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Pregnenolone 16α-Carbonitrile-Inducible P-450 Gene Family: Gene Conversion and Differential Regulation

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A cytochrome P-450 cDNA clone, designated pP450PCN2, homologous to the previously characterized pregnenolone 16α-carbonitrile (PCN)-induced P-450 cDNA (pP450PCN1; F. J. Gonzalez, D. W. Nebert, J. P. Hardwick, and C. B. Kasper, J. Biol. Chem. 260:7435–7441), was isolated from a rat liver cDNA expression library by use of a polyclonal anti-P450PCN1 antibody. This P-450 cDNA contains 2,014 base pairs and yields an open reading frame of a protein consisting of 504 amino acids (M_r = 57,760). P450PCN2 cDNA and protein shared 90% nucleotide and 89% amino acid similarity with P450PCN1 cDNA and protein, respectively. The 5′ untranslated, coding, and 3′ untranslated regions between the two cDNAs share 94, 93, and 79% similarities, respectively. Nucleotide differences in the coding regions, however, are not evenly distributed. Complete homology exists between the two mRNAs for 425 nucleotides (positions 346 through 771). Other regions of 93 nucleotides containing only one difference and 147 nucleotides containing two differences exist toward the 3′ end of the coding regions. These data suggest the possibility that a gene conversion event(s) have occurred subsequent to duplication of the ancestral P450PCN gene. Oligonucleotide probes unique for P450PCN1 and P450PCN2 cDNAs were used to examine the levels of their respective mRNAs in noninduced and PCN-induced liver cells and in male and female rats of various ages. P450PCN1 mRNA was not detectable in either male or female rats at any ages. In contrast, P450PCN2 mRNA was present at a low level in newborn rats and became elevated in both males and females at 1 week of age. Levels of P450PCN2 mRNA continued to increase in males until 12 weeks, whereas the mRNA in females reached peak levels at 2 weeks of age but declined continuously at the onset of puberty (between 4 and 12 weeks). These levels of P450PCN2 mRNA closely parallel the increases in testosterone 6β-hydroxylase activity and P450PCN2 protein level, as analyzed by Western blots. P450PCN1 mRNA was induced by PCN, dexamethasone, and phenobarbital in both male and female rats. P450PCN2 mRNA was not significantly induced by PCN or dexamethasone but was readily induced by phenobarbital. Testosterone 6β-hydroxylase activity was also induced severalfold by PCN, dexamethasone, and phenobarbital. These data demonstrate that P450PCN1 and P450PCN2 genes are differentially regulated during development and after administration of inducing compounds and furthermore suggest that both enzymes possess testosterone 6β-hydroxylase activity.

The primary components of the hepatic microsomal multisubstrate monooxygenase system are NADPH-cytochrome P-450 oxidoreductase and cytochrome P-450 (P-450). The former is a single molecular species that transfers reducing equivalents from NADPH to P-450. P-450s are a population of related enzymes that range in size from 49,000 to 58,000 daltons and contain noncovalently bound heme. In some cases, foreign compounds and endogenous substances regulate levels of specific P-450 species (22, 47). The structure (1, 5a), regulation (1, 30, 46), and role of P-450s in carcinogen metabolism (12, 36) have been reviewed previously.

Collectively, P-450s represent a superfamily of enzymes, and three families have been grouped based on their regulation. The tetrachlorodibenzo-*p*-dioxin (TCDD)-inducible P-450 family is composed of two genes in the mouse (13, 29) and rat (11, 40, 51). Another family of P-450s has at least two members that are induced by phenobarbital (2, 41). The exact number of genes in this family has not yet been determined; however, estimates based on isolation of genomic clones and Southern blots range from 9 to 20 (1, 2, 28). The pregnenolone 16α-carbonitrile (PCN)-induced P-450 gene family also contains several members. Southern blot analysis revealed the presence of multiple genomic fragments that anneal with a PCN-induced P-450 cDNA (16). This is the only cDNA (designated pP450PCN1) in the PCN family that has been characterized by complete sequence analysis (14). Interestingly, the TCDD-induced (18), phenobarbital-induced (38, 39), and PCN-induced (39) P-450 families are located on different mouse chromosomes.

The fact that three families of P-450 genes are located on different chromosomes (18, 38, 39) and share only about 30% amino acid homology suggests that these genes diverged from an ancestral P-450 early in evolution (29). In addition to random divergence, however, another factor has affected P-450 evolution. Examination of TCDD-induced (13) and phenobarbital-induced (1, 3) P-450 cDNAs reveals regions of high and low homology among family members. Recently, structural analysis of several genes in the phenobarbital gene family has shown that homology between cDNAs extends into introns (3). This evidence strongly suggests that gene conversion events have occurred in the phenobarbital-induced P-450 family (3). A gene conversion event is a nonreciprocal recombination in which a segment of one gene replaces a segment of another (19). This action can either maintain homology among gene family members or introduce more diversity (24). Gene conversion has played a role in the evolution of a number of gene families (see Discussion).

