

## Deletion of a Phenylalanine in the N-terminal Region of Human Cytochrome P-450<sub>17 $\alpha$</sub> Results in Partial Combined 17 $\alpha$ -Hydroxylase/17,20-Lyase Deficiency\*

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Steroid 17 $\alpha$ -hydroxylase and 17,20-lyase activities reside within the same polypeptide chain (cytochrome P-450<sub>17 $\alpha$</sub> ), and consequently human 17 $\alpha$ -hydroxylase deficiencies are characterized by defects in either or both of these activities. Human mutants having these deficiencies represent an excellent source of material for investigation of P-450<sub>17 $\alpha$</sub>  structure-function relationships. The CYP17 gene from an individual having partial combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency has been characterized structurally and the homozygous mutation found to be the deletion of the phenylalanine codon (TTC) at either amino acid position 53 or 54 in exon 1. Reconstruction of this mutation into a human P-450<sub>17 $\alpha$</sub>  cDNA followed by expression in COS 1 cells led to production of the same amount of immunodetectable P-450<sub>17 $\alpha$</sub>  protein as found with expression of the normal human P-450<sub>17 $\alpha$</sub>  cDNA. However, 17 $\alpha$ -hydroxylase activity of this mutant protein measured in intact cells was less than 37% of that observed upon expression of the wild-type enzyme, whereas 17,20-lyase activity of the mutant was less than 8% of that observed with the normal enzyme. When estimated in intact cells, the  $K_m$  for 17 $\alpha$ -hydroxylation of progesterone was increased by a factor of 2 in the mutant enzyme, whereas the  $V_{max}$  was reduced by a factor of 3. In order to estimate the kinetic parameters for the 17,20-lyase reaction, microsomes were isolated from transfected COS 1 cells to enrich for this activity. Surprisingly, the specific activity of the mutant 17 $\alpha$ -hydroxylase in microsomes was 3-fold less than that observed in intact cells, indicating that the structure of mutant P-450<sub>17 $\alpha$</sub>  was dramatically altered upon disruption of COS 1 cells. Apparently the deletion of a single phenylalanine in the N-terminal region of P-450<sub>17 $\alpha$</sub>  alters its folding in such a way that both enzymatic activities are dramatically decreased, leading to the partial combined deficiency observed in this individual.

The initial step common to the steroidogenic pathways in the adrenal cortex and gonads is the side chain cleavage of cholesterol catalyzed by cytochrome (P-450<sub>sc</sub>)<sup>1</sup> to form pregnenolone, which can be dehydrogenated at the 3 $\beta$ -position to form progesterone. Subsequent 17 $\alpha$ -hydroxylation of pregnenolone or progesterone is a branch point for the formation of cortisol and sex hormones in the steroidogenic pathways. The side chain of the C21 steroids 17 $\alpha$ -hydroxypregnenolone and 17-hydroxyprogesterone can be further cleaved during the 17,20-lyase reaction to yield the C19 steroid precursors of testosterone and estrogens, dehydroepiandrosterone (DHEA) and androstenedione, respectively. Evidence obtained using both purified protein samples and *in situ* COS 1 cell expression systems has proven that a single polypeptide, cytochrome P-450<sub>17 $\alpha$</sub> , can catalyze both the 17 $\alpha$ -hydroxylase and 17,20-lyase reactions (Nakajin *et al.*, 1984; Zuber *et al.*, 1986a). Accordingly, in the adrenal cortex, a fraction of the 17 $\alpha$ -hydroxysteroids is converted to C19 steroids by the 17,20-lyase reaction, the remainder of these 17 $\alpha$ -hydroxysteroids being precursors for cortisol production. In the gonads, all of the 17 $\alpha$ -hydroxysteroids are converted to C19 steroids.

Human 17 $\alpha$ -hydroxylase deficiency is an autosomal recessive disorder and is one of the causes of congenital adrenal hyperplasia (Biglieri *et al.*, 1966). In this disease, impaired production of cortisol provokes elevation of ACTH and consequently overproduction of mineralocorticoids, principally deoxycorticosterone, resulting in hypertension and hypokalemia. In addition, impaired production of sex hormones causes abnormalities of sexual development, leading to failure of virilization in the male and sexual infantilism in the female in the most severe cases. We have reported previously the molecular basis of two examples of complete combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency (Kagimoto *et al.*, 1988, 1989; Yanase *et al.*, 1988). Although many such patients show complete blocks of both 17 $\alpha$ -hydroxylase and 17,20-lyase activities, some individuals are found to have partial deficiencies of both activities leading to ambiguous external genitalia (New *et al.*, 1970; Jones *et al.*, 1982). In addition, a few patients have been reported to show deficiency of only a single activity, either 17 $\alpha$ -hydroxylase (Vargas *et al.*, 1981) or 17,20-lyase activity (Zachman *et al.*, 1982). Presumably, the elucidation of the molecular basis of these variants leading to partial

