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Paradoxical transcriptional activation of rat liver cytochrome P-450 3A1 by dexamethasone and the antiglucocorticoid pregnenolone 16α-carbonitrile: Analysis by transient transfection into primary monolayer cultures of adult rat hepatocytes

(hepatocyte culture/lipofection)

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ABSTRACT **The family 3A cytochromes P-450, among the most abundant members of this supergene family of microsomal hemoproteins expressed in animal and human liver, are inducible by glucocorticoids but also by such antiglucocorticoids as pregnenolone 16α-carbonitrile (PCN). To investigate the mechanism for this nonclassical glucocorticoid effect, we analyzed the ability of 1.5 kilobases of DNA or of its successive subsegments isolated from the 5′ flanking region of the rat *CYP3A1* structural gene to modulate transcription of a reporter gene consisting of a viral promoter coupled to the chloramphenicol acetyltransferase (CAT) structural gene (expression vector pBLCAT2) and transiently expressed in a homologous cell system consisting of primary monolayer cultures of adult rat hepatocytes in which CYP3A1 mRNA and protein are inducible. The CAT activity measured after chimeric gene constructions were transferred into the cultured rat hepatocytes by lipofection increased as much as 7.2-fold if the cells were treated with dexamethasone (DEX). One CYP3A1 fragment (positions −220 to −56; 164 base pairs), which does not contain a traditional glucocorticoid responsive element, conferred dose-dependent DEX responsiveness independent of its orientation but not its position in pBLCAT2. This construction was activated by addition of PCN to the cultures and was synergistically induced by PCN plus DEX. In contrast, induction of CAT activity in cultures containing MMTVCAT, a plasmid containing the CAT gene controlled by the mouse mammary tumor virus long terminal repeat, was unaffected by PCN treatment, required lower concentrations of DEX for a maximal response, and was inhibited by treatment with DEX plus PCN. We conclude that a primary mechanism for induction of CYP3A1 is stimulated transcription through a pathway activated by steroid hormones.**

Cytochromes P-450 represent a supergene family of ubiquitous microsomal hemoproteins that catalyze a variety of important biological oxidations of endogenous substances and also drugs, environmental chemicals, and other foreign compounds.¶ Expression of many of these genes, prominently in the liver, involves their selective induction by groups of chemically or pharmacologically related agents (reviewed in ref. 4). For example, the family 1A P-450 genes are inducible by such polycyclic aromatic hydrocarbons as 3-methylcholanthrene and dioxin, some family 2B genes are inducible by lipophilic drugs such as phenobarbital, and family 3A genes are inducible by dexamethasone (DEX) and other glucocorticoids. It would be reasonable to assume that induction of the 3A genes by the glucocorticoids would be controlled by the well known process in which the steroid hormone binds stereospecifically to the glucocorticoid receptor (GCR), altering the conformation of this protein and thereby permitting its interaction with glucocorticoid response elements (GREs) in genomic DNA, in most instances increasing the transcription of associated responsive genes (5).

However, several years ago we demonstrated that P-450p (now called CYP3A1), a family 3A P-450 in rat liver, is inducible by glucocorticoids to the exclusion of other steroid hormones both in rats (6) and in primary cultures of adult rat hepatocytes (7, 8). However, an extensive study of dose–response and agonist/antagonist relationships indicated that by comparison with classical glucocorticoid responsive genes, induction of CYP3A1 (measured as rates of *de novo* protein synthesis) required higher doses of glucocorticoids, accepted a different rank order of potency of glucocorticoid agonists, and, paradoxically, was enhanced synergistically by pregnenolone 16α-carbonitrile (PCN), an antiglucocorticoid that blocks induction of typical glucocorticoid responsive genes (7, 8). We interpreted these observations as reflecting an unusual, nonclassical glucocorticoid receptor-mediated process for induction of CYP3A1 and of a related enzyme, digitoxigenin UDP-glucuronosyltransferase (9).

