0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(2):646–657

doi: 10.1210/jc.2003-030587

Two Distinct Compound Heterozygous Constellations (R277X/IVS34–1G>C and R277X/R1511X) in the Thyroglobulin (TG) Gene in Affected Individuals of a Brazilian Kindred with Congenital Goiter and Defective TG Synthesis

VIVIANA J. GUTNISKY, CHRISTIAN M. MOYA, CARINA M. RIVOLTA, SABINA DOMENÉ, VIVIANA VARELA, JUSSARA V. TONIOLO, GERALDO MEDEIROS-NETO, AND HÉCTOR M. TARGOVNIK

Laboratorio de Biología Molecular (V.J.G., C.M.M., C.M.R., S.D., V.V., H.M.T.), Cátedra de Genética y Biología Molecular, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, 1120 Buenos Aires, Argentina; and Thyroid Unit (J.V.T., G.M.-N.), Division of Endocrinology, Hospital das Clínicas, São Paulo University School of Medicine, 05403-900 São Paulo, Brazil

In this study, we have extended our initial molecular studies of a nonconsanguineous family with two affected siblings and one of their nephews with congenital goiter, hypothyroidism, and marked impairment of thyroglobulin synthesis. Genomic DNA sequencing revealed that the index patient (affected nephew) was heterozygous for a single base change of a cytosine to a thymine at nucleotide 886 in exon 7 (886C>T, mother's mutation) in one allele and for a novel guanine to cytosine transversion at position −1 of the splice acceptor site in intron 34 (IVS34–1G>C, father's mutation) in the other allele. The two affected siblings inherited the 886C>T mutation from their mother and a previously reported cytosine to thymine transition at nucleotide 4588 in exon 22 from their father (4588C>T). The 886C>T and 4588C>T substitutions resulted in premature stop codons at amino acids 277 (R277X) and 1511 (R1511X), respectively. *In vitro* transcription analysis showed that the exon 35 is skipped entirely when the IVS34–1G>C mutation is present, whereas the wild-type allele is correctly spliced. SSCP (exon 7 and 35) and restriction analysis (exon 22) using Taq I indicated that the two affected siblings, the affected nephew, his mother, and his unaffected brother were all heterozygous for the R277X mutation. The two affected siblings, their father, and three unaffected siblings were all heterozygous for the R1511X mutation, whereas the affected nephew and his father were heterozygous for the IVS34–1G>C mutation. Moreover, in this kindred, we have characterized polymorphisms (insertion/deletion, microsatellite, and single nucleotide polymorphism) located within introns 18 and 29 and exon 44 that are associated with the described mutations. Haplotype analysis with these polymorphic markers in two unrelated Brazilian families (present family studied and previously reported family) harboring the R277X mutation suggests a founder effect for the R277X mutation. In conclusion, the affected individuals of this family are either compound heterozygous for R277X/IVS34–1G>C or R277X/R1511X. This observation further supports that thyroglobulin gene mutations display significant intraallelic heterogeneity. (*J Clin Endocrinol Metab* 89: 646–657, 2004)

CONGENITAL HYPOTHYROIDISM IS the most common hereditary endocrine disorder, which affects approximately 1 in 4000 newborns, frequently diagnosed by neonatal screening (1, 2). Patients with this heterogeneous clinical and biochemical syndrome can be divided into two groups: nongoitrous and goitrous neonatal hypothyroidism. In general, the nongoitrous group results from thyroid gland agenesis (athyreosis), ectopic thyroid tissue at the base of the tongue, or in any position along the thyroglossal tract (dysgenesis) and hypoplastic thyroid gland. In some of these patients, the congenital hypothyroidism is associated with mutations in genes responsible for the growth or development of thyroid follicular cells: TSHβ (3), thyroid transcription factor 1 (4, 5), thyroid transcription factor 2 (6), paired box transcription factor 8 (7–9), and TSH receptor genes (10–12). The presence of congenital goiter has been linked to a number of abnormalities in thyroid hormonogenesis. Mutations in the Na^+/I^- symporter (13–15), thyroglobulin (TG) (16–23), thyroperoxidase (24, 25), thyroid oxidase 2 (26), and Pendred syndrome (27, 28) genes, originate in a wide spectrum of congenital goitrous hypothyroidism, transmitted in an autosomal recessive mode.

Several mutations in the human TG gene have been reported and are associated with congenital goiter (16–23) or endemic (29) and nonendemic simple goiter (30, 31). In one such patient, there was defective synthesis of TG due to the absence of exon 4 from the major TG transcript because of a cytosine to guanine transversion at position minus 3 in the acceptor splice site of intron 3 (16). van de Graaf *et al.* (20) identified a point mutation in exon 7 that replaces the normal cytosine in nucleotide 886 with a thymine. As a result, the normal arginine in codon 277 is replaced with a stop codon

Abbreviations: Indel, Insertion/deletion; SNP, single nucleotide polymorphism; SSCP, single-strand conformation polymorphism; TG, thyroglobulin.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

(R277X). Other reports have indicated that cysteine substitutions (C1245R and C1977S in exons 17 and 33, respectively) cause an abnormal three-dimensional structure of TG and its defective intracellular transport (21). Molecular analysis in two members of a family with a history of congenital goiter reveal an aberrant splicing due to a guanine to thymine transversion at position +1 in the donor splice site of intron 30 (18, 22). More recently, we identified two siblings with fetal goitrous hypothyroidism caused by a compound heterozygous mutation (1143delC and 6725G→A [R2223H]) (23). In animals, hereditary thyroid disorders linked to TG defects have been reported in Afrikander cattle (R697X) (32), Dutch goats (Y296X) (33), cog/cog mouse (L2263P) (34), and rdw rats (G2320R) (35, 36).

We previously identified a TG nonsense mutation in members of a Brazilian family (MA) with a complex history of congenital goiter (37). An analysis of the TG transcripts in the goiter of one of the affected siblings showed a cytosine to thymine transition at nucleotide position 4588 in exon 22 that generates a stop codon at amino acid position 1511 (R1511X) (17). The nonsense mutation is thus removed from the transcripts by exon skipping, and there is a preferential accumulation in the goiter of a TG mRNA lacking exon 22. The nonsense mutation converts the TCGA sequence into TTGA, thus removing a Taq I site at this position in the mutant TG gene. The preliminary genetic studies indicate that the two affected siblings, their father, and three unaffected brothers are all heterozygous for the nonsense mutation (19). The mutation was not identified in the affected nephew, suggesting that at least one, and probably two, additional mutations of the TG gene segregate in this family.

In the present study, we extended our initial molecular studies to show that the MA family carries three different single nucleotide changes in the TG gene and that the affected individuals are either compound heterozygote for R277X/IVS34–1G>C or R277X/R1511X. This is, to our knowledge, the first time that the presence of two compound heterozygous constellations in the TG gene within a family is reported. We also present evidences that a founder effect accounts for the R277X mutation.

Subjects and Methods

Clinical report

A detailed clinical and laboratory evaluation (37), TG mRNA analysis (17), and initial genetic studies (19) of this nonconsanguineous MA family have been reported previously. The affected members, two siblings (II-1 and II-2) and one of their nephews (III-2), had goiter, congenital hypothyroidism, and a marked impairment of TG synthesis. The siblings' father, six other siblings, the nephew's brother, and the nephew's father had no goiter and normal thyroid function.

The results of thyroid function tests of the MA family members studied are shown in Table 1.

Written informed consent was obtained from all individuals involved in this study, and the research project was approved by the Ethical Committee of the University of São Paulo Medical School.

DNA sequencing

Genomic DNA was isolated from peripheral blood leukocytes following standard procedure. The complete coding sequence of the human TG gene, including regions of the TG promoter, and splicing signals and the flanking intronic regions of each intron, were amplified from the index patient III-2 by PCR as described previously (23). Forward and reverse intronic primers were specially designed for each one of the 48-TG exons (38–41). Their oligonucleotide sequences and the position of their 5′ coding sequence ends are shown in Table 2. M13 sequences (18 nucleotides long) have been incorporated at the 5′ end of the forward and reverse primers. After amplification, PCR products were run on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

DNA sequences from each amplified fragment were performed with the Taq polymerase-based chain terminator method (*fmol*; Promega, Madison, WI) using either the M13 tags linked to the PCR fragments or the TG-specific forward and reverse primers used in the DNA amplification (Table 2).

The results were analyzed using the PC gene (Intelligenetics, Geneva, Switzerland), DNASTAR (DNASTAR Inc., University of California–San Francisco, San Francisco, CA) and Nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST) software programs.

Construction and expression of the minigenes

To study the effect of the IVS34–1G>C mutation, wild-type and mutated minigenes were constructed using the exon trapping vector pSPL3 (Life Technologies, Inc., Gaithersburg, MD). Genomic DNA region from index patient III-2 containing exon 35 and intronic flanking sequences (2138 bp upstream from the 5′ exon end and 2252 bp downstream from the 3′ exon end) was amplified by Long PCR technique, as described previously (42): the forward primer (pSPL3-In34F: 5′ ccggaattctgacgtgagatgcatggta 3′) containing the EcoRI site and the reverse primer (pSPL3-In35R: 5′ ccggatatctcgagtgacagcaggatagctgaa 3′) containing XhoI and EcoRV sites. The 4453 bp PCR products were purified using GFX PCR DNA and gel band purification kit (Amersham Biosciences, Piscataway, NJ) and sequentially digested with XhoI and EcoRI. The insert was directionally cloned into the EcoRI and XhoI sites of the pSPL3 vector. Because the index patient III-2 is heterozygous for the IVS34–1G>C mutation, the wild-type and mutated alleles were thus cloned. The recombinant plasmids were amplified in HB101 competent cells and purified using Concert high purity plasmid miniprep system (Life Technologies, Inc.). The correct sequence was confirmed by sequencing (*fmol*; Promega) with the pSPL3-In34F primer.

