

**BRIEF REPORT: TESTICULAR AND OVARIAN RESISTANCE TO LUTEINIZING HORMONE CAUSED BY INACTIVATING MUTATIONS OF THE LUTEINIZING HORMONE-RECEPTOR GENE**

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**I**N normal males, luteinizing hormone (LH) regulates the function of Leydig cells and, hence, male sexual differentiation, pubertal androgenization, male sexual function, and fertility. Abnormalities in the function of Leydig cells result in primary hypogonadism and varying degrees of male pseudohermaphroditism.<sup>1-5</sup> In these patients, Leydig cells are absent, hypoplastic, or unresponsive to stimulation with human chorionic gonadotropin (hCG), and studies of testicular-biopsy samples from some patients have revealed the absence of LH receptors.<sup>2,3</sup>

In normal women, LH stimulates the theca cells to produce androgen precursors for aromatization to estradiol by granulosa cells during the follicular phase of the menstrual cycle.<sup>6</sup> Subsequently, during its midcycle surge, LH promotes follicular maturation and ovulation, and during the luteal phase, LH induces the formation of the corpus luteum and stimulates progesterone secretion. Thus, abnormalities in the LH receptor would be expected to result in partial ovarian failure characterized by defective folliculogenesis, anovulation, the absence of a luteal phase, delayed or incomplete feminization at puberty, amenorrhea, and infertility.

The human LH receptor belongs to the G protein-coupled superfamily of receptors with seven transmembrane domains.<sup>7</sup> A homozygous missense inactivating mutation in the sixth transmembrane domain of the LH-receptor gene has been identified in two male pseudohermaphrodite siblings with female phenotypes and Leydig-cell hypoplasia.<sup>8</sup> In this report we describe two unrelated kindreds with defects in the differentiation of male external genitalia in genetically male family members and amenorrhea in a genetically female family member. DNA-sequencing analysis revealed ho-

mozygous mutations of the LH-receptor gene in each kindred that impaired the function of the LH receptor and prevented it from transmitting the hormonal signal in the testes and ovaries of the affected patients.

**CASE REPORTS**

**Family 1**

The probands of this family were two phenotypically female siblings, 32 (Subject II-1) and 23 (Subject II-7) years of age, who were referred to the Hospital das Clínicas, University of São Paulo, Brazil, for lack of breast development and primary amenorrhea (Fig. 1A).<sup>5</sup> A third phenotypically female sibling (Subject II-14) was seen later, at the age of 15 years. Their parents were not related by blood. All three siblings had a eunuchoid habitus, an absence of breast tissue, and pubic-hair development of Tanner stage 4 (Subjects II-1 and II-7) and 2 (Subject II-14). They had female external genitalia, with a normal clitoris, an absence of posterior labial fusion, and separate urethral and vaginal openings. Gonads were palpable bilaterally in the inguinal regions, except that the right gonad of Subject II-1 was intraabdominal. The karyotypes of their peripheral-blood leukocytes were 46,XY. Their serum LH concentrations were elevated (49, 41, and 36 IU per liter, in Subjects II-1, II-7, and II-14, respectively; mean [ $\pm$ SD] value for normal men,  $7.7 \pm 4.7$ ), as were serum follicle-stimulating hormone (FSH) concentrations (38, 26, and 30 IU per liter; normal men,  $6.9 \pm 4.9$ ), whereas their serum testosterone concentrations were very low (27, 16, and 25 ng per deciliter [0.93, 0.55, and 0.86 nmol per liter]; normal men, 240 to 1030 ng per deciliter [8.3 to 36 nmol per liter]) and failed to increase after hCG administration. The three patients underwent bilateral gonadectomy. The respective sizes of their left and right gonads were 3 by 2 by 2 cm and 3 by 2 by 1.5 cm for Subject II-1, 3.5 by 2.5 by 2 cm and 4 by 3 by 1 cm for Subject II-7, and 1.8 by 1.2 by 1.5 cm and 1.9 by 1.5 by 1.1 cm for Subject II-14. Histologic analysis showed tubules with thickening of the basal membranes, immature Sertoli cells, and rare spermatogonia in all patients. The interstitium contained fibroblast-like cells but no mature Leydig cells. After their gonadectomy, all three patients were treated with 0.625 mg of oral conjugated equine estrogen; their breasts developed normally to Tanner stage 5. All three patients were raised as females and had a heterosexual orientation. Subjects II-1 and II-7 have each married and have reported satisfactory sexual relations with their spouses; each has adopted a child. Subject II-7 required vaginal dilations.