Verifying the presence of a nonclassical glucocorticoid pathway by detailed molecular studies would require isolation of cloned genomic DNA encoding the *CYP3A1* gene, an accomplishment that has eluded investigators to date. In addition, it would be necessary to transfer cloned segments of genomic DNA into primary monolayer cultures of adult rat hepatocytes maintained on a reconstituted basement membrane matrix (EHS gel) prepared from Engelbreth-Holms-Swarm tumor and previously called matrigel (10), the only reported culture system that responds appropriately to glucocorticoid hormonal stimuli with transcriptional induction of the cellular *CYP3A1* gene. Unfortunately, transfection of DNA into these nonproliferating hepatocytes by the use of such traditional protocols as incubation with calcium phosphate-precipitated DNA (11) produces toxicity and unacceptably low levels of transfected DNA. We have overcome these technical obstacles by successfully isolating a genomic clone encompassing the 5′ regulatory region of the *CYP3A1* gene and by devising an effective method for DNA transfer into

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Abbreviations: DEX, dexamethasone; PCN, pregnenolone 16α-carbonitrile; TK, thymidine kinase; MMTV, mouse mammary tumor virus; CAT, chloramphenicol acetyltransferase; GRE, glucocorticoid responsive element; GCR, glucocorticoid receptor; TAT, tyrosine aminotransferase.

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¶Under the recommended P-450 gene nomenclature system (1) CYP3A1, previously designated P450p (2) or P450PCN1 (3), refers to the major glucocorticoid-inducible P-450 in rat liver.

cultured rat hepatocytes by lipofection.[‖] We now demonstrate that glucocorticoids activate *CYP3A1* transcription by a process that is synergistically enhanced by PCN, an antiglucocorticoid that protects animals from the lethal (12) and carcinogenic (13) effects of many toxic chemicals.

METHODS

Animals and Materials. Adult male Sprague–Dawley rats (Dominion Laboratories, Dublin, VA) weighing 180–200 g were maintained in pairs in wire bottom cages with free access to animal chow and water for 2 weeks before use. Collagenase (type I) was purchased from Cooper Biomedical; Opti-MEM (minimal essential medium), Eagle's MEM, Lipofectin reagent {*N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride}, and restriction enzymes were from GIBCO/BRL; chloramphenicol and DEX were from Sigma; acetyl coenzyme A was from Pharmacia; and [1-^{14}C]acetyl coenzyme A was from Amersham. Reagents for oligonucleotide synthesis were from Millipore. EHS gel was prepared as described (10, 14); PCN was a gift from John Babcock (Upjohn); RU 486 was from Roussell Uclaf; pRSVCAT (15) was provided by Bruce Howard (National Institutes of Health, Bethesda, MD), pMMTVCAT (16) was provided by R. Miksicke, and pBLCAT2 (17) was provided by G. Schutz (Institute for Cell and Tumorbiology, Heidelberg, F.R.G.). Oligonucleotides were synthesized with a Biosearch Cyclone and HPLC purified as described (18).

Isolation of the Rat *CYP3A1* Gene. The 5′ cDNA fragments formed by endonuclease digestions of a full-length *CYP3A1* cDNA (19) (kindly provided by Frank Gonzalez, National Institutes of Health, Bethesda, MD) and an oligonucleotide composed of the first 63 bases from its start site of translation were used as hybridization probes to screen a library of rat genomic DNA segments cloned in the phage vector EMBL3. One clone, EMBL1-1-4, containing the first exon and 1.5 kilobases (kb) of 5′ flanking sequence of *CYP3A1* was isolated for further analysis (see Fig. 1).

DNA Sequence Analysis. *CYP3A1* gene restriction fragments were ligated into pGEM vectors for double-stranded DNA sequencing by the chain-termination method (20) with either modified T7 DNA polymerase (United States Biochemical) or *Taq* DNA polymerase (Promega) and ^{32}P-labeled oligonucleotide primers according to the manufacturers' instructions. Exonuclease III was sometimes used to prepare unilateral deletions as described (Erase-a-Base system; Promega). The GCG computer software package (21) was used to perform DNA sequence alignments.

Northern Blot Analysis. Total hepatocellular RNA (10 μg) isolated from five culture dishes was resolved by Northern blot (10, 22) and hybridized with a [^{32}P]dCTP radioactively labeled cDNA specific for *CYP3A1* (pDEX 12) (23) or tyrosine aminotransferase (TAT) (24). The bands were visualized by autoradiography.

