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# Trans-species Gene Transfer for Analysis of Glucocorticoid-Inducible Transcriptional Activation of Transiently Expressed Human *CYP3A4* and Rabbit *CYP3A6* in Primary Cultures of Adult Rat and Rabbit Hepatocytes

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**SUMMARY**

Interindividual variation in the spontaneous and in the glucocorticoid- or rifampicin-inducible expression of the *CYP3A* cytochromes P450, the dominant forms of this supergene family that catalyze the oxidation of numerous drugs and environmental chemicals in human liver, remains largely unexplained, due in part to the lack of a validated animal model. We analyzed the 5′-flanking sequences of *CYP3A* genes from the rat (*CYP3A23*, *CYP3A2*), rabbit (*CYP3A6*), and human (*CYP3A4*, *CYP3A5*, *CYP3A7*) and found variable regions separated by three areas (consensus I, II, and III) of sequence homology immediately upstream of their respective promoters. We used trans-species gene transfer *in cellulo* as a new approach for determining the basis for qualitative differences among species in liver expression of different forms of *CYP3A*. When we transfected into cultured rat hepatocytes vectors containing 5′-flanking DNA from *CYP3A23*, *CYP3A4*, or *CYP3A6* genes, we found that CAT activity was induced on treatment with dexamethasone or pregnenolone-16α-carbonitrile only if consensus II sequences were included. Rifampicin treatment had no effect. When the same constructions containing consensus II were transfected into rabbit hepatocytes, increased activity was observed on treatment of the cells with dexamethasone or with rifampicin but not with pregnenolone-16α-carbonitrile. These results suggest that the host cellular environment rather than the structure of the gene dictates the pattern of *CYP3A* inducibility. The application of this new model system will provide a unique technique for identifying mechanisms of induction and advancing the development of appropriate toxicological models for human safety assessment.

The liver plays an important role in the interaction between humans and the chemical environment. An important locus of that interaction is the supergene family of microsomal hemoproteins known collectively as the CYPs. In humans, the dominant form of liver *CYP*, now called *CYP3A3/4*[2] (1, 2), catalyzes and, in many instances, may limit the rate of the oxidative metabolism of >60% of all clinically used drugs (3) as well as a wide array of lipophilic environmental pollutants, including carcinogens and chemi-

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[2] The sequence of the 5′-noncoding region of the rat genomic clone used in this study and in previous publications (23, 25) referred to as *CYP3A1* is completely identical to that of the recently isolated *CYP RL33* (40) but different from that of *CYP 3A1* cDNA. This gene was originally assigned the name of *CYP3A1* by the Nomenclature Committee (41) based on the sequencing of a cDNA and the 5′-flanking segment of the rat gene (23). This gene, inducible by DEX, PCN, and phenobarbital, has been assigned the name *CYP3A23* (D. Nelson, personal communication). The *CYP3A1* cDNA probes used previously to study the regulation of rat liver by DEX or PCN were incapable of distinguishing between *CYP3A1* and *CYP3A23*.

**ABBREVIATIONS:** CYP, cytochrome P450; DEX, dexamethasone; PCN, pregnenolone-16α-carbonitrile; RIF, rifampicin; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; PCR, polymerase chain reaction; MEM, minimum essential medium; bp, base pair(s).

cal toxins (4). There is a wide (>10-fold) variation in the amount of *CYP3A4* protein (5, 6), mRNA (5, 7), and activity (8) among individuals [as well as polymorphic differences in expression of minor forms *CYP3A5* and *CYP3A7* (9–11)], but the factors that account for such differences remain largely unknown, due in part to the difficulties of studying such questions in humans. Although there are substantial structural and catalytic similarities among the various members of the *CYP3A* family across species lines, there are important differences in regulatory control of these genes (for a review, see Ref. 12). We and others have shown that glucocorticoids such as DEX as well as phenobarbital and phenobarbital-like agents induce expression of *CYP3A4* in human liver (13) and in primary cultures of human hepatocytes (5, 7, 14). Both these compounds and other groups of agents (macrolide antibiotics, imidazole antimycotics, and some antiglucocorticoids) induce a homologous *CYP* form, *CYP3A23*, in rat liver (15–18) and in primary cultures of adult rat hepatocytes (18, 19). However, a clear discrepancy between these two species is that the antibiotic RIF induces *CYP3A4* in human liver (13) but does not induce *CYP3A23* in rat liver (20). RIF does induce *CYP3A6*, the homologous form, in rabbit liver (21) and in rabbit hepatocyte cultures (7), yet in the rabbit, the antiglucocorticoid PCN, which induces *CYP3A23* in rat liver (20) and may induce *CYP3A4* in some but not all human livers (5, 7), does not induce *CYP3A6*. Given the widespread metabolic importance of *CYP3A*, it would be of great clinical benefit to find an appropriate animal model for use in developing a better understanding of the regulatory control and interindividual heterogeneity in liver expression of *CYP3A* in humans.

