Nuclear Receptor Involvement in the Regulation of Rat Cytochrome P450 3A23 Expression*

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Many genes of the cytochrome P450 3A (CYP3A) subfamily, including several human and rat isoforms, are inducible by glucocorticoids. In the rat CYP3A23 gene, a 110-base pair segment of the proximal 5′-flanking region mediates dexamethasone activation. Three binding sites (DexRE-1, DexRE-2, and Site A), identified by DNase I footprinting analysis, were characterized for their relative contribution to both basal activity and dexamethasone inducibility. Site-directed mutagenesis of DexRE-1 (−144 to −169) and DexRE-2 (−118 to −136) demonstrated that each contained a core imperfect AGGTCA direct repeat, which comprised a consensus nuclear receptor binding site, and was essential for dexamethasone responsiveness but was not required for basal activity. Competition gel shift and supershift analyses revealed that both sites can bind the orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor.

Site A (−85 to −110) was shown to be important for both basal activity and dexamethasone responsiveness. Point mutants displayed a reduced (2–3-fold) induction response, compared with 15-fold for wild-type, which was accompanied by a 40–60% drop in basal activity. Site A was shown to bind the liver-enriched nuclear receptor hepatocyte nuclear factor 4. Our studies demonstrate that the mechanism mediating glucocorticoid-inducible transcriptional activity of CYP3A23 involves multiple binding sites for members of the nuclear receptor superfamily.

The cytochrome P450s (CYPs) make up a superfamily of heme-containing monoxygenases that are found most abundantly in liver endoplasmic reticulum and catalyze the oxidation or reduction of both endogenous and foreign substrates, often as an essential step in their elimination (1). CYP expression is modulated by endogenous and exogenous compounds, which may reflect a homeostatic mechanism whereby normal “endogenous ligand” concentrations are maintained. In early studies, steroids were shown to regulate expression of a cytochrome P450 family that was distinct from the 3-methylcholanthrene or phenobarbital inducible P450s (2–5). Pregnenolone 16α-carbonitrile (PCN) was characterized as the prototypical inducer of the CYP3A family; however, many glucocorticoids have been shown to be more potent than PCN, whereas certain non-glucocorticoids, such as phenobarbital and macroline antibiotics, also induce the same protein (6–8). CYP3A proteins have been identified in several species, including rat, rabbit, mouse, and human (9–14). The major glucocorticoid-responsive CYP3A gene in rat is 3A23, whereas 3A4 and 3A5 are inducible in human (15, 16). CYP3A enzymes metabolize over 60% of therapeutically relevant compounds and are involved in 6β-hydroxylation of the endogenous steroids testosterone, progesterone, and cortisol (17–19).

Regulation of rat 3A genes CYP3A23 and 3A2 has been the most extensively characterized. CYP3A23 is the form expressed in uninduced animals; however, its expression displays a gender and developmental stage-dependent pattern (8, 20–22). Despite its high homology (89%) to 3A23, CYP3A2 is only weakly induced by glucocorticoids (23, 24). CYP3A23 is expressed at low levels in untreated animals but is highly inducible by glucocorticoids, such as dexamethasone, and by PCN, a synthetic steroid with no hormonal activity (16, 25). Results from nuclear run-on experiments have established that the response is transcriptional (23, 26).

Induction of CYP3A protein by glucocorticoids was shown to be unusual in comparison to other glucocorticoid-responsive genes, such as tyrosine aminotransferase (5, 27). One prominent difference was that a 100-fold greater concentration of dexamethasone was required to elicit a maximal CYP3A response than was required for tyrosine aminotransferase induction. Also, PCN antagonized the glucocorticoid induction response of tyrosine aminotransferase while acting as a strong CYP3A inducer (27). However, other characteristics of the response are typical of glucocorticoid-inducible genes, such as specificity for glucocorticoids and inhibition by the glucocorticoid receptor (GR) antagonist RU38486 (25, 29). It has been determined, though, that the region supporting dexamethasone responsiveness does not bind GR, which excludes direct GR-mediated transactivation of the gene (30, 31).

Previous characterization by this laboratory of the CYP3A23 5′-flanking region localized the glucocorticoid response to the −60 to −170 region (30). This segment contained three nuclear protein binding sites as demonstrated by in vitro footprinting analysis and conferred responsiveness onto a heterologous promoter. The distal-most site, DexRE-1, has a core sequence of AACTCAAAGGAGGTCA, an imperfect direct repeat of an AGGTCA hexamer with a spacing of four nucleotides between the motifs (DR4). The AGGTCA sequence is a characteristic core binding motif of the class of nuclear receptors that includes the receptors for thyroid hormone, vitamin D₃, retinoic acid, and
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9-cis-retinoic acid, and the orphan receptors (32, 33). Mutations that disrupted either the upstream or downstream hexamer or their spacing caused a drop in dexamethasone induction, whereas changing the site to match a perfect direct repeat enhanced the response (30). This suggested that a member of the nuclear receptor superfamily, although not GR, was acting through the DexRE-1 element.

