

## Brief Report

## A FAMILY WITH HYPOGONADOTROPIC HYPOGONADISM AND MUTATIONS IN THE GONADOTROPIN-RELEASING HORMONE RECEPTOR

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**H**YPOGONADOTROPIC hypogonadism is often associated with anosmia in a condition known as Kallmann's syndrome. The gene for the X-linked form of Kallmann's syndrome has been mapped to chromosome Xp22.3,<sup>1</sup> and several mutations have been described.<sup>2-4</sup> In idiopathic hypogonadotropic hypogonadism there is no anosmia, and the involved genes have not been characterized. One possible candidate is the gene for gonadotropin-releasing hormone (GnRH), especially since hypogonadal mice with the deletion of this gene have been identified.<sup>5</sup> However, no abnormality of the gene for GnRH has been found in several patients with idiopathic hypogonadotropic hypogonadism.<sup>6-9</sup> The gene for the GnRH receptor is another candidate in this disease. This gene was recently cloned, and its product proved to be a G-protein-coupled receptor with seven transmembrane segments and an extracellular amino terminus but no intracellular carboxy terminus.<sup>10-13</sup> Activation of this receptor results in increased activity of phospholipase C and mobilization of intracellular calcium by means of the Gq/G<sub>11</sub> group of G proteins.<sup>14</sup> The gene comprises three exons<sup>15</sup> and maps to the long arm of chromosome 4.<sup>15,16</sup>

We describe here a family with idiopathic hypogonadotropic hypogonadism with compound heterozygous mutations of the GnRH-receptor gene. One mutation, in the first extracellular loop of the receptor, dramatically decreased the binding of GnRH to

its receptor. The other mutation, in the third intracellular loop, did not modify the binding of the hormone but decreased the activation of phospholipase C.

### CASE REPORTS

The proband (Subject II-3 in Fig. 1) was a 22-year-old man who was referred because of hypogonadism. Puberty had occurred at the age of 16. His height was 180 cm, his weight 84 kg, and his arm span 186 cm. He reported impaired libido, but it was difficult to ascertain whether he had had sexual intercourse. Physical examination revealed the absence of facial hair, sparse pubic hair (Tanner stage 3), and a penis of 6 cm. He had scrotal testes; the volume of each was 8 ml (normal, 15 to 25 ml). There was no gynecomastia. He had a normal sense of smell and no mirror movements of the upper limbs, no abnormal eye movements, no color blindness, and no renal or craniofacial abnormalities. The results of audiometry and magnetic resonance imaging of the head were normal. The karyotype was 46,XY. The patient's serum testosterone concentration was 80 ng per deciliter (2.8 nmol per liter; normal range, 260 to 690 ng per deciliter [9 to 24 nmol per liter]).

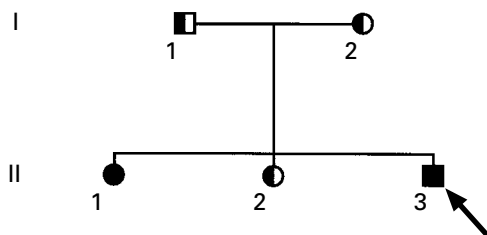
The basal serum luteinizing hormone and follicle-stimulating hormone concentrations were 4.0 IU per liter and 5.9 IU per liter, respectively (normal ranges, 1.0 to 5.0 and 0.9 to 5.7, respectively), and increased normally in response to GnRH (100 µg administered intravenously): luteinizing hormone increased to 24 IU per liter (normal range, 6 to 23), and follicle-stimulating hormone increased to 8.9 IU per liter (normal range, 1.5 to 9). Pulsatile luteinizing hormone secretion was evaluated at 10-minute intervals for 8 hours. The mean (±SE) serum luteinizing hormone concentration was 3.7±0.1 IU per liter, there were 4.5 pulses in eight hours (normal, 4.0±0.8), and the amplitude was 0.8±0.1 IU per liter (normal, 3.9±1.2) (Fig. 2). Results for growth hormone, prolactin, and pituitary-thyroid and pituitary-adrenal function were normal. The semen volume was 0.1 ml, with very low concentrations of seminal androgen markers: fructose, 0.5 µmol per liter (normal in men, >21), and citrate, 1.0 µmol per liter (normal in men, >47). Sperm density was 39.1×10<sup>6</sup> per milliliter, with 5 percent motility and 43 percent normal morphology. The serum ferritin concentration was normal.