Subsequently, one additional phenotypically female family member (Subject II-11 in Fig. 1A) was referred for evaluation of amenorrhea at the age of 22 years. Spontaneous gonadarche had occurred at the age of 13 years, and she had a single episode of vaginal bleeding at the age of 20 years. Her height and weight were normal, pubic-hair development was Tanner stage 5, and the breasts and external genitalia were those of a normal woman. Her karyotype was 46,XX. Pelvic ultrasonography revealed a small uterus (volume, 14 ml; normal, 30 to 90) and cystic ovaries of unequal sizes (right, 1.9 ml; left, 7.2 ml). The serum LH concentration was 37 IU per liter (normal value during follicular phase,  $7.1 \pm 3.0$ ), whereas serum FSH and prolactin concentrations were normal (8.7 IU per liter; normal, 3.2 to 10.0; and 16  $\mu$ g per liter; normal, 3 to 23, respectively). The serum estradiol concentration was 32 pg per milliliter (118 pmol per liter; normal value during midfollicular phase in adults,  $39 \pm 15$  pg per milliliter [ $143 \pm 55$  pmol per liter]). Serum progesterone concentrations were below 0.36 ng per milliliter (1.15 nmol per liter) on several occasions. Serum testosterone, androstenedione, and 17-hydroxyprogesterone concentrations were normal.

**Family 2**

The only affected member of Family 2 was a six-year-old phenotypically male child (Subject II-1 in Fig. 2A) who was referred as a neonate to Children's Hospital of New Jersey in Newark for evaluation of micropenis. At birth, the length of his stretched phallus was 1.5 cm (more than 2.5 SD below the normal mean for age). Both testes were descended, with a volume of approximately 1 ml each. There was no family history of male pseudohermaphroditism or hypogonadism, and no history of consanguinity for four generations. The blood leukocyte karyotype