Efforts to define the molecular genetic basis for CYP3A enzyme induction have been immeasurably assisted by the development of a system for maintaining differentiated function in primary cultures of adult rat hepatocytes maintained on a reconstituted basement membrane, Matrigel (22). In this system, it has been established unequivocally that glucocorticoids and, paradoxically, some antiglucocorticoids such as PCN act individually and synergistically to increase the amount of CYP3A23 protein and mRNA (16, 23). The cultured rat hepatocyte system has proven to be invaluable for demonstrating that accumulation of CYP3A23 mRNA is largely attributable to stimulation of *CYP3A23* gene transcription. Thus, when segments of 5′-flanking DNA isolated from *CYP3A23* and inserted into recombinant vectors upstream of a heterologous, viral promoter (TK) and a reporter gene (CAT) (24) were transiently transfected into cultured hepatocytes treated with DEX, PCN, or both, transcription of this *CYP3A23* chimeric gene was activated in a manner that mirrored completely the accumulation of endogenous *CYP3A23* mRNA in these cultured hepatocytes (23). In an extension of these studies, we determined that a 33-bp subfragment of this 5′-flanking *CYP3A23* DNA cloned into this vector in either forward or reverse orientation acts as an inducible enhancer, still supporting DEX and PCN stimulation of expressed CAT activity in cultured rat hepatocytes (25). Because this 33-bp element contains no recognizable glucocorticoid response element and fails to bind to the glucocorticoid receptor, we concluded that activation of *CYP3A23* gene transcription by glucocorticoids may involve proteins already bound to the controlling element in the *CYP3A23* gene through a mechanism in which glucocorticoid receptor in the presence of hormone does not bind directly to *CYP3A23* DNA.

In the current study, we compared sequences of the 5′-flanking DNA of human and rabbit *CYP3A* family members with those of the rat *CYP3A23* DEX/PCN enhancer and found areas of substantial homology. Still, it remained unclear whether differences in the structure of the putative control regions of human and rabbit *CYP3A* genes (i.e., *cis*-acting differences) account for the striking qualitative differences in interspecies *CYP3A* inducibility or whether *trans*-acting factors resident in the liver cell hosts control these effects. Fortunately, the present hepatocyte culture system offers a way to explore this question and to establish which animal model best represents human regulatory control of *CYP3A* (7). We transferred rabbit (*CYP3A6*) and human (*CYP3A4*) regulatory segments into vectors, expressed them transiently in cultured hepatocytes from the rat and rabbit, and found that the cellular environment rather than differences in gene structure seems to fully rationalize the observed species differences in regulatory expression of these genes.

## Experimental Procedures

**Animals and materials.** Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 190–220 g and male New Zealand White rabbits (Star Pines, Parker, CO) weighing 0.8–1.0 kg were maintained in wire-bottom cages with free access to animal chow and water. Collagenase (Type I) was purchased from Worthington Biochemical Corporation (Freehold, NJ). General tissue culture reagents, bicinchoninic acid microassay reagents, and DEX were purchased from Sigma Chemical Co. (St. Louis, MO). Opti-MEM, Eagle's MEM, and Lipofectin were purchased from Life Technologies, GIBCO-BRL (Grand Island, NY). Matrigel was prepared from the Engelbreth-Holm-Swarm tumor (26) provided by Dr. Hynda Kleinman (National Institute of Dental Research, National Institutes of Health, Bethesda, MD). Reagents used in the PCR were purchased from Perkin Elmer (Norwalk, CT). The CAT ELISA system was purchased from Boehringer Mannheim (Indianapolis, IN). PCN was a gift from John Babcock (The Upjohn Co., Kalamazoo, MI). The reporter plasmid pBLCAT2 (24) was provided by G. Schutz (Institute for Cell and Tumor Biology, Heidelberg, Germany). Oligonucleotides were synthesized with a Biosearch Cyclone DNA synthesizer and purified through high performance liquid chromatography or purchased from Macromolecular Resources (Fort Collins, CO).

**Construction of chimeric plasmids and preparation of double-stranded oligonucleotides.** *CYP3A4* 5′-flanking fragments corresponding to sequences −179 to −35 and −152 to −35 were generated through PCR with a previously isolated genomic clone (27) as template. Anchored PCR (28), an amplification technique used when only a small region of target sequence is known, was used to amplify *CYP3A4* genomic sequences extending from −2200 to −35. The specificity of the amplification reaction was ensured through the use of a specific primer complementary to a known 3′ sequence (−59 to −35) and ligating a short oligonucleotide (anchor adapter) to the complementary 5′ overhang of a filled-in *Nco*I restriction site at −2200, artificially creating a known sequence. After the first cycle of PCR, in which extension of the specific primer along the target DNA includes the ligated anchor adapter, a third oligonucleotide (anchor template/primer containing the added adapter sequences) serves as the priming site in subsequent PCR cycles. *CYP3A6* 5′-flanking fragments extending from −186 to −37 and −161 to −37 were generated through PCR with a *CYP3A6* genomic clone isolated from a rabbit EMBL3 genomic library as template. Complementary oligonucleotides spanning sequences −175 to −152 (5′-GAATATGAACTCAAAGGAGGTCA-3′) of *CYP3A4* and sequences −182 to −160 (5′-

