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REGULATION OF THE RAT UDP-GLYCOSYLTRANFERASE 1A6 BY GLUCOCORTICOIDS INVOLVES A CRYPTIC GLUCOCORTICOID RESPONSE ELEMENT

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Running Title: Regulation of rat UGT1A6 by glucocorticoids

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Abbreviations: *AhR*, aryl hydrocarbon receptor; *Ah*RE, Aryl hydrocarbon receptor response element; BA, 1,2-benzanthracene, DEX, dexamethasone, DMSO, dimethyl sulfoxide; GRE, glucocorticoid response element; MMTV LTR mouse mammary tumor virus long terminal repeat, PAH, polycyclic aromatic hydrocarbon, RU 38 486, 17β-hydroxy-11β-(4-dimethylamino-phenyl)-17α-(prop-1-ynyl)-estra-4,9-dien-3-one; UGT, UDP-glycosyltransferase

ABSTRACT

Glucocorticoids precociously induce fetal rat UDP-glycosyltransferase 1A6 (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 kb of the 5'-flanking region of the UGT1A6 non-coding leader exon (A1*), dexamethasone increased basal activity 3-to-7-fold in cells co-transfected with an expression plasmid for GR. PAH increased gene expression 23-fold, but the presence of DEX only induced PAH-dependent expression by 1.5-fold, suggesting interaction between GR and *Ah* receptor. Further, the GR antagonist RU 38 486 was a partial agonist increasing, rather than inhibiting 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 (*AhRE*) response element and both PAH and glucocorticoid-dependent gene activation were lost when this area was deleted. Mutation of a single base pair located in the *Ah*RE region simultaneously reduced induction by PAH and increased glucocorticoid induction. Thus the sequences of both the *Ah*RE and glucocorticoid response elements appear to overlap, suggesting *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 a non-classical mechanism of induction is involved in this response.

INTRODUCTION

The UDP-glycosyltransferases (UGT) are a superfamily of enzymes with molecular weight between 50-60 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 (PAH), such as benzo[a]pyrene or chlorinated compounds, such as 2,3,7,8-tetrachloro-p-benzodioxin (Emi *et al.*, 1995;Emi *et al.*, 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 PAS domain class which dimerizes with ARNT (*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 *Ah*RE is located in the region flanking the non-coding 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;Wishart and Dutton, 1977b) demonstrated that administration of glucocorticoids to pregnant rats induced microsomal UGT

activity with *o*-aminophenol, *p*-nitrophenol, 1-napthol 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;Emi *et al.*, 1996) is unusual since enzymes encoded by this gene are generated from unique first exons that encode regions of the protein that dictate substrate specificity and share exons 2-5 which encode catalytic function. A rare inherited disease, Crigler-Najjar syndrome, that 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;Emi *et al.*, 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 Dutton and Co-workers (Wishart and Dutton, 1977a). The second cluster containing exons B1-B5 encodes enzymes with substrates 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 (Prough *et al.*, 1996;Xiao *et al.*, 1995) 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 2-3 fold by inclusion of the synthetic glucocorticoid, DEX. In these studies, CYP1A1 was also potentiated, while other genes namely NAD(P)H:quinone oxidoreductase, glutathione *S*-tranferase 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 either activates or represses gene transcription depending on 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 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.

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MATERIALS AND METHODS

Materials: Restriction endonucleases, T4 ligase and pGl_3-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 (LSU Medical Center, Shreveport, LA). The expression plasmid for the chimeric GR-PPAR plasmid was provided by T.H. Rushmore as has been described previously (Boie *et al.*, 1993).

Reagents for culturing *E. coli* were purchased from Difco Laboratories (Detroit, MI). Antimycotic/antibiotic solution, non-essential amino acids and Dulbecco's modified essential medium (high modified) was obtained from Mediatech (Hernon, 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 (Pittsbutgh, PA).

BA and DEX were obtained from Sigma Chemical Co (St. Louis, MO). Nafenopin was obtained from Ciba-Geigy Chemical (Ardsley NY). RU 38 486 (17β-hydroxy-11β(4-dimethylamino-phenyl)-17α(propyl-1-ynyl)-estra-4,9-dien-3-one) was obtained from Roussel Uclaf (Romainville, Cedex, France). Chloro-phenol 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* DH5α cells were purchased from Invitogen (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 Eagles Medium (high modified) supplemented with 10% fetal bovine serum, antimycotic/antibiotic and non-essential amino acids. The cells were incubated at 37 C in a 5% carbon dioxide atmosphere and sub-cultured 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 down steam primer and either primers OKF45, OKF 46, OKF47, OKF49. To mutate the *Ah*RE, 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 *Ah*RE, 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 Mg^{2+} 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 sub-cloned into pCR2 or pCR2.1 (Invitrogen, Carlsbad, CA) and the insert sequenced at the University of Louiville Center for Genetics and Molecular Medicine core facility. The sequences in all but the mutant

constructs agreed with that previously, (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 pRPT6 2.6 BX that was sub-cloned from λRPT6 (Metz and Ritter, 1998). p1.106UGT1A6 was synthesized by initially sub-cloning a 1.1 kilobase Hind III/Eco RI fragment from pRPT6 2.6 BX into the unique restriction sites of pBSIIKS+ (Stratagene, La Jolla, CA). A Kpn I/EcoRI fragment was sub-cloned into the KpnI/EcoRI sites of a modified version of the pGL_3-basic vector (Promega, Madison, WI). p2.26UGT1A6 was produced by sub-cloning a 2.7 kb XhoI/SacI fragment from pRPT6 2.6 BX into the unique XhoI/SacI sites of pGL_3-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 sub-cloning this fragment into the EcoRI site in the modified version of pGL_3-basic. Orientation of the fragment was determined by digestion with TthIIIL/HindIII. p0.118UGT1A6 (OKF44,49) was generated by sub-cloning a KpnI/EcoRI fragment into those unique sites in the modified pGL_3-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% confluency by a calcium phosphate-based transfection technique described previously (Falkner *et al.*, 2001). The cells were treated 24 hours following transfection with various agents made up as 500 X stock solutions in DMSO (Controls received DMSO alone). After 24 hours, the cells were harvested with 100 µL of cell lysis buffer (Promega, Madison, WI) according to the manufacturer's instructions and subjected to a single freeze thaw event. All cells were co-

transfected with 500 ng of pCMV-β as a transfection control. Routinely, 125 ng of receptor expression plasmid, pCMVGR or pGR-PPAR and 1 μg of the various UGT1A6 expression plasmids were added to each well.

