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Identification of a Novel Dexamethasone Responsive Enhancer in the Human *CYP3A5* Gene and Its Activation in Human and Rat Liver Cells

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SUMMARY

The human liver cytochromes P450 3A (CYP3As), orthologous to the rat glucocorticoid inducible forms, are composed of at least four differentially expressed members. To begin the study of the molecular events in the glucocorticoid regulation of *CYP3A5*, we fused 5′ sequences of *CYP3A5* to the chloramphenicol acetyltransferase gene in a vector that contains the herpes simplex virus thymidine kinase promoter. In HepG2 cells, the largest 5′ *CYP3A5* gene fragment (1.4 kb) suppressed the TK promoter. However, suppression was overcome by addition of 10 μM dexamethasone. A series of unidirectional deletions revealed a unique 219-bp fragment (−891 to −1109 bp upstream from the transcriptional start site) that conferred dexamethasone responsiveness on the TK promoter regardless of either the distance or orientation from the promoter and thus appears to be an enhancer. Nucleotide sequence analysis of this *CYP3A5* enhancer revealed no consensus 15-bp glucocorticoid responsive element (GRE) (GGTACANNNTGTTCT); however, two GRE "half-sites" (TGTTCT) were found separated by 160 bp. Although dexamethasone stimulated the *CYP3A5* enhancer only 3–4-fold in HepG2 cells, the *CYP3A5* enhancer was stimulated 7- and 12-fold in immortalized primary human hepatocytes and primary rat hepatocyte cultures, respectively. The glucocorticoid receptor (GCR) seems to be indispensable to this process because 1) dexamethasone induction can be blocked by the antiglucocorticoid RU-486, 2) dexamethasone-dependent transcriptional activation of the *CYP3A5* enhancer in HepG2 cells required cotransfection of an expression vector containing the intact GCR, yet 3) cotransfection with a plasmid that contains a mutation in the ligand binding domain of the GCR does not activate the *CYP3A5* enhancer in the presence of dexamethasone. To further localize the dexamethasone responsive region of the 219-bp *CYP3A5* enhancer, it was subdivided and fused to the TKCAT expression vector. Transfection analysis in HepG2 cells demonstrated that neither GRE half-site can independently confer dexamethasone responsiveness on the TK promoter. Block mutations of either of the two GRE half-sites or point mutations at specific GCR binding sites eliminates dexamethasone inducibility, demonstrating the half-sites need to interact. Electromobility shift assays indicate that the *CYP3A5* 5′-GRE half-site 1) specifically binds purified GCR, 2) can displace binding of the GCR to a consensus GRE, and 3) shifts a protein in HepG2 nuclear extracts that is supershifted by GCR antibody, demonstrating that this enhancer is an authentic GRE. This is the first study to demonstrate that a member of the human *CYP3A* gene family contains an enhancer that binds the GCR and that this binding is critical to transcriptional activation by dexamethasone.

P450s are a family of hemoproteins[1] responsible for the oxidation of drugs and environmental contaminants and many endogenous compounds. More than 60 distinct forms of these enzymes (each encoded by separate genes) have been purified from rat and human liver (2), with many of these expressed simultaneously in a given tissue, such as the liver. Because the rates of metabolism of individual substrates may depend on the amounts and kinds of P450s expressed in a tissue, there is a growing belief that human variation in biological response to drugs and other chemicals is dictated by the profile of expression of P450s in each person (3, 4).

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[1] The glucocorticoid-inducible cytochrome P450s (CYP3As) and other cytochromes P450 (P450 or CYP450) are designated according to recommended nomenclature (1): *CYP3A* (italicized), the cytochrome P450 3A gene family; and CYP3A (nonitalicized), the gene products (mRNA or proteins).

ABBREVIATIONS: CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumor virus; GCR, glucocorticoid receptor; GRE, glucocorticoid responsive element (GGTACANNNTGTTCT); PCR, polymerase chain reaction; bp, base-pair(s); kb, kilobase(s); PCN, 16α-pregnenolone carbonitrile; TAT, tyrosine aminotransferase.

Among the most prominent form of human P450 found in human liver is the CYP3A family, which is composed of at least four members: CYP3A3 (HLp) (5), CYP3A4 (NF) (6), CYP3A5 (HLp2) (7, 8), and CYP3A7 (HFLA) (9, 10). These enzymes catalyze a remarkable number of oxidation reactions of clinically important drugs (e.g., quinidine, warfarin, erythromycin, cyclosporin A, midazolam, lidocaine, nifedipine, dapsone), environmental toxins (e.g., aldrin, hexachlorinated biphenyls, aflatoxins B1 and G1, benzo[*a*]pyrene-7,8-diol), and endogenous substances (e.g., cortisol, progesterone, testosterone, and dehydroepiandrosterone-sulfate) (3, 11–13). CYP3A3 and CYP3A4 differ by only 11 of 503 amino acids (6, 14) and have almost identical catalytic activities, whereas CYP3A5 (which is 83% homologous to CYP3A3 (5, 7)) metabolizes nifedipine as does CYP3A4 but is less active in the catalysis of 6β-hydroxylation of progesterone and androstendione and catalyzes only the M1 hydroxylation of cyclosporin A (8). A form isolated from fetal liver, CYP3A7 may preferentially metabolize dehydroepiandrosterone-sulfate (9). Although CYP3A4 is expressed in all postnatal livers, CYP3A5 is expressed polymorphically, being detected in only ~25% of adult livers (7, 8, 15, 16). CYP3A7 also appears to be expressed polymorphically, being found in all fetal livers but in only a small proportion (25%) of adult livers (not necessarily those expressing CYP3A5) (17). Thus, variations in the rate of metabolism of CYP3A substrates may arise because of interindividual differences in the amounts of each of these family members expressed in a given liver. We have previously characterized two cDNA clones called *CYP3A3* (HLp) and *CYP3A5* (HLp2) (5, 7). As a first step toward understanding the mechanisms of *CYP3A* regulation, we isolated a cloned segment of the human genome that contained the 5′ sequence of *CYP3A5* (7).[2] This sequence was recently published (18). In this report, we use a series of deletions of the *CYP3A5* 5′ sequence fused to a thymidine kinase promoter to identify a unique dexamethasone responsive element. Further studies revealed that transcription from the *CYP3A5* promoter can only be detected when fused with 5′ sequences that contain the dexamethasone responsive enhancer. Furthermore, this novel glucocorticoid responsive enhancer functions in the HepG2 cells as well as primary cultures of rat hepatocytes and immortalized human hepatocytes.