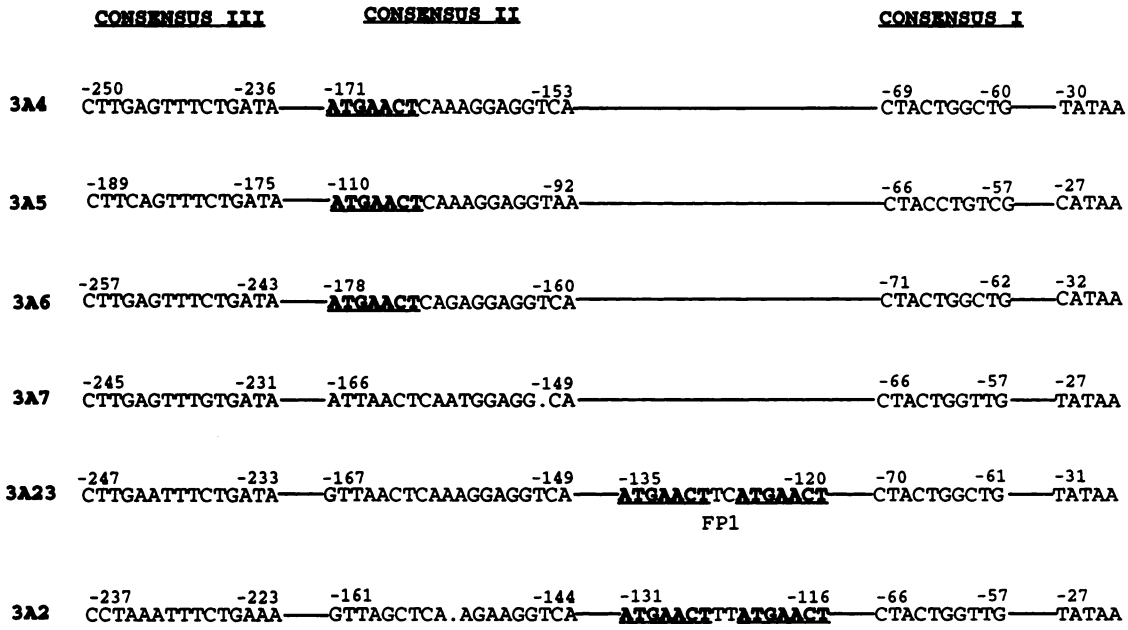


**Fig. 1.** Consensus sequences of the 5′-flanking region of the *CYP3A* genes. Nucleotides for each gene are numbered relative to their respective transcriptional start sites. *Bold underlined sequences*, the 7-bp direct repeat [located in a DNase I-resistant (footprint) site, FP1, described previously (25)] of the *CYP3A23* enhancer and the single 7-bp copy in consensus II of *CYP3A4*, *CYP3A5*, and *CYP3A6*. The flanking sequences of the *CYP3A* genes presented (other than *CYP3A23* and *CYP3A2*) do not contain the direct repeats of FP1 and are not included in the figure. *Periods within consensus sequences*, gaps in alignment.

GCACATGAACTCAGAGGAGGTCA-3′) of *CYP3A6* were annealed, and the double-stranded oligonucleotides were purified through polyacrylamide gel electrophoresis. The PCR fragments containing 5′ *Hin*dIII and 3′ *Bam*HI restriction sites and the double-stranded oligonucleotides containing *Bam*HI restriction sites at each end were then cloned in the 5′ to 3′ orientation in front of the TK promoter of the pBLCAT2 reporter plasmid (TK-CAT) and sequenced (29) to confirm the fidelity of the amplification reaction and to determine the number of copies and orientation of the double-stranded oligonucleotide. The cloning of these fragments into pBLCAT2 did not artificially create the DEX-responsive direct repeats of FP1 previously identified in *CYP3A23*. Construction of *CYP3A23* −175/−36 was as described previously (25).

**Sequence analysis.** Sequences for the 5′-flanking *CYP3A* gene comparison were obtained from the following references: *CYP3A23* (23), *CYP3A2* (30), *CYP3A4* (31), *CYP3A5* (32), *CYP3A7* (33), and *CYP3A6*.[3] A Genetics Computer Group (Madison, WI) software package was used to perform DNA sequence alignments.

**Preparation of hepatocytes and DNA transfections.** Primary cultures of rat and rabbit hepatocytes were prepared as described previously (7, 34). Hepatocytes freshly isolated through collagenase perfusion of the livers of untreated rats or rabbits were incubated in a humidified atmosphere of 95% air/5% $CO_2$ in a modification of Waymouth MB-752 medium containing 0.1 μM insulin as the only hormone. Hepatocytes ($0.38 \times 10^6$) were incubated in 35-mm plastic culture dishes (Lux) in 1 ml of medium supplemented with 5% fetal calf serum. At 24 hr, the medium was removed, and the cultures were maintained for 24–66 hr in serum-free Eagle's MEM containing insulin. At 66 hr, each dish of hepatocytes was washed with 1 ml of Opti-MEM and exposed to a mixture formed by combining 12 μg of Lipofectin with 3.5 μg of DNA as recommended by the manufacturer. After 6 hr of incubation, the Lipofectin/DNA mixture was removed, and the cultures were incubated with 1 ml of Waymouth medium containing 294 μg of Matrigel and inducers. Inducers were added as 1000-fold stocks in dimethylsulfoxide. The medium was replaced at 24-hr intervals with Waymouth containing inducers only.

**Assay of CAT protein.** After 66 hr of treatment with inducers, cell lysates were prepared as described previously (25) and assayed for CAT protein according to instructions provided by the manufacturer. Total cellular protein was measured with a bicinchoninic acid microassay. Transfection efficiency in several culture preparations was in the range of 5–10%, and CAT protein measured from duplicate plates varied by <10%.

## Results

Computer-assisted sequence analysis of the 5′-flanking regions of rat genes (CYP3A23, *CYP3A2*), human genes (*CYP3A4*, *CYP3A5*, *CYP3A7*), and a rabbit gene (*CYP3A6*) revealed three regions sharing a high degree of sequence similarity (Fig. 1). These intergene "consensus" regions were located ~60–70 (consensus I), ~150–170 (consensus II, except for *CYP3A5* at 92–110), and ~230–250 (consensus III) bp upstream of the transcription start sites. A previous deletion analysis of 5′-flanking *CYP3A23* DNA cloned into a heterologous TK-CAT expression vector and transiently expressed in primary cultures of rat hepatocytes identified a 164-bp segment (between −220 and −56 relative to the start site of *CYP3A23* transcription) that conferred induction by DEX or PCN and exhibited synergy (rather than inhibition) with treatment by DEX plus PCN (23). This segment encompassed two consensus regions.

