# Functional Characterization of Two Cytochrome P-450s within the Mouse, Male-Specific Steroid 16α-Hydroxylase Gene Family: Expression in Mammalian Cells and Chimeric Proteins

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ABSTRACT: Two cDNAs, pc16α-2 and pc16α-25, which encode P-450s from within the mouse, male-specific steroid 16α-hydroxylase (C-P-450$_{16\alpha}$) gene family, were transfected into COS-1 cells in order to study catalytic activities of the expressed P-450s. pc16α-2 was shown previously to encode the growth hormone dependent and androgen-dependent C-P-450$_{16\alpha}$ in adult male mice (Wong et al., 1987). The sequence of pc16α-25-encoded P-450 (P-450cb) was identical with gene cb within the C-P-450$_{16\alpha}$ family. There was 94% and 87% nucleotide and amino acid sequence identity, respectively, between P-450cb and C-P-450$_{16\alpha}$. We expressed both P-450s by transfecting their cDNAs into COS-1 cells and found that steroid 16α-hydroxylase activity was catalyzed by C-P-450$_{16\alpha}$ but not by P-450cb. In addition to testosterone, progesterone and estradiol were hydroxylated specifically at the 16α-position by the expressed C-P-450$_{16\alpha}$. The results indicated that a broad steroid substrate specificity with high regio- and stereoselectivity at that position was a characteristic of C-P-450$_{16\alpha}$. We constructed and expressed chimeras between the two P-450s and found that the presence of about two-thirds of the C-P-450$_{16\alpha}$ molecule from its C-terminus was necessary for the chimeric cytochrome to maintain steroid 16α-hydroxylase activity.

Steroid hormone metabolism is mediated in part by the cytochrome P-450-dependent monooxygenase system. As liver is a major organ involved in such metabolism, it contains a large number of different cytochrome P-450s which function as terminal oxidases. These enzymes determine the sex specificity of steroid metabolism activities. At the same time, this monooxygenase system also participates in metabolic oxidation of xenobiotics such as drugs and chemicals. Therefore, the metabolism of xenobiotics frequently becomes sex specific.

Expression of cytochrome P-450s is under specific, but complex, regulations by sex, age, tissues, and exogenous and endogenous inducers. Questions about cytochrome P-450 which could not have been answered previously are now being solved by recent advances in the molecular biology of cytochrome P-450.

Taking advantage of genetic homogeneity and variants of inbred mice, we began investigating the expression mechanism of mouse, male-specific steroid 16α-hydroxylase[1] by purifying the form of cytochrome P-450 (C-P-450$_{16\alpha}$) specific to the 16α-hydroxylase activity from 129/J male mice (Harada & Negishi, 1984). We then found that the male-specific expression of C-P-450$_{16\alpha}$ was regulated by growth hormone and androgens (Harada & Negishi, 1985; Noshiro & Negishi, 1986; Wong et al., 1987). The C-P-450$_{16\alpha}$ gene (gene ca) was cloned, and its structure was determined (Wong et al., 1988). Within the C-P-450$_{16\alpha}$ gene family, there were an undetermined number of related genes and at least five that were highly similar (Wong et al., 1987). Among the latter, genes cb and cc were characterized as sharing 94% nucleotide sequence similarities to gene ca (Wong et al., 1988). Northern hybridization of oligonucleotide probes with poly(A+) RNA, specific to each gene, indicated that gene expression within this family was regulated differently: gene ca (C-P-450$_{16\alpha}$) exhibited male-specific expression both in liver and in kidney; gene cb was expressed only in liver of both genders; expression of gene cc was not detected in either tissue.

For this report, we cloned cDNA encoded by gene cb. This cDNA as well as C-P-450$_{16\alpha}$ cDNA (Wong et al., 1987) was expressed in mammalian cells. Then we measured catalytic activities of the expressed C-P-450$_{16\alpha}$ and P-450cb.[2] Chimeric cytochromes between the two P-450s were expressed in order to study a structure–function relationship of C-P-450$_{16\alpha}$.

## Experimental Procedures

*Animals.* Two-month-old 129/J male mice were purchased from Jackson Laboratory (Bar Harbor, ME).