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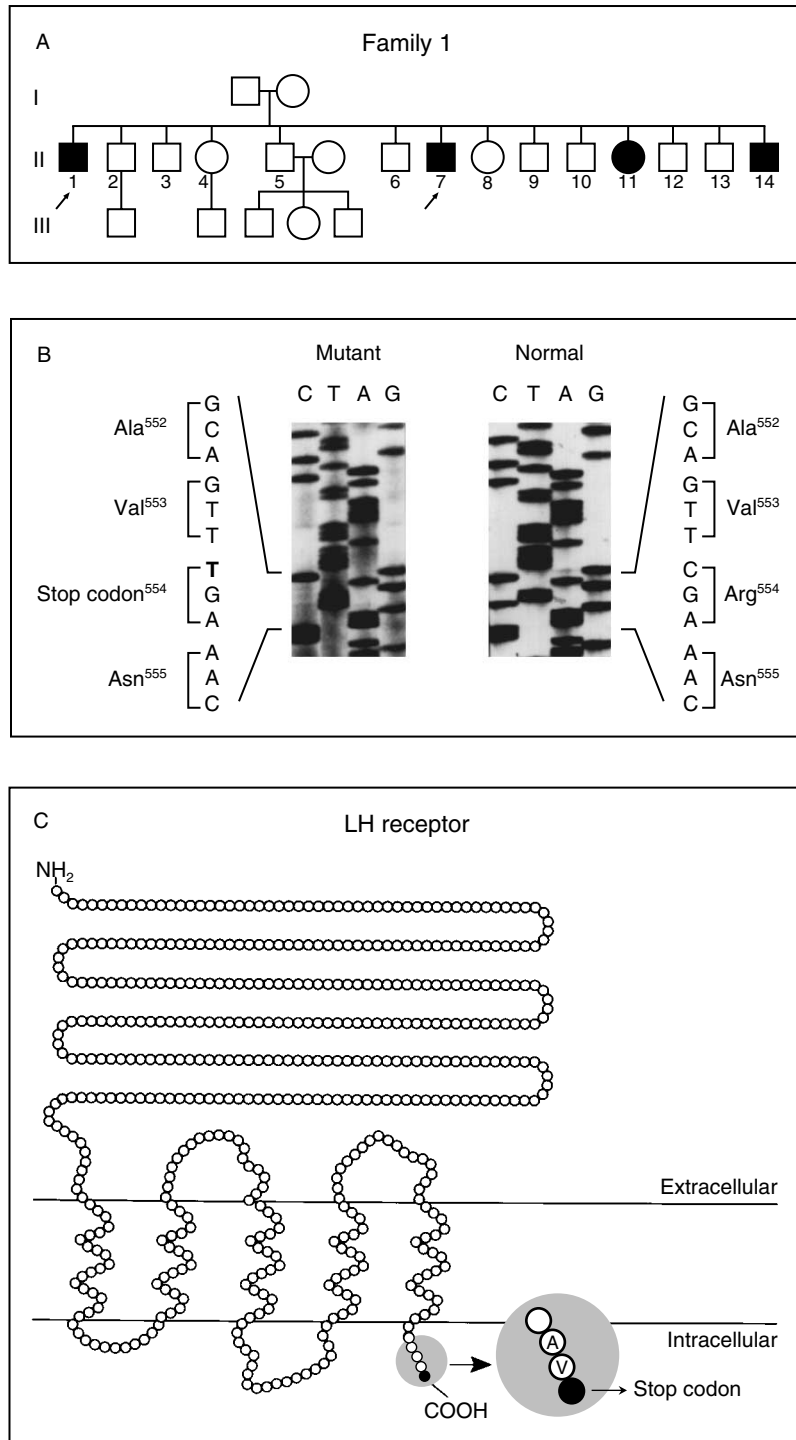


Figure 1. Pedigree of Family 1 (Panel A), Results of Direct Sequencing of the cDNA of the LH-Receptor Gene (Panel B), and the Truncated LH Receptor (Panel C).

In Panel A, the probands are indicated by arrows. Solid symbols denote affected subjects, open symbols unaffected subjects, squares male family members, and circles female family members. In Panel B, direct sequencing of the cDNA of the LH-receptor gene in all four affected family members revealed the homozygous substitution of thymine (T) for cytosine at position 1660, resulting in a stop codon (TGA) in the third cytosolic loop.

mal for age (6.3 IU per liter), and the serum FSH concentration was normal (1.3 IU per liter); both increased normally in response to the intravenous administration of 100  $\mu$ g of gonadotropin-releasing hormone. The serum inhibin concentration was 3.0 IU per milliliter (normal, 4 to 14).

#### METHODS

The study was approved by the institutional review boards of the respective institutions, and appropriate informed consent was obtained from all subjects.

#### DNA Sequencing

DNA-extraction kits (Nucleon II, Scotlab, Strathclyde, United Kingdom) were used to isolate genomic DNA from peripheral-blood samples from selected members of both families, including all affected members in Family 1 and the proband and his parents in Family 2. The entire exon 11 was amplified by the polymerase chain reaction (PCR) with the use of flanking primers as described previously.<sup>9</sup> The PCR products were used to produce single-stranded DNA, which was purified and directly sequenced by the dideoxy chain-termination method, as modified by Kadowaki et al.<sup>10</sup> Inner primers that spanned exon 11 of the LH-receptor gene were used for sequencing, and the reaction products were separated by electrophoresis on a 6 percent polyacrylamide gel.