Materials and Methods

The human hepatoblastoma cell line, HepG2, was obtained from American Tissue Culture Collection and maintained in α-minimal essential media (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum. Restriction enzymes were obtained from Life Technologies. The M13 and pGEM cloning vectors were obtained from Pharmacia (Piscataway, NJ) and Promega (Madison, WI), respectively. The chloramphenicol acetyltransferase expression vector pBLCAT2 (also known as TKCAT and containing the thymidine kinase promoter) (19) and the TAT-CAT (20) were kindly provided by Dr. Gunther Schutz (Institut fur Zell und Tumor Biologie, Heidelberg, Germany). The pSV0-CAT and pRSV-CAT (21) were obtained from Dr. Bruce Howard (National Institutes of Health, Bethesda, MD). The GCR expression vector pRShGRα (22) and the mutant GCR expression vector, I582 (23) were kindly provided by Dr. Ron Evans (Howard Hughes Medical Institute, Salk Institute for Biological Studies, La Jolla, CA). The recombinant human GCR purified from baculovirus, anti-GCR anti-sera, and the consensus tyrosine aminotransferase GRE were obtained from Affinity Bioreagents (Neshanic Station, NJ). Oct-1 and Tf2D consensus oligonucleotides were from Promega (Madison, WI). *CYP3A5* oligonucleotides were synthesized by the Center for Biotechnology core facility at St. Jude Children's Research Hospital.

For identification of the human *CYP3A5* gene, a cDNA fragment from human *CYP3A3* (bp 0–170 (5)) was used to screen an EMBL-3 human genomic library to isolate the structural gene encoding *CYP3A5* that has been previously described (7).

DNA sequence analysis. Selected *CYP3A5* 5′ sequences were subcloned into TKCAT, and each insert was characterized by double-stranded DNA sequence analysis using either the modified T7 polymerase or *Taq* polymerase primed with oligonucleotides radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The GCG computer software package was used to perform DNA sequence analysis (24).

Construction of chimeric plasmids. Chimeric gene constructions of *CYP3A5* were made according to standard recombinant DNA techniques (25). *Hin*dIII linkers were attached to the 2.8-kb *Sal*I/*Eco*RI fragment, and it was subcloned into M13. Next, unidirectional deletions were performed to generate a fragment that contained +17 bp 3′ and −1,442 bp 5′ of the transcriptional start site. This fragment was cloned into the *Hin*dIII site of TKCAT to test for a dexamethasone responsive enhancer in the *CYP3A5* gene. For construction of internal deletions the genomic clone was digested from a unique internal *Bst*eII site (at position −618 bp relative to the cap site)[2] and *Bal*31 for varying intervals. The ends were filled in with the Klenow fragment of *Pol*I and ligated with T4 ligase into TKCAT. A small *CYP3A5 Pst*I fragment (bp −298 to −1109) was subcloned into pGEM4z to generate unidirectional deletions with Exonuclease III. These deletions were subcloned into the TKCAT vector (19), and the constructions were verified by DNA restriction mapping and DNA sequencing as described above. Construct TKB/X was generated by using *Pst*I and *Sau*3AI restriction enzymes that cut at the following positions (−1109 bp, −1034 bp). The 76-bp fragment was first subcloned into pGEM7(z) followed by subcloning into the *Bam*HI/*Xba*I site in TKCAT. All plasmids used in transfections were prepared using Qiagen plasmid preparation kits (Qiagen Corp, Chatsworth, CA).

PCR-generated deletion and mutant constructs. Additional deletion and mutant constructs were developed by the use of synthetic oligonucleotide primers and PCR amplification using the *CYP3A5* 1.4-kb insert as template. The nucleotide sequence of all PCR generated plasmids was confirmed by DNA sequence analysis. The nucleotide sequence of the primers were as follows: oligonucleotide −1109, 5′-GCAGGTCATTATGTTAGGTAAAAT-3′, which represents the 5′ extent of the PCR constructs; oligonucleotide −1050, 5′-CACTTATTTGTGGG-3′, which will remove the 5′ most GRE half-site (TGTTCT); oligonucleotide −986, 5′-TACCCTAAGTACAGGGAGCACAGC-3′, which will remove the "CAAT" boxes; oligonucleotide −898, 5′-CCAACCACCCTGATG-3′, which removes the proximal GRE half-site; oligonucleotide −891, 5′-AGAACACCCAACCACCCT-3′, which represents the 3′ extent of the PCR constructs; oligonucleotide −1089 wt, 5′-AAATAAGCCAGGCACACAAAGACA-3′; oligonucleotide −1089 mt; 5′-AAATAAGCCAGGCACACAAAGACAGACATTGCAT *A*TTCT-3′, the italicized base has been changed from a G to an A; oligonucleotide −891 wt, 5′-AGAACACCCAACCACCCT-3′; and oligonucleotide −891 mt, 5′-AGAA *T*ACCCAACCACCCT-3′, the italicized base has been changed from a C to a T.

Cell culture and transfections. HepG2 cells were subcultured by trypsinization, and 3–4 × 10^5 cells were plated per 60-mm tissue culture dish. When cultures attained 40–60% confluence, cells were transfected by standard calcium phosphate coprecipitation using 10 μg of purified test plasmid DNA and, in some cases, 500 ng of wild-type (pRShGRα) or mutated (I582) GCR expression plasmid. After an 18-hr transfection, the cells were treated for 36 hr with dexamethasone, and cells were harvested for CAT assay as described (26).

Primary rat hepatocytes were prepared, cultured, and transfected with lipofectin as previously described (26). Cultures of human hepa-

[2] The nucleic acid sequence for the 5′ sequence of *CYP3A5* in this article was submitted by J.D.S. to GenBank in 1993 under Accession Number L35912.

tocytes were prepared (27), immortalized clones were generated by stable transfection of the SV40 large T-antigen gene (28), and the clones transfected by calcium phosphate coprecipitation.

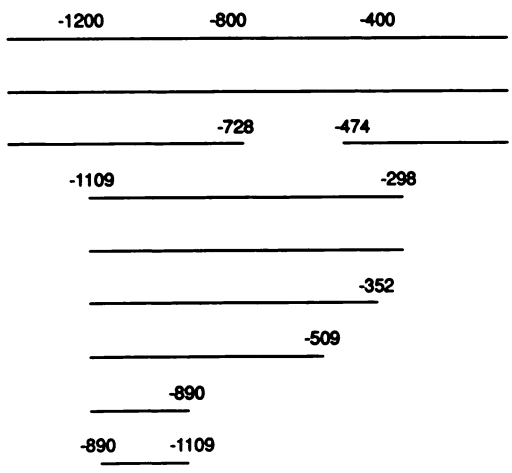
CAT assays. Cells were scraped from 60-mm dishes after washing once with phosphate-buffered saline (80 mM Na_2HPO_4, 20 mM KH_2PO_4, 100 mM NaCl, pH 7.4) and a brief incubation in a harvest buffer (150 mM NaCl, 40 mM Tris·HCl, pH, 7.4, 5 mM EDTA) as described (26). Cellular CAT activity in 60 μg of cell extract was assayed exactly as described (26, 29), except that cell extracts HepG2 cells were not heat-inactivated before CAT assay. The assays were carried out for 2 hr. The data were calculated as relative CAT activity (after subtraction of background activity obtained from mock transfected control dishes) as described in the figure legends for the individual experiments.

