# Functional Characterization of Mutant CYP17 Genes Isolated From a 17α-Hydroxylase/17,20-Lyase-Deficient Patient

Jong Ryeal Hahm, Tae Sik Jung, Sook Yong Byun, Young Nam Lee, Kon Ho Lee, and Deok Ryong Kim

**CYP17 has a dual enzymatic activity that is necessary for steroid hormone biosynthesis. It catalyzes the 17α-hydroxylation of progesterone or pregnenolone and also removes an acetyl moiety of hydroxy-progesterone or hydroxypregnenolone by its 17,20-lyase activity to produce androstenedione or dehydroepiandrosterone (DHEA), respectively. We previously isolated a compound heterozygous mutant of CYP17 from a Korean female patient: 1-base deletion and 1-base transversion mutation at 1 allele and 3-base deletion mutation at the other allele. Here we tested the functional activities of these 2 mutant CYP17 alleles using a transfection analysis in COS-1 cells with radiolabeled substrates and thin layer chromatography. Both mutant CYP17 genes lost not only 17α-hydroxylation activity, but also 17,20-lyase activity in this assay system. This nonfunctional nature of 2 mutant CYP17 genes explains the clinical manifestation of a patient who had 17α-hydroxylase deficiency.**


THE CYP17 gene, encoding cytochrome P450 17α-hydroxylase, is involved in steroid hormone biosynthesis. It is composed of a single polypeptide (508 amino acid residues) that catalyzes 2 different enzymatic steps.[1,2] First, CYP17 can transfer a hydroxy group to the 17th carbon of progesterone or pregnenolone, intermediates in steroid hormone biosynthesis, resulting in the production of hydroxyprogesterone or hydroxypregnenolone. Also, this enzyme can convert 17α-hydroxy C-21 steroids (OH-progesterone or OH-pregnenolone) to C-19 steroids (dehydroepiandrosterone [DHEA], androstenedione) by its 17,20-lyase activity. These products are subsequently used for precursors for biosynthesis of corticoids and many sex hormones in the adrenal and gonadal steroidogenesis or participate as direct mediators in many biologic reactions.[3] The simplified pathway for steroid hormone biosynthesis is depicted in Fig 1. Because of the crucial role of CYP17 in steroidogenesis, mutation in this gene causes overall defectiveness in steroid biosynthesis and then leads to 17α-hydroxylase/17,20-lyase deficiency with clinical manifestations, such as amenorrhea, sexual infantilism, and hypokalemic low aldosterone hypertension.[4,5]

Many mutant CYP17 genes have been isolated from 17α-hydroxylase/17,20-lyase-deficient patients. Most CYP17 mutants are found in the structural gene, and some of them are located at the regulatory regions or introns of the CYP17 gene.[6] These CYP17 mutant proteins and their enzymatic activities are summarized in our previous report.[7] Some missense mutations in the CYP17 gene (eg, S106P (T→C), F417C (T-C), H373L) abolish both 17α-hydroxylase activity and 17,20-lyase activity by a transient transfection analysis in COS-1 cells.[8,9] Some other mutant CYP17 proteins (eg, R96W, nonsense mutation at codon 461) contain no 17,20-lyase activity, although they showed some detectable 17α-hydroxylase activities.[10-12] According to previous reports, the C-terminal region of CYP17 (435-455 residues) contains a critical domain responsible for binding to a heme group that is absolutely required for catalytic activity in both hydroxylation and lyase reactions.[13-15] Thus, all mutant CYP17 proteins with deletions at the C-terminal region completely lose both hydroxylase and lyase activities.