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TABLE 1. Homologous segments between P450PCN1 and P450PCN2 cDNAs[a]

Nucleotide positions	Total nucleotides	Mismatched nucleotides	% similarity	Similarity index[b]
1–128	128	5	96	
129–345	212	27	87	
346–771[c]	425	0	100	1
772–1115	343	46	87	
1116–1209	93	1	99	2
1210–1351	141	21	85	
1352–1499	147	2	99	3
1500–1578	78	13	83	
1579–2020	441	95	79	

[a] Sequences are shown in Fig. 1.
[b] Rank of most significant homologies, based on length and mismatched nucleotides.
[c] Underlined regions are highly homologous.

P450PCN1 mRNA is highly induced by PCN and phenobarbital (16). Other investigators have shown that another P-450 species is also induced by glucocorticoids (47, 49, 50). This P-450 was designated P450PCN-E (47) and P450p (49). The amino-terminal sequences of P450PCN-E (37) and P450p (49) are identical and distinctively different from that of P450PCN1 (14). It is likely, however, that P450PCN-E/P450p and P450PCN1 are members of the same gene family. In addition to being inducible by PCN, P450PCN-E is also an adult-male-specific P-450 that is regulated developmentally (47).

In the present study a second member of the PCN-induced P-450 gene family (P450PCN2) was isolated and characterized. Comparison of the structure of P450PCN2 with that of P450PCN1 indicates recent gene conversions. In addition, the differential regulation of these highly homologous P-450s was studied during normal development and in the presence of various P-450-inducing compounds.

MATERIALS AND METHODS

Isolation and sequencing of P450PCN2 cDNA. A cDNA library was constructed by using 4-week-old male rat liver mRNA and the expression vector λgt11 (53). Briefly, double-stranded cDNA was synthesized with reverse transcriptase, methylated with *Eco*RI methylase, and inserted into *Eco*RI-digested λgt11 with the aid of *Eco*RI linkers. The library was screened with a polyclonal anti-P450PCN1 antibody produced in rabbits. P450PCN1 corresponds to the form previously isolated and sequenced (14, 16). The cDNA clone containing the largest insert was isolated, and the insert was sequenced by the M13 shotgun cloning (9) and dideoxy (35) methods. Full-length cDNA was subsequently isolated by hybridization and sequenced. Sequence alignments and other manipulations were carried out with the Beckman Microgenie software.

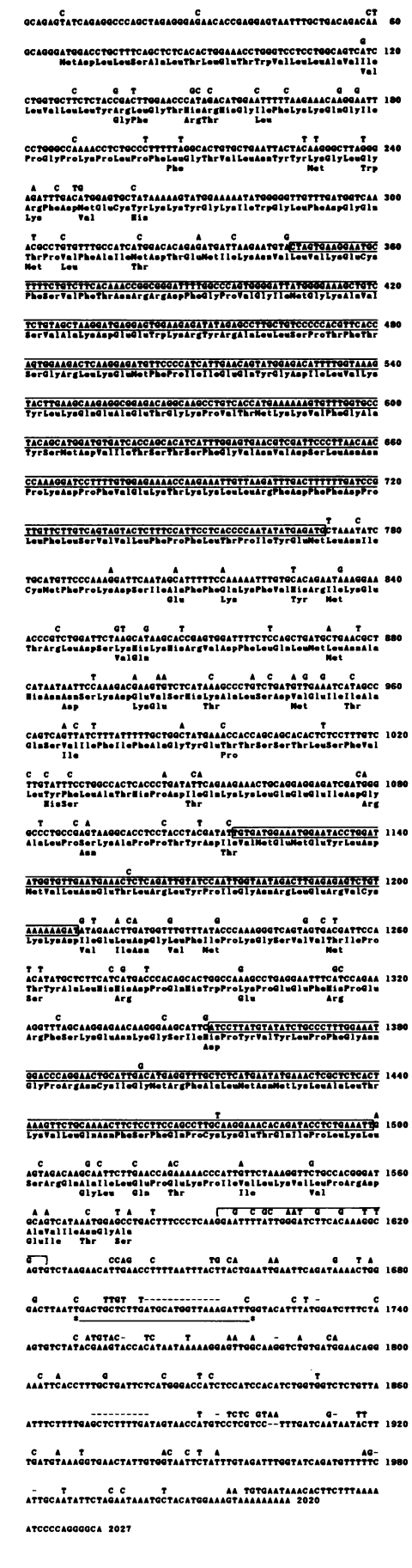
Analysis of P450PCN1 and P450PCN2 mRNA with oligonucleotide probes. Total liver RNA was isolated by the guanidine hydrochloride method (8) from rats of various ages and from rats that had received an intraperitoneal dose of one of the following: PCN (400 mg/kg of body weight), dexamethasone sulfate (500 mg/kg), 4-methylpyrazole (300 mg/kg), 3-methylcholanthrene in corn oil (30 mg/kg), phenobarbital (100 mg/kg), or isotonic NaCl. Rats received a single injection of inducing agent 12, 24, or 48 h before killing (see figure legends). RNA (10 μg) was either subjected to electrophoresis in denaturing 2.2 M formaldehyde–1.2% agarose gels with subsequent blotting or bound to nylon filters (Nytran, 0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) with the aid of the Slot Blot II apparatus (Schleicher & Schuell, Inc.). Oligonucleotides specific for P450PCN1 and P450PCN2 mRNAs were purchased from OCS Laboratories and labeled with [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham Corp., Lexington Heights, Ill.) by use of polynucleotide kinase (IBI, Inc.). Hybridizations of the 30-mer oligonucleotides to filter-bound RNA were performed as described previously (5). Hybridization and washing temperatures were 50 and 58°C, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analyses. Microsomes were prepared from rat livers by differential centrifugation as described previously (6). The protein concentration was determined by the BCA method (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as the standard. Electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate was performed essentially by the method of Laemmli (25). Western blot analyses (43) were carried out with rabbit antisera against P450PCN1 followed by incubation with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (KPL Laboratories), with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as the substrates. Testosterone 6β-hydroxylase assays were performed as described previously (52).

