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Demonstration in Multiple Species of Inducible Hepatic Cytochromes P-450 and Their mRNAs Related to the Glucocorticoid-Inducible Cytochrome P-450 of the Rat

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SUMMARY

We have recently demonstrated that P-450p, a form of rat liver cytochrome P-450 inducible by steroids such as dexamethasone and pregnenolone-16α-carbonitrile, by the macrolide antibiotic triacetyloleandomycin, and by phenobarbital, is immunochemically related to and shares 73% NH_2-terminal amino acid sequence homology with rabbit cytochrome LM3c. Extending this interspecies comparison we now report that liver microsomes prepared from the rabbit, hamster, gerbil, and mouse contain inducible cytochromes P-450 that resemble P-450p in: (a) converting triacetyloleandomycin to a metabolite that forms a distinct spectral complex with cytochrome P-450 heme, (b) catalyzing the demethylation of erythromycin, and (c) reacting on immunoblots with antibodies directed against P-450p or LM3c. These three characteristics changed in parallel within treatment groups of a given species receiving different inducers of cytochrome P-450. However, there were striking qualitative and quantitative interspecies differences in the responses to inducers. For example, rifampicin was the most efficacious inducer of LM3c in the rabbit and yet was not at all an inducer of P-450p in the rat whereas pregnenolone-16α-carbonitrile, an inducer in the rat, failed to induce LM3c in the rabbit. Immunoblot analysis of these microsomes revealed in each species except the rabbit a single immunochemically related protein. A second immunoreactive protein was present in microsomes from male and female and rifampicin- and dexamethasone-treated female rabbits. Two cloned cDNAs, which hybridized to a species of liver mRNA directing the synthesis of P-450p in a cell-free translation system, were used to probe Northern blots of liver RNAs. These revealed a single band of hybridizable mRNA in each species (except RNA from the rabbit which gave no signal even under conditions of reduced stringency) that was induced in qualitative proportions to that of the accumulated immunoreactive protein. We conclude that P-450p appears to be conserved in evolution and is represented in each of the species tested by one or more immunochemically related proteins which exhibit similar catalytic activities to those of P-450p.

INTRODUCTION

The hepatic cytochromes P-450 are a superfamily (1) of microsomal hemoproteins that catalyze the oxidation of numerous endogenous and exogenous compounds (2). The concentration in the liver of many of the individual forms of cytochrome P-450 appears to be under multifactorial regulation that involves positive (induction) or negative (repression) effects (or both) exerted by administered drugs and other foreign chemicals (3). It has been traditional to classify inducing agents as resembling either PB[3] or MC based on the type of cytochrome P-

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[3] The abbreviations used are: PB, phenobarbital; DEX, dexamethasone; PCN, pregnenolone-16α-carbonitrile; TAO; triacetyloleandomycin; MC, 3-methylcholanthrene; RIF, rifampicin; EM, erythromycin; IgG, immunoglobulin G; SDS, sodium dodecyl sulfate; P-450p, the major form of cytochrome P-450 in rats treated with DEX or PCN; LM3c, the major rabbit liver form of cytochrome P-450 in animals treated with TAO.

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450 isozymes that are induced. Thus, in the liver of PB-treated rats the separate gene products, P-450b and P-450e, are dominant (4) whereas P-450c and P-450d are the major hepatic forms in MC-treated rats (1, 3). There is now compelling evidence that glucocorticoids such as DEX and antiglucocorticoids such as PCN represent a "third class" of inducers that stimulate *de novo* synthesis of P-450p[4] (5–8), an isozyme that is biochemically and immunochemically distinct from members of the two other cytochrome P-450 families.

The characteristics of P-450p induction have been better defined recently. Among steroid hormones only glucocorticoids induce P-450p, acting through a stereospecific mechanism that excludes estrogens, androgens, and progestational compounds (5, 6). In addition, some nonsteroidal agents such as PB are capable of modestly inducing P-450p (3, 7). P-450p is also under developmental control, being present in rats of both genders at birth but disappearing from female rat liver at the onset of sexual maturity (9). Finally, TAO and other macrolide antibiotics have proven to be remarkably efficacious inducers of P-450p (10). Indeed, P-450p is unique among identified cytochromes P-450 in catalyzing EM demethylation and in converting TAO to a metabolite which binds to P-450p forming a spectrally detectable complex (10). In addition, TAO and RIF, another macrolide antibiotic, have been shown to be efficacious inducers of CO-binding hemoproteins in rabbit liver (11). Indeed, we recently noted (10) that the NH_2-terminal amino acid sequence of P-450p is strikingly similar to that published for LM3c, a form of hepatic cytochrome P-450 purified originally from untreated rabbits. Since P-450b/e and P-450c/d appear to be conserved in evolution (1, 12) and are represented by homologous cytochromes P-450 in other species (1, 13, 14), it seemed possible the same might be true for P-450p. Accordingly, we treated female rats, gerbils, hamsters, mice, and rabbits with known inducers of P-450p and examined liver microsomes from these animals for the presence of cytochromes homologous to P-450p by measuring TAO complex formation, EM demethylation, and immunoreactivity with antibodies directed against P-450p or LM3c. We also prepared cloned DNA complimentary to P-450p mRNA as a probe to test for the presence of mRNAs homologous to P-450p mRNA.

Experimental Procedures

Materials. PCN, TAO, and EM were gifts from The Upjohn Co., Pfizer, and Laboratories Roussel (Paris), respectively; DEX, RIF, PB, and NADPH were purchased from Sigma; rabbit anti-goat IgG and goat peroxidase anti-peroxidase complex from Miles Scientific (Elkhart, IN); nitrocellulose paper from Bio-Rad; 3,3′-diaminobenzidine tetrahydrochloride from Pfaltz and Bauer (Stamford, CT). Restriction enzymes and ribonuclease H were obtained from Bethesda Research Laboratories, reverse transcriptase from Molecular Genetic Resources (Tampa, FL), and polymerase I from Boehringer Mannheim. Terminal deoxynucleotidyltransferase was purchased from P-L Biochemicals, oligo(dT)-cellulose from Collaborative Research (Lexington, MA), and nuclease S1 from Miles Scientific (Napterville, IL). α-^{32}P-labeled nucleotide triphosphates with a specific activity of >200 Ci/mmol were purchased from ICN Radiochemicals (Irvine, CA). All other materials were of reagent grade or better.

