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Review

Naturally Occurring Mutations in the Thyroglobulin Gene

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Thyroglobulin (Tg) is a large glycoprotein dimer secreted into the follicular lumen. It serves as the matrix for the synthesis of thyroxine (T_4) and triiodothyronine (T_3), and the storage of thyroid hormone and iodide. In response to demand for thyroid hormone secretion, Tg is internalized into the follicular cell and digested in lysosomes. Subsequently, the thyronines T_4 (approximately 80%) and T_3 (approximately 20%) are released into the blood stream. Biallelic mutations in the *Tg* gene have been identified in several animal species and human patients presenting with goiter and overt or compensated hypothyroidism. In untreated patients, goiters are often remarkably large and display continuous growth. In most instances, the affected individuals have related parents and are homozygous for inactivating mutations in the *Tg* gene. More rarely, compound heterozygous mutations lead to a loss of function of both alleles. Molecular analyses indicate that at least some of these alterations result in a secretory defect and an endoplasmic reticulum storage disease (ERSD). This review discusses the nature and consequences of naturally occurring *Tg* gene mutations in humans and several animal species. Recent recommendations for the nomenclature of mutations have led to different numbering systems, an aspect that is discussed in order to clarify discrepancies between different publications.

Congenital Hypothyroidism and Clinical Presentation of Patients with Thyroglobulin Defects

THE PREVALENCE OF permanent congenital hypothyroidism is approximately 1 in 3000–4000 newborns, and it is one of the most common preventable causes of mental retardation. In approximately 85%, the disorder is associated with developmental abnormalities of the thyroid such as agenesis of the gland, ectopic thyroid tissue, and thyroid hypoplasia (1–5). Recently, monoallelic mutations in the paired domain transcription factor PAX-8 have been documented and characterized in sporadic and familial patients with thyroid hypoplasia or ectopy (6–8). In addition, a familial PAX-8 mutation was found in patients with congenital hypothyroidism and a normally formed thyroid gland (9). Homozygosity for recessive mutations in the forkhead/winged-helix domain transcription factor TTF2/FOXE1 cause a syndromic form of thyroid dysgenesis (thyroid agenesis, cleft palate, choanal atresia, bifid epiglottis, and spiky hair) (10,11). Despite the elucidation of the molecular pathogenesis in a subset of patients with thyroid dysgenesis, the etiology remains elusive in the vast majority of cases.

Approximately 10% of the patients with congenital hypothyroidism harbor inborn errors of metabolism in one of the steps for thyroid hormone synthesis in thyrocytes (1–5). Dyshormonogenesis can be caused by recessive defects at any of the steps required for normal thyroid hormone synthesis including mutations in the *Tg* gene (Table 1). In untreated patients, thyroid dyshormonogenesis is typically associated with goitrous enlargement of the thyroid secondary to long-term thyrotropin (TSH) stimulation. Goiters are often remarkably large and display continuous growth. Symptoms caused by compression of adjacent neck structures can occur. Tg defects can result in overt hypothyroidism or, alternatively, in compensated hypothyroidism if the available Tg yields sufficient secretion of thyroid hormone. In these patients, the radioiodine uptake is elevated indicating an activation of the iodine concentration mechanism due to

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Table 1. Known Genetic Defects Causing Congenital Hypothyroidism

Phenotype	*Gene*	*Chromosome*
Thyroid dysgenesis		
Agenesis	TTF2 (FOXE1)	9q22
Hypoplasia, hemiagenesis	PAX-8	2q12-q14
Ectopy	Unknown	
Dyshormonogenesis		
A) Hypothalamic-pituitary axis		
Combined pituitary hormone deficiency (CPHD)	PROP1	5q
	POU1F1 (PIT1)	3p11
	LHX3	9q34.3
	LHX4	1q25
	HESX1 (RPX)	3p21.2-p21.1
	Other, unidentified	
Isolated TRH deficiency	No known mutations in TRH	3p
TRH resistance	TRHR	8q23
Isolated TSH deficiency	TSH β subunit	1p13
B) Thyroid gland		
Partial or complete hypothyroidism, hypoplasia	TSHR	14q13
Pseudohypoparathyroidism Ia: hypothyroidism, hypogonadism, AHO	GNAS1	20q13.2
Other postreceptor defects	Other, unidentified	
Hypothyroidism with defective iodide uptake	NIS	19p12-13.2
Iodide organification defect	TPO	2p25
Pendred's syndrome: deafness, goiter, partial organification defect	PDS/SLC26A4	7q31
Transient or permanent hypothyroidism, defective H_2O_2 generation	THOX2	15q15
Goiter with compensated or overt hypothyroidism	TG	8q42.2–24.3
Hypothyroidism, goiter, loss of iodine through secretion of DIT, MIT	Gene unidentifie	
Thyroid dysfunction, ataxia, respiratory distress	TTF1 (NKX2.1)	14q13
Hypothyroidism with normally located thyroid of normal size	PAX-8	2q12-q14