The two additional sites within this region, designated DexRE-2 and Site A, were also implicated in the dexamethasone induction pathway (30). DexRE-2 (−118 to −136) contains an AGTTCA repeat with a nucleotide spacing of three (DR3), differing at two positions from a consensus nuclear receptor binding site. Its role relative to the other two sites was not determined in previous studies. The proximal site, Site A, displays strong homology to a hepatocyte nuclear factor 4 (HNF-4) consensus binding site (34). HNF-4 directs liver-specific expression of numerous genes, including several cytochrome P450 genes (35). In the current study, Site A was examined for its role in mediating basal expression and/or dexamethasone responsiveness.

Our studies to date provide strong evidence that the dexamethasone response of CYP3A23 is driven by a multisite mechanism. In the present study, DexRE-2 and Site A were characterized and found to be crucial for full glucocorticoid responsiveness of CYP3A23. In addition, we demonstrated that functional activity at all three sites (DexRE-1, DexRE-2, and Site A) was correlated with ability to bind proteins from H4IIE nuclear extracts. Furthermore, we demonstrated through gel shift analyses that the orphan receptor receptor chicken ovalbumin upstream promoter transcription factor (COUP-TF) binds at DexRE-1 and DexRE-2 and, by cotransfection experiments, that Site A corresponds to a functional HNF-4 site. Hence, glucocorticoids induce CYP3A23 transcription through a multisite unit that encompasses binding sites for members of the nuclear receptor superfamily.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Oligonucleotides used for both mutagenesis and gel shift reactions were synthesized by Integrated DNA Technologies (Coralville, IA). Polymerase chain reactions were performed using the GeneAmp kit and Taq polymerase (Perkin-Elmer). Restriction enzymes, T4 ligase, and T4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA). Radioactive isotopes [α-32P]dATP (1000Ci/mmol) and [γ-32P]dATP (3000Ci/mmol) were obtained from Amersham Pharmacia Biotech. Luciferase assay reagents were from Promega (Madison, WI).

Mutagenesis of CYP3A23 5′-Flanking Region—Mutagenesis was performed on the wild-type 3A23 deletion construct P3−210. Construction of the DexRE-1 mutants Δ155−158, DR3, and DR4 has been previously reported (30). Changes were made by the polymerase chain reaction overlap extension technique using complementary mutant oligonucleotides as internal primers and oligonucleotides annealing to the pGL2-Basic vector as the external primers (36). Polymerase chain reaction products were cut with SacI/HindIII, and the fragments were subcloned into the pGL2-Basic vector containing the luciferase reporter gene (Promega). All constructs were sequenced by the dideoxy-chain termination method with the Sequenase version 2.0 DNA sequencing kit as described by the manufacturer (U. S. Biochemical Corp.).

Cell Culture, Transfection, and Luciferase Assays—H4IIE rat hepatoma cells, obtained from H. Pitot (Mc Ardle Laboratory), were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with heat-inactivated 10% fetal calf serum (Hyclone, Logan, UT) at 37 °C in humidified 5% CO2. HeLa cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's minimum essential medium with 10% fetal calf serum. Plasmid DNA for transfection experiments was purified using Qiagen Maxiprep columns (Santa Clarita, CA) followed by phenol/CHCl3 extraction. H4IIE transfections were performed by overlaying a 1-ml mixture of 6 µg/ml plasmid DNA, 75 µg/ml DEAE-dextran, 25 µM Tris, and 50% serum-free Dulbecco's minimum essential medium onto subconfluent H4IIE cells that had been plated 24 h prior to transfection. After 6 h of incubation in transfection mixture, cells were shocked for 2 min with 10% MeSO in phosphate-buffered saline followed by 64−66 h incubation in medium containing 10 µM dexamethasone (Sigma) or MeSO. HeLa cells were transfected using 10 µg/ml Lipofectin reagent (Life Technologies, Inc.) for 6 h followed by addition of 10% fetal calf serum containing Dulbecco's minimum essential medium to halt the transfection. Unless otherwise specified, 3 µg/ml of luciferase reporter DNA and 0.01 µg/ml of cytomegalovirus promoter-driven expression plasmids were used in the co-transfection experiments. Cells were harvested, and luciferase assays were performed according to manufacturer's instructions (Promega). Activity was measured using a Monolight 2010 lumino- meter (Analytical Luminescence, Cockeysville, MD) and is expressed as relative light units/100 µg of protein. Fold induction by dexamethasone corresponds to the ratio of relative light units/100 µg of protein for dexamethasone-treated to relative light units/100 µg of protein for MeSO-treated cells. Protein determination was made according to a modified Lowry method.