Assays for β-galactosidase and Luciferase activity: Luciferase activity was determined using the Luciferase Assay system (Promega, Madison, WI) in a Perkin Elmer Victor^3 1420 Multilabel counter (Waltham, MA) For β-galactosidase activity, cell extracts were incubated with chlorophenol red β-galactopyranoside at 37 C for between 15-60 minutes . The activity was determined spectrophotometrically at 595 nm using a Bio-Tek μQuant universal microplate spectrophotometer (Winooski, VT).

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

RESULTS

Effects of BA, DEX and RU 38 486 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 2,360 bp of the 5-flanking region of UGT1A6 (Metz and Ritter, 1998) by 22-fold (Figure 1). This result is similar to the previous studies (Emi *et al.*, 1996) which 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 μM) significantly potentiated the PAH-dependent induction by 2.3-fold. The DEX-dependent induction required co-transfection of an expression vector for the glucocorticoid receptor (results not shown). These results are consistent with observations *in vivo* in treated animals by Dutton and co-workers (Wishart and Dutton, 1977b;Wishart and Dutton, 1977a) that glucocorticoids induce glucuronidation rates of substrates for UGT1A6.

Treatment with the glucocorticoid and progesterone receptor antagonist RU 38 486 induced the basal level of expression of p2.26UGT1A6Luc by 3.5 ± 0.4-fold, suggesting it is an agonist for this gene. RU 38 486 was also an effective antagonist of the DEX-dependent induction of this reporter system as the expression of luciferase activity with treatment with DEX in the presence of RU38 486 was not different from RU38 486 alone.

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PAHs interfere with the glucocorticoid dependent induction of luciferase activity in HepG2 cells transfected with p2.26UGT1A6luc. In Figure 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 (Figure 2A), in comparison with a classical GRE driven reporter (Figure 2B). As shown in Figure 2A, 50 μM BA induced the UGT reporter 7.8 ± 1.1-fold, 5 μM BA treatment induced this construct 4.89 ± 1.12-fold, while the lowest concentration of BA tested (0.5 μM) did not significantly induce luciferase activity. In control cells, the DEX-dependent induction was 3.44 ± 0.72 fold, while in cells treated with 50 μM BA the fold induction (1.32±0.186) was reduced by approximately 87%. In the 5 μM 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 classical glucocorticoid-reponsive reporter. The reporter, p2XDEX-Luc, has two copies of the palindromic glucocorticoid response element found in the MMTV LTR. The results, shown in Figure 2B unexpectedly indicated that BA induced this reporter construct 2.47 ± 0.42-fold and 1.54 ± 0.23- fold at 50μM and 5 μM BA, respectively. This induction by BA was unexpected because this reporter was not known to contain an *Ah*RE. 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 μM) was a reduction in the fold induction by DEX observed (54 ± 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 classical glucocorticoid responses and possibly being 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 the majority of the effect being DNA specific with the UGT1A6 gene and most likely being due to crosstalk between the GRE and *Ah*RE.

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 classical glucocorticoid receptor dependent reporter. As shown in Figure 3, a simple monotonic increase was observed for both the UGT1A6 reporter p2.26UGT1A6Luc (Figure 3A) and the "classical" glucocorticoid responsive reporter p2XDEX-Luc (Figure 3B). The IC_{50} for both processes was approximately 8.3×10^{-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 (approximately 4-fold) was considerably lower than that observed with the model MMTV-GRE containing reporter, p2XDEX-Luc (80-fold). Thus, the concentration-dependence of this response obtained in this study is consistent with the process being mediated through the glucocorticoid receptor and it is

sensitive to Ru 38 486 (Figure 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 *Ah*RE. 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 Figure 4A. As shown in Figure 4B, there was a major difference in the basal level of expression between p2.26UGT1A6Luc and p1.1UGT1A6Luc, whose basal activity is 98-fold higher than p2.26UGT1A6Luc, 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 Figure 4C, all of the constructs tested except p0.118UGT1A6Luc were responsive to glucocorticoids. This was a surprising result as 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 p0.195UGT1A6Luc and p0.141UGT1A1Luc, 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 expression of luciferase activity The *Ah*RE that is responsible for the PAH-dependent induction of this gene is located in this region between base pairs -134 and -129.

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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-dep*endent and glucocortoid-dependent induction of this gene.

Mutation of the *Ah*RE reduces induction of UGT1A6 reporters by PAHs, but increases the ability of glucocorticoids to induce gene expression. To test whether *AhR* binding to the *Ah*RE is directly involved in the suppression of the glucocorticoid-dependent induction, we mutated a single base pair in the *Ah*RE found in 5'-flanking region of the UGT1A6 gene. Previous studies (Emi *et al.*, 1996) have shown that either mutation of base pairs in the *Ah*RE 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 Figure 5.

As anticipated, BA induced reporter constructs that had an intact *Ah*RE, p2.26UGT1A6Luc and p0.141UGT1A6Luc, 13.5±0.6-fold and 16.6 ±4.6 fold, respectively. In reporter constructs that had either a deleted *Ah*RE (p0.118UGT1A6) or a mutated *Ah*RE (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 (Figure 5). Interestingly,

the *Ah*RE-mutated construct, p0.141UGT1A6LucM1, which has a single base pair mutation difference from p0.141UGT1A6Luc was induced 20.2 ± 2.4-fold; 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 *Ah*RE on glucocorticoid-dependent induction of this gene that is independent of the occupancy of *Ah*RE by the *AhR*. However, BA treatment reduced the fold-induction by glucocorticoids in all PAH responsive plasmids, suggesting 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. While the presence of DEX and BA causes a trend toward induction in the 2,260 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 co-activators to the UGT1A6 glucocorticoid responsive unit (GRU).