Recently, we isolated a compound heterozygous mutant CYP17 gene from a Korean female patient who was diagnosed with 17α-hydroxylase/17,20-lyase deficiency.[7] The patient had a high level of progesterone and very low level of dehydroepiandrosterone (DHEA) as expected. But, the adrenocorticotropic hormone (ACTH) serum concentration (52.4 pg/mL; normal, 5 to 50) of the patient was not elevated. One CYP17 allele of this patient had 1-base deletion and 1-base transversion (TAC→AA) at codon 329, resulting in the production of a premature protein (1-417 a.a.). The other allele contains 3-base deletion (TCC) at either codons 350-351 or 351-352. Here, we test the functional activity of these 2 CYP17 mutant genes and try to explain the patient's clinical manifestations leading to 17α-hydroxylase/17,20-lyase deficiency.

## MATERIALS AND METHODS

### *Cell Culture*

COS-1 cells, a kidney cell of Africa Green Monkey, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin, obtained from GIBCO BRL, Carlsbad, CA) at 37°C in 5% $CO_2$.

### *Site-Directed Mutagenesis*

Mutation of CYP17 genes was performed by a method of polymerase chain reaction (PCR)-based mutagenesis using 2 oligonucleotides as previously described.[16] A mammalian expression vector of wild-type CYP17, pMT2c17 (obtained from Dr Walter L. Miller, University of Califonia San Francisco) was used for a template for mutagenesis. For 1-base deletion and 1-base transversion mutation at codon 329, 2 primers (CYP17-3: 5′-ACT ATC AGT GAC CGT AAC CGT CTC CTG CTG GAG GCC ACC ATC CGA GAG-3′; CYP17-4: 5′-CTC TCG GAT GGT GGC CTC CAG CAG GAG ACG GTT ACG GTC ACT GAT AGT-3′) were used. For 3-base deletion mutation, 2 primers (CYP17-1: 5′-CCT CAG GTG AAG AAG CTC AAG AGG AGA

*From the Departments of Internal Medicine and Biochemistry and RINS, College of Medicine and Gyeongsang Institute of Health Science, Gyeongsang National University, JinJu; and the Division of Applied Life Science and Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, JinJu, Korea.*

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*Address reprint requests to Deok Ryong Kim, Department of Biochemistry and RINS, College of Medicine and Gyeongsang Institute of Health Science, Gyeongsang National University, JinJu, Korea 660-751.*



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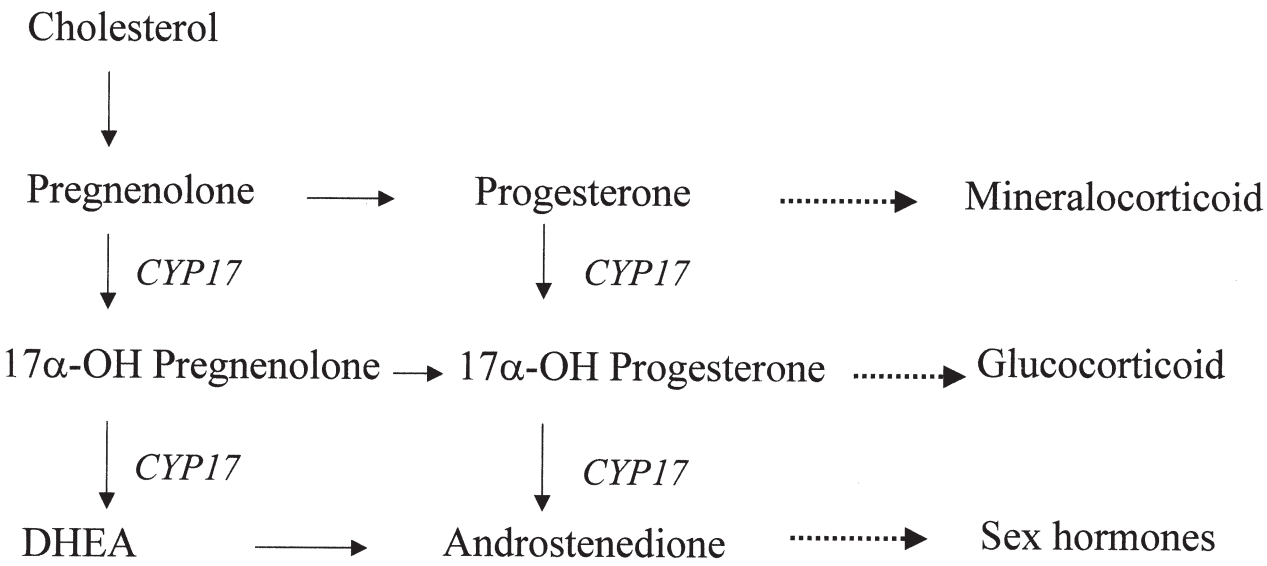