*Materials.* [4-$^{14}$C]Testosterone (50–60 mCi/mmol), [4-$^{14}$C]progesterone (57 mCi/mmol), and 17β-[4-$^{14}$C]estradiol (57 mCi/mmol) were purchased from DuPont–New England Nuclear (Boston, MA); [α-$^{32}$P]dCTP (3000 Ci/mmol), nick translation kits, and *Eco*RI–*Bam*HI adaptor were from Amersham Corp. (Arlington Heights, IL); *Escherichia coli* RNase H, DNA ligase, DNA polymerase I, T4 DNA polymerase, T4 polynucleotide kinase, *Eco*RI methylase, restriction endonucleases, and DEAE-dextran were supplied by Pharmacia LKB Biochemicals (Piscataway, NJ). M-MLV[3] reverse transcriptase was procured from Bethesda Research Labora-

[1] Originally, C-P-450$_{16\alpha}$ was purified from 129/J male mice by using testosterone 16α-hydroxylase activity as the basis for selection of fractions from columns (Harada & Negishi, 1984). We, therefore, have been calling C-P-450$_{16\alpha}$ testosterone 16α-hydroxylase. Recently, we reported that in addition to testosterone, the purified C-P-450$_{16\alpha}$ catalyzed 16α-hydroxylation of many other steroids (Harada & Negishi, 1988). This multisubstrate specificity of C-P-450$_{16\alpha}$ was confirmed by our present work with the P-450 expressed from its cDNA in COS-1 cells. From here on, we will refer to C-P-450$_{16\alpha}$ steroid 16α-hydroxylase.

[2] The p-450 encoded by gene cb was designated temporarily P-450cb. The cytochrome P-450cb and C-P-450$_{16\alpha}$ are members within P-450IID subfamily (Nebert et al., 1987).

[3] Abbreviations: M-MLV, Moloney murine leukemia virus; DMEM, Dulbecco's modified Eagle's medium; bp, base pair(s); SDS, sodium dodecyl sulfate.

tories (Gaithersburg, MD); oligo(dT)–cellulose (type II) was from Collaborative Research (Lexington, MA); packaging solution and λgt11 vector were from Promega Biotec (Madison, WI); nitrocellulose paper and nylon paper (Nytran) were from Schleicher & Schuell (Keene, NH). DMEM[3] and fetal bovine serum were obtained from GIBCO Laboratories (Long Island, NY). NADPH–cytochrome P-450 reductase was purified from phenobarbital-treated rat liver microsomes by using the procedure of Yasukochi and Master (1976).

*cDNA Cloning.* 129/J liver RNA was extracted with 8 M guanidine hydrochloride (Cox, 1986) and then enriched for poly(A+) RNA by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972); finally, liver poly(A+) RNA was incubated with M-MLV reverse transcriptase to synthesize single-stranded cDNAs. The method of Okayama and Berg (1982) was performed to make double-stranded cDNAs that first were size-selected with Sepharose 4B chromatography and then ligated to λgt11 vector by using *Eco*RI linker, packaged, and transfected to *Escherichia coli* 9623 (Young & Davis, 1982).

The cDNA library was screened by using $^{32}$P-labeled pc16α-2, cDNA encoding growth hormone dependent, androgen-dependent C-P-450$_{16\alpha}$ (Noshiro & Negishi, 1986; Wong et al., 1987). Recombinant phage DNAs positive to the cDNA probe were purified and analyzed by restriction endonucleases; pc16α-25 was characterized as a homologous cDNA. The nucleotide sequences showed that pc16α-2 and -25 shared 94% identity and also indicated the presence of a 100 bp DNA insertion in the coding region of pc16α-25. On the basis of the nucleotide sequence, this 100 bp insert was determined to be the eighth intron of gene cb. The intron's nucleotide sequences were reported in a previous paper (Wong et al., 1989). Thus, pc16α-25 represented an unprocessed mRNA of C-P-450$_{16\alpha}$. Therefore, the cDNA library was rescreened by using an oligonucleotide probe (OP-2; 5′-CGGAAAAGGAAAGACACCAAG which is reverse complement from +1441 to +1462) specific to pc16α-25 to isolate pc16α-23, an overlapped clone, which was without the insertion. These cDNA inserts were subcloned into pUC 13 plasmid.

*DNA Sequence.* Fragments generated by digesting cDNA inserts with *Pst*I, *Hind*III, or *Bam*HI, or combinations of these, were ligated to the proper site(s) of M13 vectors. The recombinant M13 phage was transfected into *Escherichia coli* JM103, and single-stranded DNA was isolated (Messing et al., 1977). Chain termination reactions (Sanger et al., 1980; Biggin et al., 1983; Tabor & Richardson, 1987) were performed to obtain nucleotide sequences by using $^{35}$S-dCTP as a radioactive precursor.

