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# Regulation of Rat Hepatic Hydroxysteroid Sulfotransferase (SULT2-40/41) Gene Expression by Glucocorticoids: Evidence for a Dual Mechanism of Transcriptional Control

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## ABSTRACT

Glucocorticoid-inducible hydroxysteroid sulfotransferase (SULT2-40/41) gene transcription was investigated in primary cultured rat hepatocytes transiently transfected with a series of SULT2-40/41 5′-flanking region-luciferase reporter constructs, with emphasis on examining the functional role of an inverted repeat-0 nuclear receptor motif (IR0). Treatment of transfected cultures with any of four glucocorticoids activated luciferase expression from a construct containing 1938 base pairs (bp) of the SULT2-40/41 gene 5′-flanking sequence, whereas deletion of bp −227 to −158 (containing the IR0 motif) largely abolished the effect. On closer analysis, treatment of hepatocyte cultures with either of the potent glucocorticoids dexamethasone [strong cytochrome P-450 3A (CYP3A) inducer] or triamcinolone acetonide (weak CYP3A inducer) produced dose-dependent increases in luciferase activity when hepatocytes were transiently transfected with a construct containing as little as 158 bp of 5′-flanking sequence or containing a mutated IR0 motif. The dexamethasone dose-dependent increase in luciferase activity continued through a dose of $10^{-6}$ M when the transfected construct contained the IR0 motif, but was maximal at $10^{-7}$ M when the transfected construct lacked the IR0 motif. In contrast, triamcinolone acetonide-induced luciferase activity was maximal at a dose of $10^{-7}$ M, irrespective of the presence or absence of the IR0 motif. Coincubation of transfected hepatocytes with $10^{-8}$ M dexamethasone and the antiglucocorticoid RU486 inhibited luciferase expression. Luciferase induction by the prototypical CYP3A inducer pregnenolone 16α-carbonitrile was restricted to constructs containing the IR0 motif. These data suggest that glucocorticoid-inducible SULT2-40/41 gene expression occurs through a dual mechanism, whose components possibly involve the glucocorticoid receptor and the pregnane X receptor.

The hydroxysteroid sulfotransferases (SULT2) play critical roles in drug metabolism, bile acid detoxication, and carcinogen activation, and in the regulation of intratissue active hormone levels. Therefore, understanding the molecular mechanisms that regulate the expression of this multigene family is important. SULT2 enzymes catalyze the sulfonation of a wide range of sulfate acceptor molecules such as hydroxysteroid hormones, bile acids, aliphatic alcohols, procarcinogens such as 5-hydroxymethylchrysene, and other endogenous and exogenous compounds (Jakoby et al., 1980; Barnes et al., 1989; Ogura et al., 1990b). Depending on the stability of the sulfate ester that is formed, SULT2-catalyzed reactions may culminate in the creation of a polar end product that is amenable to excretion and elimination (detoxication) or in the bioactivation of a procarcinogen to a highly reactive intermediate. Moreover, because sulfated hormones are generally considered to be receptor inactive, alterations in SULT2 gene expression have the potential to shift the balance of intratissue active hormone levels and affect gene expression.

In a broad-based search for hepatic genes that undergo altered expression during aging, Roy and coworkers cloned two rat senescence marker protein genes, SMP2A and SMP2B (Song et al., 1990), that were later identified as sulfotransferase genes of the SULT2 family (Ogura et al., 1990a; Watabe et al., 1994). Age- and gender-related expression of the rat hepatic SULT2 gene family has long been recognized as a key feature of these enzymes and strongly suggests that hormonal regulation is central to SULT2 gene expression. Throughout adult life, the SULT2 enzymes are more abundantly expressed in female compared with male rat liver (Runge-Morris and Wilusz, 1991; Chatterjee et al., 1994). As male rats surpass puberty, SULT2 gene expression declines in response to rising androgen levels and produces an androgenizing effect on hepatic gene expression (Chatter-

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**ABBREVIATIONS:** SULT2, hydroxysteroid sulfotransferase; Oct-1, octamer transcription factor-1; HNF1, hepatic nuclear factor 1; C/EBP, CCAAT enhancer-binding protein; CYP3A, cytochrome P-450 3A; bp, base pair; DMSO, dimethyl sulfoxide; PXR, pregnane X receptor; IR0, inverted repeat-0 nuclear receptor motif.

jee et al., 1990, 1994). Temporal fluctuations in androgen (Chatterjee et al., 1987, 1990, 1994) and pituitary growth hormone concentrations (Yamazoe et al., 1989; Ueda et al., 1997) have both been implicated to influence SULT2 gene expression, and we have found that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, an "environmental hormone" with pleiotropic effects on gene expression, also produces alterations in rat hepatic SULT2 mRNA levels (Runge-Morris, 1998).

Insights into the mechanisms responsible for SULT2 gene regulation are just beginning to emerge. Although the three known SULT2 enzyme isoforms that are present in rat liver (SULT2-40/41, -20/21, and -60) appear to have similar substrate specificities, they are independently regulated in response to steroid hormone (Liu and Klaassen, 1996) and xenobiotic treatments (Runge-Morris et al., 1998). Among the SULT2 isoforms, SULT2-40/41, which is expressed in vivo and in primary rat hepatocyte culture, has been best characterized. A recent analysis of the SULT2-40/41gene 5′-flanking region revealed that direct androgen receptor-DNA interactions are unlikely to be responsible for androgen-mediated repression of SULT2-40/41gene transcription and that the octamer transcription factor-1 (Oct-1), hepatic nuclear factor 1 (HNF1), and CCAAT enhancer-binding protein (C/EBP) transcription factors figure prominently in the control of liver-specific SULT2-40/41 gene expression (Song et al., 1998).

Glucocorticoids and steroidal "antiglucocorticoids" such as pregnenolone 16$\alpha$-carbonitrile have been shown to induce SULT2-40/41 mRNA levels in rat liver (Liu and Klaassen, 1996). Regulation of SULT2 gene expression by glucocorticoids appears to share some features with steroid-inducible cytochrome P-450 3A (CYP3A) expression, including dose-response relationships that continue increasing beyond physiological glucocorticoid doses (Schuetz and Guzelian, 1984; Schuetz et al., 1984; Runge-Morris et al., 1996). In liver, steroid-inducible CYP3A is transcriptionally controlled via a *cis*-acting ATGAACT direct repeat sequence in the 5′-flanking region of the gene (Quattrochi et al., 1995). This direct repeat sequence, which also can be viewed as the complement of a DR3 nuclear receptor motif (i.e., a direct repeat of the hexanucleotide AG$^{G}/_{T}$TCA, in which the two halves of the repeat are separated by three bases), is a binding site for the pregnane X receptor (PXR), a novel member of the nuclear receptor superfamily (Kliewer et al., 1998). The SULT2-40/41 gene contains an ATGAACT "half-site" at base pair (bp) −184 to −178, which also can be considered as part of an imperfect inverted repeat-0 nuclear receptor motif (IR0) (i.e., an inverted repeat of the aforementioned hexanucleotide, in which the two halves of the repeat are separated by zero bases). DNase I footprinting analysis suggested that this IR0 site may play a role in regulating SULT2-40/41 gene expression (Song et al., 1998). In the present study, transient transfection analyses were conducted in primary cultured rat hepatocytes to identify which regions of the SULT2-40/41 gene are responsible for conferring glucocorticoid-inducible transcriptional activation, with particular emphasis on examining the functional significance of the IR0 motif.

