

## Brief Report

## LEYDIG-CELL TUMORS CAUSED BY AN ACTIVATING MUTATION OF THE GENE ENCODING THE LUTEINIZING HORMONE RECEPTOR

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**T**HE receptor for luteinizing hormone and chorionic gonadotropin plays a major part in normal and abnormal reproductive function.<sup>1-4</sup> In males, activation of the receptor regulates the development and function of Leydig cells.<sup>5</sup> Testosterone secreted by Leydig cells promotes male sexual differentiation, pubertal androgenization, and fertility. The human luteinizing hormone receptor is a G protein-coupled receptor with a transmembrane domain composed of seven segments. Activation of the receptor by luteinizing hormone leads to activation of G<sub>s</sub>, the G protein that is coupled to adenylyl cyclase, and to an increase in cyclic AMP (cAMP). High concentrations of luteinizing hormone or of chorionic gonadotropin can also stimulate production of inositol phosphates and 1,2-diacylglycerol by phospholipase C, although the physiologic role of this secondary pathway remains unclear.<sup>1,4,6</sup>

Loss-of-function mutations of the gene for the luteinizing hormone receptor in males cause pseudohermaphroditism associated with Leydig-cell hypoplasia, supporting the concept that a functional receptor is necessary for the early development of Leydig cells.<sup>2,3,7</sup> Activating mutations of the receptor, on the other hand, cause gonadotropin-independent male-limited precocious puberty, a disorder characterized by autonomous hyperplasia and hyperfunction of Leydig cells in association with inappropriate stimulation of adenylyl cyclase and the cAMP signaling pathway,<sup>2,3,8,9</sup> but little or no activation of the phospholipase C pathway. The most common mutation is a change from aspartic acid to glycine at position

578 (Asp578Gly) in the sixth transmembrane segment of the receptor.

Leydig-cell adenomas are the most prevalent hormone-producing tumors of the testis and account for 1 to 3 percent of all testicular tumors.<sup>10-12</sup> They are usually benign, but 10 percent of tumors in adults are malignant. Boys with Leydig-cell tumors typically have signs of isosexual precocity as a result of testosterone secretion by the tumor.

The demonstrated role of the luteinizing hormone receptor in the proliferation of Leydig cells and the presence of germ-line and somatic mutations in the gene for the homologous thyrotropin receptor in familial nonimmunogenic hyperthyroidism and sporadic thyroid adenomas, respectively,<sup>13,14</sup> led us to hypothesize that some Leydig-cell adenomas might be caused by novel, activating somatic mutations of the luteinizing hormone-receptor gene. We now describe such a mutation in Leydig-cell adenomas in three boys.

### CASE REPORTS

The clinical characteristics of the three boys with isosexual precocity are summarized in Table 1. Each boy presented with early pubertal development that had begun one to two years previously. None had a family history of sexual precocity. After hormonal evaluation revealed gonadotropin-independent hypersecretion of testosterone, the possibility that the precocity was due to congenital adrenal hyperplasia or a chorionic gonadotropin-secreting tumor was excluded. The volume of the testicles was asymmetrically increased in Patients 1 and 2, and a discrete testicular mass was palpable in Patient 2. Testicular ultrasonography revealed a unilateral mass in all three patients. Unilateral orchiectomy was performed in Patients 1 and 3, and a testis-sparing surgical procedure was performed in Patient 2.

### METHODS

#### Amplification and Sequencing of DNA

Written informed consent was obtained from the patients' parents after the study protocol had been approved by the institutional review board. Slides of paraffin-embedded sections of tissue that had been stained with hematoxylin and eosin were used to identify discrete regions of interest (tumor and normal testis). Small, isolated regions of tissue were then scraped into microfuge tubes, and DNA was extracted as described elsewhere.<sup>17</sup> Genomic DNA from blood was obtained by standard methods.