RESULTS

Structure of P450PCN2. A cDNA to PCN-induced P-450 was previously isolated and completely sequenced (14). This P-450 cDNA was named pP450PCN and was shown to be a member of a new P-450 gene family. In the present report, pP450PCN is now designated pP450PCN1. Southern blot analysis revealed that more than one member of the PCN-induced P-450 gene family exists (16, 39). Indeed, immunochemical screening of a noninduced male rat liver library with polyclonal anti-P450PCN1 antibody produced another cDNA, designated pP450PCN2, that is highly similar to the original P450PCN1 cDNA (14). Comparison of nucleotides and amino acids between P450PCN1 and P450PCN2 (Fig. 1) revealed that both P450PCN1 and P450PCN2 mRNAs and proteins are 90% similar and that three regions of high nucleotide homology exist. The results, summarized in Table 1, revealed that nucleotide positions 1116 to 1209 (Table 1, similarity index 2) and 1352 to 1499 (Table 1, similarity index 3) are 98 to 99% similar and are surrounded by regions 772 to 1115 and 1210 to 1351 containing only 85 to 87% similarity. The most homologous segment (Table 1, similarity index 1, positions 346 to 771) contains 425 matches without a single difference. This nonrandom distribution of homologous segments is indicative of recent gene conversion events.

Regulation of P450PCN1 and P450PCN2. pP450PCN1 and pP450PCN2 cDNA clones were isolated from cDNA libraries constructed from PCN-induced and noninduced male rat liver mRNA, respectively. Rescreening of either library with pP450PCN1 resulted in the isolation of six P450PCN1-like cDNA clones from the induced library and six P450PCN2-like cDNA clones from the noninduced library. This was determined by partial restriction endonuclease mapping and by sequencing of the cDNA 3′ ends. Although the presence of other cDNA clones cannot be totally ruled out, these results suggested that P450PCN2 mRNA was present and



P450PCN1 mRNA was absent, or low in content, in noninduced animals.