**Fig 1. Biosynthesis pathways of steroid hormones. Cholesterol, a precursor for steroid hormones, can be converted to pregnenolone by CYP11A1. This molecule can be further transformed either to progesterone, a direct precursor for mineralocorticoid, or 17α-hydroxy pregnenolone by the 17α-hydroxylase activity of CYP17. The 17α-hydroxyprogesterone is synthesized from progesterone by the same hydroxylation activity of CYP17 and used for the glucocorticoid synthesis. Both 17α-hydroxy pregnenolone and 17α-hydroxyprogesterone are converted to DHEA and androstenedione, precursors for sex hormone biosynthesis, by the 17,20-lyase activity of CYP17.**

TTG ACC AGA ATG TG-5′; CYP17-2: 5′-CAC ATT CTG GTC AAT CTC CTC TTG AGC TTC TTC TTC ACC TGA GG-3′) were used. PCR was performed as follows: a reaction mixture (50 μL) containing pMT2c17 template plasmid and 2 primers was heated at 94°C for 1 minute for denaturation of double-stranded DNA, and 2 primers were annealed to template DNA at 58°C for 1 minute. DNA was synthesized by *pfu* DNA polymerase (Invitrogen, Carlsbad, CA) at 72°C for 14 minutes (2 min/kb DNA). The synthesized DNA was incubated with *Dpn*I at 37°C for 1 hour in PCR reaction to remove unreplicated template plasmids. Then, *Dpn*I-treated DNA was transformed into *Escherichia coli* DH5α competent cells. Mutant CYP17 DNA was confirmed by DNA sequencing. The final 2 plasmids were named pTM2c17-1bd (for 1 nucleotide deletion mutation) and pTM2c17-3bd (for 3-base deletion mutation). Also, the exon 6 region of CYP17 was amplified using 2 primers (CYP17-5: 5′-GGA CCC TGG CCT TCC-3′; CYP17-6: 5′-ACT CAC CGA TGC TGG-3′), and PCR products were subject to restriction digestion with *BseR*I or *Sml*I as previously described.[7] pTM2, a vector control, was constructed from pTM2c17 by deleting out the CYP17 gene at the *EcoR*I site and religating the vector.

### *Reverse Transcriptase-PCR*

Total RNA was isolated by QAI RNAins kit (QIAGEN, Hilden, Germany) as described in the product manual. Reverse transcription was performed in a reaction mix (20 μL) containing RNase inhibitor (20 U), deoxy nucleotide triphosphates (dNTPs) (4 μL of each 2.5 mmol/L), 200 U reverse transcriptase (RT) (Invitrogen), and total RNA (0.5 μg) annealed with 15 pmol oligo d(T) at 75°C for 10 minutes. The reaction mix was incubated at 37°C for 1 hour, then followed by denaturation at 95°C for 5 minutes and stored at −20°C until being used. PCR was performed in a reaction mix (50 μL) containing 2 μL cDNA template, 2 primers (CYP17-5 and CYP17-6, each 20 pmol), dNTPs (0.2 mmol/L final), and 2.5 U *Tag* DNA polymerase for 30 cycles (denaturation at 94°C for 1 minute, annealing 50°C for 1 minute, elongation at 72°C for 1 minute). PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide (EtBr) staining.

