

BRIEF REPORT: RESISTANCE TO THYROTROPIN CAUSED BY MUTATIONS IN THE THYROTROPIN-RECEPTOR GENE

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HORMONE-resistance syndromes can be broadly defined as conditions resulting from reduced or absent end-organ responsiveness to biologically active hormones. They are caused by defects in hormone receptors or post-receptor defects.¹⁻³ Mutations in the thyroid hormone-receptor β gene cause resistance to thyroid hormone, which is characterized by elevated serum thyroid hormone concentrations with few or no clinical and biochemical manifestations of thyroid hormone excess and, most notably, normal or slightly increased thyrotropin secretion.¹ Mutations that inactivate the thyrotropin receptor or the G (guanine nucleotide-binding) protein that couples the receptor to adenylate cyclase should cause thyrotropin resistance, resulting in either hypothyroidism or euthyroidism with increased thyrotropin secretion, depending on the completeness of the defect. There have been several reports of patients with congenital hypothyroidism,^{4,6} including some with familial hypothyroidism,⁷ and an apparent resistance to the action of thyrotropin. However, sequencing of the thyrotropin and thyrotropin-receptor genes in these patients revealed no abnormalities.⁸

We describe three siblings who were euthyroid and had normal serum concentrations of thyroid hormone but high concentrations of thyrotropin. They had mutations in both alleles of the thyrotropin-receptor gene, one inherited from each parent. The mutant thyrotropin receptor inherited from the father had almost no biologic activity, and that inherited from the mother had reduced activity.

CASE REPORTS

The proband, the second of three daughters born to unrelated parents, had a blood thyrotropin concentration of 103 mU per liter (normal, <20) on neonatal screening. Her thyroid gland was normal on radioiodide scanning. At 16 days of age, she had a serum thyrotropin concentration of 47 mU per liter and a serum thyroxine (T_4) concentration of 9.2 μ g per deciliter (119 nmol per liter); the 24-hour uptake of radioiodide by the thyroid was 23 percent (normal, 8 to 30 percent). Because of the high serum thyrotropin values, she was treated with T_4 .

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These results prompted the testing of her older sister (Daughter 1), then four years of age, whose physical and mental development was normal. Her serum thyrotropin concentration was 80 mU per liter (normal, 0.5 to 6.2), and her serum T_4 concentration was 9.8 μ g per deciliter (126 nmol per liter; normal, 6.0 to 13.0 μ g per deciliter [77 to 167 nmol per liter]). She had a normal thyroidal radioiodide scan, with a three-hour uptake of 9 percent. One year later she was treated with T_4 at a daily dose of 50 μ g, which reduced her serum thyrotropin concentration to 38 mU per liter. Four years later, the youngest daughter (Daughter 3) was also found to have a high blood thyrotropin concentration (96 mU per liter) at birth, with a normal T_4 concentration (13.0 μ g per deciliter [168 nmol per liter]). After the high thyrotropin value was confirmed by its measurement in serum (53 mU per liter), she was treated with T_4 .

All family members had thyroid glands of normal size, and none had symptoms or signs of hypothyroidism at any time. The three girls continued to develop normally without adjustment of their T_4 doses, which at the time of our study were lower than the usual replacement dose (Table 1). The results of thyroid-function tests in the three girls before and 3, 6, and 12 months after the discontinuation of T_4 therapy as well as in their parents are shown in Table 1. Additional studies in the eldest girl conducted two months after T_4 was discontinued revealed a serum glycoprotein hormone α -subunit concentration of 0.6 μ g per liter (normal, <1.0) and no serum antithyrotropin antibodies, as determined by the binding of radiolabeled thyrotropin to serum immunoglobulins. Her serum thyrotropin concentration increased from 66 mU per liter to a peak of 338 mU per liter 15 minutes after the intravenous administration of 400 μ g of thyrotropin-releasing hormone. Serum calcium, parathyroid hormone, luteinizing hormone, and follicle-stimulating hormone concentrations were all normal. The child's bone age was 14 years at a chronologic age of 12.3 years. The parents consented to these studies.