## Experimental Procedures

**Materials.** Steroids (dexamethasone, triamcinolone acetonide, betamethasone, hydrocortisone, and pregnenolone 16$\alpha$-carbonitrile) were purchased from Sigma Chemical Co. (St. Louis, MO). Custom-synthesized oligonucleotides were purchased from Genosys (The Woodlands, TX). Other supplies and reagents were obtained from the sources described previously (Runge-Morris et al., 1996; Kocarek et al., 1998).

**Preparation of Reporter Constructs.** A fragment of the SULT2-40/41gene spanning bp −1938 to +52 was prepared by polymerase chain reaction amplification, using *Pfu* polymerase (Stratagene, Inc., La Jolla, CA), rat genomic DNA as template, and primers corresponding to bp 36 to 55 and 2022 to 2005 [5′-GGACGCGTAATGTTCAACATCCTTATCA-3′ and 5′-GGCTCGAGCTCTGTGTAGGTCCTGT-3′; an *Mlu* I site and a *Xho*I site (shown underlined) were added to the 5′ ends of the forward and reverse primers, respectively] of the published SULT2-40/41 gene (GenBank accession no. M29301). The amplified fragment was ligated into the *Mlu* I and *Xho*I sites of the pGL3-basic (Promega Biotec, Madison, WI) firefly luciferase reporter plasmid (construct 1938) and sequenced completely. There were single base pair differences between our SULT2-40/41 clone and the published sequence, and the sequences were identical between bp −629 and +52. A construct containing the 1938-bp 5′-flanking sequence, but lacking the 70-bp fragment delimited by *Pst*I sites at −227 and −158 (construct Δ*Pst*I), was prepared by performing a complete *Pst*I digestion of the 1938 construct, and ligating closed the digested plasmid. Constructs containing 227 bp or 158 bp of 5′-flanking sequence (constructs 227 and 158) were prepared by first performing a complete digestion of the 1938 construct with *Kpn*I (in the pGL3-basic multiple cloning site, 5′ of the SULT2-40/41insert) followed by a partial digestion with *Pst*I, and blunt-ending and ligating closed the digested plasmid. Constructs containing 77 bp or 34 bp of 5′-flanking sequence (constructs 77 and 34) were prepared by polymerase chain reaction amplification, with construct 1938 as template. These amplified fragments were initially ligated into the pGEM-T vector (Promega Biotec), and were subsequently subcloned into the *Mlu* I and *Xho*I sites of pGL3-basic. A construct containing a mutagenized ATGAACT motif (construct IR0-Mut) was prepared with the Altered Sites II in vitro mutagenesis system (Promega Biotec) according to manufacturer's instructions. The mutagenic primer sequence (corresponding to bp −199 to −164) was 5′-TTCTGTTTGGGGGTCAactAgTTGGGCTCACAAAT G-3′, in which the ATGAACT motif (the corresponding location in the mutagenic primer is underlined; base changes are shown in lowercase letters) was changed to contain a unique *Spe*I restriction site (ACTAGT), to facilitate the identification of mutant clones. A construct lacking the IR0 motif (construct ΔIR0) was prepared by replacing the 70-bp *Pst*I fragment of the 1938 construct with a 58-bp double-stranded oligonucleotide lacking the IR0 sequence. The downstream *Pst*I site of this oligonucleotide was changed to GTGCAG to facilitate the identification of a forward orientation clone following ligation. Sequences of all constructs were verified by sequence analysis (Center for Molecular Medicine and Genetics DNA Sequencing Facility, Wayne State University).

**Transient Transfection and Treatment of Primary Cultured Rat Hepatocytes.** Isolation, primary culture, and transient transfection of rat hepatocytes were performed essentially as described previously (Kocarek et al., 1998). Hepatocytes were isolated from the livers of adult male Sprague-Dawley rats (220–300 g) and plated in standard medium, consisting of Williams' medium E supplemented with 0.25 U/ml insulin, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin, onto Vitrogen- (The Collagen Corporation, Palo Alto, CA) coated 12-well plates ($3 \times 10^5$ hepatocytes/well). At ~21 h after plating, culture medium was replaced with 0.6 ml of Opti-MEM containing a premixed complex of 5.5 $\mu$g of Lipofectin reagent (Gibco-BRL, Grand Island, NY) and 0.8 $\mu$g of reporter plasmid, in combination with 0.08 $\mu$g of the pRL-TK plasmid (Promega Biotec), which expresses the *Renilla* luciferase under the control of the herpes simplex virus thymidine kinase promoter, to allow for normalization among samples due to differences in transfection efficiency. Transfection incubations were continued for 5 h, after which culture me-

dium was replaced with standard Williams' medium E for 2 h. Culture medium was then aspirated, and hepatocytes were overlaid with 0.8 mg of Matrigel (Collaborative Research Products, Bedford, MA). After incubating the cultures at 37°C for 30 min to allow for Matrigel gelation, standard culture medium (1 ml) was added to each well, and cultures were incubated overnight. Alternatively, following the transfection incubation, 1 ml of standard medium was added to each well, and 100 $\mu$g of Matrigel was pipetted into the medium. These two methods of Matrigel addition were equally effective in restoring steroid responsiveness to the hepatocyte cultures. At 48 h after plating, fresh medium, either alone or containing steroid [or dimethyl sulfoxide (DMSO) vehicle, 0.1% final medium concentration], was added to each well. After 24-h treatment, hepatocytes were harvested for measurement of luciferase activity (firefly and *Renilla*) with the dual luciferase reporter assay system (Promega Biotec), according to the manufacturer's instructions, and a Dynex model MLX luminometer. In preliminary experiments, with a plasmid expressing $\beta$-galactosidase under the control of the cytomegalovirus promoter, transfection efficiency of the primary cultured rat hepatoctyes was estimated to be ~5%. Data were analyzed by one-way ANOVA followed by the Newman-Keuls post hoc test. $ED_{50}$ values and 95% confidence intervals were estimated by fitting dose-response data to a sigmoidal function, with Prism (version 2) software (GraphPad Software, San Diego, CA).

