 ± 0.1 -fold. With the most glucocorticoid-sensitive UGT1A6-based reporter, p0.141UGT1A6LucM1, which has a point mutation in the AhRE, DEX induced gene transcription 21 ± 0.4 -fold, whereas nafenopin only induced the construct 2.7 ± 0.6 -fold. Although the cotransfection of the receptor and treatment with nafenopin does induce transcription of these reporters in a ligand-dependent manner, the magnitude of the induction is far lower than when the glucocorticoid receptor is cotransfected and the cells are treated with DEX. This suggests that receptors with a glucocorticoid receptor DNA-binding domain can mediate this response, sequences in the ligand-binding domain must also be critical in interacting with other proteins to obtain full induction.

Discussion

The results presented in this study indicate that in transient transfection experiments in HepG2 cells the rat *UGT1A6* gene expression can be induced through action of the glucocorticoid receptor. Our previous studies in isolated rat hepatocytes (Xiao et al., 1995) and earlier work by Wishart and Dutton (1977a) and Wishart et al. (1977) using fetal rat tissue indicate that glucocorticoids induce the expression of glucuronosyltransferases (i.e., UGT1A6). Wishart and Dutton (1977a) and Wishart et al. (1977) demonstrated that UGT activities toward *o*-aminophenol, *p*-nitrophenol, 1-naphthol, and serotonin were strikingly increased after treating pregnant rats with dexamethasone (~2 mg/kg body weight i.p.). Subsequent studies have indicated that UGT1A6 is the major form of glycosyltransferase responsible for the conjugation of these compounds (Falany and Tephly, 1983). Our studies (Xiao et al., 1995) in isolated rat hepatocytes indicated that DEX potentiated the PAH-dependent induction of 4-nitrophenol conjugation and mRNA levels of UGT1A6 by approximately 2-fold.

DEX induced expression of only one of two possible RNA transcripts of *UGT1A6* in rat liver by alternate promoters (Auyeung et al., 2001). These studies indicated that DEX treatment in vivo induced only a class 1 transcript generated from the P1 promoter (Auyeung et al., 2001), whereas PAHs, such as 3-methylcholanthrene, induced both class 1 and 2 transcripts. There are significant differences between various biological systems used to study *UGT1A6* gene expression; for example, intact liver expresses both transcripts; immortalized cell lines, such as HepG2 cells, express a class 1 transcript; and isolated rat hepatocytes predominantly express a class 2 transcript (Auyeung et al., 2003). This suggests that there are subtle developmental changes in the expression of each transcript that may account for the differences observed between the studies. Another possible factor is that high concentrations of DEX were employed that may likely have activated the pregnane X receptor (Emi et al., 1995). The pregnane X receptor and glucocorticoid receptor have been shown to have opposing actions at the same response element in the glutathione *S*-transferase A2 gene (Falkner et al., 2001; Ki et al., 2005), so the high concentrations of DEX used may have masked the glucocorticoid receptor-dependent effects.

In our studies, we document that RU 38486 is a partial agonist, rather than a pure antagonist, of the glucocorticoid-dependent transcriptional activation of this gene. Hormone antagonists have been classified into two major classes. The type 1 antagonists induce minor conformational change, insufficient to allow regulatory cofactors to recognize a ligand-activated receptor. The type 2 antagonists, such as RU 38486, induce a conformation change that is similar to an agonist-activated receptor; however, these antagonists tend to recruit corepressors rather than coactivators when associated with a response element. The ability to act as an antagonist or mixed agonist/antagonist is associated with the concentration of regulatory cofactors involved such as NcoR1 (Schulz et al., 2002) and the glucocorticoid receptor (Szapary et al., 1996). In HeLa cells transfected with GREtkCAT, RU 38486 has been shown to be a partial agonist of glucocorticoid receptor function when the cells are cotransfected with an expression vector for the glucocorticoid receptor (Szapary et al., 1996). In HepG2 cells, we have found that to achieve consistent results of regulation by glucocorticoids, we had to cotransfect an expression vector for the glucocorticoid receptor to elicit high levels of expression by DEX (Falkner et al., 1998, 2001), and thus the partial agonist activity we observe in these studies may simply be an artifact of overexpression of the glucocorticoid receptor. Another possible explanation is that RU 38486 is a true partial agonist of this response. The bovine prolactin promoter contains a negative glucocorticoid response element in which RU 38486 acts as a partial agonist (Cairns et al., 1993). The effect is mediated through the ability of the glucocorticoid receptor to interact with other transcription factors, dictating whether corepressors or coactivators are recruited.

In a classic sense, glucocorticoid signaling is mediated through the glucocorticoid receptor binding its cognate response element, an imperfect palindrome of TGTCT separated by three base pairs. In transient transfection assays, at least two copies of this response element are required to facilitate a transcriptional response to glucocorticoids. In our studies, binding of the AhR and deletion or mutation of the AhRE affected the glucocorticoid responsiveness of the reporter constructs (Fig. 5), whereas mutation of the putative GRE had no effect upon Ah receptor-mediated gene activation (Fig. 6). This may reflect the differences in Ah receptor and GR function. The Ah receptor is capable of transactivating reporter constructs when its response element is present as a single copy (Rushmore et al., 1990). In contrast, GR requires either multiple copies of its response element or the interaction with other transcription factors to form a glucocorticoid-responsive unit. Thus, AhR does not require cooperation from other transcription factors to recruit coactivators, whereas the GR often does.

