would otherwise have remained undetected.

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Characterization of a cDNA for Rat P-450g, a Highly Polymorphic, Male-Specific Cytochrome in the P-450IIC Subfamily†,‡

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ABSTRACT: Cytochrome P-450g (IIC13) is a highly polymorphic, male-specific rat liver isozyme which is a member of the P-450IIC subfamily. A cDNA, c5126 (1737 bp), for P-450g was isolated from a λgt11 library synthesized from (+g) male rat liver mRNA. Sequence analysis of the clone, c5126, revealed an open reading frame of 1473 nucleotides, which encodes for a 490 amino acid polypeptide possessing the 30 NH_2-terminal residues reported for cytochrome P-450 (M-3) (P-450g) [Matsumoto et al. (1986) *J. Biochem. 100*, 1359-1371]. A high degree of sequence similarity (>70%) exists between c5126 and the published sequences of cDNAs for members of the IIC subfamily, while its sequence similarity to other subfamilies (IA, IIB, and IIIA) was much lower (<55%). RNA blot analysis utilizing an oligonucleotide probe specific for P-450g revealed that P-450g mRNA was expressed in livers of male but not female Sprague-Dawley (CD) and ACI rats, indicating that the sex difference was regulated pretranslationally. Furthermore, expression of P-450g mRNA was age dependent in livers of male ACI rats (a homozygous, phenotypically high P-450g strain). However, the mRNA for P-450g was expressed equally in livers of outbred male CD rats representing either the high (+g) or the low (-g) phenotype and of inbred ACI rats (+g) representing the high phenotype, indicating that the defect in (-g) rats does not reflect differences in expression of P-450g mRNA.

Cytochrome P-450 (P-450)[1] is a superfamily of hemoproteins which function in the oxidative metabolism of a variety of endogenous and exogenous substrates (Sato & Omura, 1978). Recently, an ad hoc committee has proposed division of this superfamily on the basis of similarities of amino acid sequence between families (Nebert et al., 1987). Currently, nine families of mammalian P-450s designated P-450I, -II, -III, -IV, -XI, -XVII, -XIX, -XXI, and -XXVI have been

† A preliminary report of this work was presented at the 72nd Annual Meeting of the Federation of American Societies for Experimental Biology (McClellan-Green et al., 1988).

‡ The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02861.

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[1] Abbreviations: P-450, cytochrome P-450; CD, Charles River Sprague-Dawley; IPTG, isopropyl β-D-thiogalactopyranoside; GAR-HRP, goat anti-rabbit IgG, human IgG absorbed, horseradish peroxidase conjugated; RIA, radioimmunoassay; SSC, standard sodium citrate; SDS, sodium dodecyl sulfate.

described [nomenclature recommended by Nebert et al. (1989)]. The P-450II family is further divided into five subfamilies. These include the phenobarbital-inducible and many constitutive and sex-specific forms of cytochrome P-450.

Recently, several cDNAs from the P-450IIC subfamily have been isolated and characterized (Yoshioka et al., 1987; Zaphiropoulos et al., 1988; Kimura et al., 1988; Gonzalez et al., 1986). These P-450s represent constitutively expressed and sex-specific P-450s from livers of adult rats, i.e., P-450 (M-1) (IIC11), P-450 15β (IIC12), P-450 PB1 (IIC6), and P-450f (IIC7).[2] These isozymes share a high degree of amino acid homology (>70%) (Nebert et al., 1987) and exhibit immunochemical cross-reactivity (McClellan-Green et al., 1987; Bandiera et al., 1985).

P-450g is a male-specific hepatic cytochrome which is immunochemically related to P-450 M-1, P-450 15β, P-450f, and PB-1 (Bandiera et al., 1985; McClellan-Green et al., 1987). P-450g is immunochemically related to human P-450 HLx, which is phenotypically variable in humans (Wrighton et al., 1987). P-450g is also highly polymorphic in outbred male Long-Evans or Sprague-Dawley (CD) rats (Bandiera et al., 1986; McClellan-Green et al., 1987). Outbred CD male rats appear to be divided into at least two populations, expressing either high (+g) or very low (−g) levels of P-450g. Surprisingly, our earlier studies indicated that the levels of the translatable mRNA for P-450g were identical in the high and low phenotypes (McClellan-Green et al., 1987). In the present study, a cDNA clone for P-450g was isolated from a λgt11 library synthesized from mRNA from the liver of a (+g) adult male rat and sequenced. The expression of the mRNA for this P-450 was then examined in livers of outbred male CD rats using a specific oligonucleotide probe, to determine whether the polymorphism was the result of a defect in transcription of P-450g mRNA in the (−g) phenotype. In addition, the age-dependent expression of P-450g and its mRNA was examined in the ACI rat, a strain of rat which is homozygous for P-450g (+g) (Rampersaud & Walz, 1987).

