# Regulation of the Rat Glutathione S-Transferase A2 Gene by Glucocorticoids: Involvement of Both the Glucocorticoid and Pregnane X Receptors

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## ABSTRACT

Glucocorticoids regulate the rat glutathione S-transferase A2 (*GSTA2*) gene in a biphasic manner in cultured hepatocytes that repress gene expression at low concentration (10–100 nM) but induce gene expression at high concentration ($>1$ $\mu$M). High concentrations of the glucocorticoid receptor (GR) antagonist RU38486 (5–10 $\mu$M) also induced the expression of *GSTA2*. These effects were reproduced in HepG2 cells transfected with a luciferase reporter containing 1.6 kilobase pairs of 5′-flanking sequence of *GSTA2* and expression plasmids for either GR, pregnane X receptor (PXR) or a combination of both. Dexamethasone *t*-butylacetate (1 $\mu$M *t*-Bu-DEX) repressed gene expression between 60 to 75% when only GR was expressed. When PXR was expressed, both basal and *t*-Bu-DEX-dependent gene expression was increased over 2-fold, respectively.

Biphasic regulation of gene expression was observed over a broad range of *t*-Bu-DEX concentrations when expression plasmids for both receptors were cotransfected. Other steroids of the pregnane class induced *GSTA2* expression as expected for a PXR-dependent process. Because no canonical responsive element for the PXR-RXR$\alpha$ heterodimer was observed in the 5′-flanking region of the construct, deletion analysis was used to identify a pregnane responsive region between base pairs $-700$ and $-683$; this 20-bp region contains the antioxidant response element (ARE). When the ARE sequence was mutated, basal, *t*-butylhydroquinone- and 17$\alpha$-hydroxypregnenolone-inducible expression were all lost. These results suggest that PXR interacts with factors binding to the ARE to elicit the pregnane inductive response for *GSTA2.*

The glutathione S-transferase A2 (*GSTA2*) gene encodes a protein that is a member of a superfamily of dimeric enzymes of subunit molecular mass between 20 and 30 kDa (Ketterer 1986; Mannervik and Danielson, 1988; Hayes and Pulford, 1995) These proteins play a major role in the detoxification of xenobiotic chemicals by catalyzing the conjugation of glutathione with the electrophilic centers of these toxicants, thus preventing protein or nucleic acid alkylation reactions. In addition, GSTA2 serves as an intracellular binding protein for nonsubstrate ligands, which includes steroids of the pregnane class (Listowsky, 1993) and bile acids including lithocholic acid (Takikawa and Kaplowitz, 1988).

The transcriptional activation of the rat *GSTA2* subunit gene by xenobiotic compounds has been well characterized by Pickett and coworkers (Rushmore et al., 1990; Rushmore et al., 1991; Nguyen and Pickett, 1992). Two functionally independent responsive elements, the *Ah*-receptor response element (*Ah*RE, Rushmore et al., 1990) and the antioxidant response element (ARE, Rushmore et al., 1991), are required for the induction of this protein subunit by xenobiotic compounds. Polycyclic and polychlorinated aromatic compounds induce gene expression via the *Ah* receptor, which binds to the *Ah*RE (Whitlock et al., 1996). The metabolites of aromatic compounds, phenolic antioxidants, phorbol esters, and hydrogen peroxide cause transcriptional activation, apparently through action of the transcription factor Nrf-2 and its heterodimerization partner, a member of the small maf family, which binds to the ARE (Itoh et al., 1999).

Glucocorticoids have been shown to modulate transcription of genes by several different mechanisms. Classically, glucocorticoids act as ligands for the glucocorticoid receptor (GR) to either enhance or repress expression of target genes (Beato, 1989). The simplest interaction of this transcription factor is through binding to a canonical sequence, TGTYCT, often found as an imperfect palindrome separated by three base pairs. This interaction is characterized by a concentration dependence for the synthetic glucocorticoid DEX similar

**ABBREVIATIONS:** GSTA2, glutathione S-transferase A2; *Ah*, aryl hydrocarbon; *Ah*RE, *Ah*-receptor response element; ARE, antioxidant response element; PXR, pregnane X receptor; kb, kilobase pair(s); DEX, dexamethasone; GR, glucocorticoid receptor; PCR, polymerase chain reaction; *t*-Bu-DEX, dexamethasone *t*-butylacetate; RU38486, 17$\beta$-hydroxy-11$\beta$-(4-dimethylamino-phenyl)-17$\alpha$-(prop-1-ynyl)-estra-4,9-dien-3-one; DMSO, dimethyl sulfoxide; bp, base pair(s); RXR$\alpha$, retinoid X-receptor-$\alpha$; DR3, direct repeat separated by 3 base pairs; QOR, NAD(P)H:quinone oxidoreductase.

to the known $K_d$ value of GR (less than $10^{-7}$ M) and by inhibition of its action by the GR antagonist RU38486.

Kliewer et al. (1998) described another member of the nuclear receptor superfamily, the pregnane X receptor (PXR), that is activated by steroids of the pregnane class and cholestatic bile acids, such as lithocholic acid and 3 keto-litho-cholic acid (Staudinger et al., 2001). In addition to these endogenous ligands, this receptor is activated by xenobiotic compounds such as imidazole derivatives and macrolide antibiotics (e.g., rifampicin). In contrast to GR, PXR binds DEX with much lower affinity and the responses to DEX are observed at concentrations greater than $1 \times 10^{-7}$ M. The ligand dependence is also different from the classical glucocorticoid response; for example, RU38486 serves as an agonist for PXR, rather than an antagonist of GR. PXR binds DNA as an obligate heterodimer with RXR$\alpha$. In rodents, this heterodimeric complex preferentially recognizes a direct repeat of the nonsteroidal nuclear receptor half-site separated by a three-nucleotide spacer (Kliewer et al., 1998). A DR3 binding sequence of AGTTCAnnnAGTTCA as observed in rat *CYP3A23* (Huss et al., 1998; Kliewer et al., 1998; Quattrochi et al., 1998) binds the PXR and is thought to account for the "nonclassical" glucocorticoid induction of rat CYP3A23 protein described by Guzelian and coworkers (Schuetz et al., 1984; Schuetz and Guzelian, 1984; Kocarek et al., 1994).

Our previous work revealed inconsistencies in the effects of glucocorticoids on the expression of *GSTA2* in different systems. In isolated rat hepatocytes, DEX at $1 \times 10^{-6}$ M induced *GSTA2* expression (Xiao et al., 1995). However, transient transfection experiments with HepG2 cells, expression of a reporter construct containing 1.62 kb of 5′ flanking region of rat *GSTA2* was suppressed by DEX at $1 \times 10^{-5}$ M (Falkner et al., 1998). The current report is based on the hypothesis that low concentrations of DEX suppress *GSTA2* gene expression via a GR-dependent mechanism, whereas high concentrations of DEX induce expression via a PXR-dependent mechanism. This report describes the biphasic concentration-dependence of the effects of DEX on the expression of *GSTA2* in primary adult rat hepatocytes. Furthermore, each phase of this response is reproduced in transient transfection experiments in HepG2 cells by cotransfection of expression plasmids for either GR or PXR.