Animals and treatments. Female Sprague-Dawley rats (Flow Labs, Dublin, VA) weighing 150 g, female Mongolian gerbils (Tumblebrook Farms, West Brookfield, MA) weighing 60 g, and female Syrian hamsters (Charles River, Wilmington, MA) weighing 100 g received daily gavage of DEX (300 mg/kg) or PCN (300 mg/kg) for 4 days, intraperitoneal injections of RIF (50 mg/kg) daily for 4 days, TAO (1% (w/w)) in diet for 10 days or PB (0.1% (w/v)) in drinking water for 6 days. Female New Zealand rabbits (Blue and Grey, Aylett, VA) weighing 2 kg received the same treatments except the dose of DEX was reduced to 150 mg/kg to avoid producing overt toxic affects. Female DBA/2 mice (Charles River, Wilmington, MA) weighing 20 g received daily intraperitoneal injections of DEX (250 mg/kg), PCN (200 mg/kg), RIF (50 mg/kg), PB (100 mg/kg), or TAO (120 mg/kg) for 4 days. After an overnight fast, the above treated female animals and female and male untreated animals of the same weight were killed and their livers were perfused with iced phosphate-buffered saline, pH 7.4. The livers were excised, homogenized, and microsomes were prepared and stored as previously described (8). Protein concentration was determined colorimetrically (15).

Purification of rat P-450p and rabbit LM3c and preparation of antibodies. Rat P-450p was purified from PCN-treated female rats, and form-specific antibodies were prepared to it as previously described (8, 10). Rabbit LM3c was purified to homogeneity and to a specific content of 17 nmol/mg protein from RIF-treated female rabbits by the method of Koop *et al.* (16). As previously reported (10), the NH_2-terminal sequence of this LM3c preparation was determined to be 73% homologous to that of rat P-450p through their first 15 amino acids. Serum containing antibodies to LM3c was obtained 8 weeks after the goat was initially immunized with 500 μg of LM3c in Freund's complete adjuvant. The initial immunization was followed by two boosts of 125 μg of LM3c at 4 and 6 weeks in Freund's incomplete adjuvant and buffer, respectively.

TAO complex formation. The amount of TAO complex formed *in vivo* was measured by diluting liver microsomes isolated from TAO-treated animals to 1 mg of protein/ml in 100 mM potassium phosphate buffer (pH 7.4). One ml of the suspension was placed into the reference and the sample cuvettes of an Aminco DW-II$_a$ spectrophotometer. After a baseline was recorded, 13.3 μl of 2 mM $K_3Fe(CN)_6$ was added to the reference cuvette and an equal volume of water to the sample cuvette. After a 10-min incubation at 25°, the spectrum was recorded to determine the difference in absorbance between 457 and 510 nm. The concentration of TAO complex was calculated using the previously determined extinction coefficient of 68 mM^{-1} cm^{-1} (10). Formation of the TAO complex *in vitro* was measured with microsomal samples other than those from TAO-treated rats. Suspensions (1 ml) of microsomes (1 mg of protein/ml) were added to the sample and to the reference cuvettes. The samples were heated to 35°, a baseline was recorded, and then 4 μl of 5 mM TAO in dimethyl sulfoxide or solvent alone was added to, respectively, the sample and reference cuvettes. NADPH (1 mM, final concentration) was then added to the sample cuvette to initiate the reaction. After 30 min when maximal complex had been formed, the spectrum was recorded and the concentration of TAO complex calculated.

Demethylation of EM. EM demethylation activity was detemined at 37° in an incubation mixture containing 0.5 mg of microsomal protein, 0.5 μmol of EM in a final volume of 0.5 ml of 100 mM potassium phosphate (pH 7.4). The reactions were initiated by the addition of 0.6 μmol of NADPH, terminated after 10 min, and formaldehyde formation measured by the method of Nash (17). Prior to assay, microsomes from TAO-treated animals were decomplexed by treatment with $K_3Fe(CN)_6$.

Immunoblot analysis. Quantitative immunoblot analysis of the electrophoretically separated microsomal proteins was carried out as previously described (5) with the following modifications. Microsomes in

[4] In previous reports (5, 10) we have demonstrated that P-450 PCN, P-450 DEX and P-450 TAO are all the same protein. Therefore, to avoid confusion, this cytochrome P-450 isoenzyme was renamed P-450p in Ref. 10.

amounts containing approximately 1 pmol of immunoreactive cytochrome P-450 were applied to 1.5-mm SDS-polyacrylamide (10%) slab gels (18) with 10 wells and were subjected to electrophoresis for 2 h at 30 mA/slab. Electrophoresis was stopped temporarily, and a second loading was made by adding to each well a different amount of purified P-450p in the range of 0.25–1.5 pmol of cytochrome P-450/well. Electrophoresis was resumed and, after 2 more hr, the separated proteins were transferred electrophoretically to nitrocellulose sheets at a current setting of 200 mA for 2 hr at 25° followed by 700 mA at 4° for 1 hr in a 50 mM Tris, 385 mM glycine, 40% methanol buffer. Under these conditions there was complete transfer of proteins (up to sample size of 35 μg) as shown by routinely staining the gel with Coomassie Blue after completing the "Western" transfer. Unreacted sites on the nitrocellulose sheets were blocked overnight at 25° with phosphate-buffered saline containing 10% dialyzed calf serum and 3% bovine serum albumin. The nitrocellulose was then treated sequentially (with 4–5 intermediate washings with phosphate-buffered saline at each step) with goat antibody directed against P-450p or LM3c, rabbit anti-goat IgG, goat peroxidase anti-peroxidase, and, finally, with 3,3′-diaminobenzidine tetrahydrochloride in hydrogen peroxide. The density and total area of the immunostained bands were measured by high resolution digitized scanning on a Colorscan C-4100 reflectance densitometer (Optronics, Intl.) controlled by an Optronics computer. The integrated density from approximately 3000 data points for a typical band was corrected for surrounding background readings by a program operating on a VAX computer. All values of integrated density fell within the linear response range as determined from densities of known cytochrome P-450p standards. Furthermore, in preliminary analyses, dilutions of a given microsomal sample produced densitometric readings which were directly proportional to the amount of applied protein.

RNA purification. A 25% (w/v) suspension of freshly excised rat liver was disrupted in 25 mM sodium acetate buffer, pH 5, containing 8 M guanidine hydrochloride and 1 mM dithiothreitol using a Brinkmann Polytron at midsetting. Total RNA was then extracted as described (19). Two cycles of oligo(dT)-cellulose chromatography was used to isolate poly(A) RNA.