Thus, co-operativity between GREs is required to facilitate this response. In native genes, this co-operativity can be achieved through interaction with other transcription factors, giving rise to the concept of a hormone-responsive unit or a glucocorticoid-responsive unit in this case. This has been most elegantly described in the PEPCK gene (Wang et al., 1999; Stafford et al., 2001), whose transcription rate is induced in liver but repressed in adipose tissue when treated by glucocorticoids. In liver, induction of the PEPCK gene by corticosteroids involves the interaction of at least four other transcription factors (namely HNF4/COUP-TF, HNF3 β , COUP-TF, and C/EBP β) whose response elements are in close proximity to the glucocorticoid receptor-binding sites. Mutation of these accessory factor response elements results in either diminution or abolishment of glucocorticoid sensitivity. Both HNF4 and C/EBP β sites have been identified in the promoter of the UGT1A6 gene (Emi et al., 1996); however, these transcription factor-binding sites are deleted in our most glucocorticoid-sensitive -141-bp reporter constructs. Relatively little is known about the transcription factor-binding sites that reside in the distal promoter responsible for generating a type 1 transcript (Auyeung et al., 2003). In our studies, deletion of the region between -141 and -118 nearly abolishes glucocorticoid sensitivity of reporter constructs, and mutation of the AhR response element located between base pairs -134 and -129 results in increased glucocorticoid-responsiveness of our reporter construct. This suggests that an accessory factor that is important in the glucocorticoid-dependent gene activation of *UGT1A6* also binds in that area.

In addition to classic glucocorticoid receptor function through its canonical response element, other modes of glucocorticoid receptor function include binding directly to other transcription factors through protein-protein interaction. This mechanism of interaction is most notably characterized in the α_1 -acid glycoprotein gene (Lorenzo et al., 1991), where the glucocorticoid receptor facilitates its response through binding to C/EBP β , and in the collagenase-3 gene, where the glucocorticoid receptor binds to AP-1 transcription factors (Jonat et al., 1990; Schule et al., 1990; Heck et al., 1994). The rat *UGT1A6* gene does not seem to contain any high-consensus AP-1 or C/EBP β elements in close proximity to the glucocorticoid-responsive unit. Determining the transcription factors that mediate promoter-1 activity and through which glucocorticoid signaling is mediated will be the focus of future studies.

In conclusion, the results presented in this study provide evidence that the glucocorticoid-responsive unit in the rat *UGT1A6* gene is located between base pairs -141 and the promoter. The cross-talk observed between the glucocorticoid and AhR is most likely due to the close proximity of the two respective response elements. An important caveat is that the position of response elements in other orthologous *UGT1A6* genes is not conserved, so the cross-talk observed in this study are likely restricted to the rat gene.

Acknowledgments

We acknowledge Mary Pendleton for expert technical assistance and Shevonda T. McGill, a Summer Medical Research fellow, for participation in the preliminary experiments of this work. We also thank Michael Mathis and Tom Rushmore for providing expression plasmids.

This work was supported by National Institutes of Health Grant DK54774 (R.A.P.) and Projects 2 (R.A.P.) and 5 (K.C.F.) of National Aeronautics and Space Administration Grant NAG5-12874 (E. Wang).

ABBREVIATIONS

UGT, UDP-glycosyltransferase
 PAH, polycyclic aromatic hydrocarbon
 Ah, aryl hydrocarbon
 DEX, dexamethasone
 CMV, cytomegalovirus
 GR, glucocorticoid receptor
 MMTV, mouse mammary tumor virus
 GRE, glucocorticoid response element
 PPAR, peroxisome proliferator-activated receptor
 PCR, polymerase chain reaction
 BA, 1,2-benzanthracene
 kb, kilobase
 DMSO, dimethyl sulfoxide
 bp, base pair(s)
 LTR, long terminal repeat
 AhR, aryl hydrocarbon receptor
 AhRE, aryl hydrocarbon receptor response element
 RU 38486, 17 β -hydroxy-11 β -(4-dimethylamino-phenyl)-17 α -(prop-1-ynyl)-estra-4,9-dien-3-one.

References

- Auyeung DJ, Kessler FK, Ritter JK. An alternative promoter contributes to tissue- and inducer-specific expression of the rat UDP-glucuronosyltransferase 1A6 gene 23. *Toxicol Appl Pharmacol* 2001;174:60–68. [PubMed: 11437649]
- Auyeung DJ, Kessler FK, Ritter JK. Differential regulation of alternate UDP-glucuronosyltransferase 1A6 gene promoters by hepatic nuclear factor-1. 16. *Toxicol Appl Pharmacol* 2003;191:156–166. [PubMed: 12946651]
- Beato M. Gene regulation by steroid hormones. *Cell* 1989;56:335–344. [PubMed: 2644044]
- Boie Y, Adam M, Rushmore TH, Kennedy BP. Enantioselective activation of the peroxisome proliferator-activated receptor. *J Biol Chem* 1993;268:5530–5534. [PubMed: 8383673]
- Cairns C, Cairns W, Okret S. Inhibition of gene expression by steroid hormone receptors via a negative glucocorticoid response element: evidence for the involvement of DNA-binding and agonistic effects of the antiglucocorticoid/antiprogestin RU486. *DNA Cell Biol* 1993;12:695–702. [PubMed: 8397827]
- Clark AG, Carrol N. Suppression of high affinity ligand binding to the major glutathione S-transferase for galleria mellonella by physiological concentrations of glutathione. *Biochem J* 1986;233:325–331. [PubMed: 3954738]
- Emi Y, Ikushiro SI, Iyanagi T. Drug-responsive and tissue-specific alternative expression of multiple first exons in rat UDP-glucuronosyltransferase family 1 (UGT1) gene complex. *J Biochem* 1995;117:392–399. [PubMed: 7608130]
- Emi Y, Ikushiro SI, Iyanagi T. Xenobiotic response element-mediated transcriptional activation in the UDP-glucuronosyltransferase family 1 gene complex. *J Biol Chem* 1996;271:3952–3958. [PubMed: 8632018]