METHODS

Tests of Thyroid Function

Serum T_4 and triiodothyronine (T_3) concentrations were measured by radioimmunoassay (Diagnostic Products, Los Angeles), and thyrotropin by chemiluminescence assay (Nichols Institute, San Juan Capistrano, Calif.). The serum free T_4 index was calculated as the product of the serum T_4 and T_4 -resin uptake values.⁹ Serum free T_4 and free T_3 concentrations were measured by equilibrium dialysis (Nichols Institute).

Clinical Studies

The responsiveness of the pituitary and peripheral tissues to thyroid hormone was evaluated in the eldest daughter (Daughter 1).¹ She was given a dose of 25 μ g of T_3 every 12 hours for three days, followed by a 50- μ g dose every 12 hours for three days. Blood samples were obtained before and 12 hours after the last 25- μ g and 50- μ g dose for the measurement of serum T_4 , T_3 , free T_4 index, thyrotropin, sex hormone-binding globulin, alkaline phosphatase, cholesterol, and creatine kinase.

Preparation of Genomic DNA, RNA, and Complementary DNA and DNA Sequencing

Genomic DNA was isolated from peripheral-blood leukocytes. Total RNA was extracted from the same source by the acid guanidinium thiocyanate technique.¹⁰ The coding regions (exons 2 and 3) of the thyrotropin β gene and exon 10 of the thyrotropin-receptor gene were sequenced, with genomic DNA used as the template. Sequences of exon 1 through the 5' end of exon 10 of the thyrotropin-receptor gene were obtained from complementary DNA (cDNA) synthesized by reverse transcription of very small amounts of thyrotropin-receptor messenger RNA from blood mononuclear cells (illegitimate transcription). DNA was amplified by the polymerase chain reaction (PCR) with specific oligonucleotide primers, subcloned into M13 bacteriophages or pBluescript plasmids, and then sequenced (Sequenase,

U.S. Biochemical, Cleveland). The sequences of the oligonucleotide primers used are available elsewhere.*

Confirmation of the Mutations and Haplotyping

To confirm the presence of each mutation and polymorphism (cytosine or adenine at position 253),¹¹ degenerate oligonucleotide primers complementary to sequences near but not overlapping the variant nucleotide were synthesized. The primers were designed so that the product of amplification would create a unique restriction site only in the presence of the variant nucleotide (endonuclease-digestion allele-specific-primer method).^{11,12}

After the subjects' genomic DNA was amplified by PCR, the DNA fragments were digested with the appropriate enzymes and then subjected to electrophoresis in a 3 percent NuSieve-1 percent agarose gel. Partial cleavage of the PCR products indicated that the mutant nucleotide was present in one of the two alleles.

Construction of Wild-Type and Mutant Thyrotropin-Receptor cDNA Expression Vectors

The full-length wild-type thyrotropin-receptor cDNA was cloned into pSVL.¹³ Appropriate DNA fragments carrying each mutation and polymorphism identified in the subjects were replaced to generate vectors expressing alanine at position 162 (Ala¹⁶²), threonine at position 52 and alanine at position 162 (Thr⁵²-Ala¹⁶²), and asparagine at position 167 (Asn¹⁶⁷). The final constructs were verified by sequencing.

The reporter-gene construct, -846 α -Luc, responsive to cyclic AMP,¹⁴ contained 846 base pairs (bp) of the 5'-flanking sequence and 44 bp of exon 1 of the human glycoprotein α -subunit gene linked to the luciferase gene in the plasmid pA3 Luc.