*Construction of Expression Plasmids Containing P-450 cDNAs.* Figure 1 shows restriction maps of pc16α-2, -23, -25, and -35. pc16α-2 was a full-length cDNA of C-P-450$_{16\alpha}$ (Wong et al., 1987). As pc16α-25 and -23 overlapped, *Eco*RI–*Bam*HI and *Bam*HI–*Eco*RI fragments prepared from pc16α-25 and -23, respectively, were ligated in order to obtain a full-length cDNA (pc16α-35) without the presence of a 100 bp insertion. These two full-length cDNAs, pc16α-2 and -35, were ligated at the *Bam*HI site of the Okayama Berg expression vector pcD, by using an *Eco*RI–*Bam*HI adaptor. The constructed plasmids containing the cDNA in a 5′ to 3′ orientation were designated pcD-16-2-1 and pcD-16-35-7, respectively. A plasmid called pcD-16-2-4, which contained the cDNA in a 3′ to 5′ orientation, was also prepared to be used as a negative control for P-450 expression in cells.

*Expression of P-450 in COS-1 Cells.* COS-1 cells (from American Type Culture Collection, Rockville, MD) were

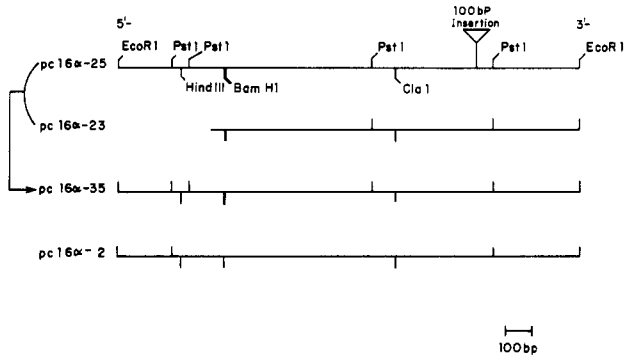


FIGURE 1: Restriction maps. *Pst*I, *Hind*III, *Bam*HI, and *Cla*I sites are indicated on each cDNA clones. The *Eco*RI sites on both ends were the cloning sites. The map of pc16α-2 was taken from previous work (Wong et al., 1987). pc16α-35 was constructed by ligating the 5′-*Eco*RI–*Bam*HI fragment of pc16α-25 to 5′-*Bam*HI–*Eco*RI of pc16α-23. The construction details were described under Experimental Procedures.

maintained in DMEM supplemented with 10% fetal bovine serum. Cells grown to subconfluency were harvested and replated in 5 times as many 10-cm dishes as originally collected. After incubation for 24 h at 37 °C, the replated cells were washed with Dulbecco's phosphate-buffered saline [containing $CaCl_2$ (0.1 g/L), $KH_2PO_4$ (0.2 g/L), $MgCl_2$ (0.1 g/L), KCl (0.2 g/L), NaCl (8 g/L), and $Na_2HPO_4$ (1.15 g/L)] and then transfected with recombinant plasmid DNA (5 μg per dish) in DEAE-dextran (500 μg/mL) for 30 min at 37 °C according to the method of Sompayac and Donna (1981). Then transfected cells were washed with Dulbecco's phosphate-buffered saline, followed by treatment with chloroquine (52 μg/mL) for 5 h at 37 °C as described by Luthman and Magnusson (1983). The transfected, chloroquine-treated cells were further incubated in DMEM containing 10% fetal bovine serum for 72 h at 37 °C. All incubations and treatments were performed under a stream of 95% air/5% $CO_2$. These cells were harvested with the use of a rubber policeman and then homogenized both by the use of a Polytron and by sonication in 10 mM Tris-HCl (pH 7.5) containing 150 mM KCl, 1 mM EDTA, and both leupeptin and pepstatin (5 μg/mL). The whole cell homogenates were subsequently used for measuring catalytic activities and immunoblotting of expressed P-450.

*Other Analytical Methods.* Testosterone, progesterone, and estradiol 16α-hydroxylase activities were measured by previously used methods (Harada & Negishi, 1984, 1988).

Protein contents were determined by Bradford's method (Bradford, 1976), and Western blot analysis was performed by the method of Domin et al (1984).