Experimental Procedures

Materials. Restriction endonucleases were purchased from Pharmacia LKB (Piscataway, NJ). λgt11 was purchased from Promega-Biotec (Madison, WI). ^{125}I-Cytochrome P-450g was prepared by ICN Radiochemical (Irvine, CA). [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) and [α-^{32}S]dATP (650 Ci/mmol) were from the Radiochemical Center (Amersham, England). All other reagents were of the highest quality available.

Animals. Eight-week-old male and female Sprague-Dawley (CD) rats were obtained from Charles River (Kingston, NY) and maintained on standard rat chow and water ad libitum. For the developmental study, late-term pregnant ACI rats (Harlan) were obtained, and the livers from the male pups were collected on the day of birth and at 2, 4, 6, 8, and 10 weeks.

Purification of P-450g. Cytochrome P-450g was purified from the liver microsomes of untreated Sprague-Dawley male rats as described (Ryan et al., 1984). Antibody to P-450g was raised in rabbits and immunospecific anti-P-450g prepared by immunoadsorption over solid-phase immunoaffinity columns prepared from solubilized female CD rat liver microsomes and male Fischer microsomes coupled to CNBr-activated Sepharose 4B as previously described (McClellan-Green et al., 1987). Prior to use of the antibody for selection of cDNA clones for P-450g, the antibody was further adsorbed over *Escherichia coli* lysate bound covalently to CNBr-activated Sepharose 4B.

Isolation of Microsomes and RNA. Microsomes were prepared from each of 14 male CD rat livers at 8 weeks and from the livers of male ACI rats at various ages, as previously described (Goldstein et al., 1982). Total RNA was isolated from rat livers by the guanidine hydrochloride method (Cox, 1968). Poly(A+) RNA was prepared from the total RNA on oligo(dT)–cellulose columns (Aviv & Leder, 1972).

cDNA Cloning Procedure. Total RNA was isolated from the liver of a high (+g) male CD rat identified by immunoblot analysis and RIA for P-450g as described below. Poly(A+) RNA was selected following two cycles over an oligo(dT)–cellulose column. Double-stranded cDNA was constructed by the method outlined by Watson and Jackson (1985). A cDNA library was constructed by the addition of *Eco*RI linkers to the double-standard cDNA, size selection, and ligation into λgt11. The λgt11 cDNA constructs were transfected to *E. coli* Y1090. Specific P-450 (+g) cDNA clones were isolated by screening the library with the immunospecific antibody to P-450g. Colonies were lysed onto nitrocellulose filters (Schleicher & Schuell) saturated with 10 mM IPTG. Immune complexes were identified by using a 1:2000 dilution of Bio-Rad GAR-HRP (Bio-Rad Laboratories, Richmond, CA) in conjunction with freshly prepared substrate, 4-chloro-1-naphthol. Approximately 10000 phages were screened, and 8 immunopositive clones for P-450g were isolated (Benton & Davis, 1977). Phage DNA was isolated (Maniatis et al., 1982) and digested with *Eco*RI. A 1.23-kb insert was isolated and radiolabeled with [α-^{32}P]dCTP to a specific activity of 1×10^8 cpm/μg by nick translation. The cDNA, c15, was then used as a probe for further screening of the λgt11 library. Fourteen additional P-450g cDNAs were isolated, and the DNA was digested with *Eco*RI or *Pvu*II. Four cDNA clones were judged to be approximately full-length based on their restriction profile. From these, the longest two were selected for further analysis. Phage DNA was subcloned into *Sma*I-digested PUC 13 and the recombinant plasmid DNA isolated by the alkaline–SDS method (Birnboim & Doly, 1979). Inserts from these clones, c515 and c5126, were characterized by restriction mapping, followed by DNA sequence analysis by the dideoxy chain termination method (Sanger et al., 1977) in conjunction with site-directed M13 cloning. In addition, the recombinant PUC 13 plasmid was purified by CsCl gradients (Maniatis et al., 1982), and some areas of the ds DNA were sequenced similarly by using a series of synthetic 20-mer primers based on the sequence of C5126. Each nucleotide was sequenced a minimum of 2 times in both directions.

RNA Blot Analysis. Five to ten micrograms of poly(A+) RNA was electrophoretically separated on 2.2 M formaldehyde–1% agarose gels (Maniatis et al., 1982). The poly(A+) RNA was then transferred to Nytran membranes (Schleicher & Schuell) and baked for 2 h at 80 °C. Alter-

[2] The P-450 isozymes described in this work have been designated as follows: P-450s b, c, d, e, f, g, and j according to Ryan et al. (1979, 1984); PB-1 according to Waxman (1984); P-450 15β as designated by MacGeoch et al. (1984) is identical with P-450i (Ryan et al., 1984). P-450 (M-1) as designated by Matsumoto et al. (1986) is identical with P-450h (Ryan et al., 1984). According to the gene nomenclature system recommended by Nebert et al. (1989), cytochrome P-450g is designated as IIC13 (gene CYP2C13), while the other P-450s referred to in this report have been designated as follows: rat P-450 forms P-450a = IIA1, P-450c = IA1, P-450d = IA2, P-450b = IIB1, P-450e = IIb2, P-450f = IIc7, P-450 (M-1)= IIC11, P-450 15β = IIC12, P-450j = IIE1, db1 = IID2, LAω = IVA1, and pcn1 = IIIA1, and rabbit form 1 = IIC5. Human P-450 forms P-450a = IIA1 have been designated as follows: human form 1 = IIC8, db1 = IID1, mp8 = IIC10, and P-450(c21)B = XXIA2 as reviewed by Nebert et al. (1989).

