Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11673–11677, December 1993
Genetics

Molecular basis of aromatase deficiency in an adult female with sexual infantilism and polycystic ovaries

YUJI ITO*†, CAROLYN R. FISHER*†, FELIX A. CONTE‡, MELVIN M. GRUMBACH‡, AND EVAN R. SIMPSON*†§

*The Cecil H. and Ida Green Center for Reproductive Biology Sciences, and †The Departments of Obstetrics/Gynecology and Biochemistry, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235; and ‡Department of Pediatrics, University of California at San Francisco, San Francisco, CA 94143

Communicated by Ronald W. Estabrook, September 10, 1993 (received for review July 20, 1993)

ABSTRACT **We identified two mutations in the *CYP19* gene responsible for aromatase deficiency in an 18-year-old 46,XX female with ambiguous external genitalia at birth, primary amenorrhea and sexual infantilism, and polycystic ovaries. The coding exons, namely exons II–X, of the *CYP19* gene were amplified by PCR from genomic DNA and sequenced directly. Direct sequencing of the amplified DNA from the patient revealed two single-base changes, at bp 1303 (C → T) and bp 1310 (G → A) in exon X, which were newly found missense mutations and resulted in codon changes of R435C and C437Y, respectively. Subcloning followed by sequencing confirmed that the patient is a compound heterozygote. The results of restriction fragment length polymorphism analysis and direct sequencing of the amplified exon X DNA from the patient's mother indicate maternal inheritance of the R435C mutation. Transient expression experiments showed that the R435C mutant protein had ≈1.1% of the activity of the wild type, whereas C437Y was totally inactive. Cysteine-437 is the conserved cysteine in the heme-binding region believed to serve as the fifth coordinating ligand of the heme iron. To our knowledge, this patient is the first adult to have described the cardinal features of a syndrome of aromatase deficiency. Recognition that such defects exist will lead to a better understanding of the role of this enzyme in human development and disease.**

The biosynthesis of estrogens from C_{19} steroids is catalyzed by an enzyme complex known as aromatase, whose activity results in aromatization of the A ring of androgens to form the phenolic A ring characteristic of estrogens, with concomitant loss of the C_{19} angular methyl group (1, 2). This enzyme complex is located in the endoplasmic reticulum of estrogen-producing cells and consists of two components. The first is a form of cytochrome P450 named aromatase cytochrome P450 (P450arom), the product of the *CYP19* gene (3). The second component, NADPH–cytochrome P450 reductase, a ubiquitous flavoprotein of the endoplasmic reticulum of most cell types, transfers reducing equivalents to the P450arom, which binds the C_{19} substrate and catalyzes the insertion of oxygen into the molecule, resulting in the formation of C_{18} estrogens (4–7). A single species of P450arom has the capacity to metabolize the three substrates—testosterone, androstenedione, and 16α-hydroxydehydroisoandrosterone—as evidenced by experiments in which placental P450arom cDNA inserts isolated in our laboratory and expressed in COS-1 monkey kidney tumor cells have been shown to be capable of catalyzing the aromatization of all three substrates (8).

In the human, *CYP19* is expressed in a number of cells and tissues including ovarian granulosa cells (9), testicular Sertoli (10) and Leydig cells (11, 12), placenta, adipose tissue of both males and females (13), and various sites of the brain including hypothalamus, amygdala, and hippocampus (14, 15). In most other species, estrogen biosynthesis does not occur in such a wide distribution of tissues, but rather is limited to at most two sites (namely, the gonads and the brain). We have presented evidence that the tissue-specific expression of the *CYP19* gene is regulated, in part, by the use of tissue-specific promoters through mechanisms involving alternative splicing (16). Thus, whereas expression in the ovary is driven by a proximal promoter, expression in placenta involves a distal element at least 40 kb upstream from the start of translation. Transcripts in adipose contain two other untranslated exons. All of these are spliced into a common 3′-splice junction upstream of the start of translation; thus, the coding region is unaffected and the protein product in each tissue site of expression is identical (17). The overall length of the gene is at least 75 kb, and it is composed of nine coding exons and at least four untranslated 5′ exons.