Electromobility shift assays. Nuclear extracts were prepared from HepG2 cells grown in 10% serum as described (30). These extracts contain GCR with GRE binding activity because sufficient endogenous glucocorticoids are present in this serum. The *CYP3A5* enhancer probe was gel purified by Qiaex (Qiagen, Chatsworth, CA) and digested with *Sau*3A1, and the fragment representing the distal GRE half-site, bp −1109 to −1034, isolated. All probes were end labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The assays were performed with probe added last to a reaction (20 μl final volume) containing poly(dIdC) (2 μg/reaction), 5 mM $MgCl_2$, 30 mM KCl, 12% glycerol, and various amounts of HepG2 nuclear extract or purified recombinant GCR. This salt concentration and poly(dIdC) disrupt any nonspecific interactions with the GCR (31). In some assays, double-stranded oligonucleotides in molar excess were added with the probe and served as competitor. In all assays, samples were electrophoresed in 4% nondenaturing polyacrylamide gels (Protogel, National Diagnostics, Atlanta, GA) (prerun for 1 hr) in 0.25× Tris/borate/EDTA buffer at 200 V and constant current. All gels were dried and exposed to autoradiographic film. The intensity of autoradiographic signals was determined by densitometry.

Results

Localization of the dexamethasone responsive region of the *CYP3A5* gene. Because the *CYP3A* gene family has been reported to be regulated by glucocorticoids (5, 32, 33) and seven sequences related to the GRE half-site (TGTTCY, Ref. 34) are distributed over the 1.4-kb 5′ sequence, we evaluated whether this fragment could confer glucocorticoid responsiveness on a heterologous promoter. We first determined that human hepatoblastoma HepG2 cells are good recipient cells for determining a gene's glucocorticoid responsiveness. HepG2 cells were transfected with the MMTV-CAT construct, a gene that responds robustly to glucocorticoids, because of the four GREs in its 5′ sequence (35) as well as other sequences (e.g., NF-1, Octamer factor) that can enhance GCR-mediated transcriptional activation (36). Because replicating cells like HepG2 are frequently deficient in the amount of *trans*-acting factors (37), such as the GCR, we first determined the optimal amount of exogenous GCR necessary for maximal steroid activation of MMTV-CAT in these cells. Cotransfection of 100–500 ng of GCR expression vector dose-dependently activated MMTV-CAT, although further increases in GCR failed to further enhance MMTV-CAT *trans*-activation (not shown). We further determined that 500 ng of GCR was optimal regardless of the amount of MMTV-CAT or dose of dexamethasone added (not shown). Therefore, the HepG2 cells were routinely cotransfected with 500 ng of GCR expression vector.

Because computer-assisted analysis of the *CYP3A5* 5′ sequence did not identify a consensus GRE (GGTACANNNTGTTCT), we performed a series of unidirectional and internal deletions on *CYP3A5* (Fig. 1). One plasmid that contained an internal deletion from CYP3A5-CAT (Exo1-TKCAT) conferred dexamethasone responsiveness on the TK promoter (Fig. 1). This construct contained an internal deletion of 254 bp (from −474 to −728 bp). To further pinpoint the dexamethasone responsive region, in the next studies we used DNA constructs modified by unidirectional deletions of *CYP3A5* with Exonuclease III. We performed unidirectional



CONSTRUCTION	CAT ACTIVITY [a]NO DEX	CAT ACTIVITY DEX	FOLD INCREASE BY DEX
CYP3A5-TKCAT	44	113	2.6
R-CYP3A5-TKCAT	[b]0	0	[c]ND
ExoΔ1-CYP3A5-TKCAT	144	306	2.1
809-CYP3A5-TKCAT	51	81	1.6
R-809-CYP3A5-TKCAT	0	0	ND
Δ4-CYP3A5-TKCAT	64	191	3.0
Δ17-CYP3A5-TKCAT	184	250	1.4
Δ26-CYP3A5-TKCAT	85	340	4.0
R-Δ26-CYP3A5-TKCAT	80	180	2.3

Fig. 1. Dexamethasone responsiveness of the human CYP3A5-TKCAT gene constructs in transient transfections of HepG2 cells. The *CYP3A5* gene 5′-flanking sequences from bases −1412 bp to +17 bp relative to the transcription start site was ligated into a *Hin*dIII linearized TKCAT plasmid. Deletions within the chimeric gene CYP3A5-TKCAT were constructed as described in Materials and Methods. The resulting clones and the positions of the deletion are indicated. HepG2 cells were transfected with the CYP3A5-TKCAT constructs, treated with 10 μM dexamethasone, and harvested, and cellular CAT activity was measured. *a*, All CAT activity values represent the average of five or six separate determinations and are expressed as a percentage of the CAT activity in TKCAT transfected cultures, which is given a value of 100. These CAT assays were performed in duplicate and varied by no more than 17% between samples; *b*, zero CAT activity; *c*, no difference.

3′ to 5′ deletions (see Materials and Methods) on an 811-bp *PstI* fragment that spanned a region of the *CYP3A5* gene from −298 bp to − 1109 bp. The deletion fragments were subcloned into TKCAT, and dexamethasone responsiveness was evaluated (Fig. 1). The reverse orientation of this 811-bp fragment (R-809-CYP3A5-CAT) suppressed activity of the TKCAT promoter; however, the forward orientation (809-CYP3A5-CAT) retained dexamethasone responsiveness. This 809-bp fragment was "cut" back, and a minimal dexamethasone responsive fragment of 219 bp (bp −891 to −1109) was identified. This fragment conferred dexamethasone responsiveness, regardless of orientation, on the TKCAT promoter (Fig. 1). Thus, the 219-bp fragment in the 5′ flanking sequence of *CYP3A5* appears to be a novel dexamethasone responsive enhancer because it can confer dexamethasone responsiveness on a heterologous promoter regardless of the orientation.