To examine P450PCN1 and P450PCN2 mRNA levels in livers of induced and noninduced rats, we used specific oligonucleotides. These probes were generated against regions corresponding to the 3′ untranslated portion of each mRNA (Fig. 1). The specificity of the oligonucleotides was verified by annealing each with both P450PCN1 and P450PCN2 cDNAs (Fig. 2B, bottom left panel). Each oligonucleotide hybridized to an mRNA approximately 2,200 nucleotides long (Fig. 2A); only the P450PNC1 oligonucleotide probe, however, hybridized to a PCN-induced mRNA, whereas the P450PCN2 mRNA level was not affected by PCN treatment (Fig. 2A). It is also evident from the results that P450PCN2 mRNA is present at a higher level in noninduced animals than the P450PCN1 mRNA (Fig. 2A). Exposure of the mRNA blot to X-ray film for 20 days did not reveal a P450PCN1 mRNA in uninduced male and female rats, suggesting that this mRNA is absent. The lack of induction of P450PCN2 by PCN is further illustrated by slot-blot analysis of induced and noninduced RNA (Fig. 2B). P450PCN2 mRNA is present at a higher level in male rats than in female rats and is not induced by either a 24- or a 48-h exposure to PCN. P450PCN1, however, was readily induced in both male and female rats even after a 24-h exposure to PCN (Fig. 2B). Because no P450PCN1 mRNA was detectable with the specific oligonucleotide probe in noninduced rats (Fig. 2A), the extent of its induction could not be quantitated. In this respect, the presence of a slight hybridization signal in the control RNA slot, hybridized with the P450PCN1 probe, probably represents background nonspecific binding of the probe to other RNA.

Earlier reports demonstrated that an mRNA that hybridizes with pP450PCN1 was induced by phenobarbital (16) and dexamethasone (50). To establish whether P450PCN1 or P450PCN2 was induced by these agents, the specific oligonucleotides were used as probes. Mature male rats were injected with dexamethasone and phenobarbital, and their total liver RNA was analyzed by Northern blot analysis (Fig. 3). Again, P450PCN1 mRNA was absent and P450PCN2 mRNA was detectable in noninduced RNA. Both P450PCN1 and P450PCN2 mRNAs were induced by phenobarbital, while only P450PCN1 mRNA was induced by dexamethasone, without an increase in P450PCN2 mRNA. Neither P450PCN1 nor P450PCN2 mRNA was induced by 3-methylcholanthrene. In fact, decreases in P450PCN2 mRNA were evident in the 3-methylcholanthrene- and dexamethasone-induced RNAs. 4-Methylpyrazole also slightly increased the level of P450PCN1 mRNA but not P450PCN2 mRNA. These results demonstrate that P450PCN1 and P450PCN2 are differentially regulated in rat liver. Another smaller RNA species was consistently detected with the pP450PCN1 probe (Fig. 2A and 3). This species could represent another P-450 mRNA in the PCN gene family that shares 3′ nucleotide homology with

FIG. 1. Comparison of the P450PCN2 and P450PCN1 cDNA and protein sequences. The complete P450PCN2 cDNA and protein sequences are presented. P450PCN1 cDNA and protein are aligned with those of P450PCN2, and only the nucleotides (top of pP450PCN2 nucleotide sequence) and amino acids (bottom of P450PCN2 amino acid sequence) of pP450PCN1 that do not match those of pP450PCN2 are presented. The regions of high conservation between the two cDNAs are surrounded by lines. Oligonucleotides specific for pP450PCN1 and pP450PCN2 were synthesized from the regions that are overlined and enclosed by brackets and underlined and enclosed by asterisks, respectively.