CV-1 cells were grown in 3.8-cm dishes in DMEM supplemented with 5% bovine calf serum and 100 U/ml penicillin-streptomycin in a 5% CO_2 atmosphere at 37 C. When cells reached approximately 90% confluence, they were transfected with 1 μg plasmid DNA (wild-type, mutant, and

TABLE 1. Thyroid function tests and serum TG (see pedigree for details)

	Family members (gender)									
	II-1 (M)[a]	II-2 (M)[a]	II-3 (M)	II-4 (F)	II-5 (M)	II-6 (F)	II-7 (M)	III-2 (M)[a]	I-1 (M)	Reference range
Serum TT_3	2.45	1.07					2.45	2.76	2.61	1.22–3.07 nmol/liter
Serum TT_4	90.1	64.3					108.1	12.1	102.9	57.9–141.6 nmol/liter
Free T_4			1.8	20.5	19.3	18.0				9.0–20.5 pmol/liter
Serum TSH	36.7	>100.0	1.7	1.3	0.9	1.0	2.3	>100.0	2.0	0.5–4.5 mU/liter
Serum TG[b]	1.8	3.4	5.6	4.8		11.9	1.2	2.1	6.1	0.5–18.0 μg/liter

Conversion to conventional units: TT_3, nmol/liter ÷ 0.01536 ng/dl; TT_4, nmol/liter ÷ 12.87 μg/dl; free T_4, pmol/liter ÷ 12.87 ng/dl. M, male; F, female.

[a] III-2, propositus; II-1 and II-2, the two affected siblings.

[b] There was no significant increase in serum TG after stimulation by exogenous TSH (affected patients).

TABLE 2. TG oligonucleotides used as primers in PCR amplification and sequence reactions

Exon no.	Forward primers		Fragment size (bp)[a]	Reverse primers	
	Position of 5′ end	Nucleotide sequence (5′→3′)		Position of 5′ end	Nucleotide sequence (5′→3′)
1	−208	*cgttctgttcccccacagtt*	338	+22	**gctccatggcctcagaactt**
2	−38	**ccacactcttctctgatgaa**	242	+95	**agttgcagggcagagctcaggaa**
3	−138	**ccactccactctctccctaa**	327	+91	**gctcagtcacccacccaaa**
4	−84	**gggaagggagcatgagttt**	355	+67	**ggatcgcagtgaggaaa**
5	−83	**gggacacgagtgcatatg**	299	+56	**agtgtgggctgcgtcaggtgat**
6	−54	**gcttgtgatgacttgcctt**	260	+99	**cagtcactctagctgtgctt**
7	−42	**ctgaatgagaccatctctgaa**	216	+30	**cacgcacattgttggcagtt**
8	−63	**gcatgctgtgaagacaccaa**	277	+28	**aaagaggaagcccccagaggaaa**
9 A	−96	**gttctggcttcttactacct**	570	1549	CTTCTTGTCTCCCTCCAT
B	1389	CCCAAAGAGACTCCAGCAAA	477	1865	GTCGTGCATGATGGGACAAA
C	1758	TGTGCCAGAAGATGTGGCAA	473	+54	**agggcctaaagagagactca**
10 A	−92	**gacggagtttggacagtt**	363	2447	TTTCCGGAAGCTGCTTCT
B	2397	TGCCAAGCTGCTAGTGAA	460	+95	**ccctcccactagacatcctt**
11	−83	**cgtgagggcacacatgctt**	404	+81	**aggatgactgaggagagac**
12	−61	**agctacagagcccacacaga**	237	+38	**tgtcacttggccacctgaa**
13	−46	**gtccctagtgcaattcctgaa**	182	+58	**tagaccagtgatgcccaccaa**
14	−39	**ccacgaccagtcctttacaa**	208	+56	**atctctgaccagcggggacaa**
15	−73	**catctgccagcagcaggtt**	259	+83	**gacacgaagccagctcttt**
16	−38	**actgattccccagcccatct**	323	+84	**cccagcttctctagtact**
17	−85	**gagaaggagacacccacaa**	326	+28	**gcaggatagatgctctgat**
18	−75	**gcagaggaaatcccaa**	278	+48	**ccctgagttcagtccaa**
19	−120	**gtagggttggtggaggatt**	327	+50	**ctcagagaggctgcatagctt**
20	−51	**ccctagcagagtacagt**	313	+43	**cacatggctccacaggagat**
21	−70	**gtgtgttctgggattgt**	264	+44	**cattgcagggcttttct**
22	−48	**gattccagaggcccatt**	280	+61	**agcccttgagactact**
23	−61	**cttctctgcagatgcccta**	220	+42	**ccacaccttcttctgagt**
24	−39	**gggaaagccaggtgagtga**	199	+44	**acagcggatgaggagcagaa**
25	−79	**ggttagggttggatgaatg**	361	+173	**agcgtgctgtgctgagtct**
26	−61	**gctctgtccaactctgccat**	326	+73	**gtgtgtcctggcttctgcat**
27	−73	**tcaggggacagagaagagt**	325	+84	**ggagggctcataagaaagt**
28	−80	**gagatggggctattgcagta**	193	+47	**catgtaatcagcgtcctgcta**
29	−60	**gaccagtggagtactacccat**	202	+61	**gcacccatttagtctctgcat**
30	−210	**gaactattcctgtctgaccc**	474	+126	**ccacagtgatccatgagttatgacac**
31	−77	**cccagagaatcctgtagaga**	313	+59	**accacagagccagcagaaca**
32	−52	**catgactacagcaaatctc**	262	+98	**gtgctgggtatgcttctgtt**
33	−93	**ccccaaagcaagaatgacta**	274	+101	**ggataggagatgctgaggat**
34	−104	**gtctgctgaacaatgtact**	283	+35	**gacgtccatatagctgtcat**
35	−148	**cccaccctgaccaataca**	321	+110	**gcgagtgatatgcaagcta**
36	−65	**cacggctgtctttgttact**	323	+123	**cccttacttccaagcatccta**
37	−79	**ggatggatgactggaaga**	278	+34	**caggatggctgcaaaggcata**
38	−74	**cagaatgccagtggagagagc**	378	+84	**ctgctactgagtcccatttgga**
39	−95	**gctttggaatggggtagtgg**	318	+129	**ggcaaatcacctaacctcagct**
40	−82	**tgtgtcaaccaagaatcaggc**	368	+126	**aaatttccactgtgtgcctag**
41	−122	**tgaggacaagagcccagagc**	423	+98	**ccaagcaattgaagccaactag**
42	−93	**gatgcagaaccctgatgtgg**	381	+123	**cagttggtcatcagcctcatg**
43	−95	**accagtattggcattcagtatgg**	319	+56	**ccagagcccttaggaaatgc**
44	−123	**ttgtgtttaatgccatgccc**	375	+70	**cctgaacaccaactgagtctgc**
45	−145	**gggaatgggaagaaggtgttc**	344	+91	**tagaggggttaagtggtgtgtcc**
46	−109	**gaagggaaagtcttggttttgg**	340	+96	**gagtctatcgatgcaaattggg**
47	−97	**tgtccctcagataccgagtgc**	443	+155	**ccccagaccatgaacaactca**
48	−60	**gaccagagaagagaagtccta**	380	+81	*acagcagcctgggattcaaa*

Exon sequences are in *capital letters*, intron sequences are in *bold lowercase letters*, and promoter and 3′ intergenic region sequences are in *italic lowercase letters*. M13 sequences have been incorporated at the 5′ end of the primers.

[a] The fragment sizes are considered without the M13 sequences.

control pSPL3)/3.8-cm dish with the Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA). Forty-eight hours later, cells were harvested and total RNA was extracted with Trizol (Invitrogen, Life Technologies). The RT-PCR was performed as described previously (17) using vector-specific primers: forward primer (pSPL3F), 5′ tctgagtcacctggacaacc 3′ and reverse primer (pSPL3R), 5′ atctcagtggtatttgtgagc 3′. Samples were heated to 95 C for 3 min, followed by 40 cycles of DNA denaturation (95 C for 30 sec), annealing (62 C for 30 sec), and polimerization (72 C for 1 min). After the last cycle, the samples were incubated for an additional 10 min at 72 C. The RT-PCR products were purified from the gel as described above, cloned into pGemT-easy vector (Promega), and then sequenced (*fmol*; Promega) with the pSPL3F primer.

Identification of 886C>T and IVS34–1G>C mutations by single-strand conformation polymorphism (SSCP) analysis

SSCP was used to screen for the presence of 886C>T and IVS34–1G>C mutations. PCR was performed using the intronic forward and reverse exon 7 and exon 35 primers (Table 2) under identical PCR conditions as reported previously (23).

The gel matrix for SSCP contained 8% (886C>T mutation) or 10% (IVS34–1G>C mutation) polyacrylamide (29:1) (Invitrogen, Life Technologies), without glycerol. Samples were electrophoresed for up to 18 h at a constant temperature (4 C). DNA was visualized by silver-staining according to standard procedures.

Identification of 4588C>T mutation by Taq I restriction analysis

The mutation detected at position 4588 in exon 22 destroys a Taq I recognition site (17, 19). The presence of the mutation was, therefore, independently analyzed by restriction analysis with Taq I. A 316-bp fragment containing exon 22 was generated by PCR, under identical PCR conditions as described previously (23), using the intronic forward and reverse exon 22 primers (Table 2). Restriction enzyme digestion with Taq I was performed as recommended by the manufacturer (New England BioLabs Inc., Beverly, MA). After digestion, the DNA fragments were separated on a 12% polyacrylamide gel. Digestion of the wild-type allele results in two fragments of 125 and 191 bp.