Functional Studies of the Thyrotropin Receptors in a Transient Transfection System

COS-7 cells were propagated in Dulbecco's modified Eagle's medium (GIBCO BRL, Gaithersburg, Md.) containing 10 percent fetal-calf serum at 37°C and 5 percent carbon dioxide. The cells were plated in 12-well dishes in concentrations of 2×10^5 cells per well and transfected 24 hours later by the calcium phosphate precipitation method¹⁵ with 1 μ g of the reporter vector, -846 α -Luc, and 1 μ g of each of the thyrotropin-receptor expression vectors described above. Eight to 12 hours after transfection, the cells were washed and incubated for 48 hours with the complete medium in the absence or presence of various amounts of recombinant human thyrotropin (Genzyme, Cambridge, Mass.). The cells were lysed and assayed for luciferase activity (Promega, Madison, Wis.). The individual data points we report are the means (\pm range) for duplicate

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Table 1. Tests of Thyroid Function in Members of a Family with Resistance to Thyrotropin.*

SUBJECT	AGE	T ₄ THERAPY	T ₄	T ₃	FREE T ₄	FREE T ₄	FREE T ₃	THYROTROPIN
			μ g/dl	ng/dl	INDEX	ng/dl	pg/dl	mU/liter
Daughter 1	12	75 μ g/day	8.5	117	8.9	0.9	250	50
		None for 3 mo	6.8	106	7.0	—	—	69
		None for 12 mo	8.0	103	8.5	—	—	66
Daughter 2	9	50 μ g/day	9.1	125	9.1	1.3	276	44
		None for 3 mo	8.2	155	6.9	—	—	88
		None for 6 mo	7.2	142	6.7	—	—	73
Daughter 3	5	25 μ g/day	9.0	137	8.5	1.6	340	46
		None for 6 mo	8.1	169	7.2	—	—	55
Father	40	None	6.4	118	8.3	1.3	286	4.3
Mother	38	None	7.8	120	7.7	1.3	268	3.9
Normal range		—	5.0–12.0	80–180†	6.0–10.5	0.8–2.7‡	260–480	0.4–3.6

*Thyroglobulin and thyroid peroxidase antibodies were not detected in any serum samples. To convert values for T₄ to nanomoles per liter and free T₄ to picomoles per liter, multiply by 12.87; to convert values for T₃ to nanomoles per liter and free T₃ to picomoles per liter, multiply by 0.015.

†The normal range in children is 90 to 210 ng per deciliter (1.38 to 3.23 nmol per liter).

‡The normal range in children is 0.8 to 2.0 ng per deciliter (10.3 to 25.7 pmol per liter).

Table 2. Responses to the Administration of T₃ in the Eldest Daughter (Daughter 1) in a Family with Resistance to Thyrotropin and in Nine Normal Subjects.*

SUBSTANCE MEASURED	BASE LINE IN DAUGHTER	50 μ g OF T ₃ PER DAY		100 μ g OF T ₃ PER DAY	
		DAUGHTER	NORMAL SUBJECTS	DAUGHTER	NORMAL SUBJECTS
Serum T ₄ — % of base line (μ g/dl)	100 (7.7)	78 (6.0)	82 \pm 10	64 (4.9)	71 \pm 9
Serum T ₃ — % of base line (ng/dl)	100 (126)	225 (284)	207 \pm 30	410 (516)	411 \pm 68
Serum free T ₄ index — % of base line	100 (7.8)	83 (6.5)	85 \pm 8	68 (5.3)	74 \pm 7
Serum thyrotropin — % of base line (mU/liter)	100 (66)	10 (6.4)	28 \pm 25	1 (0.8)	8.0 \pm 9.3
Serum sex hormone-binding globulin — % of base line (nmol/liter)	100 (9)	89 (8)	120 \pm 30	178 (16)	136 \pm 21
Serum alkaline phosphatase — % of base line (U/liter)	100 (104)	106 (110)	101 \pm 5	120 (125)	115 \pm 7
Serum cholesterol — % of base line (mg/dl)	100 (161)	92 (148)	94 \pm 7	75 (120)	78 \pm 12
Serum creatine kinase — % of base line (U/liter)	100 (123)	97 (119)	88 \pm 12	69 (85)	78 \pm 11

*To convert values for T₄ to nanomoles per liter, multiply by 12.87; to convert values for T₃ to nanomoles per liter, multiply by 0.015; to convert values for cholesterol to millimoles per liter, multiply by 0.0259. The values for the normal subjects are mean (\pm SD) percentages of their base-line concentrations.

incubation mixtures, expressed as multiples of the base-line level of luciferase activity in the absence of thyrotropin.