### *Assay for CYP17 Activity*

Both enzymatic activities (17α-hydroxylase and 17,20-lyase) of CYP17 were determined as previously described.[17-19] A total of 5 μg CYP17-expressing plasmids (wild-type or mutant) prepared by $CsCl_2$ gradient centrifugation was transfected into COS-1 cells (50% confluency) with superfectin (QIAGEN). The transfected cells were incubated at 37°C for 3 hours and washed with 1X phosphate-buffered saline (PBS) once. Cells were then further incubated in the fresh DMEM (+ 10% FBS) at 37°C for 36 hours. [$^3$H]-labeled progesterone or [$^3$H]-labeled hydroxyprogesterone ($6 \times 10^6$ cpm (16.25 nmol/L), final 1 μmol/L with nonradiolabeled substrate, Amersham Pharmacia Biotech, Buckinghamshire, England) was added into cells in 4 mL fresh media containing 10% FBS. The cells were incubated under the same condition for 6 hours. Radiolabeled steroids were extracted from the culture medium with 15 mL ethylacetate/isooctane (1:1) and concentrated by speed vacuum dryer or liquid nitrogen evaporation. Steroids were separated on TLC plates (Silica gel 60, 20 × 20 cm, MERCK, Darmstadt, Germany) with chloroform:ethyl acetate (3:1) and identified by autoradiography using EN$^3$HANCE spray (NEN, Boston, MA).

## RESULTS AND DISCUSSION

Cytochrome p450 17α-hydroxylase, encoded by the CYP17 gene, catalyzes 2 separate enzymatic reactions during steroid hormone biosynthesis.[3] It hydroxylates progesterone or pregnenolone at the 17 carbon and converts such 21-carbon steroids to 19-carbon steroids (androstenedione or DHEA) by its 17,20-lyase activity. Therefore, failures in its catalytic reaction or its gene transcription causes many steroid hormone-based disorders, such as amenorrhea, sexual infantilism, or hypertension.[4]

In this report, we analyzed the functional activity of 2 mutant CYP17 genes (1-base deletion at codon 329 and 3-base deletion at codons 350-352) previously isolated from a patient who had

a compound heterozygous mutant CYP17 causing amenorrhea, sexual infantilism, and hypokalemic low aldosterone hypertension. The enzymatic activities (17$\alpha$ -hydroxylase and 17,20-lyase activity) of CYP17 can be determined by detecting the conversion of radiolabeled steroid substrates in CYP17-expressing COS-1 cells.[5,20] A plasmid (pTM2c17) expressing a human wild-type CYP17 gene was obtained from Dr Miller (University of California at San Francisco). Two mutant CYP17-expressing plasmids: pTM2c17-3bd (3-base deletion) and pTM2c17-1bd (1-base deletion) were constructed from pTM2c17 using oligonucleotide-based mutagenesis as described in Materials and Methods. The exon 6 region (215 bp) of CYP17 of 2 mutant constructs possessing 2 mutation positions was amplified using 2 primers (CYP17-5 and CYP17-6, described in Materials and Methods) and digested with a restriction enzyme *Sml*I for 3-base deletion mutant or *BseR*I for 1-base deletion mutant as previously described.[7] The PCR product of pTM2c17-3bd can be cut by *Sml*I because of acquisition of a new *Sml*I site from the 3-base deletion mutation, whereas the PCR product of pTM2c17-1bd can not be digested by *BseR*I site due to its loss by 1-base deletion (data not shown). Furthermore, these mutant constructs were confirmed by DNA sequencing.

