Complementary DNA and Protein Sequences of Ethanol-inducible Rat and Human Cytochrome P-450s

TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF THE RAT ENZYME*

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The cDNAs encoding ethanol-inducible forms of rat and human cytochrome P-450s have been isolated, sequenced, and used to study the expression of this cytochrome P-450 during development and by various inducing agents. Polyclonal antibody against ethanol-inducible cytochrome P-450 was used to screen rat and human agt11 cDNA expression libraries. The longest cDNAs obtained from each library were completely sequenced, and the deduced amino acid sequence of the rat cDNA was found to correspond to P450j based on the published amino-terminal sequence. The rat and human cytochrome P-450s both contained 493 amino acids and calculated molecular masses of 56,634 and 56,916 daltons, respectively. Human P450j shared 75% nucleotide and 78% amino acid similarities to the respective orthologous rat cDNA and deduced amino acid sequences. Amino acid alignment also revealed that P450j was 48% similar to P450b and P450e, the major phenobarbital-inducible forms, and 54% similar to P450PB1 and P450f, two developmentally regulated forms. Southern blot analyses of rat and human genomic DNAs verified that only a single gene shared immunodetectable protein. The cDNA of P450j was found to be developmentally regulated. No immunodetectable protein or P450j mRNA was present in newborn rats; however, rapid increases in P450j mRNA and protein occurred within 1 week after birth in both male and female rats. The levels of P450j and its mRNA thereafter remained elevated up to 12 weeks of age in both sexes. The increases in both P450j and its mRNA paralleled the change in aniline hydroxylation activity during development. Run-on transcriptional analysis confirmed that these increases were due to transcriptional activation of the P450j gene. In contrast to transcriptional activation during development, induction of P450j by various agents such as pyrazole, 4-methylpyrazole, and acetone might be due to post-transcriptional events. A 4-fold elevation in both enzymatic activity and immunodetectable P450j was not accompanied by an increase in P450j mRNA level.

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The nucleotide sequences reported in this paper have been submitted to the GenBank™/EMBL Data Bank with accession numbers J02626 and J02627.

The abbreviations used are PCN, pregnenolone 16α-carbonitrile; UEP, unit evolutionary period; prP450 and pbP450 refer to the respective rat and human P450 cDNA clones.
small molecules such as ethanol and acetoate can induce enzymes which metabolize the same compounds; these agents probably do not act directly through a specific receptor.

In order to explore the mechanism of this unique induction process and to develop probes for analyzing potential individual variations in alcohol and nitrosamine metabolism, the cDNAs for rat and human P450j were isolated, sequenced, and compared with other cytochrome P-450 gene families. The rat P450j cDNA probe was utilized to study the regulatory mechanisms of expression of this enzyme during development and its induction by various agents.

MATERIALS AND METHODS

Nomenclature of Cytochrome P-450s—Except for P450PCN and P450PB1, the nomenclature used in this report is that of Ryan and co-workers (Ryan et al., 1985). P450b and P450e are the major cytochrome P-450s induced by phenobarbital in the rat (Ryan et al., 1985). cDNA libraries constructed from homologous rat probes using the methods described above and those purified by other laboratories from rats and other species have been discussed (Waxman, 1986; Black and Coon, 1986). cDNA sequences of rat cytochrome P-450s induced by 3-methylcholanthrene in rat (Ryan et al., 1985), and P450f is a constitutive form of cytochrome P-450 (Ryan et al., 1983), is also found in mionized adult animals (Waxman et al., 1985). P450f and P450PB1 share 75% amino acid similarity and are developmentally regulated (Gonzalez et al., 1986a). P450PCN is induced by phenyl-nitroimidazole (Gonzalez et al., 1985b). The relationships between the cytochrome P-450s described above and those purified by other laboratories from rats and other species have been discussed (Waxman, 1986; Black and Coon, 1986).