In spite of the size and complexity of the *CYP19* gene, at this time only one definitively characterized case of aromatase deficiency has been reported, although deficiencies of most of the other steroidogenic forms of cytochrome P450 have been well characterized. In 1991, Shozu *et al.* (18) reported a case of female pseudohermaphroditism secondary to placental aromatase deficiency in a Japanese infant, and biochemical and molecular genetic studies were conducted by Harada *et al.* (19) to characterize the molecular basis of the deficiency. P450arom cDNA isolated from a placental cDNA library from this patient was found to have an insert of 87 bp, encoding 29 amino acids, in frame, with no termination codon. Examination of genomic DNA from the patient revealed that the consensus 5′-splice acceptor sequence was mutated from GT to GC, resulting in the use of a cryptic splice acceptor site further downstream in intron 6. This resulted in the incorporation of 87 bases from intron 6, which were translated into the additional 29 amino acids present in the mature polypeptide. Further examination of genomic DNA from the patient and her parents revealed that the patient was homozygous for this mutation, and her parents were obligate heterozygotes (20).

In this paper we report the molecular basis of aromatase deficiency caused by two mutations in the *CYP19* gene, which was suspected on clinical and biochemical evidence, in an 18-year-old 46,XX female with sexual infantilism, primary amenorrhea, ambiguous external genitalia at birth, and polycystic ovaries. The clinical aspects of this case will be described in detail in a parallel publication (F.A.C. and M.M.G.). To our knowledge, this is the first definitive reported case of an adult having aromatase deficiency, which consequently represents a new syndrome.

MATERIALS AND METHODS

Preparation and Amplification of Genomic DNA. Ovarian fibroblasts were obtained from the patient at the time of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: P450arom, aromatase cytochrome P450, the product of the *CYP19* gene.

§To whom reprint requests should be addressed.

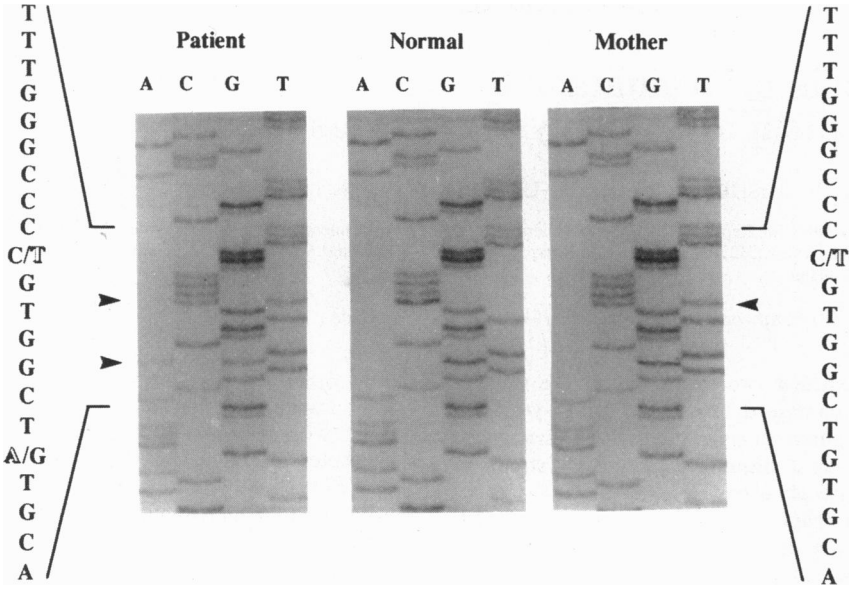

FIG. 1. Nucleotide sequence of the region of exon X of the *CYP19* gene in the patient, a normal subject, and the patient's mother. Genomic DNA was extracted from the patient's fibroblasts and lymphoblasts of a normal subject and the patient's mother; exons II–X of the *CYP19* gene were amplified by PCR and sequenced directly as described in *Materials and Methods*. In the patient, single-base changes at bp 1303 (C → T) and bp 1310 (G → A) in exon X were detected (*Left*). The maternal DNA has only a single-base change at bp 1303 (C → T) (*Right*). In the normal *CYP19* gene, only C and G were detected at bp 1303 and 1310, respectively (*Middle*).