To test the 17$\alpha$-hydroxylase activity of 2 mutant CYP17 genes, we transfected plasmids containing wild-type or mutant CYP17 genes into COS-1 cells and incubated cells in the medium with [$^3$H]-progesterone for 6 hours. Steroids extracted from culture media were then analyzed on TLC. As shown in Fig 2, both mutant CYP17 genes (3-base deletion and 1-base deletion) lost their ability to catalyze 17$\alpha$-hydroxylation of progesterone (Fig 2A, lanes 5 to 8). Instead, wild-type CYP17 converted all progesterone substrates to 17$\alpha$-hydroxyprogesterone and some 16$\alpha$-hydroxyprogesterones (Fig 2A, lanes 3 and 4). The production of 16$\alpha$-hydroxyprogesterones by overexpressed CYP17 in this COS-1 assay system has been reported by many previous studies.[5,20] In both cases of no plasmid and vector only, any products of 17$\alpha$-hydroxyprogesterones were not detected (Fig 2A, lanes 1 and 2). We tested the expression level of CYP17 in all COS-1 cells using RT-PCR for the measurement of enzymatic activity. Except for the culture media used for activity, all COS-1 cells were collected for preparation of total RNA. The mRNA level of CYP17 in each experiment was determined by RT-PCR using CYP17-specific primers (CYP17-5 and CYP17-6) above mentioned in Materials and Methods. All COS-1 cells containing CYP17 constructs (wild-type or mutants) produced CYP17 mRNA almost equally (Fig 2B).

We also tested 17,20-lyase activities of CYP17 using the same assay with [$^3$H] 17-hydroxyprogesterone as substrates. Both mutant CYP17 genes (1-base deletion and 3-base deletion) did not show any detectable 17,20-lyase activity, although wild-type CYP17 converted all substrates to androstenediones or other unknown products (Fig 3A). Androstenedione migrated faster than 17-hydroxyprogesterone as shown in a previous report.[19] The control vector (pTM2) without CYP17 gene was not able to convert the substrate to any product (Fig 3A, lane 2). The CYP17 mRNA level in all experiments was very similar (Fig. 3B).

As shown in Fig 1, CYP17 catalyzes 2 different pathways: the biosynthesis pathway from pregnenolone to DHEA ($\Delta^5$-steroid pathway) and from progesterone to androstenedione ($\Delta^4$-steroid pathway). In humans, the catalytic efficiency of 17$\alpha$-hydroxylase in both pathways was generally compatible, but the 17,20-lyase activity in the $\Delta^5$-steroid pathway was much greater than that in the $\Delta^4$-steroid pathway.[5,20] Therefore,