Does *CYP3A5* require the upstream dexamethasone responsive region for basal promoter activity? To determine if the *CYP3A5* dexamethasone responsive enhancer can confer dexamethasone responsiveness on its own promoter, we subcloned different subfragments of the *CYP3A5* 5′-flanking sequence into a promoterless CAT vector (pSV0-CAT) (21) (Fig. 2). Neither the *CYP3A5* promoter fragment (bp −266 to +17) nor the 1.4-kb 5′ sequence of the *CYP3A5* gene were transcriptionally active in either the presence or absence of dexamethasone. However, by fusing *CYP3A5* 5′ sequences from −728 bp to −1.4 kb with the *CYP3A5* promoter (−266 to +17 bp), both basal activity and dexamethasone responsiveness were restored. These studies demonstrate that in the absence of the appropriate upstream sequence, transcription from the *CYP3A5* promoter in CAT reporter vectors is undetectable.

The GCR is required for activation of the *CYP3A5* enhancer. To determine if the dexamethasone responsiveness of the 219-bp *CYP3A5* enhancer was dependent on the GCR and dexamethasone, the plasmid Δ26-CYP3A5-TKCAT was cotransfected with a human GCR expression vector or a mutant GCR (I582) that does not bind glucocorticoid (Fig. 3). In the absence of dexamethasone, neither the wild-type GCR nor the mutant GCR I582 activates the *CYP3A5* enhancer. This GCR mutant (I582) was selected because, unlike other receptor mutants, it does not have either a constitutive transcriptional activation or repressor function, yet it retains the DNA binding domain (23). On dexamethasone addition, only the intact wild-type GCR increased CYP3A5-TKCAT activity (4-fold), whereas the mutant GCR produced no effect. These studies demonstrate that dexamethasone's transcriptional activation of the *CYP3A5* dexamethasone responsive enhancer requires ligand-activated GCR.

***CYP3A5* enhancer is dexamethasone responsive in primary rat and human hepatocytes.** In contrast to HepG2 cells, which are derived from a human hepatoblastoma, primary human hepatocytes provide the most immediate approximation of normal human liver. To have a renewable resource of normal human hepatocytes, we previously immortalized human hepatocytes by stable transfection of the SV40 large T-antigen gene and generated clones 4A, 5A, and 6A. Dexamethasone readily activated Δ26-CYP3A5-TK-CAT (Table 1) transiently transfected into these human hepatocytes.

In previous studies,we established that the primary rat hepatocytes have sufficient endogenous GCR to *trans*-activate a vector containing glucocorticoid responsive sequences, including MMTV and the rat *CYP3A1* gene (26). To determine whether the human *CYP3A5* gene is dexamethasone inducible in a heterologous rat liver cell, primary cultures of rat hepatocytes were transfected with the Δ26-CYP3A5-TK-CAT plasmid (Table 2). Dexamethasone treatment readily activated the *CYP3A5* enhancer, although a brief preincubation with the antiglucocorticoid RU-486 blocked dexamethasone activation. Thus, in a heterologous hepatocyte, the human *CYP3A5* enhancer responds dose-dependently to

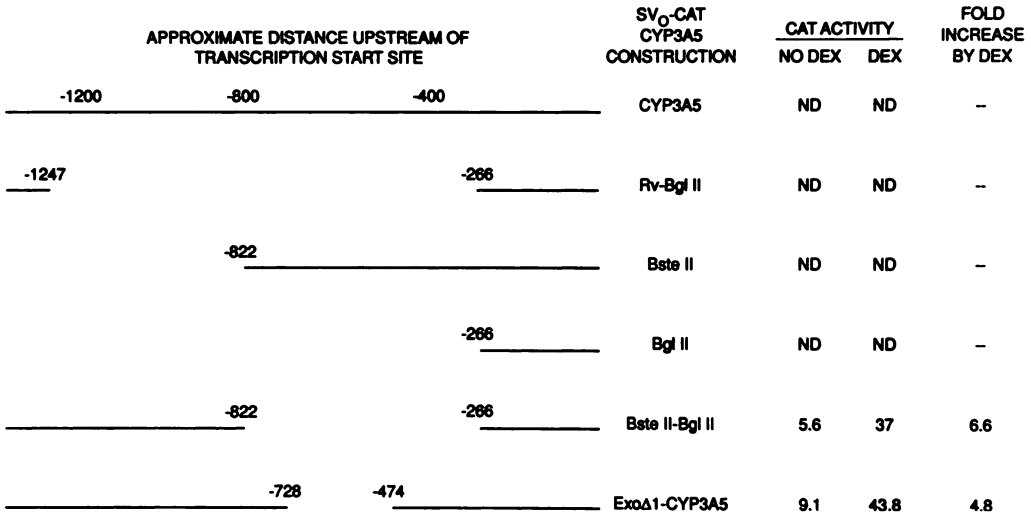


Fig. 2. Dexamethasone responsiveness of the human *CYP3A5* promoter. The *CYP3A5* 5′-flanking sequence (−1412 bp to +17 bp) was ligated into the promoterless vector pSV0-CAT at the *Hin*dIII site. Subsequent deletions were created, as indicated, by the use of convenient restriction sites (*Bgl*II, *Eco*RV, or *Bst*eII). Other deletions were created as described in Materials and Methods. HepG2 cells were cotransfected with these *CYP3A5* plasmids (10 μg) and the GCR expression vector (pRShGRα) (500 ng). Cells were treated with 10 μM dexamethasone, and CAT activity was measured. The CAT activity value represents the average of at least three separate determinations. The values shown are determined as follows: [(CAT dpm/μg protein/hr) minus CAT activity in mock transfected cells]/1000. ND, no detectable CAT activity. The promoterless vector pSV0-CAT had no detectable CAT activity.

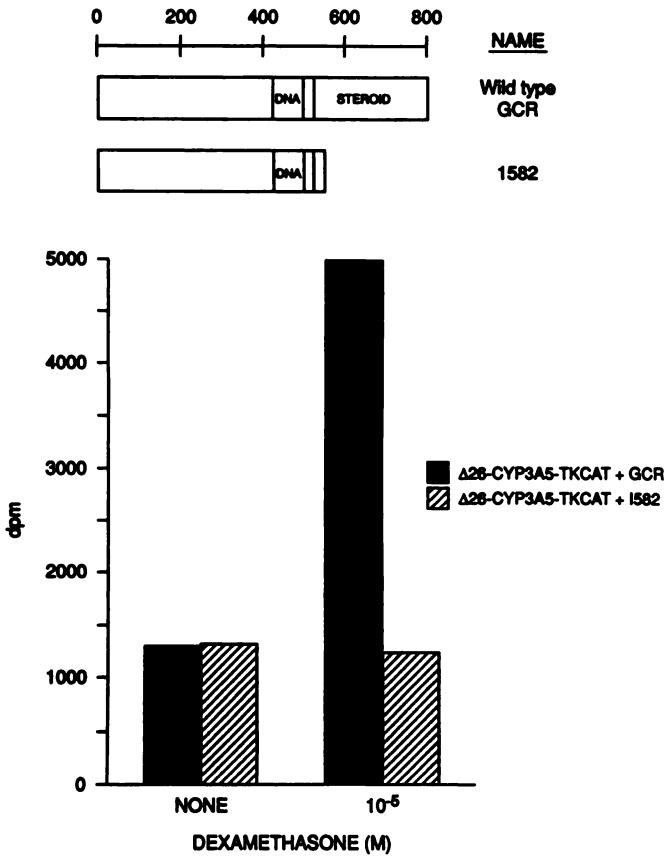


Fig. 3. Determination of the role of intact GCR in *trans*-activation of the *CYP3A5* GRE. HepG2 cells were transfected with the CYP3A5-TKCAT construction (bp −891 to −1109) (10 μg) in either the presence or absence of 500 ng of either the functional GCR expression vector or the GCR expression vector with an insertion mutation in the ligand binding domain (1582) (23). Some cells were treated with 10 μm dexamethasone, followed by harvest and assay for CAT activity. The results are representative of three separate experiments with similar results.