DNA prepared from paraffin-embedded tissue or blood was amplified by the polymerase chain reaction (PCR) in 100  $\mu$ l of reaction mixture containing 0.1 mM deoxynucleotide triphosphates, 2.5 mM magnesium chloride, *Taq* Gold polymerase buffer and 0.5 U of *Taq* Gold polymerase (Perkin-Elmer Applied Biosystems, Foster City, Calif.), and 0.1 mM upstream and downstream primers. Primers (5'TGTAAAACGACGGTTTGCAGTTCGA-AACCCAGAATTAA3' and 5'CAGGAAACAGCTATGACCTG-AAGGCAGCTGAGATGGCAAAA3') contained -21M13 and M13 sequences (underlined) at their 5' ends and were designed to amplify a short segment of exon 11 that encodes the sixth transmembrane domain. Amplification consisted of denaturation at 95°C for 10 minutes, followed first by 40 cycles of annealing at 55°C for 1 minute, extension at 72°C for 30 seconds, and denaturation at 95°C for 1 minute and then one final cycle with a 3-minute primer extension. The remainder of exon 11 was amplified with primers that have been described elsewhere.<sup>18</sup>

DNA sequencing was performed with the ABI Prism Big Dye Primer cycle-sequencing kit with Ampliqaq DNA polymerase and an ABI 377 sequencer (all Perkin-Elmer Applied Biosystems).

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**TABLE 1.** CLINICAL CHARACTERISTICS OF THREE BOYS WITH ISOSEXUAL PRECOCITY AND LEYDIG-CELL TUMORS.\*

| CHARACTERISTIC  | PATIENT 1 | PATIENT 2 | PATIENT 3 |
|---|-----------|-----------|-----------|
| Age at diagnosis (yr)                                 | 8.3       | 7.5       | 7.8       |
| Height  |           |           |           |
| Centimeters   | 142       | 154       | 140       |
| No. of SDs above the mean for age and sex             | 3.0       | 5.6       | 2.8       |
| Bone age (yr)   | 10        | 13        | 11        |
| Tanner stage for pubic hair                           | 3         | 4         | 3         |
| Testicular volume (ml)†                               |           |           |           |
| Right   | 5         | 10        | 4         |
| Left  | 2         | 6         | 4         |
| Dimensions of tumor on ultrasonography (mm)           | 8×7×6     | 15×10×10  | 8×6×2     |
| Serum testosterone (ng/dl)‡                           | 150       | 335       | 79        |
| Serum luteinizing hormone (IU/liter)                  |           |           |           |
| Base line   | <0.5      | <0.8      | 1.1       |
| Peak after intravenous gonadotropin-releasing hormone | <0.5      | 1.2       | 2.8       |
| Serum follicle-stimulating hormone (IU/liter)         | 0.7       | 0.8       | <2        |

\*Bone age, an index of skeletal maturation, was assessed as described previously by comparing radiographs of the hand and wrist with standards of maturation in a normal population.<sup>15</sup> Tanner stage was assessed as described previously.<sup>16</sup>

†In Patients 1 and 2, the mass was detected by ultrasonography in the right testicle; in Patient 3, it was detected in the left testicle.

‡To convert values for serum testosterone to nanomoles per liter, multiply by 0.035. The normal serum testosterone concentration in prepubertal boys is less than 20 ng per deciliter.

Data were analyzed with Sequence Navigator software, version 1.0.1 (Applied Biosystems).

#### Allele-Specific *BsrI* Digestion

The substitution of cytosine for guanine at nucleotide 1732 (G1732C) that encoded replacement of aspartic acid with histidine at amino acid position 578 (Asp578His) was detected by creating a new *BsrI* restriction-enzyme site (New England Biolabs, Beverly, Mass.) in the DNA sequence adjacent to the mutation. Amplification was conducted with the sense primer 5'GTGGAA-ACCACTCTCTCACAAGTCTA3' and the antisense primer 5'AAA-AAAAGAGATAGGTGCCATGCAGGTGAACT3', in which adenine at one position was replaced by a cytosine (underlined). This variant oligonucleotide primer allowed *BsrI* digestion, converting a 210-bp PCR product into 176- and 34-bp fragments, only when the mutant allele was present.

#### Characterization of the Functional Properties of Mutant Luteinizing Hormone Receptor

The Asp578His mutation was generated in the complementary DNA (cDNA) for human luteinizing hormone receptor in the expression vector pSG5 with use of the Transformer Mutagenesis Kit (Clontech, Palo Alto, Calif.), and its presence was confirmed by sequencing. The wild-type DNA and mutant DNA were prepared with plasmid-purification kits (Qiagen, Chatsworth, Calif.). Lipofectamine (GIBCO-BRL, Gaithersburg, Md.) was used for the transient transfection of COS-7 cells ( $2 \times 10^6$  in a 100-mm dish) with 10  $\mu$ g of plasmid DNA.