Electrophoretic Mobility Shift Assays—Double-stranded DNA for probes was generated by annealing complementary, single-stranded oligonucleotides for DexRE-1 (5′−CCCCGAATTGTTAACCTAAGGGAGGTCAATATGCTG-3′, 5′−AGCCTATTTGTAGCTCTTGTTAAAGGTTAAACAT-3′), DexRE-2 (5′−TGTAAGTGAACCTTCAAGTGCTG-3′, and Site A (5′−GGAAGAAGATCAAAAGTTCCAGTGGAGG-3′) (exact reverse complements) were used for opposite strands of DexRE-2 and Site A). Other pertinent sequences are provided in the figures. DNA (2−4 pmol) was end-labeled with [γ−32P]dATP using T4 polynucleotide kinase, and unincorporated nucleotides were removed by Sephadex G-50 filtration. Binding reactions contained 12 µM Hepes, 4 mM Tris, 12% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 µg of di-dC, 5−7 µg of H4IIE crude nuclear extract, and 0.05 pmol of probe (20,000 cpm). In competition reactions, excess unlabeled oligonucleotide was preincubated (10 min) at room temperature before being incubated with probe for an additional 10 min. For supershift reactions, antisera to COUP-TF or HNF-4 (1.5 dilution), generously provided by M.-J. Tsai and F. M. Sladek, respectively, was added to reactions and incubated on ice for 15 min before addition of probe. Protein-DNA complexes were resolved on a 4% nondenaturing polyacrylamide gel.

RESULTS

The activity of DexRE-1 mutants positively correlates with their affinity for nuclear binding proteins. The sequence of the CYP3A23 dexamethasone-responsive region is shown in Fig. 1. The heavy lines over each of the three regions indicate sequence patterns in DNase I footprinting analysis (30), and the consensus sequences highlighted in boldface for DexRE-1 and DexRE-2 are imperfect AGGTCA direct repeats (indicated by arrows) with four- and three-nucleotide spacing, respectively. Also shown aligned with CYP3A23 is the homologous region of the CYP3A2 gene. Within this region of the two promoters, the genes are 91% identical, but the promoters differ in the magnitude of their response to glucocorticoids (23). It is likely that mismatches between CYP3A23 and 3A2, indicated (Fig. 1) by vertical dashes between the aligned sequences, are responsible for their differential inducibility. In this study, several mutants were constructed for the analysis of DexRE-1 and DexRE-2 based on these mismatches. Previously, it was demonstrated that dexamethasone activation could be reduced or enhanced depending on the specific changes made to the degenerate DR4 of DexRE-1 (30). However, binding studies were not performed to determine whether changes in DexRE-1 activity were associated with alterations in binding to nuclear factors. To examine this point, analyses were performed using several DexRE-1 mutants that displayed either strongly decreased or enhanced dexamethasone responsiveness in transient transfection experiments in H4IIE rat hepatoma cells (Fig. 2). In the P3−210(3A2) mutant, DexRE-1 of the 3A23 gene was converted to the equivalent element of the 3A2 gene. These changes resulted in an approximate 60% decrease in the dexamethasone response compared with the wild-type, P3−210. The maximum induction response observed for any CYP3A2 promoter-driven reporter construct is 3−4-
The dexamethasone-responsive region of the CYP3A23 5’-flanking region. The sequence of the CYP3A23 dexamethasone-responsive region is compared with the homologous region of the less responsive rat CYP2A2 gene. Lines over the sequence indicate regions identified by DNase I footprinting analysis in previous work and are designated DexRE-1, DexRE-2, and Site A (30). For DexRE-1 and DexRE-2, the nucleotides in boldface type correspond to direct repeat elements that are homologous to binding sites for members of the steroid/thyroid hormone receptor superfamily, as discussed in the text. For Site A, the boldface type highlights the region with 80% homology to an HNF-4 consensus binding site.

Fig. 1. Transcriptional activity of DexRE-1 mutants positively correlates with their affinity for nuclear binding proteins. A, DexRE-1 mutations were made within the context of the P3–210 construct. Substitutions are represented by a lowercase letter, whereas single and multiple deletions are represented by a dash and /, respectively. Transient transfections were performed in H4IIE cells, and activities were assessed following 60 h of Dex (10 μM) or vehicle treatment. Activities are reported as mean fold induction ± S.D. (Dextreated/activity/control activity) and represent a minimum of four experiments. Data for Δ−155 to −158, DR3, and DR4 were taken from Ref. 30. B, competition gel shift analyses were performed in which the DexRE-1 probe was incubated with H4IIE extracts in the presence or absence of the indicated fold excesses of cold competitor. The designation of competitors corresponds with mutant names from A. The arrows labeled A and B indicate specific DNA-protein complexes. NE, H4IIE nuclear extracts. An asterisk indicates nonspecific complex.

fold; therefore, these results indicate that divergence in DexRE-1 sequence between CYP3A23 and 3A2 is, in part, responsible for the differential responsiveness of the two genes. In addition, 6-fold induction was observed for the spacing mutant, P3–210(Δ−155 to −158), whereas only a 3-fold response to dexamethasone was seen for P3–210(Δ−149 to −154), in which the downstream hexamer was disrupted. Hence, modification of the downstream AGGTCA inactivated DexRE-1, because mutants completely lacking DexRE-1 still displayed a partial (3-fold) induction response (30). Previously, we reported that conversion of the imperfect AGGTCA direct repeat of DexRE-1 to a perfect consensus DR4 or DR3 resulted in dexamethasone induction greater than that of wild-type (Ref. 30 and Fig. 2A).