laparotomy at 17 months of age and stored in liquid nitrogen. Fibroblasts were maintained in Waymouth's enriched medium containing 10% (vol/vol) fetal bovine serum, and genomic DNA was extracted (21). Blood was collected from normal subjects, the patient, and her mother, and genomic DNA was extracted from Epstein–Barr-transformed lymphoblasts (22). Exons II–X (including all of the coding region and flanking intronic sequence) of the *CYP19* gene were amplified by PCR. Amplification of DNA fragments was performed using specifically designed intronic primers to determine the complete sequence of the exons as well as the intron–exon junctions. The PCR was carried out in a total volume of 50.5 μl containing the following additions: genomic DNA template (0.28 μg), upstream and downstream oligonucleotide primers (50 pmol of each), 10× reaction buffer (5 μl), 10 mM dNTPs (1 μl), *Thermus aquaticus* (*Taq*) DNA polymerase (2.5 units; Perkin—Elmer/Cetus), and water to make a total volume of 50.5 μl. Amplification was carried out for 30 cycles; each cycle consisted of incubations of 30 sec at 94°C for denaturation, 20 sec at 55°C for annealing, and 30 sec at 72°C for extension.

Direct Sequencing of Amplified DNA. The amplified fragments were then loaded onto a 1.5% agarose gel and subjected to electrophoresis. The fragment was excised and eluted from the agarose gel using a Qiaex gel-extraction kit according to the manufacturer's instructions (Qiagen, Chatsworth, CA). The purified fragment was subjected to direct sequencing using the *fmol* DNA sequencing system (Promega) and either the primers used to generate the fragment or primers specific to the coding region of the exon

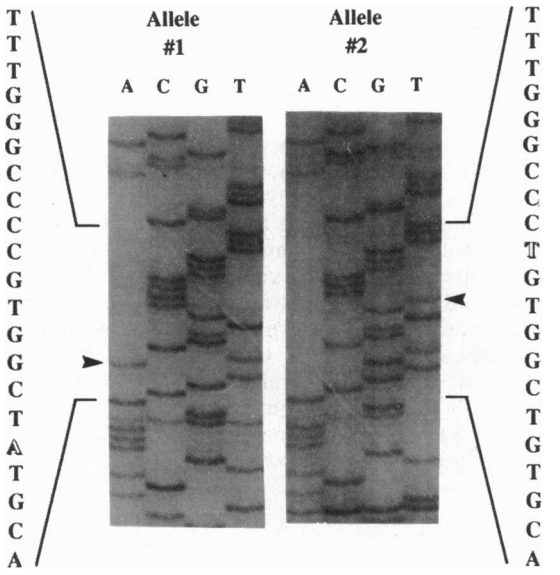

FIG. 2. Nucleotide sequence of the region of exon X of the *CYP19* gene of the patient. The amplified exon X DNA was subcloned into the vector pCR II and sequenced as described in *Materials and Methods*. One allele (#1) has the base change at bp 1310 (G → A), and the other allele (#2) has the base change at bp 1303 (C → T).