- Falany CN, Green MD, Swain E, Tephly TR. Substrate specificity and characterization of rat liver p-nitrophenol, 3 alpha-hydroxysteroid and 17 beta-hydroxysteroid UDP-glucuronosyltransferases. *Biochem J* 1986;238:65–73. [PubMed: 3099767]
- Falany CN, Tephly TR. Separation, purification and characterization of three isoenzymes of UDP-glucuronyltransferase from rat liver microsomes. *Arch Biochem Biophys* 1983;227:248–258. [PubMed: 6416180]
- Falkner KC, Pinaire JA, Xiao GH, Geoghegan TE, Prough RA. Regulation of the rat glutathione S-transferase A2 gene by glucocorticoids: involvement of both the glucocorticoid and pregnane X receptors. *Mol Pharmacol* 2001;60:611–619. [PubMed: 11502894]
- Falkner KC, Rushmore TH, Linder MW, Prough RA. Negative regulation of the rat glutathione S-transferase A2 gene by glucocorticoids involves a canonical glucocorticoid consensus sequence. *Mol Pharmacol* 1998;53:1016–1026. [PubMed: 9614203]
- Falkner KC, Xiao GH, Pinaire JA, Pendelton ML, Lindahl R, Prough RA. The negative regulation of the rat aldehyde dehydrogenase 3 gene by glucocorticoids: involvement of a single imperfect palindromic glucocorticoid response element. *Mol Pharmacol* 1999;55:659–657.
- Heck S, Kullmann M, Gast A, Ponta H, Rahmsdorf HJ, Herrlich P, Cato ACB. A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *EMBO J* 1994;13:4087–4095. [PubMed: 8076604]
- Jonat C, Rahmsdorf HJ, Park KK, Cato ACB, Gebel S, Ponta H, Herrlich P. Antitumor promotion and antiinflammation down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* 1990;62:1189–1204. [PubMed: 2169351]
- Ki SH, Cho IJ, Choi DW, Kim SG. Glucocorticoid receptor (GR)-associated SMRT binding to C/EBP beta TAD and Nrf2 Neh4/5: role of SMRT recruited to GR in GSTA2 gene repression. *Mol Cell Biol* 2005;25:4150–4165. [PubMed: 15870285]
- La Baer J, Yamamoto KR. Analysis of the DNA-binding affinity, sequence selectivity and context dependence of the glucocorticoid receptor zinc finger region. *J Mol Biol* 1994;239:664–688. [PubMed: 8014988]
- Lorenzo DI, Williams P, Ringold G. Identification of two distinct nuclear factors with DNA binding activity within the glucocorticoid regulatory region of the rat alpha-1-acid glycoprotein promoter. *Biochem Biophys Res Commun* 1991;176:1326–1332. [PubMed: 2039514]
- Mackenzie PI, Owens IS, Burchell B, Bock KW, Bairoch A, Belanger A, Fournel-Gigleux S, Green M, Hum DW, Iyanagi T, et al. The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* 1997;7:255–269. [PubMed: 9295054]
- Metz RP, Ritter JK. Transcriptional activation of the UDP-glucuronosyltransferase 1A7 gene in rat liver by aryl hydrocarbon receptor ligands and oltipraz. *J Biol Chem* 1998;273:5607–5614. [PubMed: 9488689]
- Prough RA, Xiao G-H, Pinaire JA, Falkner KC. Hormonal regulation of xenobiotic drug metabolizing enzymes. *FASEB J* 1996;10:1369–1377. [PubMed: 8903507]
- Ripp SL, Falkner KC, Pendleton ML, Tamasi V, Prough RA. Regulation of CYP2C11 by dehydroepiandrosterone and peroxisome proliferators: identification of the negative regulatory region of the gene. *Mol Pharmacol* 2003;64:113–122. [PubMed: 12815167]
- Ritter JK, Chen F, Sheen YY, Tran HM, Kimura S, Yeatman MT, Owens IS. A novel complex locus UGT1 encodes human bilirubin, phenol, and other UDP-glucuronosyltransferase isoenzymes with identical carboxyl termini. *J Biol Chem* 1992;267:3257–3261. [PubMed: 1339448]
- Ritter JK, Yeatman MT, Kaiser C, Gridell B, Owens IS. A phenylalanine codon deletion at the UGT1 gene complex locus of Crigler-Najjar type I patient generates a pH-sensitive bilirubin UDP-glucuronosyltransferase. *J Biol Chem* 1993;268:23573–23579. [PubMed: 8226884]
- Rushmore TH, King RG, Paulson KE, Pickett CB. Regulation of glutathione S-transferase Ya subunit expression: identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. *Proc Natl Acad Sci U S A* 1990;87:3826–3830. [PubMed: 2160079]
- Schule R, Rangarajan P, Kliewer S, Ransone LJ, Bolando J, Yang N, Verma IM, Evans RM. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell* 1990;62:1217–1226. [PubMed: 2169353]