RESULTS

The results of thyroid-function tests of all family members are shown in Table 1. The distinctive features of the syndrome in the three daughters were high serum thyrotropin concentrations and normal serum free T₄ index, free T₄, and free T₃ values. Both parents had slightly increased serum thyrotropin concentrations and normal serum T₄ and T₃ concentrations. Discontinuation of T₄ treatment in the three girls resulted in an increase in serum thyrotropin concentrations, though the magnitude of the increase varied. Three and six months after the discontinuation of T₄ treatment, se-

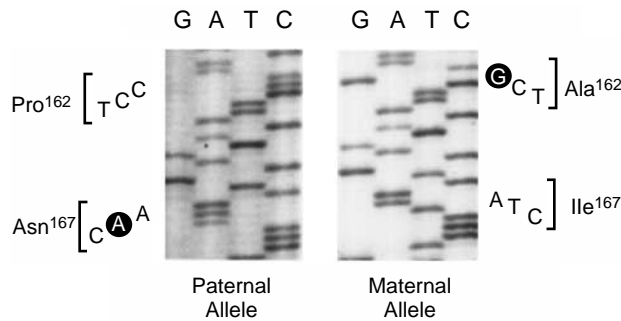


Figure 1. Sequencing Gel Showing the Mutations in Exon 6 of the Thyrotropin Receptor in the Eldest Daughter of a Family with Resistance to Thyrotropin.

G denotes guanine, A adenine, T thymine, and C cytosine. The substituted nucleotides (G for C and A for T) are circled in black.

rum T_4 and free T_4 index values were lower than those measured during therapy and, in two of the three girls, serum T_3 concentrations were higher. At one year, the serum T_4 , T_3 , and free T_4 index values equaled those measured during T_4 treatment. The girls' growth continued to be normal.

The results of administering two doses of T_3 to the eldest daughter are shown in Table 2. The fractional decrease in the serum thyrotropin concentration and the changes in serum sex hormone-binding globulin, alkaline phosphatase, cholesterol, and creatine kinase concentrations were normal, indicating normal sensitivity of the pituitary thyrotrophs and peripheral tissues to thyroid hormone. Furthermore, the decrease in serum thyrotropin was accompanied by a corresponding decline in serum T_4 and free T_4 values, indicating that the secretion of thyroid hormone was dependent on thyrotropin.

The coding region from 10 clones of the thyrotropin β gene isolated from the eldest daughter had normal sequences. This result indicated that the defect did not involve the β subunit of thyrotropin, which confers the biologic activity of the hormone, and therefore that the abnormality was most likely in a step mediating the action of thyrotropin.

The thyrotropin-receptor gene of the eldest daughter was then sequenced in its entirety. Different nucleotide substitutions were detected in each of the two alleles (Fig. 1), indicating the presence of compound heterozygosity. Both mutations were located in exon 6, which encodes the midportion of the extracellular domain of the thyrotropin receptor. In one allele the normal thymine at position 599 was replaced by an adenine, resulting in the replacement of isoleucine by asparagine at position 167. In the other allele, the normal cytosine at position 583 was replaced by a guanine, resulting in the replacement of proline by alanine at position 162. The latter allele also contained a previously described polymorphic variant in exon 1 (threonine [ACC] instead of proline [CCC] at position 52).¹¹

We confirmed the presence of the same nucleotide substitutions in all three girls. Furthermore, we traced

each of the two mutant alleles to the corresponding parent (Fig. 2). The paternal allele contained adenine at position 599, and the maternal allele guanine at position 583. The heterozygous state of each parent was confirmed by the presence of one normal allele (Fig. 2).