## Results

To determine whether the first ~2 kilobases of the SULT2-40/41 gene 5′-flanking region contained sequences conferring glucocorticoid inducibility, and to obtain preliminary information on whether the ATGAACT/IR0 motif (hereafter referred to simply as the IR0 motif) located upstream of bp −184 may be involved in any glucocorticoid-mediated effects, primary cultures of rat hepatocytes were transiently transfected with a lucerifase reporter construct containing 1938 bp of SULT2-40/41 5′ sequence (1938 construct), or with a deletion construct containing the same sequence but lacking the 70-bp fragment delimited by *Pst*I sites at −227 and −158 bp (Δ*Pst*I construct) (Fig. 1). Forty-eight hours after plating, the transfected hepatocytes were treated for 24 h with medium alone, DMSO vehicle, or with one of the following potent glucocorticoids: dexamethasone (at $10^{-7}$ or $10^{-5}$ M), triamcinolone acetonide ($10^{-5}$ M), betamethasone ($10^{-5}$ M), or hydrocortisone ($10^{-5}$ M) (Fig. 1). Relative to untreated or vehicle-treated controls, glucocorticoid treatment significantly induced luciferase reporter gene expression in the 1938-bp SULT2-40/41 5′-luciferase construct (each steroid induced luciferase activity at least 15-fold at the $10^{-5}$ M dose) (Fig. 1A). The higher dose of dexamethasone ($10^{-5}$ M) produced a 1.9-fold greater degree of induction from the 1938-bp construct compared with the lower dose of dexamethasone ($10^{-7}$ M) (significant at $p < .05$). In contrast, deletion of the 70-bp *Pst*I fragment containing the IR0 motif (Δ*Pst*I), markedly blunted glucocorticoid inducibility of the construct because the maximal increase observed following glucocorticoid treatment was 6-fold (Fig. 1B).

To examine the glucocorticoid-mediated activation of the SULT2-40/41 gene more thoroughly, and the functional significance of the IR0 motif more directly, primary rat hepatocyte cultures were transiently transfected with a series of SULT2-40/41 5′-luciferase reporter constructs, and complete dose-response relationships for the glucocorticoid inducibility of reporter gene expression were determined (Figs. 2 and 3). The test constructs (see Fig. 2 for representations of the constructs) included two plasmids that contained the intact IR0 motif (the 1938 construct and 227, containing 227 bp of 5′-flanking sequence), two plasmids that lacked the IR0 motif (the Δ*Pst*I construct and 158, containing 158 bp of 5′-flanking sequence), and one plasmid that contained 1938 bp of SULT2-40/41 5′-flanking region, but a mutagenized ATGAACT motif (IR0-Mut construct). In these experiments, transiently transfected hepatocytes were treated for 24 h with either dexamethasone, a potent glucocorticoid and efficacious inducer of CYP3A (Fig. 2), or with triamcinolone acetonide, a potent glucocorticoid that is a relatively ineffective CYP3A inducer (Fig. 3) (Schuetz and Guzelian, 1984; Kocarek and Reddy, 1998). Each steroid was delivered at doses ranging from $10^{-9}$ to $10^{-5}$ M. Consistent with the results described in Fig. 1, treatment with dexamethasone or triamcinolone acetonide produced a dose-dependent increase in luciferase activity in hepatocytes transfected with the 1938 construct (Figs. 2A and 3A), and this induction was markedly attenuated in hepatocytes transfected with the Δ*Pst*I construct (Figs. 2B and 3B). Glucocorticoid inducibility of luciferase activity was only slightly diminished in hepatocytes transfected with the 227 construct compared with that occurring in hepatocytes transfected with the 1938 construct, suggesting that sequences upstream of bp 227 were relatively unimportant for glucocorticoid inducibility (Figs. 2C and 3C). However, substantial glucocorticoid inducibility (albeit lower than that observed in hepatocytes transfected with the 1938 or 227 construct) was retained in hepatocytes transfected with the 158 construct or the IR0-Mut construct (Figs. 2, D and E and 3, D and E). Thus, glucocorticoid treatment in-

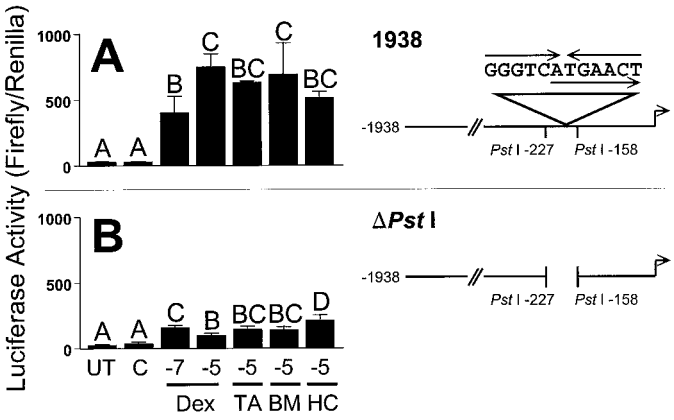


**Fig. 1.** Effects of glucocorticoid treatments on luciferase activity in primary cultured rat hepatocytes transiently transfected with SULT2-40/41 5′-luciferase reporter constructs. Primary cultured rat hepatocytes were transiently transfected with a luciferase reporter construct containing 1938 bp of the SULT2-40/41 5′-flanking sequence (construct 1938, A) or the 1938-bp sequence lacking a 70-bp fragment delimited by *Pst*I sites at −227 and −158 (Δ*Pst*I, B) and treated for 24 h with medium alone (UT) or containing 0.1% DMSO (C), $10^{-7}$ or $10^{-5}$ M dexamethasone (Dex), or $10^{-5}$ M triamcinolone acetonide (TA), betamethasone (BM), or hydrocortisone (HC). Drug concentrations are shown as the logs of the molar concentrations. After treatment, cells were harvested for measurement of luciferase activities. Each bar represents the mean normalized luciferase activity ± S.D. ($n$ = 3 wells/treatment group). Group means were compared with one-way ANOVA, followed by the Neuman-Keuls multiple comparison test. To indicate statistical comparisons among the multiple groups, each bar is labeled with one or more capital letters; groups not sharing a letter are significantly different from each other ($p < .05$). Treatment of cells transfected with an empty reporter plasmid (i.e., pGL3-basic) did not alter luciferase activity (data not shown). Representations of the reporter constructs indicate the positions of *Pst*I sites, the ATGAACT motif (→), and the IR0 motif (→←).

duced luciferase expression irrespective of whether the transfected construct contained an intact IR0 motif. Comparable dexamethasone-inducible effects on SULT2-40/41 gene transcription were observed when primary cultured female hepatocytes were transfected with the 1938, Δ*Pst*I, or 158 construct (data not shown).