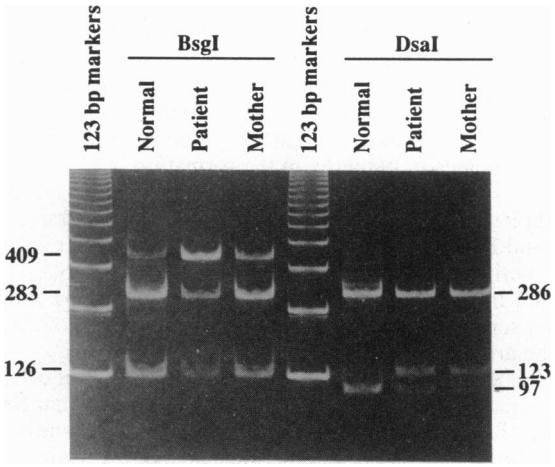

FIG. 3. Restriction fragment length polymorphism analysis of amplified exon X (*CYP19* gene) from normal, patient, and maternal genomic DNA. Amplified exon X (full-length, 409 bp) was digested with *Bsg* I and *Dsa* I. Molecular sizes of fragments generated by *Bsg* I and *Dsa* I are indicated on the left and right, respectively.

being examined. The ratio of template to primer was 1:6. Sequencing reaction mixtures were prepared according to the manufacturer's directions, placed in a preheated (94°C) thermal cycler for 2 min, and sequenced using the same conditions as employed for generation of the fragments.

Subcloning of Amplified DNA Fragments Containing Exon X. Subcloning of amplified exon X into the TA Cloning vector (pCR II; Invitrogen) and the transformation of INVαF′ competent cells was carried out according to the manufacturer's instructions. Positive colonies were identified by color selection and rescreened using end-labeled oligonucleotides. DNA was isolated from the bacterial cells by the alkaline lysis method and sequenced by the dideoxy chain termination method (23).

Restriction Enzyme Digestion. To determine the presence of mutations in other family members, the region containing exon X from normal, patient, and maternal genomic DNA was amplified and digested with *Dsa* I or *Bsg* I (New England Biolabs) and electrophoresed in a 10% polyacrylamide gel. Digested fragments were visualized by ethidium bromide staining.

Expression of P450arom cDNA in COS-1 Cells. Fragments (377 bp) containing either the R435C or C437Y mutations or the normal sequence were removed from the pCR II vector containing exon X as an insert by digestion with *Apa* I and purified from a 1.5% agarose gel by Qiaex gel extraction according to the manufacturer's directions. The fragments were ligated into *Apa* I-digested wild-type pCMVarom and transformed into DH5α-competent cells (BRL). To select for recombinants, colonies were picked and grown overnight in LB medium containing ampicillin at 100 μg/ml. Plasmid DNA was isolated from the bacterial cells using the Insta-Prep plasmid isolation system (5 Prime→3 Prime, Inc.), and recombinants were selected by *Eco*RI digestion. Since the *Apa* I fragment contained a portion of the 3′ untranslated region of exon X, these constructs were digested with *Pml* I and religated to remove this intervening sequence. The purified plasmids were resequenced to confirm that restriction enzyme digestion did not introduce a frameshift.

All three constructs and the vector control were grown in large quantities and purified by cesium chloride gradient centrifugation. COS-1 cells maintained in Dulbecco's modified Eagle's medium plus 10% (vol/vol) bovine calf serum were harvested by trypsinization when the cells were 50% confluent, and 100 μg of each plasmid was introduced into the cells by electroporation. Cells carrying each respective plasmid were plated in triplicate wells of a six-well culture dish. Forty-eight hours after transfection, the cells were assayed by the tritiated water-release assay (24). After assay, the cells were lysed in 1% deoxycholate/0.1% SDS and collected for protein determination by the BCA method (Pierce). To determine that expression levels of each plasmid were equivalent, 25 μg of total cellular protein from a representative well for each treatment was digested with EndoH (New England Biolabs) according to the manufacturer's instructions to deglycosylate the P450arom. After digestion, the proteins were precipitated with 30% (wt/vol) trichloroacetic acid, washed with cold (4°C) acetone, and resuspended in sample loading buffer for electrophoresis in a 10% SDS/polyacrylamide gel. Proteins were then transferred to an Immobilon-P membrane (Millipore) and visualized by the ECL method (Amersham) using as a primary antibody the polyclonal antibody raised against a conserved 20-residue synthetic peptide common to human, chicken, and rat P450arom (25).