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<sup>1</sup> The abbreviations used are: P-450<sub>sc</sub>, cholesterol side chain cleavage cytochrome P-450; P-450<sub>17 $\alpha$</sub> , 17 $\alpha$ -hydroxylase cytochrome P-450; P-450<sub>11 $\beta$</sub> , 11 $\beta$ -hydroxylase cytochrome P-450; P-450<sub>C21</sub>, steroid C21-hydroxylase cytochrome P-450; P-450<sub>cam</sub>, camphor hydroxylase cytochrome P-450; DHEA, dehydroepiandrosterone; ACTH, adrenocorticotropin; bp, base pair(s); kb, kilobase pair(s); SDS, sodium dodecyl sulfate; Pipes, 1,4-piperazinediethanesulfonic acid.

deficiencies holds the promise of contributing substantially to the general understanding of the structure-function relationships in P-450<sub>17α</sub>. In particular, reconstruction of mutations into the human P-450<sub>17α</sub> cDNA, expression of such mutant enzymes, and analysis of the resultant enzymatic properties should be particularly informative.

We have now investigated the molecular basis of an example of partial combined deficiency in a female Japanese patient. As a result of a phenylalanine deletion at either amino acid 53 or 54 in the N-terminal region of P-450<sub>17α</sub>, both enzymatic activities are dramatically reduced but not absent in this patient. Upon isolation of microsomes from COS 1 cells transfected with the mutant cDNA, both activities are even more dramatically reduced compared with those of the normal enzyme when compared with results of *in situ* assays carried out in intact transfected COS 1 cells. We surmise that the deletion leads to altered folding of the protein and consequently producing an enzyme having reduced 17α-hydroxylase and 17,20-lyase activities.

#### MATERIALS AND METHODS

**Case Record**—The CYP17 (Nebert *et al.*, 1989) gene examined in this study was that of a Japanese female having a 46 XX karyotype, the subject of a case report by Suzuki *et al.* (1974). In summary, the patient (JG) was 20 years old when reported and currently is 35 years old, married, and has no children. Her parents and siblings had no apparent clinical abnormalities. When she visited a hospital because of an occipital headache, she was found to suffer from hypertension. Her blood pressure was 170/110 mmHg, and she was also hypokalemic. Importantly, she had menstruation, although irregularly, and physical examination revealed hypoplastic breasts with no pubic or axillary hair. *In vivo* studies showed decreased levels of 17α-hydroxylated corticoids such as cortisol, 11-deoxycortisol, and their metabolites. Adrenal androgens and estrogens were also decreased but detectable. On the other hand, 17-deoxycorticoids such as corticosterone were increased. These data plus the presence of irregular menstruation, suggesting some level of estrogen production, indicate that this patient suffers from partial combined 17α-hydroxylase/17,20-lyase deficiency.

**DNA Extraction and CYP17 Sequence Analysis**—Heparinized blood was obtained from the patient, and genomic DNA was prepared from the leukocytes as described by Blin and Stafford (1976). Southern analysis of the CYP17 gene was performed following *Eco*RI digestion of the DNA using a nick-translated *Bam*HI insert of a human P-450<sub>17α</sub> cDNA clone (Bradshaw *et al.*, 1987). Exonic sequence analysis of the CYP17 gene was carried out following the protocol described previously by this laboratory (Kagimoto *et al.*, 1988; Yanase *et al.*, 1988). Briefly, a genomic DNA library was prepared for this patient by *Eco*RI digestion, ligation with *λ*gtWES *Eco*RI arms (Bethesda Research Laboratories), *in vitro* packaging (Packagene, Promega-Biotec), and plaque formation on agar plates. Plaque hybridization was used to screen approximately  $8 \times 10^4$  plaques with the nick-translated *Bam*HI fragment of human P-450<sub>17α</sub> cDNA. Six positive clones were identified by this procedure, three of which contained the 5.7-kb *Eco*RI fragment associated with the CYP17 gene and three of which contained the 6.9-kb *Eco*RI fragment of this gene. Together, these fragments contain all eight exons of the CYP17 gene (Kagimoto *et al.*, 1988). Both the 5.7- and 6.9-kb *Eco*RI fragments were further subcloned into pUC19 (Yanase *et al.*, 1988) and sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using universal, reverse, or appropriate custom oligonucleotide primers.

**Determination of the Patient's Genotype**—To determine the genotype of the patient, we utilized the ribonuclease A (RNase A) cleavage method originally developed for detection of single base substitutions by Meyers *et al.* (1985). A 1.9-kb *Kpn*I-*Sac*I genomic fragment containing the mutation was subcloned into Bluescript (Stratagene). After digestion with *Pvu*II, a <sup>32</sup>P-labeled RNA probe (247 bp) complementary to the 234-bp *Sac*I-*Pvu*II mutant DNA template plus 13 bp of Bluescript was prepared by transcription using T3 RNA polymerase in the presence of [ $\alpha$ -<sup>32</sup>P]UTP. The reaction mixture (20  $\mu$ l) contained 40 mM Tris-HCl, pH 7.5; 6 mM MgCl<sub>2</sub>; 10 mM NaCl; 2 mM spermidine (Sigma); 10 mM dithiothreitol (Sigma); 0.5 mM each ATP, GTP, and CTP; 12  $\mu$ M UTP; 50  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]UTP (Amersham