DIT, diiodinated tyrosine; MIT, monoiodotyrosine.

chronic stimulation by TSH. In patients evaluated with a perchlorate discharge test, there is no increased release of radioiodine after administration of the competitor, indicating that the organification process itself is not affected. The serum Tg levels are usually very low, or in the low normal range, and the presence of a low Tg level in a goitrous individual may suggest a Tg defect (12). *Tg* gene defects are inherited in an autosomal recessive manner and affected individuals are either homozygous or compound heterozygous for mutations in the *Tg* gene (Table 1).

Thyroglobulin

Thyroglobulin (Tg) serves as a matrix for the synthesis of thyroxine (T_4) and triiodothyronine (T_3), and for their subsequent storage. Tg is encoded by a single-copy gene of 270 kb (13) (GenBank accession number NT_ 008046), which has been mapped on chromosome 8q24.2–8q24.3 (14–16). It contains 48 exons separated by introns of up to 65 kb (13,17,18). The synthesis of the *Tg* gene is controlled by transcription factors such as TTF-1 (NKX2.1), TTF-2 (FOXE1), and PAX-8 (19,20). The full-length human 8.5 kb messenger RNA (mRNA) (GenBank accession number: NM 003235) contains a 41-nucleotide 5′-untranslated segment preceding an open reading frame of 8307 bases and a 3′-untranslated region ranging from 101 to 120 bp (21,22). Alternative splicing generates various transcripts and subsequently a heterogeneous population of Tg polypeptides (23). As of yet, at least 15 polymorphisms have been detected at the nucleotide level; among them, 10 result in variations in the amino acid sequence, whereas 5 are silent (23).

After translation of the mRNA, the 19-amino acid signal peptide drives the Tg molecule into the endoplasmic reticulum (ER), where the Tg polypeptide is submitted to folding and dimerization. The monomer of Tg is composed of a 19-amino acid signal peptide followed by 2749 residues containing 66 tyrosines (Fig. 1) (23). The motifs within the secreted protein are usually numbered after subtracting the 19-amino acid signal peptide. Similarly, mutations have traditionally been described using this numbering system (23). However, others have included the signal peptide (24–26). Moreover, the Recommendations for a Nomenclature System for Human Gene Mutations (27–29), a system supported by many of the major genetic journals, recommend to denote the A of the ATG encoding the initiator methionine as nucleotide +1, and the initiator methionine of the signal peptide is designated as amino acid 1 (27,28). Consequently, several publications have based the description of Tg mutations on these recommendations (30,31). Not surprisingly, the coexistence of these two numbering systems, together with several modifications of the initially published sequences (23), often lead to confusion. Figure 2, Table 2 (human Tg mutations), and Table 3 (Tg mutations in animals) summarize the currently reported mutations indicating the location of the mutation within the coding sequence, respectively in the introns, and the amino acid alterations in the precursor, as well as the mature polypeptide. Table 4 lists the known naturally occurring mutations according to the type of mutation.

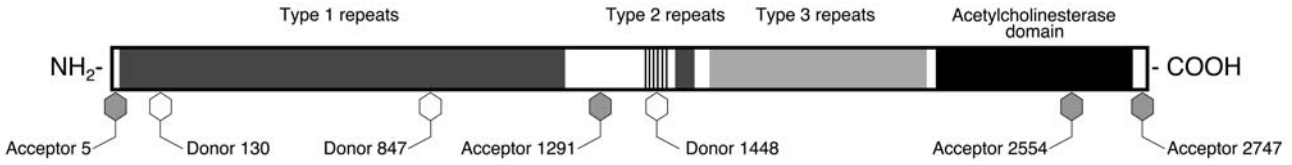

FIG. 1. Schematic structure of the thyroglobulin protein with the major hormonogenic sites.