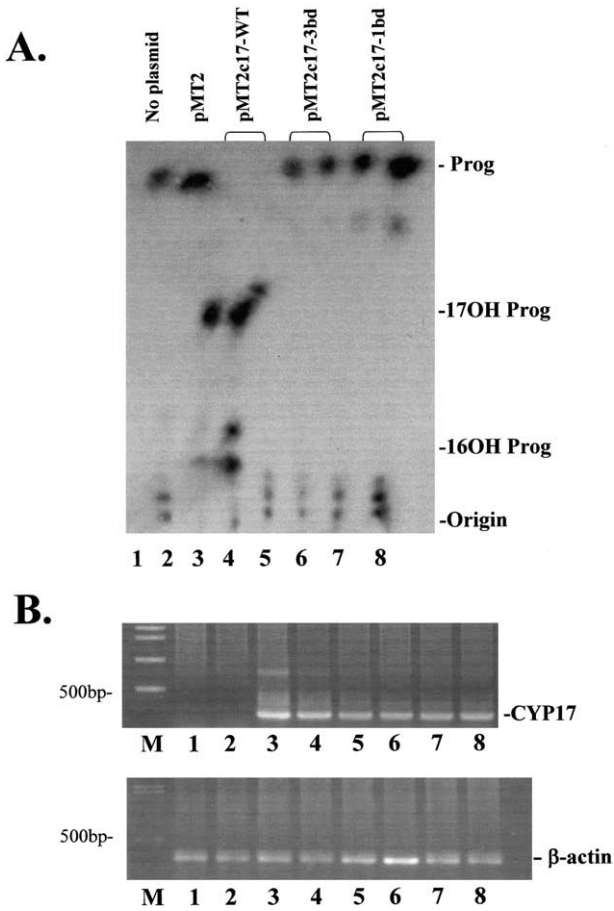


**Fig 2. Hydroxylation activity of CYP17. (A) Measurement of activities of CYP17 mutant genes on TLC. The hydroxylation activity of CYP17 mutant genes was measured using [$^3$H]-labeled progesterone as substrates. CYP17-expressing plasmids: pMT2c17, wild-type CYP17; pMT2c17-3bd, 3-base deletion mutant CYP17; pMT2c17-1bd, 1-base deletion mutant CYP17; pMT2, vector only, were transfected into COS-1 cell as described in Materials and Methods. The steroid products in culture media were extracted and separated on a TLC plate. Radiolabeled steroids were visualized by autoradiography. Lanes 3 and 4 are wild-type CYP17 and lanes 5 to 8 are mutant CYP17. The migration positions of progesterone, 17-hydroxyprogesterone, and 16-hydroxyprogesterone are indicated by Prog, 17OH-prog, 16OH-prog, respectively. (B) RT-PCR. The expression level of each experiment was determined by RT-PCR. Total RNA was isolated from COS-1 cells transfected by each CYP17-expressing plasmid. RT and PCR reactions were performed as described in Materials and Methods. The mRNA level of CYP17 was amplified by 2 primers (CYP17-5 and CYP17-6), resulting in production of 215 bp. The amount of $\beta$-actin mRNA, a control, was determined by 2 $\beta$-actin specific primers (forward: 5′-ATC CGT AAA GAC CTC TAT GC-3′; reverse: 5′-AAC GCA GCT CAG TAA CAG TC-3′). The size of its PCR products is 287 bp. M indicates DNA size markers. Lane 1, no plasmid; lane 2, pTM2 vector control; lanes 3 and 4, wild-type CYP17; lanes 5 and 6, 3-base deletion CYP17; lanes 7 and 8, 1-base deletion CYP17.**