## Experimental Procedures

**Materials.** pCMV-$\beta$ was obtained from Stratagene (La Jolla, CA). The reporter construct p2XDEX-LUC and pRSVGR, the expression vector for the human GR, were a kind gift from Michael Mathis (LSU Medical Center, Shreveport, LA). The expression vector pPXR for the mouse PXR was generously provided by Steven Kliewer (Glaxo Wellcome Research and Development, Research Triangle Park, NC). The expression vector for human RXR$\alpha$ was generously provided by Dan Noonan (University of Kentucky, Lexington, KY).

Reagents for culturing *E. coli* were obtained from Difco Laboratories (Detroit, MI). Antibiotic/antimycotic solution, nonessential amino acids and Dulbecco's modified Eagle's medium (high modified) were purchased from Mediatech, Inc (Herdon, VA). Fetal bovine serum was obtained from Harlan Bioproducts for Science, Inc. (Indianapolis, IN). DNA purification kits from QIAGEN (Chatworth, CA) were used to produce transfection quality plasmid DNA. Oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, CA). PCR reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Steroid ligands for PXR [i.e., corticosterone, DEX, hydrocortisone,

progesterone, 17$\alpha$-hydroxypregnenolone, and 6$\alpha$-methyl-17$\alpha$-hydroxy progesterone acetate (Depo-Provera)] were purchased from the Sigma Chemical Co. (St. Louis, MO). RU38486 was obtained from Roussel Uclaf (Romainville, FRANCE). Pregnenolone was obtained from Mann Research Laboratories, (New York, NY). t-Bu-DEX was purchased from Research Plus, Inc. (Bayonne, NJ). Chlorophenol red-$\beta$-D-galactopyranoside was purchased from Roche Molecular Biochemicals (Indianapolis, IN). All other reagents were purchased from commercial suppliers and were either American Chemical Society or molecular biology grade.

**Primary Hepatocyte Cell Culture** Hepatocytes were routinely prepared from male adult Sprague-Dawley rats (180–250 g; [Hsd: Sprague-Dawley SD] from Harlan Sprague-Dawley, Indianapolis, IN) by in situ liver collagenase perfusion as described previously (Xiao et al., 1995). Cell viability (>85–90%) was determined by trypan blue exclusion. Hepatocytes were suspended (approx.$1 \times 10^6$ cells/ml) in arginine-free Eagle's minimum essential medium supplemented with L-ornithine and insulin-transferrin-sodium selenite solution. Cells (3 ml) were seeded on 60-mm tissue culture dishes that had been precoated with Matrigel. Once plated, the cells were maintained at 37°C in a humidified atmosphere of 95% air/5% $CO_2$. The media were changed 2 and 24 h after plating. At 24 h, inducing agents, either DEX or RU38486, were added in DMSO. Control cells received equivalent amounts of solvent alone. At the desired time, media were removed from the dishes by aspiration, the cells were washed twice with Dulbecco's phosphate-buffered saline, and mRNA were isolated as described previously (Xiao et al., 1995).

**Analysis of mRNA Levels.** Total RNA was isolated by modification of the method of Chomczynski and Sacchi (1987). Northern blot experiments were performed after size-fractionation of the denatured RNA (25 $\mu$g) on formaldehyde-containing 1% agarose gels and transfer of the RNAs to Zetaprobe membranes (Life Science Research, Hercules, CA) by capillary transfer. GSTA2 mRNA was detected using a 290-bp fragment of the plasmid pGTB38 kindly provided by Cecil Pickett (Schering Plough, Kenilworth, NJ). Cytochrome P450 3A1 mRNA was detected using a synthetic oligonucleotide termed ON-1 by Waxman et al. (1992) [5′-ACCACATT-TCTGACGAATTCAGCAGAACTC-3′]. This probe has a single nucleotide mismatch with the sequence reported for *CYP3A23*, the major DEX-inducible cytochrome P450 in the rat (Huss et al., 1998). CYP2B1/2 mRNA was detected using a 1.7-kb *Eco*RI fragment of the plasmid pSP450, which contains the entire coding sequence for *CYP2B1* obtained from Milton Adesnick (New York University Medical Center, New York, NY). These nucleic acid probes were labeled with [$^{32}$P]dCTP using the random primer labeling procedure. Hybridization was carried out at 43°C overnight in 0.25 M sodium phosphate buffer, pH 7.2. As a control, $\beta$-actin mRNA levels were measured using a mouse cytoskeletal $\beta$-actin (Xiao et al., 1995).

**Plasmid Constructs.** Luciferase constructs containing either 0.164 or 1.651 kb of the 5′-flanking region, p0.164YaLUC and p1.62YaLUC, respectively, of the rat *GSTA2* have been described previously (Falkner et al., 1998). Deletion constructs where produced by either sequential subcloning of PCR products or introduction of ds-oligonucleotides into p0.164YaLUC. To prepare a reporter gene with the *GSTA2* promoter containing a polycloning site, primers BMYaUNI (5′-AGACT-AAGCTTGGGTTGTAAAAGAGATACTGA) and 164BGLTOP (5′-AGACT-AGATCT-CTTGTCAGCCCCTCCC-CCCA-3′) were used with p1.6YaCAT as a template to produce a 252-bp fragment containing 164 bp of the 5′-flanking region of the *GSTA2* gene. PCR was performed in a Thermolyne Amplitron II thermal cycler (Barnstead/Thermolyne, Dubuque, IA) with 2 mM $Mg^{2+}$. PCR products were generated through 20 cycles of the following steps: denaturing temperature, 94°C for 0.5 min; annealing temperature, 50°C for 1 min; and elongation temperature, 72°C for 1 min. The PCR fragments were initially subcloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and using the *Bgl*II and *Hin*dIII restriction sites, the recovered PCR fragment was subcloned into the pGL$_2$-basic vector (Promega, Madison, WI) to form p0.164bYaLUC. Other

fragments of the 5′-flanking region were generated using the primers listed in Table 1, which were subcloned into the pCR2.1 recovery vector. These fragments were subcloned into p0.164bYaLUC using either restriction sites in the 5′-flanking regions of the primers or unique restriction sites in the polycloning region of pCR2.1. No differences have been observed in the function of either p0.164YaLUC or p0.164bYaLUC.