In the follicular lumen, Tg is a present as a 19S dimeric glycoprotein of 660 kd (32–34). Analysis of the primary structure of the Tg protein for internal homology led to its division into four major regions (Fig. 1). (1) The type 1 repetitive region contains eleven type 1 motifs, a domain found in a large family of proteins (35). In the mature polypeptide lacking the signal peptide, these motifs are located between amino acids 12 and 1191, and 1492 and 1546. (2) The type 2 repetitive region, which is composed of three type 2 elements, is located between amino acids 1437 and 1484. (3) The type 3 repetitive region is characterized by five elements between residues 1584 and 2168. (4) The carboxyterminal part of the Tg monomer, encompassing residues 2192 to 2716, shares remarkable homology with acetylcholinesterase (13,21,36,37). Overall, this structure has been interpreted to indicate the possibility of a convergent origin of the *Tg* gene from two different ancestral DNA sequences (38).

The maturation of Tg is controlled by several molecular chaperones such as BiP, GRP94, Erp72, and calnexin (39). Misfolded Tg is accumulated in the ER and then translocated back into the cytoplasm to undergo degradation by the proteasome system (39). This process, referred to as endoplasmic reticulum-associated degradation or ERAD, includes removal of interchain disulfide bonds, carbohydrate dissociation, polyubiquination, and hydrolysis before complete proteasomal degradation (40). Properly folded Tg dimers migrate to the Golgi apparatus where glycosylation occurs. The Tg monomer contains 20 potential glycosylation sites; among them, 16 are known to be glycosylated (32,41), and approximately 10% of the molecular weight is accounted for by carbohydrates. Tg contains four types of carbohydrate units. Simple or type A units consist of asparagine-linked mannose and N-acetylglucosamine. Type B or complex carbohydrates contain three mannose residues and variable numbers of N-acetylglucosamine, galactose, fucose, and sialic acid residues. Two additional carbohydrate units are linked to the peptide chain through the hydroxyl group of serine or threonine. Type C carbohydrates consist chiefly of galactosamine and type D is a large chondroitin sulfate unit. Other secondary modifications of the Tg polypeptide include sulfation and phosphorylation (42,43). Recent studies indicate that consensus sequences required for tyrosine sulfation are present at most of the hormonogenic sites within Tg (44). Tyrosine sulfation may play a role in the hormonogenic process (44), a concept that remains to be corroborated by further experimental evidence.

From the Golgi apparatus, glycosylated Tg migrates to the apical membrane in small secretory vesicles and is secreted