To determine whether *cis*-acting sequences in the three consensus regions are involved in glucocorticoid regulation of *CYP3A* genes, we selected segments of DNA containing the conserved regions of a human gene, *CYP3A4*, and the rabbit gene, *CYP3A6*, for further analysis. When a 5′-flanking segment of *CYP3A4* (−2200 to −35) was cloned into the TK-CAT expression vector and transfected into cultured rat hepatocytes, the basal activity was increased 4.5- and 3.4-fold on

[3] The 5′ nucleic acid sequences used in this article have not been published; they were, however, submitted by R. H. Tukey to GenBank under accession No. U40569.

treatment with DEX ($10^{-5}$ M) or PCN ($10^{-5}$ M), respectively. In repeating this experiment with *CYP3A4* (−227 to −35), (5.5-fold, DEX; 3.2-fold, PCN) results were obtained that were similar to those we previously reported with a comparable segment of *CYP3A23* (−220 to −56) (23). This remarkable result of a trans-species gene transfer indicates that the rat hepatocyte has sufficient factors to allow inducible expression of a human *CYP3A* gene. However, we recently made subsequent deletions to more closely define the DEX/PCN-responsive sequences in *CYP3A23*, and we found a 33-bp segment located at −115 to − 148 that deletes all three of the consensus regions yet fully supports the DEX/PCN induction in cultured rat hepatocytes observed with longer segments of 5′ *CYP3A23* (25). Close inspection of the 33-bp *CYP3A23* inducible enhancer showed homology to its companion rat gene *CYP3A2* (−116 to −131, Fig. 1) but not to the other human or rabbit *CYP3A* genes. However, the 33-bp *CYP3A23* enhancer contains a 7-bp direct repeat *ATGAACT*-tc*ATGAACT* located between consensus I and consensus II (Fig. 1). One half of this possible binding site (ATGAACT) is matched exactly within the consensus II regions of *CYP3A4* and *CYP3A6*.

Based on an assumption that the ATGAACT sequence is responsible for the inducible function of the *CYP3A* DNA fragments, we transiently expressed in primary cultures of rat and rabbit hepatocytes cloned segments of DNA from both *CYP3A4* and *CYP3A6* that either contained or had deletions of consensus II. A similar fragment of *CYP3A23* served as a comparison. Measurement of CAT expression after 66-hr treatment of the rat hepatocytes with DEX or PCN showed that only the fragments containing consensus II were inducible by DEX (17–28-fold) (Fig. 2). Each fragment inducible by DEX was also inducible by PCN (4–8-fold) (Fig. 2). No inducible activity was seen in constructions in which consensus II was deleted (Fig. 2). Indeed, a *CYP3A4* construction containing either none of the three consensus regions (−70 to −152, located between consensus I and consensus II; not shown) or only consensus I (−35 to −152, Fig. 2) gave no inducible expression on treatments with DEX, PCN, or RIF. Furthermore, a construction prepared from the rabbit gene *CYP3A6* (−37 to −161), homologous to *CYP3A4* (−35 to −152) and containing only consensus I, also gave no DEX-, PCN-, or RIF-inducible expression in the cultured rat hepatocytes (Fig. 2). This pattern of transcriptional activation observed for the human and rabbit *CYP3A* genes is identical to the expression of the transfected *CYP3A23* sequences, which showed 16-fold induction by DEX and 6-fold induction by PCN but no induction by RIF (Fig. 2). In contrast, when identical constructions containing consensus II were transiently expressed in primary cultures of rabbit hepatocytes, increased CAT expression was observed on treatment of cells with DEX (~3-fold) and RIF (~3-fold) but not with PCN (Fig. 2).

To confirm the involvement of consensus II sequences in glucocorticoid induction of *CYP3A* genes, a 23-bp double-stranded oligonucleotide consisting of the sequence 5′-GAATATGAACTCAAAGGAGGTCA-3′ (spanning nucleotides −175 to −152) from the *CYP3A4* gene was ligated 5′ of the TK promoter in the TK-CAT expression vector. In primary rat hepatocyte cultures transfected with *CYP3A4* (−175 to −152) TK-CAT (Table 1), DEX treatment induced CAT expression by 27-fold, PCN treatment induced CAT expression by 6–11-fold, and RIF treatment had no effect. This 23-bp sequence also confers the synergistic effect of PCN