Binding analyses of both decreased and enhanced activity mutants are shown in Fig. 2B. In the competition gel shift assays, DexRE-1 was used as probe, and binding was competed with increasing amounts of unlabeled wild-type or mutant double-stranded oligonucleotides. DexRE-1 formed two DNA-protein complexes with H4IIE nuclear extracts (Fig. 2B, lane 2), designated A and B, that were eliminated upon incubation with excess cold self (lanes 3 and 4). Most notable was the observation that the decreased activity mutants (Δ−155 to −158 and −149 to −154) did not compete for the formation of complex B (Fig. 2B, lanes 7, 8, and 12–14), even up to 150–200-fold excess of mutant DNA. However, both mutants successfully competed for complex A formation. The Δ−155 to −158 mutant was equivalent to wild-type (Fig. 2B, compare lanes 4 and 7), whereas −149 to −154 displayed reduced affinity, because 50-fold excess mutant (lane 12) competed as efficiently as 10-fold wild-type for complex A (lane 4). The enhanced activity DR4 was as effective as wild-type in competing for both complexes A and B (Fig. 2B, compare lanes 9 and 3). Hence, a correlation exists between activity and binding to DexRE-1, although formation of complex A was less affected by changes in spacing or the downstream hexamer than was complex B.

DexRE-2 is Essential for Glucocorticoid Activation of the CYP3A23 Promoter—Our previous studies showed that two consensus sites, DexRE-1 and DexRE-2, cooperated to mediate glucocorticoid induction of the CYP3A23 gene. DexRE-2, which spans nucleotides −118 to −136, contains an AGTTC direct repeat separated by three nucleotides and bears two mismatches with the corresponding element of the less responsive CYP2A2 gene (Fig. 1). Fig. 3A shows the effects of altering the sequence of DexRE-2 on both basal and induced activity of the P3–210 construct. The C-T mismatches between CYP3A2 and CYP3A23 at positions −117 and −127 were changed individually in P3C-117T and P3C-127T, respectively. The downstream substitution caused a moderate decrease in dexamethasone inducibility, whereas the mutation at −127 had no effect. In contrast, mutations that decreased the spacing between direct repeats or disrupted either the upstream or downstream motif virtually eliminated the glucocorticoid induction response. The most active mutant of this type was P3–122 to −124, which retained only a 2.2-fold induction response to dexamethasone.

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2 J. M. Huss and C. B. Kasper, unpublished observation.
Interestingly, none of the DexRE-2 mutations resulted in any significant change in basal activity, suggesting that this element plays an important role in glucocorticoid induction but does not contribute to basal gene expression.

The relative binding characteristics of each mutant element were assessed by competition gel shift assays (Fig. 3B). DexRE-2 formed a single specific complex with extracts from H4IIE cells that was eliminated upon addition of cold self (lane 3). A positive correlation was observed between glucocorticoid responsiveness and binding affinity. Whereas low activity mutants of 2131 to 2133 and 2122 to 2124 displayed reduced affinity for the protein(s) binding to DexRE-2 (Fig. 3B, compare lanes 3, 4, and 7), point mutants that retained significant activity displayed binding affinities equivalent to that of wild-type (compare lanes 3, 5, and 6). These data demonstrate that the direct repeat core is the essential component of DexRE-2, which when mutated results in the reduction in both functional activity and protein binding affinity.

The Nuclear Receptor COUP-TF Binds to Both DexRE-1 and DexRE-2—Gel shift analyses of DexRE-1 and DexRE-2 using H4IIE nuclear extract were performed to compare patterns of specific complex formation at the respective sites (Fig. 4). As observed above, DexRE-1 formed two DNA-protein complexes (A and B), and DexRE-2 formed the expected single complex. When the two probes were analyzed on the same gel, it became apparent that the DexRE-2 complex migrated at the same mobility as the DexRE-1 complex A (lanes 2 and 8), suggesting that the sites might be interacting with a common protein(s). To test this possibility, reciprocal competition gel shift experiments were performed using DexRE-1 as probe and excess cold DexRE-2 as competitor, and vice versa. Interestingly, both sites appeared to bind the same protein(s). DexRE-2 efficiently competed for binding with DexRE-1, but only for complex A, whereas cold DexRE-1 completely eliminated DexRE-2 complex formation when added at a 50-fold excess. Therefore, DexRE-1 and DexRE-2 can bind to a common factor(s), although the results indicate that DexRE-2 has a higher affinity for the factor(s) than DexRE-1.