The pARE-LUC plasmid containing the sequences from −722 to −682 bp was consructed in a method identical with p0.164YaLUC (Falkner et al., 1998), except that ARE-CAT (Rushmore et al., 1991) was used as a template rather than 0.164YaCAT. The construct −703 to −683 was produced by annealing oligonucleotides OKF82 (5′-TAATGGTGACAAAGCAACTT-3′) and OKF83 (5′-TAAAGTT-GCTTTGTCACCAT-3′) and subcloning a single copy of the ds-oligonucleotide into the *Nde*I site of p0.164YaLUC. Likewise, the −722 to −682 ARE mutant construct was produced by subcloning a single copy of the annealed oligonucleotides OKF 80 (5′-TAAAGTTGCTTT-TGTCCCCATTAGCAATGCCATTTCCAAGCTC-3′) and OKF81(5′-TAGA-GCTTGGAAATGGCATTGCTAATGGGGACAAAGCAACTT-3′) into the *Nde*I site of p0.164YaLUC.

**Cells and Culture Conditions.** The human hepatoblastoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with antibiotic/antimycotics, nonessential amino acids and 10% fetal bovine serum. The hepatoma cells were incubated at 37°C in a 5% carbon dioxide atmosphere and were subcultured every 2 to 3 days.

**Transfection of Hepatoblastoma Cells.** HepG2 cells were plated into 12-well tissue culture plates and transfected using the calcium phosphate method of Gorman et al. (1982) at 40% cell confluence. DNA-CaCl$_2$-HEPES buffered precipitates were added to the cells and, after 24 h, the media were changed. The cells were subsequently treated with various agents as $500\times$ concentrated stocks in DMSO; controls received an equivalent volume of DMSO alone. After an additional 24 h, the cell protein was harvested with 100 $\mu$l of cell lysis buffer (Promega Corp., Madison, WI) according to manufacturer's instructions and subjected to a single freeze/thawing event. All cells were cotransfected with 500 ng of pCMV-$\beta$ as a transfection control. Routinely, 125 ng of receptor expression plasmids (pPXR or pRSVGR) and 1 $\mu$g of the various *GSTA2* reporter constructs, including p1.62YaLUC, were added to each well.

**Assays of $\beta$-Galactosidase and Luciferase Activity.** Luciferase activity of cell extracts was determined using the luciferase assay system from Promega (Madison, WI) in a Berthold Model LB9501 Lumat luminometer (Wallac, Inc., Gaithersburg, MD). For the $\beta$-galactosidase assays, cell extracts were incubated with chlorophenol red $\beta$-galactopyranoside at 37°C for 30 min. Activity was determined spectrophotometrically at 574 nm with a $\mu$Quant microplate spectrophotometer (Biotek Instruments Inc., Winooski, VT).

**Statistical Analysis.** The data were analyzed using a Student's *t* test.

## Results

**Varying Concentrations of Glucocorticoids Cause Biphasic Changes in *GSTA2* Expression in Cultured Rat Hepatocytes.** The glucocorticoid DEX decreases expression of the GSTA2 mRNA in cultured rat hepatocytes at concentrations up to $1 \times 10^{-7}$ M (Fig. 1, A and B). However, at concentrations above $1 \times 10^{-6}$ M, an increase in mRNA levels was observed, demonstrating two phases of response to DEX for the *GSTA2* gene. Because induction occurred with a concentration-dependence consistent with a PXR-dependent process, we tested the effects of DEX on the expression of *CYP3A23* and *CYP2B1/2* genes as shown in Fig. 1C. The effects of glucocorticoids between $1 \times 10^{-11}$ and $1 \times 10^{-7}$ M for *CYP2B1/2* expression was similar to that observed with

*GSTA2* expression. Therefore, within the concentration range associated with activation of the GR (up to $1 \times 10^{-7}$ M), DEX exerts a concentration-dependent repression of both *GSTA2* and *CYP2B1/2* message levels. In our hands, basal expression of *CYP3A23* in cultured rat hepatocytes cultured for 24 to 48 h was very low.

At concentrations above $1 \times 10^{-6}$ M, a 2- to 3-fold increase in mRNA levels was observed for *GSTA2* and *CYP2B1/2*

TABLE 1
Oligonucleotides used for PCR to make fragments of the *GSTA2* promoter

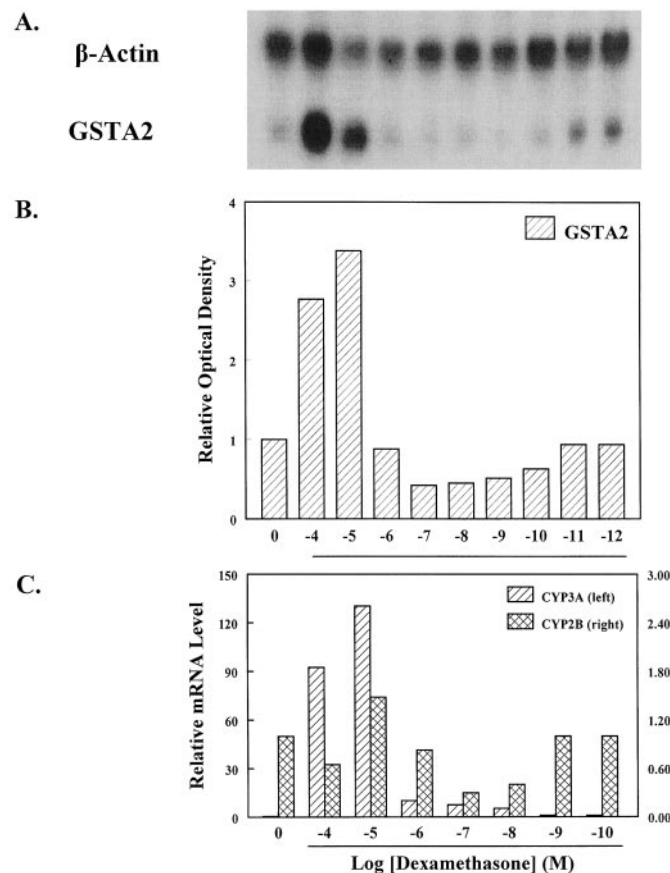| | |
|---|---|
| −1652 | 5′-GGAATTC–CATATG–TTCTCAGTTCTTTACTGTTCATC-3′ |
| −1082 | 5′-AGCTCG–GTGCAGGCAGGCTTGAAGAA-3′ |
| −914 | 5′-TCAGGCATGTTGCGTGCATC-3′ |
| −786 | 5′-GATGACCTTGTTGTGGATAA-3′ |
| −164 | 5′-AGACT–AAGCTTGGGTTGTAAAAGAGATACTGA-3′ |
| +65C | 5′-AGACT–AGATCT–CTTGTCAGCCCCTCCCCCCA-3′ |
| −638C | 5′-AAGCTT–AGGTCTCAGTGCAGCCTGTG-3′ |
| −868C | 5′-TTACCTAGTGATCTGGCTGG-3′ |
| −786C | 5′-CACCGATGGCTCCACTCACA-3′ |



**Fig. 1.** Effect of DEX on levels of mRNAs specific for *GSTA2*, *CYP3A23*, and *CYP2B1/2* in cultured rat hepatocytes. Total RNA was isolated from cultured hepatocytes treated 72 h with either vehicle (DMSO) or varying concentrations of DEX. A and B, Northern analysis of *GSTA2* and $\beta$-actin mRNAs was performed as described under *Experimental Procedures*. Absorbance of relevant bands on the autoradiograms were measured using a Bio-Rad model 620 videodensitometer and normalized with the absorbance values obtained from the autoradiogram of the loading control, $\beta$-actin. The data shown are representative of three separate experiments summarized in Table 2. C, Northern analysis was performed after hybridization of total mRNA to $^{32}$P-labeled cDNA probes for *CYP2B1/2* and $\beta$-actin or ds-oligonucleotide probe specific for *CYP3A23*. The data shown are representative of two to three separate experiments and is summarized in Table 2.