TABLE 1

Immortalized human hepatocytes

Human hepatocyte clone	pRSV-CAT activity	Δ26-TK-CAT activity	
		Untreated	10 μM dexamethasone
4A	31,012	4,662	27,921 (5.99)
5A	50,586	9,062	34,099 (3.76)
6A	46,291	6,974	47,325 (6.79)

Numbers in parentheses indicate fold-increase over untreated control.

TABLE 2

The human *CYP3A5* 219-bp glucocorticoid enhancer responds to dexamethasone in primary rat hepatocyte cultures and this induction is blocked by RU-486

Dose of dexamethasone	Fold-increase over control[a]	+RU-486
log molar		
Untreated control	1.0 ± 0.2	0.75[b]
−7	9.74 ± 6.75	0.26
−6	12.95 ± 8.1	0.23

[a] Average value ± range of two separate experimental determinations.
[b] Relative to untreated control cultures.

dexamethasone, and this activation is readily blocked by RU-486 treatment.

Because we have previously shown that the *CYP3A1* gene contains a glucocorticoid and PCN responsive enhancer (26), we tested whether the *CYP3A5* enhancer was responsive to PCN in primary rat hepatocytes. Despite activation by dexamethasone, the *CYP3A5* enhancer was not transcriptionally activated by PCN (not shown).

Comparison of MMTV and *CYP3A5* dexamethasone dose response. Our previous studies in rat liver and human hepatocytes have shown that the maximum glucocorticoid induction of the *CYP3A* genes requires pharmacological amounts of dexamethasone to achieve transcriptional activation (32). We compared in HepG2 cells the dexamethasone responsiveness of the *CYP3A5* gene enhancer (Δ26-CYP3A5-CAT) with that of the MMTV enhancer, a gene that is potently induced by glucocorticoids (Fig. 4). Dexamethasone readily activated MMTV-CAT and achieved a >8-fold induction at 0.1 μM. In contrast, the *CYP3A5* enhancer (forward orientation) was maximally induced 4-fold at 10 μM dexamethasone. The reverse orientation *CYP3A5* enhancer was slightly less responsive to dexamethasone (2.5-fold). Thus, Although the *CYP3A5* dexamethasone responsive enhancer is dose-dependently activated by dexamethasone; however, it is not as responsive to glucocorticoids as the MMTV enhancer, requiring >100-fold higher amounts of dexamethasone to achieve maximal activation.

Electromobility shift assays. Gel retardation studies were performed to determine whether the GCR interacts with the glucocorticoid responsive *CYP3A5* enhancer. Addition of recombinant GCR to a consensus GRE from the TAT gene produced a GRE complex consisting of a major band (faster migrating) and a minor band (slower migrating) (Fig. 5A). Binding of both bands could be dose-dependently suppressed by unlabeled TAT GRE at 50- and 100-fold molar excess. In contrast, oligonucleotides for the consensus Oct-1

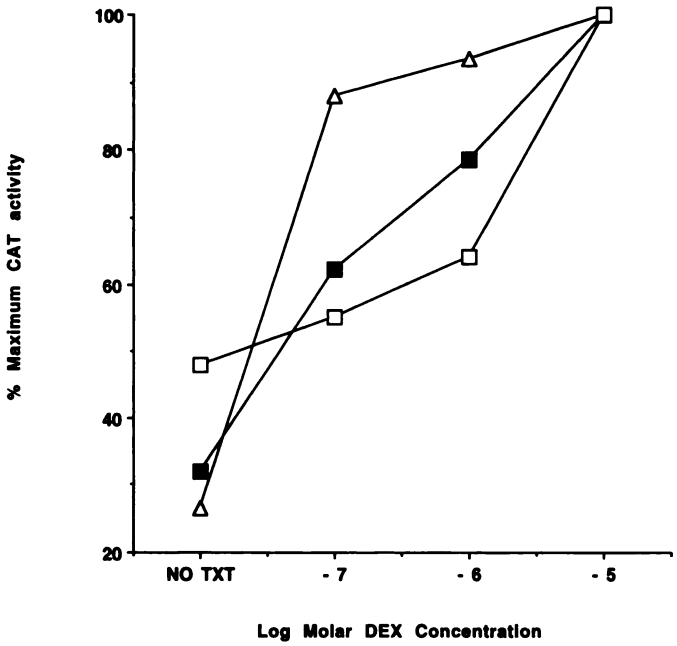


Fig. 4. Comparison of the dexamethasone dose-response of the *CYP3A5* gene enhancer and the MMTV enhancer. HepG2 cells were transfected with MMTV-CAT (△) or with the Δ26-CYP3A5-TKCAT construction (bp−891 to −1109) in the forward (■) or reverse orientation (□). After transfection, cells were treated with dexamethasone (100 nM to 10 μM) for 20 hr, harvested, and assayed for CAT activity. Each point represents the mean of three separate determinations.