Corp.); 1  $\mu$ g of linearized template DNA; 20 units of RNasin (Promega); and 50 units of T3 RNA polymerase (Bethesda Research Laboratories). Incubation was carried out at 37 °C for 60 min. The sample was then diluted with 100  $\mu$ l of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% SDS, and used as a probe. A similar RNA probe (250 bp) complementary to the normal DNA fragment was also prepared by the same procedure. A mixture of *Eco*RI-digested genomic DNA (5  $\mu$ g) and either the mutant or wild-type cRNA probe ( $5 \times 10^5$  cpm) was heated at 90 °C for 10 min and annealed in 30  $\mu$ l of a hybridization buffer (80% formamide, 40 mM Pipes, pH 6.4, 0.4 M NaCl, and 1 mM EDTA) at 45 °C for 12 h, after which 350  $\mu$ l of a solution containing RNase A (40  $\mu$ g/ml) in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 200 mM NaCl, and 100 mM LiCl was added and incubated at 25 °C for 30 min. The mutant or normal cRNA probe ( $1 \times 10^5$  cpm) was also hybridized with 50 ng of mutant or normal cloned DNA (5.7-kb *Eco*RI fragment containing CYP17 exon 1) at 45 °C for 60 min, and these hybrids were also subjected to RNase A treatment. The reaction was stopped by the addition of 10  $\mu$ l of 20% SDS and 10  $\mu$ l of proteinase K (10 mg/ml) (EM Biochemicals) followed by incubation at 37 °C for 15 min. After phenol extraction and ethanol precipitation, the samples were subjected to electrophoresis on a denaturing polyacrylamide gel (5%) (Maniatis *et al.*, 1982), and the results were visualized by autoradiography.

**Construction of Mutant P-450<sub>17α</sub> cDNA**—The overall procedure for constructing the TTC (phenylalanine) deletion in our full length human P-450<sub>17α</sub> cDNA is summarized in Fig. 1. Basically, the N-terminal region of a genomic clone containing this mutation was joined with a fragment of the cDNA containing the remainder of the human P-450<sub>17α</sub> sequence. A 1.6-kb *Pst*I-*Pst*I fragment of the 5.7-kb genomic subclone (see Yanase *et al.*, 1988) containing the TTC deletion at amino acid 53 or 54 in exon 1 was inserted into pUC19 (pUCJG17α-1). pUCJG17α-1 was then cut with *Nae*I (5'-flanking region) and *Sma*I (pUC19 polylinker) and blunt end ligated into pUC19 (pUCJG17α-2). A 188-bp *Kpn*I-*Pst*I fragment of pUCJG17α-2 was further cloned into Bluescript (pBSJG17α-1). The full length human P-450<sub>17α</sub> cDNA in Bluescript (pBSH17α-1) was prepared from a *Bam*HI-*Bam*HI fragment of pCD17α-H (Bradshaw *et al.*, 1987) by using *Bal*31 digestion followed by blunt end ligation into the *Sma*I site. A 143-bp *Pst*I-*Xba*I cDNA fragment was cloned from pBSH17α-1 into pBSJG17α-1, giving pBSJG17α-2. A 1410-bp *Xba*I-*Hind*III cDNA fragment of pBSH17α-1 was cloned into pUC19, giving pUCH17α-1. The mutant cDNA construct was completed by ligation of a 325-bp *Xba*I-*Kpn*I fragment of pBSJG17α-2 into pUCH17α-1, producing pUCJG17α-3. The cDNA construct was sequenced by the dideoxy chain termination method to validate its structure. Finally, the mutant cDNA was inserted in an expression vector, pCMV, developed by David W. Russell at this institution (Andersson *et al.*, 1989) using the *Kpn*I and *Hind*III sites, the construct being named pCMVJG17α-H. Wild-type human P-450<sub>17α</sub> cDNA in pCMV was constructed from pBSH17α-1, a *Bam*HI-*Kpn*I fragment containing the full length 17α-cDNA being cloned from pBSH17α-1 into pCMV using the *Bgl*II and *Kpn*I sites (pCMV17α-H).