Upon closer inspection of the data, differences were observed among the dose-response relationships that were obtained in hepatocyte cultures transfected with the SULT2-40/41 constructs that either contained or lacked the IR0 motif. Luciferase activity was maximal at a dexamethasone dose of ~$10^{-7}$ M when the SULT2-40/41 construct lacked an intact IR0 motif [i.e., constructs Δ*Pst*I, 158, and IR0-Mut (Fig. 2, B, D, and E)], but continued to increase through a dexamethasone dose of $10^{-6}$ M when the construct contained the IR0 motif [i.e., constructs 1938 and 227 (Fig. 2, A and C)]. Thus, after transfection with either of the two IR0-containing constructs, the luciferase activity measured in hepatocytes treated with $10^{-6}$ M dexamethasone was at least 30% greater [i.e., 30.8% greater for the 1938 construct and 51.5% greater ($p < .05$) for the 227 construct] than that measured in cultures treated with $10^{-7}$ M dexamethasone (Fig. 2, A and C). In contrast, the largest increase in luciferase activity that was measured at $10^{-6}$ compared with $10^{-7}$ M dexamethasone treatment in hepatocytes transfected with a construct lacking an intact IR0 motif was 12.6% (Fig. 2E).

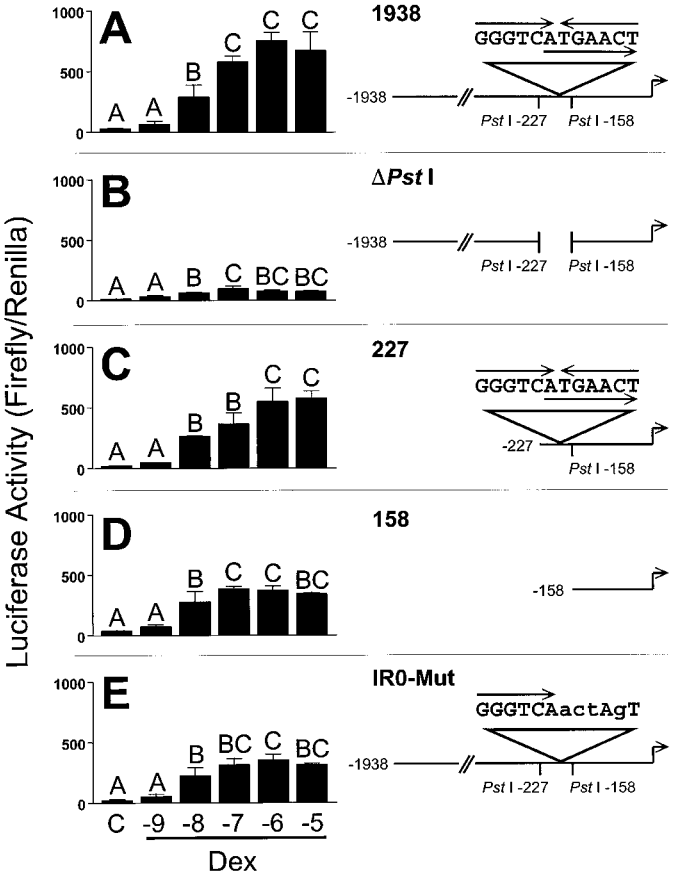


**Fig. 2.** Dose-dependent effects of dexamethasone treatment on luciferase activity in primary cultured rat hepatocytes transiently transfected with SULT2-40/41 5′-luciferase reporter constructs. Primary cultured rat hepatocytes were transiently transfected with a luciferase reporter construct containing 1938 bp of the SULT2-40/41 5′-flanking sequence (construct 1938, A); the 1938-bp sequence lacking a 70-bp fragment delimited by *Pst*I sites at −227 and −158 (Δ*Pst*I, B); 227 bp of SULT2-40/41 5′-flanking sequence (227, C); 158 bp of SULT2-40/41 5′-flanking sequence (158, D); or the 1938-bp sequence containing a mutagenized ATGAACT motif (IR0-Mut, E), treated for 24 h with medium containing 0.1% DMSO (C) or dexamethasone (Dex) at concentrations ranging from $10^{-9}$ to $10^{-5}$ M (shown as log molar concentrations), and harvested for measurement of luciferase activities. Each bar represents the mean normalized luciferase activity ± S.D. ($n$ = 3 wells/treatment group). Group means were compared with one-way ANOVA, followed by the Neuman-Keuls multiple comparison test. To indicate statistical comparisons among the multiple groups, each bar is labeled with one or more capital letters; groups not sharing a letter are significantly different from each other ($p < .05$). Treatment of cells transfected with an empty reporter plasmid (i.e., pGL3-basic) did not alter luciferase activity (data not shown). Representations of the reporter constructs indicate the positions of *Pst*I sites, the ATGAACT motif (→), and the IR0 motif (→←).