The patient's older sister (Subject II-1) was a 37-year-old woman with a history of primary amenorrhea and infertility. Spontaneous thelarche had occurred at the age of 14 years. She had a single episode of uterine bleeding at the age of 18, and afterward received combined oral contraceptive treatment. This treatment was interrupted when she desired children. However, amenorrhea and absence of pregnancy led to ovulation-inducing treatment, which resulted in two normal pregnancies and the births of a girl and a boy currently four and seven years old. After each pregnancy, she had persistent amenorrhea, and oral contraceptive treatment was resumed. At physical examination, her height was 165 cm, and her weight 66 kg. Pubic-hair development was at Tanner stage 5, and her breasts and external genitalia were those of a normal woman. Hormonal evaluation of the pituitary-gonadal axis was performed six weeks after the withdrawal of estrogen and progestin treatment. The plasma estradiol concentration was 35 pg per milliliter (128 pmol per liter; normal range during the early follicular phase, 25 to 90 pg per milliliter [90 to 320 pmol per liter]). The plasma luteinizing hormone and follicle-stimulating hormone concentrations were 5.0 IU per liter and 5.2 IU per liter, respectively (normal ranges, 1.1 to 5.4 and 2.3 to 6.0, respectively). Pelvic ultrasonography showed a normal uterus but two small ovaries (right ovary, 1.8 ml, and left ovary, 1.6 ml) with no dominant follicle larger than 10 mm.

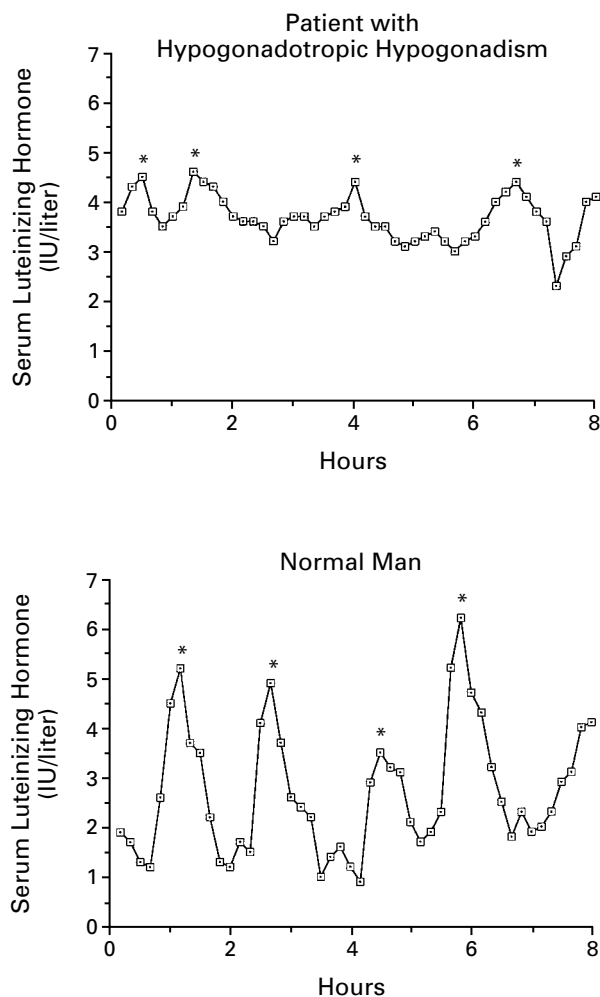
The patient's 62-year-old mother (Subject I-2) and his younger sister (Subject II-2), who was 34 years old, had normal pubertal development and regular menstrual cycles, and Subject II-2 had three children. The women's serum gonadotropin and estradiol concentrations were normal. The 64-year-old father of the proband (Subject I-1) was normally virilized, and his serum gonad-

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**Figure 1.** Pedigree of the Propositus and His Family. The propositus is indicated by an arrow. Solid symbols denote affected subjects, half-solid symbols unaffected heterozygotes, circles female family members, and squares male family members.