**Transfection of COS 1 Cells and Enzymatic Assays**—Transfection of COS 1 cells with plasmids purified by CsCl density gradient centrifugation was carried out as described previously (Zuber *et al.*, 1986a). 17α-Hydroxylase and 17,20-lyase activities were assayed by conversion of pregnenolone/progesterone to 17α-hydroxypregnenolone/17α-hydroxyprogesterone and of 17α-hydroxypregnenolone to DHEA, respectively. The medium (4 ml) contained <sup>3</sup>H-labeled substrate (10<sup>5</sup> cpm/ml) plus 1.0 or 2.5  $\mu$ M substrate. At times after addition of substrate, aliquots of culture media were removed (6 and 12 h, 0.5 ml; 24 h, 1.0 ml), steroids extracted and subjected to analysis by thin layer chromatography (TLC). For estimation of *K<sub>m</sub>* and *V<sub>max</sub>* in intact cells, 3 ml of medium was used rather than 4 ml. The steroids from 0.5 ml of media were extracted with 5 ml of methylene chloride by Vortex mixing and centrifuging for 10 min at 1000 rpm. The aqueous layer was removed, and the organic phase was evaporated to dryness, dissolved in 0.1 ml of methylene chloride, and chromatographed by TLC. Pregnenolone, 17α-hydroxypregnenolone, and DHEA were separated in chloroform:ethyl acetate (95:5) and detected with a sulfuric acid spray. Progesterone, 17α-hydroxyprogesterone, and 16α-hydroxyprogesterone were isolated in chloroform:ethyl acetate (80:20) and detected using short wave UV light. The radioactivity in each TLC lane was determined following separation of the lane into 11 equal portions, which were placed into scintillation vials and counted after the addition of 10 ml of OCS (Amersham) containing 2% methanol.

**Immunoblots of Cellular Proteins**—COS 1 cells were rinsed once



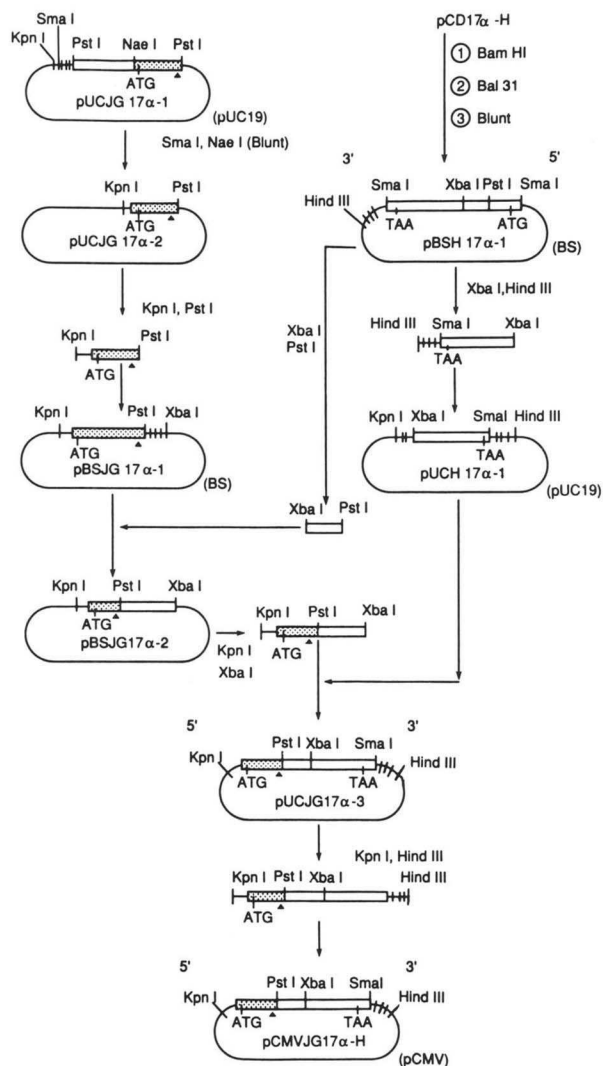


FIG. 1. Schematic description of the construction of a eukaryotic expression vector containing the mutant P-450<sub>17α</sub> cDNA (pCMVJG17α-H). pCD17α-H contains the full length P450<sub>17α</sub> cDNA prepared in this laboratory (Bradshaw *et al.*, 1987). BS, Bluescript; ATG and TAA, the initiator and terminator codons, respectively, of the human P-450<sub>17α</sub>; ▲, the location of the phenylalanine deletion. The pCMV expression vector contains the major immediate early promoter of cytomegalovirus (Thomsen *et al.*, 1984).

with phosphate-buffered saline and were collected by scraping with a rubber policeman using a lysis solution containing 0.1% SDS and 1% cholate. Solubilization of total cell protein was accomplished by repeated cycles of freezing and thawing. Following protein assay (BAC kit, Pierce Chemical Co.), 200 μg of cellular protein was placed in a loading buffer (0.05 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 2 mM EDTA, and 0.05% bromophenol blue) and boiled for 2 min. The samples were loaded on 10% SDS-polyacrylamide gels and electrophoresed in a running buffer (50 mM Tris, 385 mM glycine, 0.5 M EDTA, and 10% SDS). Molecular weight markers (Bethesda Research Laboratories) were run on each gel. The proteins were then transferred electrophoretically to nitrocellulose paper at 35 V for 12 h using 20% methanol, 20 mM Tris-base, and 150 mM glycine and immunoblotted essentially as described by Towbin *et al.* (1979). For blocking, the paper was first incubated for 2 h at room temperature with 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.2% Nonidet P-40, and 5% nonfat milk powder, after which an antibody against porcine testis P-450<sub>17α</sub> (kindly provided by Dr. J. I. Mason of this institution) was added for a 2-h incubation at room temperature. The paper was washed six times with washing buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.2% Nonidet P-40, 0.25% sodium deoxycholate, and 0.1% SDS) and then incubated with <sup>125</sup>I-labeled protein A (Du Pont-New England Nuclear) for 30 min. The paper was washed six times with the same washing buffer, and the specific bands corre-

sponding to P-450<sub>17α</sub> were visualized by autoradiography.