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 (Figure 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 figure 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 which 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 EMSA studies. We utilized 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 μM DEX treatment and 13.1 ± 3.18-fold by BA treatment as is shown in Fig 6B. In the GRE mutant construct p0.141UGT1A6LucM2, the basal level of the reporter activity (44,729 ± 10,804 Relative light units) was relatively unchanged from the wild type construct (35,044 ± 11,803 RLU), 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 μM 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 appears independent of mutation of GRE and therefore, GRE occupancy. However, as

seen in Figure 5, this is not the case for GR binding, which probably involves a multi-protein complex, like the GRU 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 glucocorticoid receptor ligand binding domain and the peroxisome proliferator activated receptor ligand binding domain. This receptor can robustly induce a response through a classical GRE as is shown in Figure 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 "classical" 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 co-transfected with the UGT1A6 reporters and the cells 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, while 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 *Ah*RE, DEX induced gene transcription 21 ± 0.4 fold, while nafenopin only induced the construct 2.7 ± 0.6. Although the co-transfection 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 co-transfected 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 paper 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 Dutton and co-workers (Wishart and Dutton, 1977a;Wishart *et al.*, 1977) using fetal rat tissue indicate that glucocorticoids induce the expression of glucuronosyltransferases (*i.e.*, UGT1A6). Dutton and co-workers (Wishart and Dutton, 1977a;Wishart *et al.*, 1977) demonstrated that UGT activities toward with *o*-aminophenol, *p*-nitrophenol, 1-napthol and serotonin were strikingly increased after treating pregnant rats with dexamethasone (approx. 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., 2001b). These studies indicated that DEX treatment *in vivo* induced only a class 1 transcript generated from the P1 promoter (Auyeung et al., 2001a), while 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 predominately express a class 2 transcript (Auyeung et al., 2003b). 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 RU38 486 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 one antagonists induce minor conformational change, insufficient to allow regulatory cofactors to recognize a ligand-activated receptor. The type two antagonists, such as RU 38 486, induce a conformation change that is similar to an agonist-activated receptor; however, these antagonists tend to recruit co-repressors rather than co-activators, when associated with a response element. The ability to act as a 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 38 486 has been shown to be a partial agonist of glucocorticoid-receptor function when the cells are co-transfected 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 co-transfect an expression vector for the glucocorticoid receptor to elicit high levels of expression by DEX (Falkner et al., 1998;Falkner et al., 2001) and thus the partial agonist activity we observe in these studies may simply be an artifact of over-expression of the glucocorticoid receptor. Another possible explanation is that RU38 486 is a true partial agonist of this response. The bovine

prolactin promoter contains a negative glucocorticoid response element in which RU38 486 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 co-repressors or coactivators are recruited.

Classically, glucocorticoid signaling is mediated through the glucocorticoid receptor binding its cognate response element, an imperfect palindrome of TGTYCT 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 *Ah*RE affected the glucocorticoid responsiveness of the reporter constructs (Figure 5), while mutation of the putative GRE had no effect upon *Ah*-receptor mediated gene activation (Figure 6). This may reflect the differences in *Ah*-receptor and GR function. The *Ah*-receptor is capable of trans-activating 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 the cooperatively with other transcription factors to recruit co-activators, while 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 (Stafford *et al.*, 2001;Wang *et al.*, 1999) 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, HNF3β, COUP-

TF and C/EBPβ) 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/EBPβ 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 factors binding sites that reside in the distal promoter responsible for generating a type 1 transcript (Auyeung et al., 2003a). 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 to –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 classical 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/EBPβ and in the collagenase-3 gene, where the glucocorticoid receptor binds to AP-1 transcription factors (Heck *et al.*, 1994;Jonat *et al.*, 1990;Schule *et al.*, 1990). The rat UGT1A6 gene does not appear to contain any high consensus AP-1 or C/EBPβ 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 paper 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 paper are likely restricted to the rat gene.

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FOOTNOTE

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Legends for Figures

Figure 1. Effects of DEX, BA and RU486 on expression of a full length rat UGT IA6 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 in Materials and Methods. Cells were treated with either 0.1 μM Dex (DEX), 50 μM BA (BA), or a combination of both compounds (BA+DEX) in the absence or presence of 1 μM RU38 486. 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 ± SD of three samples. * $P<0.05$ statistically difference than control cells, ** $P<0.05$ statistically different than control or DEX-treated cells.

Figure 2. Concentration-dependence of the glucocorticoid-dependent induction of UGT reporter luciferase activity in HepG2 cells. HepG2 cells were transfected with either p2.26UGT1A6Luc (Panel A) or p2XDex-Luc (Panel B) and 24 hours 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 co-expressed with the reporter vectors, as described in Materials and Methods. Cells were treated with varying concentrations of BA in the absence or presence of 0.1 μM 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 ± SD 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 classical GRE reporter p2XDex-Luc. Luciferase and β-galactosidase assays were performed on lysed HepG2 cells that had been transfected with either p2.26UGT1A6Luc (Panel A) or p2XDex-Luc (Panel B), a control vector pCMV-β and an expression vector for the glucocorticoid receptor pRSV-GR, as described in 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 ± SD of three samples. * $P<0.05$ statistically significant difference from control cells

Figure 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 in Materials and Methods. The cells were treated either with vehicle (CON) or 0.1μM DEX (DEX) for 24 hrs prior to harvest. Basal level luciferase is shown in Figure 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 ± SD of three samples. * $P<0.05$ statistically significant difference from control cells

Figure 5. Effects of mutation or deletion of the *Ah*RE 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 *Ah*RE deletion constuct p0.118UGT1A6Luc

(p0.118UGT), p0.141UGT1A6Luc (p0.141UGT) or a *Ah*RE mutated version of p0.141UGT1A6LucM1 (p0.141UGTM1). All vectors were co-transfected with a control vector pCMV-β, and an expression vector for the glucocorticoid receptor pRSV-GR, as described in Materials and Methods. Cells were treated with varying concentrations of 0.1 μM DEX (DEX), 50 μM BA (BA) or a combination of both compounds (BA + DEX). Control cells (CON) received DMSO alone. The normalized luciferase activity ± SD of three samples. * $P<0.05$ statistically significant effect of DEX, ** $P<0.05$ statistically significant effect of BA.