Insertion/deletion (Indel) polymorphism analysis

The large Indel polymorphism of 1464 bp localized in intron 18 of the human TG gene (42) was analyzed by multiplex PCR. The reactions were performed in 50 μl, using a standard PCR buffer (Highway Molecular Biology, Tandil, Argentina), containing 200–300 ng DNA, 2.5 mmol/liter $MgCl_2$, 200 μmol/liter of each dNTP, 1 U Taq polymerase (Highway Molecular Biology), and 100 pmol of each primer. The primers used were 18 forward primer (Table 2), i18–3-F (intronic forward primer, located in the 1464 Indel region) (42) and i18–1-R (intronic reverse primer) (42).

Samples were heated to 94 C for 2 min, followed by 35 cycles of DNA denaturation (94 C for 30 sec), annealing (55 C for 30 sec), and polymerization (72 C for 1 min). After the last cycle, the samples were incubated for an additional 10 min at 72 C. The amplified fragments were analyzed in a 2% agarose gel.

The amplification generates two fragments of 374 (between 18 forward primer and i18–1-R) and 541 (between i18–3-F and i18–1-R) bp, indicating the exclusion or inclusion of the Indel polymorphic region, respectively.

Microsatellite analysis

The TGrI29 microsatellite, localized in intron 29 of the human TG gene, was amplified using the primers and PCR conditions described previously (43). PCR products were resolved by electrophoresis in 6% polyacrylamide denaturing gels.

Single nucleotide polymorphism (SNP) analysis

Taq I endonuclease was used to screen for the presence of the 7589G>A SNP in exon 44. The primers and PCR conditions were described previously (44). The samples were cleaved with Taq I restriction endonuclease according to the specifications of the manufacturer (Invitrogen, Life Technologies) and analyzed by electrophoresis in 12% polyacrylamide gel. The fragment contains two Taq I sites (positions 7587 polymorphic and 7667 not polymorphic). Taq I restriction showed two fragments (94 and 107 bp) in the homozygous form with absence of the 7587 Taq I site, three fragments (27, 80, 94 bp) in the homozygous form with presence of the 7587 Taq I site, and four fragments (27, 80, 94, and 107 bp) in the heterozygous state.

Results

Sequence analysis of the TG gene

All 48 exons of the TG gene from index patient III-2 (affected nephew) from the MA family were analyzed as well as 180 bp of the TG promoter and all the flanking regions of each intron. A total of 15,000 bases were analyzed. A heterozygous cytosine to thymine transition at nucleotide position 886 in exon 7 (886C>T) was found in the proposita. Its position in the gene near the end of the exon is schematically given in Fig. 1, and the expected amino acid sequence after translation is also shown. Instead of encoding for an arginine residue at amino acid position 277, the triplet harboring the mutation encodes for a premature stop codon (R277X). The same nonsense mutation had been reported previously in the offspring of a consanguineous Brazilian kindred with multiple occurrence of congenital hypothyroidism with goiter (20). The GT-AG splicing consensus sequences are rigorously respected in all introns, except for the acceptor splice site of intron 34. A novel heterozygous G to C transversion was observed at position −1 (IVS34–1G>C), compared with the expected sequence (AC instead of AG) (Fig. 2).

Analysis by direct sequencing of PCR products of exons 7 (Fig. 1) and 35 (Fig. 2) from III-2, his mother II-8, his father II-9, and his unaffected brother III-1 showed that III-2 inherited one copy of the 886C>T mutation from his mother and one copy of the IVS34–1G>C mutation from his father. The heterozygous state of each parent was confirmed by the presence of one wild-type allele. The healthy brother (III-1), who has normal thyroid function, was heterozygous for the IVS34–1G>C mutation and did not carry the 886C>T mutation. This finding indicates that III-2 is a compound heterozygous for R277X/IVS34–1G>C.

We reported previously that both affected siblings II-1 and II-2 were heterozygous for the 4588C>T mutation (R1511X) (17). In contrast, because their sister II-8 carries the R277X mutation, we speculated that II-1 and II-2 also carry this mutation together with the R1511X mutation. To test this hypothesis, we performed direct sequencing of PCR products of exons 7 (Fig. 1) and 22 (data not shown) from II-1, II-2, II-3 (unaffected siblings' brother), and their father I-1. The results indeed indicated that II-1 and II-2 have inherited one copy of the R277X from their mother (I-2) and one copy of the R1511X mutation from their father. Their healthy brother (II-3) is heterozygous for the R1511X mutation and does not carry the R277X mutation. These results are in perfect agreement with the data obtained previously in the thyroid tissue of II-2 (17) and in direct and indirect segregation analyses (19).

Minigenes analysis

We tested a minigene containing the IVS34–1G>C mutation for abnormal splicing using the exon trapping system. *In vitro* transcription showed that the exon 35, which consists of 63 bp, is skipped entirely when the IVS34–1G>C mutation is present, whereas the minigene that contains the wild-type allele is spliced correctly (Fig. 3).

Segregation analyses of the mutations in the TG gene

Genomic DNA from each MA family member was amplified in separated PCR reactions with use of the sense and antisense primers for exons 7, 22, and 35. The PCR fragments were analyzed by SSCP (exon 7 and 35) or restriction analysis (exon 22) using Taq I.

As shown in Fig. 4, individuals II-1, II-2, II-8, III-1, and III-2 were heterozygous for the presence of an aberrant fragment in SSCP analysis of exon 7. These results indicate that the three affected individuals (III-2, II-1, II-2), the nephew's mother (II-8), and the unaffected nephew (III-1) are all heterozygous for the R277X mutation.

The two affected siblings, II-1 and II-2, their father, and three unaffected siblings, II-3, II-6, and II-7 are all heterozygous for the R1511X mutation in exon 22 (Fig. 4). The mutation was not observed in III-2 because Taq I digestion

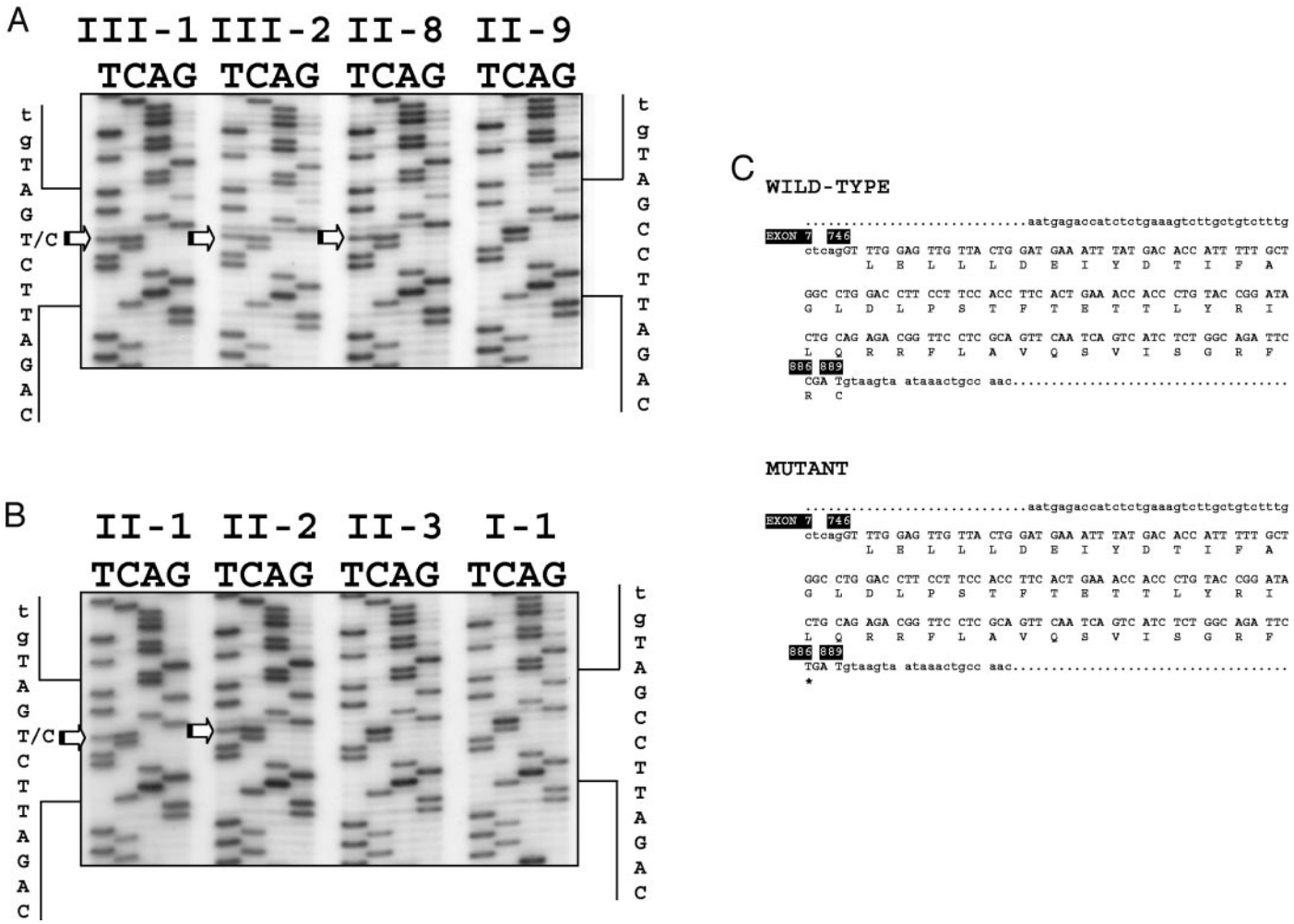

FIG. 1. Identification and family analysis of the heterozygous 886C>T (R277X) mutation in exon 7 of the TG gene. A and B, Partial DNA sequence (coding strands) of exon 7. The *arrows* point to the 886C>T transition in the unaffected nephew (III-1), affected nephew (III-2), nephew's mother (II-8), and two affected siblings (II-1 and II-2). Wild-type alleles coexist in each case with the mutated allele. The nephew's father (II-9) and the siblings' brother (II-3) and father (I-1) have only the wild-type sequences at this position. C, Partial nucleotide and deduced amino acid sequences of exon 7 and intron 6/exon 7/intron 7 junction sequences from wild-type and mutant TG genes. Exon 7 maps between nucleotides 746 and 889 of the TG mRNA (41). Exonic sequences are in *capital letters* and intronic sequences are in *lowercase letters*. The site of a premature stop codon is indicated by an *asterisk*. Nucleotide sequence is given in the upper line, and amino acid translation (represented by *single-letter code*) is given below their respective codons.

indicates that the wild-type sequence is present at this site in both alleles.