**Preparation of Microsomes from COS 1 Cells**—COS 1 cells were washed once with phosphate-buffered saline, collected by scraping with a rubber policeman, suspended in 24 volumes (w/v) of ice-cold medium containing 0.5 M sucrose, 1 mM EDTA, 0.04 units/ml aprotinin (Sigma), and 0.1 μg/ml leupeptin (Boehringer Mannheim), and homogenized by a motor-driven homogenizer. The homogenization was by 10 strokes at 2,500 rpm and 5 strokes at 5,000 rpm. Following homogenization, the sample was centrifuged at 760 × *g* for 15 min to remove cell debris and nuclei. The supernatant was then centrifuged at 11,000 × *g* for 15 min to remove mitochondria. To increase the recovery, this pellet was resuspended and resedimented (11,000 × *g* for 15 min). The pooled 11,000 × *g* supernatants were then sedimented at 206,000 × *g* for 60 min using a TL-100 Ultracentrifuge (Beckman). The recovery of the microsomes was approximately 300 μg of protein/culture dish (100 mm). The microsomal pellet was finally suspended in 0.05 M Tris-HCl buffer (pH 7.5) and frozen at -70 °C. The microsomal 17α-hydroxylase activity was assayed by measuring conversion of progesterone to 17α-hydroxyprogesterone in incubations carried out at 37 °C in 500 μl of 0.05 M Tris-HCl buffer (pH 7.5) containing 1 mM NADPH (Sigma), 10 mM glucose 6-phosphate (Sigma), 2.4 units/ml glucose-6-phosphate dehydrogenase (Sigma), 3 mM MgCl<sub>2</sub>, <sup>3</sup>H-labeled (10<sup>5</sup> cpm/ml) and nonlabeled progesterone, and 400 μg/ml COS 1 cell microsomes. The reaction was started by the addition of microsomes and stopped by putting samples on ice and the subsequent addition of 5 ml of methylene chloride. Steroids were then analyzed by TLC as described.

## RESULTS

Southern blot analysis of genomic DNA from the patient following *Eco*RI digestion showed 5.7- and 6.9-kb *Eco*RI fragments that are identical to those of the normal human CYP17 gene (data not shown). This indicates that no large deletions or structural alterations exist within the P-450<sub>17α</sub> gene of this patient. Sequence analysis of the patient's P-450<sub>17α</sub> gene revealed a deletion of a phenylalanine codon (TTC) at either amino acid position 53 or 54 in exon 1 (Fig. 2). In the normal gene, tandem TTC triplets are found at this position, and it is unknown which one has been deleted in the patient's gene. The sequence of the region containing the deletion was confirmed in both the 5' → 3' and the 3' → 5' directions using the universal primer and a custom synthesized oligonucleotide primer. As a result of this deletion, the mutant P-450<sub>17α</sub> (P-450<sub>17α</sub><sup>ΔPhe53 or 54</sup>) is 1 amino acid shorter (507) than the normal P-450<sub>17α</sub> (508). The mutation was found in all three 5.7-kb fragments analyzed. However, to be certain that this

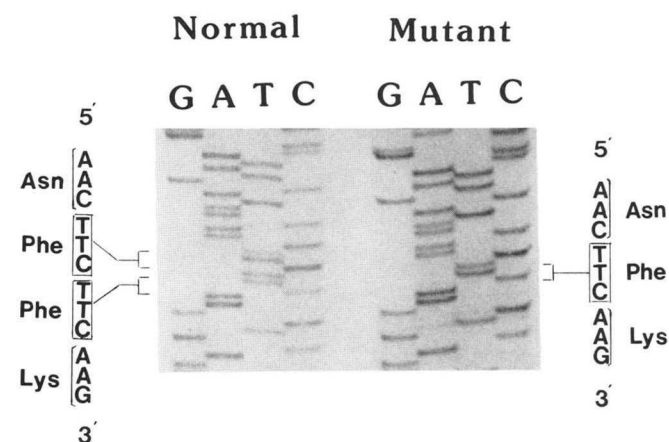


FIG. 2. Nucleotide sequences of the region of exon 1 of the CYP17 genes showing the deletion of a phenylalanine codon (TTC) at the position of amino acid 53 or 54. In the normal CYP17 gene, codons 53 and 54 are both TTC (phenylalanine). The CYP17 gene of this patient also contained two silent mutations in exon 1, as reported previously (Kagimoto *et al.*, 1988). Histidine 46 is encoded by CAC rather than CAT, and serine 65 is encoded by TCG in place of TCT.



