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Dexamethasone responsiveness of a major glucocorticoid-inducible CYP3A gene is mediated by elements unrelated to a glucocorticoid receptor binding motif

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ABSTRACT **Elements responsible for dexamethasone responsiveness of CYP3A23, a major glucocorticoid-inducible member of the CYP3A gene family, have been identified. DNase I footprint analysis of the proximal promoter region revealed three protected sites (sites A, B, and C) within the sequence defined by −167 to −60. Mutational analysis demonstrated that both sites B and C were necessary for maximum glucocorticoid responsiveness and functioned in a cooperative manner. Interestingly, neither site contained a glucocorticoid responsive element. Embedded in site C was an imperfect direct repeat (5′-AACTCAAAGGAGGTCA-3′), showing homology to an AGGTCA steroid receptor motif, typically recognized by the estrogen receptor family, while site B contained an ATGAACT direct repeat; these core sequences were designated dexamethasone response elements 1 and 2 (DexRE-1 and -2), respectively. Neither element has previously been associated with a glucocorticoid-activated transcriptional response. Conversion of the DexRE-1 to either a perfect thyroid hormone or vitamin D_3 responsive element further enhanced induction by dexamethasone. Gel-shift analysis demonstrated that glucocorticoid receptor did not associate with either DexRE-1 or -2; hence, glucocorticoid receptor does not directly mediate glucocorticoid induction of CYP3A23. These unusual features suggest an alternate pathway through which glucocorticoids exert their effects.**

The cytochromes P450 (CYP) are a superfamily of heme-containing mono-oxygenases that catalyze the oxidative, peroxidative, and reductive metabolism of both exogenous and endogenous substrates. Fourteen families comprised of 26 subfamilies have been identified in mammals (1). The first four P450 families consist of enzymes that primarily convert foreign lipophilic compounds to more polar, readily excretable products, although certain substrates are metabolized to more toxic species (2). Expression of these xenobiotic-metabolizing P450 enzymes is frequently modulated by exogenous compounds at the transcriptional and posttranscriptional levels.

The major glucocorticoid-inducible class of cytochromes P450 is the CYP3A family. Members of this family have been identified in several species, including mice, rats, and humans. The mouse and human genes are localized to chromosomes 6 and 7, respectively (3–5). There is evidence that four distinct CYP3A forms are expressed in rats treated with various inducing compounds (6). The most thoroughly characterized forms in rat, CYP3A1 and CYP3A2, display distinct expression patterns (7–9). CYP3A1 has low constitutive expression, and is highly inducible by glucocorticoids, such as dexamethasone, but not by other classes of steroids (10). Induction occurs at the transcriptional level (11, 12), with a concomitant increase in protein and enzyme activity (13, 14). CYP3A2 is expressed constitutively in immature rats of both sexes, but it is not detectable in mature females (7, 9). Furthermore, although CYP3A2 is inducible by glucocorticoids, the magnitude of the response is lower than that for CYP3A1.

Two genes belonging to the CYP3A family have been cloned in our laboratory from a Wistar–Furth genomic library (S.I.W. and C.B.K., manuscript in preparation). The first is the male-specific form, CYP3A2, which corresponds to the gene isolated by Miyata *et al.* (15), whereas the second gene, designated CYP3A23, encodes a protein that is homologous to the deduced amino acid sequence of two previously reported cDNAs, cDex (16) and RL33 (17). In the present study, the regulation of CYP3A23 by dexamethasone is examined.

MATERIALS AND METHODS

Enzymes and Reagents. PCR reactions were performed using a GeneAmp kit and *Taq* DNA polymerase (Perkin–Elmer/Cetus). Restriction enzymes and T4 polynucleotide kinase were obtained from New England Biolabs. Luciferase assay reagents, including luciferin substrate, and 5x cell lysis buffer were from Promega.