Figure 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 Figure 6A. Mutated base pairs are indicated with *. In Figure 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 co-transfected with a control vector pCMV-β, and an expression vector for the glucocorticoid receptor pRSV-GR, as described in Materials and Methods. Cells were treated with varying concentrations of 0.1 μM Dex (DEX), 50 μM BA (BA) or a combination of both compounds (BA+DEX). Control cells (CON) received DMSO alone. The normalized luciferase activity ± SD of three samples. * $P<0.05$ statistically significant effect of DEX, ** $P<0.05$ statistically significant effect of BA.

Figure 7. Comparision 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 co-transfected with a control vector pCMV-β and receptor expression vectors, pRSV-GR or pGR-PPAR as described in Materials and Methods. Cells co-transfected with pRSV-GR were treated with 0.1 μM DEX while cells transfected with pGR-PPAR were treated with 50 μM 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 ± SD of three samples. *P< 0.05 statistically different from untreated cells.

Table 1. Oligonucleotide PCR primers used generate UGT1A6 reporter constructs

Name	Sequence	Position
OKF44	5'-TTAGAGACCTGCGACGTGA-3'	102 to122 C
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'-ACAGCACTCTCTCCCTCAGCTGC-3'	-95 to -72
OKF155	5'-TGCTGTTTGCATCAAAGAACT-3'	-100 to -89C

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Table 2. List of UGT 5-flanking Constructs in pGL_3-basic used in transient transfection assays

Name	5'-flanking sequence	Mutation site
p2.26UGT1A6Luc	-2260 to + 431	
p1.10UGT1A6Luc	-1,106 to +27	
p0.434UGT1A6Luc	-434 to +27	
p0.191UGT1A6Luc	-191 to +27	
p0.141UGT1A6Luc	-141 to +27	
p0.141UGT1A6LucM1	-141 to +27	-134 -tgc**c**tga- -128
p0.141UGT1A6LucM2	-141 to +27	-95 -a**c**a**g**ca- -90
p0.118UGT1A6Luc	-118 to +27	

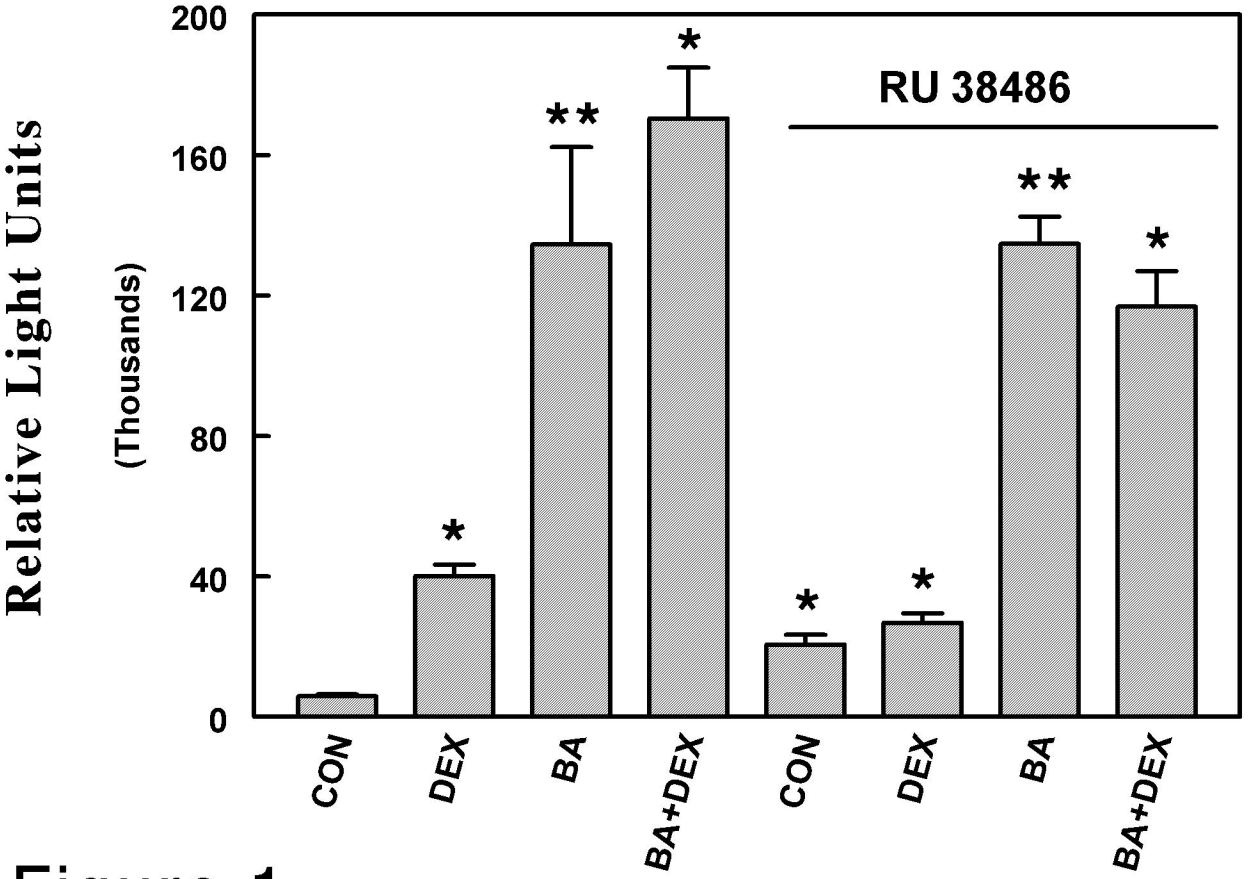
Relative Light Units
(Thousands)
200
160
120
80
40
0
RU 38486
*
**
*
*
*
**
*
CON
DEX
BA
BA+DEX
CON
DEX
BA
BA+DEX
Treatment