Agarose gels run under denaturing conditions, as described by Thomas (1980), were used for electrophoresis of RNA and its transfer to Nytran paper. Hybridization conditions with $^{32}$P-labeled cDNA were previously described (Harada & Negishi, 1985).

## RESULTS

*P-450cb Sequences and Comparison with C-P-450$_{16\alpha}$.* The nucleotide and deduced amino acid sequences of pc16α-25 are shown in Figure 2. Only those that are different are shown for the C-P-450$_{16\alpha}$. The initiation codon was located at 50 bp downstream from the 5′ end, and the polyadenylation site (AATAAA) was present at 18 bp upstream from the poly(A) tail. Since the pc16α-25 sequence exactly matched the gene cb exonic sequence (Wong et al., 1988), this cDNA represents the P-450 mRNA encoded by the gene. Thus, the name P-450cb was given temporarily to this cytochrome.

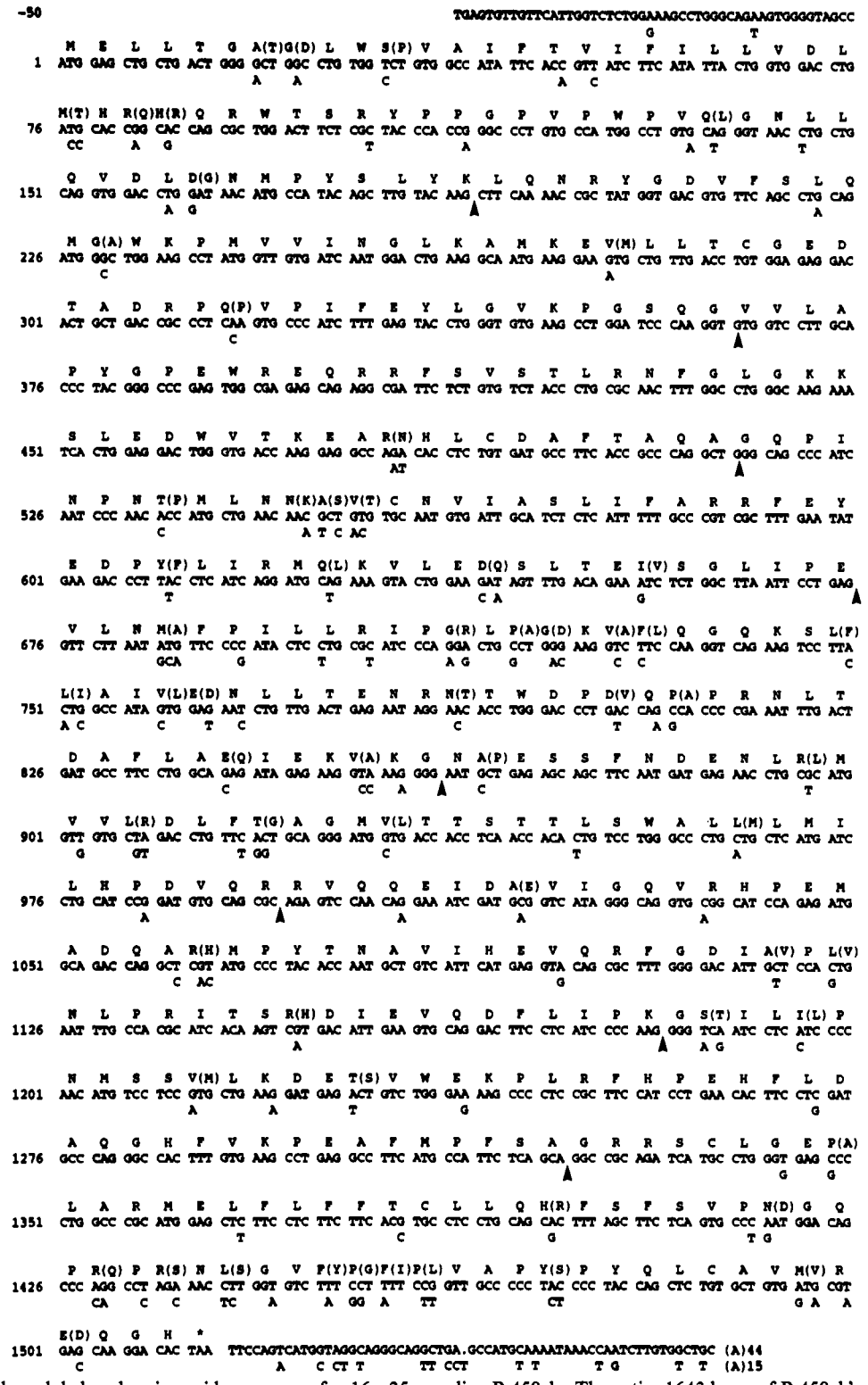

FIGURE 2: Nucleotide and deduced amino acid sequences of pc16$\alpha$-25 encoding P-450cb. The entire 1643 bases of P-450cb's nucleotide sequence and its deduced amino acid sequence are shown here. Only those nucleotides (under the P-450cb's sequence) and amino acid residues (in parentheses) that were different were written for the C-P-450$_{16\alpha}$. The asterisk indicates the termination codon, and the exon–exon junctions are pointed by arrows.

There was 94% nucleotide sequence identity between the pc16$\alpha$-2 and -25 coding regions which were translated to C-P-450$_{16\alpha}$ and P-450cb. Both cytochromes consisted of 504 amino acid residues, in which there were 63 substitutions between the P-450s (87% sequence identity). It was noted that the identity was at least 7% lower in the amino acid sequence than in the nucleotide sequence, indicating a higher number of nonsynonomous substitutions. Exon-by-exon comparisons of nonsynonomous and synonomous substitutions between C-P-450$_{16\alpha}$ and P-450cb are summarized in Table I. Interestingly, the higher rates of nonsynonomous substitutions were concentrated in exons 4, 5, and 9. The highest rate was found in exon 4 in which all of the eight mutated codons altered amino acid residues. Exons 5 and 9 showed only 66% and 68% amino acid sequence identity, respectively, and were the most diverse exons between the two P-450s. The nonsynonomous substitutions in these exons occurred, respectively, 2.5 and 1.9 as many times as the synonomous substitutions. In the other