Construction and Assay of Promoter Mutants. Deletion constructs P3-1400, P3-738, P3-422, P3-281, P3-144, and P3-60 were prepared using promoter fragments generated from the natural 5′ restriction sites *Hin*dIII, *Ssp*I, *Hin*fI, *Sty*I, *Hin*cII, and *Bam*HI, respectively, and a common 3′ *Fok*I site at +112. Site-directed mutagenesis was performed by the PCR overlap extension method (18). Fragments were cloned into the pGL2-Basic vector containing the luciferase reporter gene and the resulting plasmids were transfected into H4IIE (19) cells that were subsequently treated with either 10 μM of dexamethasone (Sigma) or dimethyl sulfoxide. Cells were harvested after 60 to 64 h and cell lysates were assayed for luciferase activity (Promega) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego). Activity is expressed as relative light units per 100 μg of protein. Heterologous promoter constructs were prepared by cloning flanking region sequences upstream of the thymidine kinase (tk) promoter (−110 to +50) in the pGL2-Basic vector. All constructs were sequenced by the dideoxy chain termination method (20).

DNase I Footprinting and Gel-Shift Assays. Liver nuclear extracts from both control and dexamethasone-treated animals (80 mg/kg) were prepared by the method of Gorski *et al.* (21) and stored at −80°C. Male Sprague–Dawley rats (Harlan–Sprague–Dawley) weighing 140–160 g were used in this study.

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Abbreviations: CYP, cytochrome P450; PCN, pregnenolone 16α-carbonitrile; TK, thymidine kinase; GRE, glucocorticoid responsive element; DexRE-1 and -2, dexamethasone response elements 1 and 2, respectively; GR, glucocorticoid receptor.

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The footprinting probe was a 365-bp promoter segment generated from P3–422 by digestion with *Sac*I/*Bam*HI (−422/−57). The 3′ recessed *Bam*HI site was labeled with [α-^{32}P] GTP using Sequenase version 2.0 (United States Biochemical). For gel-shift analysis dexamethasone response element 1 (DexRE-1) probe (5′-ATGTTAACTCAAAGGAGGTCAAAATAGGCT-3′) was prepared by annealing complementary oligonucleotides (Fisher Scientific). The glucocorticoid responsive element (GRE) probe was from Promega and had the following sequence: 5′-TCGACTGTACAGGATGTTCTAGCTACT-3′. Probes were end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. Gel-shift and footprint procedures were carried out as described (22). Monoclonal antibody to the glucocorticoid receptor (GR), BuGR2 (Affinity Bioreagents, Neshanic Station, NJ), or mouse pre-immune serum was added to reactions before addition of probe. Protein–DNA complexes were resolved by electrophoresis on a 4% polyacrylamide gel.

RESULTS

Effects of 5′ Flanking Region Deletions on Dexamethasone Induction. To identify regions important for dexamethasone responsiveness, various lengths of the CYP3A23 5′ flanking region were cloned upstream of the luciferase structural gene (Fig. 1). The transcriptional response to dexamethasone treatment remained high upon successive deletion from −1400 to −167; however, a substantial drop from 9- to 3.5-fold induction was observed when the region between −167 and −144 was deleted. Upon further deletion to −60, dexamethasone responsiveness was abolished. This stepwise decrease in transcriptional activity implies that either separate elements located between −167 and −60 contribute to induction or that one element located around −144 exists and was partially inactivated in preparing the P3-144 construct. The localization of dexamethasone responsiveness to this region agrees with studies by Burger *et al.* (23) on what was described as the CYP3A1 promoter. Inspection of this promoter sequence suggests that it belongs to the CYP3A23 gene rather than CYP3A1. Importantly, basal activity for constructs P3-1400 through P3-144 remained unchanged; hence, induction levels were not influenced by fluctuations in basal activity. However, deletion of nucleotides between −144 and −60 elicited an 80% drop in basal transcription (data not shown), suggesting that additional cis-acting elements important for basal expression of CYP3A23 may reside in this region.