Construction of Chimeric CYP3A1–pBLCAT2 Genes. A 1.47-kb *Sal* I/BamHI fragment of the *CYP3A1* gene containing 5′ flanking sequences from −1525 to −56 base pairs (bp) upstream of the transcription start site was ligated in the 5′ to 3′ orientation (forward) upstream of the thymidine kinase (TK) promoter of the pBLCAT2 expression vector or into the plasmid vector pGEM3Z. Deletion mutants of 1.47CYP3A1–pGEM3Z were prepared by digestions with exonuclease III or by use of CYP3A-specific primer pairs and the polymerase chain reaction (PCR). Several DNA fragments generated by exonuclease digestion [spanning positions −56 to −595 (539 bp; −595R TKCAT); −1265 to −56 (1209 bp; −1265 TKCAT); −908 to −56 (852 bp; −908 TKCAT)] were ligated into the upstream multiple cloning site of pBLCAT2 (R, reverse orientation; CAT, chloramphenicol acetyltransferase). Products of PCRs containing 1.47CYP3A1–pGEM3Z as a template and primers spanning bp −70 to −56 and −220 to −206 of *CYP3A1* were subcloned into the multiple cloning site of pBLCAT2 in forward and reverse orientation to generate the plasmids −220 TKCAT and −220R TKCAT, while primers −631 to −614 and −285 to −269 were used to generate −631 to −269 TKCAT. DNA sequencing was used to confirm the identity of all CYP3A1 fragments.

Hepatocyte Cultivation and Lipofection of Chimeric CYP3A1–pBLCAT2 Genes. Primary cultures of adult rat hepatocytes (10) were prepared by incubating hepatocytes freshly isolated by collagenase perfusion of the livers of untreated rats in a humidified atmosphere (35°C) of 95% air/5% CO_2 in our standard medium (10), a modification of Waymouth MB-752 medium containing insulin (0.1 μM) as the only hormone. For transfection experiments, 1.3×10^6 hepatocytes were incubated in 3 ml of medium supplemented with 5% fetal calf serum in Lux 5220 dishes (60 mm). The medium was renewed on days 1 and 2 of culture with serum-free Eagle's MEM. On day 3, each dish of hepatocytes was washed with 3 ml of Opti-MEM and then exposed to a mixture formed by combining 30 μg of Lipofectin with 5 μg of DNA (25). Control dishes were mock transfected in the same mixture without DNA. In some experiments, we included dishes transfected with pRSVCAT (15) to estimate transfection efficiency or with pMMTVCAT (16) to monitor glucocorticoid responsiveness. After 6 hr of incubation with the cells, the Lipofectin/DNA mixture was removed and the cultures were incubated with 3 ml of our standard Waymouth medium supplemented with 50 μl of EHS gel per ml of medium. Drugs were added to the medium in dimethyl sulfoxide. On day 4, the medium was renewed as described above.

Assay of CAT Activity in Transfected Hepatocytes. On the 5th culture day, 15 hr after the last addition of drugs, the hepatocytes in a single dish were harvested for assay of CAT activity as described by Sleigh (26). Duplicate CAT assays performed on a given cell extract varied by <4%. The average CAT activity in extracts from hepatocytes transfected with TKCAT was 1332.0 ± 134.5 dpm (n = 6). While MMTVCAT (a plasmid containing the CAT gene controlled by the mouse mammary tumor virus long terminal repeat) demonstrated greater variation in basal CAT activity (849.3 ± 314.1 dpm) (n = 3) the -fold induction by 10 μM DEX was 45.6 ± 1.2 (n = 3).

RESULTS

We used a *CYP3A1* cDNA to screen a library of rat liver genomic DNA cloned into the EMBL3 vector and isolated a recombinant bacteriophage that contained 16 kb of inserted DNA. A 1.8-kb digestion fragment of the genomic clone that hybridized on Southern blots with the most 5′ part of the *CYP3A1* cDNA was ligated into a pGEM vector and its DNA sequence was determined. The cloned genomic segment contained the first exon of the *CYP3A1* structural gene plus ≈1.5 kb of 5′ flanking DNA and part of intron 1 (Fig. 1). The nucleotide sequence of the genomic clone exactly matched that published for *CYP3A1* cDNA (19) from bp 15 (67 bp upstream of the translational start site) to the intron–exon border at bp 153 (Fig. 1). Moreover, the nucleotide sequence of *CYP3A1* within the first exon and intron is significantly different from the recently identified *CYP3A2* genomic clone (27). Primer extension of mRNA isolated from the liver of DEX-treated rats, carried out to establish the actual transcriptional start site of the *CYP3A1* gene, produced several minor products (data not shown). Because the longest product (90 bp) had a purine nucleotide, G, typical of an RNA cap

[‖]The sequence reported in this paper has been deposited in the GenBank data base (accession no. M86850).