Figure 1

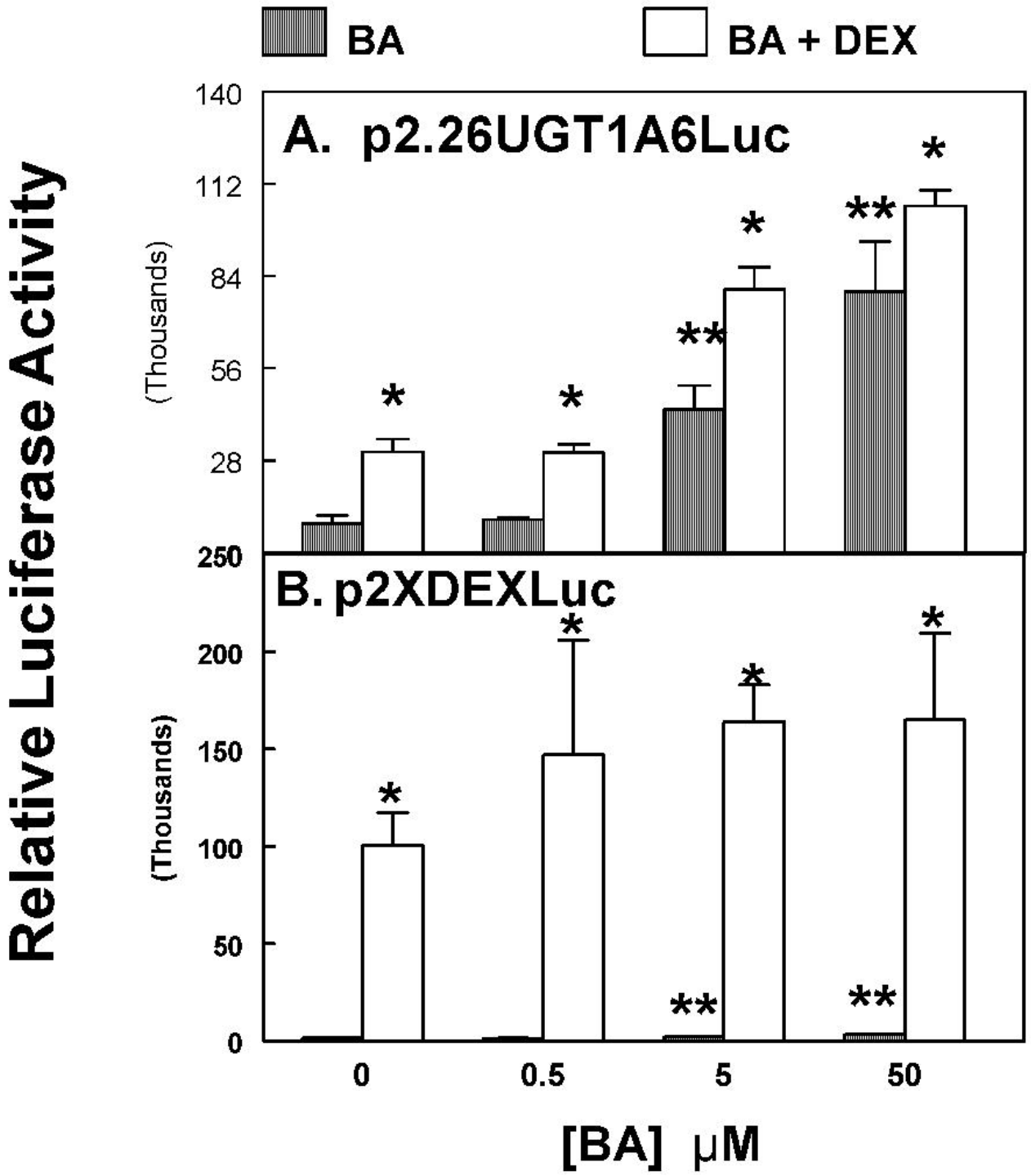
BA
BA + DEX
A. p2.26UGT1A6Luc
B. p2XDEXLuc
Relative Luciferase Activity
(Thousands)
140
112
84
56
28
250
200
150
100
50
0
0
0.5
5
50
[BA] µM

Figure 2

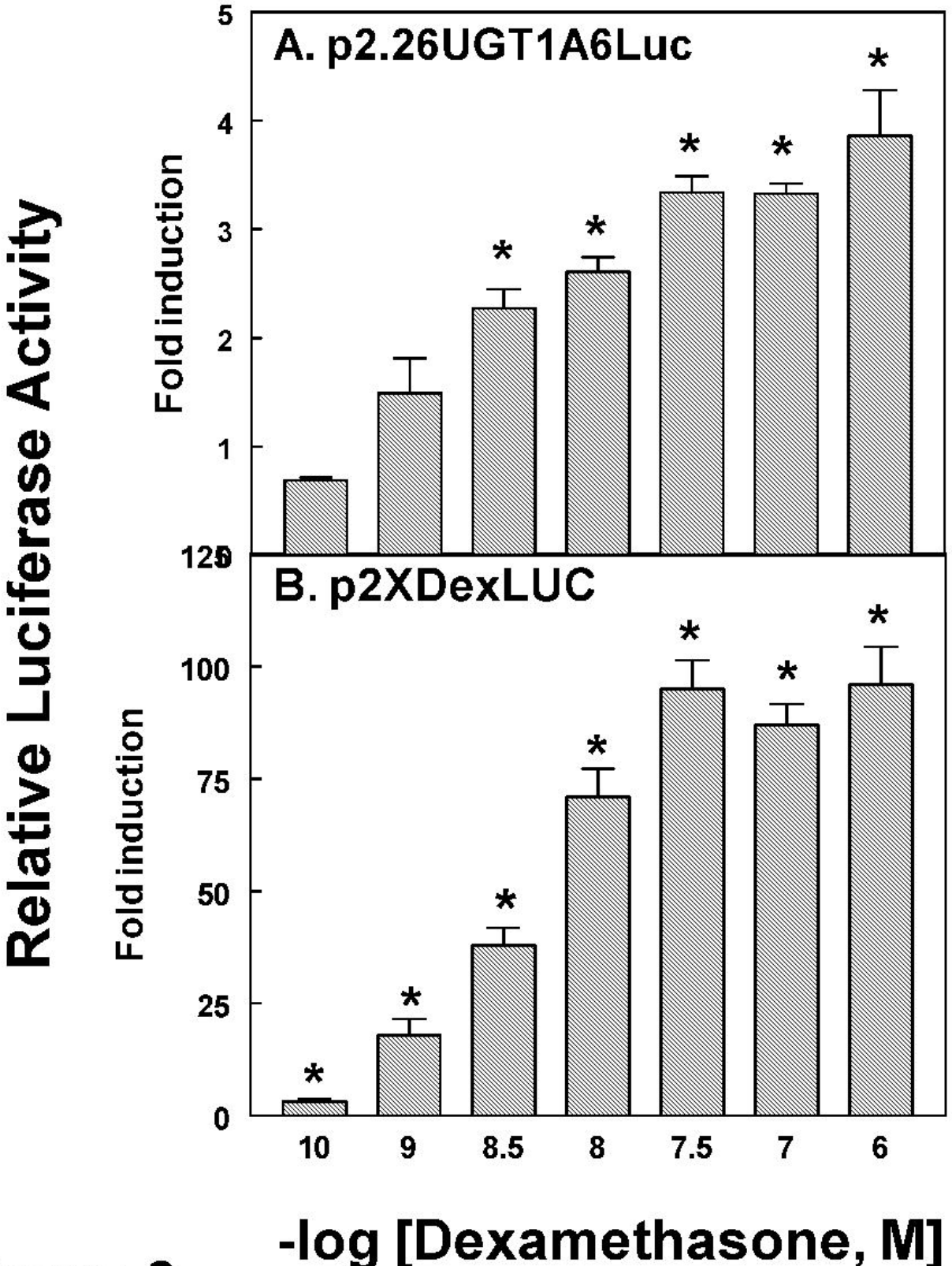
A. p2.26UGT1A6Luc
B. p2XDexLUC
Fold induction
Fold induction
Relative Luciferase Activity
-log [Dexamethasone, M]
10
9
8.5
8
7.5
7
6
0
25
50
75
100
125
1
2
3
4
5
*