Next, experiments were performed to identify the protein(s) present in complex A. When DexRE-2 sequence was compared with reported nuclear receptor binding sites, an extension of the DR3 was found to closely match a consensus element that binds both orphan receptors, COUP-TF and HNF-4. The DexRE-2 sequence, CAGTTCAtgAAGTTCA, differs by one nucleotide from the consensus element, which consists of a 5’- (A/G)(A/G)G(G/T)TCA-3’ heptamer direct repeat (mismatch in boldface) (37). Although considerable sequence overlap exists between HNF-4 and COUP-TF response elements, not all HNF-4 binding sites bind COUP-TF (35); therefore, to distinguish between these two possible binding activities for DexRE-2, several oligonucleotides specific for either COUP-TF or HNF-4 were used in competition gel shift experiments with DexRE-2 probe (Fig. 5A). TREpal and TREpal12 are idealized palindromic arrangements of GGTCA separated by 0 and 12
nucleotides, respectively, that specifically bind with COUP-TF but not HNF-4 (38). The COUP oligonucleotide corresponds to the natural COUP-TFI binding site found in the chicken ovalbumin promoter and comprises a DR2 (39). These oligonu-
cleotides competed with labeled DexRE-2 for complex forma-
tion as would be predicted if COUP-TF were the binding pro-
tein (Fig. 5B). That is, competition by TREpal and COUP, both of which are specific high affinity COUP-TF binding sites, was comparable to that of wild-type DexRE-2 (Fig. 5B, compare 
lanes 3, 4, and 8), whereas the low affinity site, TREpal12, only decreased DexRE-2 binding when present at 50-fold excess 
(lane 7). Finally, the α1-antitrypsin element from the α1-anti-
trypsin gene that specifically binds HNF-4 but not COUP-TF 
proteins was unable to compete with DexRE-2 (Fig. 5B, lanes 
10–12). The same experiment was performed with DexRE-1, 
and an identical pattern of competition was observed for com-
plex A, providing further support that DexRE-1 and DexRE-2 
bond to the same protein (data not shown).

Additionally, supershift analyses were performed using an-
tibodies for COUP-TF and HNF-4 (Fig. 5C). COUP-TF antibody 
recognizes both COUP-TFI and COUP-TFII, which share 98% 
homology in their DNA binding domains and have indistin-
guishable binding characteristics (40). The COUP probe formed 
a complex with H4IIE nuclear extracts with a mobility identi-
cal to that of complex A and, as expected, formed a supershifted 
complex with COUP-TF antibody. (Fig. 5C). Identical results 
were obtained in the DexRE-2 reaction; however, anti-HNF-4 
had no effect on complex A. In addition, reactions performed 
with in vitro synthesized protein revealed that recombinant 
COUP-TFI, but not HNF-4, complexed with DexRE-1 and 
DexRE-2 (data not shown). These results indicate that either 
COUP-TFI, COUP-TFII, or an antigenically related factor is an 
integral part of complex A. In addition, HNF-4 does not bind to

![Fig. 4. DexRE-1 and DexRE-2 complex with a common protein in H4IIE nuclear extracts.](image)

DexRE-1 or DexRE-2 elements were used as probes in gel shift reactions using H4IIE nuclear extract. In lanes 1–6, the DexRE-1 probe was competed with both cold self and DexRE-2 at the indicated fold molar excesses. In lanes 7–12, the reciprocal experiment was performed using the DexRE-2 probe. The two arrows labeled A and B indicate specific DNA-protein complexes. The asterisk indicates nonspecific complex.

![Fig. 5. DexRE-2 interacts with COUP-TF.](image)

A, the sequences of competing consensus elements. TREpal, TREpal12, and COUP are COUP-TF binding sites, whereas α1-antitrypsin specifically binds HNF-4. B, labeled DexRE-2 was incubated with H4IIE extracts in the presence of the indicated molar excess of cold self or competitor double-stranded oligonucleotides. The specific complex is indicated by an arrow. C, supershift experiments were performed with the COUP element (from panel A) or DexRE-2 as probe. Each element was incubated with H4IIE extracts in the absence or presence of COUP-TF antibody (Ab). Additionally, DexRE-2-protein complex was reacted with HNF-4 antibody. The specific DexRE-
2-protein complex is indicated by an arrow, and the supershifted complex is indicated by an arrowhead. An asterisk indicates nonspecific complex.
Figure 6. Site A, an HNF-4 binding site, contributes to both dexamethasone responsiveness and to basal CYP3A23 activity. A, gel shift reactions were performed with the Site A probe, which was competed with either cold self, the α1-antitrypsin gene HNF-4 element (α1-AT), or the cAMP-responsive element (CRE) at the indicated fold excesses (lanes 1–6). Supershift reactions were performed in lanes 7–8 with either HNF-4- or COUP-TF-specific antibodies. The major complexes are indicated by arrows. The supershifted complex is indicated by an asterisk. B, Site A mutants were made in the P3–210 deletion construct; the wild-type sequence is shown at the top, and alterations are indicated in lowercase boldface letters. Mutants were transiently transfected into H4IE cells and assayed for luciferase activity after 60–64 h treatment with 10 μM dexamethasone. Basal activity is reported relative to wild-type in gray bars (□), and fold dexamethasone induction is shown in striped bars (■). Columns represent mean ± S.D. of at least three experiments.

either DexRE-1 or 2.