#### Transfection and Functional Studies

Wild-type and mutant (Tyr<sup>616</sup>) LH-receptor complementary DNA (cDNA) from Family 2 were prepared as previously described.<sup>9</sup> The wild-type and mutant LH-receptor cDNA was cloned into pSVL. Then, 25  $\mu$ g of each pSVL-based construct was added to cuvettes containing  $2 \times 10^7$  COS-7 cells and electroporated.<sup>11</sup> The transfected cells were plated in Dulbecco's modified Eagle's medium (serum-free) with 10 percent fetal-calf serum in 12-well plates ( $4 \times 10^5$  cells per well) for binding studies and 48-well plates ( $10^5$  cells per well) for cyclic AMP (cAMP) assays. Binding of LH to the transfected COS-7 cells and stimulation of cAMP release from the cells were determined 48 hours after electroporation.

Before the binding studies, the transfected cells were washed with binding buffer (serum-free Dulbecco's modified Eagle's medium with 0.1 percent bovine serum albumin). Human LH was iodinated (specific activity, 54.2  $\mu$ Ci per microgram) by the lactoperoxidase meth-

was 46,XY. Treatment with testosterone enanthate at a dose of 25 mg intramuscularly every three weeks for three months resulted in an increase in the length of the phallus to 4 cm. At the age of five years, serum testosterone was undetectable ( $< 10$  ng per deciliter [0.3 nmol per liter]) and remained unmeasurable after hCG stimulation. Serum concentrations of cortisol, 17-hydroxypregnenolone, 17-hydroxyprogesterone, progesterone,  $\Delta$ 4-androstenedione, and estradiol were normal and increased normally in response to 0.25 mg of cosyntropin given intravenously. The serum LH concentration was at the upper limit of nor-

od.<sup>12,13</sup> Binding of LH after two hours of incubation was measured in duplicate at 37°C in 0.4 ml of binding buffer containing human LH labeled with iodine-125 ( $3 \times 10^5$  cpm) and increasing concentrations of unlabeled human LH. The computer program Ligand and a single high-affinity binding-site model, with the best fit of all the data, were used to calculate the binding affinity and maximal binding capacity of LH to transfected cells.<sup>14</sup>

Before cAMP was measured, the transfected cells were washed twice with serum-free Dulbecco's modified Eagle's medium and incubated without LH and then with increasing concentrations of LH (specific activity, 5900 IU per milligram; National Hormone and Pituitary Program, Baltimore) in 200  $\mu$ l of serum-free Dulbecco's modified Eagle's medium containing 0.25 mM 3-isobutyl-1-methyl-xanthine and 0.1 percent bovine serum albumin (Sigma Chemical, St. Louis). In each case samples were incubated in triplicate. The extent of the extracellular accumulation of cAMP in the medium was determined by radioimmunoassay after one hour of incubation at 37°C.<sup>11</sup>

The experiments were repeated with three different batches of transfected cells. The values in each experiment were corrected for the amount of cell protein as determined with the Pierce BCA protein assay (Pierce, Rockford, Ill.), with bovine serum albumin used as the standard.<sup>15</sup>

### Ribonuclease Protection Assay and Reverse-Transcriptase PCR

Total RNA was isolated from COS-7 cells transfected with wild-type LH receptor cDNA, mutant (Tyr<sup>616</sup>) LH receptor cDNA, or pSVL, with Trizol used as a reagent (GIBCO BRL, Life Technologies, Gaithersburg, Md.). The expression of LH-receptor messenger RNA (mRNA) in COS-7 cells transfected with wild-type or mutant LH-receptor cDNA was confirmed by a ribonuclease protection assay (Ambion, Austin, Tex.), which used a complementary RNA probe labeled with phosphorus-32, and reverse-transcriptase PCR (first-strand cDNA-synthesis kit for reverse-transcriptase PCR, Boehringer-Mannheim, Indianapolis).