Figure 3

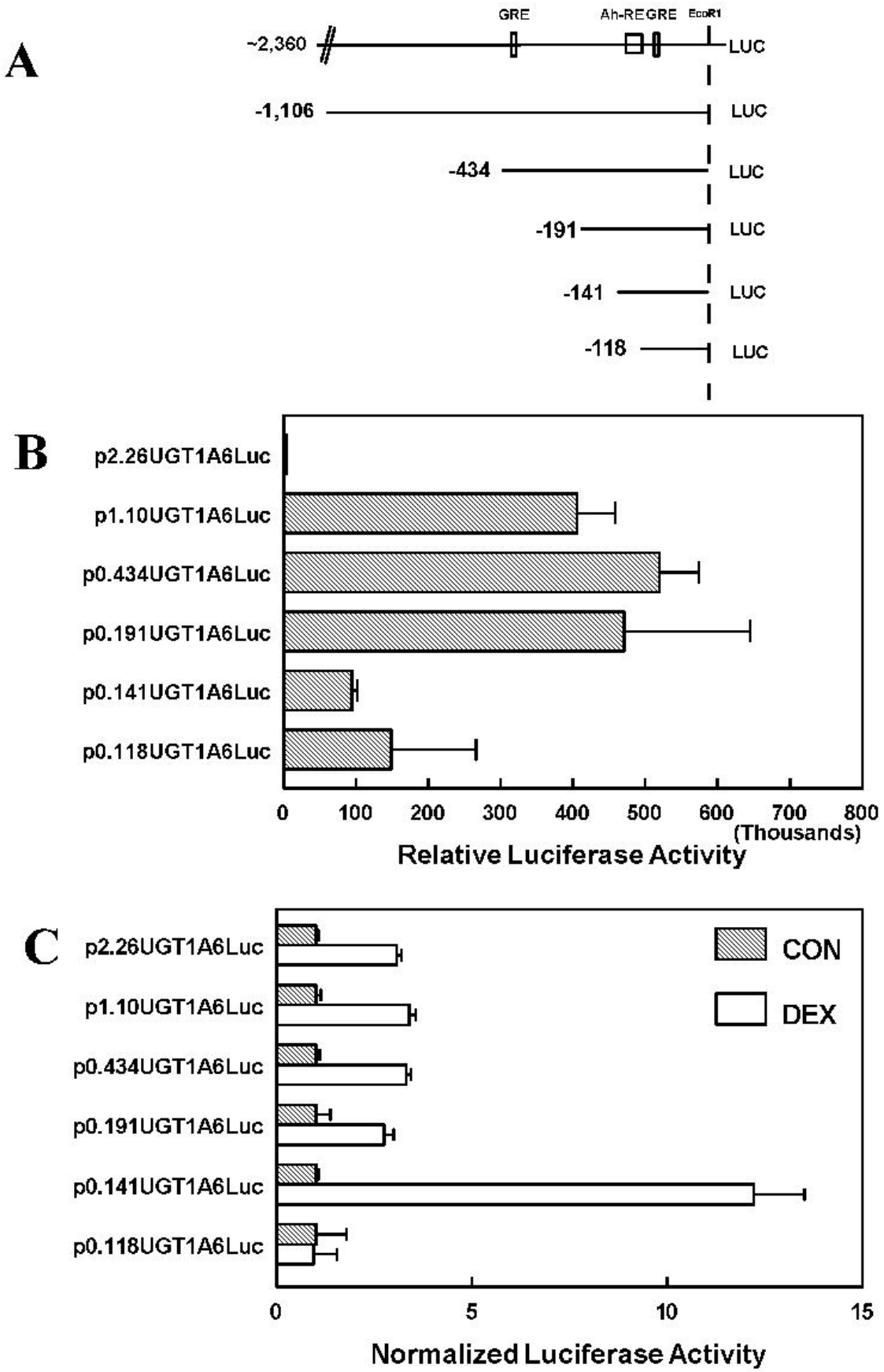
A
GRE
Ah-REGRE
EcoR1
-2,360
LUC
-1,106
LUC
-434
LUC
-191
LUC
-141
LUC
-118
LUC
B
p2.26UGT1A6Luc
p1.10UGT1A6Luc
p0.434UGT1A6Luc
p0.191UGT1A6Luc
p0.141UGT1A6Luc
p0.118UGT1A6Luc
0
100
200
300
400
500
600
700
800
(Thousands)
Relative Luciferase Activity
C
p2.26UGT1A6Luc
p1.10UGT1A6Luc
p0.434UGT1A6Luc
p0.191UGT1A6Luc
p0.141UGT1A6Luc
p0.118UGT1A6Luc
CON
DEX
0
5
10
15
Normalized Luciferase Activity