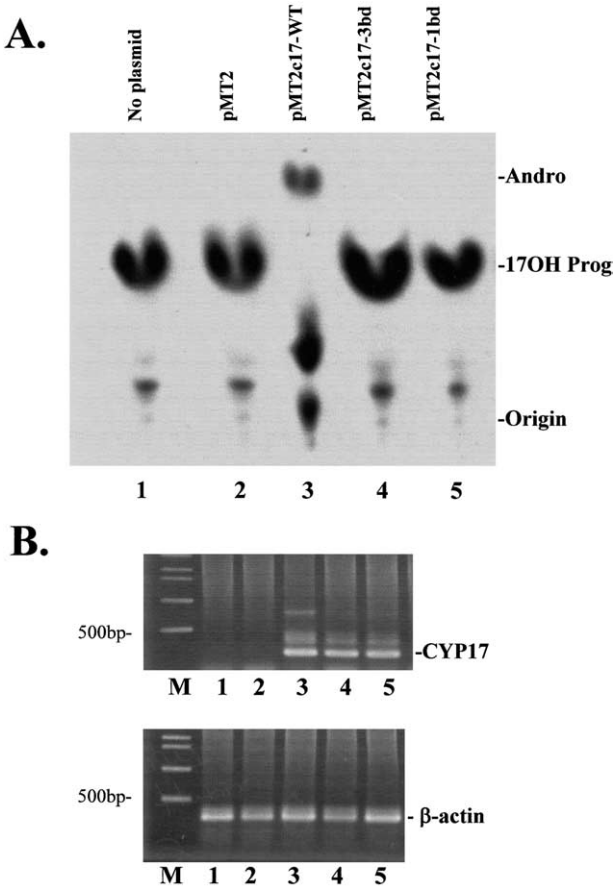


**Fig 3. 17,20-Lyase activity of CYP17. (A) Measurement of activities of CYP17 mutant genes on TLC. The lyase activity of CYP17 mutant genes was measured using [$^3$H]-labeled hydroxyprogesterone as substrates. CYP17-expressing plasmids: pMT2c17, wild-type CYP17; pMT2c17-3bd, 3-base deletion mutant CYP17; pMT2c17-1bd, 1-base deletion mutant CYP17; pMT2, vector only, were transfected into COS-1 cell as described in Materials and Methods. The steroid products in culture media were extracted and separated on a TLC plate. Radiolabeled steroids were visualized by autoradiography. Andro indicates the migration position of androstenedione. (B) RT-PCR. The expression level of each experiment was determined by RT-PCR. Total RNA was isolated from COS-1 cells transfected by each CYP17-expressing plasmid. RT and PCR reactions were performed as described in Materials and Methods. The mRNA level of CYP17 was amplified by 2 primers (CYP17-5 and CYP17-6), resulting in production of 215 bp. The amount of $\beta$-actin mRNA, a control, was determined by 2 $\beta$-actin specific primers (forward: 5′-ATC CGT AAA GAC CTC TAT GC-3′; reverse: 5′-AAC GCA GCT CAG TAA CAG TC-3′). The size of its PCR products is 287 bp. M indicates DNA size markers. Lane 1, no plasmid; lane 2, pTM2 vector control; lane 3, wild-type CYP17; lane 4, 3-base deletion CYP17; lane 5, 1-base deletion CYP17.**

DHEA, an intermediate in the $\Delta^5$-steroid pathway, is a major precursor for sex hormone biosynthesis. In contrast, the catalytic efficiency of CYP17 17,20-lyase in both pathways in other animals, such as rats, mice, guinea pigs, and Xenopus has been shown to be similar.[21] Unfortunately, in this report, we could not test both CYP17 activities in the $\Delta^4$-steroid pathway: conversion from 17 hydroxypregnenolone to DHEA by 17,20-lyase activity and from pregnenolone to 17 hydroxypregnenolone by 17$\alpha$-hydroxylase activity, because radiolabeled substrates were not commercially available.

From these functional analyses, we expected that the mutant protein produced from the 1-base deleted CYP17 gene had no functional activity because a base deletion at codon 329 caused the reading frame shift and, in turn, produced a premature protein (1-417 a.a.). This mutant protein does not contain a heme-binding site (435-455 a.a.) that is crucial for catalysis.[13] On the contrary, the activity of 3-base deletion mutant CYP17 has been expected to be reserved rather because the mutation does not lead to a frame shift and delete out only 1 internal amino acid on the protein. In addition, as reported in our previous study,[7] the patient did not have too high a level of ACTH, suggesting that CYP17 activities might not be completely blocked. However, we did not see any detectable activities of 3-base deletion mutant CYP17 in both hydroxylation and lyase reaction. One leucine deletion of 4 consecutive leucines by the mutation might result in changing the overall protein structure leading to be a nonfunctional protein. No elevation of the ACTH level in the patient might be explained in some other way. The high concentration of deoxycorticosterone may activate the cortisol receptor and, in turn, control the ACTH level in the patient. These 2 CYP17 mutants have no detectable 17$\alpha$-hydroxylase and 17,20-lyase activities, although other CYP17 mutants possess different levels of both catalytic activities as summarized in our previous report.[7] While some mutants, such as H373L, F417C, and R440H completely lost both activities, the R496C mutant protein showed its decreased activity in 17$\alpha$-hydroxylase and 17,20-lyase reactions.[9,10,15]

In conclusion, both CYP17 mutant genes isolated from a patient lost its ability to catalyze hydroxylation at 21-carbon steroids and removal of an acetyl group from 17$\alpha$-hydroxylated steroids and, in turn, led to the 17$\alpha$-hydroxylase deficiency shown in our previous report.[7]

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