Eighteen hours after transfection, the cells were replated for binding assays and assays of cAMP and inositol phosphate production, as previously described.<sup>19</sup> Forty-eight hours after transfection, cAMP was measured by iodine-125 radioimmunoassay (Biomedical

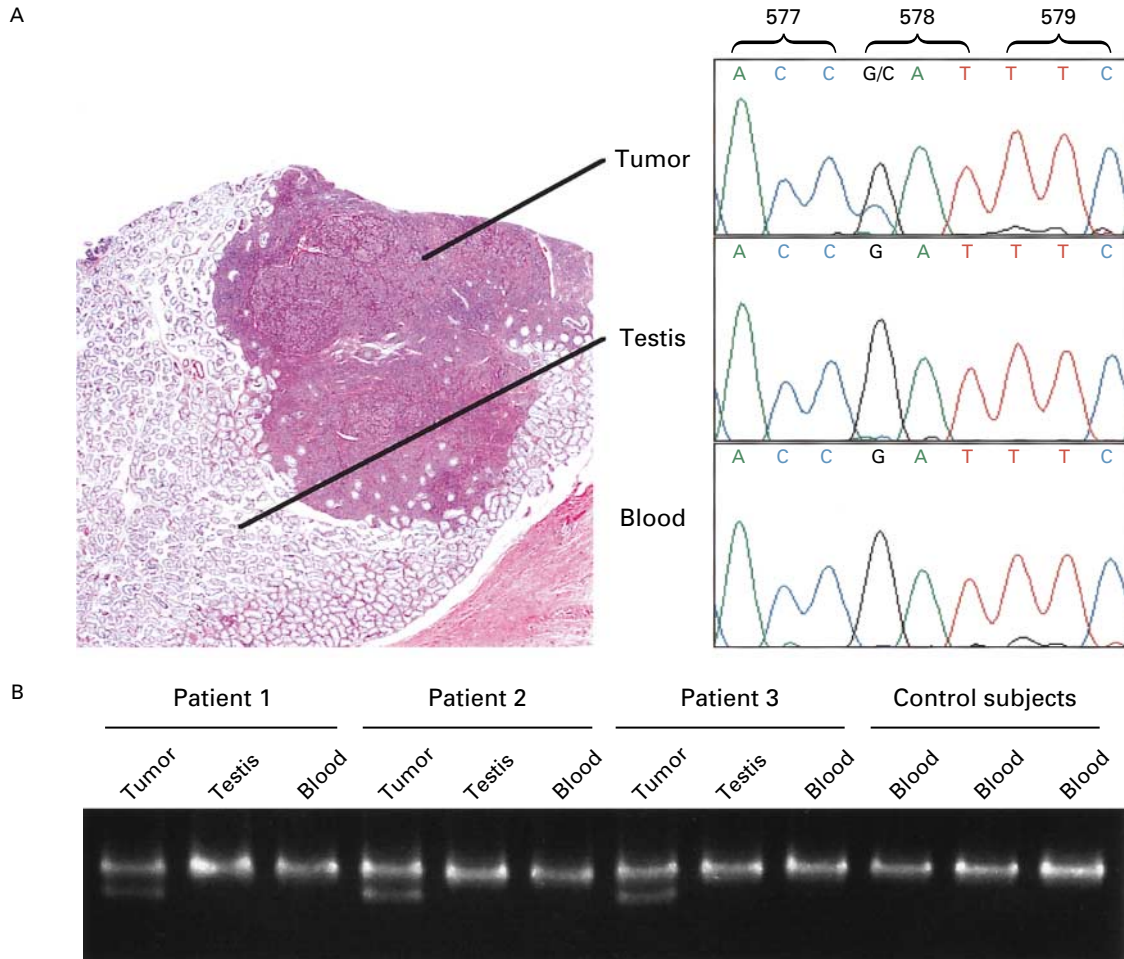
Technologies, Stoughton, Mass.). Total inositol phosphates were measured by means of anion-exchange column chromatography (Dowex AG1-X8, Bio-Rad, Richmond, Calif.). Data were analyzed with Prism software (version 2.0, GraphPad, San Diego, Calif.).