Identification of DNA-Protein Interactions Within the Dexamethasone Responsive Region. In close proximity to the

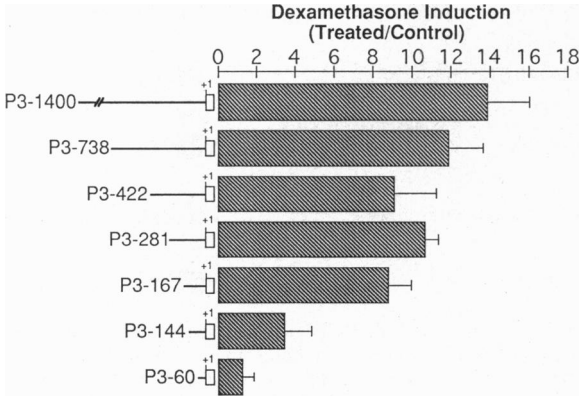

FIG. 1. Deletion analysis of the 5′ flanking region of the CYP3A23 gene. Promoter constructs were prepared as described in *Methods and Materials* and are named according to their 5′ terminus. All constructs have a common 3′ end (+112). Activities are expressed as mean dexamethasone induction ±SD for a minimum of three experiments.

−167 to −60 region implicated in the glucocorticoid response are several consensus sequences, including a TGTTC motif at −186 to −190 that resembles a GRE half-site, a CACCC-box (−173 to −177) and an imperfect AGGTCA direct repeat (−149 to −164) that resembles a steroid/hormone receptor consensus binding site (24–26). DNase I footprinting analysis of the −60 to −250 region revealed a protected region, site C, corresponding to the imperfect AGGTCA direct repeat (Fig. 2*A*), but no DNA–protein interactions were detected at either the putative GRE half-site or the CACCC consensus sites. Two additional protected regions, sites A and B, were identified 3′ of site C. Nuclear extracts from control and dexamethasone-treated animals gave qualitatively and quantitatively similar results, suggesting that the binding proteins, if inducible, were not in limiting concentrations. The nucleotide sequences corresponding to each footprint are underlined in Fig. 2*B*. The direct repeat within site C is highlighted by arrows. The site B footprint extends from −118 to −136 and contains a repeated ATGAACT sequence (arrows) that does not match any previously described cis-acting consensus element. The most proximal site, at −91 to −110 (site A), shares over 80% identity with an HNF-4 (hepatic nuclear factor) consensus element, RGRXCAAAGTXCAXYY, shown in other P450 genes to play a role in liver-specific constitutive expression (27–30). Interestingly, all three sites are located in the region implicated by deletion analysis to be important for dexamethasone responsiveness (Fig. 1). To determine the relative contribution of each element, additional reporter constructs were designed to test the functional importance of individual binding sites.

Cooperative Interaction of Sites B and C in the Dexamethasone Response. The ability of sites A, B, and C, either individually or in combination, to confer dexamethasone responsiveness to a heterologous promoter was tested by linking segments of the flanking region to a TK-luciferase reporter gene (Fig. 3). The −170TK construct, which possesses all three protein binding sites identified by footprint analysis, was induced about 8-fold in response to dexamethasone treatment. Removal of site A in the −170/−110TK construct did not significantly decrease the induction response, indicating that sites B and C are sufficient to maintain dexamethasone inducibility. However, when site C was placed alone upstream of the TK promoter (−170/−140TK), no significant induction was observed, suggesting both sites B and C are required to elicit a full response. A similar result was demonstrated for site B. The −144TK construct, containing both sites B and A, displayed more than a 3-fold induction, which is in agreement with P3-144 activity reported above (Fig. 1). This response was lost when site B was deleted in the −125TK construct, demonstrating the importance of site B to dexamethasone responsiveness. Site B by itself, however, cannot support induction, because the −144/−110TK construct displayed background activity similar to the TK-luciferase vector. Therefore, neither site C nor site B alone can mediate a dexamethasone response, but together they confer sensitivity to a heterologous promoter comparable to that observed with the CYP3A23 promoter. For the purpose of further delineating the responsive sequences, the imperfect direct repeat contained within site C (AACTCAAAGGAGGTCA) and the ATGAACT direct repeat contained in site B were designated DexRE-1 and -2, respectively.

Modulation of the Dexamethasone Response. Because the DexRE-1 resembles the AGGTCA class of steroid responsive elements that would not be predicted to mediate a glucocorticoid response (see *Discussion*), we wanted to investigate its unique role in the CYP3A23 activation pathway more closely. A set of mutants was made to determine the effects of altering the DexRE-1 element on dexamethasone induction. Four mutant constructs showed a marked reduction in their sensitivity to dexamethasone relative to wild-type P3-210 (Fig. 4*A*).