Preparation of cDNA. Using liver poly(A) RNA isolated from a DEX-induced rat as template, a cDNA library was constructed according to the protocol described by Fagan *et al.* (20). Incorporation of 0.3 mCi/mmol of [α-^{32}P]dCTP into first strand cDNA by reverse transcriptase was extended to 60 min at 42°. The isolated cDNA was made double stranded with *Escherichia coli* DNA polymerase I by incubation with 0.2 Ci/mmol of [α-^{32}P]dCTP for 22 hr at 15°. Poly(dC) was added to the 3′ termini of S1-treated double-stranded cDNA by reaction with terminal transferase at 37° for 7 min. The tailed cDNA was annealed to an equimolar amount of *Pst*I-linearized pBR322 previously tailed with deoxyguanosine residues in a 200-μl reaction containing 10 mM Tris, pH 7.8, 100 mM NaCl, and 0.1 mM EDTA. The annealing reactions were heated to 70° for 8 min followed by sequential 1-hr incubations at 57, 42, and 22°. *E. coli* strain HB101 was transformed with 20-μl aliquots of the annealed plasmid mixture. One hundred ninety-four tetracycline-resistant ampicillin-sensitive transformants were screened by hybrid-selected translations.

Hybrid selection. Plasmid DNA isolated from 5-ml cultures using the alkaline lysis procedure (21) was dissolved in 100 μl of 10 mM Tris, pH 8, 1 mM EDTA, boiled for 10 min, and then denatured with 150 μl of 1 M NaOH. Following a 20-min incubation at room temperature the solutions were neutralized with 900 μl of 10× SSC containing 0.25 M Tris, pH 7.5, and 0.25 N HCl. The DNA was immediately bound to a nitrocellulose membrane previously soaded in 6× SSC. The filter was allowed to air dry and then baked 2 hr at 80° in a vacuum oven. Individual discs of the recombinant plasmids were pooled and hybridized to poly(A) RNA from DEX-treated rat liver according to Fagan *et al.* (20). The hybrid-selected RNA was translated in a reticulocyte lysate (New England Nuclear), and the products were analyzed by SDS-polyacrylamide gel electrophoresis (18) and fluorography (22).

Preparation of a second cDNA library. A second cDNA library was constructed from poly(A) RNA isolated from DEX-treated rat liver according to a protocol similar to that used to construct the first cDNA library except that S1 nuclease treatment was omitted and second strand synthesis was achieved with the use of RNase H and DNA polymerase I (23). The recombinant plasmids (23) were cloned in *E. coli* strain MM294 that had been rendered competent for transformation (24).

Screening of second cDNA library with pDEX3.22. Bacterial colonies harboring recombinant plasmids grown on LB agar were replicated on nitrocellulose filters as described (21). After baking (2 hrs at 80° *in vacuo*) filters were washed for 1 hr at 65° in 4× SSC containing 1 × Denhardt's reagent. Bacterial debris was removed from the filters by wiping with tissue paper and then prehybridized with 4× SSC containing 1 × Denhardt's reagent, 0.1% SDS, and 20 μg/ml salmon sperm DNA in heat-sealed bags for 2 h at 65°. A probe was prepared by isolating the cDNA insert from pDEX3.22 by digestion with *Pst*I followed by purification from agarose gels (21). This insert was radiolabeled with ^{32}P by nick translation (21) and was added (10 μg/ml) to the filters in heat-sealed bags in the same buffer for 16 hr at 65°. The filters were then washed twice with 2× SSC containing 0.1% SDS, twice with 1× SSC plus 0.1% SDS, and twice with 0.5× SSC plus 0.1% SDS, all at 65° with gentle shaking. The filters were autoradiographed overnight at −80° with 2 intensifying screens. Of 1 × 10^5 colonies screened, 35 gave positive signals when hybridized to pDEX3.22 and, after rescreening, these were grown as 5-ml cultures, plasmids were isolated (21), the inserts were excised by digestion with *Pst*I, and the longest of these, termed pDEX12, was used in further studies.

Northern blots. Total liver RNA (10 μg) or poly(A)-selected RNA (0.5 μg) was subjected to electrophoresis in agarose gels in 10 mM sodium phosphate buffer containing 1.1 M formaldehyde and transferred to nitrocellulose all as described in the manufacturer's (Schleicher and Schuell) specification sheet 352-354. Hybridizations were carried out with ^{32}P-labeled DNA inserts from pDEX3.22 or pDEX12 prepared by nick translation of excised cDNA purified from 5% polyacrylamide gels with the use of D-Gel apparatus (Epigene, Baltimore, MD).

RESULTS

Measurements of P-450p functions in liver microsomes from various species. We have recently demonstrated using a number of criteria that rat P-450p is uniquely responsible for the conversion of TAO into a metabolic intermediate that forms a spectrally detectable complex with P-450p (10). Therefore, measuring the formation of the TAO complex *in vitro* provides a simple way to estimate the amount of P-450p in microsomes. In order to further study the regulation of the induction of P-450p we examined TAO complex formation by liver microsomes isolated from female gerbils, hamsters, rabbits, mice, and rats that had been treated with inducers of cytochrome P-450p at their maximally tolerated doses or at doses previously demonstrated to result in the induction of other cytochromes P-450. It was found that the amount of P-450p in rat microsomes as determined by the ability to form TAO complex was induced more than 40 times by treatment of female rats with TAO, more than 20 times by treatment with DEX or PCN, less than two times by treatment with PB, and not at all by treatment with RIF when compared to microsomes from untreated males (Fig. 1). There was no detectable TAO complex formed by microsomes from untreated female rats, a finding consistent with other reports that demonstrate that P-450p is a "male-specific" form of cytochrome P-450 (9). In all other species tested maximal induction of microsomal TAO complex-forming activity

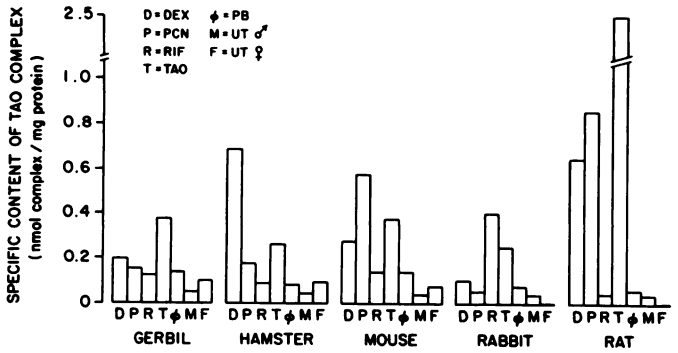

FIG. 1. *TAO complex formation in liver microsomes from various species treated with inducers of rat P-450p*

Microsomes were prepared as indicated in Experimental Procedures from the livers isolated from untreated males (*M*) and females (*F*) and from female animals treated with DEX (*D*), PCN (*P*), RIF (*R*), TAO (*T*), or PB (*ϕ*). A single animal was used for each of the indicated treatments. TAO complex was measured directly (TAO-treated animals) or as formed upon incubation with TAO *in vitro* as described in Experimental Procedures. Results are presented as maximal amount of complex formed per mg of protein and are the average of two replicate determinations for each microsomal preparation, except for female rabbit (*F*) which was not measured.