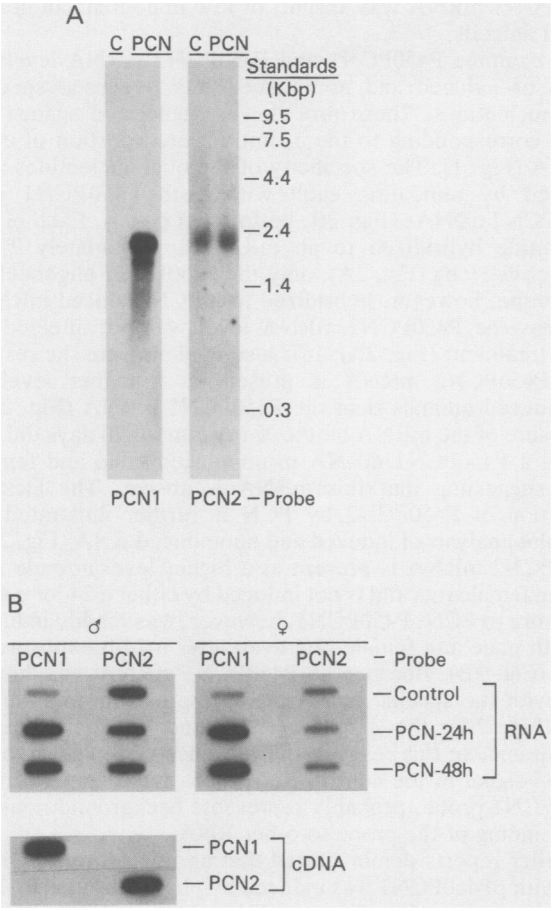


FIG. 2. (A) Analysis of P450PCN1 and P450PCN2 mRNA by use of oligonucleotides. Total liver RNAs (10 μg) from 8-week-old untreated control rats (C) and from rats treated for 24 h with PCN (PCN) were subjected to electrophoreses on 2.2 M formaldehyde–1.2% agarose gels. Blots of the gels were hybridized to pP450PCN1 (PCN1)- and pP450PCN2 (PCN2)-specific oligonucleotides. RNA molecular weight standards were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). (B) Total RNA (10 μg) from 8-week-old control rats and rats administered a single injection of PCN for 24 h (PCN-24h) or two 24-h injections of PCN (PCN-48h) were bound to nylon filters with a Slot-Blot II device and hybridized with pP450PCN1 (PCN1)- and pP450PCN2 (PCN2)-specific oligonucleotides as described in Materials and Methods. The two filters at the bottom of the figure both contain denatured P450PCN1 (left side) and P450PCN2 (right side) cDNAs. The filters were exposed for 48 h at −70°C with the aid of a Du Pont Cronex Lightning-Plus intensifying screen.

P450PCN1 mRNA, or it may be an mRNA degradation product.

Analysis of mature female rats showed the same pattern of inducibility as that found for males (data not included). However, even though P450PCN1 mRNA was again absent, P450PCN2 mRNA was diminished relative to the levels in adult male rats. To explore this sex difference further, total liver RNA was isolated from male and female rats of various ages and analyzed for P450PCN1 and P450PCN2 mRNA. Slot-blot analysis with the P450PCN2 oligonucleotide revealed an unusual sex difference in the expression of P450PCN2 mRNA (Fig. 4). P450PCN1 mRNA was absent in liver RNA from male and female rats of various ages (data not included). P450PCN2 mRNA (analyzed with the P450PCN2 oligonucleotide probe), however, was present at low levels in livers of newborn male and female rats and increased two- to threefold before 1 week of age. P450PCN2 mRNA remained elevated at 2 weeks of age in males and females before decreasing at the onset of puberty of female rats between 4 and 12 weeks of age. In contrast, in male rats, P450PCN2 mRNA remained elevated at 4 weeks and increased threefold at 12 weeks of age.

To verify that P450PCN2 mRNA levels correlate with immunodetectable P450PCN protein levels during development, we performed Western blot analyses with microsomal proteins of male and female rats of various ages using polyclonal anti-P450PCN1 antibody (Fig. 5). Based on the data of mRNA blots previously described, levels of P450PCN2, while clearly undetectable in newborn rats, become elevated during the first week of age, and remain elevated in mature male rats, but continuously decline in female rats as they reach puberty (Fig. 5, lower panel). The levels of NADPH-cytochrome P-450 oxidoreductase, however, do not decrease but gradually increase in both male and female rats during development (Fig. 5, upper panel).

Finally, the microsomes were assayed for testosterone 6β-hydroxylase activity. This activity was previously found to correlate with immunodetectable levels of another PCN-induced cytochrome P-450, P450PCN-E (47). Testosterone 6β-hydroxylase activity was induced by dexamethasone, PCN, and phenobarbital (Table 2). Enzyme activity was absent in newborn rats, but increased at 1 and 2 weeks of age in male and female rats and then declined in females at 12

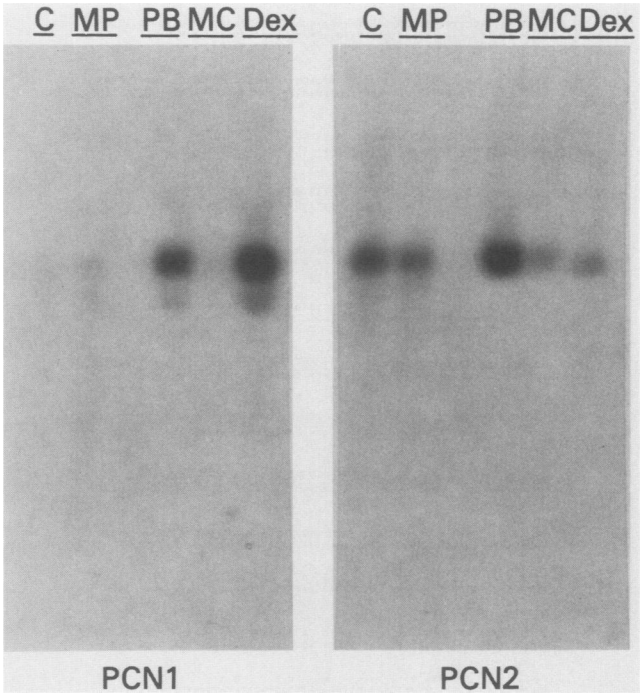


FIG. 3. Analysis of P450PCN1 and P450PCN2 mRNA after administration of various P-450-inducing agents. Total liver RNAs (10 μg) from control (C), 4-methylpyrazole-treated (MP), phenobarbital-treated (PB), 3-methylcholanthrene-treated (MC), and dexamethasone-treated (Dex) rats were analyzed by Northern blot analysis with P450PCN1 (left panel) and P450PCN2 (right panel) mRNA-specific oligonucleotides. The filters were exposed for 48 h at −70°C with the aid of a Du Pont Cronex Lightning-Plus intensifying screen.