The functional activities of the mutant thyrotropin receptors and the wild-type receptor are shown in Figure 3. In cells transfected with the wild-type thyrotropin receptor, the maximal luciferase activity induced by thyrotropin was 20 times the basal level. Approximately 10 times more thyrotropin was required for an equal effect in cells transfected with maternal mutant thyrotropin receptor (Thr⁵²-Ala¹⁶²). The thyrotropin responses of cells containing the thyrotropin receptor with Ala¹⁶² and the usual proline at position 52 (Pro⁵²-Ala¹⁶²) were similar to those of the maternal mutant thyrotropin receptor (Thr⁵²-Ala¹⁶²). Cells containing the paternal mutant thyrotropin receptor (Asn¹⁶⁷) had almost no thyrotropin-inducible activity (Fig. 3A). Cotransfection of the wild-type thyrotropin receptor with each of the mutant thyrotropin receptors, to simulate the heterozygous state of the parents, resulted in responses to low thyrotropin concentrations indistinguishable from those of cells expressing the wild-type

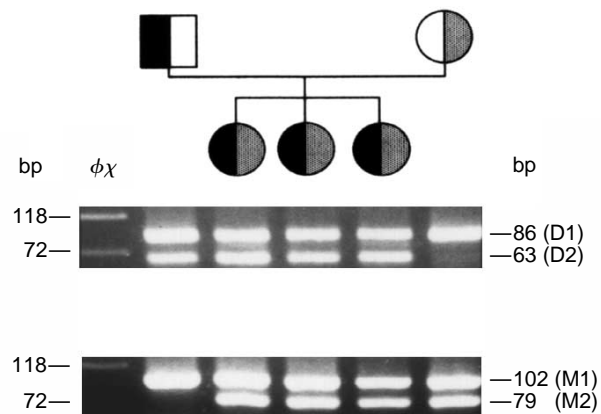


Figure 2. Confirmation of the Mutations in the Thyrotropin-Receptor Gene in Members of the Study Family.

Genomic DNA from peripheral-blood leukocytes was amplified in separate PCR reactions with the use of the same antisense primer (5'-actggaataactcacAGTGCA3') and with two degenerated sense primers: 5'ACAGACAACCCTTACATGACITIAA3', which produces a *Dra*I restriction site in the presence of adenine at position 599, and 5'ctctgcagTGAAATTG CAGAC3', which produces a *Mwo*I restriction site in the presence of guanine at position 583 (the degenerated nucleotides are underlined, and intronic sequences are lowercase). The upper gel shows that the father and his three daughters all have a mutant allele containing adenine at position 599 that is digested with *Dra*I (D2), producing a 63-bp fragment of DNA, and an allele resistant to digestion with *Dra*I (D1) containing the thymine normally found at position 599. The lower gel shows the mutant allele containing guanine at position 583 found in the mother and her three daughters that is digested with *Mwo*I to produce a 79-bp fragment of DNA (M2) as well as an allele containing the cytosine normally found at position 583 that resists digestion (M1). The pedigree above the gels shows the pattern of inheritance of the mutant thyrotropin-receptor alleles. The DNA size marker ($\phi\chi$) was digested with *Hae*III.

