REGULATION OF GLUCOCORTICOID-INDUCIBLE HYDROXOSTEROID SULFOTRANSFERASE (SULT2A-40/41) GENE TRANSCRIPTION IN PRIMARY CULTURED RAT HEPATOCYTES: ROLE OF CCAAT/ENHANCER-BINDING PROTEIN LIVER-ENRICHED TRANSCRIPTION FACTORS

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ABSTRACT:

The mechanism responsible for glucocorticoid receptor (GR)-mediated induction of rat hepatic hydroxysteroid sulfotransferase (SULT2A-40/41) gene transcription was investigated. We previously reported that the region of the SULT2A-40/41 5′-flanking region delimited by −158 to −77 nucleotides relative to the transcription start site was sufficient to support GR-inducible expression. This region of the SULT2A-40/41 gene does not contain a consensus glucocorticoid receptor-responsive element, but does contain two consensus sites for liver-enriched CCAAT/enhancer-binding protein (C/EBP) transcription factors. In the present study, incubation of primary cultured rat hepatocytes with a GR-activating concentration (10⁻⁷ M) of a potent glucocorticoid, dexamethasone or triamcinolone acetonide (TA), rapidly produced increases in C/EBPα and C/EBPβ nuclear protein contents, as measured by Western blot or in vitro DNA-binding activity analysis, that preceded increases in SULT2A-40/41 mRNA and protein levels. Transient cotransfection of SULT2A-40/41 reporter plasmids with a dominant negative C/EBP expression plasmid completely blocked TA-inducible SULT2A-40/41 reporter gene expression. Linker scanning and site-directed mutagenesis of the proximal SULT2A-40/41 5′-flanking region, complemented by in vitro DNA-binding analyses, indicated that the more distal C/EBP site was important for controlling SULT2A-40/41 promoter activity. These data support a role for GR-inducible C/EBPα and C/EBPβ expression in the transactivation of hepatic SULT2A-40/41 expression.

Achieving a clear understanding of the molecular events that regulate the expression of hepatic hydroxysteroid sulfotransferase (SULT2A) enzymes has important implications for xenobiotic metabolism and metabolic disease mechanisms. SULT2A enzymes play a central role in hepatic detoxification of bile acids (Kitada et al., 2003) and xenobiotics such as α-hydroxytamoxifen (Davis et al., 2000) and the synthetic steroid cyproterone acetate (Kasper and Mueller, 1999). SULT2A enzymes transfer a sulfonate group from the physiological sulfate donor, 3'-phosphoadenosine-5'-phosphosulfate, to a wide range of diverse, metabolically important substrates including hydroxysteroids, such as the sex hormone precursor dehydroepiandrosterone (Lyon et al., 1981). The formation of polar sulfonate conjugates generally results in the elimination of toxic species (detoxication). By contrast, the formation of unstable sulfonate esters produces reactive DNA-damaging intermediates, and SULT2A enzyme activity has been implicated in the bioactivation of several classes of carcinogens, including polycyclic hydroxymethylarenes (Ogura et al., 1990b) and benzylic and allylic alcohols (Surh, 1998). Of the three structurally similar SULT2A isoforms that are expressed in rat liver, the transcriptional regulation of the SULT2A-40/41 gene, which is expressed both in vivo and in primary cultured rat hepatocytes, has been best characterized (Runge-Morris et al., 1999; Sonoda et al., 2002). Alternative names for SULT2A-40/41 include (RAT)SULT2A3 (Blanchard et al., 2004), BAST-I (Barnes et al., 1989), HST-a (Runge-Morris, 1998), SULT2-40/41 (Runge-Morris et al., 1999), ST-40/41 (Liu and Klaassen, 1996a), STA (Ogura et al., 1990a), Std (Song et al., 1998), and SMP-2 (Chatterjee et al., 1987).

We and others have shown previously that rat hepatic SULT2A-40/41 gene expression is glucocorticoid-inducible (Liu and Klaassen, 1996b; Runge-Morris et al., 1996). In primary cultured rat hepatocytes, the induction of SULT2A-40/41 transcription by glucocorticoids occurs through a dual transcription control mechanism that involves both the GR and the PXR nuclear receptors (Runge-Morris et al., 1999; Sonoda et al., 2002). At higher concentrations of glucocorticoid (e.g., DEX, 10⁻⁶ M), SULT2A-40/41 gene transcription is

ABBREVIATIONS: SULT2A, hydroxysteroid sulfotransferase; C/EBP, CCAAT/enhancer-binding protein; DEX, dexamethasone; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; GR, glucocorticoid receptor; GRE, glucocorticoid response element; PEPCK, phosphoenolpyruvate carboxykinase; PXR, pregnane X receptor; RT-PCR, reverse transcription-polymerase chain reaction; TA, triamcinolone acetonide; nt, nucleotide(s); HNF, hepatocyte nuclear factor; Ct, cycle threshold; LS, linker scanning mutant; AP-1, activator protein-1.
transactivated by the PXR transcription factor, which binds to an inverted repeat enhancer sequence with zero intervening bases located at nt –189 to –178 within the 5′-flanking region of the SULT2A-40/41 gene (Runge-Morris et al., 1999; Sonoda et al., 2002). By contrast, GR, but not PXR, is required for the inducible SULT2A-40/41 expression that is produced by physiological concentrations of glucocorticoids (Runge-Morris et al., 1999).