FIG. 1. Nucleotide sequence of the 5′ flanking region of the *CYP3A1* gene. Nucleotides are numbered in negative numbers from the mRNA start site indicated by +1. Numbers above the sequence indicate the beginning or end of fragments used for construction of chimeric genes (see Table 1). The first exon, the deduced amino acid sequence, and the exon/intron border are indicated. Nucleotide symbols above the sequence indicate positions with nucleotide mismatches between *CYP3A1* and the *CYP3A2* cDNA, a closely related family member (19). Putative GREs are underlined and labeled. In addition, positions with similarity to consensus sequences for binding sites of transcriptional regulatory factors are indicated. (−), Sequences found on the opposite strand. Nucleotides at −1336, −1335, and −1000 could not be resolved unambiguously even after repeated sequencing at high temperatures.

site, this locus was designated as +1 (Fig. 1). Computer-assisted sequence analysis of the 5′ flanking region revealed a consensus sequence for a TATA box (−31 bp) (28), and the presence of putative GREs on both strands similar to the TGTYC (Y, pyrimidine) motif of the GRE consensus half-palindrome (29), but none with the complete TGTYCT hexamer motif (5) or the consensus 15-bp palindrome (5) essential for optimal response to glucocorticoids (Fig. 1). Two regions (−936 to −797 and −219 to −146) both contain GRE-like sequences in close proximity to sequences known to bind transcription factors that interact with the GCR such as NF1 (30), octamer factor (31), and CACCC box binding factor (32) (Fig. 1).

Next, we tested the ability of *CYP3A1* DNA segments to modulate transcription of an artificial gene consisting of a viral (TK) promoter coupled to the *E. coli* CAT structural gene (expression vector pBLCAT2) transiently expressed in the intact rat hepatocyte. This approach excludes the possibility that upstream *CYP3A1* elements require interaction with the homologous *CYP3A1* promoter for mediating steroid induction, although preliminary experiments with a related gene, *CYP3A5*, indicate that such induction occurs with constructions containing either promoter (33). A 1.5-kb *CYP3A1* fragment (−1525 to −56) was ligated upstream of the TK promoter in pBLCAT2 (position −1525) (−1525 TK-CAT). This recombinant vector was combined with the cationic transfer agent Lipofectin and transfected into 3-day-old primary cultures of rat hepatocytes (Table 1). The resultant hepatocellular CAT activity was lower than that in control hepatocytes transfected with pBLCAT2 (TKCAT) containing no inserted *CYP3A1* DNA (Table 1). Plasmids containing more proximal portions of the inserted 1.5-kb *CYP3A1* fragment produced the expected amount of CAT activity (−220R TKCAT) when transfected into hepatocyte cultures (Table 1). To test for the presence of glucocorticoid responsive regions in *CYP3A1* DNA, cultures harboring these transfected chimeric gene constructions were treated for 48 hr with DEX. In each instance, CAT activity increased, as high as 7.2-fold (−1265 TKCAT; Table 1). One *CYP3A* fragment (−220 to −56; 164 bp) conferred DEX responsiveness when it was ligated in pBLCAT2 in both the forward (−220 TKCAT; 4.6-fold) and reverse (−220R TKCAT; 4.2-fold) orientations (Table 1). However, when a plasmid containing −220 TKCAT ligated into the second multiple cloning site of pBLCAT2 (downstream of the CAT structural gene and the simian virus 40 polyadenylylation site) was transfected into hepatocyte cultures, no stimulation of CAT activity occurred after DEX treatment of the cells (data not shown). In this same set of experiments, we also tested the effect of treating transfected hepatocyte cultures with PCN.

Table 1. CAT activity of chimeric *CYP3A1*-TKCAT genes

Chimeric gene	Basal CAT activity, relative units (TKCAT = 1.0)	CAT induction, relative units (untreated = 1.0)	
		DEX	PCN
−1525 TKCAT	0.3 ± 0.1*	5.8 ± 1.2†	2.3 ± 0.3†
−1265 TKCAT	0.4 ± 0.1†	7.2 ± 0.6†	2.9
−908 TKCAT	0.5 ± 0.3*	5.6 ± 0.5†	1.3 ± 0.2†
−511 TKCAT	1.5 ± 0.1*	5.2 ± 1.0†	1.3
−220 TKCAT	1.6 ± 0.3*	4.6 ± 1.7*	2.1 ± 1.0*
−220R TKCAT	0.8 ± 0.4*	4.2 ± 2.0†	2.9
−595R TKCAT	2.0 ± 0.3*	2.5 ± 0.2†	NT
TKCAT	1.0	1.3 ± 0.5*	1.5 ± 0.4

Hepatocyte cultures were transfected with CYP3A1-pBLCAT2 plasmids and assayed for CAT activity. Left column indicates the chimeric gene used for transfection. Basal CAT activity of dishes without addition of drugs is expressed relative to the activity of the vector pBLCAT2. CAT induction by 10 μM DEX or 10 μM PCN is expressed relative to the activity of untreated control dishes. NT, not tested.