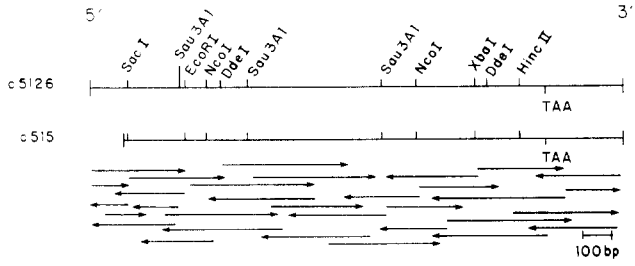

FIGURE 1: Restriction map and sequence strategy. The cleavage sites of restriction endonucleases used for the sequence analysis are indicated. Restrictions fragments of the inserts c5126 and c515 were subcloned into M13 mp18 or mp19 and sequenced following the chain termination dideoxy method (Sanger et al., 1977). All sequences were determined in both directions. In regions where fragments were not readily generated by restriction digest, ds DNA was sequenced by using a series of synthetic 20-mer primers based on the previously determined sequence of an adjacent area of c5126.

natively, 0.5, 1.0, and 3.0 μg of poly(A+) RNA was denatured in 6.15 M formaldehyde–10 × SSC at 67 °C for 15 min and then directly bound to Nytran membranes by use of the Miniblot II apparatus (Schleicher & Schuell). A specific oligonucleotide of the anticoding strand (the 498th–517th nucleotides) was labeled at the 5′ end to a specific activity of 1×10^8 cpm/μg with [γ-^{32}P]ATP (Zarbl et al., 1985). The filters were prehybridized at 42 °C in 6 × SSC, 4 × Denhardt's solution, 0.5% SDS, and 10 μg/mL sonicated salmon sperm DNA for 2 h. Filters were then hybridized overnight at 42 °C in 6 × SSC, 4 × Denhardt's solution, 0.1% SDS, and 10 μg/mL sonicated salmon sperm DNA. The filters were washed 4 times at room temperature in 2 × SSC–0.1% SDS and exposed to X-ray film overnight.

Immunoblots and Radioimmunoassays for P-450g. Microsomal protein was determined by the method by Lowry et al. (1951). Hepatic P-450g was measured in a double-antibody radioimmunoassay (RIA) similar to that previously described for P-450c and P-450d (Luster et al., 1983). SDS–polyacrylamide gel electrophoresis of liver microsomal proteins was performed according to the method of Laemmli (1970), and the proteins were transferred to nitrocellulose and immunoblotted as previously described (Yeowell et al., 1985).

Results

Nucleotide Sequence of the P-450 (+g) cDNA. The cDNA inserts prepared from the two longest clones, c5126 (2300bp) and c515 (1900 bp), were mapped for cleavage sites by several restriction endonucleases (Figure 1). Appropriate fragments were then subcloned into M13 mp18 and mp19 and sequenced by using the dideoxy chain termination method. Figure 2 shows the nucleotide and deduced amino acid sequences for the c5126 cDNA for P-450 (+g) mRNA. An open reading frame consisting of 1473 nucleotides was found to code for a 490 amino acid polypeptide with a calculated molecular weight of 55 859. Clone c515 did not contain the 5′-NH_2-terminal nucleotides (1–63) but was otherwise identical with c5126.

The first 15 amino acids encoded by the nucleotide sequence were identical with those reported for P-450g (Haniu et al., 1984), and the first 30 amino acids were identical with those reported for P-450 (M-3) (Matsumoto et al., 1986). This led us to conclude that we had indeed isolated the cDNA corresponding to P-450 (+g) mRNA. There were at least 13 nucleotides in the 5′ noncoding leader sequence prior to the open reading frame and 251 nucleotides in the 3′ noncoding trailer sequence. Two stop codons, TAA and TGA, were found in tandem at positions 1471–1476. The consensus sequence, AATAAA, for the poly(A) addition signal is located at position 1690–1695, 15 nucleotides upstream of the poly(A) tail.