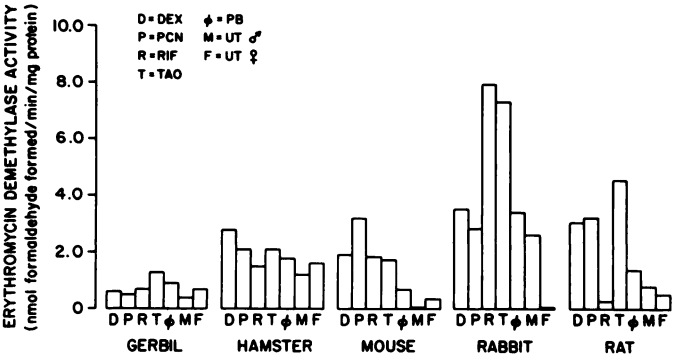

FIG. 2. *Erythromycin demethylase activity in liver microsomes from various species treated with inducers of rat P-450p*

Microsomes were prepared as outlined in Fig. 1 and were analyzed for EM demethylase activity as described in Experimental Procedures. Results are expressed as nanomoles of formaldehyde formed per min per mg of protein and represent the average of two separate determinations, except for female rabbit (*F*) which was not measured.

was less than in the rat (Fig. 1), and the relative efficacy of the inducers was strikingly different. For example, in the rabbit, RIF gave the greatest induction of the ability to form TAO complex whereas PCN produced little, if any, induction of this catalytic activity (Fig. 1). The mouse resembled the rat in the profile of inducers of TAO complex formation, except that TAO gave less induction than did the steroids, and complex formation was slightly higher in females than in males. In the hamster DEX maximally increased the TAO complex formation whereas in the gerbil TAO was the only strong inducer of a form of P-450 responsible for TAO complex formation (Fig. 1).

With the same microsomal preparations used for the results in Fig. 1, we measured EM demethylase, a catalytic activity characteristic of rat P-450p and its homologous form in rabbits, LM3c (10). In rats, the relative ability of the tested inducers to increase EM demethylase activity (Fig. 2) was similar to that for induction of TAO complex formation. However, the magnitude of increase of EM demethylase activity by TAO (5.5-fold), by PCN (3.8-fold), and by DEX (3.6-fold) relative to untreated male rat controls was less than that for TAO complex formation. Low levels of EM demethylase activity were detected in microsomes from RIF-treated and untreated female rats (Fig. 2). The latter finding suggests that EM demethylation may also be catalyzed by isozymes other than P-450p since P-450p is a "male-specific" form (9). Among all species the greatest increase in EM demethylase activity was in the rabbit where RIF and TAO treatments resulted in the largest increases. The profiles for induction of EM demethylase in the gerbil and mouse were similar to that for TAO complex formation (except for lack of EM demethylase activity in untreated male mouse microsomes), whereas in the hamster, little induction was apparent because the basal levels of EM demethylase in untreated male and female controls were higher than in the other species. Thus, it may be concluded that there is an excellent parallelism between EM demethylase and TAO complex formation within a species (correlation coefficients for EM demethylation *versus* TAO complex formation within a species are: rat, 0.90; mouse, 0.97; gerbil, 0.89; hamster, 0.91; and rabbit, 0.87); however, the actual turnover numbers for these reactions differed markedly among the species we tested.

Identification and measurement of proteins immunochemically related to P-450p. Qualitative immunoblots of electrophoretically separated proteins of the various microsomes developed with anti-P-450p IgG revealed, in most cases, only a single narrow band (Figs. 3A and 4A). The only samples which lacked detectable amount of immunoreactive protein at all concentrations of microsomes examined were from untreated and RIF-treated female rats. Rat P-450p has an apparent molecular weights of 51,000 (8) whereas the immunochemically related proteins in mouse, gerbil, hamster, and rabbit have molecular weights of 52,000, 51,000, 51,000 and 53,000, respectively (Fig. 3A). In the rabbit a second immunoreactive band migrating as a larger protein than the 53,000-dalton band was clearly present in microsomes from untreated males and females and in DEX- and RIF-treated females (Figs. 3A and 4A). Only the smaller of the two immunoreactive proteins (probably LM3c) is present in microsomes isolated from rabbits treated with TAO, PCN, or PB suggesting that these compounds repress the accumulation of the higher molecular weight protein. We confirmed these results by developing another set of blots with antibodies to rabbit LM3c (Figs. 3B and 4B), a protein that is immunochemically and structurally related to P-450p (10). The pattern of proteins intensely stained in blots developed with anti-LM3c IgG was identical to that obtained with anti-P-450p (Figs. 3A and 4A). However, there were additional lower molecular weight proteins visualized on blots developed with anti-LM3c IgG that were not recognized by anti-P-450p IgG (Figs. 3 and 4). Thus, from immunoblot analyses it may be concluded that each of the species tested contain at least one, and in the case of the rabbit,

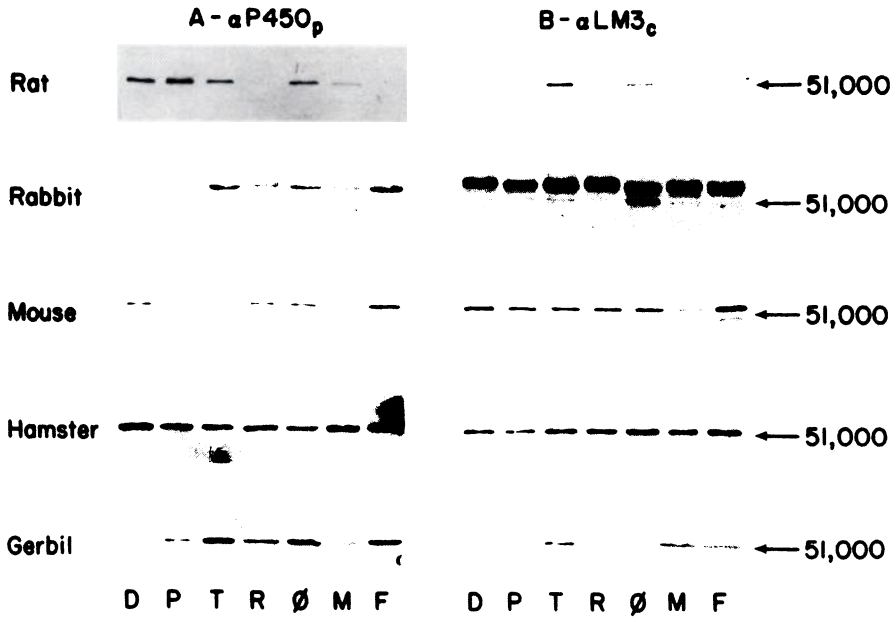

FIG. 3. *Qualitative immunoblots of proteins from various species reactive with antibodies to P-450p or LM3c*

Various concentrations of the liver microsomal preparations from the indicated species and treatment groups (Fig. 1) were analyzed by immunoblotting as described in Experimental Procedures. The blots were developed with either antibody to rat P-450p (A) or rabbit LM3c (B). The indicated size (51,000 daltons) was determined from interpolation of the mobility of molecular weight standards.

at least two inducible proteins immunochemically related to P-450p and LM3c.