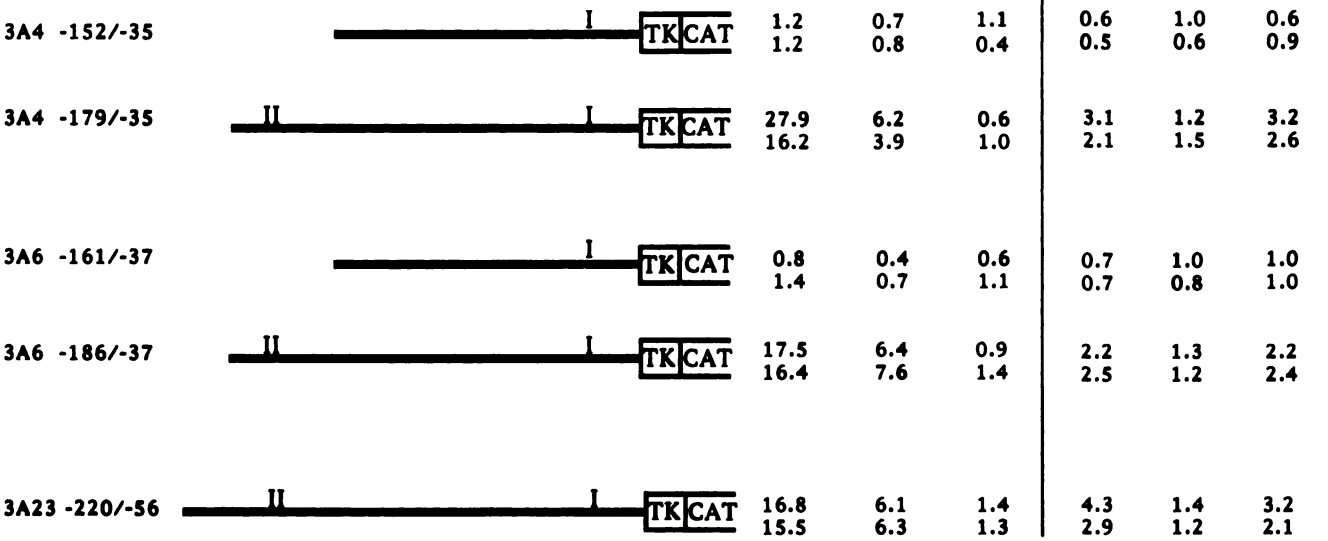


**Fig. 2.** Expression of *CYP3A4* and *CYP3A6* chimeric plasmids in primary cultures of rat and rabbit hepatocytes. *CYP3A4* and *CYP3A6* 5′-flanking sequences generated by PCR were cloned into pBLCAT2 (TK-CAT) as described in Experimental Procedures. Each construction contains either consensus I plus consensus II sequences or only consensus I sequences. Transfected hepatocytes were treated for 48–66 hr with 10 μM DEX (rat), 50 μM DEX (rabbit), 10 μM PCN, or 100 μM RIF and analyzed for CAT expression. CAT induction is presented as the ratio of CAT protein of induced cells to uninduced control cells from two independent transfection experiments.

TABLE 1

**Expression of chimeric *CYP3A4* TK-CAT and *CYP3A6* TK-CAT plasmids in primary hepatocytes**

Double-stranded oligonucleotides spanning sequences −175 to −152 of *CYP3A4* and −182 to −160 of *CYP3A6* cloned into TK-CAT as described in Experimental Procedures were compared with *CYP3A4*- TK-CAT (−179 to −35) in transient transfection assays. Transfected hepatocytes were treated for 66 hr with 10 μM DEX (rat), 50 μM DEX (rabbit), 10 μM PCN, or 100 μM RIF and analyzed for CAT expression. CAT induction is expressed as the ratio of CAT protein of induced cells to uninduced control cells from one (*CYP3A6*) or two (*CYP3A4*) independent transfection experiments in each species.

| Chimeric plasmids | CAT induction | | | | | |
|---|---|---|---|---|---|---|
| | DEX | | PCN | | RIF | |
| | Rat | Rabbit | Rat | Rabbit | Rat | Rabbit |
| | *fold increase relative to untreated transfected hepatocytes* | | | | | |
| CYP3A4 (−179 to −35 TK-CAT) | 27.9<br>16.2 | 2.1<br>3.1 | 6.2<br>3.9 | 1.0<br>1.2 | 0.6<br>1.0 | 2.1<br>3.2 |
| CYP3A4 (−175 to −152 TK-CAT) | 26.7<br>27.3 | 5.0<br>6.4 | 5.7<br>10.9 | 1.8<br>0.8 | 0.8<br>0.5 | 3.3<br>3.6 |
| CYP3A6 (−182 to −160 TK-CAT) | 20.4 | 4.2 | 7.2 | 0.6 | 1.8 | 3.3 |
| TK-CAT | 0.8<br>0.9 | 0.6<br>0.7 | 0.8<br>0.3 | 1.0<br>0.6 | 1.2<br>1.8 | 0.5<br>0.7 |

on *CYP3A4* induction by DEX demonstrated previously for *CYP3A23* (23, 25) (not shown). In primary rabbit hepatocyte cultures transfected with *CYP3A4* (−175 to −152) TK-CAT, DEX treatment induced CAT expression by 6-fold, RIF treatment induced CAT expression by 4-fold, and PCN treatment had no effect (Table 1). The same host-specific induction responses to treatments with DEX, PCN, or RIF were observed in rat and rabbit hepatocytes transiently expressing the homologous *CYP3A6* (−160 to −182) TK-CAT construction (Table 1).

## Discussion

In the current study, we used sequence analysis to find three "consensus" areas of DNA homology in the 5′-flanking regions of human, rat, and rabbit members of the *CYP3A* family (Fig. 1). Deletion analysis of the 5′-flanking region of *CYP3A23* transiently expressed in primary cultures of rat hepatocytes disclosed a minimal DEX/PCN-responsive element (located between consensus I and consensus II) containing a heptameric direct repeat (*ATGAACT*tc*ATGAACT*) (Fig. 1) that binds rat liver nuclear proteins based on footprint and gel-shift analyses (25). Finding ATGAACT as a single copy in consensus II of each of the other members of the *CYP3A* family suggested a region of regulatory significance. We verified this hypothesis by showing that consensus II segments alone (or the relevant oligonucleotides) of human *CYP3A4* and rabbit *CYP3A6* cloned into the TK-CAT expression vector and transferred into cultured rat and rabbit hepatocytes were inducible by DEX treatment of the cells (Fig. 2). More importantly, treatment of the rat cells with PCN also induced rabbit *CYP3A6* and human *CYP3A4* to an extent equal to that observed for the homologous *CYP3A23* gene. Indeed, transfection of *CYP3A6* and *CYP3A4* constructions into primary cultures of rabbit hepatocytes were inducible by treatment with RIF but not by PCN treatment, a pattern that mirrors the response of intact rabbit liver to these two *CYP3A* inducers. We conclude that the failure of PCN to induce liver *CYP3A* in rabbits (20) and in some humans (7) is not attributable to a deficit in the DNA structure of the controlling sequences of *CYP3A6* or *CYP3A4* but more likely reflects a deficiency of essential cellular factors resident in the rat hepatocyte. By analogy, the rat hepatocyte seems to lack essential factors that permit the rabbit liver cell to respond to RIF with induction of *CYP3A23* and other *CYP3A* forms. The lack of induction by RIF of any of the *CYP3A* genes we transfected into cultured rat hepatocytes is fully consistent with the hypothesis that the host cell dictates the pattern of *CYP3A* gene induction because RIF is not an inducer of liver *CYP3A* in rats (20) but is well known to induce *CYP3A6* in rabbits (20, 21) and *CYP3A4* in humans (5, 7).