Site A Binds Specifically to the Orphan Receptor, HNF-4—Site A was predicted to correspond to an HNF-4 binding site, because it bears 80% homology to an HNF-4 consensus identified in several CYP2C genes (34). This hypothesis was tested in Site A gel shifts by competition with the specific HNF-4 element from the α1-antitrypsin gene (Fig. 6A). With a Site A oligonucleotide as probe, two main complexes formed with H4IE nuclear extracts that were competed by excess cold self (Fig. 6A, lane 3). Furthermore, the α1-antitrypsin oligonucleotide was able to efficiently compete for binding to both complexes at a 10-fold molar excess, similar to wild-type, whereas a cAMP-responsive element could not decrease binding when added at 100-fold excess. In the same experiment, when HNF-4 antibody was added to the Site A reaction, a supershifted complex formed, indicating that HNF-4 is a component of the DNA/protein complex (Fig. 6A, lane 7). In contrast to the results of the competition experiment, the upper main band was retarded, leaving the lower band unaffected. This may be the result of proteolytic degradation of HNF-4 causing reduced reactivity with antibody. Because COUP-TF and HNF-4 have been shown to share binding sites in several genes (35), supershift experiments were also performed with COUP-TF antibody. Band intensity was not decreased, and no supershifted complex was observed, indicating no interaction between COUP-TF proteins and Site A (Fig. 6A, lane 8). Therefore, Site A of CYP3A23 corresponds to a specific binding site for the orphan receptor HNF-4.

The HNF-4 Binding Site Contributes to Both Basal Regulation and Dexamethasone Responsiveness of the CYP3A23 Promoter—In previous work, deletion of the −144 to −60 region, which encompasses DexRE-2 and HNF-4 sites, resulted in both an 80% drop in basal expression as well as a loss of dexamethasone inducibility (30). The present study demonstrated that mutation of DexRE-2 eliminated dexamethasone induction without affecting basal expression (Fig. 3A), suggesting that the HNF-4 element exclusively regulates basal CYP3A23 activity. To further examine this issue, the HNF-4 site was mutated within the P3–210 construct, and both basal activity and responsiveness to dexamethasone were analyzed relative to wild-type (Fig. 6B). Alterations were based on mutations of HNF-4 sites in several P450 genes that have been shown to decrease protein binding and activation (34). Mutation of the CYP3A23 HNF-4 site decreased both basal activity and responsiveness to dexamethasone. In each mutant (P3–96/98 and P3C94G), a 40–60% reduction in basal activity was observed, along with a marked drop (>75%) in dexamethasone inducibility. Therefore, the HNF-4 site does, in fact, function in dexamethasone regulation, but it is distinct from DexRE-1 and DexRE-2 in that it has a major role in regulating basal expression.

HNF-4 Expression Activates CYP3A23 Expression in a Nonhepatic Cell Line—In order to definitively establish a role for HNF-4 in CYP3A23 regulation, the receptor was transiently expressed in HeLa cells, a cell line lacking endogenous HNF-4, to determine whether HNF-4 could activate expression of CYP3A23. When the P3–210 construct was co-transfected with the HNF-4 expression plasmid, pCMVHNF-4, a 2.9-fold activation was observed (Fig. 7A). However, the Site A mutant, P3C94G, displayed only a 1.5-fold induction response, whereas P3–96/98 did not respond to HNF-4 co-expression. Furthermore, the HNF-4 site conferred HNF-4 responsiveness when placed upstream of a heterologous promoter (Fig. 7B). Site A(2×)-TK, which contains two copies of the HNF-4 element cloned upstream of the thymidine kinase promoter, displayed an approximate 4-fold induction response to HNF-4 that was otherwise not observed in the parent TK-Luc construct or the 96/98(3×)-TK construct, which contains three copies of the mutated HNF-4 element. These data demonstrate that activation of CYP3A23 by HNF-4 specifically relies on the presence of an intact HNF-4 binding site.

Discussion

Three major regulatory elements within a 110-bp segment of the proximal CYP3A23 5′-flanking region, previously defined as the glucocorticoid-responsive region, have been demonstrated to all be essential for glucocorticoid inducibility. Previously, we showed that DexRE-1 played a definitive role in the glucocorticoid response and demonstrated that the two downstream binding sites, DexRE-2 and Site A, may cooperate with DexRE-1 in the induction process (30). The current study demonstrates that all three elements are essential, because disruption of any individual site within the responsive region decreased dexamethasone inducibility. Hence, this region of the CYP3A23 gene represents a multisite glucocorticoid-responsive unit. This multisite mechanism for glucocorticoid inducibility differs from other previously described mechanisms in that the CYP3A23-responsive unit lacks a GR binding site but rather binds to nuclear receptors of the estrogen receptor subclass (30, 31, 41).
were cotransfected into HeLa cells with an HNF-4 expression vector

moter constructs (3

Site A element. Data reflect the mean 

two copies of the wild-type Site A element or three copies of a mutated
ator sequence. The heterologous TK promoter constructs contain either 

to a minimal promoter construct containing a TATA box and an initi- 
ator sequence. The heterologous TK promoter constructs contain either 
two copies of the wild-type Site A element or three copies of a mutated Site A element. Data reflect the mean ± S.D. of four transfection experiments carried out in triplicate.