Immunoblot analyses of electrophoretically separated microsomal proteins can be used to measure the amount of a given form of P-450 by comparing the staining intensities of an unknown sample to a standard curve developed with known quantities of the purified proteins (3). Since all the treated animals were female, the amounts of the immunoreactive protein(s) in the various treatment groups were expressed relative to the value in the untreated female of the indicated species (Fig. 5). P-450p was strongly induced by TAO (over 350-fold) and the steroids (over 100-fold), was weakly induced by PB (18-fold), and was not at all induced by RIF when compared to the minimum detectable value of 0.2 pmol of P-450p in female rats. The pattern of induction of P-450p protein is similar to that for induction of TAO complex formation (Fig. 1) and EM demethylase activity (Fig. 2) in the rat. However, the absolute correlations are not as strong as the correlations between EM demethylation and TAO complex formation possibly due to the presence of apocytochrome. The patterns of relative induction of the immunoreactive forms in the other species also closely parallel those seen for the induction of TAO complex formation (Fig. 1) and EM demethylase activity (Fig. 2). Thus, in rabbits RIF was the best inducer while the steroids were the weakest whereas in hamsters DEX was the strongest and RIF the weakest. In gerbils, TAO was the strongest inducer. In mice there were similar increases produced by all inducers except PB (Fig. 5). Because we lacked purified proteins for use as standards with gerbils, hamsters, and mice all results have been expressed as the ratio of the value for the treated female animals or male untreated animal relative to that of the female untreated controls.

Use of cloned cDNAs to detect mRNAs related to P-450p mRNA. The first cDNA library was prepared by isolating poly(A) RNA from the liver of DEX-treated rats for use as template for synthesis of cDNAs. These were converted to double-stranded cDNAs and were inserted into the *Pst*I site of pBR322 by homopolymer tailing and were cloned in *E. coli* hosts. The recombinant plasmids from 50 of the resulting colonies were isolated, immobilized on nitrocellulose filters, and hybridized with liver RNA prepared from a DEX-treated rat. Specifically hybridized mRNA was eluted from each filter and used to program a cell-free translation system. Analysis of the total radioactive translation products separated by gel electrophoresis and visualized by fluorography (Fig. 6) revealed substantial enrichment over background absorption of a band migrating as a 51,000-dalton protein from the mRNA "selected" by pDex3.22. This protein was immunoprecipitated by antibodies directed against P-450p or LM3c but not by nonimmune IgG nor by specific antibodies directed against P-450b or P-450c (not shown).

pDex3.22 which contained approximately 400 nucleotides of cDNA inserted at the *Pst*I site was excised and radiolabeled with ^{32}P by nick translation and hybridized as a probe for P-450p mRNA to "Northern" blots of rat liver RNA. As shown by autoradiography, when [^{32}P] pDex3.22 was hybridized to blots of electrophoretically separated liver RNA extracted from rats treated with DEX, PCN, or TAO, a single band was visualized with apparent size of 2.1 kilobase pairs on formaldehyde gels (Fig. 7). This size is in good agreement with the reported

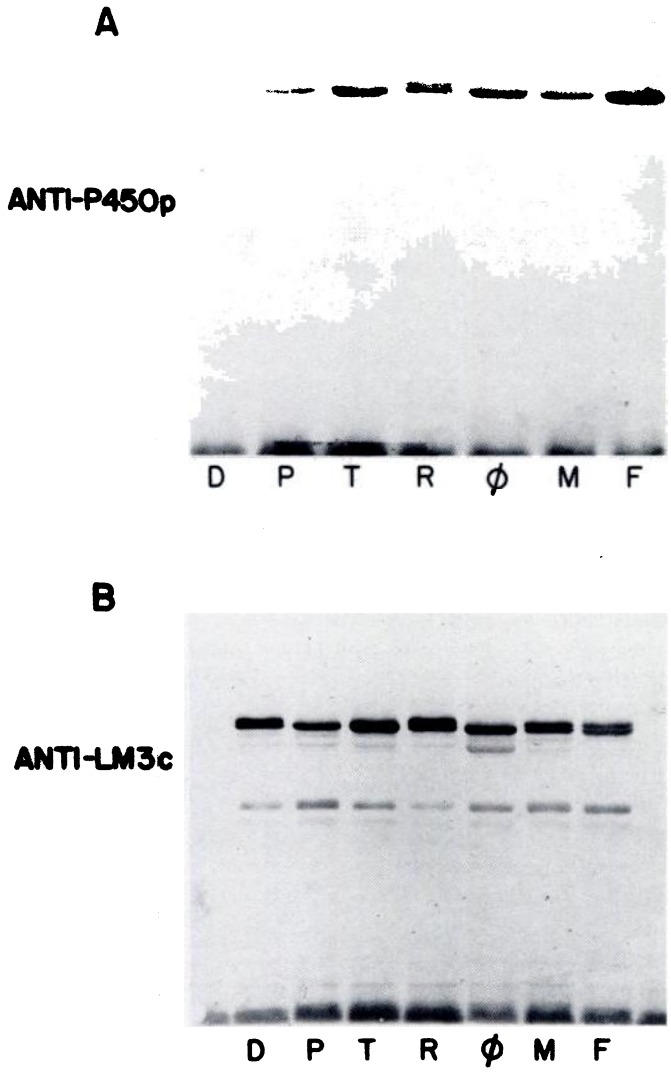

FIG. 4. *Qualitative immunoblots of rabbit microsomal proteins immunoreactive with antibodies to P-450p or LM3c*

Various concentrations of the rabbit liver microsomal preparations from the indicated treatment groups (Fig. 1) were analyzed by immunoblotting as described in Experimental Procedures. The blots were developed with either antibody directed against rat P-450p (A) or rabbit LM3c (B).

message size of 2038 nucleotides for the PCN-inducible cytochrome P-450 isolated by Gonzalez *et al.* (25). Weaker signals were observed for RNA from PB-treated rats or from male controls (Fig. 7), and no response was detected for RNA from β-naphthoflavone-treated or female untreated rats (Fig. 7). Although these results are qualitative, it may reasonably be concluded that pDex3.22 hybridizes to a species of rat liver mRNA with induction characteristics similar to those of P-450p protein (Fig. 5).