Table I: Exon-by-Exon Comparisons of Nonsynonomous and Synonomous Substitutions between C-P-$450_{16\alpha}$ and P-450cb[a]

| exon | Ns | Ss | Ns/Ss |
|---|---|---|---|
| 1 | 8 | 7 | 1.1 |
| 2 | 3 | 2 | 1.5 |
| 3 | 1 | 0 | |
| 4 | 8 | 0 | |
| 5 | 15 | 6 | 2.5 |
| 6 | 6 | 4 | 1.5 |
| 7 | 5 | 4 | 1.2 |
| 8 | 4 | 3 | 1.3 |
| 9 | 13 | 7 | 1.9 |

[a] Nonsynonomous substitution (Ns) implied a nucleotide substitution(s) by which the encoded amino acid was changed to a different amino acid, whereas the amino acid was conserved by nonsynonomous substitution(s). These substitutions were determined by the amino acid and nucleotide sequences shown in Figure 2.

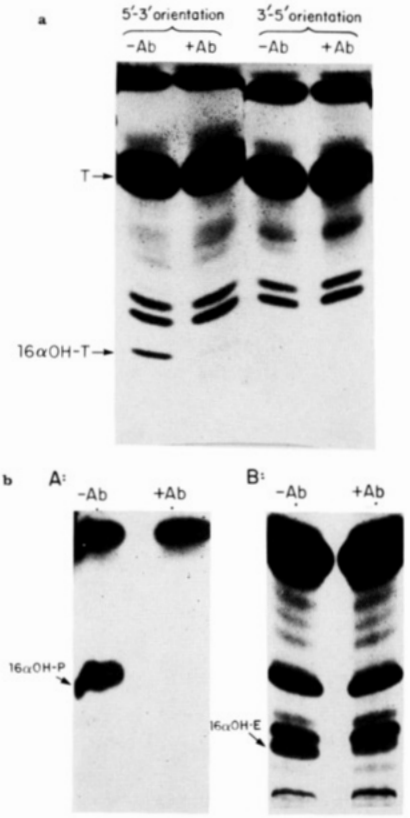


FIGURE 3: Steroid hydroxylation activity of transfected cell homogenates. (a) pcD-16-2-1 (5'–3' orientation) and pcD-16-2-4 (3'–5' orientation) were separately transfected into COS-1 cells according to the procedure described under Experimental Procedures. From each transfection, the cells harvested from 16 plates were homogenized. First, small portions of the homogenates were taken for Western blot; then the rest of cell homogenates were divided into two parts for measuring testosterone 16α-hydroxylase activity in the presence and absence of anti-C-P-$450_{16\alpha}$. The metabolites were extracted and analyzed by thin-layer silica gel chromatography. The silica gels were exposed to X-ray films for 48 h. T indicates testosterone; 16αOH-T, 16α-hydroxytestosterone. (b) The hydroxylation activity of pcD-16-2-1-transfected cells was measured by using progesterone or estradiol as substrate. A picture of thin-layer chromatography is shown here for progesterone or estradiol metabolites, respectively. The solvent conditions for chromatography were described previously (Harada & Negishi, 1988). 16αOH-P and 16αOH-E indicate 16α-hydroxyprogesterone and estriol, respectively.