Animals—Sprague-Dawley rats were obtained from the National Institutes of Health Veterinary Resources Branch and maintained on Purina NIH Open Formula Rat chow and water ad libitum. For the developmental study, male and female rats of various ages were obtained and killed within 2 days. Late term pregnant rats were obtained, and livers from newborn rats were collected on the day of birth. The inducing agents, pyrazole, 4-methylpyrazole, and imidazole were purchased from Sigma and administered intraperitoneally to the rats (Ryan et al., 1985). The rat liver cytochrome P-450 was purified as described by Ryan et al. (1980). These antibodies were used as probes in conjunction with goat anti-mouse or anti-rat IgG antisera against ethanol-induced rat cytochrome P-450 purified according to the procedure described by Patten et al. (1986). Immune complexes were identified by using a second goat anti-mouse IgG followed by mouse monoclonal antibody (against goat IgG) conjugated with peroxidase-anti-peroxidase (Miles Scientific, Naperville, IL) in conjunction with freshly prepared substrate, 4-chloro-1-naphthol (3 mg/ml in 20% methanol with 0.01% H2O2). Approximately 10,000 phage from each library were screened, and 12 rat and 12 human P450j CDNA were isolated. Phage DNA was isolated by standard protocols (Maniatis et al., 1982) and their inserts subcloned into EcoRI-digested pUC18 (Pharmacia P-L Biotechomicals). Plasmid DNA was prepared from cultures of bacteria by alkaline lysis method (Bernheim and Doly, 1979). DNA sequencing was performed by the dideoxy chain terminator method (Sanger et al., 1977). Random sonicated DNA fragments were produced from concatenated cDNA inserts (Deininger, 1983) and cloned into the Smal site of M13mp19. Sequence was displayed on standard 0% polyacrylamide, 50% urea gels with and without a salt gradient (Biggin et al., 1983). Each nucleotide was read an average of 9 times and at least twice in both directions. Sequence data were assembled and analyzed by the use of the Microgenie software (Beckman Instruments).

RESULTS

Structure of Rat and Human P450j—During the course of preparing monoclonal antibodies against an ethanol-inducible rat cytochrome P-450, polyclonal antisera was collected from the immunized mice. This antisera was used to screen rat and human liver cDNA expression libraries. Twelve immunologically positive clones were isolated from each of the rat and human liver libraries, and their DNAs were purified. Analysis of the rat clones revealed the presence of the rat cDNA probe (Hamada et al., 1982); P450j cDNA (Gonzalez and Kasper, 1982), and P450PCN cDNA (Gonzalez et al., 1985b).

The results of the identification of the clone by using the antisera from the rat and human cDNA libraries and the screening of the rat cDNA expression libraries were similar, indicating that the cDNA sequence from the rat and human cDNA libraries was highly homologous. The results of the screening of the rat cDNA expression libraries indicated that the cDNA sequence from the rat and human cDNA libraries was highly homologous. The results of the screening of the rat cDNA expression libraries indicated that the cDNA sequence from the rat and human cDNA libraries was highly homologous.
Structural and Regulatory Characteristics of P450j

The amino acid sequences of rat and human P450j have two general features common to all microsomal cytochrome P-450 proteins sequenced to date; a hydrophobic amino terminus and a conserved cysteine-containing fragment around amino acid position 440–460. This cysteine residue is proposed to be the 5th thiolate ligand to the heme iron in the enzyme’s active site (White and Coon, 1980). P450j shows considerable similarity with other sequenced cytochrome P-450s in this region since several residues surrounding Cys are identical or are conservative substitutions of the residues observed in other cytochrome P-450s sequenced to date (Fig. 1, arrows; see Gonzalez et al., 1985b).

Rat P450j shows some similarity with both P450e (Kumar et al., 1983) and P450PB1 and P450f (Gonzalez et al., 1986a). This data is best illustrated by a dot matrix analysis of amino acid sequences (Fig. 2). Although the protein sequence of rat P450j is 48 and 54% similar to those of P450b/e and P450PB1, respectively, the similarities exist in several distinct segments throughout the primary sequence. Results similar to the P450PB1 and P450j comparison were obtained with P450f and P450j. The amino acid similarities between the above cytochrome P-450s are also reflected in the nucleotide sequence. Areas of higher similarity, however, are not sufficient for the prP450j probe to cross-hybridize with P450b/e or P450PB1 mRNAs. In addition, both polyclonal and monoclonal antibodies against P450j do not cross-react with P450b/e or P450e or P450PB1/P450f and vice versa (data not included). No significant similarity was detected between P450j and P450e (Yabusaki et al., 1984), P450d (Kawajiri et al., 1984), and P450PCN (Gonzalez et al., 1986b), except around Cys, as mentioned above.