*Means ± SD of three independent transfection experiments.

†Averages ± range of two independent transfection experiments.

This antiglucocorticoid consistently increased the CAT activity (≈2-fold) in cultures that received plasmids with the longest (−1525 TKCAT) and the shortest (−220 TKCAT) fragments of *CYP3A1* DNA, whereas no induction was seen with some fragments of intermediate length (−908 TKCAT, −511 TKCAT; Table 1) or with a 359-bp fragment spanning positions −621 to −269 inserted into TKCAT (data not shown). DEX treatment of cells containing the last plasmid also failed to produce CAT induction, an important control showing that inclusion of the −220 to −56 sequence is essential for steroid hormone responsiveness. The possible causes for the lack of effect of PCN on some of these partial deletions were not further investigated. Additions of either of these steroids to cultures transfected with pBLCAT2 (Table 1) or with RSVCAT (data not shown) produced no important change in CAT activity.

We recognized that this system offers a unique opportunity to determine whether *CYP3A1* transcription is mediated by the nonclassical GCR-mediated pathway for induction of CYP3A1 protein synthesis by steroids (7, 8). Accordingly, we compared the effects of DEX, PCN, or RU 486, a potent and well-characterized GCR antagonist (34), on the production of CAT activity in cultured hepatocytes transfected with −220 TKCAT (Fig. 2*B*) or with MMTVCAT (Fig. 2*A*), a powerful DEX-responsive promoter typical of GCR-regulated genes (16). DEX produced dose-dependent increases in CAT activity for both MMTVCAT and −220 TKCAT, although maximal stimulation of MMTVCAT (50-fold) was observed at a concentration of DEX (10 μM) (Fig. 2*A*) that stimulated −220 TKCAT only 5.5-fold (Fig. 2*B*). The maximal response of MMTVCAT was ≈9-fold greater than that of −220 TKCAT (Fig. 2*B*). DEX induction of MMTVCAT in the presence of equimolar to a 1000-fold excess of an antagonist was either completely abolished (RU 486) or substantially reduced (PCN) as compared to cultures receiving DEX alone (Fig. 2*A*). Additions of antiglucocorticoids alone gave no effect on MMTVCAT. In contrast, PCN induced −220 TKCAT synergistically (5-fold induction) in the presence of low concentrations of DEX (10 nM) that alone produced little, if any, induction of −220 TKCAT (Fig. 2*B*). Unlike PCN, RU 486 attenuated the DEX response of −220 TKCAT, although to a lesser extent than the antagonism of DEX induction of MMTVCAT. Similar DEX dose–response curves were obtained for the −164-bp fragment cloned in the reverse orientation (data not shown).

In developing a protocol for efficient and consistent transient transfection of DNA into cultured rat hepatocytes, it became necessary to modify the conditions of culture (e.g., addition of soluble EHS gel) as compared to our standard method (10). To verify that the endogenous *CYP3A1* cellular gene retained its glucocorticoid and PCN responsiveness under the conditions we used for transiently transfecting chimeric genes into primary hepatocytes, RNA was isolated from steroid-treated and untreated transfected hepatocytes and was analyzed on Northern blots probed with cDNAs for TAT (24) and CYP3A1 (23). Under these culture conditions, DEX treatment produced a dose-dependent increase in the amounts of TAT mRNA and CYP3A1 mRNA (Fig. 3), although half-maximal induction of CYP3A1 mRNA required ≈100 times higher DEX concentration (Fig. 3). Moreover, mirroring exactly the effects on rates of synthesis of CYP3A1 protein in cultured hepatocytes (7, 8), the present results showed that PCN is an efficacious inducer of CYP3A1 mRNA (Fig. 3), fails to increase TAT mRNA, and, when combined with low concentrations of DEX, synergistically increases CYP3A1 mRNA while inhibiting the induction of TAT mRNA. This set of experiments confirms that, under the conditions used for transfer of DNA into cultured hepatocytes by lipofection, a parallelism is retained between expression of the endogenous *CYP3A1* and *TAT* genes, as judged by accumulation of their respective mRNAs, and transcriptional activation of the *CYP3A1* gene and the classical glucocorticoid gene MMTV, as reflected in CAT activity.