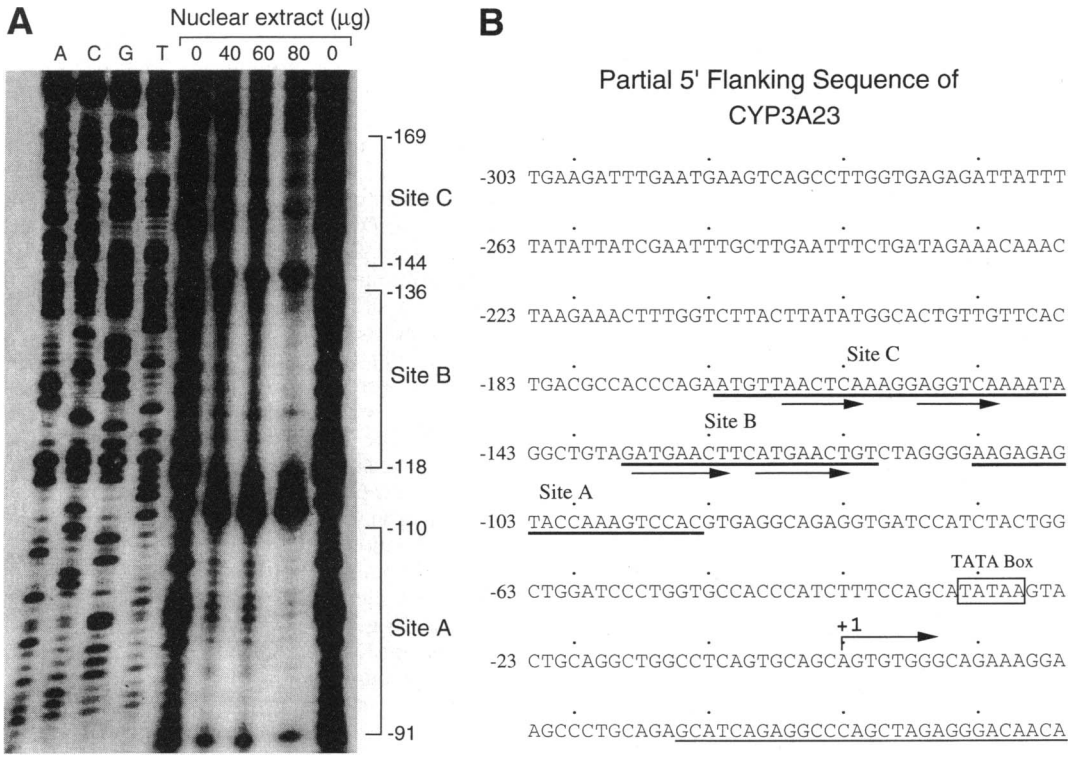

FIG. 2. Sequence and protein-binding regions of the CYP3A23 promoter. (*A*) DNase I footprint analysis of the 5′ flanking region using liver nuclear extract from dexamethasone-treated rats. The reactions were subjected to PAGE on a 6% denaturing gel in parallel with a sequencing reaction in the first four lanes. (*B*) Partial sequence of the CYP3A23 promoter. The protected regions are underlined and labeled sites A, B, and C. Arrows highlight direct repeat elements. The +1 position was determined by primer extension.

The P3-210(Δ-158) and P3-210(Δ155-158) constructs had removed one or all of the intervening nucleotides between the direct repeat elements. These mutants displayed 41 and 43% of wild-type induction, respectively, demonstrating the negative impact of altering direct repeat spacing. In P3-210(A163G), the first core motif of the direct repeat was altered from AACTCA to AGCTCA based on a mismatch at this site between CYP3A23 and CYP3A2. This single base change caused nearly a 50% drop in activity. Data from the 5′ deletion (Fig. 1) and TK promoter (Fig. 3) experiments showed that a 3- to 4-fold induction response was observed after deletion of the −167 to −144 region containing DexRE-1. To definitively show that all of the dexamethasone responsiveness conferred by the −167 to −144 region could be localized to DexRE-1, the entire direct repeat element was deleted (Fig. 4*A*). As expected, the P3-210(Δ149–164) construct showed 3.8-fold induction, which was equivalent to activities observed for both P3-144 (Fig. 1) and −144TK (Fig. 3) constructs. These results demonstrate that DexRE-1 plays a crucial role in mediating much of the dexamethasone-sensitive transcriptional activity in CYP3A23.