The identity is unknown of the postulated factor(s) in the rat liver cells that confers PCN inducibility on human and rabbit genes that are not normally inducible by PCN in their homologous cellular hosts. Studies of rat liver nuclear proteins that bind to the *CYP3A2* gene (35) revealed a site (called 6βA-A) in the 5′-flanking region (−106 to −87) that is thought to bind HNF-4 (or a related protein), a unique "orphan" member of the family of ligand-dependent nuclear transcription factors that reside in the nucleus and have an unknown ligand (for a review, see Ref. 36). Through transfection of this fragment into cultures of a human hepatoma, HepG2, the authors concluded that this site (located between consensus I and consensus II, downstream of the direct repeat (*ATGAACT*tc*ATGAACT*; see Fig. 1) was essential for basal expression of the homologous or of a viral promoter (inducible expression was not tested). The immediate upstream region of *CYP3A2* that does contain the direct repeat (called 6βA-B) was found to bind an unknown rat liver nuclear protein, as we found for the homologous segment of *CYP3A23* (25), but was not essential for *CYP3A2* gene transcription (35). In contrast, we found that the segment of *CYP3A23* containing the direct repeat (free from the equivalent 6βA-A or the further upstream binding site, 6βA-C) is fully sufficient to support basal as well as DEX- or PCN-inducible transcription of a viral promoter in cultured rat hepatocytes (Fig. 2) (25). Caution should be exercised before assigning physiological significance to 6βA-A because HepG2 cells express basal and DEX-inducible expression of *CYP3A7* (5) but not *CYP3A4* and because HNF-4 is a type IV orphan receptor that preferentially interacts as a homodimer to hexameric direct repeats (36) that are lacking in 6βA-A.

The direct repeat sequence ATGAACT, found as a possible DEX/PCN-responsive core element in *CYP3A23*, has not to our knowledge been identified previously as a binding site for a known transcription factor. Although there is sequence similarity with the "promoter coupling element" of the rat α-fetoprotein gene, the liver proteins that bind to these sequences do not appear to be of the same size.[4] Because hepatic nuclear proteins from control or from DEX-treated rats give the same pattern of binding to the *CYP3A23* direct repeat (25), we believe that induction of *CYP3A23* by glucocorticoids involves a multilayered process in which bound transcription factors are acted on directly or indirectly by glucocorticoids to stimulate *CYP3A23* gene transcription. Finding that consensus II regions of *CYP3A4* and *CYP3A6*

[4] J. Locker, personal communication; L. C. Quattrochi, P. S. Guzelian, unpublished observations.

are DEX or PCN inducible when transiently expressed in cultured rat hepatocytes and DEX or RIF inducible when transiently expressed in cultured rabbit hepatocytes (Fig. 2) but have only one ATGAACT site strongly argues that the *CYP3A23* DEX-response element (interpreted as hexameric direct repeats, TGAACT, with three intervening nucleotides, or as heptameric direct repeats, ATGAACT, with two intervening nucleotides) may not be essential for inducible transcriptional activity and that the putative transcription factor or factors may fall into the category of group III orphan receptors that bind as monomers (36). We noted that consensus II regions of *CYP3A4* and *CYP3A6* contain a second hexamer (AGGTCA) associated with group II orphan receptor binding (36). However, its location (six interspersed nucleotides) would be unprecedented for an orphan receptor acting through heterodimerization (37). Although we are unaware of a precedent for RIF exerting glucocorticoid effects, the activation by both this antibiotic and by DEX of −175 to −152 *CYP3A4*TK-CAT (Table 1) in rabbit hepatocytes suggests a common mechanism of action. The existence of a separate "RIF-response element" now seems less likely. It may be noted that through transient expression of *CYP3A5* 5′-flanking sequences in HepG2 cells, a 219-bp upstream (−891 to −1109) DEX-responsive enhancer element containing traditional glucocorticoid receptor element "half-sites" was identified (38). Inasmuch as the *CYP3A5* consensus II sequence was not tested in that study (or in the current study) in rat or rabbit hepatocytes, further experiments are needed to determine whether *CYP3A5* is transcriptionally activated by the nontraditional receptor element we identified for other *CYP3A* genes in this study and, if so, whether the upstream *CYP3A5* enhancer assists in glucocorticoid induction. Notwithstanding the useful insights gained through examination of trans-species gene expression as "mutations of convenience," several models of inducible expression of these *CYP3A* genes remain viable alternatives until additional directed mutagenesis studies and isolation and purification of the putative transcription factors have been completed.

There is substantial heterogeneity among humans in the amount of liver *CYP3A4* measured either directly (1, 39) or noninvasively with the use of the erythromycin breath test (8). In addition, treatment of humans with RIF results in induction of the erythromycin breath test activity or in excretion of urinary 6β-hydroxycortisol, both of which are noninvasive measures of *CYP3A4* activity, which is itself extremely heterogeneous (8). We and others have found that treatment of primary cultures of adult human hepatocytes with DEX or RIF consistently induces *CYP3A4* mRNA (7, 14). However, a few of the human hepatocyte cultures responded to PCN with induction of *CYP3A4* mRNA (7). If this finding is verified, then there may be two populations resident among humans, one responsive to PCN and therefore illustrative of the rat as a model for induction and the other represented by the rabbit, in which PCN is not an inducer. Our results suggest that it is unlikely that *CYP3A4* allelic differences will account for such qualitative heterogeneity of inducibility but rather that differences in formation of liver transcription factors may account for the differences among animal species and the heterogeneity of inductive response in humans. Analysis of this postulate should be technically feasible with the availability of modern recombinant DNA techniques in the use of the primary cultures of mammalian hepatocytes as the appropriate differentiated hosts.