We previously showed that retention of a short 158-nt fragment located just proximal to the SULT2A-40/41 core promoter is sufficient to support GR-inducible SULT2A-40/41 gene transcription (Runge-Morris et al., 1999). The 5′-flanking region of the SULT2A-40/41 gene lacks a consensus GRE but contains three consensus recognition sites for liver-enriched C/EBP transcription factors (Song et al., 1998). Roy’s research group (Song et al., 1998) demonstrated previously that over-expression of C/EBPα in the human HepG2 hepatocellular carcinoma cell line produces a strong activation of a cotransfected rat SULT2A-40/41 reporter gene. They also definitively demonstrated that the expression of a SULT2A-40/41 reporter gene can be transcriptionally activated in nonhepatic 3T3 fibroblasts by cotransfecting expression plasmids that produce the liver-enriched hepatocyte nuclear factor-1α (HNF1α) and C/EBPα transcription factors (Song et al., 1998), suggesting that liver-enriched transcription factors are especially important to the maintenance of basal SULT2A-40/41 expression in the liver. The C/EBPα and C/EBPβ transcription factors are robustly expressed in the liver (Alam et al., 1992; Crosson et al., 1997), and the expression of both of these transcription factors has been reported to be glucocorticoid-inducible in hepatic cells (Matsumo et al., 1996; Ramos et al., 1996; Crosson et al., 1997; Gotoh et al., 1997). Since glucocorticoids induce SULT2A-40/41 gene transcription in the absence of a consensus GRE (Runge-Morris et al., 1999), the present study was designed to test the hypothesis that GR-inducible C/EBPs are commandeered to transactivate GR-inducible SULT2A-40/41 gene transcription in primary cultured rat hepatocytes.

Materials and Methods

Materials. DEX, TA, and Poncane S solution were purchased from Sigma-Aldrich (St. Louis, MO). Matrigel was purchased from BD Biosciences Discovery Labware (Bedford, MA). Vitrogen was purchased from Cohesion Technologies (Palo Alto, CA). Recombinant human insulin (Novolin R) was purchased from Novo Nordisk U.S. (Princeton, NJ). Culture medium, Lipo- fectin reagent, and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). Custom-synthesized oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The NE-PER (Nuclear and Cytoplasmic Extraction Reagents) kit was obtained from Pierce Endogen (Rockford, IL). Hybond-P-PVDF membranes were purchased from Amersham Biosciences Inc. (Piscataway, NJ). ECL-Plus Western blotting kit and reagents were obtained from Amersham Biosciences Inc. Real-time RT-PCR reagents were purchased from Applied Biosystems (Foster City, CA). The QuickChange II XL Site-Directed Mutagenesis kit was purchased from Stratagene (La Jolla, CA). The TransAM C/EBPα/β Transcription Factor Assay Kit and protein standards were purchased from Active Motif Inc. (Carlsbad, CA). Other supplies and molecular biology grade reagents were obtained from the sources indicated below or previously described (Runge-Morris et al., 1999).

Primary Culture of Rat Hepatocytes. Hepatocytes were isolated from adult male Sprague-Dawley rats (250–350 g; Harlan, Indianapolis, IN), as previously described (Runge-Morris et al., 1996). Following isolation, 3 million adult male Sprague-Dawley rats (250–350 g; Harlan, Indianapolis, IN), as previously described (Runge-Morris et al., 1999). The 5′-flanking region of the SULT2A-40/41 gene (Runge-Morris et al., 1999). The 5′-flanking region of the SULT2A-40/41 gene contains a consensus GRE but contains three consensus recognition sites for liver-enriched C/EBP transcription factors (Song et al., 1998), suggesting that liver-enriched transcription factors are especially important to the maintenance of basal SULT2A-40/41 expression that is produced by physiological concentrations of glucocorticoids (Runge-Morris et al., 1999; Sonoda et al., 2002). By contrast, GR, but not PXR, is required for the inducible SULT2A-40/41 gene (Runge-Morris et al., 1999; Sonoda et al., 2002), but with the following modifications. Nuclear and cytosolic extracts were prepared from primary cultured rat hepatocytes (six pooled dishes) using the NE-PER kit according to the manufacturer’s instructions (Pierce Endogen). For Western blot analysis, 10 μg of nuclear (for C/EBPs) or cytosolic (for SULT2A-40/41) protein were loaded into each lane. Rat liver nuclear extract, used as a standard on the C/EBP immunoblots (loaded at 3.5 μg), was purchased from Active Motif Inc. Uniform protein loading and transfer onto Hybond-P-PVDF membranes was confirmed using Peroxone S staining. Rabbit anti-rat C/EBPα (sc-61) and rabbit anti-rat C/EBPβ (sc-150) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and were both used at 1:1000 dilutions. Rabbit anti-rat SULT2A (antibody to STA, used at 1:10,000) (Sheng and Dufell, 2000) and a purified expressed rat SULT2A protein standard (loaded at 5 ng) were generous gifts from Dr. Michael Dufell (University of Iowa, Iowa City, IA). A secondary peroxidase-conjugated goat anti-rabbit antibody was purchased from Jackson Immunoresearch Laborato ries Inc. (West Grove, PA), and was used at a dilution of 1:10,000, 1:15,000, and 1:3000 for C/EBPα, C/EBPβ, and SULT2A, respectively. Visualization of immunoreactive bands was accomplished using the ECL-Plus Western blotting kit, according to the manufacturer’s instructions (Amersham Biosciences Inc.). Chemiluminescent band intensities were estimated by scanning laser densitometry, using a Molecular Dynamics Personal Densitometer SI (Amersham Biosciences Inc.) equipped with ImageQuant Version 5.2 software.