- Schulz M, Eggert M, Baniahmad A, Dostert A, Heinzel T, Renkawitz R. RU486-induced glucocorticoid receptor agonism is controlled by the receptor N terminus and by corepressor binding. *J Biol Chem* 2002;277:26238–26243. [PubMed: 12011091]
- Stafford JM, Waltner-Law M, Granner DK. Role of accessory factors and steroid receptor coactivator 1 in the regulation of phosphoenolpyruvate carboxykinase gene transcription by glucocorticoids. *J Biol Chem* 2001;276:3811–3819. [PubMed: 11069927]
- Szapary D, Xu M, Simons SS. Induction properties of a transiently transfected glucocorticoid-responsive gene vary with glucocorticoid receptor concentration. *J Biol Chem* 1996;271:30576–30582. [PubMed: 8940029]
- Wang JC, Stromstedt PE, Sugiyama T, Granner DK. The phosphoenolpyruvate carboxykinase gene glucocorticoid response unit: identification of the functional domains of accessory factors HNF3 β (hepatic nuclear factor-3 β) and HNF4 and the necessity of proper alignment of their cognate binding sites. *Mol Endocrinol* 1999;13:604–618. [PubMed: 10194766]
- Wells PG, Mackenzie PI, Chowdhury JR, Guillemette C, Gregory PA, Ishii Y, Hansen AJ, Kessler FK, Kim PM, Chowdhury NR, et al. Glucuronidation and the UDP-glucuronosyltransferases in health and disease. *Drug Metab Dispos* 2004;32:281–290. [PubMed: 14977861]
- Whitlock JP Jr, Ma Q, Dong L. DNA binding by the heterodimeric Ah receptor. *J Biol Chem* 1996;271:7942–7948. [PubMed: 8626473]
- Wishart GJ, Dutton GJ. Precocious development in utero of certain UDP-glucuronyltransferase activities in rat fetuses exposed to glucocorticoids. *Biochem Biophys Res Commun* 1977a;75:125–129. [PubMed: 403913]
- Wishart GJ, Dutton GJ. Precocious development of foetal glucuronidating enzymes induced by glucocorticoids in culture and in utero. *Nature* 1977b;266:183–184. [PubMed: 404564]
- Wishart GJ, Goheer MA, Leakey JEA, Dutton GJ. Precocious development of uridine diphosphate glucuronosyltransferase activity during organ culture of foetal rat liver in the presence of glucocorticoids. *Biochem J* 1977;166:249–253. [PubMed: 410407]
- Xiao GH, Pinaire JA, Rodrigues AD, Prough RA. Regulation of the Ah gene battery via Ah receptor-dependent and independent processes in cultured adult rat hepatocytes. *Drug Metab Dispos* 1995;23:642–650. [PubMed: 7587946]

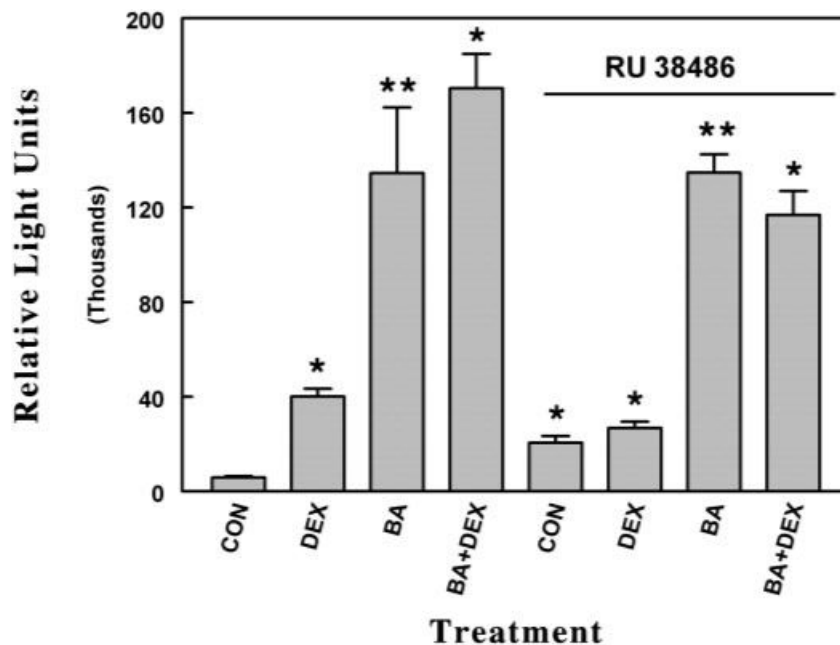


Fig. 1.

Effects of DEX, BA, and RU 38486 on expression of a full-length rat UGT 1A6 reporter in HepG2 cells. Luciferase and β -galactosidase assays were performed on lysates from HepG2 cells transfected with p2.26UGT1A6Luc, a control vector pCMV- β and an expression vector for the glucocorticoid receptor pRSV-GR, as described under *Materials and Methods*. Cells were treated with either 0.1 μ M DEX, 50 μ M BA, or a combination of both compounds (BA + DEX) in the absence or presence of 1 μ M RU 38486. Control cells (CON) received DMSO alone. The normalized luciferase activity is expressed as the relative light units divided by β -galactosidase activity and is the mean \pm S.D. of three samples. *, $P < 0.05$ statistically different than control cells; **, $P < 0.05$ statistically different than control or DEX-treated cells.