Table 1. Expression of mutant P450arom cDNAs in COS-1 cells

cDNA	[1β-^{3}H]Androstenedione conversion,* pmol per mg of protein per 2 hr
Normal	127.0 ± 8.2 (100%)
R435C	1.4 ± 0.1 (1.1%)
C437Y	0.05 ± 0.01 (0%)
Vector	0.02 ± 0.05

pCMV vectors containing wild-type or mutant cDNA inserts or no insert were transfected into COS-1 cells, and P450arom activity was assayed by the release of tritium as ^3H_2O from [1β-^{3}H]androstenedione as described in *Materials and Methods*. The data presented are the mean ± SD of triplicate replicates. The numbers in parentheses are the percent relative to the value for the normal cDNA.
*Average of three determinations.

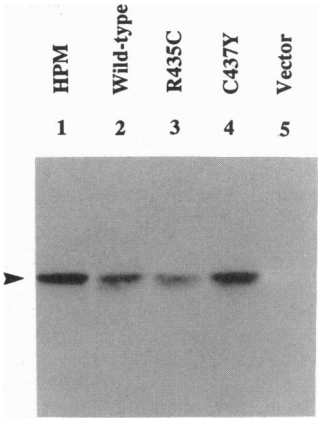

FIG. 4. Western blot analysis of human P450arom in lysates from COS-1 cells transfected with pCMV vector containing wild-type (lane 2), R435C (lane 3) or C437Y (lane 4) mutant cDNA, or vector only (lane 5). Lane 1 contained 5 μg of a sample of human placental microsomal protein (HPM) to serve as a positive control. Lanes 2–5 contained 25 μg of protein. Samples in all lanes were treated with EndoH as described in *Materials and Methods*. After SDS/polyacrylamide electrophoresis, proteins were immunoblotted and probed with a polyclonal peptide-generated antibody as described in *Materials and Methods*.

RESULTS AND DISCUSSION

The patient, EB, at the time of this investigation was an 18-year-old girl. At birth she presented with nonadrenal female pseudohermaphroditism. Chromosomal analysis revealed a 46,XX karyotype. At 17 months, normal internal female genital structures were identified at laparotomy; the ovaries were grossly and microscopically normal. At age 14 she failed to exhibit breast development and had primary amenorrhea. The clitoris had progressively enlarged to 4 × 2 cm, and pubic and axillary hair was Tanner stage III. Basal plasma testosterone levels were elevated at 95 ng/dl, Δ^4-androstenedione levels were 285 ng/dl, and plasma estrone and estradiol levels were undetectable. Adrenocorticotrophin and dexamethasone tests indicated a nonadrenal source of the testosterone and androstenedione. Plasma follicle-stimulating hormone and luteinizing hormone levels were markedly elevated. Quantification of urinary steroids by gas chromatography–mass spectrometry indicated normal levels of C_{19} and C_{21} steroids but very low levels of estrone and estradiol. Sonography and magnetic resonance imaging showed multiple bilateral 4- to 6-cm ovarian cysts. Estrogen treatment resulted in a decrease in plasma gonadotropins, breast development, a pubertal growth spurt, menarche, and regression of the ovarian cysts.

We extracted genomic DNA from the patient's fibroblasts and amplified the individual exons of the *CYP19* gene by PCR. Primers were chosen in the introns flanking each exon so that it would be possible to determine the complete sequence of the exons as well as the intron–exon junctions. Direct sequencing (Fig. 1) of the amplified DNA revealed single-base changes at bp 790 (T → C) in exon VII and bp 1303

FIG. 5. Comparison of DNA sequence and derived amino acid sequence of the heme-binding region of the normal and patient's *CYP19* gene. Indicated by the boxed area is the putative heme-binding region. Within this sequence is a cysteine, indicated by underlining, that is common to all cytochrome P450s and is believed to be the fifth coordinating ligand of the heme iron. One allele has a base change at bp 1310 (G → A), indicated in boldface type, which encodes tyrosine instead of cysteine 437, and the other allele has a missense mutation at bp 1303 (C → T), which encodes cysteine instead of arginine-435, indicated in boldface type.