Southern Blot Analysis of the P450j Gene Family—To determine whether P450j has closely related family members, Southern blot analysis was performed. Rat and human DNAs were digested with four restriction enzymes, subjected to agarose gel electrophoresis, and blotted to nylon membranes. After hybridization with respective prP450j and phP450j inserts only a few fragments were detected for both rat (Fig. 3A) and human (Fig. 3B) DNAs. These simple patterns of hybridization are indicative of a single copy gene without multiple closely related members. All of the fragments detected in Fig. 3, A and B, have been isolated from rat and human genomic libraries, sequenced, and shown to contain the coding sequences of rat and human P450j, respectively. These results verify that under the hybridization conditions of Church and Gilbert (1984) prP450j and phP450j will only detect rat and human P450j mRNAs, respectively.

Induction of P450j by Various Agents—Young adult male rats were treated with various cytochrome P-450 inducing agents as described under “Materials and Methods.” Microsomes and total RNA were then isolated and analyzed for P450j protein, catalytic activity, and mRNA content. P450j migrated with an $M_r$ of 52,000 on this gel system and was detected by Western blots in noninduced control rats (Fig. 4, middle panel). It was elevated approximately 4-fold by treatment with acetone, 4-methylpyrazole, and pyrazole. No increase in P450j was detected in rats treated with imidazole. In contrast, P450PCN ($M_r$, 54,000) was not elevated by any of these treatments and was actually decreased by some agents such as acetone and pyrazole (Fig. 4, bottom panel). Cytochrome P-450b ($M_r$, 53,000) was induced by acetone and 4-methylpyrazole, but not by pyrazole treatment (Fig. 4, top panel).

2 M. Umeno, unpublished results.
The increase in P450j is also reflected in the elevation of aniline hydroxylase activity (Table I). The 4-fold increase in the enzymatic activity detected after acetone, 4-methylpyrazole, and pyrazole treatment paralleled the increase in P450j protein as determined by Western blot analysis (Fig. 4). Benzphetamine N-demethylase activity (Ryan et al., 1980) increased approximately 2-fold by acetone, 4-methylpyrazole, and pyrazole treatment.

In order to explore the mechanism by which P450j is induced by acetone, 4-methylpyrazole, and pyrazole, the content of P450j mRNA in total RNA was measured. Northern blot analysis revealed a single mRNA of approximately 1700 nucleotides in noninduced liver (Fig. 5, lane 1). Its level was not increased by either imidazole, 4-methylpyrazole, or pyrazole (Fig. 5, left panel, lanes 2, 3, and 4, respectively). In contrast, the level of P450b/e mRNA was elevated approximately 4-fold after 4-methylpyrazole treatment but was not increased by pyrazole or imidazole (Fig. 5, left panel, lanes 6, 7, and 8, respectively). Treatment of rats with either acetone or ethanol for 7 days induced P450j 4-fold, but did not induce P450j mRNA (data not included).

Studies were also done using slot blot analysis of total RNA from control and treated rats (Fig. 5, right panel). P450j mRNA was not increased by any of the treatments (Fig. 5, right panel, row A). However, P450b/e mRNA was elevated by 4-methylpyrazole, phenobarbital, and PCN (Fig. 5, right panel, row B). P450PCN was induced by PCN and phenobarbital treatment but was not elevated by any of the other agents (Fig. 5, right panel, row C). These results suggest that rat P450j is post-transcriptionally regulated in contrast to P450c/d, P450b/e, and P450PCN. Interestingly, P450b/e was increased by 4-methylpyrazole probably through an induction of its mRNA. Although P450b and P450e have considerable benzphetamine N-demethylase activity (Ryan et al., 1980) the increase in this activity in microsomes by 4-methylpyrazole or pyrazole is only 2-fold instead of 4-fold as expected from P450b/e protein and mRNA induction. This result suggests that other P450s that are not regulated by these agents are demethylating benzphetamine.

Developmental Regulation of Rat P450j—Several forms of cytochrome P-450 become elevated during rat development (Waxman et al., 1985; Gonzalez et al., 1986a, 1986b). Therefore, rP450j was analyzed for sex and developmental differences in its expression. Microsomes and RNA were isolated from rats of various ages and analyzed for levels of rP450j and its mRNA. Western blot analyses showed that P450j was undetectable in newborn rat livers but increased dramatically within 1 week after birth and remained elevated throughout 12 weeks of age in both male and female rats (Fig. 6, bottom panel).
However, P450b was detectable in newborn male and female rats although the quantity is less than that of adult rats (Fig. 6, top panel). The level of P450b increased continuously 1 week after birth and seemed to reach the maximum level at around 4 weeks old in both male and female rats. There is another immunodetectable band (M, of 49,000) recognized by anti-P450b polyclonal antibody in both male and female 4-week-old rats, but the nature of this immunodetectable band is unknown (Fig. 6, top panel).