Attempts to probe "Northern blots" of rabbit liver RNA with pDex3.22 failed to produce evidence of specific binding even under low stringency conditions (not shown). It was possible that pDex3.22 was not sufficiently long to encompass areas of homology that might

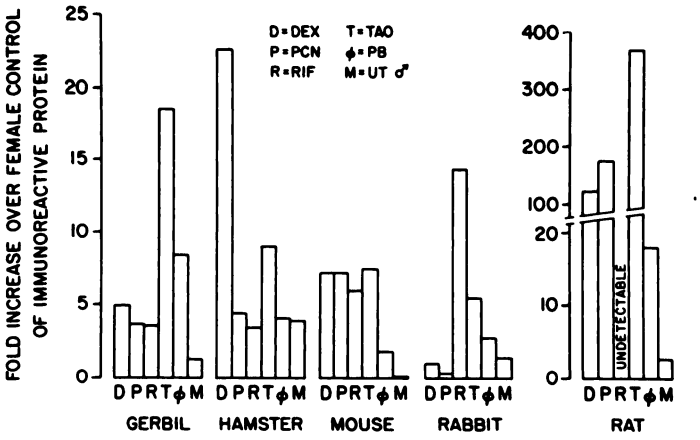

FIG. 5. *Immunochemical quantitation of P-450p and related proteins in various species treated with inducers of rat P-450p*

The same microsomal samples utilized in Figs. 1 and 2 were analyzed by quantitative immunoblot analysis as described in Experimental Procedures. The results were expressed as relative induction over and above the values in untreated female controls or the minimum detectable level which was 0.2 pmol of rat P-450p.

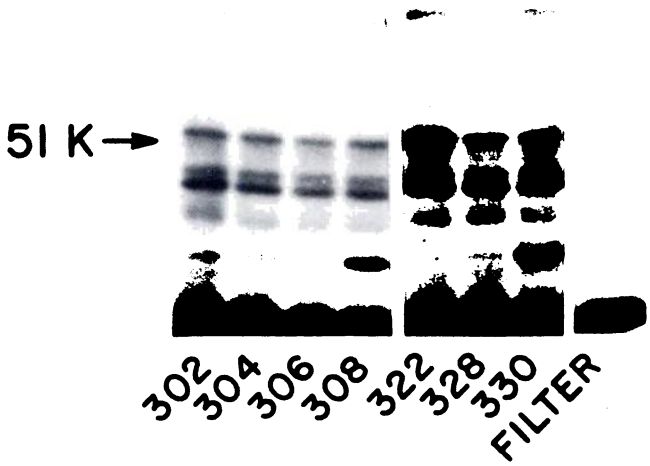

FIG. 6. *Fluorographic analysis of total translation products programmed by mRNA hybrid selected by cloned cDNAs*

Recombinant plasmids were extracted from individual colonies of a cDNA library constructed from DEX-induced rat liver mRNA, were affixed to nitrocellulose filters, and hybridized with the total RNA used to construct the library. After washing, the specifically hybridized mRNA was eluted and used to program a reticulocyte lysate translation system containing [^{35}S]methionine (see Experimental Procedures). Total translation products were separated by polyacrylamide gel electrophoresis and visualized by fluorography. The numbers indicate examples of the translation products produced by "hybrid selected" mRNAs by individual plasmids. "Filter" refers to a control filter containing no plasmid.

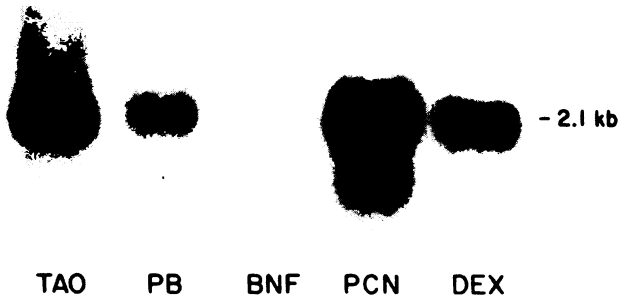

FIG. 7. *Northern blot analysis of liver RNAs from rats treated with various inducers*

Total liver RNA extracted from rats treated with the indicated inducers (Fig. 1) was processed by electrophoresis on agarose gels, transferred to nitrocellulose paper, and hybridized with [^{32}P]pDEX3.22 as described in Experimental Procedures. The apparent size of mRNA on the autoradiograph was determined by the interpolation of the mobility of RNA standards. *BNF*, β-naphthoflavone.

be expected for the mRNAs of immunochemically related P-450p and LM3c. Therefore, we constructed a second cDNA library from DEX-induced rat liver RNA according to a different protocol. The resulting recombinant plasmids cloned in *E. coli* hosts and were screened by hybridizing the colonies with ^{32}P-labeled pDex3.22. Of the plasmids recovered from the positive colonies, pDEX12 contained an inserted cDNA of approximately 1000 nucleotides and, like pDex3.22, hybridized to an mRNA that, when translated in a cell-free system, produced a 51,000-dalton protein immunoprecipitable by anti-P-450p IgG (not shown). The nucleic acid sequence of pDEX12 (Table 1) is composed of approximately half 3′ coding information and half 3′ untranslated regions of the mRNA (25).

Use of pDex12 as a probe for "Northern hybridizations" of rat liver RNA produced similar results (Fig. 8) to those obtained with pDex3.22 (see Fig. 7) in that an intense band was observed for RNA from rats treated with DEX or TAO, a weaker band (of similar mobility) from rats treated with PB, and no reaction for RIF-treated or female untreated rats. However, under equivalent conditions of stringency and washing (Fig. 8) and even with reduced stringency or with additional RNA applied to the blot (not shown), pDEX12 like pDex3.22 failed to hybridize to any of the rabbit RNAs tested. On the same blots hamster RNA from DEX-, RIF-, TAO-,

TABLE 1

Nucleic acid sequences of pDex3.22 and pDex12

pDEX3.22 was partially sequenced by the Maxam-Gilbert technique (21). The cloned cDNA in pDEX12 was excised by double digestion with *Pst*I and *Eco*RI restriction endonucleases, the fragments were subcloned into *Pst*I/*Eco*RI-digested phage vectors M13p18 and M13p19 (Pharmacia Molecular Biologicals, Piscataway, NJ) and were sequenced by the Sanger dideoxy method (Amersham Corp.). The indicated sequence and numbers are taken from the full length cDNA clone of a PCN-inducible protein as reported by Gonzalez and associates (25). The underlined sequences represent pDEX12. The overlined sequences are pDEX3.22. Discrepancies between pDEX12 and the full length clone are represented by the latter being superscripted. The boldface letters represent the *Eco*RI restriction site.