## RESULTS

### Sequencing of the LH-Receptor Gene

Direct sequencing of the PCR products from the three 46,XY siblings in Family 1 who had female external genitalia and from their 46,XX sister with amenorrhea revealed a homozygous substitution of thymine for cytosine at nucleotide 1660 of the LH-receptor cDNA (Fig. 1B). This mutation changed codon 554 from one coding for arginine (CGA) to a stop codon (TGA) within the third cytosolic loop of the LH receptor (Fig. 1C).

Direct sequencing of the PCR products from the proband of Family 2, who had micropenis, revealed a homozygous substitution of adenine for cytosine at nucleotide 1847 of the LH-receptor cDNA (Fig. 2B). This mutation changed codon 616 from one coding for serine (TCT) to one coding for tyrosine (TAT) within the seventh transmembrane region of the LH receptor (Fig. 2C). The normal mother and father of this subject were heterozygous for this mutation.

### LH Binding and Responsiveness in Cells Transfected with Wild-Type and Mutant LH-Receptor cDNA

Cells transfected with the wild-type LH-receptor cDNA bound labeled LH with high affinity. In contrast, no specific LH binding was found in the cells transfected with mutant (Tyr<sup>616</sup>) LH-receptor cDNA (Fig. 3A).

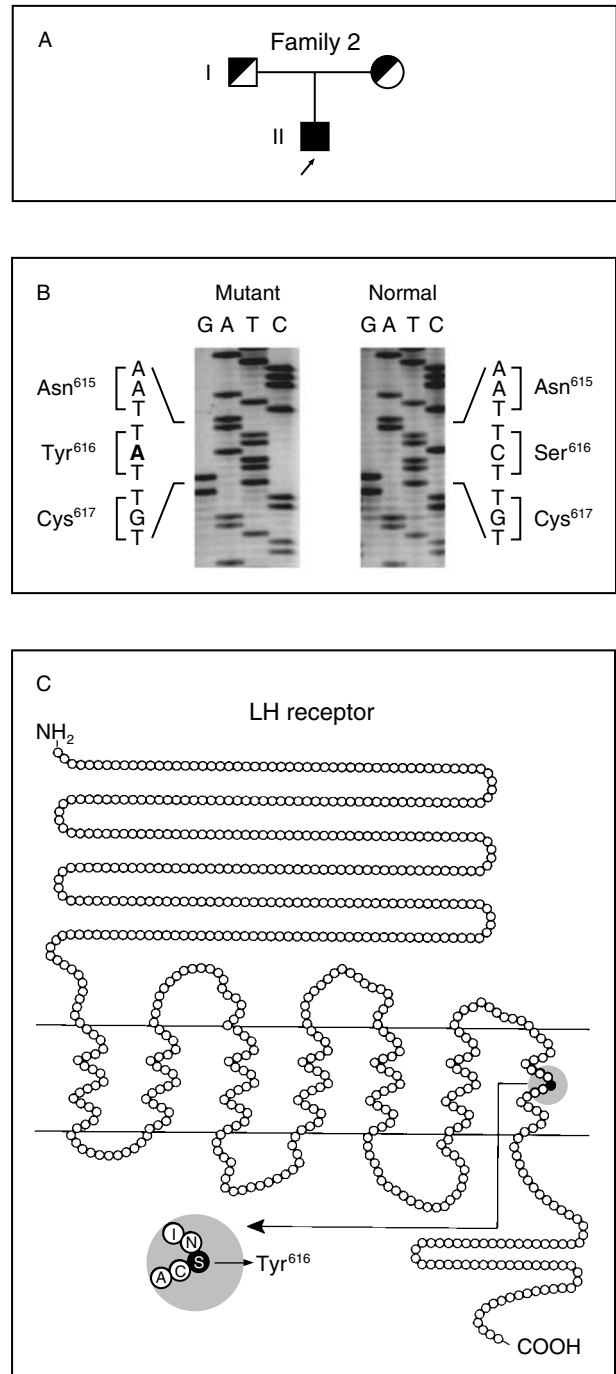


Figure 2. Pedigree of Family 2 (Panel A), Results of Direct Sequencing of the cDNA of the Proband's LH-Receptor Gene (Panel B), and the Mutant Receptor (Panel C).

In Panel A, the proband is indicated by an arrow. Half-solid symbols denote heterozygotes, the solid symbol a homozygote, squares male family members, and the circle a female family member. In Panel B, direct sequencing of the cDNA of the proband's LH-receptor gene revealed the homozygous substitution of adenine (A) for cytosine at position 1847 of the cDNA, resulting in the substitution of tyrosine for serine at position 616 in the seventh transmembrane domain. The parents were heterozygous for this mutation.

The concentrations of basal and LH-stimulated cAMP were compared in COS-7 cells transfected with mutant (Tyr<sup>616</sup>) or wild-type LH-receptor cDNA and cells transfected with pSVL vector alone (Fig. 3B). The basal concentrations were similar in all three types of cells. Cells transfected with the wild-type LH-receptor cDNA responded in a dose-dependent fashion, with a

peak increase of cAMP that was 38 times the base-line value. Cells transfected with the mutant receptor or vector alone did not respond to LH.

#### Expression of LH-Receptor mRNA in Transfected Cells

The ribonuclease protection assay (Fig. 3C) and reverse-transcriptase PCR (Fig. 3D) identified mRNA