Although the DexRE-1 mediates glucocorticoid activation, its sequence, AACTCAnnnnAGGTCA, is homologous to a class of steroid responsive elements recognized by the estrogen receptor family. To determine if a perfect consensus could also mediate dexamethasone responsiveness in CYP3A23, two additional mutants were constructed. DexRE-1 was converted to either a vitamin D_3 or a thyroid hormone responsive element with AGGTCA direct repeats spaced by either 3 or 4 nucleotides, respectively. Both mutant constructs showed an enhanced transcriptional response compared to wild type (Fig. 4*B*). The P3-210(DR4) construct displayed nearly a 38-fold induction, which is 2.5 times the activity of wild type, while P3-210(DR3), which contains the three nucleotide spaced direct repeat, displayed a 26-fold induction. It is intriguing that a mutant containing the DR4 consensus site, which differs from P3-210 by two nucleotides, shows enhanced dexamethasone responsiveness, while the P3-210(A163G), which differs by only one nucleotide, has a decreased induction response.

Lack of GR Association with the DexRE-1. The CYP3A23 gene shows a strong transcriptional enhancement in response to not only dexamethasone but also to other GR ligands (P.M. and C.B.K., unpublished results). However, the elements identified as necessary for the dexamethasone response show

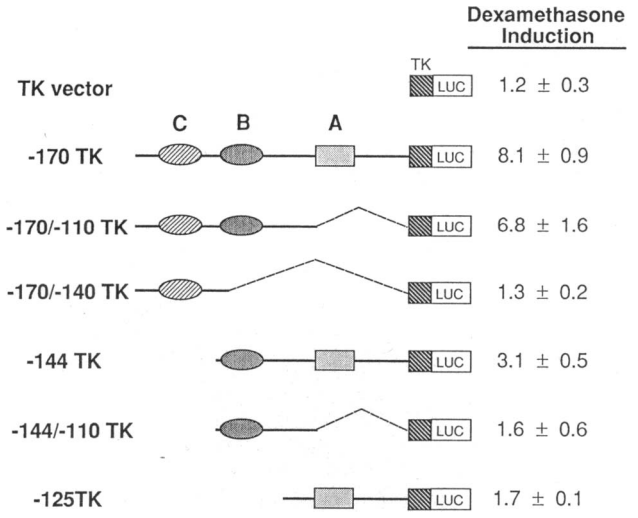

FIG. 3. Ability of CYP3A23 flanking sequences to confer dexamethasone responsiveness to a heterologous promoter. Constructs −170TK, −144TK, and −125TK are identified by their 5′ base; all have −60 as their common 3′ terminus. Other constructs such as −170/−110TK are identified by their 5′/3′ termini. This defines the CYP3A23 flanking segment cloned upstream of the TK promoter. Sites A, B, and C are those identified by footprint analysis in Fig. 2. Activities are given as mean dexamethasone induction ±SD for at least three experiments.