**Figure 2.** Endogenous Luteinizing Hormone Secretion Determined at 10-Minute Intervals in the Patient with Hypogonadotropic Hypogonadism and a Normal 24-Year-Old Man. Asterisks denote luteinizing hormone pulses. The patient's serum testosterone concentration was 80 ng per deciliter (2.8 nmol per liter), and the normal man's was 930 ng per deciliter (32 nmol per liter).

otropin and testosterone concentrations were normal. There was no indication of parental consanguinity. All the subjects gave written informed consent for the studies.

## METHODS

### Luteinizing Hormone

Serum luteinizing hormone was measured by immunoradiometric assay (Cis-Bio, Gif-sur-Yvette, France). The intraassay and interassay coefficients of variation were 1.5 and 5.2 percent, respectively. The limit of detection was 0.15 IU per liter. Pulses were analyzed according to the method of Thomas et al.<sup>17</sup>

### DNA Sequencing

Since the intronic sequences close to the exon-intron junctions were not available, we cloned (with the XL PCR kit, Perkin Elmer, Branchburg, N.J.) and sequenced the human GnRH-receptor gene. Eight sequencing primers were designed to amplify the three exons of the GnRH-receptor gene from genomic DNA (information on the primers is available elsewhere\*).

### Transfection and Functional Studies

GnRH-receptor complementary DNA (nucleotides 663 to 2030)<sup>18</sup> was amplified by the polymerase chain reaction (PCR) from a human-pituitary complementary DNA library (Clontech, Palo Alto, Calif.) and cloned into the expression vector PSG5 (Stratagene, La Jolla, Calif.). The mutations were reproduced by oligonucleotide-mediated mutagenesis with PCR or by exchanging a DNA fragment amplified by PCR from the genomic DNA of the propositus.

COS-7 cells in 12-well plates were transfected with 1  $\mu$ g of plasmid and 4 to 6  $\mu$ g of lipofectamine (GIBCO BRL, Gaithersburg, Md.). GnRH binding and the accumulation of inositol phosphates were studied 48 hours after transfection. Binding studies were performed on intact transfected cells with 100,000 cpm of [<sup>125</sup>I]des-Gly<sup>10</sup>, 3-[<sup>125</sup>I]monoiodo Tyr<sup>5</sup>, D-Ala<sup>6</sup>, Pro-ethylamide<sup>9</sup>]-GnRH (<sup>125</sup>I-GnRH-A, 1734 Ci per millimole) as described elsewhere.<sup>19</sup> The affinity for the ligand and the concentration of receptor sites were calculated with Prism software (Graphpad Software, San Diego, Calif.). Triplicate measurements were made of each hormone concentration. The experiment was repeated twice. The accumulation of inositol phosphates in transfected COS-7 cells exposed to various concentrations of GnRH (Sigma Chemical, St. Louis) was measured as described elsewhere.<sup>20</sup>

## RESULTS

### Sequencing of the GnRH-Receptor Gene

Direct sequencing of the PCR products amplified from the DNA of the propositus and his affected sister revealed two heterozygotic mutations in the GnRH-receptor gene. A substitution of guanine for adenine at nucleotide 317 yielded a Gln106Arg mutation in the first extracellular loop of the receptor. The second mutation involved the substitution of adenine for guanine at nucleotide 785, yielding an Arg262Gln mutation in the third intracellular loop (Fig. 3). Both Subject II-1 and Subject II-3 were

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compound heterozygotes. The two parents and the unaffected sister (Subject II-2) were heterozygotes (the mother carrying only the Gln106Arg mutation, and the father and the sister only the Arg262Gln mutation). Neither of these substitutions was found in 20 normal subjects.