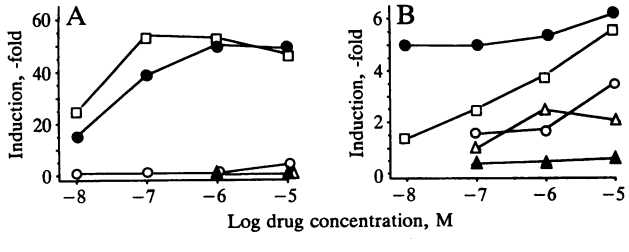

FIG. 2. Comparison of the effect of PCN and RU 486 on DEX induction of the chimeric gene MMTVCAT and −220 TKCAT. MMTVCAT (*A*) and −220 TKCAT (*B*) were transfected into cultured hepatocytes. DEX was added to the medium at the indicated concentrations alone (□) or with 10 μM PCN (●) or 10 μM RU 486 (○), respectively, 6 hr after transfection on days 3 and 4. Control dishes contained PCN (△) or RU 486 (▲) in the absence of DEX. Cells were harvested and CAT activities were assayed. Data were calculated as -fold induction relative to CAT activities obtained from dishes without any drug addition.

DISCUSSION

Pharmacologic induction of CYP3A1 can involve many steps apart from stimulation of gene transcription including changes in the amounts or the translatability of CYP3A1 mRNA or the stability of CYP3A1 protein or holocytochrome. To analyze how glucocorticoid-responsive cytochromes P-450 are induced selectively, we isolated the 5′ flanking region of the rat *CYP3A1* structural gene and analyzed its expression by developing a convenient means for DNA transfer into a homologous cell system consisting of primary monolayer cultures of adult rat hepatocytes. Unlike culture-adapted cell lines derived from malignant liver, rat hepatocytes cultured on EHS gel maintain highly differentiated functions such as the inducibility of various cytochromes P-450 including the class 3A family (10). Reproducible results were achieved by transfection of hepatocytes in an EHS gel-free environment followed by addition of soluble EHS gel to the culture medium (35). Fortunately, these manipulations did not impair inducible expression of CYP3A1 inasmuch as treatments with DEX produced a dose-dependent stimulation of *CYP3A1* transcription in cultured hepatocytes (Fig. 2, Table 1) accompanied by accumulation of CYP3A1 mRNA (Fig. 3) (10) and protein (7, 8). The 5- to 6-fold activation of CAT activity corresponds well with reports of experiments in DEX-treated living rats where transcription was assayed by less direct methods of measuring CYP3A1 mRNA in intranuclear RNA and monitoring run off of initiated nascent transcripts from isolated liver nuclei (36). We were unable to compare our results from transfection analysis with those from a run off of nuclei from

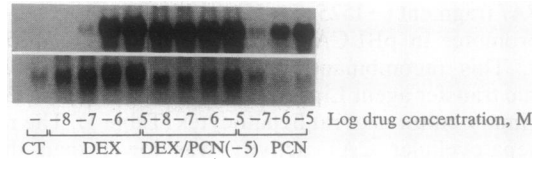

FIG. 3. Induction of CYP3A1 and TAT mRNAs by DEX and PCN. Hepatocyte cultures were treated on day 3 with soluble EHS gel, DEX, or PCN at the indicated concentrations, or with DEX plus 10 μM PCN (DEX/PCN). RNA isolated from the cells harvested on day 5 was analyzed on Northern blots and hybridized with a cDNA probe for either CYP3A1 (*Upper*) or TAT (*Lower*). CT, untreated control.

hepatocyte cultures because the values were too low to be reliably quantified and because minor differences in technique for nuclear preparation seemed to produce qualitative differences in outcome.