FIG. 3. RNA·DNA heteroduplex analysis by RNase A cleavage demonstrating the homozygous presence of the TTC deletion in the patient's genomic DNA. Probe (JG) and Probe (N) indicate mutant cRNA (247 bases) and normal cRNA (250 bases), respectively. Clone (JG) and clone (N) indicate the 5.7-kb *Eco*RI fragment containing CYP17 exon 1 from the mutant gene and the normal gene, respectively. Genomic (JG) indicates the total genomic DNA from the patient following complete digestion with *Eco*RI. The faint band below the 81-nucleotide band, best visualized in the probe (N) + clone (JG) lane, probably results from cleavage of codon 53, whereas the 81-nucleotide fragment results from cleavage at codon 54. Both the JG and N DNAs contained the silent mutations noted in the legend to Fig. 2. nt, nucleotides.

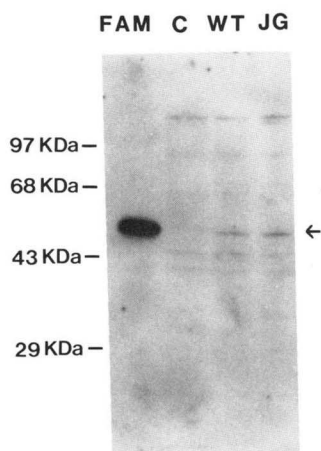
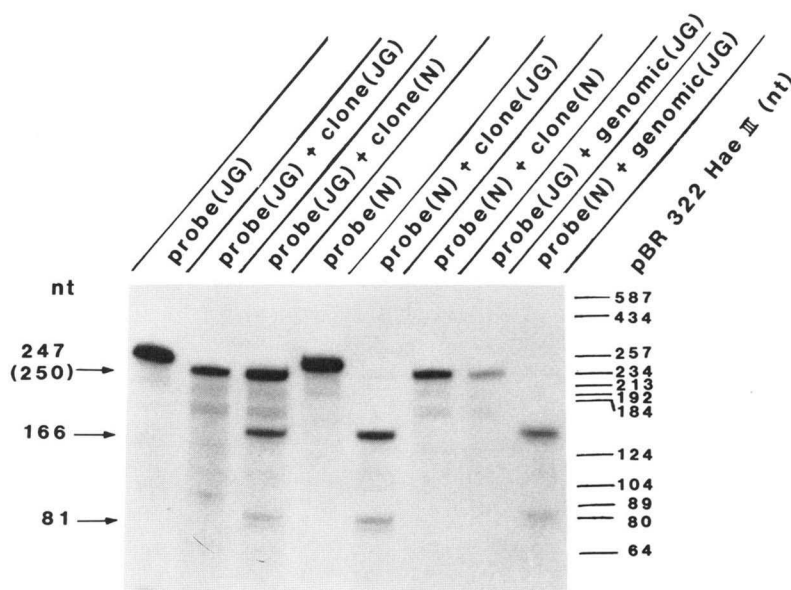


FIG. 4. Immunoblot analysis of P-450<sub>17α</sub> following expression in COS 1 cells. C, WT, and JG indicate COS 1 cells transfected with no plasmid, pCMV17α-H (wild type), and pCMVJG17α-H (mutant), respectively. Each of these lanes contained 200 μg of protein. Fifteen μg of human fetal adrenal microsome (FAM) was also run as a positive control. Molecular masses of standard proteins electrophoresed on the same gel are marked at the left of the panel. The arrow indicates the position of P-450<sub>17α</sub>.

mutation was present on both alleles (homozygous), we used a RNase A cleavage analysis based on the principle that a mismatch in a RNA·DNA heteroduplex is cleaved by RNase A. When a mutant cRNA probe complementary to the 234-bp *Sac*I-*Pvu*II DNA from exon 1 of the mutant CYP17 gene was hybridized to genomic DNA from the patient or the 5.7-kb CYP17 fragment from this individual cloned into pUC, the radiolabeled cRNA was completely protected from RNase A cleavage (Fig. 3). However, when this probe was hybridized to the normal cloned 5.7-kb CYP17 fragment, cleavage led to two fragments, 166 and 81 nucleotides, indicating cleavage at the site of the TTC deletion. When the experiment was reversed, and a normal cRNA probe was used with either genomic DNA from the patient or her cloned 5.7-kb CYP17 fragment, complete cleavage leading to 166- and 81-nucleotide fragments was observed (Fig. 3). This result clearly indicates that both alleles of the CYP17 gene in this patient contain the phenylalanine deletion.