Table I: Percent Homology of P-450g cDNA to Other P-450 cDNAs[a]

	nucleic acid sequence	deduced amino acid sequence
M-1 (IIC11)	71.7	66.1
PB1 (IIC6)	76.8	70.0
f (IIC7)	76.8	69.0
15β (IIC12)	88.1	79.8
human P-4501 (IIC8)	73.7	68.1
d (IA2)	43.5	28.9
e (IIB2)	54.1	46.5
a (IIA1)	52.2	44.1
j (IIE1)	57.7	50.4
db1 (IID1)	47.7	36.3
pcn1 (IIIA1)	39.9	23.2
LAω1 (IVA1)	39.0	19.8

[a] The homology of the nucleotide sequences and of the deduced amino acid sequences was analyzed by using the alignment algorithm of Wilbur and Lipman (1983) and the University of Wisconsin Genetics Computer Analysis Package (Devereux et al., 1984), respectively. All P-450 cDNAs were rat with the exception of IIC8 (human).

Structure of Rat P-450 (+g). The primary structure of P-450 (+g) was compared to other forms of P-450 known to be immunochemically similar to this isozyme as well as other families of P-450. Table I shows the relatively high degree of nucleotide and amino acid sequence similarity that exists between the P-450 (+g) cDNA and the published sequences of other P-450IIC members, namely, P-450 (M-1), P-450 PB-1, P-450f, and P-450 15β. On the basis of these similarities, this P-450 has been termed P-450IIC13 and the gene CYP2C13 according to the nomenclature of Nebert et al. (1989). In addition, P-450 (+g) cDNA demonstrates a high degree of similarity to human P-450 1 (IIC8) (Okino et al., 1987). Much less similarity exists between the P-450 (+g) cDNA and sequences reported for the IIB (b and e), IA (c and d), IIA (a), IIe1 (j), IID (db1), IVA (LAω1), and IIIA (pcn) subfamilies.

The portion of the amino acid sequence containing the putative heme binding cysteine, 428–448, was highly conserved, as were known conserved regions from the 28th–41st positions and 345th–357th positions (Nelson & Strobel, 1988). The conserved region from the 345th to the 357th position in the alignment of Nelson and Strobel (1988) corresponds to a proposed steroid binding region of P-450c17 (Picado-Leonard & Miller, 1988). However, the consensus sequence given by Picado-Leonard and Miller (1988) more closely resembles the amino acid sequence occurring in the 319th–326th positions. A variable region occurs in the putative substrate binding site, 199th–219th position; however, a more highly variable region exists between positions 230 and 240, similar to that reported for P-450 15β (Zaphiropoulous et al., 1988). Comparisons of homology in these regions between members of the IIC subfamily are given in Table II.

Analysis of (±g) CD Rats. A complementary oligonucleotide probe (5′-ATCCCATGATGAATTGG-3′) (see Figure 2) was synthesized to a unique region of the P-450 (+g) cDNA sequence. This probe verified the presence of P-450g mRNA in livers of 8-week-old male CD rats but not female CD rats on Northern blots (Figure 3). This shows that the oligoprobe discriminates between the mRNAs for P-450g and P-450s PB-1, f, and i since the latter three P-450s are not male-specific. In addition, the nucleotide differences between the oligonucleotide probe and P-450 (M-1) (a difference of 6 out of 17 nucleotides), P-450 PB-1 and F (8 differences), and P-450i (2 differences) indicate that the probe is specific for P-450g mRNA.