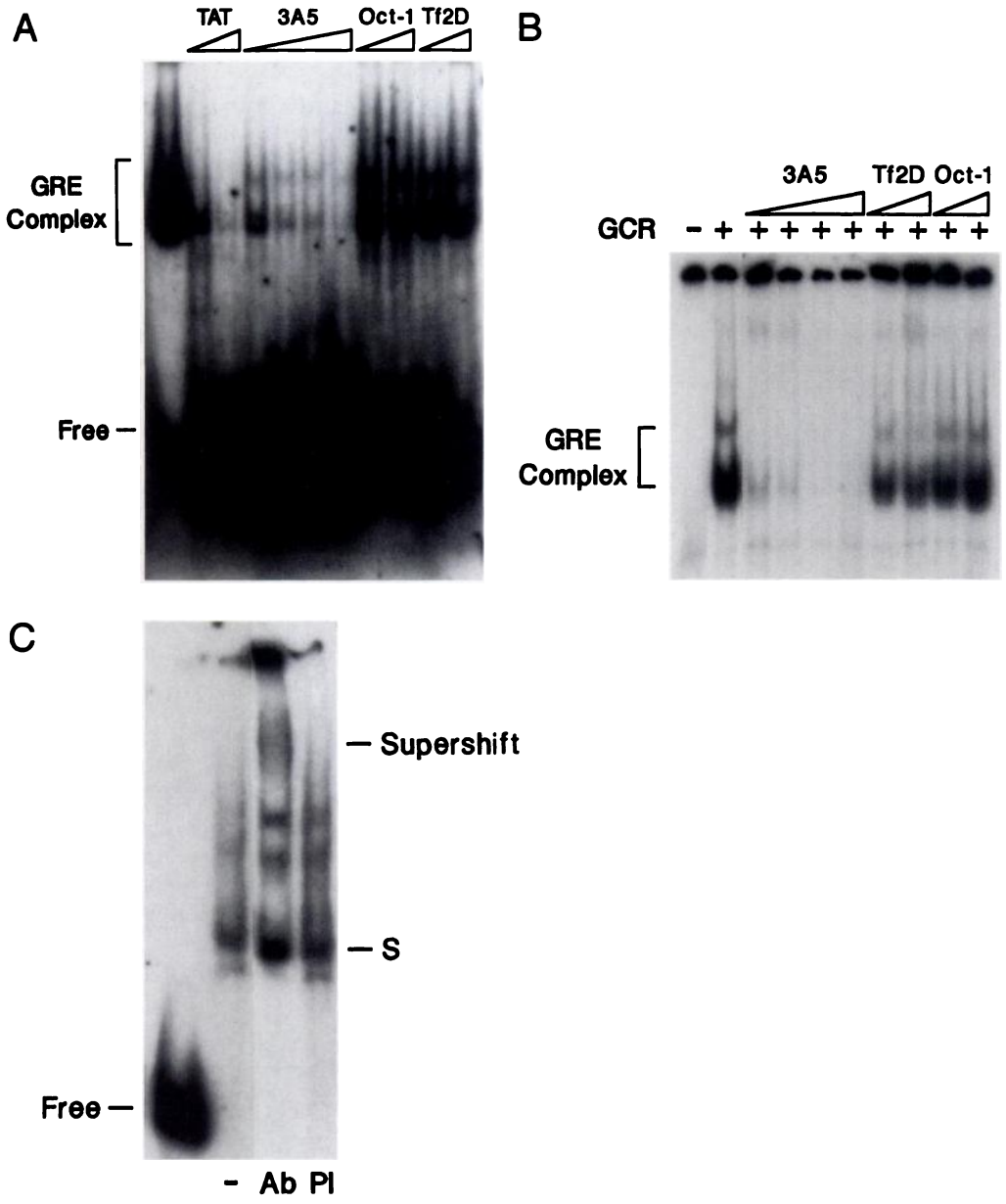


Fig. 5. Interaction of the GCR with the *CYP3A5* enhancer. A, The band shift complex of a consensus TAT GRE probe with purified recombinant GCR. *First lane*, TAT GRE in the presence of purified GCR and the GCR-GRE complex (two bands); *other lanes*, presence of increasing amounts of competitor, either the TAT GRE (50- and 100-fold molar excess), the *3A5* distal GRE half-site (3.6- to 17.5-fold molar excess), or Oct-1 or Tf2D oligonucleotides (29- to 62-fold molar excess). B, Specific binding of the distal *CYP3A5* GRE half-site to purified GCR. *Lane* −, the DNA probe in the absence of GCR protein; *lanes* +, presence of purified GCR. In some lanes, increasing amounts of *CYP3A5* distal GRE half-site (10–50-fold molar excess), Tf2D, or Oct-1 (86–182-fold molar excess). The specific GCR-GRE complex is indicated. C, HepG2 nuclear extracts were incubated with the distal *CYP3A5* GRE half-site in the absence of antibody (−). The major band of the GRE double complex is indicated (*S*). Addition of anti-GCR antibody (*Ab*) but not preimmune serum (*PI*) resulted in a unique supershifted band.

and Tf2D site did not produce suppression of GCR binding to the TAT GRE, even at 58- to 62-fold molar excess. In contrast, molar excess (3.6- to 17.5-fold) of the *CYP3A5* 5′ distal GRE half-site readily and potently displaced the GCR from the TAT GRE (Fig. 5A).

In a parallel study, the end-labeled *CYP3A5* 5′ distal GRE half-site was incubated with the GCR, and the specificity of this binding was assessed by competition analysis (Fig. 5B). Similar to the results in Fig. 5A, the *CYP3A5* GRE half-site incubated with purified GCR produced a GRE complex consisting of a major and a minor band (Fig. 5B). Addition of molar excess (86- to 182-fold) of Tf-2D or Oct-1 did not suppress *CYP3A5* binding to the GCR. However, addition of 10- to 50-fold molar excess of unlabeled *CYP3A5* 5′ distal GRE half-site readily and dose-dependently displaced the *CYP3A5* GRE from the GCR, further demonstrating that the *CYP3A5* enhancer can bind authentic GCR. In additional studies, we found the *CYP3A5* 5′ distal GRE half-site binds a GRE displaceable protein complex in HepG2 nuclear extracts and that this band shift comigrates with GRE complex produced by this fragment and the purified GCR (not shown).

We assessed the immunological identity of the proteins in the band shift complex produced with the HepG2 nuclear extracts and the *CYP3A5* distal GRE half-site (−1109 to −1034 bp) by incubation with polyclonal anti-sera directed against the GCR (Fig. 5C). Addition of preimmune serum appears to produce an additional band (nonspecific). However, addition of anti-GCR antibody produced not only the nonspecific band but also a much larger "supershifted" complex with much of the supershifted complex barely migrating from the well. Thus, the band shift produced by the *CYP3A5* enhancer fragment and HepG2 nuclear extract represents the interaction between the *CYP3A5* enhancer and a nuclear protein indistinguishable from the authentic GCR.