For analysis of SULT2A-40/41 mRNA levels, following treatment, the dishes of hepatocytes representing each treatment group (three dishes per treatment group) were pooled for preparation of total RNA, using TRIzol reagent, according to the manufacturer’s instructions (Invitrogen). Northern blot analysis was performed as described previously (Runge-Morris et al., 1996), using a rat SULT2A cDNA probe (trivial name, BAST-I cDNA) from Dr. Charles N. Falany (University of Alabama at Birmingham).

Samples of rat hepatocyte total RNA (5 μg) were reverse-transcribed, using SuperScript II according to the manufacturer’s (Invitrogen) instructions. PCR was performed using SYBR Green and the ABI Prism 7000 Sequence Detection System, according to a protocol supplied by Applied Biosystems. PCR primers were designed using Primer Express software (version 2.0; Applied Biosystems) to amplify a fragment of the SULT2A-40/41 cDNA. The upper primer (5′-CCCTTTCCTGGCTTGGGAT-3′, located in exon 1) corre- sponded to positions 68 to 87, whereas the lower primer (5′-CAATGCAGAT-3′, spanning the exon 1-exon 2 boundary) corre- sponded to positions 188 to 166 of the published SULT2A-40/41 cDNA sequence (GenBank accession number M33329). The upper primer contains one mismatch to SULT2A-20/21 (GenBank accession number M33633) and two mismatches to SULT2A-60. PCRs contained cDNA corresponding to 10 ng of RNA, 25 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems), 45 pmol of upper primer, and 15 pmol of lower primer (primer concentrations were optimized in a preliminary experiment) in a total volume of 50 μl. Samples were incubated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Following data acquisition, cycle threshold (Ct) values were determined for each PCR, using SDS software (Applied Biosystems). Amounts of cDNA in samples from TA-treated hepatocyte cultures were determined relative to those present in cultures harvested before treatment (time 0), by subtracting all Ct values from the mean time 0 Ct value and raising 2 to the power corresponding to that difference.
Mutagenesis of the SULT2A-40/41 5' Flanking Region. A series of linker scanning mutants passing through the glucocorticoid-responsive region of the SULT2A-40/41 5' flanking region, containing the two C/EBP sites previously designated as C1 and C2 (Song et al., 1998) (see Fig. 4), was prepared using the method described by Gustin and Burk (2000). The mutants were prepared by replacing successive 6-nt segments of the SULT2A-40/41 sequence with BamHI sites. The 5' end of each linker scanning mutant was −542 relative to the SULT2A-40/41 transcription start site. Briefly, for each mutant, two PCRs were initially performed using the primers indicated in Table 1, the SULT2A-40/41 1938 plasmid as template, and Pfu polymerase (Stratagene). One PCR contained primers 1 and 2, while the second contained primers 3 and 4. Following PCR, the individual amplified products were gel-purified and digested with BamHI I. Aliquots of the two reactions were then combined and ligated together. The ligated product then served as template for a final PCR that contained primers 1 and 4. This product was gel-purified, digested with MluI and XhoI, and ligated into pGEL3-Basic (Promega, Madison, WI). The corresponding wild-type control reporter plasmid (termed LS0) was prepared using primers 1 and 4 only.

Site-directed mutations (2-nt changes) were introduced into the core regions of the two C/EBP sites (C1 and C2), using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer’s instructions. For preparation of the C1 mutant, the mutagenic primer pairs were 5'-CAATAAC-CTTTGACTGTTGTCAAAATTTATATATCACACAG-3' and complement. For preparation of the C2 mutant, the mutagenic primer pairs were 5'-GTTCTTTGTTGAGTGGAAATTCATCAATACAAT-3' and complement (the nt that were changed are underscored). For preparation of individual mutants, the SULT2A-40/41 158 plasmid was used as template. For preparation of a C/EBP C1/C2 double mutant, the plasmid containing the C2 mutation was used as template in combination with the C1 mutagenic primer.