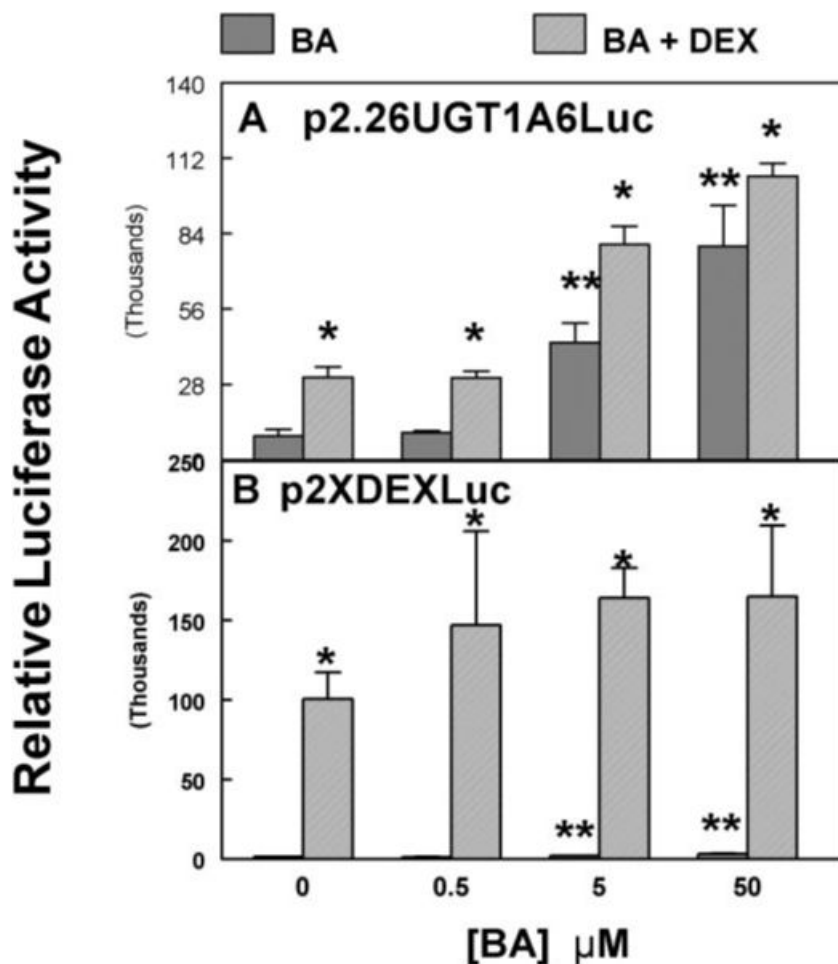


Fig. 2.

Concentration dependence of the glucocorticoid-dependent induction of UGT reporter luciferase activity in HepG2 cells. HepG2 cells were transfected with either p2.26UGT1A6Luc (A) or p2XDex-Luc (B) and 24 h after treatment with BA and/or DEX, luciferase and β -galactosidase assays were performed on lysed lysates. A control vector, pCMV- β , and an expression vector for the glucocorticoid receptor, pRSV-GR, were coexpressed with the reporter vectors as described under *Materials and Methods*. Cells were treated with varying concentrations of BA in the absence or presence of 0.1 μ M DEX. Control cells (CON) received DMSO alone. The normalized luciferase activity is expressed as the relative light units divided by β -galactosidase activity and is the mean \pm S.D. of three samples. *, $P < 0.05$ statistically significant effect of DEX; **, $P < 0.05$ statistically significant effect of BA.