SSCP analysis of exon 35 confirmed that III-2 and his father are heterozygous for the IVS34–1G>C mutation (Fig. 4). IVS34–1G>C was not detected in 50 unaffected unrelated control subjects by SSCP analysis.

Segregation analyses of polymorphic markers in the TG gene

The previously characterized markers—Indel located in intron 18 (42), the microsatellite in intron 29 (TGrI29) (43), and the 7589G>A SNP in exon 44 (44)—proved to be interesting and informative polymorphisms for indirect segregation analysis of the mutations in the MA family.

The results show that the two affected siblings, II-1 and II-2, are homozygous for the 541 bp Indel allele and the 29.3 microsatellite allele (Fig. 5); I-1 (father), II-3, II-6,and II-7 (carriers of the R1511X mutation and normal thyroid function) are heterozygous for the 374- and 541-bp alleles in the Indel system and 29.2 and 29.3 alleles in the microsatellite, whereas II-4 and II-5 (noncarriers of any nonsense mutations) are homozygous for the 374- and 29.2-bp alleles.

As shown in Fig. 5, individuals II-1, II-2, II-3, II-6, and II-7 are homozygous for the presence of the Taq I site in the SNP analysis; subjects I-1, II-4, and II-5 are heterozygous, revealing that the wild-type allele in I-1 is associated with the presence of adenine at position 7589 (Taq I site is destroyed).

The unaffected nephew's mother, II-8 (carrier of the R277X mutation), displays heterozygosity for the 374- and 541-bp Indel alleles and for 29.2 and 29.3 microsatellite alleles (Fig. 5), whereas the healthy nephew III-1 (carrier of the R277X mutation) is homozygous for the 541-bp and 29.3 alleles. The unaffected nephew's father, II-9 (carrier of the IVS34–1G>C mutation), and the affected nephew, III-2, are homozygous

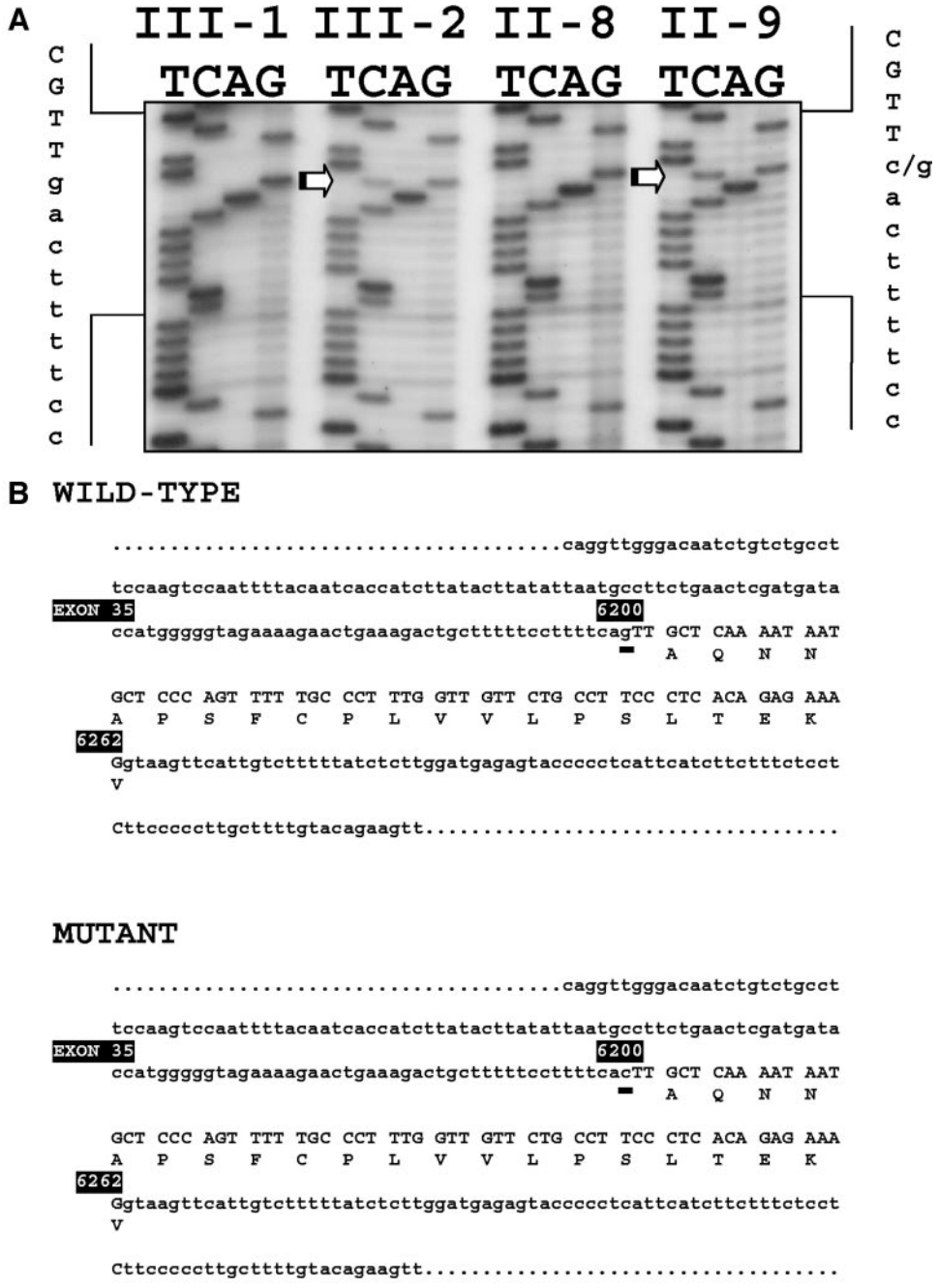

FIG. 2. Identification and family analysis of the heterozygous IVS34–1G>C mutation in intron 34 of the TG gene. A, Partial DNA sequence (coding strands) of exon 35. The *arrows* point to the IV34–1G>C mutation in the affected nephew (III-2) and his father (II-9). Wild-type alleles coexist in each case with the mutated allele. The unaffected nephew (III-1) and his mother (II-8) have only the wild-type sequences at this position. B, Nucleotide and deduced amino acid sequences of exon 35 and intron 34/exon 35/intron 35 junction sequences from wild-type and mutant TG genes. Exon 35 maps between nucleotides 6200 and 6262 of the TG mRNA (41). Exonic sequences are in *capital letters* and intronic sequences are in *lowercase letters*. The IV34–1G>C mutation is *underlined*. Nucleotide sequence is given in the upper line and amino acid translation (represented by *single-letter code*) is given below their respective codons.

for the 541-bp Indel allele and heterozygous for the 29.2 and 29.3 microsatellite alleles. This implies that the IVS34–1G>C mutation is associated with the 541-bp Indel and 29.2 microsatellite alleles.

The two brothers, III-1 and III-2, and their father, II-9, are homozygous for the presence of the Taq I site in the 7589G>A SNP analysis (Fig. 5), whereas their mother, II-8, is heterozygous because she inherited a wild-type allele with adenine at position 7589 from her father.

The results of haplotype analyses of the R277X, R1511X, and IVS34–1G>C mutations and Indel, microsatellite, and SNP markers in the TG gene of MA family members are shown in Fig. 6. An important observation is that II-8 has a clearly distinct haplotype. The mother's (I-2) haplotype data were inferred with the aid of her siblings.