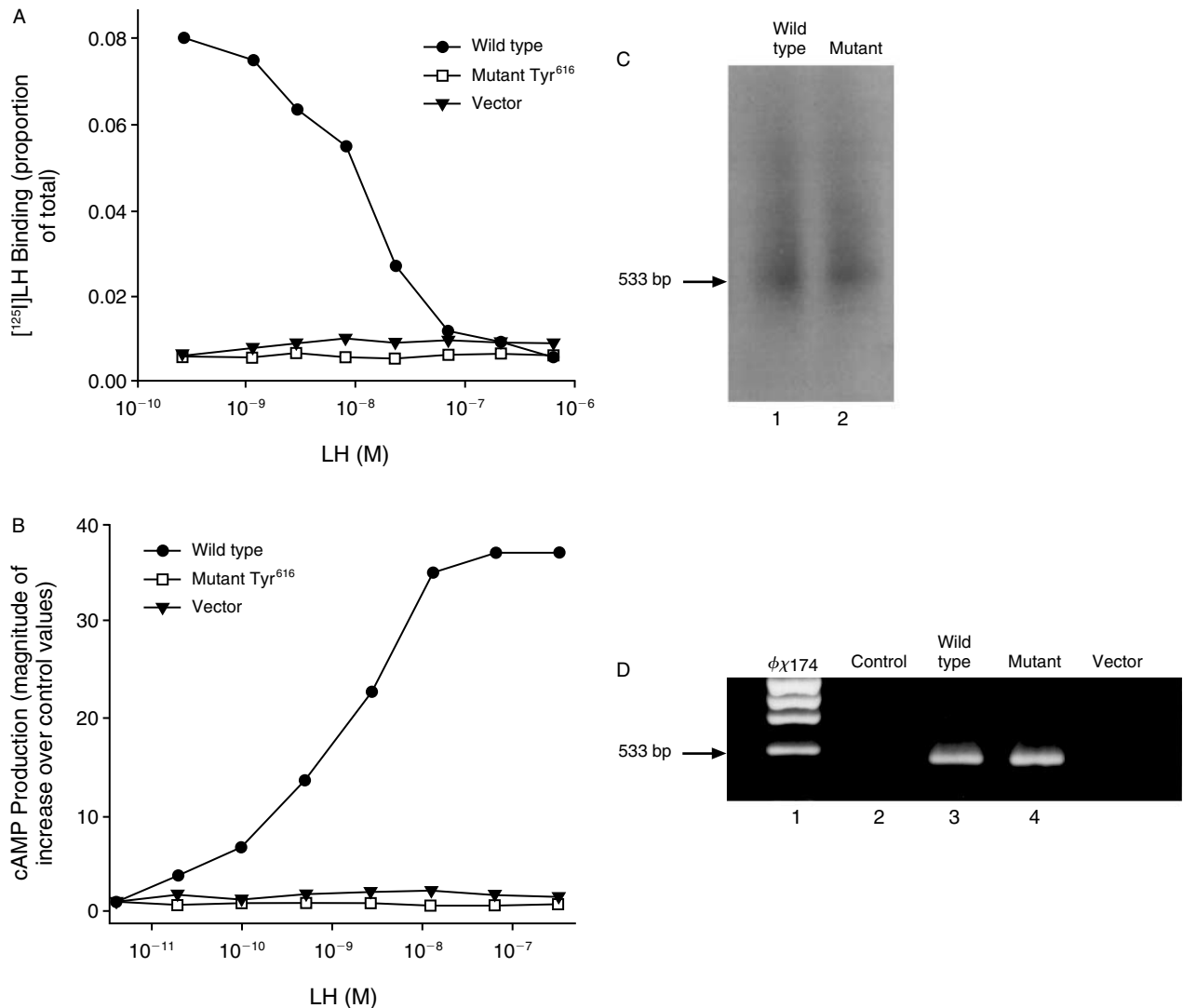


Figure 3. Studies of the Transfected Mutant Receptor in Family 2.

Panel A shows the extent of the displacement of [<sup>125</sup>I]LH by unlabeled LH in COS-7 cells transfected with the wild-type LH receptor, the mutant (Tyr<sup>616</sup>) receptor, or vector alone. Cells transfected with the wild-type receptor had a mean ( $\pm$ SE) maximal binding capacity of  $4.8 \pm 1.1 \times 10^{-9}$  M per milligram and a binding affinity of  $74 \pm 1.9 \times 10^{-9}$  M. No specific binding was seen to cells transfected with the mutant receptor or vector alone. Values are mean results of three experiments involving duplicate incubation mixtures.

Panel B shows the response of cAMP to LH in COS-7 cells transfected with the wild-type LH-receptor cDNA, mutant LH-receptor cDNA, or vector alone. In cells transfected with the wild-type receptor, the mean ( $\pm$ SE) half-maximal elevation of cAMP was  $1.1 \pm 0.5 \times 10^{-9}$  M. There was no response in cells transfected with mutant-receptor cDNA or vector alone. Values are mean results of three experiments involving triplicate incubation mixtures.

Panel C shows the results of a ribonuclease protection assay. A 533-base-pair (bp) antisense RNA probe for the LH-receptor hybridized with RNA from cells transfected with wild-type and mutant (Tyr<sup>616</sup>) cDNA.

Panel D shows the results of reverse-transcriptase PCR with mRNA from cells transfected with wild-type or mutant (Tyr<sup>616</sup>) LH-receptor cDNA or vector alone in 1.6 percent agarose gel. Lane 1 shows a lane marker ( $\phi\chi 174$  RF DNA/*Hae*III), lane 2 a negative control, lane 3 wild-type LH-receptor cDNA, lane 4 mutant LH-receptor cDNA, and lane 5 vector alone.

from both the wild-type and mutant (Tyr<sup>616</sup>) LH receptor in the transfected cells. Levels of the mutant LH-receptor mRNA were normal, but the receptors were apparently unable to bind LH in the transfected cells.