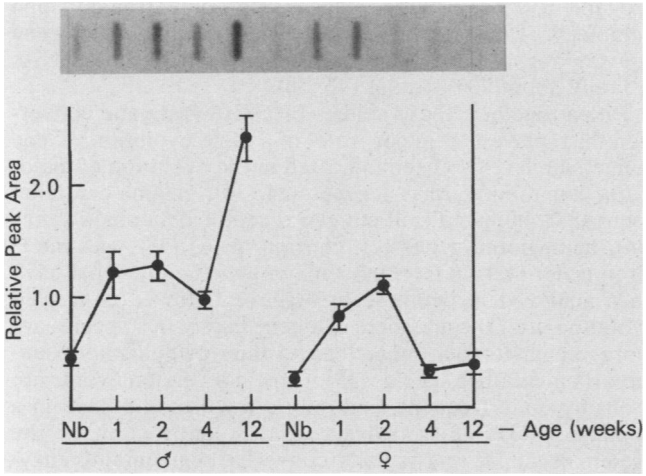


FIG. 4. Analysis of P450PCN2 mRNA of male and female rat livers of various ages. Total RNA (10 μg) of male and female rats from birth (Nb) to 12 weeks was subjected to slot-blot analysis as described in Materials and Methods and the legend to Fig. 2. Filters were hybridized with the P450PCN2 oligonucleotide probe and exposed to a film for 48 h at −70°C with the aid of a Du Pont Cronex Lightning-Plus intensifying screen. Autoradiographs were scanned with a densitometer, and the areas of each slot were plotted. The brackets in panel A represent the mean ± standard deviation of the areas from three separate filter autoradiographs.

weeks while continuing to increase in male counterparts (Fig. 6). This enzyme activity was completely inhibited by inclusion of anti-P450PCN1 antibody in the enzyme assay mixture. The developmental differences in males and females suggest that testosterone 6β-hydroxylase activity in noninduced rats is catalyzed by P450PCN2. In addition, the results indicate that P450PCN1, the PCN-inducible form, is also responsible for PCN-induced testosterone 6β-hydroxylase activity. This conclusion is supported by the fact that anti-P450PCN1 inhibits testosterone 6β-hydroxylase activity in PCN-induced microsomes (data not included). It cannot be excluded, however, that other immunorelated cytochromes, such as P450PCN-E (47), catalyze testosterone 6β-hydroxylase activity.

TABLE 2. Induction of testosterone 6β-hydroxylase activities in microsomes from male 4-week-old rats

Treatment[a]	Sp. act[b]
None	0.62
PCN	8.20
Dexamethasone	7.80
Phenobarbital	6.50

[a] Rats received a single dose of inducing agent each day for two days before killing.

[b] Nanomoles per minute per milligram of protein. Each value represents the average of duplicate determinations from three separate rats.

DISCUSSION

Cytochrome P-450s represent several enzymes that form the terminal components of the microsomal multisubstrate monooxygenase system. The precise number of P-450s in the rat is still unclear; however, protein purification data would suggest that more than 13 forms exist in rat liver (46a). Although the TCDD-induced P-450s make up two distinct forms in the mouse (13, 29), rat (40, 51), and rabbit (32), the number of phenobarbital- and PCN-induced P-450s is still unknown. Several genomic clones homologous to phenobarbital-induced P-450e cDNA have been isolated and characterized (1, 2, 28), leading to the suggestion that the phenobarbital-inducible gene family may contain from 9 to 20 active genes (3). Some of these genes may only be expressed in small amounts in liver or extrahepatic tissues; therefore, purification of their gene products may be difficult. The pP450PCN2 described herein represents a cDNA for a P-450 that has not been previously purified from rats. The above studies establish that many uncharacterized P-450s may yet be identified through homology with existing P-450s and their cDNAs. Although its complete enzymatic specificity and role in cellular metabolism are unclear, P450PCN2 shares extensive homology with the PCN-inducible P450PCN1 (14) and is probably the major testosterone 6β-hydroxylase in noninduced rat liver.