**References**


1. Molowa, D. T., E. G. Schuetz, S. A. Wrighton, P. B. Watkins, P. Kremers, G. Mendez-Picon, A. Parker, and P. S. Guzelian. Complete cDNA sequence of a cytochrome P-450 inducible by glucocorticoids in human liver. *Proc. Natl. Acad. Sci. USA* **83**:5311–5315 (1986).
2. Beaune, P. H., D. R. Umbenhauer, R. W. Bork, R. S. Lloyd, and F. P. Guengerich. Isolation and sequence determination of a cDNA clone related to human cytochrome P-450 nifedipine oxidase. *Proc. Natl. Acad. Sci. USA* **83**:8064–8068 (1986).
3. Cholerton, S., A. K. Daly, and J. R. Idle. The role of individual human cytochromes P450 in drug metabolism and clinical response. *Trends Pharmacol. Sci.* **13**:434–439 (1992).
4. Watkins, P. B. Role of cytochrome P450 in drug metabolism and hepatotoxicity. *Semin. Liver Dis.* **10**:235–250 (1990).
5. Schuetz, E. G., J. D. Schuetz, S. C. Strom, M. T. Thompson, R. A. Fisher, D. T. Molowa, D. Li, and P. Guzelian. Regulation of human liver cytochromes P450 in family 3A in primary and continuous cultures of human hepatocytes. *Hepatology* **18**:1254–1262 (1993).
6. Daujat, M., L. Pichard, I. Fabre, P. Thierry, G. Fabre, C. Bonfils, and P. Maurel. Induction protocols for cytochromes P450IIIA *in vivo* and in primary cultures of animal and human hepatocytes. *Methods Enzymol.* **206**:345–353 (1991).
7. Kocarek, T. A., E. G. Schuetz, S. C. Strom, R. A. Fisher, and P. S. Guzelian. Comparative analysis of P450 3A induction in primary cultures of rat, rabbit and human hepatocytes. *Drug Metab. Dispos.* **23**:415–421 (1994).
8. Watkins, P. B., S. A. Murray, L. G. Winkelman, D. M. Heuman, S. A. Wrighton, and P. S. Guzelian. The erythromycin breath test as an assay of glucocorticoid-inducible liver cytochromes P-450. *J. Clin. Invest.* **83**:688–697 (1989).
9. Wrighton, S. A., D. T. Molowa, and P. S. Guzelian. Identification of a cytochrome P-450 in human fetal liver related to glucocorticoid-inducible cytochrome P-450HLp in the adult. *Biochem. Pharmacol.* **37**:3053–3055 (1988).
10. Schuetz, J. D., L. Beach, and P. S. Guzelian. Selective expression of human liver cytochromes P450 CYP3A mRNAs in embryonic and adult human liver. *Pharmacogenetics* **4**:11–20 (1994).
11. Hakkola J., M. Pasanen, R. Purkunen, S. Saarikoski, O. Pelkonen, J. Mäenpää, A. Rane, and H. Raunio. Expression of xenobiotic-metabolizing cytochrome P450 forms in human adult and fetal liver. *Biochem. Pharmacol.* **48**:59–64 (1994).
12. Gonzalez, F. J. Molecular genetics of the P-450 superfamily. *Pharmacol. Ther.* **45**:1–38 (1990).
13. Watkins, P. B., S. A. Wrighton, P. Maurel, E. G. Schuetz, G. Mendez-Picon, G. A. Parker, and P. Guzelian. Identification of an inducible form of cytochrome P-450 in human liver. *Proc. Natl. Acad. Sci. USA* **82**:6310–6314 (1985).
14. Pichard, L., I. Fabre, M. Daujat, J. Domergue, H. Joyeux, and P. Maurel. Effect of corticosteroids on the expression of cytochromes P450 and on cyclosporin A oxidase activity in primary cultures of human hepatocytes. *Mol. Pharmacol.* **41**:1047–1055 (1992).
15. Wrighton, S. A., P. Maurel, E. G. Schuetz, P. B. Watkins, B. Young, and P. S. Guzelian. Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450p. *Biochemistry* **24**:2171–2178 (1985).
16. Schuetz, E. G., S. A. Wrighton, J. L. Barwick, and P. S. Guzelian. Induction of cytochrome P-450 by glucocorticoids in rat liver. I. Evidence that glucocorticoids and pregnenolone 16 *alpha*-carbonitrile regulate *de novo* synthesis of a common form of cytochrome P-450 in cultures of adult rat hepatocytes and in the liver *in vivo*. *J. Biol. Chem.* **259**:1999–2006 (1984).
17. Hostetler, K. A., S. A. Wrighton, D. T. Molowa, P. E. Thomas, W. Levin, and P. S. Guzelian. Coinduction of multiple hepatic cytochrome P-450 proteins and their mRNAs in rats treated with imidazole antimycotic agents. *Mol. Pharmacol.* **35**:279–285 (1989).
18. Watkins, P. B., S. A. Wrighton, E. G. Schuetz, P. Maurel, and P. S. Guzelian. Macrolide antibiotics inhibit the degradation of the glucocorticoid-responsive cytochrome P-450p in rat hepatocytes *in vivo* and in primary monolayer culture. *J. Biol. Chem.* **261**:6264–6271 (1986).
19. Kocarek, T. A., E. G. Schuetz, and P. S. Guzelian. Expression of multiple forms of cytochrome P450 mRNAs in primary cultures of rat hepatocytes maintained on Matrigel. *Mol. Pharmacol.* **43**:328–334 (1993).
20. Wrighton, S. A., E. G. Schuetz, P. B. Watkins, P. Maurel, J. Barwick, B. S. Bailey, H. T. Hartle, B. Young, and P. Guzelian. Demonstration in multiple species of inducible hepatic cytochromes P-450 and their mRNAs related to the glucocorticoid-inducible cytochrome P-450 of the rat. *Mol. Pharmacol.* **28**:312–321 (1985).
21. Potenza, C. L., U. R. Pendurthi, D. K. Strom, and R. H. Tukey. Regulation of the rabbit cytochrome P-450 3c gene: age-dependent expression and transcriptional activation by rifampicin. *J. Biol. Chem.* **264**:16222–16228 (1989).
22. Schuetz, E., D. Li, C. J. Omiecinski, U. Muller-Eberhard, H. K. Kleinman, B. Elswick, and P. Guzelian. Regulation of gene expression in adult rat