exons, the rates were between 1.1 to 1.5. With the whole molecules, there was approximately a 2:1 ratio of nonsynonomous to synonomous substitutions, a rate approximately twice as high as the substitutions between mouse P-450s encoded by pf26 and pf3/46 (Noshiro et al., 1988).

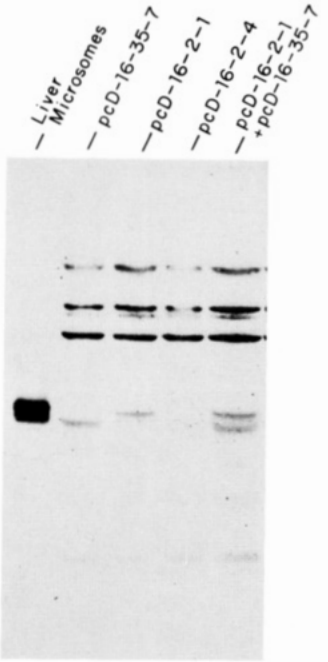


FIGURE 4: Western blot of transfected cell homogenates. One hundred micrograms of the cell homogenates was electrophoresed on an SDS–polyacrylamide gel (9%), transferred to nitrocellulose paper, and then immunostained with anti-C-P-$450_{16\alpha}$. Liver microsomes (2 μg) from 129/J male mice were used as the standard.

*Functional Expression of C-P-$450_{16\alpha}$ and P-450cb in COS-1 Cells.* The recombinant plasmids, pcD-16-2-1 and pcD-16-35-7, were transfected into COS-1 cells in order to express their encoded C-P-$450_{16\alpha}$ and P-450cb. The recombinant plasmid (pcD-16-2-4), containing the pc16α-2 cDNA in a 3' to 5' orientation, was also transfected separately as the control. Homogenates prepared from these transfected cells were used to measure steroid hydroxylation activities and to perform Western blotting analysis of the expressed P-450s.

Figure 3a shows the thin-layer chromatography of testosterone metabolites formed by the homogenates of cells transfected with pcD-16-2-1 or pcD-16-2-4. After the incubation with testosterone, the cell homogenate transfected with pcD-16-2-1 produced 16α-hydroxytestosterone as the only major metabolite. Furthermore, the 16α-hydroxytestosterone formation was completely inhibited by anti-C-P-$450_{16\alpha}$. Expectedly, the cell homogenate transfected with pcD-16-2-4 exhibited no catalytic activity toward testosterone. When progesterone or estradiol was tested as the substrate, the expressed C-P-$450_{16\alpha}$ catalyzed the formation of 16α-hydroxyprogesterone or 16α-hydroxyestradiol (estriol) (Figure 3b). The specific activities were 20, 19, and 8.4 pmol of product formed $h^{-1}$ (mg of protein)$^{-1}$ for testosterone, progesterone, and estradiol 16α-hydroxylation, respectively. The expressed P-450cb, on the other hand, exhibited no detectable hydroxylation activity toward any of these steroids.

The intensity of the P-450 bands on the Western blot indicated that the amounts expressed in COS-1 cells were similar for both cytochromes (Figure 4). Since the antibody was made against the C-P-$450_{16\alpha}$, P-450cb might have been underestimated. The former appeared larger than the latter on an SDS–polyacrylamide gel, although the molecular weight calculated from the deduced amino acid sequence as 56 948, smaller than 57 229 for P-450cb. Mouse liver microsomes contained both bands, C-P-$450_{16\alpha}$ and P-450cb. The purified mouse C-P-$450_{16\alpha}$ (Harada & Negishi, 1984) migrated with the higher molecular weight band on the Western blot (not shown).