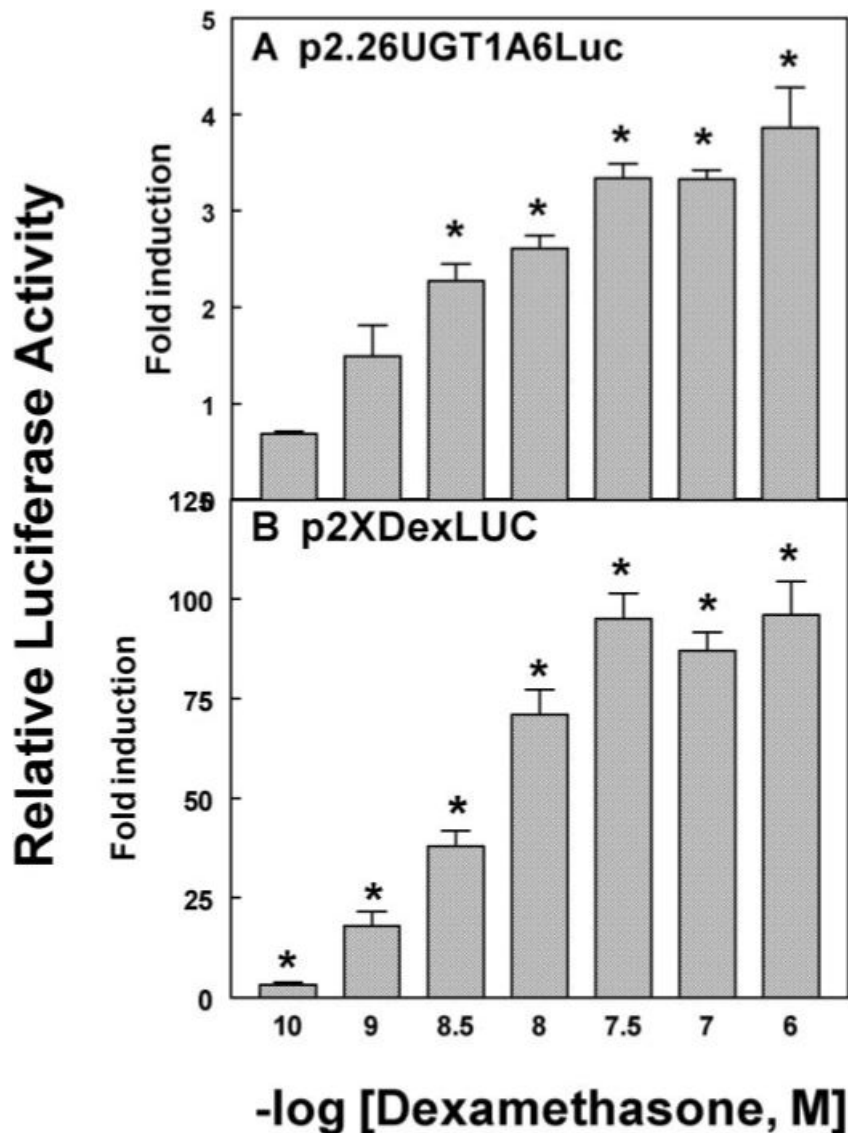


Fig. 3. DEX concentration dependence on the expression of luciferase activity in HepG2 cells transfected with either a UGT1A6 gene reporter, p2.26UGT1A6Luc, or a classic GRE reporter, p2XDex-Luc. Luciferase and β -galactosidase assays were performed on lysed HepG2 cells that had been transfected with either p2.26UGT1A6Luc (A) or p2XDex-Luc (B), a control vector, pCMV- β , and an expression vector for the glucocorticoid receptor, pRSV-GR, as described under *Materials and Methods*. Cells were treated with varying concentrations of 0.1 μM DEX. Control cells received DMSO alone. The normalized luciferase activity is expressed as the relative light units divided by β -galactosidase activity and is the mean \pm S.D. of three samples. *, $P < 0.05$ statistically significant difference from control cells.

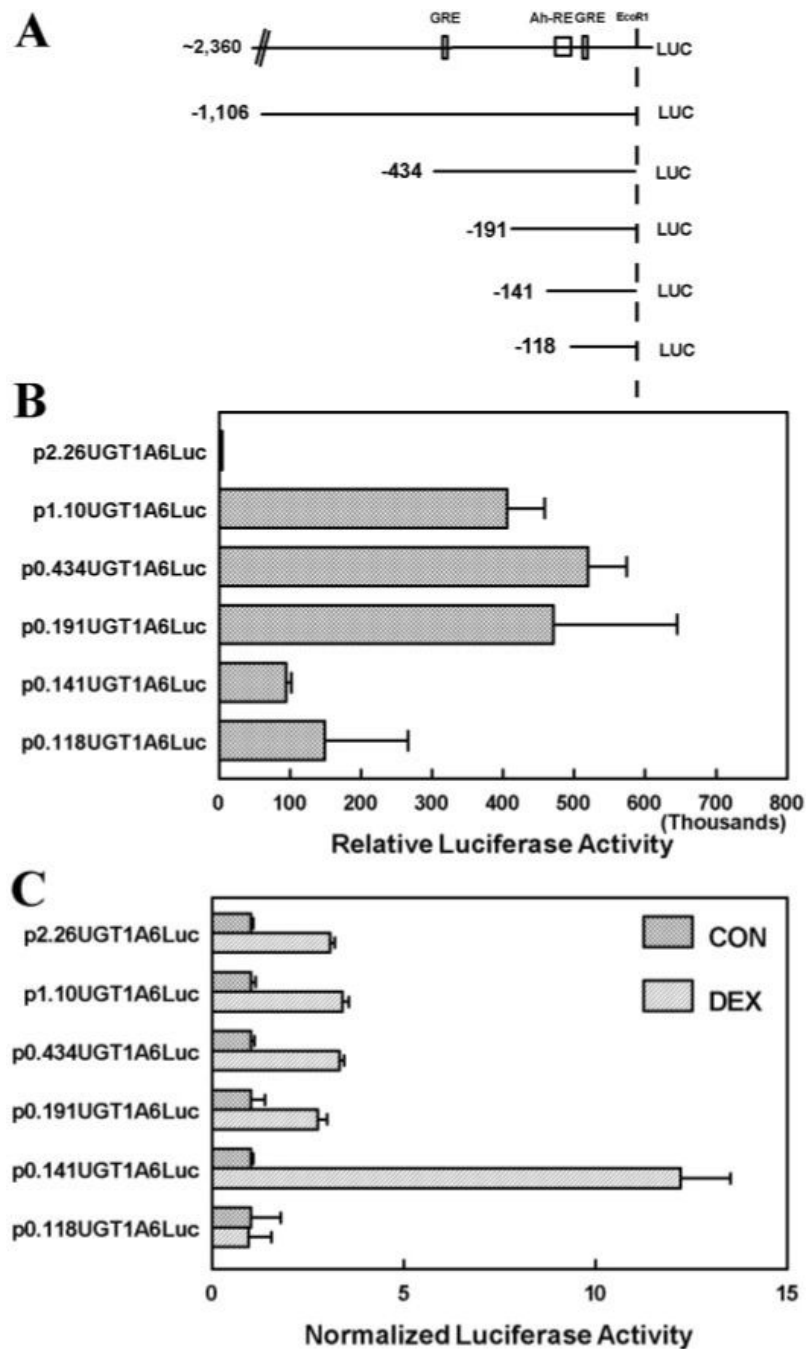


Fig. 4. Deletion analysis of the 5'-flanking region of the UGT1A6 gene. A series of luciferase constructs were prepared with deletions in the 5'-flanking regions of the UGT1A6 gene (Fig. 4A) and were tested by transient transfection in HepG2 cells as described under *Materials and Methods*. The cells were treated either with vehicle (CON) or 0.1 μ M DEX for 24 h prior to harvest. Basal level luciferase is shown in Fig. 4B. The effects of DEX treatment are shown in Fig. 4C. The normalized luciferase activity is expressed as the relative light units divided by β -galactosidase activity and is the mean \pm S.D. of three samples. *, $P < 0.05$ statistically significant difference from control cells.