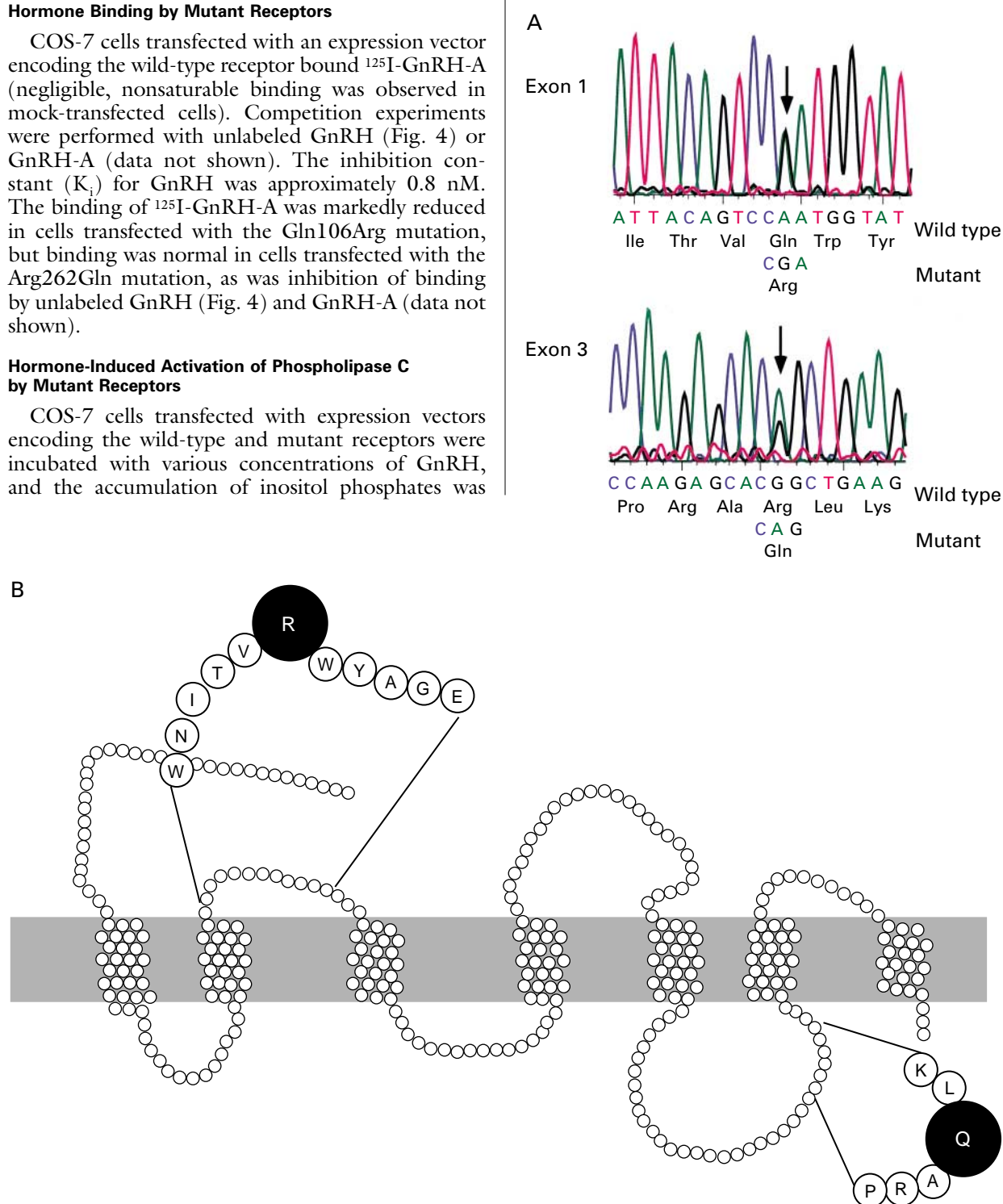
#### Hormone Binding by Mutant Receptors