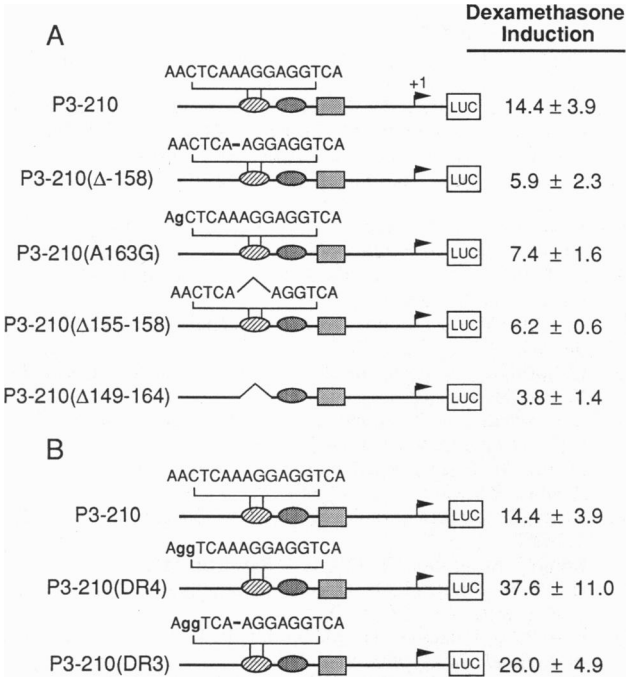

FIG. 4. Mutational analysis of DexRE-1 contained in site C. In each case, the sequence is given and specific nucleotide changes are shown in lowercase boldface letters, and single base deletions are denoted by a dash. Deletions of more than one nucleotide are represented by a ^. All constructs have identical 5′ (−210) and 3′ (+112) termini. Fold induction is expressed as ±SD for at least three experiments. (*A*) Alterations in DexRE-1 that lead to decreased dexamethasone induction. (*B*) Conversion of DexRE-1 to steroid responsive elements having a perfect AGGTCA direct repeat with n = 3 and 4, respectively.

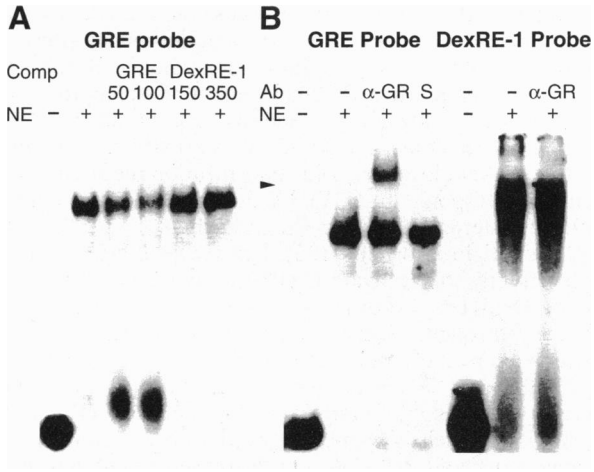

FIG. 5. Gel-shift analysis of DexRE-1 and comparison to a GRE. (*A*) Competition of DexRE-1 with GRE for complex formation. Unlabeled competitors (Comp) were present at the indicated fold excesses. (*B*) Reaction of anti-GR antibody (Ab) with GRE and DexRE-1 protein complexes. S, pre-immune serum: NE, nuclear extract.

no homology to a GRE, which is required for high-affinity binding of activated GR. Although GR would not be predicted to associate with either DexRE-1 or -2, it was necessary to investigate whether it might have a novel association with a nonconsensus element in the CYP3A23 gene. Gel-shift assays were performed with oligonucleotides corresponding to the DexRE-1 and to a consensus GRE to examine the interaction of each with GR (Fig. 5). In competition experiments, labeled GRE, when incubated with rat liver nuclear extract from dexamethasone-treated animals, produced a retarded complex that was inhibited by a 50-fold excess of unlabeled GRE but was unaffected by a 350-fold excess of unlabeled DexRE-1 (Fig. 5*A*). This suggests that the DexRE-1 does not have a measurable affinity for GR present in this complex. Addition of GR antibody to the GRE binding reaction produced a supershift that is consistent with the presence of GR in the GRE-protein complex (Fig. 5*B*). The DexRE-1 probe also formed a complex with nuclear extract that was distinct from the GRE-protein complex and did not supershift with the GR antibody (Fig. 5*B*). Furthermore, supershift experiments performed with a DexRE-2 probe showed no evidence of GR binding (data not shown). Collectively, these results demonstrate that GR does not bind to either DexRE-1 or -2 and, therefore, cannot act directly as a ligand-activated transcription factor in the dexamethasone induction of the CYP3A23 gene.