Finally, to explore whether the recurrent mutation R277X was due to a founder effect or to a mutational hot spot, we examined the haplotypes of some members of the previously reported family who also carry the mutation R277X (20) by genotyping Indel, TGrI29 microsatellite, and 7589G>A SNP markers. The analysis showed that the three affected siblings, homozygous for the R277X mutation, are also homozygous for the 541-bp Indel allele and 29.3 microsatellite allele and the presence of the Taq I site in the 7589G>A SNP system (data not shown). This finding is identical to what was observed in the study MA family. Consequently, these results

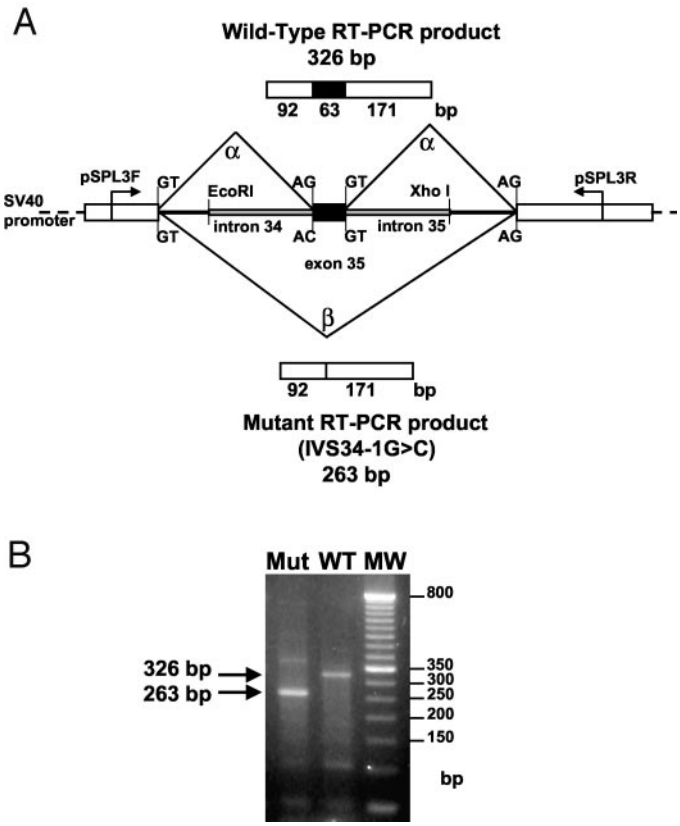

FIG. 3. *In vitro* expression of the wild-type and mutant (IVS34–1G>C) minigenes. A, Schematic representation of the genomic organization of the wild-type and mutant minigenes and their RT-PCR products. The 4453-bp PCR amplified fragments from index patient III-2 were directionally cloned into the EcoRI and Xho I sites of the exon-trapping pSPL3 vector, which was expressed in CV-1 cells. Vector and genomic DNA splice donor (GT) and acceptor (AG) sites are shown. cDNA was synthesized from transcribed mRNA and amplified with pSPL3F and pSPL3R primers complementary to flanking vector sequences. The processing of the wild-type transfected sequences (α-splicing event) produces a PCR product of 326 bp. In contrast, the IVS34–1G>C mutation results in the skipping of exon 35 (β-splicing event), producing a 263-bp fragment. B, Agarose electrophoresis of the RT-PCR products. The RT-PCR fragments from wild-type (WT) and mutant (Mut) minigenes were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light. The DNA size marker (MW) was a 50-bp DNA ladder (Invitrogen, Life Technologies).

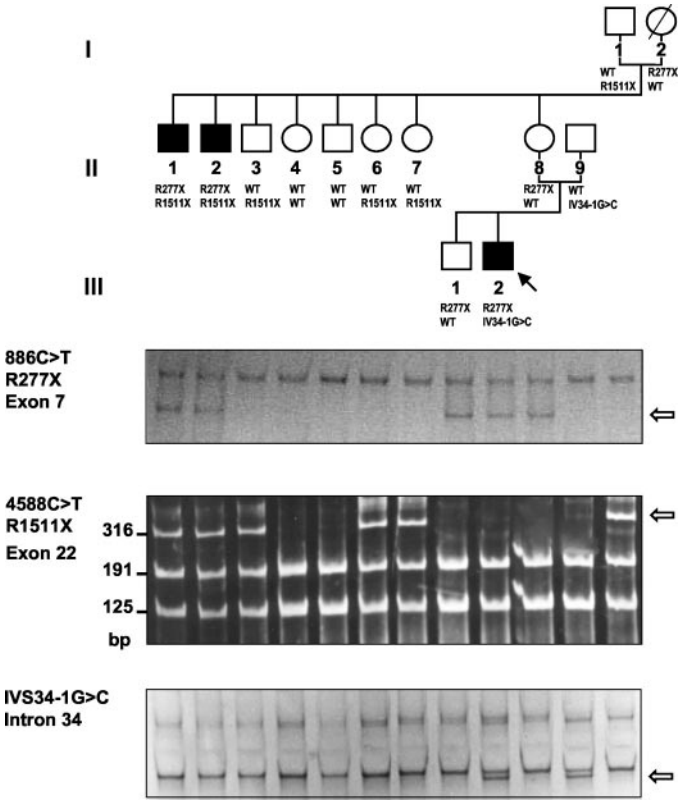

FIG. 4. Segregation analyses of the 886C>T (R277X), 4588C>T (R1511X) and IVS34–1G>C mutations in the TG gene in members of the MA family. The pedigree shows the genotype for each individual. *Squares* indicate male members, and *circles* indicate female members. *Black arrows* point to the index patient, and the *filled symbols* indicate the patients with congenital hypothyroidism and goiter. Lanes correspond to the individuals in the pedigree. The presence of the 886C>T and IVS34–1G>C mutant alleles result in SSCP fragments with an aberrant migration pattern on PAGE. The detected mutation at nucleotide position 4588C>T in exon 22 eliminates a Taq I restriction site. Digestion of the wild-type allele results in two fragments of 191 and 125 bp. PCR-SSCP fragments were stained with silver and Taq I restriction fragments with ethidium bromide. Fragments corresponding to the mutant alleles are indicated by *white arrows*.

indicated that all affected individuals in the two families shared a common haplotype at three polymorphic markers located within the TG gene.

Discussion

Impaired TG synthesis due to TG gene mutations is one of the causes for thyroid dyshormonogenesis. This type of primary congenital hypothyroidism is characterized by intact iodide trapping, normal organification of iodide, and aberrant TG protein expression (1, 2). In this kindred, all three affected members had clinical and biochemical criteria suggestive of congenital goiter associated with TG deficiency: presence of goiter, hypothyroidism, high iodide ^{131}I uptake, low-serum TG, and elevated-serum TSH with simultaneous low or normal serum T_4 and T_3 levels (1, 2). Molecular analyses indicated that the affected individuals are either compound heterozygous for R277X/IVS34–1G>C or R277X/R1511X.

We previously characterized the structure of the TG gene and defined the intronic sequence adjacent to each of the 48 coding exons (38–41) to provide tools for detecting mutations and sequence variants by either direct sequence analysis or other methods of mutation detection such as SSCP. We also identified polymorphic markers within introns and exons of the TG gene that should be useful in either linkage or association studies (42–44). TG is encoded by a single gene of 270 kb located on chromosome 8q24 and contains an 8.5-kb coding sequence (38–41). The preprotein monomer is composed of a 19-amino acid signal peptide, followed by a 2749-residue polypeptide. Eighty percent of the monomeric primary structure is characterized by the presence of three types of repetitive units (Fig. 7A). The remaining 20%, which constitutes the carboxy-terminal domain of the molecule, is not repetitive and shows a striking homology with acetylcho-

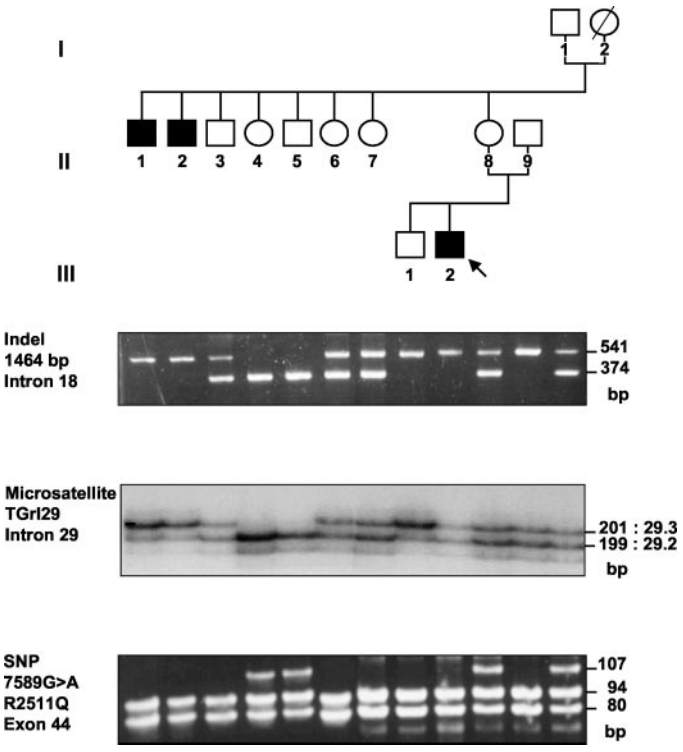

FIG. 5. Segregation analyses of the Indel, a microsatellite and a SNP marker in the TG gene in members of the MA family. Gels show Indel, microsatellite, and SNP analysis. Indel and SNP PCR fragments were stained with ethidium bromide, and microsatellite fragments were visualized by autoradiography. The SNP gel shows that the unaffected siblings II-4 and II-5, the nephew's mother II-8, and the siblings' father I-1 all have an allele containing guanine at position 7589 that is digested with Taq I, producing two fragments of 27 (data not shown) and 80 bp and an allele resistant to digestion with Taq I (107 bp) containing adenine at this position. The amplified fragment contains two Taq I sites (positions 7587 polymorphic and 7667 not polymorphic), and the band of 94 bp results from digestion at position 7667.

linesterase (41). The monomer structure includes 11 type 1, three type 2, and five type 3 repeat motifs. TG is glycosylated and forms a dimer. A correct three-dimensional structure is essential for thyroid hormonogenesis.