(C → T) and bp 1310 (G → A) in exon X. The base change at bp 790 (T → C) was a homozygous missense mutation, which results in encoding arginine instead of cysteine-264. However, this change has been found in the DNA from a normal subject and in the placental P450arom cDNA of healthy women (26, 27), and we believe it to be a genetic polymorphism. The other two base changes were newly found missense mutations encoding cysteine instead of arginine-435 (bp 1303) and tyrosine instead of cysteine-437 (bp 1310). The patient was heterozygous for these mutations as judged by the fact that bands corresponding to both C and T at nucleotide 1303 and G and A at nucleotide 1310 could be detected in the sense strand by direct sequencing (Fig. 1). In contrast, when we determined the nucleotide sequence of DNA from normal subjects, only C and G were detected at bp 1303 and 1310, respectively (Fig. 1).

Amplified exon X DNA from the patient was subcloned into the vector pCR II (Invitrogen) and sequenced to verify the presence of the mutations on different alleles, indicating that the patient is a compound heterozygote (Fig. 2). In addition, these single-base changes provide convenient restriction fragment length polymorphisms with which to examine DNA from other family members. Exon X from normal, patient, and maternal genomic DNA was amplified and digested with *Bsg* I and *Dsa* I. Since the C437Y mutant allele is lacking the *Bsg* I site present in exon X of the normal *CYP19* gene and the R435C mutant allele is lacking one of the *Dsa* I sites, restriction fragment length polymorphism analysis could be performed (Fig. 3). *Bsg* I digestion produced 283- and 126-bp bands from PCR-amplified exon X from a normal subject and the patient's mother, and 409 (full-length)-, 283-, and 126-bp bands from the patient. *Dsa* I digestion produced 286-, 97-, and 26 (not shown in the figure)-bp bands from a normal subject, and 286-, 123-, 97-, and 26-bp bands from the patient and her mother. The 123-bp band corresponds to the undigested mutant fragment, and the other bands result from digestion of the normal fragment. Despite repeated attempts, *Bsg* I did not fully digest the normal allele; however, the more intense staining of the 409-bp band in the patient's lane indicates the mutant allele is undigested. These results indicate the maternal inheritance of the R435C mutation, and this finding was confirmed by sequencing amplified exon X DNA from the patient's mother (Fig. 1). The maternal DNA had only one base change, at bp 1303 (C → T), which corresponds to the R435C mutation. Most likely the C437Y mutation was inherited from the patient's father, who however is deceased. There are no other siblings.

Expression of mutant cDNAs in COS-1 cells revealed that the activity of the mutant proteins was dramatically reduced. In the presence of the same amount of total cellular protein, the R435C mutant protein showed ≈1.1% of the activity of wild-type P450arom, while the C437Y protein was essentially inactive (Table 1).

Whole cell protein extract was subjected to Western blot analysis to ensure that P450arom was expressed at similar levels in each transfection. Both of the mutant proteins were expressed at an equivalent level to the normal cDNA (Fig. 4).

These missense mutations reside within the heme-binding region of the protein (Fig. 5). Since cysteine-437 is the conserved cysteine that makes up the fifth coordinating ligand of the heme iron, the gene product of the C437Y allele would be expected to be inactive. Arginine-435 is also a highly conserved residue within the heme-binding region among mammalian P450s with the exception of cholesterol 7α-hydroxylase cytochrome P450 in which it is a threonine (28). At the analogous position in *Pseudomonas putida* P450cam, as well as in some other bacterial P450 isoforms, this residue is a histidine. Only one other documented mutation (R448H) of this arginine has been reported, in 11β-hydroxylase deficiency. This mutation is present in the majority of mutant alleles in Moroccan Jews, among whom 11β-hydroxylase deficiency occurs relatively frequently (≈1 in 5000 births). Using a transient transfection assay, Curnow *et al.* (29) confirmed that with the R448H mutation 11β-hydroxylase activity is undetectable. Therefore a mutation at this position in P450arom would also be expected to result in a substantial loss of enzymatic activity, which indeed was the case.