1141	ATGGAATACC	TGGATATGGT	GTTGAATGAA	ACCCTCAGAT	TGTATCCAAT	TGGTAATAGA
1201	CTTGAGAGAG	TCTGTAAAAA	AGATGTTGAA	ATCAATGGTG	TGTTTATGCC	CAAAGGGTCA
1261	GTGGTCATGA	TTCCATCTTA	TGCTCTTCAC	CGTGATCCAC	AGCAGTGGCC	AGACCCTGAG
1321	GAATTTCGCC	CAGAAAGGTT	CAGCAAGGAG	AACAAGGGCA	GCATTGATCC	TTATGTATAT
1381	CTGCCCTTTG	GAAATGGACC	CAGGAACTGC	ATTGGCATGA	GGTTTGCTCT	CATGAATATG
1441	AAACTCGCTC	TCACTAAAGT	TCTGCAAAAC	TTCTCCTTCC	AGCCTTGTAA	GGAAACACAG
1501	ATACCTCTGA	AATTAAGCAG	ACAAGGACTT	CTTCAACCAA	CAAAACCCAT	TATTCTAAAG
1561	GTTGTGCCAC	GGGATGAAAT	CATAACTGGA	TCATGATTTT	CCCTCAAGGA	GTTCTGCTGA
1621	ATTC$^{G}_{T}$TCAGA	AATGTGGTGT	CTAAGAACAC	CAGACCCTTT	AATTTATGTC	ATGAATAAAA
1681	TTCAGATGAA	ATTAGGGCTT	AATCGACTTT	GTTTTGATTC	GGTACATCTT	TGATCTTTCT
1741	CAGTGTCTAC	AATGTACCCA	TCTAATATAA	AGGAAATGAC	AAGTCAGTGA	CAGAACAGGA
1801	ACTTAACCTT	TGGTGATTCT	CA$^{C}_{T}$GGGACTA	CCTCCATCCA	CATCTGGTTG	TCTCTGTTAA
1861	TTTCTTTTGA	TAGTAACCTT	G$^{CTCTC}_{TCTCT}$TGTA	ATTTGATCAG	AATTTTTCAT	GAAAATGTGA
1921	ACTATTCTGA	CACCTTTAAT	TGTAGATTTG	GTATCAGATG	TTTAGATGCA	TTATTCTACA
1981	CTAAATGTTA	CATGGAAAAA	ATGTGAATAA	ACACTTCTTT	AAAAATCCCC	AGGGGCA

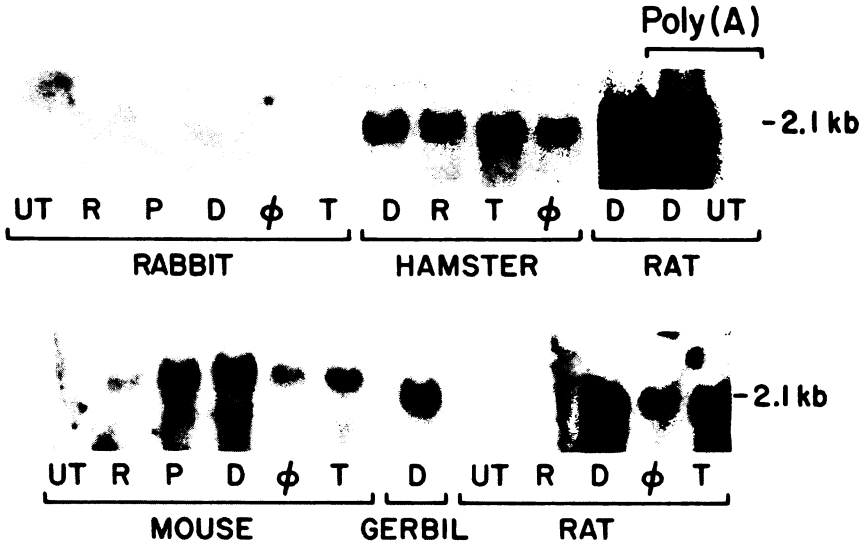

FIG. 8. *Northern hybridizations of liver RNAs from various species to pDex12*

Total liver RNA or poly(A) RNA as indicated were isolated from the indicated species and treatment groups (Fig. 1). The RNA was processed by electrophoresis on agarose gels, transferred to nitrocellulose filters, hybridized with [^{32}P]pDEX12, and visualized by autoradiography.

and PB-treated animals each gave a single band slightly faster in mobility when compared to that of rat RNAs (Fig. 8). RNA from DEX-treated gerbils also reacted with a single band, with equal mobility to the species of rat RNA (Fig. 8). Finally, "Northern" hybridization of mouse RNA (Fig. 8) with pDEX12 showed a single band, slower in mobility than that of rat RNA. This finding is consistent with the observation (Fig. 3A) that the immunochemically related mouse protein is apparently larger than P-450p. Although Northern blot analysis is qualitative, the most intense signals resulted from RNA extracted from mice treated with DEX or PCN, somewhat weaker reactions with those treated with TAO, PB, and RIF. No band was detected with untreated female controls (Fig. 8) even though the immunochemically related protein was present in female untreated mouse liver (Fig. 3).

DISCUSSION

The results show that proteins homologous to rat P-450p exist in all of the species we tested based on the criteria of TAO complex formation, EM demethylase activity, immunoblot analysis, apparent molecular weight, and inducibility by agents known to induce P-450p. These proteins are not only structurally and catalytically similar to P-450p but are also encoded by mRNAs of sufficient homology (except for the rabbit) to hybridize specifically to cloned cDNAs to P-450p mRNA. The present results may also extend to man since human liver contains an inducible protein equivalent to the rat P-450p (26). Definitive proof for homology among these immunochemically related proteins would require their purification and amino acid sequence determination. To date, this has only been partially accomplished in that we have demonstrated that the NH_2-terminal sequences of P-450p and rabbit LM3c are 73% homologous through 15 amino acids (10). Nevertheless, pending such detailed analyses it seems reasonable to conclude that P-450p is a conserved hepatic cytochrome expressed in most mammalian species. This conclusion is in keeping with emerging results from other families of the cytochrome P-450 enzyme system. There is a high degree of sequence homology among the MC-inducible forms in the rat, rabbit, and mouse (1, 13, 14) and extensive sequence homology between the phenobarbital-inducible forms in the rat and rabbit (1, 27). Our results also disclosed some striking species specificities in the inducibility of the immunorelated proteins equivalent to rat P-450p.