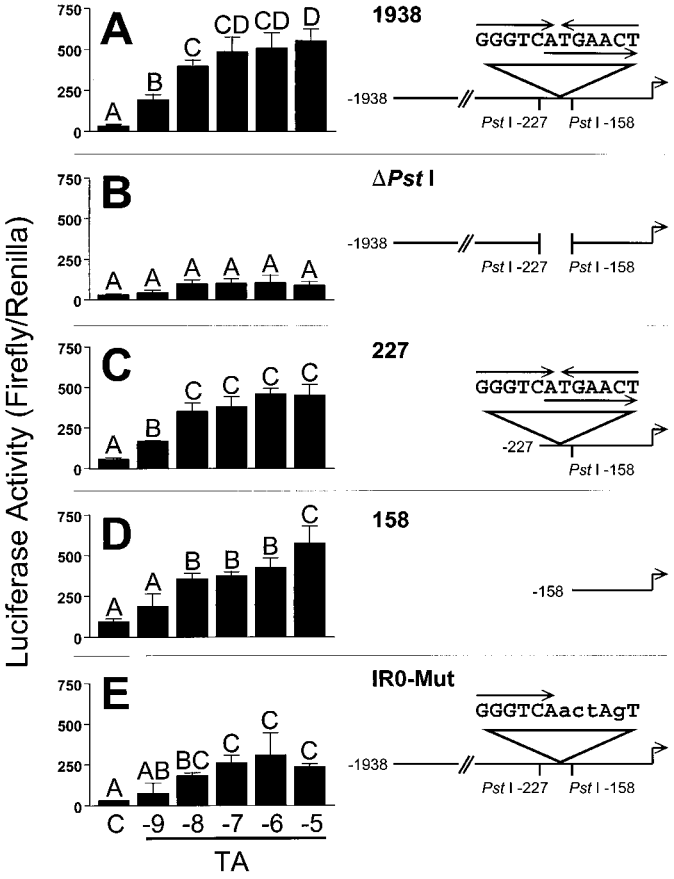


**Fig. 3.** Dose-dependent effects of triamcinolone acetonide treatment on luciferase activity in primary cultured rat hepatocytes transiently transfected with SULT2-40/41 5′-luciferase reporter constructs. Primary cultured rat hepatocytes were transiently transfected with a luciferase reporter construct containing 1938 bp of the SULT2-40/41 5′-flanking sequence (construct 1938, A); the 1938-bp sequence lacking a 70-bp fragment delimited by *Pst*I sites at −227 and −158 (Δ*Pst*I, B); 227 bp of SULT2-40/41 5′-flanking sequence (227, C); 158 bp of SULT2-40/41 5′-flanking sequence (158, D); or the 1938-bp sequence containing a mutagenized ATGAACT motif (IR0-Mut, E), treated for 24 h with medium containing 0.1% DMSO (C) or triamcinolone acetonide (TA) at concentrations ranging from $10^{-9}$ to $10^{-5}$ M (shown as log molar concentrations), and harvested for measurement of luciferase activities. Each bar represents the mean normalized luciferase activity ± S.D. ($n$ = 3 wells/treatment group). Group means were compared with one-way ANOVA, followed by the Neuman-Keuls multiple comparison test. To indicate statistical comparisons among the multiple groups, each bar is labeled with one or more capital letters; groups not sharing a letter are significantly different from each other ($p < .05$). Treatment of cells transfected with an empty reporter plasmid (i.e., pGL3-basic) did not alter luciferase activity (data not shown). Representations of the reporter constructs indicate the positions of *Pst*I sites, the ATGAACT motif (→), and the IR0 motif (→←).

In addition, when sigmoidal functions were fit to the dexamethasone dose-response data, the $ED_{50}$ values that were calculated for hepatocytes transfected with constructs containing the IR0 motif (i.e., 18.1 and 41.1 nM for constructs 1938 and 227, respectively) were greater than the upper 95% CL that were calculated for the hepatocytes transfected with the constructs lacking the IR0 motif, with the exception of the Δ*Pst*I construct, which gave a relatively flat dose-response relationship and wide confidence interval (Table 1). Likewise, the $ED_{50}$ values for the IR0-lacking constructs were smaller than the lower 95% CL for the IR0-containing constructs. In comparison, the dose-dependent increases in luciferase activity that were produced by triamcinolone acetonide were maximal at $10^{-8}$ to $10^{-7}$ M, irrespective of the presence of the IR0 motif (Fig. 3). Thus, with one exception, the $ED_{50}$ values for triamcinolone acetonide-induced luciferase expression from the SULT2-40/41 constructs ranged from 1.98 to 6.52 nM and were comparable to the $ED_{50}$ values obtained for dexamethasone-induced expression from the IR0-lacking constructs (Table 1). An outlying $ED_{50}$ value was calculated for the Δ*Pst*I construct, which again gave a very low response and wide confidence interval (Table 1). For the 158 construct, the response occurring after treatment with $10^{-5}$ M triamcinolone acetonide, which was significantly greater than the response observed after treatment with $10^{-6}$ M triamcinolone acetonide (Fig. 3D), was excluded from the analysis because an increased response at the $10^{-5}$ M dose was not reproduced in a subsequent experiment (data not shown). Collectively, these data suggest that dexamethasone, which is not only a potent glucocorticoid but also an efficacious inducer of CYP3A, activates expression of the SULT2-40/41 gene through both a low dose- and a high dose-mediated component, whereas triamcinolone, which is a potent glucocorticoid but a relatively ineffective CYP3A inducer, activates SULT2-40/41 expression only through the low-dose component. Although these analyses do not resolve the low- and high-dose components to allow estimations of their relative affinities and contributions to overall dexamethasone-inducible SULT2-40/41 expression, they do indicate that when the IR0 motif is present, a high-dose component is present that is of sufficient magnitude to modify the shape of the dexamethasone dose-response relationship for SULT2-40/41 expression.

TABLE 1

Effects of steroid treatment on SULT2-40/41 reporter constructs

$ED_{50}$ estimations for dexamethasone and triamcinolone acetonide-inducible reporter gene expression in primary cultured hepatocytes transiently transfected with SULT2-40/41 5′-luciferase constructs.

Data shown in Figs. 2 and 3 were fit to a sigmoidal function, and $ED_{50}$ values with 95% confidence intervals were calculated.

| SULT2-40/41 Construct | $ED_{50}$ (95% Confidence Interval) | |
|---|---|---|
| | Dexamethasone | Triamcinolone acetonide |
| | *nM* | |
| 1938 | 18.1 (8.4–48.0) | 4.95 (1.11–22.1) |
| Δ*Pst*I | 4.57 (0.42–47.4) | 0.12[a] |
| 227 | 41.1 (11.6–146) | 3.78 (0.75–19.2) |
| 158 | 2.87 (0.70–11.7) | 1.98[b] (0.08–49.9) |
| IR0-Mut | 4.96 (1.50–16.4) | 6.52 (0.58–73.9) |

[a] Because the dose-response relationship for this group was essentially flat, the calculated confidence interval was very wide, $3.68 \times 10^{-29}$ to $4.17 \times 10^{+26}$ nM.

[b] The response measured at $10^{-5}$ M triamcinolone acetonide was excluded from this analysis because the increased response that was seen at this dose was not reproduced in a subsequent experiment.

The low-dose component of dexamethasone-inducible SULT2-40/41 gene activation is suggestive of a classical glucocorticoid receptor-mediated mechanism. To test this possibility, hepatocyte cultures were transiently transfected with either the 227 or 158 construct (i.e., either containing or lacking the IR0 motif), and then treated with $10^{-8}$ M dexamethasone (i.e., a dose sufficient to activate the low-dose component, but not expected to activate the high-dose component), either in the absence or presence of the glucocorticoid receptor antagonist RU486, at $10^{-6}$ M [higher RU486 doses have been shown to activate the PXR (Kliewer et al., 1998; Lehmann et al., 1998; Schuetz et al., 1998)]. As shown in Fig. 2, treatment with $10^{-8}$ M dexamethasone produced comparable increases in luciferase expression in hepatocytes transfected with either of the two constructs (Fig. 4). Treatment with $10^{-6}$ M RU486 alone had no effect on luciferase activity, whereas cotreatment with RU486 significantly inhibited (by ~53%, $p < .05$) dexamethasone-induced luciferase expression from both constructs. These findings suggest that low-dose dexamethasone-inducible SULT2-40/41 expression is mediated through a glucocorticoid receptor-mediated mechanism, and that this effect does not require the IR0 motif.