In an attempt to verify the levels of P450j and P450b determined by Western blot analyses, several enzyme activities at the same time points were monitored for both male and female rats (Fig. 7). Aniline hydroxylase activity was almost undetectable at birth but elevated at 1 week of age and remained at the same level in up to 12-week-old male and female rats (Fig. 7). Benzphetamine N-demethylation activity was low but detectable in newborn rats and then increased continuously for the next 4 weeks and stayed at the same level up to 12 weeks of age in both males and females (Fig. 7). The level of NADPH-cytochrome P-450-oxidoreductase activity was also slightly low but detectable in newborn rats and gradually increased during development in both male and female rats (Fig. 7).

In order to investigate the mechanism for this developmental activation, P450j mRNA levels were examined by slot blot analysis. P450j mRNA was undetectable at birth and increased dramatically at 1 week of age in both males and females (Fig. 8, panel A). In contrast, actin mRNA levels were invariant with age (data not shown). P450b/e mRNA was low but detectable at birth and then became elevated between birth and 1 week of age, followed by a gradual increase until about age 4 weeks and a decline at 12 weeks in both male and female rats (Fig. 8, panel B).

The increase in P450j mRNA between birth and 1 week of age could be due to mRNA stabilization or transcripational activation of the P450j gene. Therefore, nuclear run-on transcription analysis was performed to distinguish between these possibilities. Nuclei isolated from rats of various ages were incubated in the presence of [α-32P]UTP, and the resultant labeled transcripts were hybridized to P450j, P450PB1, and actin probes bound to nylon filters. Labeled P450j transcripts were not detectable in newborn rats, but their levels markedly increased between birth and 1 week of age, while actin gene transcription did not change during the same period (Fig. 9). The rapid increase in P450j gene activation within 1 week of birth is in distinct contrast to the developmental activation of P450PB1 and P450f which occurs after 2 weeks of age in male and female rats (Gonzalez et al., 1986a). These results establish that elevation of P450j during development is regulated at the transcriptional level.

**DISCUSSION**

P450j cDNA clones were isolated from rat and human liver cDNA expression libraries. Based on the deduced protein sequence, the amino terminus of the rat cDNA, prP450j, was exactly identical to the 18 amino acid residues of sequence derived from purified rat P450j (Ryan et al., 1985). This protein is probably orthologous to the ethanol- and imidazole-inducible rabbit P450LM3a (Koop et al., 1982, 1984, and 1985). Since only 12 nucleotides precede the initiator methi-
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FIG. 7. Changes in cytochrome P-450 activities during rat development. Male (open) and female (closed) liver microsomes isolated from rats of different ages were analyzed for benzphetamine N-demethylase (A), aniline hydroxylase (B), and NADPH-cytochrome P-450-oxidoreductase (C) activities.

Online codon, it is likely that the prP450j cDNA clone does not represent the complete mRNA sequence. Attempts to isolated clones with longer 5' untranslated regions were not successful.

The divergence between two orthologous proteins can be used to calculate the unit evolutionary period (UEP). This value represents the time in millions of years required for a 1% change in amino acid sequence between two orthologous proteins and is calculated by dividing the number of years after speciation by the percent of dissimilar amino acid residues between the two proteins. Values obtained by comparing orthologous genes between rodents range from 2.1 to 2.4 million years (Leighton et al., 1984; Kimura et al., 1984). Comparison of the human P450 cDNA sequence with its mouse counterpart yields a UEP of 4.1 (Jaiswal et al., 1985). Similarly the speciation estimate of rodents and humans (80 million years ago) divided by the 22% difference in amino acid sequence between rat and human P450s yields a UEP of 3.6. In general, therefore, human cytochrome P-450s appear not to diverge as much relative to their rodent counterparts.