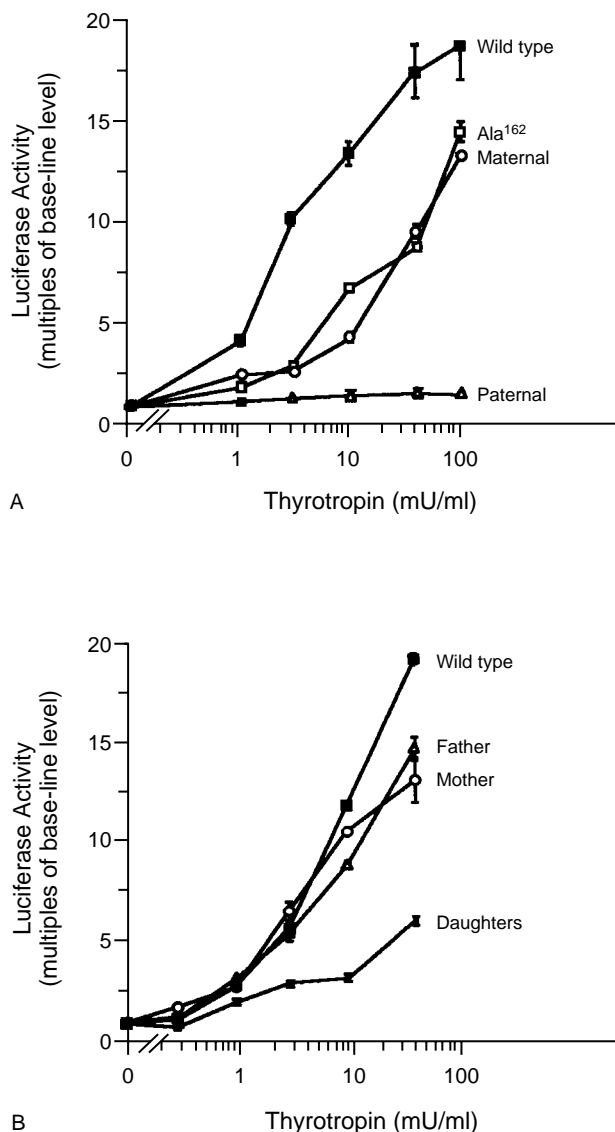


Figure 3. Biologic Function of the Mutant Thyrotropin Receptors and the Wild-Type Thyrotropin Receptor.

Thyrotropin receptors, cloned into a pSVL expression vector, were cotransfected with a cyclic AMP-responsive reporter vector into COS-7 cells. The mean (\pm range) responses to thyrotropin are expressed as multiples of the base-line level of cyclic AMP-dependent luciferase activity. Panel A shows the thyrotropin-inducible activity of the vector expressing the wild-type thyrotropin receptor (Wild type), the vector expressing alanine at position 162 (Ala¹⁶²), the vector expressing threonine at position 52 and alanine at position 162 (Maternal), and the vector expressing asparagine at position 167 (Paternal). Panel B shows the thyrotropin-inducible activity of the wild-type thyrotropin receptor coexpressed with each of the mutant thyrotropin receptors to simulate the heterozygous state of the parents (Mother and Father) and coexpression of equal amounts of the maternal and paternal mutant thyrotropin receptors to simulate the compound heterozygous state of their three daughters. Note that thyrotropin responsiveness is nearly normal in the conditions simulating the heterozygous state of the parents and that almost 20 times more thyrotropin was required in the presence of both mutant thyrotropin receptors, as found in the daughters, to produce the effect mediated by the wild-type thyrotropin receptor alone.

thyrotropin receptor alone and a slightly reduced response at high thyrotropin concentrations. In contrast, in cells transfected with equal amounts of mutant maternal and paternal thyrotropin receptors, to simulate the compound heterozygous state of the three daughters, almost 20 times more thyrotropin was required to produce the same effect as in cells transfected with the wild-type thyrotropin receptor alone (Fig. 3B).

DISCUSSION

Thyrotropin exerts its biologic action by binding to the extracellular domain of the thyrotropin receptor located on the plasma membrane of thyroid follicular cells. This interaction is believed to cause a structural change in the intracellular domain of the receptor. The main effect of the latter is activation of the α_s subunit of the G protein, which stimulates the activity of adenylate cyclase and leads to the generation of cyclic AMP, which mediates virtually all the biologic effects of thyrotropin.^{16,17} The thyrotropin receptor is encoded by a single gene located on chromosome 14.¹⁶ Recently, somatic^{18,19} and germ-line²⁰ mutations have been reported in the thyrotropin-receptor gene (Fig. 4) as well as in the G-protein gene^{2,21} that conferred constitutive activation of adenylate cyclase, resulting in autonomous hyperthyroidism.