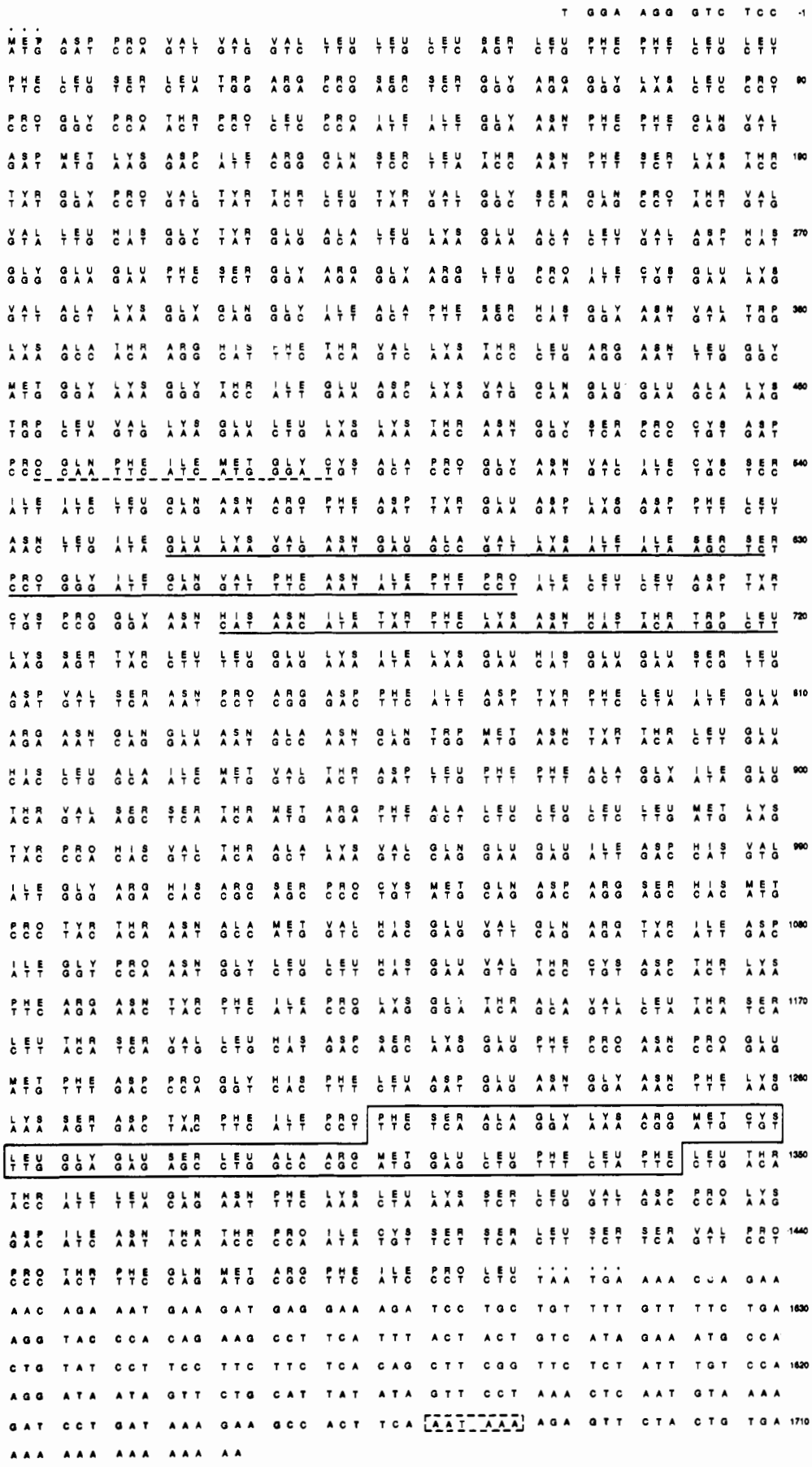

FIGURE 2: Nucleotide and amino acid sequence of P-450g cDNA. The nucleotide sequence was determined by the dideoxy chain termination method as described. The positions of the oligonucleotide probe are marked by a dashed line. The highly conserved putative heme-binding region (HR2) is enclosed by a solid line. The highly variable putative substrate binding regions are underlined by a solid line. The initation and termination codons are starred. A putative poly(A) addition signal (AATAAA) in enclosed by a dashed line.

Table II: Percent Similarity of Regions of the Deduced Amino Acid Sequence of P-450g (IIC13) cDNA to Related P-450s[a]

	constant regions		putative substrate regions		constant region	putative heme binding region
	28–41	165–185	199–219	230–240	345–357	428–448
M-1 (IIC11)	100	81	43	36	77	81
PB1 (IIC6)	93	81	52	27	77	91
f (IIC7)	100	67	67	27	85	81
15β (IIC12)	93	81	62	18	92	81
mp8 (IIC10)	93	76	62	36	85	80
Hp1-1 (IIC8)	100	76	48	36	77	86
j (IIE1)	79	52	38	27	69	81
db (IIDI)	57	33	28	9	85	85
a (IIAI)	85	33	19	18	62	71

[a] Selected regions of the deduced amino acid sequence for P-450g were compared to those of other related P-450 cDNAs.

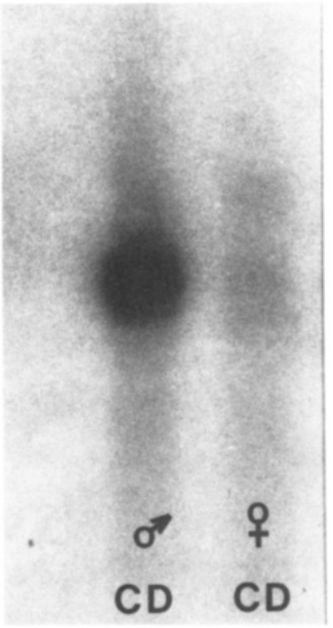

FIGURE 3: Northern blot analysis of P-450g mRNA. An oligonucleotide probe was synthesized to a unique region of the cDNA. Poly(A+) RNA (10 μg) isolated from adult male and female CD rats was subjected to electrophoresis is a denaturing formaldehyde–agarose gel and transferred to a Nytran membrane. The filter was hybridized with the specific oligoprobe and autoradiographed for 24 h utilizing a Dupont Lightning Plus intensifying screen.