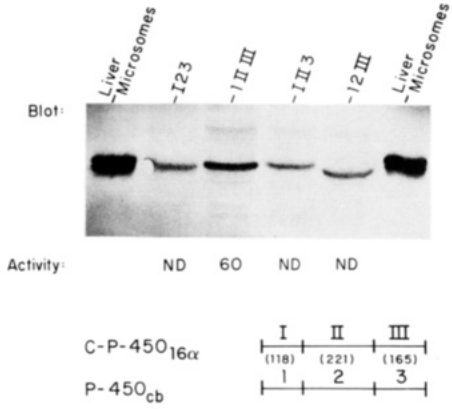


FIGURE 5: Construction and expression of chimeric P-450s. The constructed chimeras between pcD-16-2-1 and pcD-16-35-7 are illustrated. The fragments I(1), II(2), and III(3) were generated by digestion of pcD-16-2-1 (pcD-16-35-7) with *Bam*HI and *Cla*I. These fragments were religated to construct pcD-16-2/35-3, -16-2/35-34, -16-2/35-16, and -16-2/35-21 containing I 2 3, I II 3, and 1 2 III, respectively. The number of amino acid residues in each fragment is also indicated. Each chimeric recombinant was transfected into COS-1 cells. Western blot was performed with the cell homogenates (each 100 $\mu$g), and their testosterone 16$\alpha$-hydroxylase activity was measured as the activity inhibited by anti-C-P-450$_{16\alpha}$. The activity was expressed as picomoles of product formed per hour per milligram of protein. The C-P-450$_{16\alpha}$-dependent testosterone 16$\alpha$-hydroxylase activity in mouse liver microsomes was 0.62 nmol min$^{-1}$ (mg of protein)$^{-1}$. Assay limit was at the level of 0.1 pmol h$^{-1}$ (mg of protein)$^{-1}$. ND, not detectable.

*Expression of Chimeric P-450s.* Digestion with *Bam*HI and *Cla*I separated pc16$\alpha$-2 or pc16$\alpha$-35 into three fragments designated I(1), II(2), and III(3), respectively. I(1) fragment contained the N-terminal 118 amino acids, II(2) 221 amino acids in the middle portion of the molecule, and III(3)C-terminal 165 amino acids. The Roman numerals represented the fragments of C-P-450$_{16\alpha}$ and the Arabic numerals the P-450cb. Between each corresponding fragment, there were 11, 30, and 22 amino acid substitutions, respectively.

The chimeric recombinants were constructed and ligated to pcD vectors by using these *Bam*HI and *Cla*I sites. pcD-16-2/35-3, pcD-16-2/35-34, pcD-16-2/35-16, and pcD-16-2/35-21 consisted of I 2 3, I II 3, 1 II III, and 1, 2 III, respectively. Figure 5 shows a picture of Western blotting and testosterone 16$\alpha$-hydroxylase activities associated with the expressed chimeric P-450s. The only chimera that exhibited testosterone 16$\alpha$-hydroxylase activity was 1 II III. When II or III were changed to their corresponding P-450cb fragment, testosterone 16$\alpha$-hydroxylase activity was abolished, indicating that both II and III fragments were essential for the C-P-450$_{16\alpha}$ to be active. As expected from the evidence obtained above, chimeric P-450s 1 II III and I 2 3 did not catalyze the activity at all, although Western blot indicated these chimeras were expressed in COS-1 cells (data not shown). It should be noted that the activity of C-P-450$_{16\alpha}$ was enhanced consistently by replacing the C-P-450$_{16\alpha}$'s fragment I with the corresponding fragment of P-450cb.

## Discussion

We studied the steroid hydroxylase properties of two similar P-450s, C-P-450$_{16\alpha}$ and P-450cb, from within the male-specific steroid 16$\alpha$-hydroxylase gene family. To that aim, we employed the COS-1 system (Okayama & Berg, 1982) previously used by Zuber et al. (1986) to express adrenal P-450s and by MacKenzie (1986) for the UDP–glucuronyltransferase.

Recently, we reported that purified C-P-450$_{16\alpha}$ catalyzes the 16$\alpha$-hydroxylation of testosterone, progesterone, and estradiol as well as numerous other steroids at various rates (Harada & Negishi, 1988). These experiments, however, could not rule out the possibility that multiple substrate specificity was due to contamination by other P-450s in the purified C-P-450$_{16\alpha}$. To eliminate this possibility, in the present experiments we used the C-P-450$_{16\alpha}$ expressed in COS-1 cells as the enzyme source to measure the hydroxylations of testosterone, progesterone, and estradiol. The results proved undoubtedly that a single P-450, C-P-450$_{16\alpha}$ catalyzes the hydroxylation of multiple steroid substrates only at their 16$\alpha$-positions. A low stringency of steroid substrate specificity, combined with extremely high regio- and stereoselectivity at the 16$\alpha$-position, was an enzymatic characteristic of the C-P-450$_{16\alpha}$. Regardless of their structures, many steroids were hydroxylated by the cytochrome specifically at the 16$\alpha$-position at various rates. This observation indicated that the 16$\alpha$-hydroxylation was a common step in steroid hormone degradation in adult male mice, in which the C-P-450$_{16\alpha}$ played a major role.