## RESULTS

In all three cases, the tumor was well circumscribed and was composed of nests of polygonal cells with abundant eosinophilic cytoplasm and round or ovoid nuclei. Mitotic activity was low, and Reinke's crystalloids were not seen. Immunohistochemical staining for p53 protein<sup>20</sup> was negative. Genomic DNA was extracted from the Leydig-cell adenoma, adjacent normal testis tissue, and blood leukocytes from Patient 1 (Fig. 1A). PCR products encoding exon 11 of the luteinizing hormone-receptor gene, which includes the region of the gene that encodes the sixth transmembrane segment, were generated from these templates. DNA sequencing revealed a heterozygous guanine-to-cytosine mutation at nucleotide 1732 in the tumor only. This novel somatic mutation, resulting in the change of GAT to CAT, encodes replacement of the aspartic acid at position 578 of the receptor with histidine (Asp578His) (Fig. 1A).

Screening for *BsrI* digestion indicated that the Leydig-cell tumors from Patients 2 and 3 were also composed of cells that were heterozygous for the somatic mutation Asp578His (Fig. 1B), findings that were subsequently confirmed by direct DNA sequencing. PCR products generated from 50 unaffected control subjects did not have evidence of digestion with *BsrI* (data not shown), indicating that the mutation is not a common polymorphism of the luteinizing hormone-receptor gene.

To assess the functional effects of the Asp578His mutation, wild-type and mutant forms of the luteinizing hormone receptor were transiently expressed in COS-7 cells, and the binding of chorionic gonadotropin and the production of cAMP and inositol phosphates were measured. The characteristics of receptors containing the Asp578His mutation were directly compared with those of receptors containing a tyrosine residue at this position in place of aspartic acid (Asp578Tyr) (Fig. 2). The Asp578Tyr mutation is associated with severe precocious puberty in boys, characterized by diffuse Leydig-cell hyperplasia.<sup>9,19,21</sup> Binding experiments with iodine-125-labeled chorionic gonadotropin revealed that the receptor containing the Asp578His mutation was more highly expressed at the cell surface than the wild-type receptor (mean [ $\pm$ SE] maximal binding capacity,  $402 \pm 78$  percent of wild type in five experiments) or that containing the Asp578Tyr mutation (maximal binding capacity,  $279 \pm 17$  percent of wild type), but its affinity for chorionic gonadotropin (dissociation constant, 1 nM) was similar to that of the latter two receptors (data not shown). The Asp578His mutation caused an agonist-independent increase in the basal production of cAMP ( $28 \pm 3$  times the basal pro-



**Figure 1.** Microscopical Appearance and Results of DNA Analysis of Testicular Tissue from Boys with Leydig-Cell Tumors.

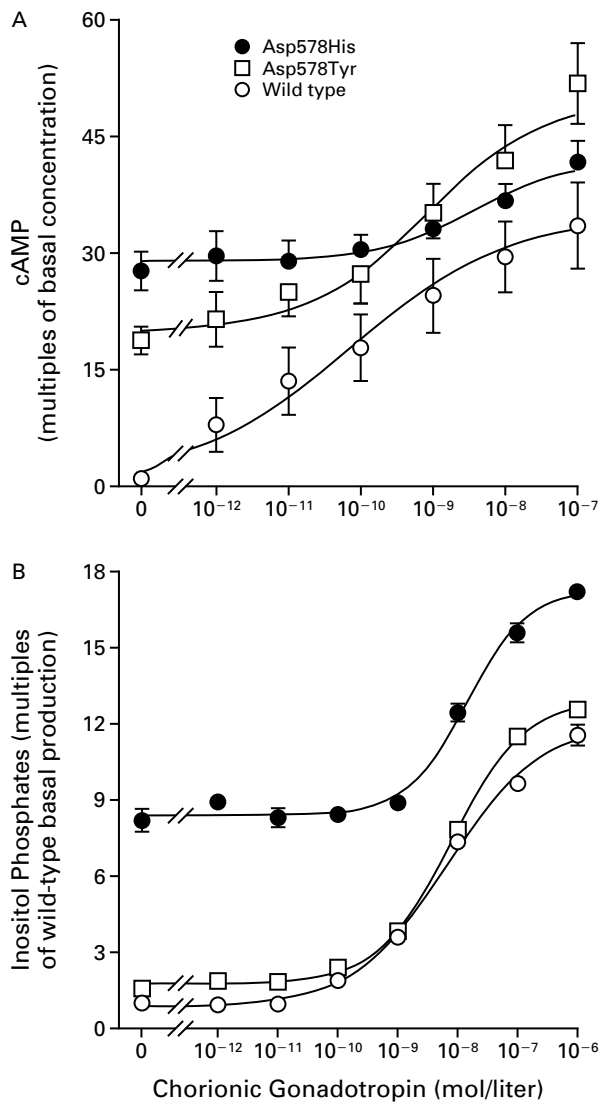
On the left-hand side of Panel A, a cross section of a paraffin-embedded section of testis from Patient 1 is shown (hematoxylin and eosin,  $\times 2$ ). Areas of tumor and adjacent normal testis tissue were dissected and analyzed separately. The results of direct sequencing of a short segment of the gene for the luteinizing hormone receptor from tumor, normal testis, and blood, after PCR amplification, are shown on the right-hand side of Panel A. A heterozygous guanine-to-cytosine (G/C) mutation that results in the replacement of aspartic acid with histidine at codon 578 was found only in the tumor specimen, indicating that the mutation is somatic. Panel B shows *BsrI* restriction-enzyme analysis of PCR products generated with a variant primer from specimens of tumor, normal testis, and blood from the three patients and blood from three unaffected control subjects. Only the tumor samples show the presence of both uncut (210-bp) and digested (176-bp) fragments.