Stimulated *CYP3A1* transcription appears to account, at least in part, for two major characteristics of CYP3A1 protein and mRNA induction that distinguish *CYP3A1* from classical glucocorticoid responsive genes such as *TAT* and MMTV: induction of *CYP3A1* requires higher than normal glucocorticoid concentrations such as might be found under conditions of stress (37), and CYP3A1 is induced, paradoxically, by PCN, a synthetic steroid, that blocks DEX-mediated induction of *TAT* and MMTV. Another antiglucocorticoid, RU 486, partially antagonized *CYP3A1* transcription in DEX-treated cultures (Fig. 2), in contrast to PCN, which interacts with DEX to induce *CYP3A1* transcription synergistically (Fig. 2). A more expansive survey of other active antiglucocorticoids in our system (7, 8) may provide insight into structural requirements for CYP3A1 inducer agonists.

The smallest fragment of *CYP3A1* DNA we tested (the 164 bp cloned in −220 TKCAT) still conferred full DEX responsiveness when transiently expressed in cultured hepatocytes (Fig. 2, Table 1). Nevertheless, the *CYP3A1* fragment in −220 TKCAT displays only limited (if any) sequence homology to the 15-bp palindromic consensus nucleotide sequence for GREs (5), although it does contain a DNA motif (TGTTCACT) related to a region in the tryptophan oxygenase enhancer, which has been footprinted by the glucocorticoid receptor (38). Lack of a high-affinity, GCR-binding GRE in the *CYP3A1* gene might explain the shift to the right of the dose–response curve for induction by DEX of CYP3A1 as compared to MMTV (Fig. 2) or TAT (Fig. 3). Another possibility is that the *CYP3A1* gene does not bind the GCR directly but rather is activated through ancillary DNA regulatory proteins produced by other glucocorticoid responsive genes. Such a secondary induction mechanism has been proposed because glucocorticoid responsiveness of α_2-microglobulin (39) and α_1-acid glycoprotein (40) genes was suppressed by blocking protein synthesis with cycloheximide. However, *CYP3A1* does not contain glucocorticoid-regulated nucleotide sequences similar to those identified in these genes and cycloheximide treatment failed to inhibit DEX-induced accumulation of CYP3A1 mRNA in cultured hepatocytes (22). Further adding to the complexities of this system is the observation that −220 TKCAT also responds positively to PCN (Fig. 2), unlike MMTV (Fig. 2) or TAT (Fig. 3). The similarities among hormone response elements of many steroid-inducible genes indicate a conserved strategy for the hormonal control of transcription by steroids (41). Nevertheless, our previous postulate of a pathway for *CYP3A1* induction that involves either a separate GCR with altered ligand binding characteristics (PCN receptor) or the classical GCR interacting directly or indirectly with altered GREs associated with PCN responsive genes (7, 8) has been reinforced by the present findings of a synergistic transcriptional control for CYP3A1 regulation by DEX and PCN. The presently available systems should make it possible to elucidate the fine molecular details involved in *CYP3A1* gene control, providing a better understanding of this important P-450 family both in animals and in humans (42, 43) and expanding our recognition of mechanisms by which steroid hormones regulate mammalian genes.