On the contrary, the P-450cb did not catalyze any hydroxylation of these three measured steroids. This inability was not due to the presence of the apo-cytochrome, since the P-450cb as well as C-P-450$_{16\alpha}$ in COS-1 cells exhibited specific benzphetamine N-demethylation activity at 14 and 20 pmol h$^{-1}$ (mg of protein),$^{-1}$ respectively. The evidence indicated the profound differences in the enzymatic activity of the two P-450s within the same gene family.

It was surprising to find a 7% lower identity in amino acid than in nucleotide sequences between C-P-450$_{16\alpha}$ and P-450cb, indicating a high nonsynonomous substitution rate. The substitutions are distributed heterogeneously in the molecule; exons 4, 5, and 9 show particularly high frequencies of this type of substitution. Consequently, exons 5 and 9 appear to be the most diverse regions in the entire P-450 molecule. Hughes and Nei (1988) found that high numbers of nonsynonomous substitutions are concentrated in the highly polymorphic antigen recognition site of the major histocompatibility complex. They proposed, therefore, that an overdominant selection mechanism might explain the gene divergency. In this regard, the similar selection mechanism worked in these P-450 exons during their evolution to create the diverse new cytochrome which possesses a different catalytic activity. Although there was no direct evidence to indicate that any enzymatic functions resided in these exons, our present study with the chimeric cytochrome between the P-450s suggests this possibility.

When we replaced the middle portion of 221 amino acids (which included exons 4 and 5) with its corresponding P-450cb portion, C-P-450$_{16\alpha}$ steroid hydroxylase activity is abolished. A similar response was observed when we exchanged the C-terminal portion of 165 amino acids (which includes exon 9). Two-thirds (middle to C-terminus) of the C-P-450$_{16\alpha}$ molecule is necessary to produce this activity. This finding, however, does not imply necessarily that there was an absence of critical amino acid residues for catalytic or substrate binding sites in the C-P-450$_{16\alpha}$ molecule, as it was shown that the two amino acid substitutions abolishes the P-450's aryl hydrocarbon hydroxylase activity (Kimura et al., 1987). The necessity of two-thirds of the P-450$_{16\alpha}$ contrasts with an earlier report by Sakaki et al. (1987) in which the chimeric P-450s between rat P-450c and P-450d were expressed in yeast cells. They proved that only the middle portions of the molecules are important for the substrate specificities of the enzymes.

The amount of this chimeric P-450, 1 II III, was always higher than others on Western blots, which might partially

explain the enhancement of the C-P-$450_{16\alpha}$'s steroid 16$\alpha$-hydroxylase activity by the replacement of its N-terminal peptide with the P-450cb. If this is the case, the N-terminal portions of the P-450s might be involved in the efficiency of membrane insertion and/or the stability of newly synthesized molecules.

In conclusion, we expressed C-P-$450_{16\alpha}$ and P-450cb in COS-1 cells and determined their steroid substrate specificity and regio- and stereoselectivity. These two P-450s are both members of the steroid 16$\alpha$-hydroxylase gene family, yet only C-P-$450_{16\alpha}$ catalyzes steroid 16$\alpha$-hydroxylations. The enzyme characteristically has a low stringency of steroid substrate specificity, combined with extremely high regio- and stereoselectivity. The C-P-$450_{16\alpha}$ and P-450cb will be excellent models for studying the regulatory and functional differences in the large P-450 gene family.

## Acknowledgments

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**Registry No.** Cytochrome P-450, 9035-51-2; DNA (mouse cytochrome P-450 as cb mRNA complementary), 120523-68-4; cytochrome P-450cb (mouse protein moiety reduced), 120523-64-0; steroid 16$\alpha$-hydroxylase, 37364-16-2; testosterone, 58-22-0; progesterone, 57-83-0; estradiol, 50-28-2.

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