Our study was not designed to definitively establish the hierachy of potency and efficacy of inducers of proteins equivalent to rat P-450p. This would require a complete dose-response testing for each inducer in each species. Nevertheless, using doses at or near those maximally tolerated for each species an apparent rank order of efficacy shows striking differences among the species. Most noteworthy is the tentative identification of two species "specific" inducers. RIF, the best inducer of rabbit immunoreactive proteins was not at all an inducer of P-450p in the rat. Just the opposite is the case for PCN, a moderate inducer of P-450p in the rat which fails to induce LM3c in the rabbit. The mouse, hamster, and gerbil responded to all of these inducers; however, the responses were of different magnitudes among the species. Whereas there are species differences in the magnitude of response to members of the phenobarbital or MC "classes" of inducers (14) the total absence of induction of a defined protein as seen in the rat and rabbit is to our knowledge unprecedented. We have suggested that in rat liver, induction of P-450p by steroids of the glucocorticoid class is mediated through a stereospecific mechanism (5, 6). Such a "receptor," if it exists in the rabbit, may not recognize PCN. RIF is an inducer in rabbits but not in rats and is not known to be a modulator of glucocorticoid responses. Therefore, RIF may act

through a separate mechanism. Since RIF does not induce in rats where PB is an inducer, the PB response may represent a third distinct mechanism. With the present results in which species have been identified that respond to some or all these inducers it should be possible using DNA recombinant technology to define the genetic differences in the molecular mechanisms of these induction processes.

An unexpected result was the finding of two immunoreactive proteins in rabbit liver microsomes. These proteins could be clearly distinguished on immunoblots developed with both anti-P-450p and anti-LM3c IgGs and, furthermore, exhibited strikingly different induction characteristics. Both proteins were present in untreated animals. The larger of the two which probably represents a protein other than LM3c was induced in female rabbits by only RIF and DEX and actually disappeared upon treatment with TAO, PCN, and PB. The fact that PCN decreased this protein suggests that the lack of induction of LM3c discussed in the preceding paragraph is not due to impaired uptake of PCN by rabbit liver. If these proteins are the products of separate genes, then the rabbit may serve as a valuable model for comparing intraspecies differences in the expression of structurally similar forms of cytochrome P-450. However, at present, we cannot exclude the possibility that the higher molecular weight protein is a precursor converted to LM3c by a process differentially affected by inducers.

Our studies of hamster liver provide strong evidence for the presence of an inducible cytochrome P-450 in females with the immunochemical and catalytic characteristics of P-450p and an inducible mRNA hybridizable with cDNAs to P-450p mRNA. Thus, it seems that the reported failure to find a PCN-inducible protein in hamster microsomes analyzed by two-dimensional gel electrophoresis concerns a protein other than P-450p (28). These data have important implications for the possible physiologic role of P-450p which has recently been reported to catalyze 6β-hydroxylation of steroids (29). Hence, if P-450p were confined to the rat and mouse, species which exclusively produce β-muricholic acid, a 6β-hydroxy derivative of chenodeoxycholic acid as the dominant trihydroxy bile acid, P-450p could be the enzyme responsible for catalyzing this reaction. Indeed, studies on rat hepatoma cultures have revealed the presence of a DEX-inducible enzyme catalyzing conversion of bile acids to β-muricholic acid (30). However, in light of the present results that proteins equivalent to P-450p exist in the hamster, rabbit, gerbil, and man, species which synthesize cholic acid as their dominant trihydroxy bile salt (31), it seems unlikely that the exclusive role of P-450p is production of β-muricholic acid.

In all of the species examined there was a striking disparity in the magnitude of induction of immunoreactive protein as compared to the magnitude of induction of TAO complex formation and EM demethylase activity (Figs. 1, 2, and 5). Even in the rat where the availability of purified standards provides the basis for definitive quantitation, the fold increase in protein levels (measured by immunoblot analyses) exceeds the increases of the oxidative activities associated with P-450p after treatment with the various inducers. It is possible that this may be explained by the presence of proteins immunoreactive with anti-P-450p IgG that are not resolved by immunoblot analysis and, yet, do not form the TAO complex or metabolize EM. It is also possible that inducers increase the amount of apoprotein in vast excess of holocytochrome which is measured specifically by TAO complex formation and EM demethylase activity. This phenomenon also has been proposed by others to explain lack of correspondence between the amount of immunoreactive cytochromes and total CO-binding heme protein (3). In addition, we have not yet demonstrated that the P-450p-related proteins in all the species are solely responsible for the metabolism of TAO. It is doubtful that this discrepancy is attributed simply to systematic errors in immunoquantitation because in primary cultures of adult rat hepatocytes the rates of *de novo* synthesis and accumulation of P-450p protein are stimulated by DEX and PCN to a far greater extent than is the amount of accumulated holocytochrome P-450 or drug-oxidizing activity (5, 6, 32).

Important supplementary information for the presence in species other than the rat of proteins equivalent to P-450p came from Northern blot analyses of liver RNA. Evidence that the cDNAs we used were directed against rat P-450p mRNA include the fact that they were isolated from libraries that were constructed with DEX-induced mRNA, that they hybridized to a mRNA-directing synthesis of a translation product of the appropriate size which was immunoreactive with anti-P-450p and anti-LM3c, and that they hybridized to rat liver mRNAs induced in parallel with P-450p protein. Strong evidence that pDEX3.22 and pDEX12 hybridize specifically to P-450p mRNA is that their sequence is in excellent agreement with that of a full length cDNA clone of a mRNA encoding a PCN-inducible protein (25). However, there may be, in fact, a family of "P-450p-like" proteins, since the reported cloned cDNA (25) contains an NH_2-terminal sequence totally different from that of P-450p (10). In the Northern blot analysis of the liver poly(A) RNA isolated from a PCN-treated rat there appears a second species of RNA hybridizing with our probes. We are currently investigating whether this band represents a second mRNA or a breakdown product of the P-450p mRNA. Lack of recognition of rabbit mRNAs may be due to insufficient sequence homology in the areas of the message spanned by our probes. It may also represent a more distant sequence homology between the proteins, even though these proteins retained immunoreactivity. It is interesting that species comparison of genomic Southern blots with a cDNA probe to a PCN-inducible cytochrome P-450 reacted only weakly in the rabbit as compared to other species (33). These intriguing findings provide an additional incentive to proceed with trans-species genetic analyses of this unique family of steroid-inducible cytochromes P-450.

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