The second orphan receptor playing a role in CYP3A23 reg-

model, mutants in which DexRE-1 was deleted or disrupted, leaving DexRE-2 and the HNF-4 site intact, displayed a 3-fold induction (30). However, a mutant in which DexRE-2 was replaced with the DexRE-1 also displayed a 3-fold response (data not shown). Therefore, DexRE-1 and DexRE-2 can mediate similar induction levels when placed in equivalent positions relative to the HNF-4 site. Because all three elements within the dexamethasone-responsive unit are in close proximity to one another, it is possible that the binding proteins interact to promote or stabilize each other’s interactions.

The interplay between DexRE-1 and DexRE-2 is an interesting point of speculation, because both sites were observed to bind to a common protein, the orphan receptor COUP-TF. Two human COUP-TF genes have been cloned, COUP-TFI and COUP-TFII (also known as Ear3 and Arp-1, respectively), for which homologs exist in several other mammalian species (40). The consensus binding element for COUP-TFs is described as a DR1; however, empirical evidence has shown that COUP-TFs interact with GGTCA direct repeats, as well as with palindromes with variable spacing (38, 43). This flexibility in binding allows COUP-TFs to compete with other nuclear receptors for their consensus binding elements and inhibit trans-activation mediated by these receptors (44). This type of COUP-TF antagonism has been observed for retinoic acid receptor, retino- 

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The second orphan receptor playing a role in CYP3A23 reg-

model, mutants in which DexRE-1 was deleted or disrupted, leaving DexRE-2 and the HNF-4 site intact, displayed a 3-fold induction (30). However, a mutant in which DexRE-2 was replaced with the DexRE-1 also displayed a 3-fold response (data not shown). Therefore, DexRE-1 and DexRE-2 can mediate similar induction levels when placed in equivalent positions relative to the HNF-4 site. Because all three elements within the dexamethasone-responsive unit are in close proximity to one another, it is possible that the binding proteins interact to promote or stabilize each other’s interactions.

The interplay between DexRE-1 and DexRE-2 is an interesting point of speculation, because both sites were observed to bind to a common protein, the orphan receptor COUP-TF. Two human COUP-TF genes have been cloned, COUP-TFI and COUP-TFII (also known as Ear3 and Arp-1, respectively), for which homologs exist in several other mammalian species (40). The consensus binding element for COUP-TFs is described as a DR1; however, empirical evidence has shown that COUP-TFs interact with GGTCA direct repeats, as well as with palindromes with variable spacing (38, 43). This flexibility in binding allows COUP-TFs to compete with other nuclear receptors for their consensus binding elements and inhibit trans-activation mediated by these receptors (44). This type of COUP-TF antagonism has been observed for retinoic acid receptor, retinoid X receptor, peroxisome proliferator-activated receptor, HNF-4, estrogen receptor, thyroid hormone receptor, vitamin D receptor, and SF-1 mediated transcriptional activation and has been observed in P450 regulation (40). Cairns et al. (45) showed that CYP2D6 has a dual binding site for HNF-4 and COUP-TF through which HNF-4 transactivates, whereas COUP-TF can inhibit HNF-4 action. In the case of CYP3A23, no dual binding sites were identified. Gel shifts using both specific antibodies (Figs. 5 and 6) as well as recombinant HNF-4 and COUP-TF (data not shown) revealed that HNF-4 did not interact at the COUP-TF binding sites, nor could COUP-TF bind at the HNF-4 site (Site A). Therefore, it is likely that COUP-TF has no direct antagonistic effect on HNF-4 transactivation of CYP3A23. Indeed, our data support a trans-activation role for COUP-TF in CYP3A23 regulation, because the activity of DexRE-2 mutants positively correlated with their binding to COUP-TF in H4IE nuclear extracts. However, given the preponderance of instances in which COUP-TF antagonizes the action of another receptor rather than activating the gene to which it is binding, COUP-TF may not be directly mediating the CYP3A23 dexamethasone response. Alternatively, COUP-TF could be sharing a binding site with an unidentified member of the nuclear receptor superfamily that is directly activated by dexamethasone and PCN. This hypothesis is particularly appropriate for DexRE-1, because we demonstrated that complex A corresponds to COUP-TF but have yet to determine the identity of complex B. Until a link can be made between COUP-TF and dexamethasone, the theory of an alternate receptor must be considered. In support of this hypothesis, an orphan receptor, designated pregnane X receptor, has recently been shown to also bind to the CYP3A23 DexRE-2 and is activated by both dexamethasone and PCN (46). COUP-TF involvement in transcriptional induction by glucocorticoids has been reported for the phosphoenolpyruvate carboxykinase gene; however, it acts as an accessory factor within a multisite unit that includes a functional glucocorticoid-responsive element, so that dexamethasone specificity is conferred by GR (41, 47). Furthermore, glucocorticoids were shown to cause no change in COUP-TF expression in lymphoid cells (48); however, the effect of glucocorticoids on COUP-TF expression in liver or liver-derived cultured cells has not been reported.