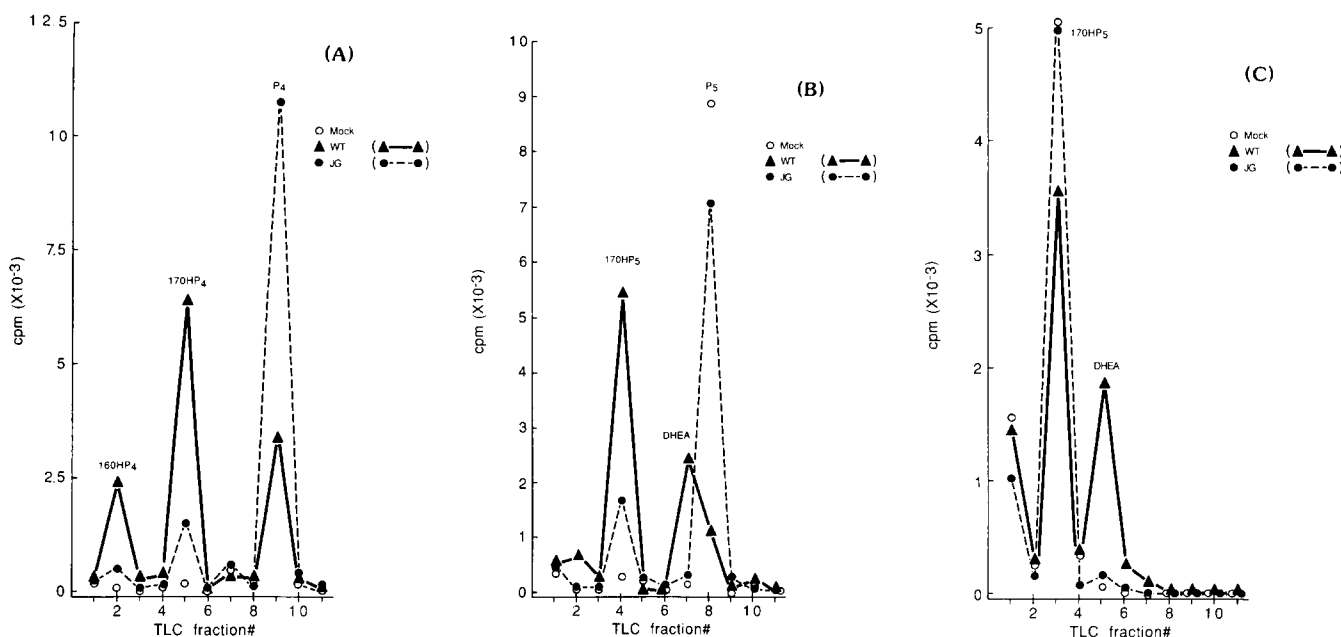
In order to evaluate the function of the P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup>, a

mutant cDNA was constructed in a eukaryotic expression vector (Fig. 1) and transfected into nonsteroidogenic cells (COS 1 cells). Transfection with the mutant P-450<sub>17α</sub> cDNA construct led to the same amount of immunodetectable P-450<sub>17α</sub> protein as found upon expression of the wild-type P-450<sub>17α</sub> cDNA (Fig. 4). In the presence of the same amount of protein, the activities of P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> in COS 1 cells were shown to be dramatically reduced (Fig. 5 and Table I). In addition to the catalysis of both the 17α-hydroxylase and 17,20-lyase reactions (Bradshaw *et al.*, 1987), normal human P-450<sub>17α</sub> cDNA is also known to catalyze 16α-hydroxylation of progesterone.<sup>2</sup> 17α-Hydroxylase and 16α-hydroxylase activities of P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> were detectable by 6 h and continued to increase to at least 24 h (Table I). However, 17,20-lyase activity of P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> was low, being detectable only at longer times of incubation. At 24 h, the 17α-hydroxylase and 16α-hydroxylase activities of the P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> were 37% or less of those of wild-type levels, whereas 17,20-lyase activity of the mutant was found to be less than 6% of that of wild type (Table II). However, because the 17,20-lyase activity is so low, it was not possible to say with certainty from these *in situ* measurements that it is altered in the P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> disproportionately to the 17α-hydroxylase activity, even though every experiment carried out showed this trend. The data in Tables I and II also indicate that the substrate preference (pregnenolone *versus* progesterone) does not appear to be significantly affected by the phenylalanine deletion.

The  $K_m$  and  $V_{max}$  for the 17α-hydroxylation of progesterone were estimated in transfected COS 1 cells using substrate concentrations of 0.5, 1.0, 2.5, 4.0, and 5.0 μM. Assays of P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> were all carried out at 6 h of incubation, whereas those of the wild-type P-450<sub>17α</sub> were all carried out at 2 h of incubation. In two separate experiments utilizing different transfections, the wild-type P-450<sub>17α</sub>  $K_m$  was found to be 1.67 and 1.47 μM, whereas the  $K_m$  of P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> was found to be 3.35 and 3.09 μM. The  $V_{max}$  for the wild-type P-450<sub>17α</sub> was estimated to be 0.86 and 0.53 nmol/h/dish, whereas the  $V_{max}$  of P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> was estimated to be 0.21 and 0.16 nmol/h/dish. Thus, both the  $K_m$  and  $V_{max}$  appear to be altered in P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup>.

In order to establish with certainty whether the 17,20-lyase

<sup>2</sup> J. I. Mason, unpublished observations.