Dissection of the *CYP3A5* enhancer. The *CYP3A5* 219-bp enhancer is glucocorticoid-inducible (Fig. 4, Tables 1 and 2) and requires a ligand-activated GCR (Fig. 3). Because gel shift analysis indicated that the GCR binds both the

5′-GRE half-site of the *CYP3A5* 219-bp enhancer fragment (Fig. 5) and the 3′ GRE half-site (not shown), the 219-bp *CYP3A5* enhancer was computer analyzed for putative regulatory sequences that might participate in glucocorticoid regulation of the *CYP3A5* gene (Fig. 6). The regulatory sequences identified included two GRE half-sites (*TGTTCT*) and a number of DNA binding sites that have been shown to enhance the transcriptional response of consensus GREs, two CAAT boxes and a region 75% homologous with an octamer binding site. Although it is well known that these coactivators participate in the transcriptional regulation of a GRE, it is unknown if these sequences might be important in transcriptional regulation from GRE half-sites. To test the possibility that 1) the GRE half-sites were functional when separated into independent 3′ and 5′ halves and that 2) adjacent sequences might allow these half-sites to function independently, specific oligonucleotides were designed and PCR was used (see Materials and Methods) to generate deletions of the putative regulatory sequences in the *CYP3A5* enhancer (Fig. 6, Table 3). These deletions were then fused to the TKCAT expression vector and transfected into HepG2 cells, and the response of these deletions was measured against the full *CYP3A5* enhancer (Δ26CYP3A5-TK-CAT) (Fig. 7). Neither TKB/X (Table 3, Fig. 7), which contains only the distal GRE half-site (located between bp −1051 and −1056), nor FWD 199, which contains only the proximal GRE half-site (located between bp −891 and −896), was responsive to the addition of dexamethasone. Appending additional upstream sequences (octamer and CAAT box sites) to the proximal GRE half-site to generate plasmid 199 did not restore dexamethasone responsiveness. The plasmid vector, which contained neither a distal nor a proximal GRE half-site (designated 196) but only internal DNA sequences from the 219-bp CYP3A5 enhancer, was also unresponsive to dexamethasone. Thus, neither the 5′ nor 3′ half-site or sequences internal to the GRE half-sites are sufficient to independently confer glucocorticoid responsiveness.

Because neither of the *CYP3A5* GCR half-sites could function independently (Fig. 7), it suggested that these half-sites interact, especially because both of these sites appear to bind the GCR, perhaps similar to what has been reported for some EREs (30). To specifically test the role of the half-sites in the activation of the *CYP3A5* glucocorticoid responsive enhancer, we developed a series of site-directed half-site mutants (we substituted a G for an A in the TGTTCT GRE half-site of these mutants because the G residue is an essential site for hydrogen bonding between the GRE and the zinc-fingers of the GCR; Ref. 38). These mutants were fused to the TKCAT reporter plasmid. We also tested the idea that an adjacent nonconsensus GRE half-site on the opposite strand, TGACCT (bp −1107 to −1102) (31), might be important in the function of the *CYP3A5* enhancer. This was done by removing 20 bp from the 5′-end of the *CYP3A5* enhancer (bp −1109 to −1090; see Materials and Methods). To test for interaction between the half-sites, we individually mutated either the distal 5′ or proximal 3′ half-site. In addition, double mutants were created that contained both 5′ and 3′ half-site mutations. In most cases, because some orientation dependence was shown (Figs. 1 and 4), we cloned these fragments in either orientation. Site-directed mutations of either the 5′ or 3′ or both 5′ and 3′ half-sites eliminated the glucocorticoid-dependent induction of the TK promoter by the *CYP3A5* enhancer (Table 4). It is of interest to note that removal of the

TABLE 3
Preparation of constructs subdividing the *CYP3A5* dexamethasone responsive enhancer

TKCAT plasmid	Nucleotides	Size	Distal GRE	Octamer factor	CAAT box	Proximal GRE
		bp				
Δ26-CYP3A5	−1109/−891	219	+	+	+	+
TKB/X	−1109/−1034	76	+	−	−	−
196	−1050/−898	153	−	+	+	−
199	−1050/−891	160	−	+	+	+
FWD 199	−986/−891	97	−	−	−	+

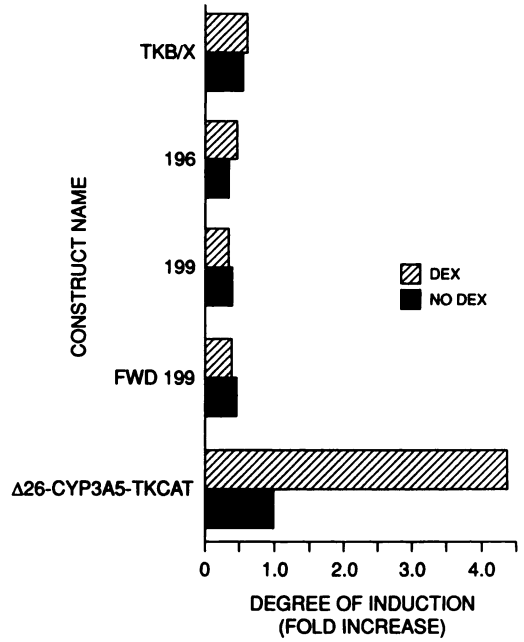


Fig. 7. Effect of subdivision of the *CYP3A5* dexamethasone responsive enhancer on dexamethasone response. The different deletion constructs (Table 3) of the *CYP3A5* dexamethasone responsive enhancer were transfected into HepG2 cells along with 500 ng of glucocorticoid expression vector (pRShGRα). After transfection, the cells were treated with 10 mM dexamethasone (*DEX*) and harvested for CAT activity. The results are the average of two separate experiments, each performed in duplicate.

***CYP3A5* Enhancer**

```
                                        -1109 GCAGGTCATT  ATGTTAGGTA
                                              GRE-HEXAMER
-1089 AAATAAGCCA GGCACACAAA GACAGACATT GCATGTTCTC ACTTATTTGT
                                                    .
                                                   1050
          Octamer-factor CAAT-box
-1039 GGGATgTaCA AATCAAAaCA ATTGAGCTAA TGTCTGGGTC TTAGTCAATT
         .
        1034
                                                  HBV-enhancer
 -989 TTGTACCCTA AGTACAGGGA GCACAGCCAT TAGAATACAT GATGAATGCT
       .
      986
                                                  GRE-HEXAMER
 -939 TTAATACAGG AATGAATAGG TGAGAGGCAT CAGGGTGGTT GGGTGTTCT
                                                   .     .
                                                  898   891
```

Fig. 6. *CYP3A5* dexamethasone enhancer sequence. The nucleotide sequence of the *CYP3A5* 219-bp enhancer is indicated with potential transcription factor binding sites indicated by underlining (*lower case letters*, mismatches). Boundary nucleotides for contructs used in Table 3 and Fig. 6 are *bold and numbered*.