Dexamethasone treatment induces CYP3A expression at higher doses than those required to saturate the classical glucocorticoid receptor. Recently, certain steroids, including dexamethasone, and other chemicals that induce CYP3A have been shown to activate a newly described member of the nuclear receptor superfamily, termed the PXR (Kliewer et al., 1998; Lehmann et al., 1998; Schuetz et al., 1998). The

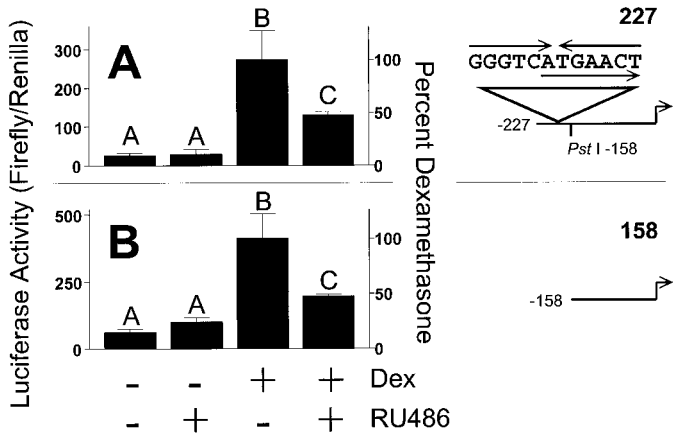


**Fig. 4.** Effect of RU486 treatment on dexamethasone-inducible luciferase activity in primary cultured rat hepatocytes transiently transfected with SULT2-40/41 5-luciferase reporter constructs. Primary cultured rat hepatocytes were transiently transfected with a luciferase reporter construct containing 227 bp of SULT2-40/41 5′-flanking sequence (construct 227, A) or 158 bp of SULT2-40/41 5′-flanking sequence (158, B) and treated for 24 h with medium containing DMSO (−/− control), $10^{-6}$ M RU486, $10^{-8}$ M dexamethasone (Dex), or $10^{-8}$ M Dex + $10^{-6}$ M RU486. After treatment, cells were harvested for measurement of luciferase activities. Each bar represents the mean normalized luciferase activity ± S.D. (left-hand axis) or the percentage of the response to Dex treatment (right-hand axis) ($n = 3$ wells/treatment group). Group means were compared with one-way ANOVA, followed by the Neuman-Keuls multiple comparison test. To indicate statistical comparisons among the multiple groups, each bar is labeled with one or more capital letters; groups not sharing a letter are significantly different from each other ($p < .05$). Treatment of cells transfected with an empty reporter plasmid (i.e., pGL3-basic) did not alter luciferase activity (data not shown). Representations of the reporter constructs indicate the positions of *Pst*I sites, the ATGAACT motif (→), and the IR0 motif (→←).

prototypical CYP3A inducer pregnenolone 16α-carbonitrile has no glucocorticoid receptor agonist activity. Nevertheless, pregnenolone 16α-carbonitrile is among the most efficacious activators of the mouse PXR (Kliewer et al., 1998). To examine whether the IR0 motif, implicated in mediating the high-dose component of dexamethasone-inducible SULT2-40/41 expression, might confer transcriptional responsiveness to pregnenolone 16α-carbonitrile, primary cultured rat hepatocytes were transiently transfected with a panel of SULT2-40/41 5′-luciferase constructs and treated with either $10^{-8}$ or $10^{-6}$ M dexamethasone, or with $10^{-5}$ M pregnenolone 16α-carbonitrile. The SULT2-40/41 constructs consisted of those described in Figs. 2 and 3, and an additional construct containing the 1938-bp 5′-flanking sequence, but specifically lacking the entire IR0 motif (ΔIR0). In contrast to dexamethasone, pregnenolone 16α-carbonitrile-inducible reporter gene activation was restricted only to hepatocytes that were transfected with SULT2-40/41 5′-luciferase reporter constructs containing the intact IR0 motif. Thus, pregnenolone 16α-carbonitrile treatment produced significant increases in luciferase activity of 3.1- and 2.8-fold in hepatocytes transfected with the 1938 or 227 construct, respectively (Fig. 5, A and C), but failed to induce reporter gene expression in hepatocytes transfected with any of the other constructs (Fig. 5, B and D–F).

Because glucocorticoid-inducible luciferase activity was still preserved when primary cultured rat hepatocytes were transfected with construct 158, which contained the least amount of 5′ information, two additional constructs were prepared, extending to bp −77 or −34 relative to the tran-