(Fig. 1, B and C), whereas a 10- to 11-fold increase was observed in the expression of the *CYP3A23* gene. In addition, a 2- to 3-fold induction of GSTA2 protein and enzyme activity was observed upon treatment of primary adult rat hepatocytes with concentrations of DEX at or above $1 \times 10^{-6}$ M (data not shown). There was a striking similarity of both the shape and magnitude of the concentration-response curves for *GSTA2* and *CYP2B1/2*. These results confirm those published by Kocarek et al. (1994) for the expression of *CYP2B1/2* and *CYP3A23*. The results presented show that glucocorticoids such as DEX are capable of regulating the expression of the rat

*GSTA2* gene in a biphasic manner and are consistent with our hypothesis that low concentrations of DEX suppress *GSTA2* gene expression via a GR-dependent mechanism, whereas high concentrations of DEX induce expression via a PXR-dependent mechanism. These results also resolve the apparent conflict between our earlier published work (Xiao et al., 1995; Falkner et al., 1998). The data shown in Fig. 1 are reproducible and a statistical analysis of the induction of GSTA2, CYP3A23, and CYP2B1/2 mRNA levels are shown in Table 2.

**The GR Antagonist RU38486 Induces GSTA2 mRNA Levels in Cultured Rat Hepatocytes.** Fig. 2 shows the concentration-dependent effects of RU38486 on *GSTA2* mRNA. GSTA2 mRNA is induced approximately 3-fold by treatment of the hepatocytes with high concentrations (10 $\mu$M) of RU38486. Although RU38486 is a potent antagonist of GR at 5–10 $\mu$M, this compound is an agonist for the PXR (Kliewer et al., 1998) and, at these concentrations, has been shown to induce both CYP3A23 and CYP2B1/2 (Kocarek et al., 1994). Thus, the ability of RU38486 to serve as an agonist for GSTA2 induction correlates well with its known function as a PXR ligand. The results shown in Fig. 2 for *GSTA2* are nearly identical with the changes in CYP2B1/2 mRNA when primary adult rat hepatocytes were treated with RU38486 (Kocarek et al., 1994). The data shown in Fig. 2 are reproducible and a statistical analysis of the effect of RU38486 on GSTA2 mRNA is shown in Table 2. The similarity of these concentration-response curves suggests that DEX and RU38486 regulate *GSTA2* and *CYP2B1/2* through two different mechanisms, probably due to action of GR and PXR.

**Cotransfection of GR and PXR Accounts for the Biphasic Regulation of a *GSTA2* Reporter Construct by *t*-Bu-DEX.** To test the hypothesis that the biphasic regulation of *GSTA2* was mediated by both a classical GR-dependent mechanism and a nonclassical (PXR-dependent) mechanism, experiments were carried out in HepG2 cells transiently transfected with various plasmids. This system allows one to manipulate expression of individual receptors and to reproduce the results seen in primary cultures of rat hepatocytes. HepG2 cells were transiently transfected with p1.62YaLUC, a luciferase reporter gene containing 1.62 kb of the 5′-flanking region of *GSTA2*, and expression vectors for either the human GR and/or murine PXR in the absence or presence of $1 \times 10^{-5}$, $1 \times 10^{-7}$, and $1 \times 10^{-10}$ M *t*-Bu-DEX. Because the $K_d$ value of GR for DEX is approximately 10 to 50 nM, we expected little effect of the lowest concentration of *t*-Bu-DEX ($1 \times 10^{-10}$ M) and a maximal glucocorticoid effect at $1 \times 10^{-7}$ M. Likewise, at $1 \times 10^{-7}$ M *t*-Bu-DEX or less, we anticipated little effect on the PXR. However, we anticipated that mPXR would be activated at the highest concentration

of *t*-Bu-DEX used in this experiment, $1 \times 10^{-5}$ M. As shown in Fig. 3, *t*-Bu-DEX at any concentration had no effect on the expression of the *GSTA2* reporter in the absence of coexpressed receptors. This suggests that the endogenous expression of these receptors is not sufficiently high in HepG2 cells to modulate reporter gene expression in transfection experiments. Consistent with our earlier results (Falkner et al., 1998) when GR was cotransfected with 1.65YaLUC in HepG2

TABLE 2
Statistical analyses of Northern blots of *GSTA2*, *CYP3A23*, and *CYP2B1/2* mRNA levels in rat hepatocytes treated with DEX or RU38486 for 72 hours in culture

Rat hepatocytes were isolated and treated as shown in the Figure 1. mRNA was isolated and quantified as described in Xiao et al. (1995). The average message levels of two to three separate experiments were averaged and the standard deviations determined.

| | GST Ya (n = 3) | *CYP3A23* (n = 2) | *CYP2B1/2* (n = 2) |
|---|---|---|---|
| Control | $1.00 \pm 0.10$ | $1.00 \pm 0.10$ | $1.00 \pm 0.10$ |
| DEX $1 \times 10^{-4}$ M | $3.10 \pm 0.53^*$ | $6.59 \pm 0.95^{**}$ | $0.54 \pm 0.09^*$ |
| DEX $1 \times 10^{-5}$ M | $2.56 \pm 0.78^*$ | $10.73 \pm 0.45^{**}$ | $1.96 \pm 0.45$ |
| DEX $1 \times 10^{-6}$ M | $0.74 \pm 0.10^*$ | $2.81 \pm 0.15^{**}$ | $1.01 \pm 0.13$ |
| DEX $1 \times 10^{-7}$ M | $0.51 \pm 0.11^*$ | $1.29 \pm 0.16$ | $0.50 \pm 0.05^*$ |
| Ru486 $1 \times 10^{-4}$ M | $2.75 \pm 0.13^{**}$ | N.D. | N.D. |
| Ru486 $1 \times 10^{-5}$ M | $1.53 \pm 0.17^{**}$ | N.D. | N.D. |
| Ru486 $1 \times 10^{-6}$ M | $1.36 \pm 0.21$ | N.D. | N.D. |
| Ru486 $1 \times 10^{-7}$ M | $1.07 \pm 0.26$ | N.D. | N.D. |