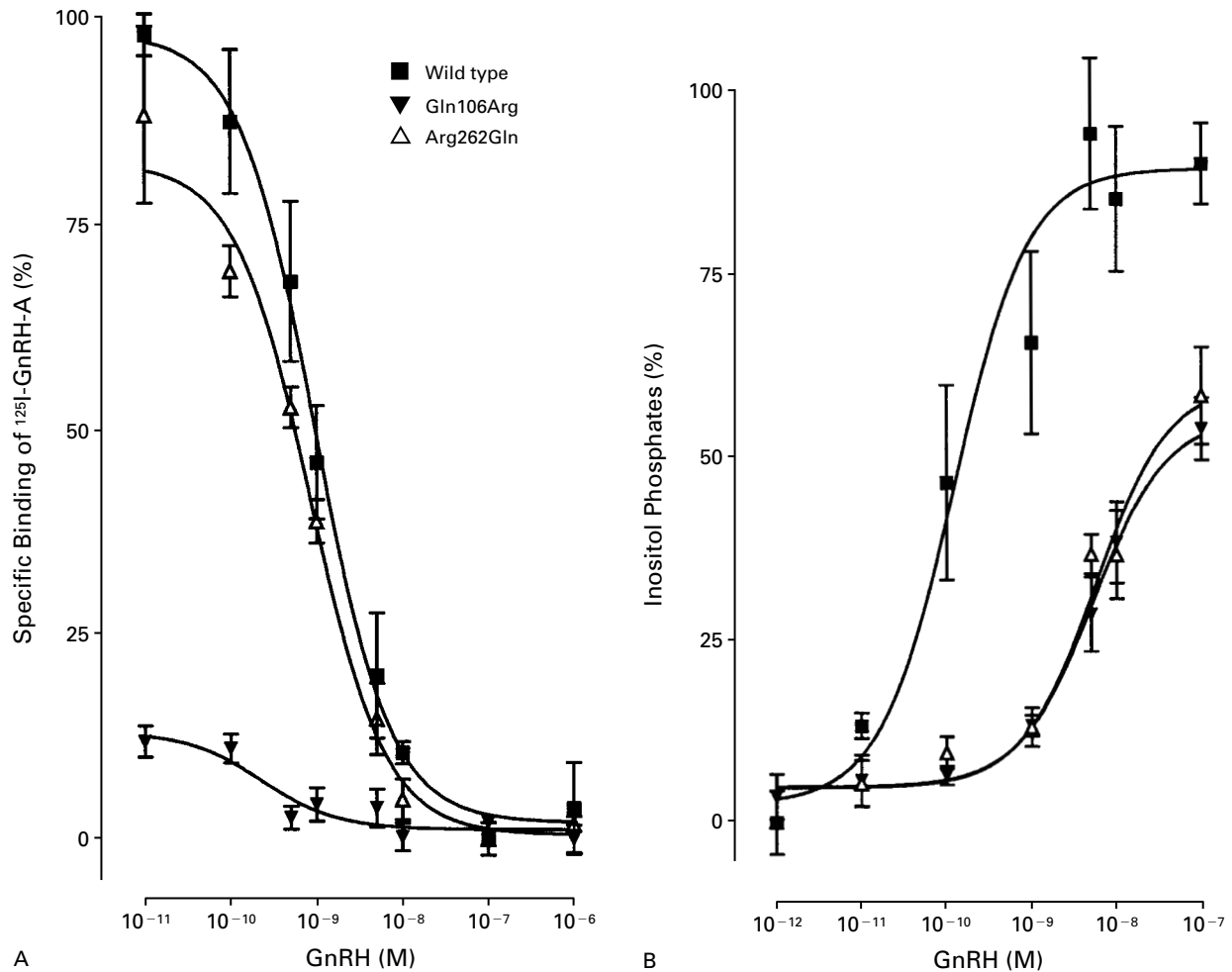
COS-7 cells transfected with an expression vector encoding the wild-type receptor bound  $^{125}\text{I}$ -GnRH-A (negligible, nonsaturable binding was observed in mock-transfected cells). Competition experiments were performed with unlabeled GnRH (Fig. 4) or GnRH-A (data not shown). The inhibition constant ( $K_i$ ) for GnRH was approximately 0.8 nM. The binding of  $^{125}\text{I}$ -GnRH-A was markedly reduced in cells transfected with the Gln106Arg mutation, but binding was normal in cells transfected with the Arg262Gln mutation, as was inhibition of binding by unlabeled GnRH (Fig. 4) and GnRH-A (data not shown).

#### Hormone-Induced Activation of Phospholipase C by Mutant Receptors

COS-7 cells transfected with expression vectors encoding the wild-type and mutant receptors were incubated with various concentrations of GnRH, and the accumulation of inositol phosphates was

**Figure 3.** Automatic DNA Sequencing (Panel A) and Location (Panel B) of the Two Amino Acid Substitutions in the Propositus. The heterozygotic mutations are indicated by arrows in Panel A. In exon 1, the lines for A (green) and G (black) are superimposed at the mutation. In the diagram of the receptor (Panel B), the solid symbols indicate the mutated amino acids arginine (R) and glutamine (Q).