It was of interest to compare the rat P450j amino acid sequence with other rat cytochrome P-450 sequences. This was done by use of a dot matrix analysis program that compares global similarities between two sequences using the preset parameters of sequence window size and similarity. No significant similarity was found with P450c (Yabusaki et al., 1984), P450d (Kawajiri et al., 1984), or P450PCN (Gonzalez et al., 1985b). However, similarity was detected with P450e (Kumar et al., 1983) and P450PB1 and P450f (Gonzalez et al., 1986a). Direct amino acid alignment revealed that P450j was 48 and 54% similar to P450c and P450PB1, and most of this similarity existed in discrete segments of residues. The cytochrome P-450 UEP of 2.1-4.1 (Leighton et al., 1984; Jaiswal et al., 1985; Kimura et al., 1984) and 52-46% divergence can be used to estimate the approximate time when P450e, P450PB1, and P450j split from a common ancestor (52 to 46

FIG. 8. Analysis of P450j (A) and P450b/e (B) mRNA levels in rats of various ages. RNAs from different ages of males (lanes 1–5) and females (lanes 6–10) were subjected to slot blot analysis as described under "Materials and Methods." Lanes 1 and 6, newborn; lanes 2 and 7, 1 week old; lanes 3 and 8, 2 weeks old; lanes 4 and 9, 4 weeks old; lanes 5 and 10, 12 weeks old. The membrane-bound RNAs were then hybridized with 32P-labeled probes of prP450j (panel A) and prP450b/e (panel B), and the resulting autoradiographs were scanned by use of a laser densitometer, and the density of each band was plotted.

X 2.1 to 4.1 = 100–200 million years ago). The discrete areas of high similarity may remain due to functional conservation of structural domains and/or gene conversion events. Gene conversion has probably play a role in the striking 5' homologous region observed in the mouse P450 and P450 genes (Kimura et al., 1984), rat P450e and P450d genes (Sogawa et al., 1985), the phenobarbital-induced P450b and P450e gene family (Atchison and Adesnik, 1986; Adesnik and Atchison, 1986), and the pregnenolone 16α-carbonitrile gene family
amino acid similarity exist between P450e/P450PBl and chrome P-450s. Considering that there is 50% nucleotide sequence similarity between P450j and P450e, it is not unreasonable to assume that the P450j gene is located in the sequence similarity between P450j and P450e. The latter shares little immunochemical cross-reactivity which appears different from those of several other cytochrome P450j, the latter is not induced by imidazoles (Petell and Doyle, 1985) and phenobarbital-inducible gene family (Atchison and Adesnik, 1983; Hardwick et al., 1983b) is also probably due to transcriptional regulation. The P450j gene, however, may be post-transcriptionally regulated. This is suggested by the 4-fold elevation of enzymatic activity and immunodetectable P450j protein after treatment with acetone, pyrazole, and 4-methylpyrazole which is not accompanied by an increase in P450j mRNA. Of interest is the fact that P450j is not induced by imidazoles, but the rabbit orthologous P450LM3a is readily induced by this agent (Koop et al., 1985). This suggests an interesting species difference in inducibility that should be examined further. The increase in enzyme content could be due to protein stabilization or an increase in rate of translation. Experiments are in progress to distinguish between these possibilities.

In contrast to a possible post-transcriptional regulation by inducing compounds, P450j is also transcriptionally regulated during development. This transcriptional activation occurs within 1 week of age and is probably mediated by a surge in the level of some endogenous component or hormone. Other hepatic proteins are also elevated during the first week of age after birth in the rat including asialoglycoprotein receptor (Petell and Doyle, 1985). The increase in hepatic proteins soon after birth could be associated with their role in liver metabolic function, i.e. hepatocyte specific enzymes associated with highly differentiated cells.

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Fig. 9. Transcriptional run-on analysis of the ratP450j and actin genes during rat development. Nuclei from rats of various ages were allowed to synthesize [32P]RNA in vitro. The newly synthesized transcripts were then hybridized to prP450j, prP450PB1, and actin probes bound to nylon membranes. The autoradiographs were scanned, and the P450j/actin transcript ratio (shown in numbers at the bottom) was determined for each sample.

No signal was detected (ND) for P450j transcripts in newborn rats (Nb).

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Relative Density
P450j/Actin Transcripts

Identification and Regulation of Ethanol-inducible Cytochrome P-450
Structure and Regulation of Ethanol-inducible Cytochrome P-450


Fig. 1. Nucleotide and deduced amino acid sequences of rP450j and hP450j. The complete DNA and deduced amino acid sequences of rP450j are presented and aligned with hP450j nucleotide (top line) and amino acid (bottom line) sequences. The upper vertical arrow shows the beginning of the phP450j nucleotide sequence. The lower vertical arrow shows the beginning of the rP450j nucleotide sequence.