Although a suspected patient was described some years ago (30), there is only one other definitively documented case of placental aromatase deficiency, in a Japanese infant. In 1991, Shozu *et al.* (18) described a 24-year-old primigravida who showed progressive virilization during the third trimester. Maternal serum levels of estrogens were low, and those of C_{19} steroids were high in the third trimester. The woman delivered vaginally a live full-term infant who exhibited female pseudohermaphroditism. Cord serum levels of estrogens were extremely low, while those of C_{19} steroids were high. In contrast, the mother of our patient did not show any virilization during pregnancy except acne, despite the presentation with nonadrenal female pseudohermaphroditism of the infant patient. According to the Japanese case report, serum levels of androgens, progesterone, and 17α-hydroxyprogesterone in the infant returned rapidly to normal after birth. It is anticipated that the patient may exhibit almost the same features as our patient in the future; however, it is still unknown to what degree the aromatase deficiency of the Japanese patient will affect her at puberty.

On the other hand, our case already has the cardinal features of a syndrome of aromatase deficiency due to molecular defects in the *CYP19* gene. She is now 18 years old and displays the symptoms of nonadrenal female pseudohermaphroditism. The fetal masculinization can be ascribed to defective placental conversion of C_{19} steroids to estrogens

and resulting increased levels of C_{19} steroids, which can be converted to testosterone peripherally. The pubertal failure and the development of multicystic ovaries at the normal age of puberty can be attributed to ovarian aromatase deficiency and consequent elevated follicle-stimulating hormone and luteinizing hormone levels. We suggest that aromatase deficiency may be responsible for some cases that might be thought as variants of polycystic ovarian syndrome—namely, those characterized by normal insulin sensitivity and failure to produce estrogens.

In spite of the possibility that some cases of aromatase deficiency may have been overlooked, it appears to be a rare condition. In a gene as large and complex as *CYP19*, with a number of untranslated exons subject to alternative splicing in a tissue-specific fashion, the occurrence of mutational events would seem to be quite likely. The possibility must be considered that a complete absence of P450arom activity is potentially lethal, perhaps because of a need for estrogens at the time of implantation of the blastocyst. However, in spite of this, it is now apparent that individuals with mutations in P450arom do exist, and based on the structure of the gene it is possible to predict not only defects due to mutations in the coding region but also tissue-specific defects caused by mutations in specific promoter regions as well as in splice junctions. Thus, for example, a mutation in the 3′-splice site upstream of the start of translation should permit normal expression in the ovary but prevent placental expression. It remains to be determined if such conditions are in fact a reality.

In conclusion, we identified two mutations (R435C and C437Y) in the *CYP19* gene responsible for aromatase deficiency in an adult female with sexual infantilism, primary amenorrhea, ambiguous external genitalia at birth, and multicystic ovaries. Moreover, we confirmed that mutation of the conserved cysteine, C437Y, totally inactivates P450arom. On the other hand, the mutation, R435C, gives rise to a protein with only marginal activity. We have also demonstrated the heritability of one of these two alleles. Therefore, we suggest that some cases of infertility in women may be accounted for by mutations in the *CYP19* gene.

We wish to thank Ms. Melissa Meister for her excellent editorial assistance. This work was supported, in part, by U.S. Public Health Service Grants AG08174 and CA51119. Y.I. was supported, in part, by Juzen Medical Society Grant H41222.