**FIG. 5. Analysis of normal P-450<sub>17α</sub> and P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> activities by thin layer chromatography.** After chromatography, TLC plates were fractionated into 11 equal sized pieces, and the radioactivity in each fraction was counted. The x axes and y axes indicate the fraction number and the radioactivity (cpm), respectively. **A**, conversion of progesterone ( $P_4$ ) to 17 $\alpha$ -hydroxyprogesterone (17OHP<sub>4</sub>) and 16 $\alpha$ -hydroxyprogesterone (16OHP<sub>4</sub>). **B**, conversion of pregnenolone ( $P_5$ ) to 17 $\alpha$ -hydroxypregnenolone (17OHP<sub>5</sub>) and DHEA. **C**, conversion of 17 $\alpha$ -hydroxypregnenolone to DHEA. *Mock*, COS 1 cells put through the transfection protocol without the addition of DNA; *WT*, COS 1 cells transfected with the wild-type (normal) P-450<sub>17α</sub> cDNA; *JG*, COS 1 cells transfected with P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> cDNA.

TABLE I

Rates of formation of steroid products in COS 1 cells

The formation is expressed as percent of total radioactivity. Two substrate concentrations, 1.0 and 2.5  $\mu$ M, were tested. *Mock*, *WT*, and *JG* represent no plasmid, pCMV17 $\alpha$ -H (wild type), and pCMVJG17 $\alpha$ -H (mutant), respectively.  $P_4$ , progesterone; 17OHP<sub>4</sub>, 17 $\alpha$ -hydroxyprogesterone; 16OHP<sub>4</sub>, 16 $\alpha$ -hydroxyprogesterone;  $P_5$ , pregnenolone; 17OHP<sub>5</sub>, 17 $\alpha$ -hydroxypregnenolone. The substrate concentration used in the mock experiment was 1  $\mu$ M.

	$P_4$	$\rightarrow$	17OHP <sub>4</sub>	(or) 16OHP <sub>4</sub>	$P_5$	$\rightarrow$	17OHP <sub>5</sub>	$\rightarrow$	DHEA	17OHP <sub>5</sub>	$\rightarrow$	DHEA
	%				%							
Mock												
6 h	92.5		0.29	0.17	87.0		0.50		1.6	66.4		2.4
12 h	93.1		0.35	0.19	87.0		0.20		2.1	62.7		2.2
24 h	92.6		1.42	1.0	88.3		2.9		1.8	64.4		1.4
WT												
6 h	76.0/76.9		13.9/11.2	1.7/1.1	59.8/74.0		23.2/15.2		4.0/1.5	69.9/71.7		6.6/4.3
12 h	45.2/61.7		34.7/20.1	8.0/5.5	48.2/47.7		34.3/25.6		10.6/4.2	50.8/56.2		12.1/7.2
24 h	28.1/38.5		43.4/37.4	16.1/11.7	20.0/33.2		48.1/45.4		21.4/8.8	44.3/54.0		23.2/10.6
JG												
6 h	86.4/89.1		3.6/2.3	0.64/0.30	83.2/87.5		4.5/1.9		1.6/1.4	76.6/71.5		2.1/2.7
12 h	84.8/87.3		5.0/3.5	1.5/1.3	77.4/87.9		6.9/3.8		2.1/1.8	63.7/57.0		2.5/3.1
24 h	74.5/78.8		9.6/7.8	3.2/2.4	68.1/75.5		14.4/10.7		2.8/2.2	63.2/62.2		2.2/2.1

TABLE II

Conversion of 1  $\mu$ M substrate in 24 h by transfected COS 1 cells

*Mock*, *WT*, and *JG* represent no plasmid, pCMV17 $\alpha$ -H (wild type), and pCMVJG17 $\alpha$ -H (mutant), respectively.  $P_4$ , progesterone; 17OHP<sub>4</sub>, 17 $\alpha$ -hydroxyprogesterone; 16OHP<sub>4</sub>, 16 $\alpha$ -hydroxyprogesterone;  $P_5$ , pregnenolone; 17OHP<sub>5</sub>, 17 $\alpha$ -hydroxypregnenolone.

Cells	$P_4 \rightarrow 17OHP_4$	(or) $\rightarrow 16OHP_4$	$P_5 \rightarrow 17OHP_5$	17OHP <sub>5</sub> $\rightarrow$ DHEA
	%			
Mock	1.8	2.0	0.7	0.4
WT	58.1	19.6	54.3	36.8
JG	22.8	7.9	20.4	2.4

activity was affected more severely than the 17 $\alpha$ -hydroxylase by the phenylalanine deletion, microsomes from transfected COS 1 cells were isolated and used for enzymatic assays. Much to our surprise as shown in Fig. 6, the 17 $\alpha$ -hydroxylase of P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> in isolated microsomes was much lower than expected relative to the activity of the wild-type enzyme. Thus, the ratio of 17 $\alpha$ -hydroxylase activity of the wild-type P-450<sub>17α</sub> versus P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> was several times greater than that measured in intact cells (at 6 h using 1  $\mu$ M progesterone, this ratio was 17 in microsomes, whereas it was 6 when measured in intact cells at the same time using the same substrate concentration). The 17,20-lyase activity of the P-450<sub>17α</sub><sup>ΔPhe(53 or 54)</sup> was also reduced accordingly in microsomes making careful examination of the effect of the mutation on the 17,20-lyase activity relative to the 17 $\alpha$ -hydroxylase activ-

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