The nucleotide similarities between P450PCN2 and P450PCN1 mRNAs are not evenly distributed. A region of 425 nucleotides exists without a single difference. The most likely explanation for this phenomenon is a gene conversion event. In fact, two other smaller regions of 98 to 99% homology between the mRNAs may also be remnants of

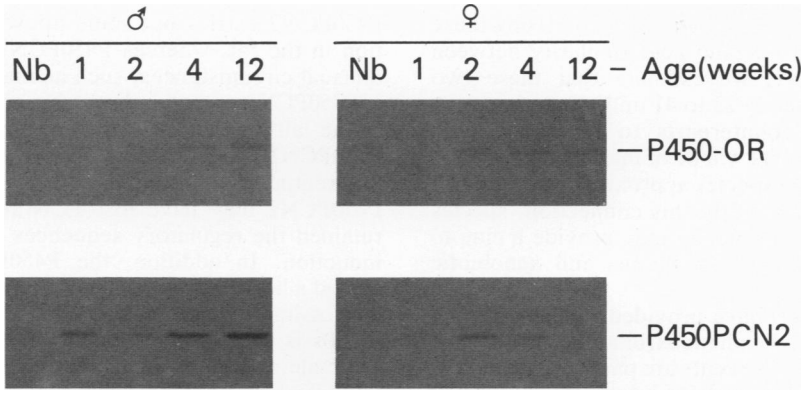


FIG. 5. Western blot analysis of P450PCN2 in rats of various ages. Microsomes were isolated from male and female rats from birth (Nb) to 12 weeks. Microsomal proteins were subjected to electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Duplicate blots were incubated with antibodies against NADPH-cytochrome P-450-oxidoreductase (P450-OR) and P450PCN1 (P450PCN2). The antibody binding was detected with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase.

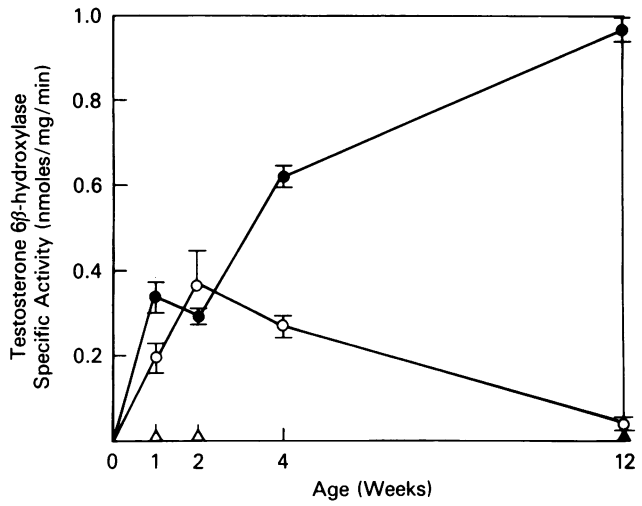


FIG. 6. Changes in testosterone 6β-hydroxylase activity during development. Microsomes were prepared from livers of rats of various ages and immediately assayed for testosterone 6β-hydroxylase activity as described previously (51). The activities were measured in the absence of antibody for the microsomes of male (●) and female (○) rats in the absence of antibody. The activities measured in the presence of antibody are also shown for 12-week-old males (▲) and 1- and 2-week-old females (△). Values represent means ± standard deviations of three determinations.

earlier gene conversion events. For example, the PCN-induced ancestor gene may have duplicated, followed by one or two gene conversions, to give rise to the two short homologous regions. More recently another conversion event may have resulted in the 425-nucleotide homology.

These gene recombinations complicate the assignment of evolutionary divergence of gene family members. However, for the purpose of comparison, divergence can roughly be estimated by using the unit evolutionary period (UEP). The sequence of an orthologous protein in two species and the time of speciation can be used to calculate the UEP, which is the time required in millions of years for a 1% change in amino acid sequence. Because the UEPs for immunoglobulins, histones, globins, and other gene families are reasonably constant, these values can be used to estimate the time when two genes in a gene family within a single organism split from a common ancestor. A UEP of between 2.2 and 4.1 million years ago was calculated for P-450 based on comparisons between rabbit and rat (26), mouse and rat (23), and mouse and human (21) orthologous P-450s. Using these values and the overall 90% amino acid similarity between P450PCN1 and P450PCN2, we estimate that these two P-450s diverged approximately 22 to 41 million years ago. A search for orthologous counterparts to P450PCN1 and P450PCN2 in mice and rabbits will be of interest because the rat diverged from these two species approximately 17 and 60 million years ago, respectively. In this connection, species differences in P-450 gene complexity may provide a clue to the role of certain P-450s in endogenous and xenobiotic metabolism.