hepatocytes cultured on a basement membrane matrix. *J. Cell Physiol.* **134:**309–323 (1988).
23. Burger, H.-J., J. D. Schuetz, E. G. Schuetz, and P. S. Guzelian. Paradoxical transcriptional activation of rat liver cytochrome P-450 3A1 by dexamethasone and the antiglucocorticoid pregnenolone 16a-carbonitrile: analysis by transient transfection into primary monolayer cultures of adult rat hepatocytes. *Proc. Natl. Acad. Sci. USA* **89:**2145–2149 (1992).
24. Luckow, B., and G. Schutz. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Res.* **15:**224–220 (1987).
25. Quattrochi, L. C., A. S. Mills, J. L. Barwick, C. B. Yockey, and P. S. Guzelian. A novel cis-acting element in a liver cytochrome P450 3A gene confers synergistic induction by glucocorticoids plus antiglucocorticoids. *J. Biol. Chem.* **270:**28917–28923 (1995).
26. Kleinman, H. K., M. L. McGarvey, J. R. Hassell, V. L. Star, F. B. Cannon, G. W. Laurie, and G. R. Martin. Basement membrane complexes with biological activity. *Biochemistry* **25:**312–318 (1985).
27. Schuetz, J. D., D. T. Molowa, and P. S. Guzelian. Characterization of a cDNA encoding a new member of the glucocorticoid-responsive cytochromes P450 in human liver. *Arch. Biochem. Biophys.* **274:**355–365 (1989).
28. Roux, K. H., and P. Khanarajan. A strategy for single site PCR amplification of dsDNA: priming digested cloned or genomic DNA from an anchor-modified restriction site and a short internal sequence. *BioTechniques* **8:**48–57 (1990).
29. Sanger, F., S. Nicklen, and A. R. Coulsen. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:**5463–5467 (1977).
30. Telhada, M. B., T. M. Pereira, and M. C. Lechner. Effect of dexamethasone and phenobarbital on run-on transcription rate and CYP3A mRNA concentration in rat liver: changes during development. *Arch. Biochem. Biophys.* **298:**715–725 (1992).
31. Hashimoto, H., K. Toide, R. Kitamura, M. Fujita, S. Tagawa, S. Itoh, and T. Kamataki. Gene structure of CYP3A4, an adult-specific form of cytochrome P450 in human livers, and its transcriptional control. *Eur. J. Biochem.* **218:**585–595 (1993).
32. Jounaidi, Y., P. Maurel, M. J. Vilarem, and P. S. Guzelian. Sequence of the 5′ region of human CYP3A5, a gene related to the glucocorticoid inducible liver cytochromes P450. *Biochem. Biophys. Res. Commun.* **205:**1741–1747 (1994).
33. Itoh, S., T. Yanagimoto, S. Tagawa, H. Hashimoto, R. Kitamura, Y. Nakajima, T. Okochi, S. Fujimoto, J. Uchino, and T. Kamataki. Genomic organization of human fetal specific *P*-450IIIA7 (cytochrome *P*-450HFLa)-related gene(s) and interaction of transcriptional regulatory factor with its DNA element in the 5′ flanking region. *Biochim. Biophys. Acta* **1130:**133–138 (1992).
34. Li, D., E. G. Schuetz, and P. S. Guzelian. Hepatocyte culture in study of P450 regulation. *Methods Enzymol.* **206:**335–344 (1991).
35. Miyata, M., K. Nagata, Y. Yamazoe, and R. Kato. Transcriptional elements directing a liver-specific expression of P450/6βA (CYP3A2) gene-encoding testosterone 6b-hydroxylase. *Arch. Biochem. Biophys.* **318:**71–79 (1995).
36. Jiang, G., L. Nepomuceno, K. Hopkins, and F. M. Sladek. Exclusive homodimerization of the orphan receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. *Mol. Cell Biol.* **15:**5131–5143 (1995).
37. Umesono, K., K. K. Murakami, C. C. Thompson, and R. M. Evans. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* **65:**1255–1266 (1991).
38. Schuetz, J. D., E. G. Schuetz, J. V. Thottassery, P. S. Guzelian, S. Strom, and D. Sun. Identification of a novel dexamethasone responsive enhancer in the human CYP3A5 gene and its activation in human and rat liver cells. *Mol. Pharmacol.* **49:**63–72 (1996).
39. Guengerich, F. P., M. V. Martin, P. H. Beaune, P. Kremers, T. Wolff, and D. J. Waxman. Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.* **261:**5051–5060 (1986).
40. Nelson, D. R., T. Kamataki, D. J. Waxman, P. Guengerich, R. W. Estabrook, R. Feyereisen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda, and D. N. Nebert. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **12:**1–51 (1993).
41. Komori, M., and Y. Oda. A major glucocorticoid-inducible P450 in rat liver is not P450 3A1. *J. Biochem.* **116:**114–120 (1994).

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