Figure 4

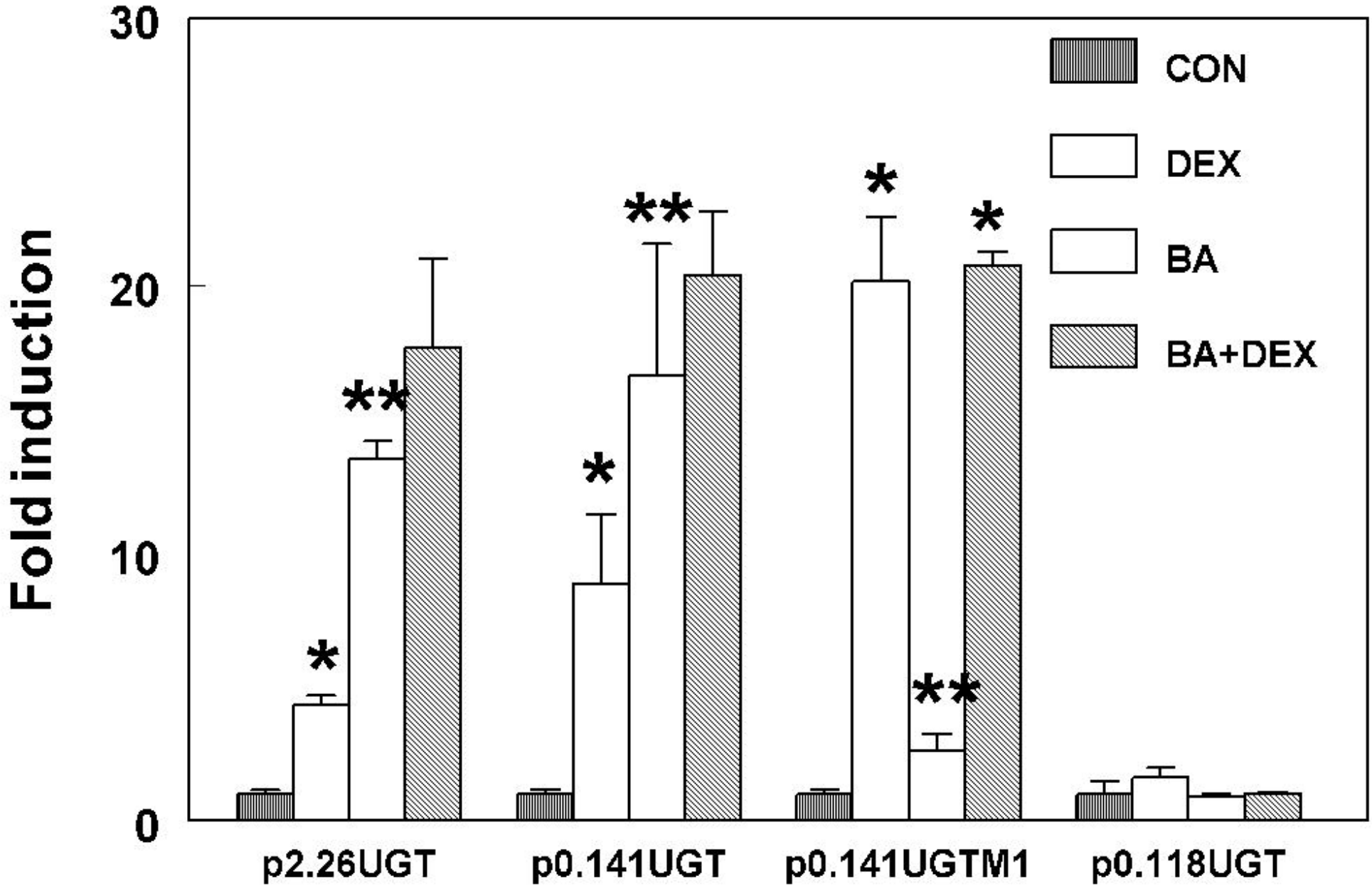
30
20
10
0
Fold induction
CON
DEX
BA
BA+DEX
*
**
**
*
*
**
p2.26UGT
p0.141UGT
p0.141UGTM1
p0.118UGT
Transfected Vector

Figure 5

A.

UGT1A6 GRE	-tgcaaAGAACActcTCTCCCtcagc-
UGT1A6 GRE mut	-tgcaaACAGCActcTCTCCCtcagc-
	* *
Consensus GRE	-nnnnnAGAACAnnnTGTTCTnnnnn-