### DISCUSSION

We report two novel homozygous inactivating nonsense and missense mutations of the LH-receptor gene — Arg<sup>554</sup>→stop codon<sup>554</sup> (TGA) and Ser<sup>616</sup>→Tyr<sup>616</sup>, respectively — in three pseudohermaphrodite 46,XY siblings with Leydig-cell hypoplasia and a 46,XX sister with amenorrhea, and a boy with micropenis and primary hypogonadism. An analysis of multiple generations of the two families provided no evidence of consanguinity, but the families came from small, remote villages in Brazil (in the case of Family 1) and Puerto Rico (in the case of Family 2); thus, common ancestry in each family was possible.

The stop codon found in the third intracellular loop of the LH receptor in Family 1 should cause premature interruption of the translation process of the LH-receptor mRNA and consequently eliminate a large part of the receptor (Fig. 1C). Even if expressed in the membrane of target cells, this truncated mutant receptor would be unable to transduce the hormonal signal.<sup>16</sup> Similar mutations introducing stop codons in the third cytosolic loop of the corticotropin receptor, rhodopsin, or vasopressin V2 receptor are associated with hereditary isolated glucocorticoid deficiency, retinitis pigmentosa, and nephrogenic diabetes insipidus, respectively.<sup>17-20</sup>

A complete lack of masculinization of the external genitalia at birth signifies early primary testicular failure. Indeed, the clinical presentation, male pseudohermaphroditism with female external genitalia, and the absence of identifiable mature Leydig cells in the gonads of the three affected 46,XY homozygotes in Family 1 are compatible with complete resistance of Leydig cells to LH. In these patients, male external genitalia failed to develop in utero, but there was some development of pubic hair during and after puberty, most likely in response to normally increasing concentrations of adrenal androgens in this period.

Amenorrhea in fully developed genetically female subjects has been noted in other families with Leydig-cell hypoplasia.<sup>4,8</sup> The LH-receptor mutation of Subject II-11 with a karyotype of 46,XX and amenorrhea in Family 1 apparently compromised the ovulation and luteinization processes while allowing normal apparent pubertal feminization. This subject had a small uterus and cystic ovaries of unequal sizes. The former indicates a decreased cumulative effect of estrogen on the uterus, and the latter may reflect the presence of non-luteinized degenerating follicles. An ovarian biopsy in a 46,XX female subject with amenorrhea who had genetically male siblings with Leydig-cell hypoplasia revealed the absence of a corpus luteum or albicans but a normal number and size of follicles for her age (unpublished data). The normal pubertal feminization in

our subject suggests that in girls LH does not have a major role in pubertal development.

The presence of micropenis at birth signifies nearly normal production and action of testosterone in the first trimester of pregnancy, when the external genitalia form, but suboptimal production or action in the second and third trimesters, when most of the penile growth occurs.<sup>21</sup> Our subject with micropenis had clinical evidence of adequately functioning Leydig cells in the first trimester, but this function failed during the second and third trimesters of gestation and postnatally. These findings could be explained by the defect of the LH-receptor gene described here, which made this receptor unable to bind LH properly.

The unusually large extracellular domain of the LH receptor is responsible for the recognition and high binding affinity of LH. Deletion of the region between residues 616 and 631 of the rat LH receptor (corresponding to residues 612 to 627 in the human LH receptor) caused trapping of the receptor within the endoplasmic reticulum, precluding its appearance on the outer surface of the cell and binding to the ligand.<sup>22-24</sup> The Tyr<sup>616</sup> mutation in Family 2 resided within this crucial region of the LH receptor.

Because the heterozygous parents of the patient with micropenis (Subject II-1 in Family 2) were normal, we conclude that one defective LH-receptor allele causes no abnormality in either sex. This also was true for the obligate heterozygote parents of the affected siblings in Family 1.

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