In the family we studied, all three siblings had high serum thyrotropin concentrations and normal T₄ concentrations. The abnormality was demonstrated at birth in two of the three siblings. The persistence of the high serum thyrotropin concentrations was not compatible with transient infantile hyperthyrotropinemia.²² The normal growth and development of the eldest girl (Daughter 1), who did not receive thyroid hormone until the age of five years, suggested that her increased thyrotropin secretion was not due to primary hypothyroidism. The persistent hyperthyrotropinemia in the three siblings suggested that the disorder was inherited. The borderline elevation of serum thyrotropin concentrations in the parents indicated that the inheritance was recessive. Nevertheless, there was no history of consanguinity, a contention supported by the different ethnic origins of the parents (a German father and an Italian-Bohemian mother).

Among the possible defects, that involving the G protein, as has been described in pseudohypoparathyroidism,² was least likely, since the abnormality was confined to the thyroid. Short-term administration of T₃ demonstrated intact regulation of thyrotropin secretion and normal responses of peripheral tissues, ruling out abnormalities of the thyroid hormone receptor. Furthermore, thyroid secretion was thyrotropin-dependent. Thus, a variant thyrotropin molecule with reduced biologic activity or a defective thyrotropin receptor was the most likely cause of the abnormality in this family. An abnormality in the α subunit of thyrotropin was unlikely because of its normal concentration in serum as well as because of the normal serum luteinizing hormone and follicle-stimulating