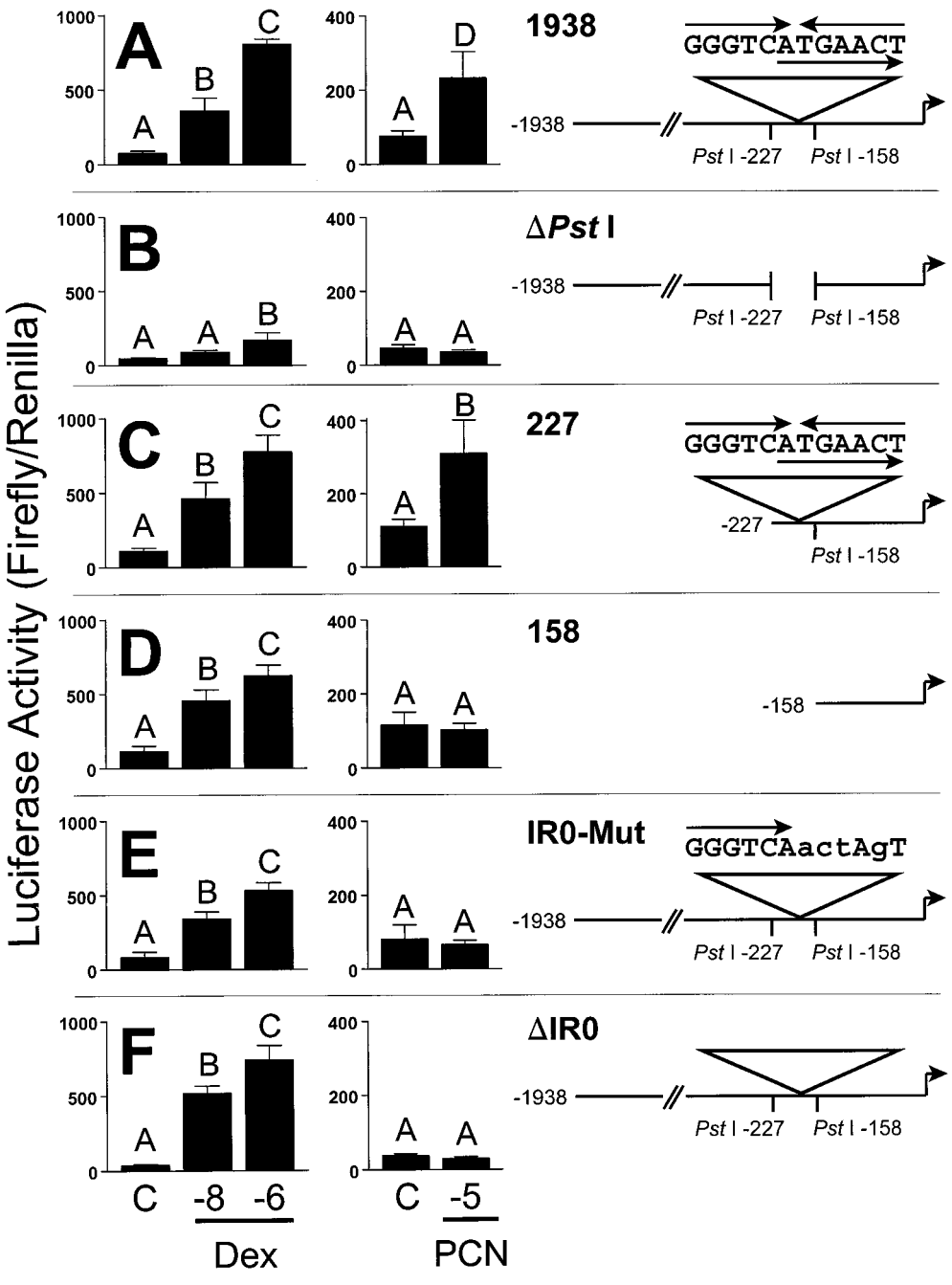


**Fig. 5.** Effects of dexamethasone and pregnenolone 16α-carbonitrile treatments on luciferase activity in primary cultured rat hepatocytes transiently transfected with SULT2-40/41 5′-luciferase reporter constructs. Primary cultured rat hepatocytes were transiently transfected with a luciferase reporter construct containing 1938 bp of the SULT2-40/41 5′-flanking sequence (construct 1938, A); the 1938-bp sequence lacking a 70-bp fragment delimited by *Pst*I sites at −227 and −158 (Δ*Pst*I, B); 227 bp of SULT2-40/41 5′-flanking sequence (227, C); 158 bp of SULT2-40/41 5′-flanking sequence (158, D); the 1938-bp sequence containing a mutagenized ATGAACT motif (IR0-Mut, E); or the 1938-bp sequence lacking the IR0 motif (ΔIR0, F) and treated for 24 h with medium containing 0.1% DMSO (C), $10^{-8}$ or $10^{-6}$ M dexamethasone (Dex) or $10^{-5}$ M pregnenolone 16α-carbonitrile (PCN). After treatment, cells were harvested for measurement of luciferase activities. Each bar represents the mean normalized luciferase activity ± S.D. ($n$ = 3 wells/treatment group). The Dex and PCN data are shown plotted against differently scaled axes to facilitate visualization of the differences in PCN treatment effects among transfection groups. Group means were compared with one-way ANOVA, followed by the Neuman-Keuls multiple comparison test. To indicate statistical comparisons among the multiple groups, each bar is labeled with one or more capital letters; groups not sharing a letter are significantly different from each other ($p < .05$). Treatment of cells transfected with an empty reporter plasmid (i.e., pGL3-basic) did not alter luciferase activity (data not shown) Representations of the reporter constructs indicate the positions of *Pst*I sites, the ATGAACT motif (→), and the IR0 motif (→←).

scription start site (constructs 77 and 34). These particular constructs were designed based on a recent report by Roy and coworkers (Song et al., 1998) that the promoter proximal ~140 bp of the SULT2-40/41 gene contained four sites (termed A, B, $C_1$, and $C_2$) that were protected in DNase I footprint experiments with rat liver nuclear extracts. Thus, construct 77 contained only sites A and B, whereas construct 34 did not contain any of the footprinted sites. When these constructs were tested in transient transfection assays, neither the 77 nor the 34 construct conferred any glucocorticoid-inducible reporter gene activity, suggesting that sequences contained within domains $C_1$ and/or $C_2$ are critical for achieving glucocorticoid responsiveness (data not shown).

## Discussion

We previously reported that the administration of dexamethasone to adult male rats significantly induced SULT2 mRNA and protein levels in the liver (Runge-Morris et al., 1996). In primary cultured rat hepatocytes, SULT2 mRNA expression continued to increase as the dexamethasone dose was augmented from $10^{-8}$ to $10^{-5}$ M (Runge-Morris et al., 1996). These findings contrasted sharply with those obtained for expression of tyrosine aminotransferase, a gene that is regulated via a classical glucocorticoid receptor-mediated mechanism (Shinomiya et al., 1984). In hepatocytes, lower doses of dexamethasone ($10^{-8}$ M compared with $10^{-5}$ M) produced more substantial increases in tyrosine aminotransferase mRNA expression, and cotreatment with dexamethasone and the glucocorticoid receptor antagonist RU486 had a greater inhibitory effect on dexamethasone-induced tyrosine aminotransferase than on dexamethasone-induced SULT2 mRNA expression (Runge-Morris et al., 1996). These data implicated the involvement of an "alternative" relative to a "classical" glucocorticoid receptor-mediated mechanism in the control of glucocorticoid-inducible SULT2 gene expression.

In this study, we have extended our earlier findings by characterizing the transcriptional regulation by glucocorticoids of expression of the SULT2-40/41 gene, the best characterized of the SULT2 family members, by conducting transient transfection analyses with a series of SULT2-40/41 5′-luciferase reporter constructs. These transient transfection studies were performed with primary cultured rat hepatocytes as the recipient cell, and the particular hepatocyte culture model that was used has been shown, through a variety of criteria, to exhibit a highly differentiated phenotype, and to recapitulate many of the effects of endogenous and xenobiotic substances on gene expression that are produced in liver in vivo. Findings of the present study suggest that glucocorticoids, such as dexamethasone, induce SULT2-40/41 gene transcription via a dual mechanism, a low-dose effect that is probably transmitted through the glucocorticoid receptor, and a high-dose effect that may be mediated through the newly described PXR (Kliewer et al., 1998). Several pieces of evidence are offered in support of these assertions. Dose-response analysis suggested that dexamethasone, a potent glucocorticoid and efficacious CYP3A inducer, activated SULT2-40/41 transcription through both a low -dose- and a high-dose-mediated mechanism. The low-dose effect was observed in constructs containing as little as 158 of the 5′ sequence, whereas the high-dose effect was observed only in constructs containing the IR0 motif. In contrast, triamcinolone acetonide, a potent glucocorticoid that is not an effective CYP3A inducer, produced only the low-dose-type inductive effect. Because the low dose-mediated effect was produced by two potent glucocorticoids, we postulated that this effect may occur through a glucocorticoid receptor-mediated mechanism. We have provided evidence in support of this hypothesis by demonstrating that coincubation of transfected primary cultured rat hepatocytes with the glucocorticoid receptor antagonist RU486 significantly inhibited SULT2-40/41 gene activation by low-dose dexamethasone treatment, irrespective of whether the transfected construct contained the IR0 motif.