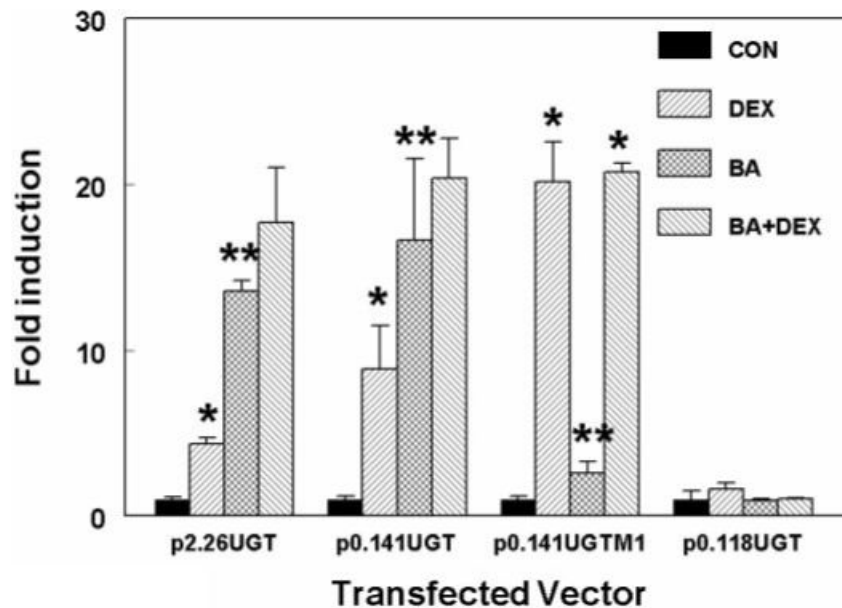


Fig. 5. Effects of mutation or deletion of the AhRE on reporter gene induction of luciferase activity by either BA, DEX, or a combination of both compounds. Luciferase and β -galactosidase assays were performed on lysed HepG2 cells that had been transfected with either p2.26UGT1A6Luc (p2.26UGT), the AhRE deletion construct p0.118UGT1A6Luc (p0.118UGT), p0.141UGT1A6Luc (p0.141UGT), or an AhRE-mutated version of p0.141UGT1A6LucM1 (p0.141UGTM1). All vectors were cotransfected with a control vector, pCMV- β , and an expression vector for the glucocorticoid receptor, pRSV-GR, as described under *Materials and Methods*. Cells were treated with varying concentrations of 0.1 μ M DEX, 50 μ M BA, or a combination of both compounds (BA + DEX). Control cells (CON) received DMSO alone. The normalized luciferase activity is the mean \pm S.D. of three samples. *, $P < 0.05$ statistically significant effect of DEX; **, $P < 0.05$ statistically significant effect of BA.

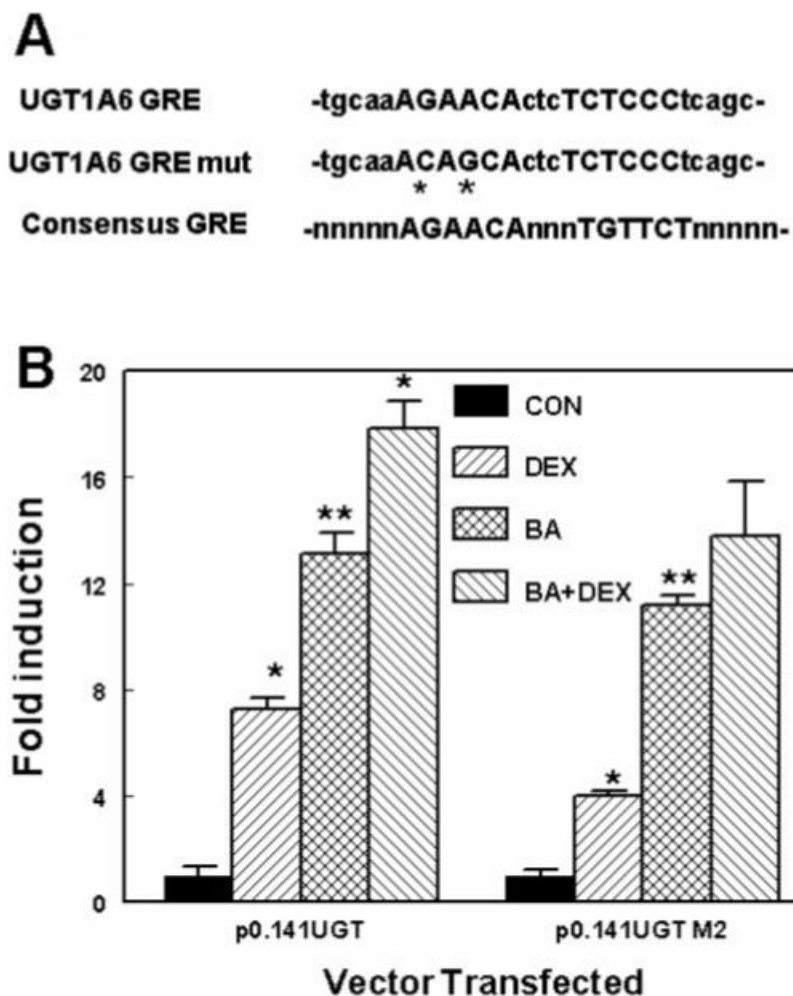


Fig. 6.