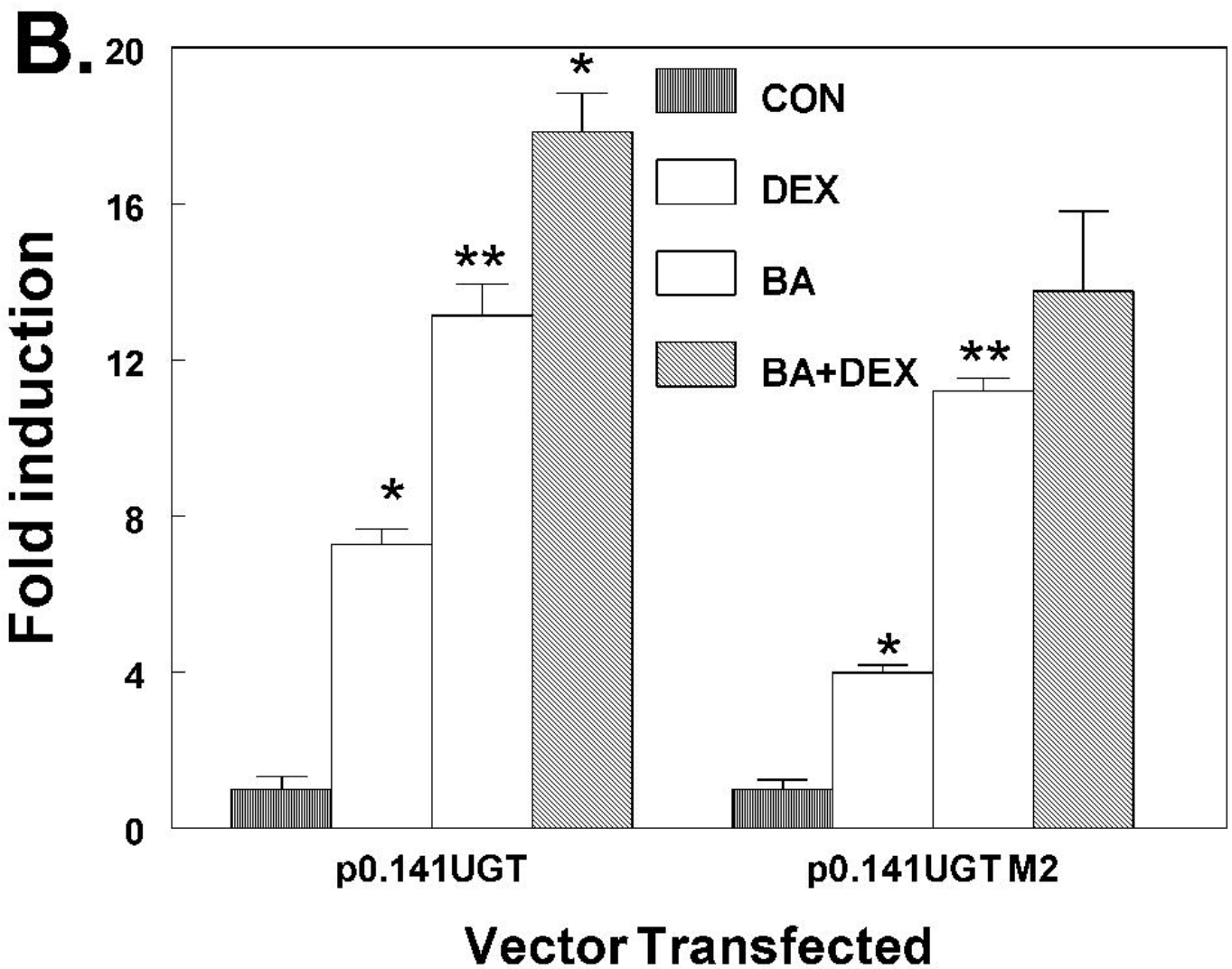

Figure 6

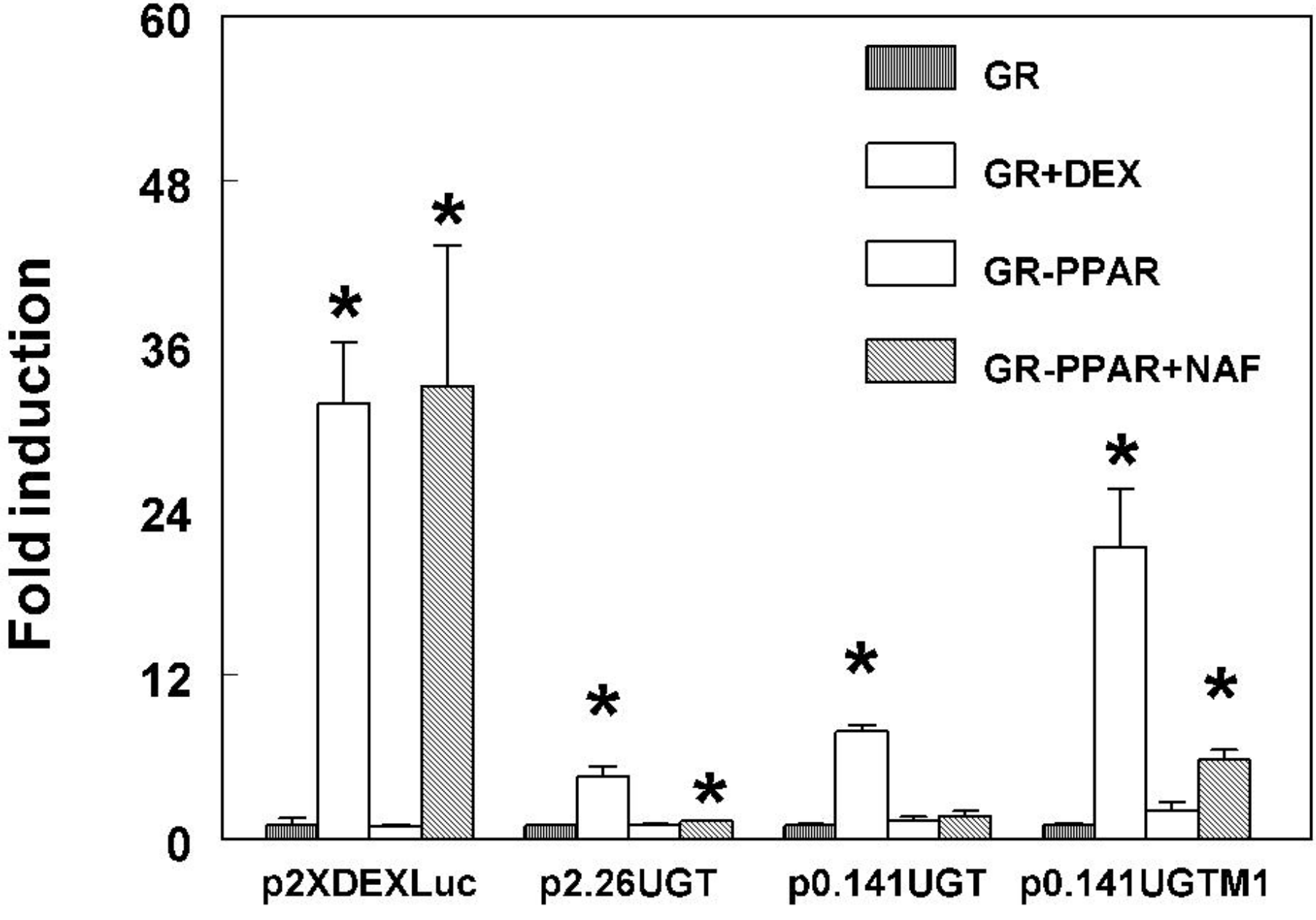
60
48
36
24
12
0
Fold induction
GR
GR+DEX
GR-PPAR
GR-PPAR+NAF
*
p2XDEXLuc
p2.26UGT
p0.141UGT
p0.141UGTM1
Reporter

Figure 7