Because the high dose-mediated effect on SULT2-40/41 expression was produced only by a glucocorticoid that is also an efficacious CYP3A inducer, we postulated that the high-dose effect may be mediated through the PXR, a newly described member of the nuclear receptor superfamily that has been shown to mediate CYP3A induction by a variety of steroidal and nonsteroidal chemicals (Kliewer et al., 1998; Lehmann et al., 1998; Schuetz et al., 1998). In support of this possibility, we showed that treatment of hepatocytes with the prototypical CYP3A inducer and PXR agonist pregnenolone 16$\alpha$-carbonitrile increased reporter gene activity, but only when the transfected SULT2-40/41 construct contained an intact IR0 motif. The activated PXR has previously been shown to interact with the direct repeat-3 and everted repeat-6 nuclear receptor motifs found in the CYP3A23 (rat) and CYP3A4 (human) genes, respectively (Kliewer et al., 1998; Lehmann et al., 1998). Our findings raise the possibility that the PXR also may interact with the SULT2-40/41 IR0 motif. However, until such an interaction is verified experimentally, it remains possible that nuclear receptors other than, or in addition to, PXR may mediate glucocorticoid-inducible SULT2-40/41 gene transcription. In this regard, the constitutive androstane receptor was recently shown to bind to and activate transcription through the same CYP3A4 everted repeat-6 nuclear receptor motif that interacts with PXR (Sueyoshi et al., 1999).

Although our data suggest that low doses of glucocorticoids activate SULT2-40/41 transcription through a glucocorticoid receptor-mediated mechanism and that the responsive *cis*-acting element resides within the 158 bp immediately upstream of the transcription start site (specifically within the fragment located at −77 to −158 relative to the transcription start site), this region of the SULT2-40/41 gene does not contain a typical glucocorticoid response element, suggesting that the glucocorticoid receptor may not directly interact with SULT2-40/41 DNA. A possible explanation for these findings may relate to the findings of Roy and coworkers (Song et al., 1998), who reported that each of the four DNase I-protected sites that are contained within the first 140 bp of the SULT2-40/41 gene (i.e., sites A, B, $C_1$, and $C_2$; the glucocorticoid-responsive −77 to −158 fragment includes sites $C_1$ and $C_2$) interacts with liver-enriched C/EBP and/or HNF1 transcription factors. In addition, transient overexpression of C/EBP$\alpha$ activated SULT2-40/41 5′-reporter gene transcription in HepG2 cells, whereas coexpression of both C/EBP$\alpha$ and HNF1$\alpha$ synergistically activated reporter gene expression in NIH-3T3 fibroblasts (Song et al., 1998). Of particular note, certain C/EBP family members (e.g., $\alpha$ and $\beta$) are re-

ported to be glucocorticoid-inducible transcription factors (Takiguchi, 1998).

Our data suggest that sequences upstream of bp −227 are not essential for glucocorticoid-inducible SULT2-40/41 transcription. However, the Δ*Pst*I construct consistently displayed the lowest glucocorticoid inducibility of any of the constructs. If sequences upstream of bp −227 have no role in regulating glucocorticoid-mediated SULT2-40/41 gene transcription, glucocorticoid-induced transcription of the Δ*Pst*I construct should have been as great as it was from the 158 construct. Also, if the IR0 motif is the only sequence contained within the *Pst*I fragment that participates in glucocorticoid inducibility, transcription of the Δ*Pst*I construct should have been as great as it was from the IR0-Mut construct. Therefore, our findings suggest that one or more *cis*-acting elements located upstream of bp −227 may exert a negative effect on glucocorticoid-inducible SULT2-40/41 expression, but that this suppressive effect is only revealed upon deletion of an element, other than the IR0, that is contained within the *Pst*I fragment. Of possible significance in this regard, Roy and coworkers (Song et al., 1998) implicated a DNase I-protected region between −231 and −292 in mediating androgen-repressible SULT2-40/41 gene transcription. This region contained several binding sites for Oct-1 and C/EBP, but did not bind to the androgen receptor. In addition, Chandran et al. (1999) recently provided evidence that glucocorticoid-repressible transcription of the gonadotropin-releasing hormone gene is mediated through a multiprotein complex in which the glucocorticoid receptor does not directly bind to the negative regulatory region, but rather is tethered to DNA-bound Oct-1.

In the liver, members of the SULT2 gene family are involved in bile acid detoxication and drug metabolism, and in the regulation of intratissue active hormone levels. It stands to reason that disruption or deregulation of SULT2 gene expression may have serious consequences for hepatic cholestasis, xenobiotic detoxication, and hormone response mechanisms. Although the deduced amino acid sequences corresponding to individual SULT2 isoforms maintain a close (~86.3 to 99.6%) structural identity (Watabe et al., 1994; Yamazoe et al., 1994; Runge-Morris et al., 1998), we and others have shown that the mRNA expression of separate SULT2 isoforms is differentially regulated in response to hormone or xenobiotic treatment. For example, noncoordinate regulation by growth hormone of the rat hepatic SULT2-40/41 and SULT2-20/21 isoforms in growth hormone-deficient rats has been previously described (Ueda et al., 1997). In addition, male and female rat liver displayed very different patterns of SULT2 isoform-specific mRNA expression following in vivo treatment with pharmacologic doses of dexamethasone or pregnenolone 16α-carbonitrile (Liu and Klaassen, 1996). Similarly, we found that SULT2-40/41 mRNA levels were induced, whereas amounts of SULT2-20/21 mRNA were concomitantly suppressed when male rats were treated with CYP2B-inducing doses of phenobarbital (Runge-Morris et al., 1998).

The reasons for heterogeneity in the expression and regulation of individual SULT2 isoforms are not yet clear, but suggest that despite their overlapping substrate specificities, the SULT2-40/41, -20/21, and -60 isoforms may have important differences in their substrate specificity profiles and consequent biological activities in vivo. The existence of dual mechanisms for the regulation of steroid-inducible SULT2-40/41 expression may presage the enzyme's physiological role. Thus, physiological levels of circulating glucocorticoid [$\sim 10^{-8}$ to $7 \times 10^{-7}$ M corticosterone in rat (Oster et al., 1988; De Boer and Van der Gugten, 1987)] would primarily activate the low-dose, glucocorticoid receptor-mediated component of SULT2-40/41 transcription, which may be essential for maintaining basal liver-specific SULT2-40/41 gene expression. Alternatively, higher doses of glucocorticoids, as occur during periods of physiological stress, may augment SULT2-40/41 gene expression via activation of the PXR. Additional molecular studies are required to define the precise identities, interactions, and functional significance of the nuclear receptor transcription factors that govern glucocorticoid-inducible SULT2-40/41 gene expression.

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