DISCUSSION

The current study describes the identification and characterization of the dexamethasone responsive region of the CYP3A23 gene, a member of the CYP3A family. CYP3A23 is homologous to, but distinct from, CYP3A1 (31) another glucocorticoid-inducible member of the CYP3A gene family. While the complete CYP3A1 gene remains to be cloned, the CYP3A23 gene has been isolated and characterized (S.I.W. and C.B.K., manuscript in preparation) and the promoter region of this gene is the focus of this investigation.

Earlier studies by Guzelian and coworkers (10, 32) established that glucocorticoid induction of the CYP3A family differed in several significant aspects from the induction response of the prototypical glucocorticoid-inducible gene, tyrosine amino transferase. These differences were manifested in (*i*) time course of induction, (*ii*) order of responsiveness to various glucocorticoids, (*iii*) the dexamethasone concentration required for optimal induction, and (*iv*) the response to pregnenolone 16α-carbonitrile (PCN). In the latter case, PCN, an antiglucocorticoid, inhibited tyrosine amino transferase induction by dexamethasone but stimulated the glucocorticoid induction pathway for CYP3A (32).

Two major unresolved issues in the regulation of CYP3A genes are (*i*) the role of GR in this nonclassical glucocorticoid response and (*ii*) the identity and role of regulatory proteins participating in the induction pathway. In characterizing the dexamethasone responsive region of the CYP3A23 promoter, GR was shown not to have a direct role in transactivation. Two binding sites, DexRE-1 and -2, were demonstrated by functional analysis to mediate dexamethasone induction in a cooperative fashion. Neither of these sites contain a consensus GRE, and gel-shift analyses demonstrated a lack of association of GR with either site. Instead, DexRE-1 resembles a thyroid responsive element (26) having an AGGTCA imperfect direct repeat separated by 4 nucleotides, and DexRE-2 has an ATGAACT direct repeat, an element not previously identified with a glucocorticoid transcriptional response.

Our results suggest a role for members of the estrogen receptor family that recognize an AGGTCA core motif. This family includes vitamin D_3, thyroid hormone, retinoic acid, and retinoid X receptors as well as numerous orphan receptors (26, 33). When DexRE-1 is converted to either a perfect thyroid hormone or vitamin D_3 (spacing of three nucleotides) responsive element, dexamethasone inducibility of the CYP3A23 promoter is markedly enhanced. Since H4IIE cells require transfection with thyroid receptor to elicit a response with T_3 (K. A. O'Leary, P.M., and C.B.K., unpublished results), it is unlikely that transcriptional enhancement of the DR4 construct (Fig. 4) involves thyroid receptor; however, another receptor of this family may mediate this response.

Several models can be proposed that incorporate previously defined aspects as well as the characteristics of the mechanism

elucidated by the present work. Because our data demonstrate that elements important for the dexamethasone response are binding sites for nuclear proteins immunochemically unrelated to GR, one possibility is the involvement of a receptor having reduced affinity for glucocorticoids and an altered ligand specificity when compared to GR. This would help explain the 100-fold higher glucocorticoid concentration required to elicit a maximal response for CYP3A23 as compared to tyrosine amino transferase as well as the inducibility of CYP3A23 by PCN, an antiglucocorticoid (32). This receptor ligand complex might directly transactivate CYP3A23 by means of DexRE-1 and/or DexRE-2. Alternatively, this receptor may function indirectly through a signal transduction pathway to activate downstream transcription factors that in turn act directly at the CYP3A23 locus. An issue unresolved by these models, however, is the possible indirect involvement of GR in this mechanism, which is supported by the observation that the antiglucocorticoid, RU 486, can decrease dexamethasone induction of members of the CYP3A family (23).

Our findings demonstrate that multiple cis-acting elements present in the 5′ flanking region of CYP3A23, which are distinct from the consensus GRE, cooperatively mediate the glucocorticoid response of this gene. Therefore, the CYP3A23 induction pathway may be an example of an alternate mode through which glucocorticoids exert their effects. Future studies will focus on characterizing the interaction between these multiple elements and elucidating the role of steroid receptors in the induction process.

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