duction in the wild-type receptor, or 80 percent of the maximal effect of chorionic gonadotropin in the wild-type receptor in four experiments), which was greater than that caused by Asp578Tyr ( $19 \pm 2$  times the basal production in the wild-type receptor in four experiments) or any other known mutation of the luteinizing hormone receptor.<sup>2,3,8,9,19</sup> Moreover, the basal production of inositol phosphates in the Asp578His mutant was seven times that in the wild-type receptor (70 percent of the maximal effect of chorionic gonadotropin in four experiments). The maximal chorionic gonadotropin-stimulated produc-

tion of inositol phosphates was also greater than that in the wild type. Basal inositol phosphate production was also increased in cells expressing a low concentration of Asp578His receptors (data not shown), indicating that a high level of constitutive activity is an intrinsic property of the Asp578His receptor and not merely a result of the overexpression of receptors.

#### DISCUSSION

Leydig-cell adenomas are one cause of gonadotropin-independent sexual precocity in boys, a condition that may also be mediated by virilizing forms of



**Figure 2.** Comparison of Receptors with the Asp578His or Asp578Tyr Mutation and the Wild-Type Receptor.

Mean ( $\pm$ SE) basal and chorionic gonadotropin-stimulated accumulation of cAMP (Panel A) and inositol phosphates (Panel B) is shown in COS-7 cells transfected with DNA for the wild-type luteinizing hormone receptor or receptors with the Asp578Tyr or Asp578His mutation. Data in Panel A are means ( $\pm$ SE) from four experiments. Data in Panel B are from one representative experiment performed in triplicate. Basal activity of the wild-type luteinizing hormone receptor is the same as that of the pSG5 expression vector alone.<sup>19</sup> Basal cAMP production of the wild-type receptor was  $1.1 \pm 0.2$  pmol per  $10^5$  cells, and basal inositol phosphate production was  $431 \pm 67$  cpm per  $10^5$  cells.

adrenal hyperplasia, adrenal tumors, tumors that produce chorionic gonadotropin, male-limited precocious puberty, and McCune–Albright syndrome (due to an activating mutation of arginine at position 201 in the  $\alpha$  subunit of  $G_s$ ).<sup>22-24</sup> In boys with male-limited precocious puberty, signs of sexual development usually appear before the age of four years,<sup>23</sup> but in boys with Leydig-cell tumors, these signs typically appear later, between the ages of five and nine.<sup>11</sup> Because small testicular masses may not be palpable, all boys with gonadotropin-independent hypersecretion of testosterone and no family history of precocious puberty should undergo testicular ultrasonography.