**Figure 4.** Functional Studies of the Wild-Type and Mutant GnRH-Receptor Gene Products.

COS-7 cells were transfected with expression vectors encoding the wild-type or mutant receptors. Panel A shows ligand binding. The cells were incubated with <sup>125</sup>I-GnRH-A (100,000 cpm) alone or in the presence of increasing concentrations of unlabeled GnRH. Each point represents the mean ( $\pm$ SE) of triplicate determinations. The concentration of receptors on the cell surface was measured in two independent experiments. The mean concentrations were  $450 \pm 80$  and  $402 \pm 106$  fmol per milligram of protein for the wild type and the Arg262Gln mutant, respectively. Panel B shows GnRH-induced activation of phospholipase C, expressed in terms of the accumulation of inositol phosphates (100 percent equals the maximal inositol phosphate synthesis in the presence of wild-type receptor). Transfected cells were incubated for 30 minutes with increasing amounts of GnRH.

measured (Fig. 4). The responses were similar with both mutant receptors. The concentration of GnRH needed to produce half-maximal increases with the two mutant receptors ( $6 \times 10^{-9}$  M) was approximately 50 times higher than the concentration needed with the wild-type receptor, and the maximal response of cells transfected with each mutant receptor was decreased by about 50 percent as compared with the maximal response of cells transfected with the wild-type receptor (Fig. 4).

#### DISCUSSION

We report here two loss-of-function mutations of the GnRH receptor in a man and his sister present-

ing with partial hypogonadotropic hypogonadism. Both patients were compound heterozygotes. The parents and one sister were heterozygotes and had a normal phenotype. The disorder was thus transmitted as an autosomal recessive trait. Since administration of GnRH increases the secretion of follicle-stimulating hormone and luteinizing hormone in patients with idiopathic hypogonadotropic hypogonadism, it was previously considered unlikely that the disease could result from mutations in the GnRH receptor.<sup>21</sup> However, partial-loss-of-function mutations have been reported in the genes for other G-protein-coupled receptors, such as the thyrotropin receptor<sup>22-24</sup> and the luteinizing hormone re-

ceptor.<sup>25-27</sup> This led us to search for GnRH-receptor defects in familial cases of incomplete idiopathic hypogonadotropic hypogonadism. Moreover, a defect of the thyrotropin-releasing hormone receptor has recently been described.<sup>28</sup>

The male proband presented with a typical case of incomplete idiopathic hypogonadotropic hypogonadism. The study of luteinizing hormone pulsatility revealed a normal pulse frequency with a decreased amplitude, findings compatible with a partial defect of the receptor. However, the patterns are similar in men with isolated GnRH deficiency and men with the so-called fertile eunuch syndrome.<sup>29,30</sup> In addition, results of the GnRH stimulation test were normal, probably because the high dose of GnRH overcame the partial receptor defect. The older sister of the proband had primary amenorrhea, infertility, and small ovaries without follicular maturation.

Diseases caused by natural mutations provide many insights into the relations between structure and function in receptors. The Gln106Arg substitution is located in the first extracellular loop of the GnRH receptor. An experimentally produced mutation in the same region (Asn102Ala) results in nearly complete abolition of GnRH binding with a decreased biologic response, much like the Gln106Arg mutation in this family.<sup>31</sup> The conservation of a partial biologic response may be due to the formation of relatively unstable hormone-receptor complexes that do not withstand the washing procedures used in the binding studies. It is not known whether this region of the first extracellular loop interacts directly with the ligand or whether it has a conformational role in the protein.

The second mutation in the patients (Arg262Gln) affected a residue located in the third intracellular loop. Hormone binding was unchanged, but signal transmission was impaired. An experimental mutation of Ala261 results in impaired G-protein coupling and receptor internalization.<sup>32</sup> In a variety of other G-protein-coupled receptors, the third intracellular loop is critical for signal transmission.<sup>33</sup> The incomplete phenotype in our patients is compatible with the partial impairment of the hormonal response of the mutant receptors in transfection experiments. It is worth noting that the two amino acids mutated in these patients are totally conserved in the GnRH receptors of a variety of species.<sup>14</sup> In conclusion, these studies show that familial hypogonadotropic hypogonadism may be due to mutations in the GnRH receptor.

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*A View of New York*

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