EMSA. EMSA was used to 1) evaluate the presence of C/EBP and AP-1 transcription factors capable of binding their respective consensus response elements in rat hepatocyte nuclear extracts (prepared using the NE-PER kit, as described above), 2) estimate the relative binding affinities of nuclear extract proteins for various C/EBP motifs (i.e., consensus, SULT2A-40/41 C1 and C2, and C1 and C2 mutants), and 3) determine the abilities of a purified AP-1 transcription factor (Fos/Jun) to bind to the potential SULT2A-40/41 AP-1 site. The following double-stranded oligonucleotides were used for C/EBP EMSA: 1) consensus, 5'-TCTGCGATATTGCGCAATTGTCAACA-3' (forward and reverse oligonucleotides were identical); the 5'-TC overhangs were added to permit fill-in radiolabeling); 2) SULT2A-40/41 C1, 5'-TGTTGTTTACAAAATTTATTTATT-3' and complement; 3) SULT2A-40/41 C1 mutant, 5'-TGTTGTTTCATAATTTATT-3' and complement (the mutated nt are underscored); 4) SULT2A-40/41 C2, 5'-TGGAAAATTCATCAATACAAT-3' and complement; and 5) SULT2A-40/41 C2 mutant, 5'-TGGAAAATTCATCAATACAAT-3' and complement (mutated nt are underscored). The following double-stranded oligonucleotides were used for AP-1 EMSA: 1) consensus, 5'-TCCGGCTTGTGACTGACCTGCGGAA-3' and 5'-TCTGCGATATTGCGCAATTGTCAACA-3' (the 5'-TC overhangs were added to permit fill-in labeling); 2) SULT2A-40/41 AP-1, 5'-TACACTTTTGACTGTTGTTGTA-3' and complement; and 3)
SULT2A-40/41 AP-1 mutant, 5'-TAACCTTGTACTGTGTTAC-3' and complement (mutated nt are underscored). The C/EBP and AP-1 consensus probes were 3'-end-labeled by filling in with Klenow DNA polymerase and [α-32P]dATP. Rat hepatocyte nuclear extract proteins (5.4 μg) or recombinant c-Fos and c-Jun proteins (25 ng each; Active Motif Inc.) were incubated with 20,000 cpm of labeled probe for 20 min at room temperature in buffer containing 12 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, and 2 μg of poly(dI-dC) in a total volume of 10 μl. DNA-protein complexes were separated on 4% native polyacrylamide gels in 0.5× Tris borate-EDTA running buffer, at 30 mA for 1.5 h. Bands were visualized by autoradiography.

ELISA. The amounts of C/EBPα and C/EBPβ capable of binding to a consensus C/EBP motif in rat hepatocyte nuclear extracts were quantified using an ELISA kit (TransAM C/EBPα/β Transcription Factor Assay Kit), according to the manufacturer's instructions (Active Motif Inc.). Each sample was assayed in duplicate, using 5.4 μg of nuclear extract protein per well.

Results

The temporal effects of a GR-activating concentration of DEX (10−7 M) on the total nuclear contents of C/EBPα and C/EBPβ were examined in primary cultured rat hepatocytes by Western blot analysis (Fig. 1A). The antibody to C/EBPα detected two bands in rat liver nuclear protein, measuring 42 kDa and 30 kDa (Fig. 1A, upper and lower bands, respectively). These protein bands represent the expected polypeptide sizes of C/EBPα splice variants (Ramji and Foka, 2002). The antibody to C/EBPβ detected three protein bands corresponding to the expected sizes of the known 38-kDa, 35-kDa, and 20-kDa C/EBPβ isoforms (Ramji and Foka, 2002) (Fig. 1A, upper, middle, and lower bands). DEX treatment increased the nuclear levels of the C/EBPα and C/EBPβ isoforms in primary cultured rat hepatocytes. At the 3-h and 6-h time points, DEX treatment increased the amount of the 30-kDa C/EBPα isoform relative to DMSO-treated controls, up to approximately 2-fold (Fig. 1A, lower band). Similarly, increased amounts of the 35-kDa and 20-kDa C/EBPβ were detected at 3 h and 6 h after DEX treatment (Fig. 1A, middle and lower bands), and the level of the 20-kDa isoform remained elevated at 22 h (Fig. 1A, lower band). By contrast to C/EBPα and C/EBPβ, DEX treatment did not detectably induce the amount of nuclear C/EBPβ in primary cultured rat hepatocytes (data not shown).

Relative to DMSO-treated controls, DEX treatment of primary cultured rat hepatocytes produced a marked increase in the amount of SULT2A mRNA at 24 h after DEX treatment, which was somewhat attenuated at 48 h (Fig. 1, B and D). A DEX-induced increase in cytosolic SULT2A protein content was observed at 48 h (Fig. 1, C and D). To date, transcriptional regulation studies of rat liver SULT2A expression have focused on the SULT2A-40/41 gene (Song et al., 1998; Runge-Morris et al., 1999). Given that the sequence similarities among the three rat hepatic SULT2A isoforms range from 86.3% to 99.6% (Watabe et al., 1994), the SULT2A cDNA probe and polyclonal antibody that were applied in the Northern and Western blot analyses could not be expected to yield isospecific information with regard to SULT2A expression. However, in corroboration of Northern and Western blot analyses, real-time RT-PCR analysis, using primers designed to amplify SULT2A-40/41 preferentially, demonstrated increased SULT2A-40/41 mRNA content in primary cultured rat hepatocytes at 24 h after DEX treatment, relative to time-matched DMSO-treated controls (Fig. 1E). By this analysis, the amount of SULT2A-40/41 mRNA was found to return to the control level at 48 h after DEX treatment (Fig. 1E).