TABLE 4

Effect of point mutations in the *CYP3A5* enhancer on its glucocorticoid-dependent activation

CAT construct	Orientation[a]	Position of mutation[b]		Relative to control[c]
		5′	3′	
6 B/S	>	MT[e]	MT	0.7 ± 0.04 (4)[d]
6 H/X	<	MT	MT	0.7 ± 0.02 (4)
23 H/X	>	N[f]	MT	1.1 ± 0.05 (4)
67 H/X	>	N	N	1.6 ± 0.3 (5)
68 B/S	<	N	N	2.0 ± 0.1 (5)
53 H/X	>	MT	N	0.8 ± 0.03 (3)
53 B/S	<	MT	N	0.6 ± 0.04 (3)
TK				0.7 ± 0.04 (3)
Δ26-CYP3A5	>	N	N	3.1 ± 0.25 (4)
MMTV				28.6 ± 5.7 (3)

[a] > denotes forward orientation 5′ to 3′; < denotes 3′ to 5′.
[b] GCR half-sites 5′-TGTTCT-3′ had a point mutation introduced to convert the half-sites to 5′-TATTCT-3′.
[c] Cells were incubated with 10 μM dexamethasone for a 24-hr interval.
[d] Standard error with number of independent experimental determinations in parentheses.
[e] MT, mutated sequence.
[f] Normal sequence.

20 bases −1109 to −1090 5′ of the most distal 5′-GRE half-site (constructs 67H/X and 68B/S) had no effect on basal activity of the TK promoter, there was, however, a diminished induction by dexamethasone (relative to Δ26-CYP3A5), possibly due to the loss of the nonconsensus GRE (bp −1107 to −1102). Thus, these data further support the concept that the half-sites interact to confer dexamethasone activation.

Discussion

The CYP3As are the most abundant P450 in human liver (39). Previous studies from our laboratory have established that this class of CYP450 responds to glucocorticoids in humans and rats (5, 32, 40). Moreover, studies in isolated primary human hepatocytes have demonstrated that glucocorticoids can induce not only *CYP3A4* (27) but also *CYP3A5*.[3] We isolated the *CYP3A5* gene and 1.4 kb upstream of the transcriptional start site to understand the role of glucocorticoids in the regulation of *CYP3A5* in human cells. We constructed recombinant plasmids containing fragments of the 5′-flanking sequence of the *CYP3A5* gene linked to either a promoterless vector pSV0-CAT or a minimal promoter of TKCAT to locate nucleotide sequence information conferring dexamethasone responsiveness. Transfection of the CYP3A5-TKCAT plasmids into the human hepatoblastoma, HepG2 cell line permitted us to identify a 219-bp dexamethasone responsive enhancer in the *CYP3A5* gene that responded dose-dependently to dexamethasone and induced a heterologous promoter 4–5-fold above untreated cells. The *CYP3A5* enhancer is unique because it appears to require both intact GRE half-sites separated by 160 bp to provide the glucocorticoid response. Moreover, these studies demonstrated that the *CYP3A5* promoter is not transcriptionally active unless the dexamethasone enhancer sequences are appended upstream. Thus, the dexamethasone responsive element is required for the basal and dexamethasone activation of the *CYP3A5* promoter.

[3] P. Maurel. Personal communication.

Two *CYP3A5* GRE half-sites have been identified that are necessary for glucocorticoid activation of a heterologous promoter and its own promoter. This is not unusual because many genes require multiple GREs to confer glucocorticoid effects, particularly if the GREs are far removed from the transcription start site (41). The data described in the present study support the concept that the *CYP3A5* GRE half-sites interact. The interaction between the two elements is supported by these findings: neither half-site alone can confer glucocorticoid responsiveness on the heterologous promoter and point mutations in either half-site eliminate glucocorticoid induction. Because each GRE half-site can bind purified GCR, these GRE half-sites must interact to reconstitute a functional *CYP3A5* enhancer element. The cooperative interaction between GREs has also been described for genes, such as TAT (20). Future studies are being directed toward how the *CYP3A5* half-sites might cooperatively interact with GCRs to form a glucocorticoid responsive enhancer.

The two half-sites in CYP3A5 are separated by 160 bp that contains putative DNA transcription factor binding sites (Fig. 6, Table 3). These other proteins are probably required for transcriptional activation by the two widely spaced GRE half-sites. For example, in the tryptophan oxygenase gene, a CACCC box binds a factor that interacts with GCRs bound by a nearby GRE (42). Our data showing that a deletion of 20 bp from the 5′ extent of the *CYP3A5* enhancer attenuates the magnitude of dexamethasone induction appears to support this idea as would preliminary data showing this 199-bp CYP3A5 fragment is not as potent as the full-length 219-bp *CYP3A5* enhancer in competition studies with GRE binding to GCR. Thus, half-site GREs, like those in *CYP3A5*, may require interactions with binding sites for other transcription factors to stabilize the glucocorticoid/GCR/GRE complex for glucocorticoids to activate *CYP3A5* gene transcription.

Functionally, the *CYP3A5* GREs mediate glucocorticoid-dependent activation of either a heterologous promoter or its own promoter (Figs. 1–4). Although we have previously identified a glucocorticoid responsive region in the *CYP3A1* gene that activates a heterologous promoter (26), comparison of the *CYP3A5* enhancer sequence with the 5′ sequences for *CYP3A4* and *CYP3A1* (accession numbers D11131 and M86850, respectively) reveals no sequence similarity. Sequence comparison of the *CYP3A5* enhancer with *CYP3A4* in this upstream region reveals only 47% homology compared with 82% identity between the proximal −644 bp of *CYP3A5* and −690 bp of *CYP3A4*. Thus, this 219-bp enhancer element is unique to *CYP3A5*.

Unlike other glucocorticoid-regulated genes in liver, such as TAT, CYP3As are unique in that they require much greater amounts (stress levels) of glucocorticoids for their induction (32, 33), and maximal transcriptional activation is achieved only at pharmacological doses of steroid (26) (Fig. 4). Inasmuch as the CYP3As are the dominant human liver forms and are induced by conditions of stress (32, 43), the unique spacing of the GRE half-sites may contribute to the unusual responsiveness of CYP3A to glucocorticoids (32, 33). The limited homology of the *CYP3A5* gene enhancer to the consensus 15-bp GRE suggests that the biological response of *CYP3A5* to dexamethasone activation may be related to the extent of the homology to the palindromic 15-bp GRE. Computer-assisted analysis of the minimal dexamethasone enhancer for the *CYP3A5* gene revealed only limited homology

with two of the GREs found in MMTV. The *CYP3A5* 219-bp enhancer has two hexameric (TGTTCT) GRE half-site sequences, and these sequences have been reported to weakly bind GCR (44). Because both GRE half-sites in the *CYP3A5* enhancer can bind purified GCR yet neither half-site can independently activate a heterologous promoter and because point mutations in either half-site eliminate dexamethasone responsiveness, we speculate that additional factors are cooperating with the GCR. Future studies will elucidate the relationship among these different factors. In summary, these studies provide the first evidence that a human *CYP3A* gene contains the necessary sequence information for regulation by glucocorticoids.

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