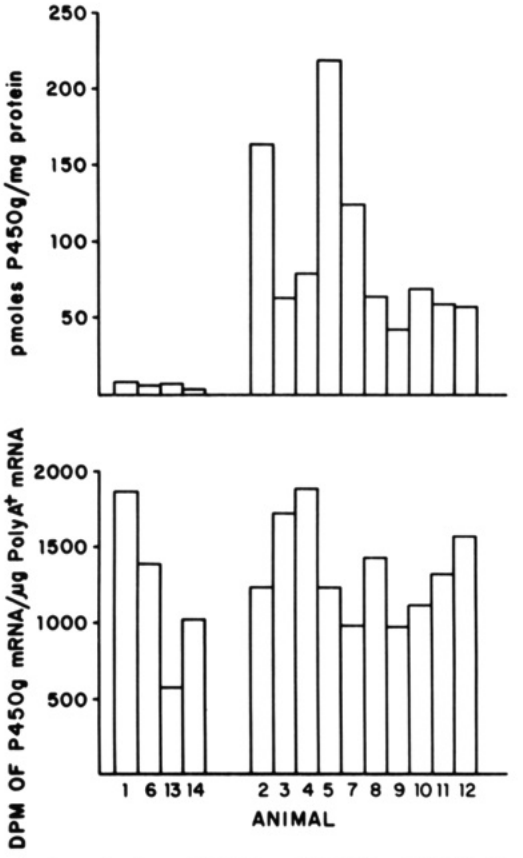

FIGURE 4: Quantitation of P-450g and P-450g mRNA in livers of 14 adult male CD rats. Cytochrome P-450g content of the liver microsomes from 14 individual male rats was measured by RIA (see Experimental Procedures). Poly(A+) RNA (0.5, 1.0, and 3.0 μg) was applied to a Nytran membrane and hybridized with a specific oligonucleotide probe for P-450g (Figure 2). Each dot was then excised and counted to obtain the average dpm per microgram. Duplicate slots, to which no RNA was added, were used as negative controls.

P-450g has been shown to be phenotypically variable in livers of adult male CD and male Long Evans rats (Bandiera et al., 1986; McClellan-Green et al., 1987). When the P-450g content in liver microsomes of 14 individual adult male CD rats was determined by RIA in the present study, at least 2 phenotypic populations, possibly 3 could be detected: those with high levels of P-450g (+g) (100–250 pmol/mg), those with intermediate levels (+g) (30–100 pmol/mg) of P-450g, and those with very low levels (–g) (1–7 pmol/mg) of P-450g. It is noteworthy that Western blots of (–g) rats also detected a faint polypeptide band with the same molecular weight as P-450g, suggesting that a small amount of P-450g was probably present in (–g) animals. When poly(A+) RNA from each of the 14 male CD rats was analyzed by Northern blotting, no significant differences were seen in the P-450g mRNA content of the (+g) and (–g) populations. The P-450 (+g) mRNA group had a mean value of 1349 ± 306 dpm/μg while that of the P-450 (–g) group was 1210 ± 544 dpm/μg (Figure 4).

Developmental Expression of P-450g in Male Rats. Because cytochrome P-450g is polymorphic in the outbred male CD rat, a rat strain (ACI) which is homozygous for P-450 (+g) (Rampersaud & Walz, 1987) was selected for investigation of the age-specific expression of P-450g in male rats. Figure 5A shows the results of RIA and RNA blot analysis. No P-450g was observed in any of the livers up to 4 weeks. However, essentially adult levels of P-450g were detected at 6, 8, and 10 weeks. RNA blot analysis, using the synthetic oligonucleotide probe, gave similar results. The major RNA species that hybridized to the probe was located around 2000 bp, which agreed well with the size of the P-450 (+g) cDNA (Figure 5B).

Discussion

In the present study, we have examined the sequence similarity between cytochrome P-450g and immunochemically related members of the P-450IIC subfamily. We isolated a