Since these initial experiments indicated that glucocorticoid treatment of primary cultured rat hepatocytes increased the levels of total nuclear C/EBPα and C/EBPβ at times that preceded increases in SULT2A mRNA and protein content, we sought further confirmation that such treatments could produce comparable temporal increases in the levels of nuclear C/EBPαs capable of specific binding to a C/EBP motif. In these and all remaining studies, we used the potent glucocorticoid, TA (10−7 M), rather than DEX, since our previous study demonstrated that TA activates SULT2A-40/41 transcription exclusively through the “low-dose,” GR-dependent mechanism (Runge-Morris et al., 1999). By EMSA, the amount of nuclear C/EBP binding activity was increased at the earliest TA treatment time examined (1 h), and at all subsequent times (Fig. 2A). The TA-mediated changes in nuclear C/EBPα and C/EBPβ binding activities were quantified using an ELISA, in which nuclear C/EBPαs were first bound to a C/EBP consensus motif and then detected with a C/EBPα- or C/EBPβ-specific antibody (Fig. 2B). By this assay, the nuclear binding activities of both C/EBPα and C/EBPβ were observed to change during the course of incubation of hepatocytes in control medium...
containing 0.1% DMSO (Fig. 2B, white bars). Thus, during the period 48 to 72 h post plating (i.e., treatment times 0 through 24 in Fig. 2B), the nuclear contents of these C/EBPs decreased in a time-dependent manner, by up to ~50%. Then, following medium change, the C/EBP levels detected at 96 h post plating (i.e., treatment time 48 h) were found to be ~70% of those detected at 48 h post plating (i.e., treatment time 0). At all time points, the C/EBPα and C/EBPβ binding activities were greater in the TA-treated hepatocytes than in the DMSO-treated cultures, with maximal fold increases of ~2-fold at treatment times of 12 and 24 h (Fig. 2B). Also, the nuclear C/EBPα and C/EBPβ binding activities in the TA-treated hepatocytes reached levels that were ~50% greater than those detected at treatment time 0 (Fig. 2B). Corresponding measurements of SULT2A-40/41 mRNA content by real-time RT-PCR showed that, in agreement with the

DEX-mediated effects shown in Fig. 1E, TA treatment produced a detectable increase in SULT2A-40/41 mRNA levels at 6 h after treatment, which was maximal by 12 h and had declined to the control level at 48 h (Fig. 2C). Thus, the TA-mediated changes in nuclear C/EBP binding activities preceded and largely paralleled the changes in SULT2A-40/41 mRNA content.

To test the hypothesis that C/EBP activity is required for the induction of SULT2A-40/41 gene transcription by GR-activating concentrations of glucocorticoid, cotransfection studies were performed in primary cultured rat hepatocytes that were treated with DMSO or TA. Relative to DMSO-treated controls, the treatment of primary rat hepatocytes with TA produced significant increases in luciferase transcription from either of two SULT2A-40/41 reporter plasmids that contained three C/EBP response elements located proximal to the

Fig. 2. Time-dependent effects of TA treatment on C/EBPα and C/EBPβ nuclear binding activities in primary cultured rat hepatocyte nuclear extracts and on SULT2A-40/41 mRNA levels. Forty-eight-hour-old primary rat hepatocyte cultures were either harvested (0 h) or treated with either DMSO (0.1%) or TA (10⁻⁷ M) for 1, 3, 6, 12, 24, or 48 h. Following treatment, hepatocytes were harvested for the preparation of nuclear extracts or total RNA. A, EMSA showing the binding of nuclear extract proteins from DMSO- or TA-treated hepatocytes to a consensus C/EBP oligonucleotide probe. Positions of the major retarded band and the free probe are indicated. The binding reaction for the lane marked NE did not contain nuclear extract. B, the relative amounts of C/EBPα and C/EBPβ in nuclear extracts from DMSO- or TA-treated hepatocytes capable of binding to a consensus C/EBP motif were determined by ELISA. Each bar represents the mean ± range of duplicate determinations. C, the relative levels of SULT2A-40/41 mRNA in DMSO- or TA-treated hepatocyte cultures were determined by real-time RT-PCR analysis.
SULT2A-40/41 core promoter, (Fig. 3, upper panels). As is postulated for the regulation of rat SULT2A-40/41, glucocorticoid-inducible PEPCk gene transcription is mediated by the C/EBPβ transcription factor (Yamada et al., 1999). TA treatment increased luciferase expression from a PEPCk reporter plasmid, as well as from a reporter containing a simple GRE, which is regulated through direct GR binding (Fig. 3, lower panels). Cotransfection of hepatocyte cultures with a dominant negative C/EBP expression plasmid effectively inhibited TA-inducible SULT2A-40/41 (Fig. 3, upper panels) and PEPCk reporter gene expression (Fig. 3, left lower panel), but, as expected, had no effect on TA-inducible expression from the consensus GRE reporter construct (Fig. 3, right lower panel).