The 886C>T transition in exon 7 produces a predicted premature stop codon, which results in a grossly truncated protein of 276 amino acids (R277X) with limited ability to generate thyroid hormone (Fig. 7B). Five hormonogenic acceptor tyrosines have been identified and localized at positions 5, 1291, 2554, 2568, and 2747, and several other tyrosines localized at positions 130, 847, and 1448 have been proposed as donor sites that transfer a iodophenoxyl group to an acceptor iodotyrosine (41). The most important hormonogenic acceptor site is at tyrosine 5, after coupling with the donor tyrosine at position 130 (41). The truncated form of TG described here harbors both the acceptor tyrosine 5 and the donor tyrosine 130 residues. However, the premature stop codon eliminates the carboxy-terminal hormonogenic domain, resulting in the loss of thyroid hormone formation. We reported previously that the RT-PCR analysis (20) excluded an alternative splicing mechanism, by exon skipping, to restore the normal reading frame disrupted by the nonsense mutation and eliminate the stop codon, which would truncate the protein.

The 4588 C>T transition in exon 22 is also characterized by a predicted premature stop codon, which results in a truncated protein of 1510 amino acids (R1511X) (Fig. 7C). However, this mutation is not present in most of the TG mRNA transcripts because the detected TG mRNA lacks 171 nucleotides corresponding to exon 22. Interestingly, skipping of mutated exon 22 (17) in the pre-mRNA restores the reading frame allowing translation to reach the normal stop codon (Fig. 7D). This alternative splicing is also present in mRNA from normal thyroid tissue, but it represents a minor fraction of the total TG transcripts (17). The excision of exon 22 in the TG mRNA results in an in-frame deletion of 57 amino acid residues, which is localized in the TG type 1 repeat motif (subtypes: 1–11, Fig. 6A). A TTG leucine codon is generated by the junction. The nonsense mutations in exon 7 and 22 occur in a CpG dinucleotide sequence and could be caused by deamination of a methylated cytosine resulting in a thymine. The CGA arginine codon is considered a hot spot for mutations in mammalian DNA.

The third mutation found in this family is a novel G to C transversion at position −1 in the acceptor site of intron 34. This infers the possibility that the splice site mutation might generate a total elimination of exon 35 of the TG gene (Fig. 7E) because removal of 63 nucleotides maintains the reading frame. It is known from other genes that mutations in this site, which alter the consensus acceptor site, can result in exon skipping. We ruled out the possibility that the IVS34–1G>C mutation could be a common polymorphism because it was not detected in 100 chromosomes from the general population by SSCP analysis. In contrast, exon 35 is the smallest exon of the TG gene and is flanked by two large introns (intron 34: 10,608 bp; intron 35: 28,488 bp) (40). It is conceivable that intron size could affect the splicing; however, the presence of this alternative transcript was not detected in humans by RT-PCR reactions (17, 41).

Because the thyroid tissue from III-2 is unavailable, we used an *in vitro* exon-trapping system to evaluate whether the IVS34–1G>C mutation produces an abnormal transcript by a defect in exon splicing. Minigenes were constructed using the pSPL3 vector, which has a minimal gene organization: the SV40 promoter followed by an exon-intron-exon structure with a multiple cloning site located inside the intron. When a fragment cloned in the multiple cloning site contains functional exons with their corresponding splicing sites, they are included in the mature mRNA. We found that in *in vitro* transcription the mutation in the acceptor splice site caused skipping of the exon 35 (Fig. 3). The excision of exon 35 in the TG mRNA results in an inframe deletion of 21 amino acid residues, which are located in the TG type 3 repeat motif (subtypes 3b-2, Fig. 7A). An ATG methionine codon is generated by the junction. Therefore, the minigenes and the CV-1 cells are an adequate genetic and cellular environment to recreate the splicing pattern of TG exon 35.

The functional consequences of the deletion of exon 22 or 35 could be structural changes in the protein molecule that alter the normal protein folding and assembly, leading to a marked reduction in the ability to export the protein from the endoplasmic reticulum with massive induction of selective

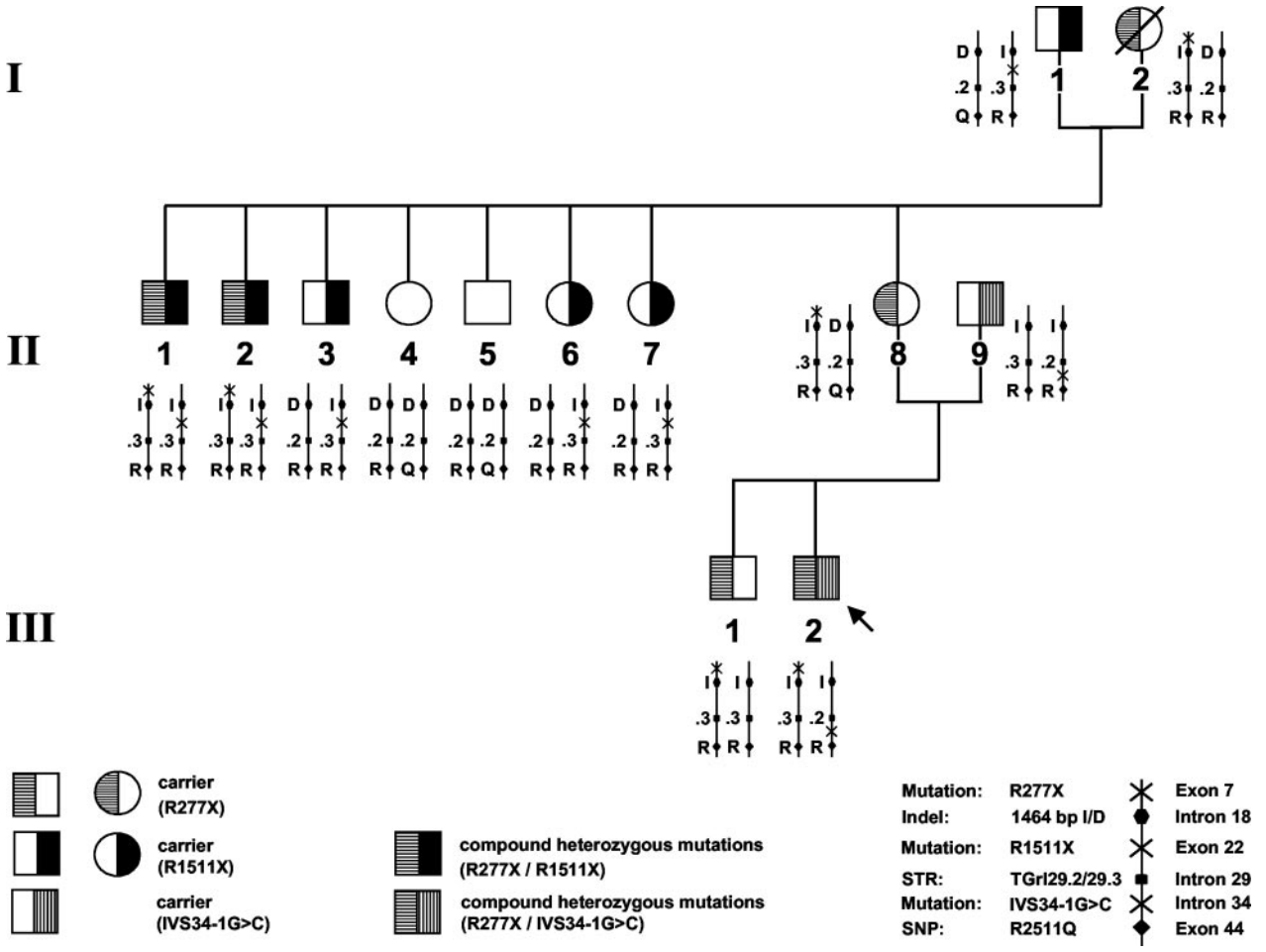

FIG. 6. Pedigree of the MA family showing haplotype analyses using the three polymorphisms and the three detected mutations. The pedigree shows the pattern of inheritance of the mutant TG alleles. All data are aligned with each individual symbol on the pedigree. Note that both affected siblings II-1 and II-2 have inherited one copy of the R277X mutation from their mother (I-2) and one copy of the R1511X mutation from their father (I-1). The affected nephew III-2 inherited one copy of the R277X mutation from his mother (II-8) and one copy of the IVS34–1G>C mutation from his father (II-9).

synthesis of molecular chaperones, which bind to the misfolded exportable protein (34, 45). Alternatively, it is possible that the elimination of exons containing repeat motifs by alternative splicing or splicing errors would result in an altered ability to transfer an iodophenoxyl group from the donor site to the acceptor iodotyrosine in the coupling machinery. Cysteines are thought to play an important role in the tertiary structure of TG, and five cysteine residues are localized in exon 22 and one in exon 35. Exon 22 contains a tyrosine residue, at position 1510, that might be involved in hormonogenesis.

In this kindred, we also characterized polymorphisms (Indel, microsatellite, and SNP) located within introns 18 and 29 and exon 44 (42–44) that are associated with the described mutations. The availability of informative polymorphic markers will allow indirect disease diagnosis by linkage or association studies, such as in cases with no identified mutations and for rapid identification of affected newborns or gene carriers in families with TG mutations. Additionally, these three polymorphic markers are well suited for routine use in laboratories engaged in linkage or association analysis in families with autoimmune thyroid diseases. Interestingly, all six affected individuals from the two unrelated Brazilian families with congenital goiter and hypothyroidism harboring the mutation R277X, either in a homozygous state (20) or as a compound heterozygous mutation with R1511X or IVS34–1G>C, had an identical haplotype for these three polymorphic markers. This suggests that both families may have a distant common ancestor and, consequently, that R277X is a putative founder mutation.