The identification of a novel somatic mutation, Asp578His, in the gene encoding the luteinizing hormone receptor in three boys with Leydig-cell tumors provides a new example of how activating mutations of G protein-coupled receptors can cause disease.<sup>25</sup> As with the homologous thyrotropin receptor,<sup>14</sup> it appears that somatic mutations of the gene for the luteinizing hormone receptor may be associated with a more highly activating phenotype than the hereditary mutations. Spontaneous Leydig-cell tumors are common in laboratory animals, and drugs that cause high serum luteinizing hormone concentrations can increase the incidence of Leydig-cell hyperplasia and adenomas in rodents.<sup>26</sup> In humans, Leydig-cell hyperplasia and tumors appear to be distinct entities.<sup>22,27</sup> Boys with precocious puberty due to activating mutations in the gene for the luteinizing hormone receptor have not been reported to be at increased risk for Leydig-cell tumors,<sup>22</sup> although there is one documented occurrence of a testicular seminoma in such a patient.<sup>28</sup>

The causes of Leydig-cell tumors are likely to be heterogeneous. Although luteinizing hormone signaling plays a major part in Leydig-cell proliferation, alterations in other local stimuli, including müllerian-duct inhibitory factor, inhibin, growth factors, and temperature, may also create conditions favorable to tumorigenesis.<sup>5,29,30</sup> The activating arginine-to-cysteine mutation Arg201Cys in the  $\alpha$  subunit of  $G_s$  was recently detected in Leydig-cell tumors from three adult women and one man, but not in a tumor from a boy with sexual precocity.<sup>31</sup> In a preliminary screening, we have found the Asp578His mutation in three of five additional Leydig-cell tumors, but none had a mutation of arginine at position 201 (unpublished data). Although Leydig-cell tumors are not common in patients with McCune–Albright syndrome or male-limited precocious puberty, it is plausible that somatic mutations that promote increased cellular production of cAMP and an increased rate of cell division represent one early event in the neoplastic process.<sup>32</sup>

The adenoma-associated mutation occurs at the conserved aspartic acid residue at position 578, where substitution by glycine has been found to cause the

most common form of male-limited precocious puberty (characterized by patchy Leydig-cell hyperplasia) and where substitution by tyrosine has been associated with a more severe clinical phenotype (characterized by diffuse Leydig-cell hyperplasia).<sup>9,21</sup>

Under normal conditions, hormone-mediated activation of the luteinizing hormone receptor in Leydig cells probably does not result in stimulation of the phospholipase C pathway.<sup>1,4</sup> Because the main feature that distinguishes the Asp578His mutation from receptor mutations associated with Leydig-cell hyperplasia is its ability to activate this pathway (Fig. 2B), it is tempting to speculate that neoplastic transformation of Leydig cells involves inappropriate costimulation or synergism of the cAMP and phospholipase C pathways. Costimulation of these pathways by a constitutively active mutant  $\alpha_{1B}$ -adrenergic-receptor transgene has been implicated in animals with thyroid neoplasia.<sup>33</sup> Furthermore, a somatic mutation corresponding to Asp578His in the thyrotropin receptor (Asp633His) has been described in a patient with a rare, autonomously hyperfunctioning insular thyroid carcinoma that had metastasized to a cervical lymph node and to the lungs.<sup>34</sup> The Asp633His mutation in the thyrotropin receptor also has been found to cause constitutive activation of both the cAMP and phospholipase C pathways (unpublished data). Many other receptors coupled to the activation of phospholipase C stimulate cell proliferation and transformation, although these effects may in fact be independent of traditional signaling pathways.<sup>35,36</sup>

Supported by grants from the American Cancer Society (IRG-93-037-04, to the Robert H. Lurie Comprehensive Cancer Center, and Illinois Division grant 98-31, to Dr. Shenker), the National Institutes of Health (5T32-DK07169-19A1), the French Ministry of Foreign Affairs (a Lavoisier grant, to Dr. Duranteau), and the Philippe Foundation (to Dr. Duranteau). Dr. Shenker is the Crown Family Young Investigator in Developmental Systems Biology.

We are indebted to Dr. David Waltherhouse, Dr. Barry Rich, Dr. Jean Bienaymé, Dr. Bernard Boudailliez, and Michele Irving for referral of patients; to Dr. Ritu Nayar, Dr. Susan Crawford, Dr. Pauline Chou, Dr. Frank Gonzalez-Crussi, and Dr. Patrick Barbet for performing the histologic analyses; to Tom Kotlar, Dr. Peter Kopp, Dr. Larry Jameson, and Dr. Angelo DiGeorge for valuable suggestions; and to the National Hormone and Pituitary Program for supplying chorionic gonadotropin.

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