To identify the 5'-flanking sequences within the proximal SULT2A-40/41 5'-flanking region that are essential to the mediation of GR-inducible expression, systematic linker scanning mutagenesis analysis of this region was performed. Linker scanning mutants LS2 through LS6 demonstrated striking reductions in basal (i.e., DMSO-treated) and TA-inducible luciferase expression relative to those detected with the corresponding wild-type construct (LS0) (Fig. 4). The LS2 and LS3 mutations disrupted sequences within a C/EBP site in the 5'-flanking region of SULT2A-40/41 that was designated as C2 (Song et al., 1998) (Fig. 4). Specifically, the LS2 mutation disrupted the entire core region of the C2 C/EBP site, whereas the LS3 mutations introduced 3-nt departures from the C/EBP matrix sequence, but did not affect promoter activity as much as did the LS2 and LS3 mutations (Fig. 4). In particular, the LS7 mutation, in which the entire core region of the C/EBP site was disrupted, had little effect on promoter activity. LS9 through LS11, which included no C/EBP sequences, were all expressed comparably to LS0.

Fig. 3. Effects of dominant negative C/EBP expression on glucocorticoid-inducible SULT2A-40/41 gene transcription. Twenty-four-hour-old primary rat hepatocyte cultures were transiently transfected with the indicated luciferase reporter plasmids, including SULT2A-40/41 1938, a C/EBP-responsive reporter plasmid (containing C/EBP-responsive regions within the PEPCk gene), or a reporter plasmid containing a consensus GRE. Some cultures were cotransfected with either an empty expression vector (CMV500-4hep) or a plasmid expressing dominant negative C/EBP (CMV500-4hep-CEBP). At 48 h, transfected cultures were treated with either 0.1% DMSO (vehicle control) or TA (10^{-7} M). Following 24 h of treatment, hepatocytes were harvested for the determination of luciferase activities. The data are representative of two independent experiments and are presented as the mean normalized luciferase activity ± S.D., n = 3 per treatment group. Group means were compared using a one-way analysis of variance followed by the Newman-Keuls post hoc test. Groups that do not share a capital letter are significantly different from each other (p ≤ 0.05).

Fig. 4. Linker scanning mutagenesis analysis of TA-inducible SULT2A-40/41 transcription in primary cultured rat hepatocytes. Linker scanning mutants LS1 through LS12, spanning the glucocorticoid-responsive region of the SULT2A-40/41 gene, were prepared as described under Materials and Methods. The sequence of the SULT2A-40/41 5'-flanking region (to −158) is shown in the top panel. The positions and nucleotide changes for each linker scanning mutant are shown in boxes below the SULT2A-40/41 sequence. The matrix sequence for a C/EBP site (VSCEBPB_01) is shown in the boxes above the SULT2A-40/41 sequence, which mark the locations of the C1 and C2 C/EBP sites. Within these sequences, capitalized letters indicate the core portions of the sites. Standard nucleotide abbreviations are h = A/T/C; k = G/T; m = C/A; n = A/T/G/C; y = C/T. The locations of possible binding sites for HNF1, HNF4, AP-1 and HNF3β, predicted by computational analysis (MatInspector) are indicated (arrows represent the orientation of the binding site). Primary rat hepatocytes were transfected with pGL3-Basic (pGL3, as negative control), wild-type SULT2A-40/41 reporter plasmid (LS0) or one of the linker scanning mutants, and treated with either DMSO (0.1%) or TA (10^{-7} M). After 24 h treatment, hepatocytes were harvested for luciferase activity measurements. The data are representative of five independent experiments, and are presented as mean normalized luciferase activity ± S.D., n = 3 per treatment group.
FIG. 6. Competitive binding analysis of rat hepatocyte nuclear extract proteins to normalized luciferase activity data are representative of three independent experiments and are presented as mean 

scanning linker mutant, which affected sequences that were just proximal to the SULT2A-40/41 core promoter, also demonstrated a markedly reduced basal and TA-inducible reporter expression (Fig. 4). These results implicated the more distal C/EBP site (C2) as being particularly important in the regulation of TA-inducible SULT2A-40/41 transcription.

A site-directed mutagenesis approach was next used to test specifically the relative contributions of the C1 and C2 sites to TA-inducible SULT2A-40/41 gene transcription. In accord with scanning linker mutant data which demonstrated that the LS7 mutation (disrupting the core region of the C1/C2 base pair) produced little repression of TA-inducible SULT2A-40/41 expression (Fig. 4), introduction of a 2-nt change (AC to CA) into the core region of C1 failed to suppress basal or TA-inducible reporter expression (Fig. 4B). These results were confirmed using the above-described ELISA (Fig. 6A). Also consistent with our functional data, competitor containing the mutated C2 sequence failed to compete (Fig. 6A). Also consistent with our functional data, competitor containing the LS6 mutant reporter failed to compete (Fig. 6A). These results were confirmed using the above-described ELISA (Fig. 6B).