CYP3A23 Regulation by Nuclear Receptors

The steroid/thyroid hormone receptors are ligand activated transcription factors that act via binding to closely related response elements. The receptor subclass that includes estrogen receptor, thyroid hormone receptor, vitamin D receptor, retinoic acid receptor, and the orphan receptors typically binds as dimers to elements containing two AGGTCA core half-sites, although binding specificity for different receptors is determined by the exact sequence, spacing, and relative arrangement of the hexamer motifs. For example, the vitamin D receptor, thyroid hormone receptor, and retinoic acid receptor bind as heterodimers with retinoid X receptor to direct repeats of the AGGTCA motif spaced by 3, 4, and 5 nucleotides (DR3, DR4, and DR5), respectively (32, 42). For DexRE-1 and DexRE-2, which contain an imperfect DR4 and DR3, respectively, it was demonstrated that the degenerate direct repeats were essential for function. A decrease in dexamethasone responsiveness was observed when either the upstream or downstream core motif of DexRE-1 was mutated or when spacing between motifs was eliminated (Ref. 30 and present study). Similarly, alterations in either AGGTCA motif of DexRE-2, as well as in hexamer spacing, caused almost a complete loss of dexamethasone inducibility, whereas changes in spacer nucleotide sequence had no detrimental effect on activity (data not shown). Finally, a positive correlation between nuclear protein binding and function was demonstrated for DexRE-1 and DexRE-2, indicating that nuclear receptor binding was important for the induction response.

Interestingly, DexRE-2 mutations eliminated the dexamethasone induction response of the wild-type construct, P3-210, whereas DexRE-1 mutants never displayed an induction response below 3–4-fold (Ref. 30 and present study). This may be due to the relative position of the two sites because disrupting DexRE-1 leaves a functional DexRE-2 situated close to the HNF-4 site and other downstream elements, such as the TATA box; however, disrupting DexRE-2 spatially cuts off DexRE-1 from these downstream sites that may cooperate in a distance-dependent manner to mediate the response. In support of this

FIG. 7. HNF-4 activation is dependent on Site A. CYP3A23 promoter constructs (3 µg) (A) or heterologous TK promoter constructs (B) were cotransfected into HeLa cells with an HNF-4 expression vector (0.01 µg). Luciferase activities are reported as the ratio of activity in the presence of HNF-4 to activity with empty pCMV5 vector. Site A mutant constructs are described in the legend to Fig. 6. The TI Luc corresponds to a minimal promoter construct containing a TATA box and an initi-
ulation is HNF-4, which is found in especially high levels in the liver and contributes to the liver-specific expression of numerous genes (35). CYP3A23 joins an expanding list of cytochrome P450 genes harboring HNF-4 sites that likely mediate tissue-specific expression, including over 20 members of the CYP2 family (49). Within the CYP3A subfamily, 3A23 and 3A2 are highly homologous members that share 91% nucleotide similarity within the region conferring dexamethasone responsiveness (−60 to −170). We showed that HNF-4 was able to bind at Site A (−85 to −110) of CYP3A23 and to activate the gene, whereas other factors involved in liver-specific gene expression, HNF-1, HNF-3 and C/EBPβ did not bind (data not shown). The homologous binding site in CYP3A2 (−87 to −106) may also correspond to a functional HNF-4 element, because it was found to be necessary for basal activity in a liver cell line (50). However, CYP3A23/CYP3A23 comparison studies have shown that their HNF-4 sites are not functionally equivalent, despite having only two mismatches. CYP3A2 displayed lower basal activity than 3A23 in H4IIE cells that was enhanced when the 3A2 Site A sequence was altered to match the 3A2 sequence. 2 This is not surprising, because minimal changes in an HNF-4 site can substantially alter binding of its receptor and, therefore, functional activity (34).

An ambiguous aspect of the CYP3A23 induction mechanism is the involvement of GR. In the most probable model, dexamethasone or a metabolite acts through a receptor, although not necessarily GR. We have previously ruled out a direct involvement of GR; therefore, if GR is involved, it acts indirectly. To address whether GR is absolutely required for dexamethasone induction of CYP3A4, the expression of the gene in response to dexamethasone treatment could be investigated in GR−/− mice (51). If the mechanism is GR-independent, then a distinct receptor activated by dexamethasone and PCN, such as pregnane X receptor, could be involved. It is possible that more than one mechanism could be operative. Concentrations of dexamethasone that activate GR but do not induce CYP3A23 can potentiate the CYP3A23 induction observed with alternate inducers, such as PCN and metyrapone, suggesting that these inducers may act through a GR-independent pathway that is augmented by dexamethasone acting through GR (31, 28). If dexamethasone and PCN do function via slightly different pathways, the pathways must converge because both dexamethasone and PCN responses are localized to the same region of CYP3A23. 2

Much of what has been learned about the CYP3A glucocorticoid induction mechanism presents a unique pathway through which dexamethasone is mediating its effects. Activation occurs through cis-acting elements that do not serve as GR binding sites but rather bind to other members of the nuclear receptor superfamily. Two distinct DNA/protein complexes were formed with DExRE-1. The slower migrating complex contains COUP-TF, whereas the proteins corresponding to complex B remain to be identified.

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