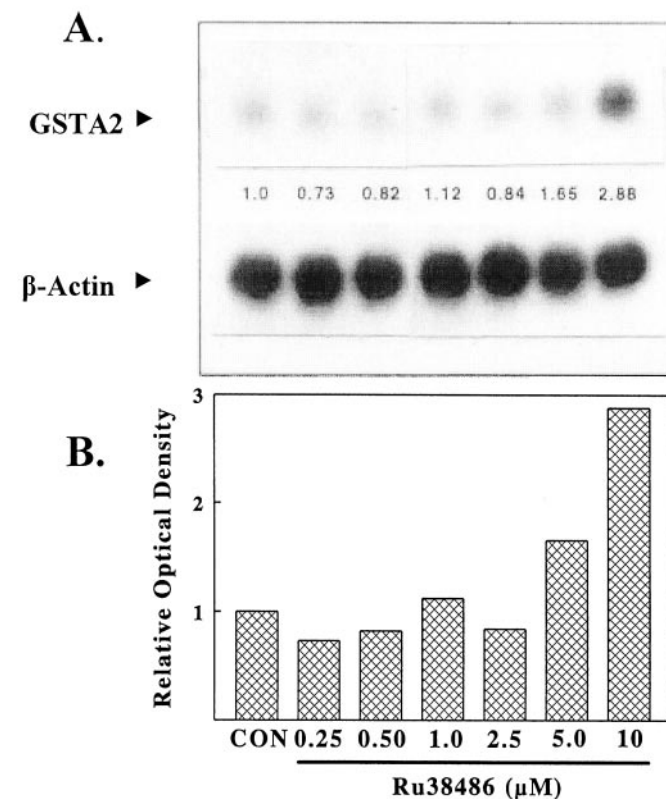$^*$ $p < 0.04$.
$^{**}$ $p < 0.02$.



**Fig. 2.** Effects of the GR antagonist RU38486 on levels of mRNAs specific for *GSTA2* in cultured rat hepatocytes. Total RNA was isolated from cultured hepatocytes treated 72 h with either vehicle (DMSO) or varying concentrations of RU38486. Northern analysis of the mRNAs was performed as described under *Experimental Procedures*. Absorbance of relevant bands on the autoradiograms were measured and normalized to the Absorbance values of the loading control, $\beta$-actin. The data shown are representative of three separate experiments and is summarized in Table 2.
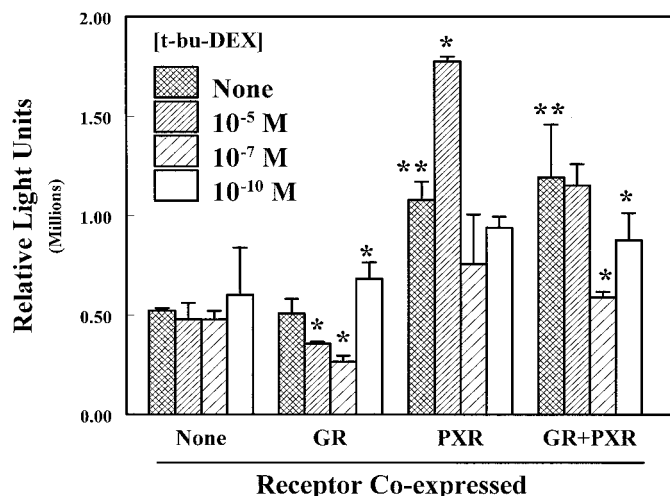
**Fig. 3.** Receptor-dependent effects of *t*-Bu-DEX on luciferase activity in HepG2 cells transiently transfected with the plasmid p1.62YaLUC. Luciferase and β-galactosidase assays were performed on lysed HepG2 cells that had been transiently transfected with p1.62YaLUC, the control vector pCMV-β and as specified expression vectors for the nuclear receptors, pRSVGR or pPXR as described under *Experimental Procedures*. Cells were treated with vehicle or varying concentrations of *t*-Bu-DEX for 24 h. The normalized luciferase activity is expressed as relative light units divided by β-galactosidase activity and is the mean ± S.D. of three wells. *$p < 0.05$, significantly different from its control; **$p < 0.005$, significantly different from cells without receptor.

cells and the cells were treated with *t*-Bu-DEX, negative regulation of this reporter was observed.

In contrast to the results with GR, there was a doubling of the basal expression of the reporter gene when PXR was coexpressed in HepG2 cells (Fig. 3), possibly reflecting either the presence of PXR ligands in the fetal bovine serum or a
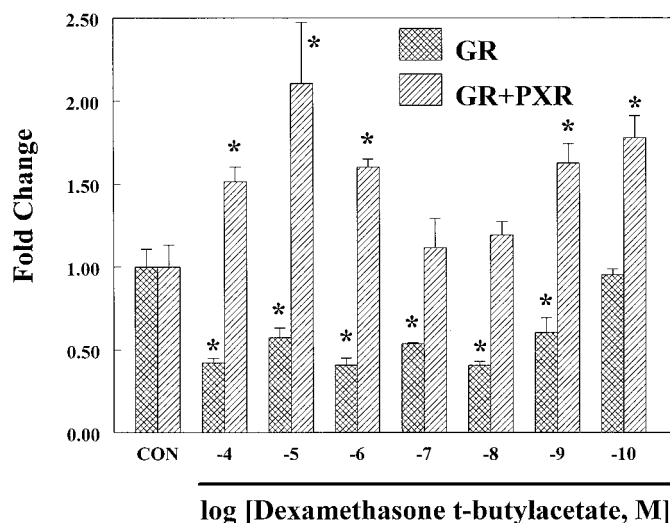
**Fig. 4.** Concentration-dependent effects of *t*-Bu-DEX on luciferase activity in HepG2 cells transiently transfected with the plasmid p1.62YaLUC. Luciferase and β-galactosidase assays were performed on lysed HepG2 cells that had been transiently transfected with p1.62YaLUC, the control vector pCMV-β, and expression vectors for the nuclear receptors, pRSVGR or pPXR as described under *Experimental Procedures*. Cells were treated with vehicle or varying concentrations of *t*-Bu-DEX for 24 h. The normalized luciferase activity is expressed as relative light units divided by β-galactosidase activity and is the mean ± S.D. of three wells. Basal expression in the presence of cotransfected PXR was 1.8-fold higher than that seen in the presence of cotransfected GR. *$p < 0.01$, statistically different from cells not treated with *t*-Bu-DEX.
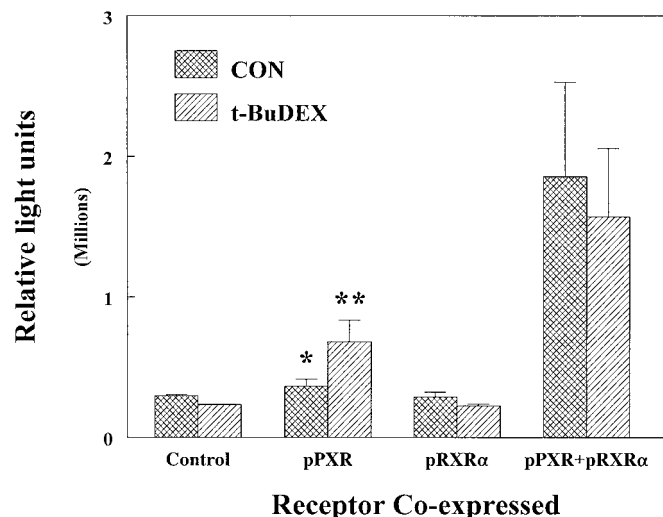
**Fig. 5.** Effect of cotransfection of RXRα on the PXR-dependent *t*-Bu-DEX induction of luciferase activity in HepG2 cells transiently transfected with the plasmid p1.62YaLUC. Luciferase and β-galactosidase assays were performed on lysed HepG2 cells that had been transiently transfected with p1.62YaLUC, the control vector pCMV-β and as specified, expression vector for the nuclear receptors pRXRα or pPXR as described under *Experimental Procedures*. Cells were treated with vehicle or *t*-Bu-DEX (10 μM) for 24 h. The normalized luciferase activity is expressed as relative light units divided by β-galactosidase activity and is the mean ± S.D. of three wells. *$p < 0.09$, , significantly different from control cells; **$p < 0.03$, significantly different from cells expressing PXR.