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1. Nebert, D. W., Nelson, D. R., Coon, M. J., Estabrook, R. W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R. & Waxman, D. J. (1991) *DNA Cell Biol.* **10**, 1–14.
2. Elshourbagy, N. A. & Guzelian, P. S. (1980) *J. Biol. Chem.* **255**, 1279–1285.
3. Gonzalez, F. J., Nebert, D. W., Hardwick, J. P. & Kasper, C. B. (1985) *J. Biol. Chem.* **260**, 7435–7441.
4. Okey, A. B. (1990) *Pharmacol. Ther.* **45**, 241–298.
5. Beato, M., Chalepakis, G., Schauer, M. & Slater, E. P. (1989) *J. Steroid Biochem.* **32**, 737–748.
6. Heuman, D. M., Gallagher, E. J., Barwick, J. L., Elshourbagy, N. A. & Guzelian, P. S. (1982) *Mol. Pharmacol.* **21**, 753–760.
7. Schuetz, E. G. & Guzelian, P. S. (1984) *J. Biol. Chem.* **259**, 2007–2012.
8. Schuetz, E. G., Wrighton, S. A., Barwick, J. L. & Guzelian, P. S. (1984) *J. Biol. Chem.* **259**, 1999–2006.
9. Schuetz, E. G., Hazelton, G. A., Hall, J., Watkins, P. B., Klaassen, C. D. & Guzelian, P. S. (1986) *J. Biol. Chem.* **261**, 8270–8275.
10. Schuetz, E. G., Li, D., Omiecinski, C. J., Muller-Eberhard, U., Kleinman, H. K., Elswick, B. & Guzelian, P. S. (1988) *J. Cell. Physiol.* **134**, 309–323.
11. Pasco, D. S. & Fagan, J. B. (1989) *DNA* **8**, 535–541.
12. Selye, H. (1971) *J. Pharm. Sci.* **60**, 1–28.
13. Conney, A. H. (1982) *Cancer Res.* **42**, 4875–4917.
14. Kleinman, H. K., McGarvey, M. L., Hassell, J. R., Star, V. L., Cannon, F. B., Laurie, G. W. & Martin, G. R. (1986) *Biochemistry* **25**, 312–318.
15. Gorman, C., Padmanabhan, R. & Howard, B. H. (1983) *Science* **221**, 551–553.
16. Cato, A. C. B., Miksicek, R., Schutz, G., Arnemann, J. & Beato, M. (1986) *EMBO J.* **5**, 2237–2240.
17. Lukow, B. & Schutz, G. (1987) *Nucleic Acids Res.* **15**, 5490.
18. Schuetz, J. D., Molowa, D. T. & Guzelian, P. S. (1989) *Arch. Biochem. Biophys.* **274**, 355–365.
19. Gonzalez, F. J., Song, B. J. & Hardwick, J. P. (1986) *Mol. Cell. Biol.* **6**, 2969–2976.
20. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
21. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
22. Burger, H.-J., Schuetz, E. G., Schuetz, J. D. & Guzelian, P. S. (1990) *Arch. Biochem. Biophys.* **281**, 204–211.
23. Wrighton, S. A., Schuetz, E. G., Watkins, P. B., Maurel, P., Barwick, J., Bailey, B. S., Hartle, H. T., Young, B. & Guzelian, P. (1985) *Mol. Pharmacol.* **28**, 312–321.
24. Shinomiya, T., Scherer, G., Schmid, W., Zentgraf, H. & Schutz, G. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1346–1350.
25. Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. & Danielsen, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7413–7417.
26. Sleigh, M. J. (1986) *Anal. Biochem.* **156**, 251–256.
27. Miyata, M., Nagata, K., Yamazoe, Y. & Kato, R. (1991) *Biochem. Biophys. Res. Commun.* **177**, 68–73.
28. Nussinov, R. (1986) *J. Theor. Biol.* **120**, 479–487.
29. Evans, R. M. (1988) *Science* **240**, 889–895.
30. Bruggemeier, U., Rogge, L., Winnacker, E.-L. & Beato, M. (1990) *EMBO J.* **9**, 2233–2239.
31. Schule, R., Muller, M., Kaltschmidt, C. & Renkawitz, R. (1988) *Science* **242**, 1418–1420.
32. Schule, R., Muller, M., Otsuka-Murakami, H. & Renkawitz, R. (1988) *Nature (London)* **332**, 87–90.
33. Schuetz, J. D., Molowa, D. T., Schuetz, E. G. & Guzelian, P. S. (1988) *Fed. Proc. Fed Am. Soc. Exp. Biol.* **2**, A1785.
34. Svec, F. (1988) *Endocrinology* **123**, 1902–1906.
35. Caron, J. M. (1990) *Mol. Cell. Biol.* **10**, 1239–1243.
36. Simmons, D. L., McQuiddy, P. & Kasper, C. B. (1987) *J. Biol. Chem.* **262**, 326–332.
37. Gala, R. & Westphal, U. (1965) *Endocrinology* **77**, 841–851.
38. Danesch, U., Gloss, B., Schmid, W., Schule, R. & Renkawitz, R. (1987) *EMBO J.* **6**, 625–630.
39. Addison, W. R. & Kurtz, D. T. (1989) *J. Biol. Chem.* **264**, 21891–21895.
40. Klein, E. S., DiLorenzo, D., Posseckert, G., Beato, M. & Ringold, G. M. (1988) *Mol. Endocrinol.* **2**, 1343–1351.
41. Martinez, E., Givel, F. & Wahli, W. (1991) *EMBO J.* **10**, 263–268.
42. Watkins, P. B., Wrighton, S. A., Maurel, P., Schuetz, E. G., Mendez-Picon, G., Parker, G. A. & Guzelian, P. S. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6310–6314.
43. Molowa, D. T., Schuetz, E. G., Wrighton, S. A., Watkins, P. B., Kremers, P., Mendez-Picon, G., Parker, G. A. & Guzelian, P. S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5311–5315.