Atchison and Adesnik (3) have provided strong evidence for gene conversion within the phenobarbital-inducible P-450 gene family. Gene conversion events are particularly evident between exons 7 and 8 and the joining intron of a number of P-450-like genes (3). The reason for this localization of high homology is unclear. It is noteworthy that the two TCDD-induced P-450s in the mouse (23) and rat (40) have regions of 420 and 350 base pairs, respectively, of extremely high homology. These areas probably also resulted from gene conversions, because the remaining portions of the mRNAs are only about 70% similar (23, 40).

Taken together, these studies establish that gene conversion has played a major role in P-450 evolution. Gene conversion has also been indicated in the evolution of major histocompatibility class I genes (24, 34), gamma-crystallin genes (27), human T-cell antigen receptor beta-chain genes (44), hemoglobin genes (7), chorion genes (33), and interferon genes (42). In fact, most major gene families that have been analyzed in detail seem to have followed concerted evolution, i.e., the members of a gene family in a species are more similar to each other than to their orthologous counterparts in another species (19). Gene conversion events are probably quite frequent, and only a few become fixed in a population (31, 45). Analysis of the contribution of the protein sequence (particularly converted segments) to enzymatic activity will help to elucidate the relationship between gene conversion and P-450 multiplicity. In this regard, only two regions of the P-450 primary sequence have been ascribed a potential function. The highly hydrophobic amino terminus probably serves as a noncleavable "signal sequence" (4) for insertion of the enzymes into lipid bilayers. In addition, a Cys-containing fragment located at about residue 440 in the P-450 sequence is highly conserved among the different P-450 gene families and is probably the fifth thiolate ligand to the heme iron in the active site of the enzyme (1, 14, 48). The exact locations of other important regions (such as the NADPH-cytochrome P-450 oxidoreductase-binding sites and the substrate-binding sites) are still a mystery. Particularly important is the relationship between primary structure and the broad substrate specificity exhibited by many forms of P-450 (5a, 12, 36). This broad specificity explains the vast metabolic potential for xenobiotic detoxification. In this regard, gene conversion could increase or stabilize P-450 diversity.

The present study reports an example of two highly homologous P-450s that are differentially regulated. Two expressed genes in the phenobarbital-induced family (2, 17) and both genes in the TCDD-induced family (15, 20) are regulated through transcriptional activation. In the case of the PCN-induced family, only P450PCN1 mRNA is induced, whereas P450PCN2 mRNA remains refractive to induction by PCN. Although it is not induced by PCN, the P450PCN2 mRNA is still expressed at measurable levels in noninduced rat liver, whereas the P450PCN1 mRNA is virtually undetectable in noninduced animals. These data suggest that P450PCN2 carries out some housekeeping metabolic function in the rat, whereas P450PCN1 is required only under unusual circumstances, such as massive stress.

P450PCN2 is not induced by either PCN or dexamethasone although it is induced by phenobarbital, while P450PCN1 is induced by all three agents. This unusual differential regulation suggests that after gene duplication, P450PCN2 may have lost PCN regulatory sequences but retained the regulatory sequences governing phenobarbital induction. In addition, the P450PCN2 gene could have gained a hormonally responsive region that regulates its own expression during development. Although neither of these P-450s is clearly associated with testosterone metabolism, the male specificity of P450PCN2 suggests that it has constitutive hepatic testosterone 6β-hydroxylase activity. The fact that this activity is elevated by PCN administration suggests that P450PCN1 also metabolizes testosterone at the 6 position. This result would not be surprising in view of high

structural homology between P450PCN1 and P450PCN2. However, another highly homologous member of the P450PCN gene family could carry out this function. In this connection P450PCN-E (47) has been implicated as testosterone 6β-hydroxylase. This P-450 has an amino terminus (37) that is the same as that of P450p (10, 49, 50), which is distinct from both P450PCN1 and P450PCN2. Interestingly, levels of this P-450 correlate with levels of P450PCN2 mRNA in both males and females during development (47). In addition, antibodies against P450PCN-E inhibit testosterone 6β-hydroxylase activity (47). These data suggest that P450PCN2 and P450PCN-E are immunologically very similar, and in view of the data herein, a gene conversion event could have resulted in a protein that has a totally different amino terminus yet shares substantial homology with P450PCN2 and P450PCN1. It is likely that the protein detected by Waxman et al. (47) in noninduced male and female rats is actually P450PCN2 and that P450PCN-E is only an inducible protein. In this connection, the smaller RNA species detected by P450PCN1 oligonucleotides (Fig. 2A and 3) may code for another PCN-induced P-450. The exact nature of P450p/P450PCN-E, however, awaits further studies, including cDNA cloning and sequence determination.

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