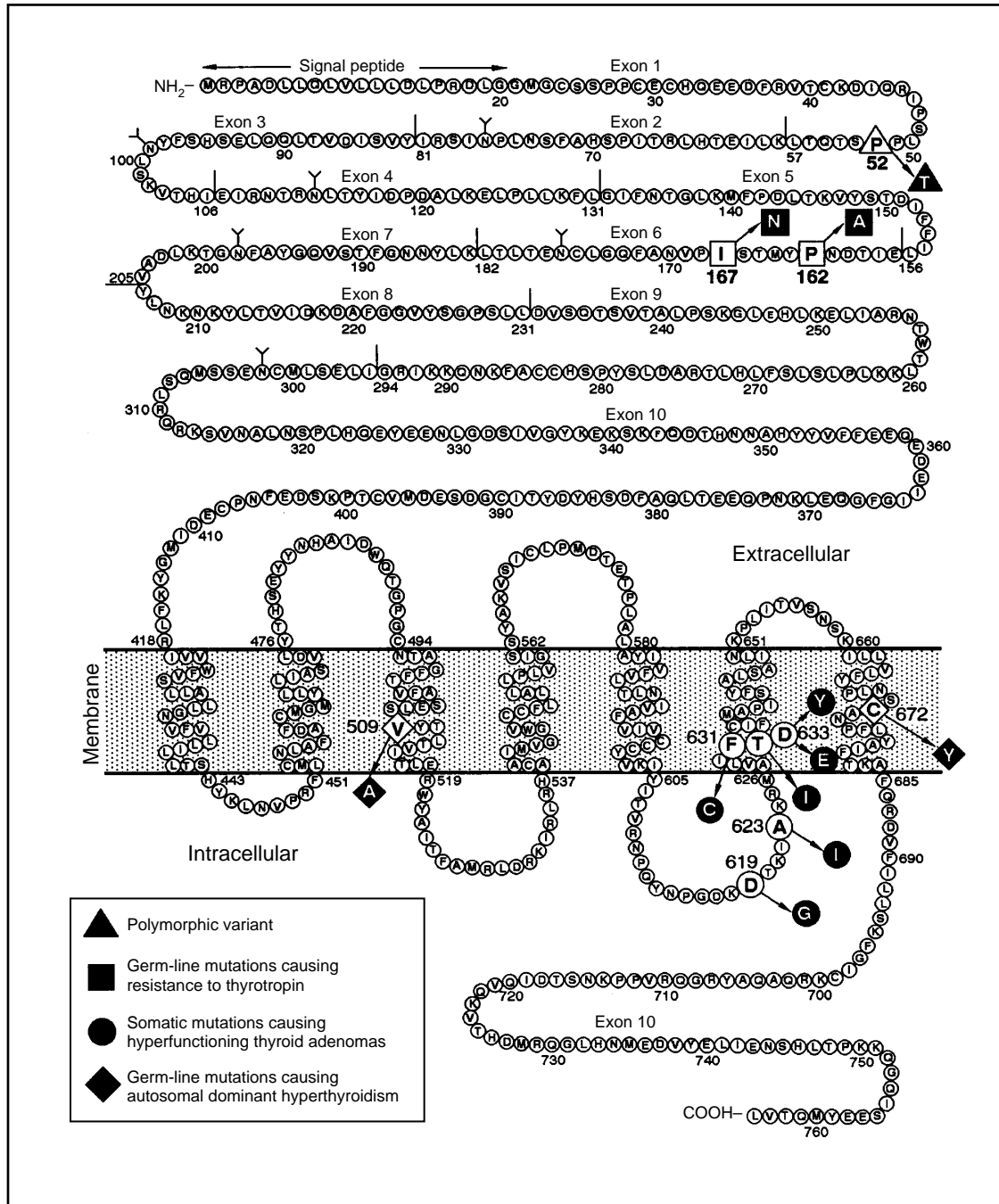


Figure 4. Structure of the Thyrotropin Receptor and Location of Known Mutations.

The amino acids are indicated by the single-letter code and numbered consecutively starting with the transcription-initiation codon. The Y on asparagine residues (N) identifies potential sites of glycosylation. The vertical lines indicate exon boundaries.

hormone concentrations. Gene sequencing revealed a normal thyrotropin β coding sequence.

Complete sequencing of the thyrotropin-receptor gene revealed a different point mutation in each of the two alleles in the three girls, one allele derived from each parent. This finding established the compound heterozygous inheritance of the defect and the recessive manifestation of the phenotype. The likelihood

that both mutant alleles would be transmitted to each of the three daughters is $(1/4)^3$, or 1.6 percent. The two mutations are five amino acids apart in exon 6, which encodes the midportion of the extracellular domain of the thyrotropin receptor (Fig. 4).

Important areas of thyrotropin binding and signal transduction of the thyrotropin receptor have been mapped in the extracellular, transmembrane, and in-

tracellular domains of the molecule.^{17,23-26} Although the functional importance of the region encoded by exon 6 of the thyrotropin receptor has not been studied in detail, the substitution of leucine for the normal proline at position 162 slightly decreased the responsiveness to thyrotropin.²⁷ In this family, functional assays demonstrated that the paternal mutant thyrotropin receptor had almost no thyrotropin-inducible activity and that the maternal mutant thyrotropin receptor had 1/10 the normal activity. Replacement of the polymorphic variant threonine at position 52 in the maternal mutant thyrotropin receptor with the more common proline did not alter the defect. Cells transfected with equal amounts of wild-type and mutant paternal or maternal thyrotropin receptors, to simulate the condition of the heterozygous parents, had normal responses to low thyrotropin concentrations and slightly reduced responses to high concentrations. These findings are compatible with the presence of slightly elevated serum thyrotropin concentrations in the parents. Cells cotransfected with the mutant maternal and paternal thyrotropin receptors, as inherited in the three daughters, required almost 20 times more thyrotropin to produce the level of activity observed in cells transfected with wild-type thyrotropin receptor alone.

These observations explain the 20-fold elevation in serum thyrotropin concentrations in the three daughters that was necessary to maintain normal thyroid hormone secretion. However, the precise mechanisms responsible for the impaired signal transduction and maintenance of a high serum thyrotropin concentration remain unknown. Since the mutations are located in the extracellular domain of the thyrotropin receptor, they may reduce the binding affinity for thyrotropin, a hypothesis we were unable to verify because of a low level of thyrotropin-receptor expression in the transient expression system. It is also possible that the substituted amino acids could alter signal transduction without affecting thyrotropin binding.²⁴ The mechanism enabling these subjects to maintain high serum thyrotropin concentrations despite their normal serum thyroid hormone concentrations is a matter of speculation, but possibilities include mild, subclinical hypothyroidism, increased frequency of pulses of thyrotropin secretion, and a resetting of the threshold for thyroid hormone-induced suppression of thyrotropin secretion.²⁸

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