role for PXR/RXRα in basal expression. When charcoal treated serum was utilized, PXR still had an effect on basal expression (data not shown). Upon addition of $1 \times 10^{-5}$ M *t*-Bu-DEX, there was a further 65% increase in expression; no statistically significant difference in expression was seen at $1 \times 10^{-7}$ or $1 \times 10^{-10}$ M *t*-Bu-DEX. These results demon-
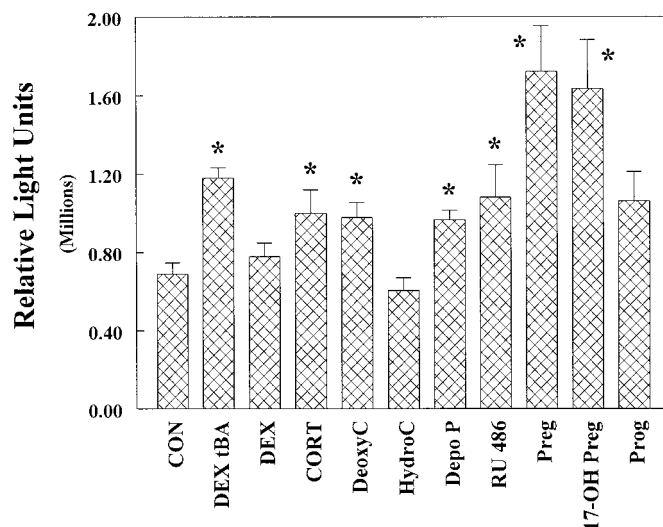
**Fig. 6.** Effect of treatment with various steroids on the PXR-dependent induction of luciferase activity in HepG2 cells transiently transfected with the plasmid p1.62YaLUC and pPXR. Luciferase and β-galactosidase assays were performed on lysed HepG2 cells that had been transiently transfected with p1.62YaLUC, the control vector pCMV-β and pPXR as described under *Experimental Procedures*. Cells were treated with vehicle or 10 μM concentration of each steroid for 24 h. The normalized luciferase activity is expressed as relative light units divided by β-galactosidase activity and is the mean ± S.D. of three wells. *$p < 0.02$; statistically different from control cells.
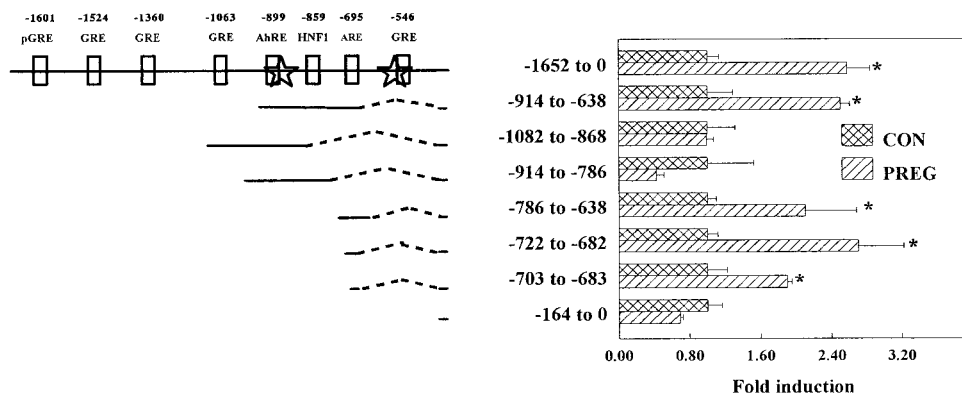
**Fig. 7.** Deletion analysis of the 5′-flanking region of the *GSTA2* gene. A series of luciferase constructs were prepared with deletions in the 5′-flanking region of the *GSTA2* gene and were tested by transient cotransfection with pPXR in HepG2 cells as described under *Experimental Procedures*. The cells were treated for 24 h. with either dimethyl sulfoxide or 17-hydroxypregnenolone in DMSO. The normalized luciferase activity is expressed as relative light units divided by β-galactosidase activity and is the mean ± S.D. of three wells. *$p < 0.03$, statistically different from its control.

strate a role for PXR in *t*-Bu-DEX-dependent induction of *GSTA2* expression.

Intermediate effects were observed when both GR and PXR were cotransfected (Fig. 3). The basal rate was increased, but negative regulation by concentrations of *t*-Bu-DEX which activate GR was noted. This effect was reversed to control levels of expression with $1 \times 10^{-5}$ M *t*-Bu-DEX. Because GR is activated by either *t*-Bu-DEX (Fig. 3) or DEX (Falkner et al., 1998), the negative effect must be caused by *t*-Bu-DEX functioning as a GR ligand, and the reversal at $1 \times 10^{-5}$ M must be caused by its action as a ligand for PXR. These results demonstrate that the biphasic response of *GSTA2* gene expression is caused by *t*-Bu-DEX acting through two independent nuclear receptor-mediated mechanisms.