During our linker scanning mutational analysis, we also found that disruption of the region between the C1 and C2 sites substantially inhibited promoter function, with transcription from the LS6 mutant being particularly low (Fig. 4). To determine a possible reason for this very low expression, we performed a computer-based transcription factor binding site analysis of this region (MatInspector; Genomatix, Inc., Cincinnati, OH) (Quandt et al., 1995). Sequences with similarity to an HNF4 matrix (Transfac matrix V$HNF4.01; core similarity 1.00, overall matrix similarity 0.827) or an AP-1 matrix (VSAP1FJ.01; core similarity 1.00, matrix similarity 0.880) were identified (Fig. 4; Table 2). Although suggestive of a possible functional role for one of these factors in the regulation of SULT2A-40/41 transcription, the core region of each of these factors is located within the LS5-mutated region, rather than the LS6-mutated region. Also, the
HNF4 site contained in the LS6 construct was predicted to be almost equivalent to the HNF4 binding site (i.e., matrix similarity 0.821) contained in the wild-type SULT2A-40/41 sequence (matrix similarity 0.827). Similarly, the AP-1 site in the LS6 mutant was predicted to be an even better AP-1 binding site (i.e., matrix similarity 0.897) than that contained in the wild-type gene (matrix similarity 0.880). We also noted that the LS6 mutation caused the introduction of a predicted binding site for cyclic AMP response element binding protein. Thus, the reason for the especially low expression in the LS6 mutant is presently unclear, and will require a systematic evaluation of the functional and binding properties of the candidate transcription factors. In this regard, we performed a preliminary evaluation of the ability of the AP-1 factor, c-Fos/c-Jun, to bind to the SULT2A-40/41 AP-1 site in vitro. Although some binding was detected, the affinity of c-Fos/c-Jun for this site was low when compared with that of a consensus AP-1 motif (data not shown).

An additional finding from our linker scanning mutagenesis analysis was that transcription from the LS8 construct was somewhat lower than that occurring from the constructs containing the neighboring mutations (Fig. 4). Computational analysis indicated the presence of an HNF3B binding site in this region (VSHNF3B0.01: core similarity 1.00, matrix similarity 1.00), whose core sequence was contained within the LS8-mutated region (Fig. 4; Table 2), raising the possibility that HNF3B also contributes to SULT2A-40/41 regulation.

### Discussion

Through a complex network of coordinated stimulatory and repressive effects on target gene expression, hetero- and homodimeric partnerships between the multiple C/EBP transcription factor family members orchestrate the development of normal differentiated cell function in a variety of tissues and cell types (Lekstrom-Himes and Xanthopoulos, 1998). The genes for six classes of C/EBP transcription factors have been identified, including C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ (Lekstrom-Himes and Xanthopoulos, 1998; Ramji and Foka, 2002). Of these, C/EBPα, C/EBPβ, and C/EBPδ are the principal C/EBP's that are expressed in the liver (Lekstrom-Himes and Xanthopoulos, 1998). The C/EBP transcription factors are members of the basic leucine zipper group of transcription factors that contain a conserved basic leucine zipper dimerization domain in association with a DNA-binding domain (Ramji and Foka, 2002). The disruption of C/EBPα in knockout mouse models has profound effects on liver glycogen storage and produces severe abnormalities in liver acinar structure and function (Wang et al., 1995; Flodby et al., 1996; Lekstrom-Himes and Xanthopoulos, 1998). C/EBPβ is constitutively expressed in hepatocytes, and its loss in knockout mice is deleterious to the mobilization of critical hepatic glycogen stores, as well as to survival of the animals (Lekstrom-Himes and Xanthopoulos, 1998; Croniger et al., 2001). The C/EBP family of transcription factors plays an important role in the regulation of target genes that control hepatocellular energy metabolism (Roesler, 2001). It follows, therefore, that the determinants of cellular energy homeostasis such as insulin, glucagon and glucocorticoids should figure prominently in the regulation of C/EBP expression (Matsumo et al., 1996; Lekstrom-Himes and Xanthopoulos, 1998; Ramji and Foka, 2002). Both C/EBPα and C/EBPβ have been reported to display inducible expression in response to glucocorticoid stimulation in hepatic cells (Matsumo et al., 1996; Rams et al., 1996; Crosson et al., 1997; Gotoh et al., 1997). For example, glucocorticoid-inducible C/EBPα has been shown to mediate steroid-inducible G1 cell cycle arrest in rat hepatoma cells (Rams et al., 1996), and glucocorticoid-inducible C/EBPβ expression in mouse hepatocytes is a prerequisite for the glucocorticoid-mediated activation of genes encoding members of the ornithine enzymatic cycle (Kimura et al., 2001).