Effects of mutation of a putative GRE on activation of reporter gene activity by either DEX, BA, or a combination of both compounds. The sequence of the putative GRE located between base pairs -81 and -95 in the 5'-flanking region and the subsequent mutation strategy employed is shown in Fig. 6A. Mutated base pairs are indicated with an asterisk. In Fig. 6B, luciferase and β -galactosidase assays were performed on lysed HepG2 cells that had been transfected with either p0.141UGT1A6Luc (p0.141UGT) and p0.141UGT1A6LucM2 (p0.141UGTM2), which contains a mutated GRE sequence. All vectors were cotransfected with a control vector, pCMV- β , and an expression vector for the glucocorticoid receptor, pRSV-GR, as described under *Materials and Methods*. Cells were treated with varying concentrations of 0.1 μ M DEX, 50 μ M BA, or a combination of both compounds (BA + DEX). Control cells (CON) received DMSO alone. The normalized luciferase activity is the mean \pm S.D. of three samples. *, $P < 0.05$ statistically significant effect of DEX; **, $P < 0.05$ statistically significant effect of BA.

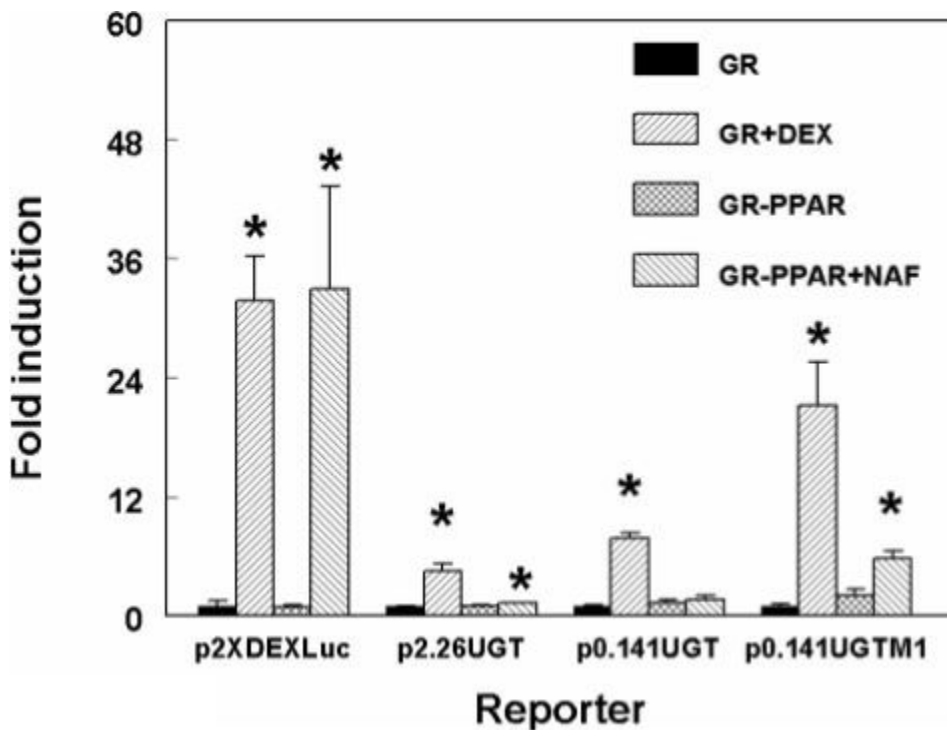


Fig. 7. Comparison of the glucocorticoid receptor and a chimeric receptor (GR-PPAR) on reporter gene induction of luciferase activity by either DEX or nafenopin. Luciferase and β -galactosidase activities were performed on lysed HepG2 cells transfected with either p2XDex-Luc or the UGT1A6 reporters p2.26UGT1A6Luc, p0.141UGT1A6Luc, or p0.141UGT1A6LucM1. All vectors were cotransfected with a control vector, pCMV- β , and receptor expression vectors, pRSV-GR or pGR-PPAR, as described under *Materials and Methods*. Cells cotransfected with pRSV-GR were treated with 0.1 μ M DEX, whereas cells transfected with pGR-PPAR were treated with 50 μ M nafenopin (NAF). Control cells received DMSO alone. The normalized luciferase activity is expressed as the relative light units divided by β -galactosidase activity and is the mean \pm S.D. of three samples. *, $P < 0.05$ statistically different from untreated cells.

TABLE 1

Oligonucleotide PCR primers used to generate UGT1A6 reporter constructs

Name	Sequence	Position
OKF44	5'-TTAGAGACCTGCGACGTGA-3'	102 to 122C
OKF45	5'-CAAGGTTAATGCAAGCGCTG-3'	-191 to -171
OKF46	5'-GGCTAAGGACAGGTACGAGG-3'	-434 to -414
OKF47	5'-GAGAATGTGCGTGACAAG-3'	-141 to -123
OKF48	5'-GAGAATGTGCCTGACAAG-3'	-141 to -123
OKF49	5'-GGTACC-GTGACCAGTTCTTTGATG-3'	-118 to -100
OKF154	5'-ACAGCACTCTCCCTCAGCTGC-3'	-95 to -72
OKF155	5'-TGCTGTTGCATCAAAGAACT-3'	-100 to -89C

TABLE 2

List of UGT 5'-flanking constructs in pGL₃-basic used in transient transfection assays

Mutated bases are shown in bold.

Name	5'-Flanking Sequence	Mutation Site
p2.26UGT1A6Luc	-2260 to +431	
p1.10UGT1A6Luc	-1106 to +27	
p0.434UGT1A6Luc	-434 to +27	
p0.191UGT1A6Luc	-191 to +27	
p0.141UGT1A6Luc	-141 to +27	
p0.141UGT1A6LucM1	-141 to +27	-134 -tgcctga- -128
p0.141UGT1A6LucM2	-141 to +27	-95 -acagca- -90
p0.118UGT1A6Luc	-118 to +27	