Five different conclusions emerged from our study. First, the sequencing of the entire coding sequence of the human TG gene, including part of the TG promoter, the splicing signals, and the flanking intronic regions of each intron revealed that three different mutations are present in the MA family: 886C>T (R277X), 4588 C>T (R1511X), and IVS34–1G>C. Second, this finding established the inheritance of two compound heterozygous constellations (R277X/IVS34–1G>C or R277X/R1511X). Third, *in vitro* transcription analysis confirmed that exon 35 is skipped entirely when the IVS34–1G>C mutation is present. Fourth, analysis of Indel, microsatellite, and SNP in the MA family showed association between the mutant TG alleles and the polymorphic markers. Finally, the two unrelated families that carry the R277X mu-

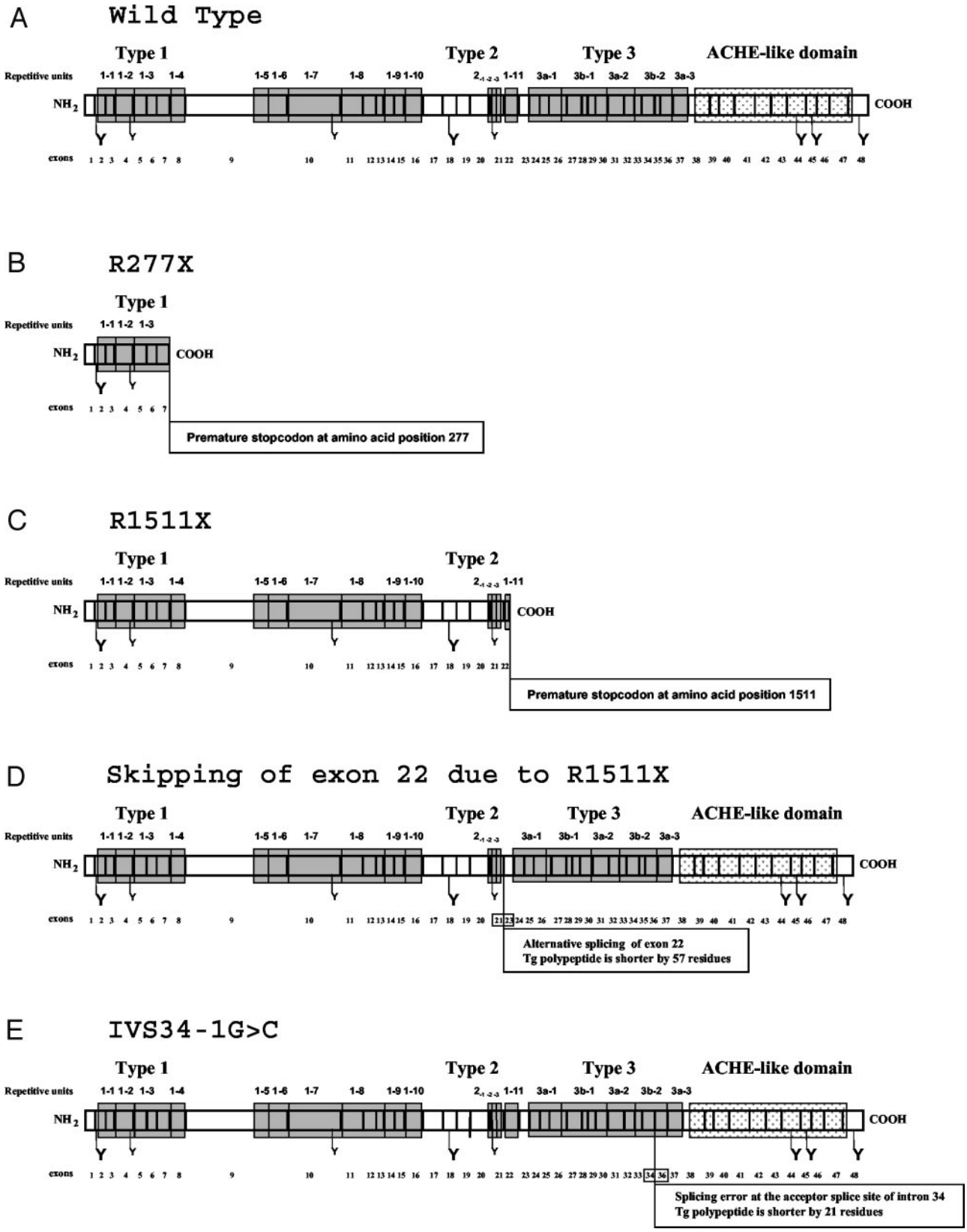

FIG. 7. Schematic representation of the exon organization and its correlation with repetitive, acetylcholinesterase-homology (ACHE-like domain), and hormonogenic domains in the normal (A) and putative mutant TG proteins (B–E). The exons are indicated by *white boxes* and, the repetitive units (types 1, 2, and 3) and acetylcholinesterase-homology domain by *shaded* and *dotted boxes*, respectively. Tyrosine residues, involved as acceptor (Y) and donor (y) sites in thyroid hormone synthesis, are shown.

tation had an identical haplotype for the three markers analyzed, suggesting a founder effect for the R277X mutation. These studies confirm the allelic heterogeneity of TG gene mutations, alterations that may provide insight into the structure-function relationship of TG.

Acknowledgments

We express our appreciation to Peter Kopp, M.D. (Northwestern University, Chicago, IL), for critical reading and suggestions about this manuscript.

Received April 3, 2003. Accepted October 16, 2003.

Address all correspondence and requests for reprints to: Dr. Héctor M. Targovnik, Laboratorio de Biología Molecular, Cátedra de Genética y Biología Molecular, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Av. Córdoba 2351, 4to piso-sala 5, 1120 Buenos Aires, Argentina. E-mail: htargovn@huemul.ffyb.uba.ar.

This work was supported by grants from Universidad de Buenos Aires (B 087/2001), Argentine National Research Council (0853/98), Fondo para la Investigación Científica y Tecnológica (05-08838/ PICT 2000/2001), and Fundação de Amparo à Pesquisa do Estado de São Paulo (99/03570-3; to G.M.-N.), São Paulo, Brazil.

C.M.M. is a research fellow of the Universidad de Buenos Aires. C.M.R. is a research fellow of the Argentine National Research Council. H.M.T. is an established investigator of the Argentine National Research Council. J.V.T. presently is a postdoctoral fellow supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (201571/88-91) at Northwestern University (Chicago, IL).

V.J.G. and C.M.M. contributed equally to the study.