Previous analysis of hepatic nuclear factors binding to the SULT2A-40/41 (alternative name Std) gene, and transient transfection evidence in hepatocellular and fibroblast cell lines, indicated that C/EBP and HNF1 liver-enriched transcription factors are capable of driving SULT2A-40/41 expression (Song et al., 1998). The results of the current analysis suggest that in primary cultured rat hepatocytes, GR-activating concentrations of glucocorticoid induce the expression of nuclear C/EBPα and C/EBPβ transcription factors, which secondarily transactivate SULT2A-40/41 gene transcription. Linker scanning and site-directed mutagenesis experiments indicated that a critical C/EBP-responsive element (C2) in the proximal SULT2A-40/41 5'-flanking region (Fig. 5) is required for the support of GR-inducible SULT2A-40/41 gene transcription. The observation that introduction of linker scanning or site-directed mutations disrupting the C2 site decreased both basal and TA-inducible levels of promoter activity is consistent with the conclusion that C/EBPs are essential for obtaining the basal level of gene transcription, and that the levels of these factors are increased following TA treatment. Thus, as shown in Figs. 1 and 2, C/EBPα and C/EBPβ are present in the nucleus of untreated hepatocytes, and their levels are increased following glucocorticoid treatment with a time course that precedes SULT2A-40/41 expression. This scenario is consistent with a “secondary” or indirect activation of GR-inducible SULT2A-40/41 transcription by either C/EBPα or C/EBPβ.

The C/EBPs are involved in the regulation of a number of genes in the hepatic energy metabolism network, and “bile acid sulfotransferase” or SULT2A-40/41 is numbered among them. Bile acids facilitate the absorption of lipids and fat-soluble vitamins, and the elimi-
nation of cholesterol (Holt, 1972). SULT2A enzymes modify the bile acid pool by sulfonating hydrophobic secondary bile acids, such as lithocholic acid (Kitada et al., 2003), as a protective measure against the development of intrahepatic cholestasis (Yousef et al., 1997; Kitada et al., 2003). Both C/EBPs and C/EBPβ function to orchestrate glucose homeostasis in the liver. Hepatic glucose utilization is strongly modulated in response to alterations in intrahepatic glucocorticoid levels (Williams et al., 2000), and the expression of 11β-hydroxysteroid dehydrogenase type I, an enzyme that catalyzes the interconversion of active and inactive glucocorticoid in the liver, is strongly activated by the C/EBPα transcription factor (Williams et al., 2000). Similarly, PEPCK represents the rate-limiting enzyme in the hepatic gluconeogenesis pathway (Rognstad, 1979). Like SULT2A-40/41, the activation of hepatic PEPCK expression by glucocorticoids involves a complex cis-acting glucocorticoid-responsive unit that consists of several accessory factor domains, two GREs, and a cAMP response element (Yamada et al., 1999). The achievement of full-strength glucocorticoid-inducible PEPCK expression is transactivated by the C/EBPβ transcription factor operating at a cis-acting cAMP response element in the 5′-flanking region of the PEPCK gene (Yamada et al., 1999).

Our analysis clearly supported the importance of C/EBPs and the C2 site in the regulation of basal and glucocorticoid-inducible SULT2A-40/41 gene transcription, but also suggested contributory roles for other liver-enriched transcription factors. As described above, a possible binding site for HNF4 was identified in the region between the C1 and C2 C/EBP sites. Also, a possible HNF3b binding site was identified that partially overlapped the C1 C/EBP site and extended just downstream, and our linker scanning mutational data suggested that this site, rather than the C1 C/EBP site, may contribute to basal and glucocorticoid-inducible SULT2A-40/41 transcription. In addition, although our computational analysis, using MatInspector, did not reveal the HNF1 site that was previously described by Song et al. (1998), a different HNF1 binding site was indicated (Fig. 4; Table 2; VSHNF1.01, core similarity 1.00, matrix similarity 0.810), which partially overlapped the C2 C/EBP site and extended just downstream. Transcription from LS4 (in which the HNF1 core sequence was disrupted) was somewhat greater than that from the LS2 mutant (in which the C2 C/EBP core sequence was disrupted), supporting the principal importance of C/EBPs in the regulation of SULT2A-40/41. Nevertheless, as supported by the previous study (Song et al., 1998), HNF1 likely contributes to the regulation of SULT2A-40/41 expression.

It thus appears likely that transcription of the SULT2A-40/41 gene is controlled by several liver-enriched transcription factors, anchored by the C/EBPs, that function as an integrating unit to control the basal and glucocorticoid-inducible hepatic expression of this sulfotransferase. Future mechanistic studies will focus on establishing the relative roles of the individual C/EBPs and C/EBPβ isoforms, as well as the additional transcription factors that function in glucocorticoid-inducible SULT2A-40/41 expression, and on establishing the functional interactions among them, for example, in coactivator-based transactivation. Information on the complex signaling events that regulate SULT2A-40/41 will provide added insights on the integrated role of SULT2A-40/41 in the hepatic energy metabolism cascade.

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