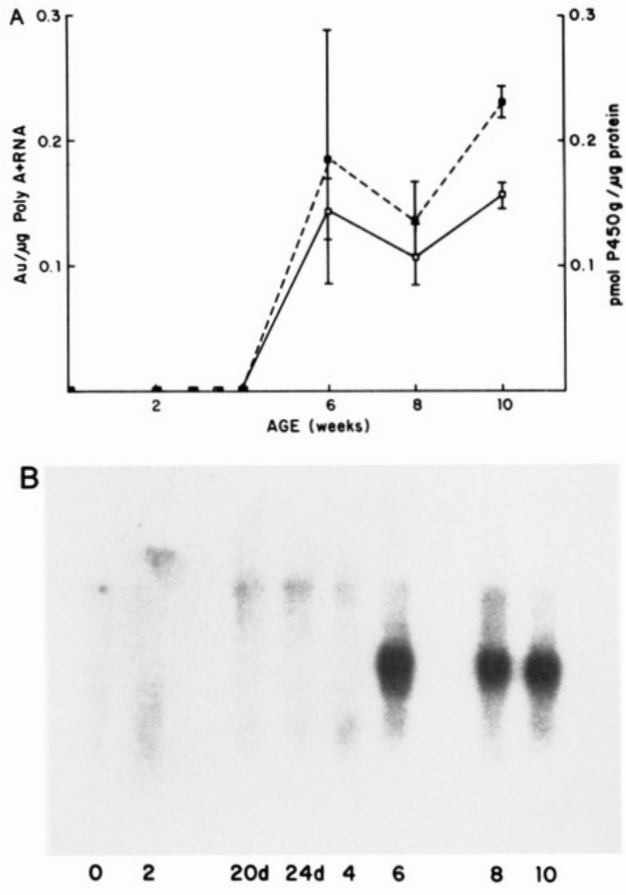

FIGURE 5: (A) Developmental expression of P-450g (□) and its mRNA (■) in livers of male rats. Total liver microsomes and poly(A+) RNA were prepared from the livers of male rats at birth (0) and 2, 4, 6, and 10 weeks of age. Cytochrome P-450g content of the liver microsomes from four male rats per age group was measured by RIA. Poly(A+) RNA (0.5, 1.0, and 3.0 μg) was applied to Nytran membranes and hybridized with the labeled oligonucleotide probe to P-450g overnight. The filters were autoradiographed overnight and films scanned by using a laser scanner (LKB Instruments). The values of the integrated peaks are plotted as mean absorbance units per microgram of poly(A+) RNA. (B) Radioautograph of developmental expression of P-450g mRNA. Ten micrograms of poly(A+) RNA from individual rats was separated by electrophoresis in a denaturing formaldehyde–agarose gel, transferred to Nytran filters, and hybridized as described above.

full-length P-450 (+g) cDNA clone by screening a cDNA library from a high (+g) CD male rat liver. Sequence analysis and the resultant deduced amino acid sequence revealed that the N-terminus was identical with the NH_2-terminal 15 amino acid residues from purified rat P-450g (Haniu et al., 1984) and the 30 amino acid residues of the NH_2 terminus from purified rat P-450 (M-3) (Matsumoto et al., 1986), indicating that the cDNA codes for P-450 (+g).

The cDNA for P-450g has >76% nucleotide similarity to P-450s PB-1 (IIC6) and f (IIC7), approximately 72% similarity to P-450 (M-1) (IIC11), and 88% similarity to P-450 15β (IIC12). In addition, c5126 has 74% nucleotide sequence similarity to human form P-450 1 (IIC8) (Okino et al., 1987). Comparison of the amino acid seqeunces for all of these cytochromes belonging to the IIC subfamily revealed an overall 66–80% similarity. With the cytochrome P-450 UEP of 2.1–4.1 (Leighton et al., 1984; Jaiswal et al., 1985), a 20–34% divergence would indicate that P-450g split from a common ancestor 42–140 million years ago. This split would have occurred approximately the same time as that of PB-1, f, (M-1), and 15β (Gonzalez et al., 1986; Zaphiropoulous et al., 1988).

It is interesting to observe the conserved nature of the heme binding region (428th–448th positions) as well as the other constant regions (Nelson & Strobel, 1988). It is also of interest that in the highly conserved region, 165th–185th positions, P-450g has a leucine at residue 183, while all other P-450s reported thus far possess phenylalanine in this position (Nelson & Strobel, 1988). In addition, P-450g has glycine at position 175, yet most P-450s contain cysteine at this position with the exception of some phenobarbital and 3-methylchloranthene-inducible P-450s and the 17α and LAω P-450s. P-450g also contains a histidine at position 125, while 14 other P-450s (a, b, c, d, e, f, PB-1, i, M-1, LAω, PCN1, PCN2, db1, and db2) contain an arginine in this position (Matsunaga et al., 1988). It would be interesting to determine whether these changes alter the activity of cytochrome P-450g. The remaining 37 of the 39 residues which Matsunaga et al. (1988) found to be conserved among these 14 rat P-450 amino acid sequences were also conserved in P-450g. The arginine at position 125 is part of a recognition site (Arg-Arg-Phe-Ser) for cAMP-dependent protein kinase in P-450 IIB1 (Mueller et al., 1985) and presumably other P-450s. There is evidence that phosphorylation of this serine can modulate catalytic activity (Pyrein et al., 1984). P-450g contains the substitution of a tyrosine for the serine at this site, suggesting that the catalytic activity of P-450g would not be affected by cAMP-dependent protein kinase.

Cytochrome P-450g is male-specific and phenotypically variable in livers of outbred strains of rat. RNA blot analysis using a specific oligonucleotide probe as well as immunoblot analysis of liver microsomal preparations confirmed that the sex-specific expression of P-450g is the result of pretranslational differences in synthesis of this protein. However, the phenotypic variability in livers of outbred male Sprague-Dawley rats could not be attributed to differences in expression of its mRNA, since equivalent amounts of P-450g mRNA were detected in both the (+g) and (−g) phenotypes. These results confirm the conclusions of an earlier study in our laboratory which showed no differences between the amount of translatable mRNA for P-450g in liver from the high and low phenotypes (McClellan-Green et al., 1987).