An extended concentration-response with *t*-Bu-DEX was performed in this reporter gene system in the presence of



**Fig. 8.** Effect of mutation of a *GSTA2* pARE-LUC reporter gene on induction of luciferase activity by either *t*-butylhydroquinone or 17-hydroxypregnenolone. Luciferase and β-galactosidase assays were performed on lysed HepG2 cells that had been transiently transfected with ARE-LUC, the control vector pCMV-β, and the expression vector pPXR as described under *Experimental Procedures*. Cells were treated for 24 h with vehicle or 50 μM of *t*-butylhydroquinone or 17α-hydroxypregnenolone either alone or in combination for 24 h. The normalized luciferase activity is expressed as relative light units divided by β-galactosidase activity and is the mean ± S.D. of three wells. *$p < 0.07$, statistically different from control cells.

either GR alone or GR plus PXR (Fig. 4). As anticipated, when GR alone was cotransfected, treatment with *t*-Bu-DEX caused a simple monotonic decline in expression of p1.62YaLUC. In the presence of both GR and PXR, more complex biphasic regulation of expression was observed. Although we normalized the basal activity for cells expressing either GR or PXR plus GR, basal expression in the presence of PXR was increased approximately 2-fold. There was negative regulation up to $1 \times 10^{-7}$ M *t*-Bu-DEX consistent with this compound mediated its effects acting through the GR. At higher concentrations up to $1 \times 10^{-5}$ M, a 2- to 2.5-fold induction of expression over basal expression of this gene was observed, again consistent with the involvement of PXR. We observed that $1 \times 10^{-4}$M *t*-Bu-DEX was toxic to HepG2 cells as observed by decreases in cell number and viability, which probably accounts for the decline in expression observed at $1 \times 10^{-4}$ M *t*-Bu-DEX.

Interestingly, in cells cotranfected with PXR and GR, the level of expression of the *GSTA2* reporter constructs was greater at the lowest concentration of *t*-Bu-DEX ($1 \times 10^{-10}$) tested than the control value. Although this response was variable, it was observed both in transient transfection experiments with HepG2 cells and in isolated rat hepatocytes. Several groups (Huss and Kasper, 2000; Pascussi et al., 2000) have reported that the expression several nuclear receptors, including RXRα and PXR, are under glucocorticoid control. Thus, the elevation of basal expression of *GSTA2* reporter expression in cells transfected with both PXR and GR may reflect increased expression of RXRα which affects basal expression.

**Over-Expression of PXR and RXRα Increases Basal Expression of a *GSTA2* Reporter Gene.** Because PXR heterodimerizes with RXRα to bind to its responsive element (Kliewer et al., 1998), we tested the effects of cotransfection of an expression vector for RXRα on expression of p1.62YaLUC. As is shown in Fig. 5, cotransfection of a expression vector for RXRα had no effect on luciferase expression on either basal or *t*-Bu-DEX treated cells. As anticipated, cotransfection of PXR caused an increase in basal level expression of approximately 1.5-fold and a significant increase in expression was observed in cells treated with $1 \times 10^{-5}$ M *t*-Bu-DEX. When both RXRα and PXR were coexpressed, the basal expression rate was markedly increased, but *t*-Bu-DEX-dependent increases in expression were not
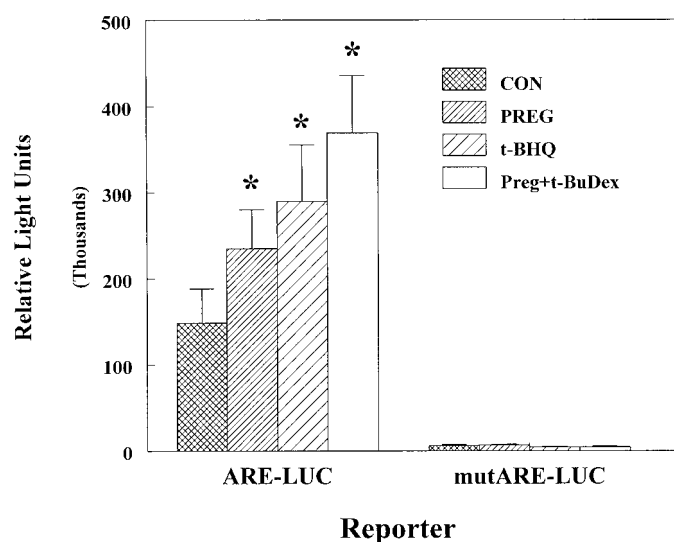
observed suggesting that PXR-RXR$\alpha$ apparently interact to increase basal expression of *GSTA2* reporter constructs. In cells cotransfected with both receptors, the level of increase of basal expression was greater than the *t*-Bu-DEX-dependent expression when PXR alone was cotransfected. The loss of ligand-inducibility suggests that corepressors may be limiting when RXR$\alpha$ and PXR are overexpressed.

**PXR-Ligands Induce Expression of a *GSTA2* Reporter Gene.** To establish which steroids may regulate this gene at concentrations $1 \times 10^{-5}$ M, we performed the reporter gene expression assay using known activators/ligands for PXR as shown in Fig. 6. The best ligands were the pregnanes pregnenolone (2.5-fold) and 17$\alpha$-hydroxypregnenolone (2.4-fold). These pregnanes were the best naturally occurring ligands for PXR in the induction of expression of a DR3 reporter in CV-1 cells. The ligands *t*-Bu-DEX (1.7-fold), RU 38486 (1.6-fold), progesterone (1.5-fold), cortisol (1.5-fold), deoxycortisone (1.4-fold), and 6$\alpha$-methyl-17$\alpha$-hydroxy progesterone acetate (1.4-fold) also caused significant induction in enzyme activity. In contrast, DEX (1.1-fold) and hydrocortisone (0.87-fold) did not significantly induce reporter gene expression via PXR. The steroids that caused a significant induction in activity have all been shown to be ligands for the PXR when tested as *trans*-activators of a DR3 response element-reporter system in transient transfection assays with CV-1 cells (Kliewer et al., 1998). There is, however, a difference in potency between the responses, in that *t*-Bu-DEX is a much better inducer of DR3-mediated reporters in CV-1 cells than the naturally-occurring pregnanes, 17$\alpha$-hydroxypregnenolone and pregnenolone, where as the reverse is true for the *t*-Bu-DEX-dependent regulation of *GSTA2* in HepG2 cells (Fig. 6).

**Deletion Analysis of the 5′-Flanking Region of *GSTA2* to Define the Location of the PXR-Responsive Element.** Because the 5′-flanking region of the *GSTA2* gene does not contain sequences with identity to or high similarity to the canonical DR3 binding sequence for the murine PXR, we undertook a deletion strategy to identify the element through which the PXR-dependent induction process was acting. As seen in Fig. 7, our initial deletion construct that included the area of 5′-flanking region between −914 and −638 bp relative to the transcription start site was responsive to 17$\alpha$-hydroxypregnenolone-dependent induction. This construct contains the elements that are responsible for induction of this gene by xenobiotic compounds, namely the *Ah*RE and ARE responsive elements. Further deletion analysis identified the region between base pairs −703 and −683 as responsible for the pregnane-dependent induction of the gene. Constructs that did not contain this sequence were not pregnane-responsive. The ARE is located between base −703 and −683; thus, our results show PXR acting within the same 20-bp region containing the ARE.