References

1. **Medeiros-Neto G, Targovnik HM, Vassart G** 1993 Defective thyroglobulin synthesis and secretion causing goiter and hypothyroidism. Endocr Rev 14: 165–183
2. **Medeiros-Neto G, Knobel M, DeGroot LJ** 2002 Genetic disorders of the thyroid hormone system. In: Baxter JD, ed. Genetics in endocrinology. Philadelphia: Lippincott, Williams and Wilkins; 375–402
3. **Pohlenz J, Dumitrescu A, Aumann U, Koch G, Melchior R, Prawitt D, Refetoff S** 2002 Congenital secondary hypothyroidism caused by exon skipping due to a homozygous donor splice site mutation in the TSHβ-subunit gene. J Clin Endocrinol Metab 87:336–339
4. **Pohlenz J, Dumitrescu A, Zundel D, Martiné U, Schönberger W, Koo E, Weiss RE, Cohen RN, Kimura S, Refetoff S** 2002 Partial deficiency of thyroid transcription factor 1 produces predominantly neurological defects in humans and mice. J Clin Invest 109:469–473
5. **Krude H, Schütz B, Biebermann H, von Moers A, Schnabel D, Neitzel H, Tönnies H, Weise D, Lafferty A, Schwarz S, De Felice M, von Deimling A, van Landeghem F, Di Lauro R, Grüters A** 2002 Choreoathetosis, hypothyroidism, and pulmonary alterations due to human NKX2–1 haploinsufficiency. J Clin Invest 109:475–480
6. **Clifton-Bligh RJ, Wentworth JM, Heinz P, Crisp MS, John R, Lazarus JH, Ludgate M, Chatterjee VK** 1998 Mutation of the gene encoding human TTF-2 associated with thyroid agenesis, cleft palate and choanal atresia. Nat Genet 19:399–401
7. **Macchia PE, Lapi P, Krude H, Pirro MT, Missero C, Chiovato L, Souabni A, Baserga M, Tassi V, Pinchera A, Fenzi G, Gruters A, Busslinger M, Di Lauro R** 1998 PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. Nat Genet 19:83–86
8. **Vilain C, Rydlewski C, Duprez L, Heinrichs C, Abramowicz M, Malvaux P, Renneboog B, Parma J, Costagliola S, Vassart G** 2001 Autosomal dominant transmission of congenital thyroid hypoplasia due to loss-of-function mutation of PAX8. J Clin Endocrinol Metab 86:234–238
9. **Congdon T, Nguyen LQ, Nogueira CR, Habiby RL, Medeiros-Neto G, Kopp P** 2001 A novel mutation (Q40P) in PAX8 associated with congenital hypothyroidism and thyroid hypoplasia: evidence for phenotypic variability in mother and child. J Clin Endocrinol Metab 86:3962–3967
10. **Sunthornthepvarakul T, Gottschalk ME, Hayashi Y, Refetoff S** 1995 Resistance to thyrotropin caused by mutations in the thyrotropin-receptor gene. N Engl J Med 332:155–160
11. **Abramowicz MJ, Duprez L, Parma J, Vassart G, Heinrichs C** 1997 Familial congenital hypothyroidism due to inactivating mutation of the thyrotropin receptor causing profound hypoplasia of the thyroid gland. J Clin Invest 99:3018–3024
12. **Tonacchera M, Agretti P, Pinchera A, Rosellini V, Perri A, Collecchi P, Vitti P, Chiovato L** 2000 Congenital hypothyroidism with impaired thyroid response to thyrotropin (TSH) and absent circulating thyroglobulin: evidence for a new inactivating mutation of the TSH receptor gene. J Clin Endocrinol Metab 85:1001–1008
13. **Fujiwara H, Tatsumi K-i, Miki K, Harada T, Miyai K, Takai S-I, Amino N** 1997 Congenital hypothyroidism caused by a mutation in the Na^+/I^- symporter. Nat Genet 16:124–125
14. **Pohlenz J, Medeiros-Neto G, Gross JL, Silveiro SP, Knobel M, Refetoff S** 1997 Hypothyroidism in a Brazilian kindred due to iodide trapping defect caused by homozygous mutation in the sodium/iodide symporter gene. Biochem Biophys Res Commun 240:488–491
15. **Pohlenz J, Rosenthal IM, Weiss RE, Jhiang SM, Burant C, Refetoff S** 1998 Congenital hypothyroidism due to mutations in the sodium/iodide symporter. Identification of a nonsense mutation producing a downstream cryptic 3′ splice site. J Clin Invest 101:1028–1035
16. **Ieiri T, Cochaux P, Targovnik HM, Suzuki M, Shimoda S-I, Perret J, Vassart G** 1991 A 3′ splice site mutation in the thyroglobulin gene responsible for congenital goiter with hypothyroidism. J Clin Invest 88:1901–1905
17. **Targovnik HM, Medeiros-Neto G, Varela V, Cochaux P, Wajchenberg BL, Vassart G** 1993 A nonsense mutation causes human hereditary congenital goiter with preferential production of a 171-nucleotide-deleted thyroglobulin ribonucleic acid messenger. J Clin Endocrinol Metab 77:210–215
18. **Targovnik H, Vono J, Billerbeck AEC, Cerrone GE, Varela V, Mendive F, Wajchenberg BL, Medeiros-Neto G** 1995 A 138-nucleotide deletion in the thyroglobulin ribonucleic acid messenger in a congenital goiter with defective thyroglobulin synthesis. J Clin Endocrinol Metab 80:3356–3360
19. **Targovnik HM, Frechtel GD, Mendive FM, Vono J, Cochaux P, Vassart G, Medeiros-Neto G** 1998 Evidence for the segregation of three different mutated alleles of the thyroglobulin gene in a Brazilian family with congenital goiter and hypothyroidism. Thyroid 8:291–297
20. **van de Graaf SAR, Ris-Stalpers C, Veenboer GJM, Cammenga M, Santos C, Targovnik HM, de Vijlder JJM, Medeiros-Neto G** 1999 A premature stop codon in thyroglobulin mRNA results in familial goiter and moderate hypothyroidism. J Clin Endocrinol Metab 84:2537–2542
21. **Hishinuma A, Takamatsu J, Ohyama Y, Yokozawa T, Kanno Y, Kuma K, Yoshida S, Matsuura N, Ieiri T** 1999 Two novel cysteine substitutions (C1263R and C1995S) of thyroglobulin cause a defect in intracellular transport of thyroglobulin in patients with congenital goiter and the variant type of adenomatous goiter. J Clin Endocrinol Metab 84:1438–1444
22. **Targovnik HM, Rivolta CM, Mendive FM, Moya CM, Medeiros-Neto G** 2001 Congenital goiter with hypothyroidism caused by a 5′ splice site mutation in the thyroglobulin gene. Thyroid 11:685–690
23. **Caron P, Moya CM, Malet D, Gutnisky VJ, Chabardes B, Rivolta CM, Targovnik HM** 2003 Compound heterozygous mutations in the thyroglobulin gene (1143delC and 6725G→A[R2223H]) resulting in fetal goitrous hypothyroidism. J Clin Endocrinol Metab 88:3546–3553
24. **Abramowicz MJ, Targovnik HM, Varela V, Cochaux P, Krawiec L, Pisarev MA, Propato FEV, Juvenal G, Chester HA, Vassart G** 1992 Identification of a mutation in the coding sequence of the human thyroid peroxidase gene causing congenital goiter. J Clin Invest 90:1200–1204
25. **Bakker B, Bikker H, Vulsma T, de Randamie JSE, Wiedijk BM, de Vijlder JJM** 2000 Two decades of screening for congenital hypothyroidism in the Netherlands: TPO gene mutations in total iodide organification defect (an update). J Clin Endocrinol Metab 85:3708–3712
26. **Moreno JC, Bikker H, Kempers MJE, van Trotsenburg ASP, Baas F, de Vijlder JJM, Vulsman T, Ris-Stalpers C** 2002 Inactivating mutations in the gene for thyroid oxidase 2 (THOX2) and congenital hypothyroidism N Engl J Med 347:95–102
27. **Kopp P** 2000 Pendred's syndrome and genetic defects in thyroid hormone synthesis. Rev Endocr Metab Disord 1:109–121
28. **Borck G, Roth C, Martiné U, Wildhardt G, Pohlenz J** 2003 Mutations in the PDS gene in german families with Pendred's syndrome: V138F is a founder mutation. J Clin Endocrinol Metab 88:2916–2921
29. **Pérez-Centeno C, González-Sarmiento R, Mories MT, Corrales JJ, Miralles-García JM** 1996 Thyroglobulin exon 10 gene point mutation in a patient with endemic goiter. Thyroid 6:423–427
30. **Corral J, Martín C, Pérez R, Sánchez I, Mories MT, San Millan JL, Miralles JM, González-Sarmiento R** 1993 Thyroglobulin gene point mutation associated with non-endemic simple goitre. Lancet 341:462–464
31. **González-Sarmiento R, Corral J, Mories MT, Corrales JJ, Miguel-Velado E, Miralles-Garcia JM** 2001 Monoallelic deletion in the 5′ region of the thyroglobulin gene as a cause of sporadic nonendemic simple goiter. Thyroid 11: 789–793
32. **Ricketts MH, Simons MJ, Parma J, Mercken L, Dong Q, Vassart G** 1987 A nonsense mutation causes hereditary goitre in the Afrikander cattle and unmasks alternative splicing of thyroglobulin transcripts. Proc Natl Acad Sci USA 84:3181–3184
33. **Veenboer GJM, de Vijlder JJM** 1993 Molecular basis of the thyroglobulin synthesis defect in Dutch goats. Endocrinology 132:377–381
34. **Kim PS, Hossain SA, Park Y-N, Lee I, Yoo S-E, Arvan P** 1998 A single amino acid change in the acetylcholinesterase-like domain of thyroglobulin causes congenital goiter with hypothyroidism in the cog/cog mouse: a model of

human endoplasmic reticulum storage diseases. Proc Natl Acad Sci USA 95:9909–9913
35. **Kim PS, Ding M, Menon S, Jung C-G, Cheng J-M, Miyamoto T, Li B, Furudate S-I, Agui T** 2000 A missense mutation G2320R in the thyroglobulin gene causes non-goitrous congenital primary hypothyroidism in the WIC-rdw rat. Mol Endocrinol 14:1944–1953
36. **Hishinuma A, Furudate S-I, Masamichi O-I, Nagakubo N, Namatame T, Ieiri T** 2000 A novel missense mutation (G2320R) in thyroglobulin causes hypothyroidism in rdw rats. Endocrinology 141:4050–4055
37. **Targovnik H, Propato F, Varela V, Wajchenberg BL, Knobel M, D'Ambrozo HF, Medeiros-Neto G** 1989 Low levels of thyroglobulin messenger ribonucleic acid in congenital goitrous hypothyroidism with defective thyroglobulin synthesis. J Clin Endocrinol Metab 69:1137–1147
38. **Mendive FM, Rivolta CM, Vassart G, Targovnik HM** 1999 Genomic organization of the 3′ region of the human thyroglobulin gene. Thyroid 9:903–912
39. **Moya CM, Mendive FM, Rivolta CM, Vassart G, Targovnik HM** 2000 Genomic organization of the 5′ region of the human thyroglobulin gene. Eur J Endocrinol 143:789–798
40. **Mendive FM, Rivolta CM, Moya CM, Vassart G, Targovnik HM** 2001 Genomic organization of the human thyroglobulin gene. The complete intron-exon structure. Eur J Endocrinol 145:485–496
41. **van de Graaf SAR, Ris-Stalpers C, Pauws E, Mendive FM, Targovnik HM, de Vijlder JJM** 2001 Up to date with human thyroglobulin. J Endocrinol 170:307–321
42. **Moya CM, Varela V, Rivolta CM, Mendive FM, Targovnik HM** 2003 Identification and characterization of a novel large insertion/deletion polymorphism of 1464 base pair in the human thyroglobulin gene. Thyroid 13:319–323
43. **Rivolta CM, Moya CM, Mendive FM, Targovnik HM** 2002 Genotyping and characterization of two polymorphic microsatellite markers located within introns 29 and 30 of the human thyroglobulin gene. Thyroid 12:773–779
44. **Mendive F M, Rossetti LC, Vassart G, Targovnik H M** 1997 Identification of a new thyroglobulin variant: a guanine-to-adenine transition resulting in the substitution of arginine 2510 by glutamine. Thyroid 7:587–591.
45. **Medeiros-Neto G, Kim PS, Yoo SE, Vono J, Targovnik HM, Camargo R, Hossain SA, Arvan P** 1996 Congenital hypothyroid goiter with deficient thyroglobulin. Identification of an endoplasmic reticulum storage disease with induction of molecular chaperones. J Clin Invest 98:2838–2844

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.