Previous work on the regulatory mechanisms of P-450g has suggested the possible presence of a cis-acting regulatory gene (Rampersaud et al., 1987). This hypothesis was based on the additive inheritance exhibited by (+g) × (−g) hybrids. However, our results show that the mRNA levels for P-450g are, in fact, identical in the two phenotypes. Therefore, a cis-acting regulatory element does not affect P-450g at the transcriptional level. It seems more likely that the (−g) phenotype may reflect the presence of an alternatively spliced or mutated mRNA for P-450g in the (−g) individual. Several laboratories have recently reported the isolation of P-450 cDNA containing inserts or alternately spliced introns. For example, Noshiro et al. (1988) reported the isolation of several cDNAs for the female-specific, mouse liver I-P-$450_{16\alpha}$, one of which contained a 27 bp insert. This insert did not alter the reading frame for the amino acid sequence but had the potential to form a loop structure. Thus, a larger than normal polypeptide would be expected to result from this cDNA. Alternatively, Wong et al. (1987) reported the isolation of a cDNA for C-P-$450_{16\alpha}$, the mouse, male-specific testosterone 16α-hydroxylase, which contained a 142 bp deletion, which appeared to correspond to the sixth exon of the gene. The resultant protein from such a deletion would not contain the conserved cysteine for the fifth ligand on the heme binding site. However, the translational products of liver polysomes

from (−g) male rats immunoprecipitated with antibody to P-450g were the same molecular weight as that of translational products from (+g) rats products (McClellan-Green et al., 1987). Moreover, a faint polypeptide with the same molecular weight as P-450g is detected on Western blots of liver microsomes from (−g) rats. These data suggest that faulty splicing of P-450g is not the most likely defect.

A more likely possibility is the presence of mutations occurring in the P-450 (−g) which would produce a defective mRNA. Missense mutations have been reported for the P-450 (c21)B (CYP21B) genes (Higashi et al., 1988). Here, the amino acid residues isoleucine, valine, and methionine were changed to asparagine, glutamic acid, and lysine, respectively, in the sixth exon of the B gene. These mutations were in the putative substrate binding region and thus could result in production of a catalytically inactive protein. Mutations could also conceviably produce a protein which turns over more rapidly. Therefore, isolation and sequencing of a (−g) cDNA and possibly the gene for IIC13 (CYP2C13) from the (+g) and (−g) rats are needed to determine the mechanism responsible for the P-450g polymorphism. Such work is now being conducted in our laboratory.

The phenotypic variation of P-450g is of particular interest since it may be a common phenomenon across species. Human P-450 HLx, which is immunochemically similar to P-450g, is also phenotypically variable in humans (Wrighton et al., 1987). Human HLx appears to be identical with human form IIC8 on the basis of its N-terminal sequence (Okino et al., 1987), an enzyme which is 74% homologous to rat liver P-450g. Human form IIC8 is also homologous (81%) to rabbit form P-4501, which is phenotypically variable in rabbits. It is not yet clear whether the phenotypic variation in all three species arises from a similar mechanism. Differences in metabolism due to phenotypic variation in other P-450s such as the form which metabolizes debrisoquine have been well documented (Gonzalez et al., 1988). It will also be of interest to determine how the phenotypic variations in human form HLx or rat form P-450g affect metabolism of xenobiotics or endogenous substances.

Like many of the P-450IIC cytochromes, P-450g is absent in the neonate and developmentally expressed in livers of male adult rats. The specific oligonucleotide probe for P-450g was employed to examine the age-specific development of P-450g mRNA in the liver of the homozygous ACI (+g) rat. No hybridization was observed with any of the poly(A+) RNA preparations up to 4 weeks after birth. Essentially maximal amounts of hybridizable poly(A+) RNA were detected from livers at 6, 8, and 10 weeks of age. The major mRNA species that hybridized to the oligonucleotide was approximately 2000 bases, which agreed well with the size of the c5126 cDNA and the RNA seen in adult male CD rats. The time course for the male-specific expression of P-450g mRNA coincided with the appearance of P-450g as detected by RIA, indicating that age-dependent expression of P-450g depends on expression of the mRNA for P-450g. These results were similar to those seen for the expression of other male-specific forms, including P-450 (M-1) (IIC11) and P-450 RLM2 (IIA2) (Yoshioka et al., 1987; Waxman et al., 1988). The mechanism of hormonal control of P-450g requires additional study.

Conclusions

In conclusion, the message for P-450g (IIC13) is expressed in equivalent amounts in both the (+g) and (−g) phenotypes of the outbred male CD rat. The lack of correlation of P-450g with its mRNA demonstrates that phenotypic differences in P-450g expression do not reflect alterations in the levels of P-450g mRNA. The production of an abnormally spliced polypeptide or a mutation resulting in a protein containing an altered amino acid sequence is possible explanation for the manifestation of the (−g) phenotype; however, the isolation and sequencing of a (−g) cDNA are needed to clarify this issue. The expression of P-450g is developmentally regulated in an age- and sex-specific manner in the inbred homozygous (+g) ACI rat strain. In contrast to the phenotypic variability, the sex- and age-specific expression of P-450g is dependent on expression of its mRNA.

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