**Mutation of the *GSTA2* ARE in a pARE-LUC Reporter Oblates Induction of Expression by PXR Ligands and *t*-Butylhydroquinone.** To further define the element through which the pregnane effect occurs, we used a plasmid that contains a single base pair mutation in the ARE core sequence (GTGACnnnGC to G**G**GACnnnGC) that has been previously shown to cause a loss of basal level activation and prevent *t*-butylhydroquinone-dependent induction (Rushmore et al., 1991). As is shown in Fig. 8, both *t*-butylhydroquinone (an ARE-activating chemical) and 17$\alpha$-hy-

droxy-pregnenolone (a PXR-activating chemical) induced expression of this wild-type reporter construct apparently in an additive manner. As expected from the work of Rushmore et al. (1991), the reporter with the mutated ARE had much lower basal activity. The mutated reporter construct was also not responsive to treatment with either 17$\alpha$-hydroxypregnenolone or *t*-butylhydroquinone which suggests that the mutated base pair is critical for either inductive process (i.e., activation via either Nrf2 or PXR).

## Discussion

The results presented show that glucocorticoids such as DEX are capable of regulating the expression of the rat GSTA2 gene in a biphasic manner. These results resolve the apparent conflict between our earlier published work (Xiao et al., 1995) in which glucocorticoids at $1 \times 10^{-6}$ M induced expression of GSTA2 message in primary rat hepatocytes, whereas identical concentrations of the synthetic corticosteroid repressed expression (Falkner et al., 1998) of reporter constructs transiently transfected into HepG2 cells in a GR-dependent process. Our results show that the regulation of expression in primary hepatocytes is biphasic and that at concentrations of DEX greater than $10^{-7}$M gene expression of *GSTA2* is induced. However, at lower concentrations of DEX, there is a suppression of gene activation consistent with the involvement of GR. Our studies using transient transfection of reporter plasmids in HepG2 cells showed that neither the negative GR-dependent nor the positive PXR-dependent DEX effects are observed if the respective receptors are not cotransfected. Negative regulation was observed only when GR was cotransfected and likewise, positive regulation was only observed when PXR was cotransfected.

The prototypical model of gene induction at high concentrations of DEX has been well documented for expression of *CYP3A23* in the rat. Studies by Guzelian and coworkers (Schuetz et al., 1984; Schuetz and Guzelian, 1984) have shown that this gene is induced by supraphysiological concentrations of glucocorticoids at the transcriptional level. Subsequent studies (Huss et al., 1998; Quattrochi et al., 1998) have shown that the response is mediated through a DR3 element that can bind a PXR/RXR$\alpha$ heterodimer. In addition, other compounds that have been shown to be ligands for the PXR also activate this response element (Kliewer et al., 1998). These compounds include other pregnanes, RU38486, the macrolide antibiotic rifampicin, and imidazole derivatives. Recently, Staudinger et al. (2001) have shown that cholestatic bile acids, such as lithocholic acid and 3-ketolithocholic acid, are excellent ligands for PXR and suggest a role for this receptor in bile acid synthesis and excretion. The ligand-mediated activation of this receptor usually occurs only at high, supraphysiological concentrations of these ligands, consistent with a role for this receptor in detoxication, a reaction catalyzed by CYP3A23.

GSTA2 is a member of a superfamily of proteins that catalyzes the reaction between the sulfhydryl group of the tripeptide glutathione and electronegative centers found in lipophilic molecules (Ketterer, 1986; Mannervik and Danielson, 1988). In addition to its role as an active catalyst, this abundant protein also acts as a binding protein for steroids (Listowsky, 1993) and cholestatic bile acids (Takikawa and Kaplowitz, 1988); as such, it was once named ligandin (Habig

et al., 1974). The role as a binding protein may be to facilitate transport of its potentially toxic ligands in the cytosolic compartment, either to specific membrane-spanning transporters or to membrane-associated cytochromes P450 to increase the rate of degradation of these ligands. Thus, the presence of an element capable of supporting a "nonclassical" or PXR-mediated mechanism of induction may be a reflection of the importance of the ligandin role this protein plays.

The transcriptional regulation of *GSTA2* by xenobiotic compounds has been intensively studied by Picket and co-workers (Rushmore et al. 1990, 1991; Nguyen and Pickett, 1992). Two elements have been identified, namely the *Ah*RE and the ARE. The work presented in this article indicates that the response element that mediates the PXR-dependent induction of *GSTA2* gene expression is located within the ARE, because mutation of the ARE core sequence (G**T**GA-CaaaGC to G**G**GACaaaGC) caused a loss of both *t*-butylhydroquinone- and PXR-dependent responses. This sequence is required for induction of GSTA2 message and protein by metabolites of aromatic compounds, phenolic antioxidants, phorbol esters, and hydrogen peroxide. The ARE sequence is thought to bind the Cap-*N*-Collar family transcription factor, Nrf-2 and its obligate heterodimerization partner, a member of the small maf family (Itoh et al., 1999). The mutation of the core sequence above has been shown to result in loss of protein binding (Nguyen and Pickett, 1992).

In contrast, rat *QOR* gene expression in hepatocytes was negatively regulated in a monotonic manner and this response was blunted by RU38486, suggesting a role for GR alone (J. A. Pinaire and R. A. Prough, unpublished results; Xiao et al., 1995). No induction of the QOR gene products was observed. Since *GSTA2* and *QOR* both contain ARE sequences and only GSTA2 expression is affected by high concentrations of DEX in rat hepatocytes, the regulation can not simply be one in which PXR interacts with Nrf-2 to bring about this response. This suggests a novel mechanism for PXR action, namely one in which PXR must interact with another transcription factor.

Interestingly, the tamoxifen-dependent induction of the human *QOR* gene expression in MCF-7 cells has also been shown to be mediated by an electrophilic element or ARE (Montano and Katzenellenbogen, 1997; Montano et al., 1998). However, unlike the ARE in the *GSTA2* gene, the ARE in the 5′-flanking region of the *QOR* gene contains a perfect canonical AP-1 site. This effect, which requires ER$\beta$, involves protein-protein interaction with the human prevents mitotic catastrophe 2 (hPMC2) gene (Montano et al., 2000). At present, it is not known whether the PXR effect observed with *GSTA2* is mediated through interaction with the Nrf-2/maf, hPMC2 or another transcription factor. This 20-bp sequence contains neither a nuclear receptor half site nor an AP1 sequence. The data presented here demonstrate that expression of native rat *GSTA2* genes in cultured rat hepatocytes is biphasic and suggest that GR and PXR account individually for the 2 phases.

In contrast to the rodent models, relatively little is known about the effects of glucocorticoids or pregnanes on the expression of human glutathione *S*-transferases. Although GR is highly conserved between rodents and humans, PXR has diverged considerably (Jones et al., 2000). Therefore, we cannot easily predict whether the mechanism presented here is conserved in the human. PXR in humans has different ligand specificity and even a different canonical binding element sequence than the rodent PXRs. The human PXR binds preferentially to an inverted repeat of AGGTCA half-sites separated by six nucleotides (IR6) in contrast to the rodent DR3. Therefore, examination of the species difference in the response, the sequence requirements for the ARE, and the transcription factors involved will be the focus of future studies.

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