1. Goto, J. & Fishman, J. (1977) *Science* **195,** 80–81.
2. Cole, P. A. & Robinson, C. H. (1988) *J. Am. Chem. Soc.* **110,** 1284–1285.
3. Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K. & Nebert, D. W. (1993) *DNA Cell Biol.* **12,** 1–51.
4. Thompson, E. A., Jr., & Siiteri, P. K. (1974) *J. Biol. Chem.* **249,** 5373–5378.
5. Mendelson, C. R., Wright, E. E., Porter, J. C., Evans, C. T. & Simpson, E. R. (1985) *Arch. Biochem. Biophys.* **243,** 480–491.
6. Nakajin, S., Shimoda, M. & Hall, P. F. (1986) *Biochem. Biophys. Res. Commun.* **134,** 704–710.
7. Kellis, J. T., Jr., & Vickery, L. E. (1987) *J. Biol. Chem.* **262,** 4413–4420.
8. Corbin, C. J., Graham-Lorence, S., McPhaul, M., Mason, J. I., Mendelson, C. R. & Simpson, E. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8948–8952.
9. McNatty, K. P., Baird, D. T., Bolton, A., Chambers, P., Corker, C. S. & MacLean, H. (1976) *J. Endocrinol.* **71,** 77–85.
10. Fritz, I. B., Griswald, M. D., Louis, B. F. & Dorrington, J. H. (1976) *Mol. Cell. Endocrinol.* **5,** 289–294.
11. Valladares, L. E. & Payne, A. H. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4460–4463.
12. Tsai-Morris, C. H., Aquilana, D. R. & Dufau, M. L. (1985) *Endocrinology* **116,** 38–46.
13. Grodin, J. M., Siiteri, P. K. & MacDonald, P. C. (1973) *J. Clin. Endocrinol. Metab.* **36,** 207–214.
14. Naftolin, F., Ryan, K. J., Davies, I. J., Reddy, V. V., Flores, F., Petro, Z., Kuhn, M., White, R. J., Takaoka, Y. & Wolin, L. (1975) *Rec. Prog. Horm. Res.* **31,** 295–319.
15. Roselli, C. E., Horton, L. E. & Resko, J. A. (1985) *Endocrinology* **117,** 2471–2474.
16. Mahendroo, M. S., Means, G. D., Mendelson, C. R. & Simpson, E. R. (1991) *J. Biol. Chem.* **266,** 11276–11281.
17. Mahendroo, M. S., Mendelson, C. R. & Simpson, E. R. (1993) *J. Biol. Chem.* **268,** 19463–19470.
18. Shozu, M., Akasofu, K., Harada, T. & Kubota, Y. (1991) *J. Clin. Endocrinol. Metab.* **72,** 560–566.
19. Harada, N., Ogawa, H., Shozu, M., Yamada, K., Suhara, K., Nishida, E. & Takagi, Y. (1992) *J. Biol. Chem.* **267,** 4781–4785.
20. Harada, N., Ogawa, H., Shozu, M. & Yamada, K. (1992) *Am. J. Hum. Genet.* **51,** 666–672.
21. Davis, L. G., Dibner, M. & Battey, J. (1986) *Methods in Molecular Biology* (Elsevier, New York), pp. 44–46.
22. Miller, G. & Lipman, M. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 190–194.
23. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
24. Lephart, E. D. & Simpson, E. R. (1991) *Methods Enzymol.* **206,** 477–483.
25. Sanghera, M., Simpson, E. R., McPhaul, M. J., Kozlowski, G., Conley, A. J. & Lephart, E. D. (1991) *Endocrinology* **129,** 2834–2844.
26. Harada, N. (1988) *Biochem. Biophys. Res. Commun.* **156,** 725–732.
27. Toda, K., Terashima, M., Mitsuuchi, Y., Yamasaki, Y., Yokoyama, Y., Nojiona, S., Ushiro, H., Maeda, T., Yamamoto, Y., Sagara, Y. & Shizuta, Y. (1989) *FEBS Lett.* **247,** 371–376.
28. Noshiro, M. & Okida, K. (1990) *FEBS Lett.* **268,** 137–140.
29. Curnow, K. M., Slutsker, L., Vitek, J., Milkelsen, M., Cole, T., Speicer, P. W., New, M. I., White, P. C. & Pascoe, L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4552–4556.
30. Mango, D., Montemurro, A., Scirpa, P., Bompiani, A. & Menini, E. (1978) *Eur. J. Obstet. Gynaecol. Reprod. Biol.* **8,** 65–71.