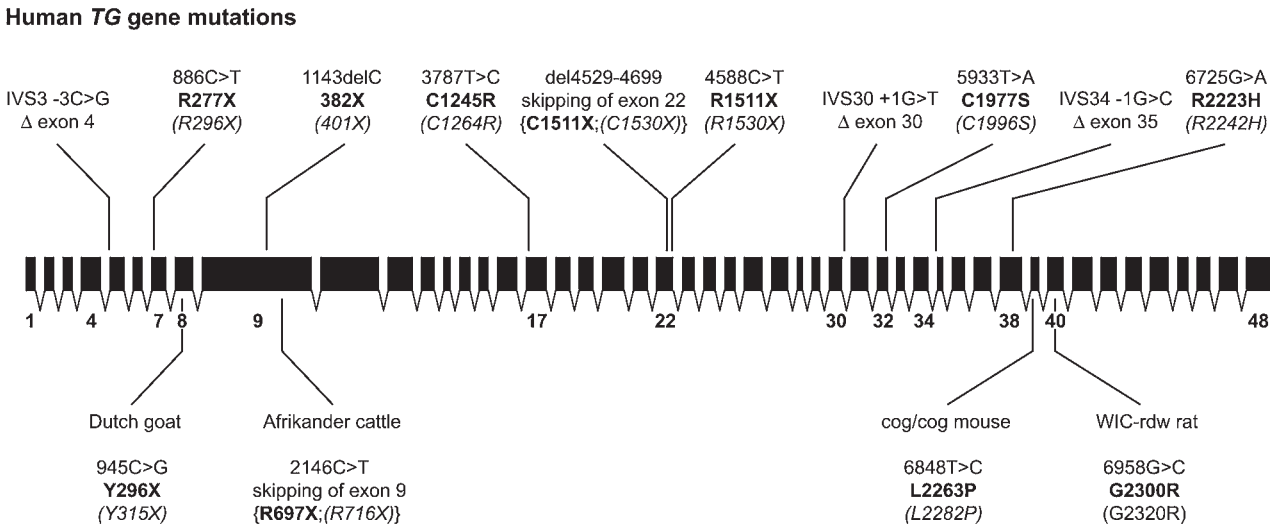

FIG. 2. Naturally occurring mutations in the *Tg* gene in humans and animal species. Mutations at the nucleotide level are described denoting the A of the initiator ATG as +1 (27,28). At the amino acid level the location is described after cleavage of the 19 amino acid signal peptide (**bold**) and in the precursor protein (*italics*). Premature stop codons resulting in nonsense-mediated altered splicing are indicated with brackets { }. For detailed sequence information see: GenBank data base accession numbers AH008122, AH007064, AF237421, AF255396, AH008090, AY053519, NT_008046; EMBL accession numbers ENSG00000042832, ENST00000220616.

Table 2. Human *Tg* Gene Mutations

Nucleotide	*Amino acid*	*Functional consequence*	*Phenotype*	*Consanguinity*	*Comment*	*Reference*
IVS3-3C>G	Deletion of exon 4	Skipping of exon 4	Goiter, hypothyroidism	Yes		Ieiri et al. (58)
4588C>T Nonense-mediated exon skipping: del4529-4699	R1511X Nonense-mediated exon skipping of exon 22	Skipping of exon 22	Goiter, hypothyroidism	No	Originally described as 4626C>T, C1510X Two distinct compound heterozygous constellations	Targovnik et al. (60) Gutnisky et al. (62)
886C>T IVS34-1G>C	R277X Deletion of exon 35	Truncated TG Skipping of exon 35			Heterozygous mutation on second allele identified	
IVS30+G>T	Deletion of exon 30	Skipping of exon 30 Retention in ER	Goiter, hypothyroidism	Yes	Mutation on second allele not identified	Targovnik et al.
886C>T	R277X	Truncated Tg and partial secretion of thyroid hormones	Goiter, mild hypothyroidism	Yes	Originally described as R277X	Van de Graaf et al. (64)
3790T>C	C1245R	Impaired intracellular transport of Tg	Patient 1: Goiter, euthyroidism Patient 2: Mild hypothyroidism	No Yes	Originally described as 3787T>C, C1263R	Hishinuma et al. (24,25)
5986T>A	C1977S	Partial retention in ER	Euthyroid adenomatous goiter and increased serum TG levels	No	Serum TG levels slightly increased Originally described as 5983T>A, C1995S	Hishinuma et al. (25)
1143delC 6725G>A	FS362>382X R2223H	No detailed characterization Possibly truncated Tg No detailed characterization Possibly intracellular retention	(Fetal) goiter, hypothyroidism	No	Compound heterozygous mutations	Caron et al. (71)
886C>T	R277X	Truncated Tg	Goiter, Hypothyroidism	Not known	Independently recurring mutation	Rivolta et al. (72)

Mutations at the nucleotide level are described denoting the A of the initiator ATG as +1 (27, 28). At the amino acid level the location is described in the mature polypeptide, i.e., after cleavage of the 19 amino acid signal peptide. Figure 2 shows the numbering with and without the signal peptide.

Tg, thyroglobulin; ER, endoplasmic reticulum.

Table 3. *Tg* Gene Mutations and Putative *Tg* Defects in Animal Species

Species	*Nucleotide*	*Amino acid*	*Phenotype*	*Functional consequence*	*Reference*
Afrikander cattle	2146C>T Nonsense-mediated exon skipping of exon 9	R697X Nonsense-mediated exon skipping of exon 9	Goiter, euthyroidism	Deletion of exon 9	Ricketts et al. (65)
Dutch goat	945C>G	Y296X	Goiter, hypothyroidism	Impaired intracellular transport of TG	Veenboer et al. (85)
cog/cog mouse	6848T>C	L2263P	Goiter, hypothyroidism	Impaired intracellular transport of TG	Kim et al. (30, 31)
WIC-rdw rat	6958G>C	G2300R	No goiter, hypothyroidism	Impaired intracellular transport of Tg	Hishinuma et al., Kim et al. (30, 31)
		Putative Tg defects			
Merino sheep	Not characterized	Not characterized	Hypothyroidism	Abnormal Tg (175 kDa)	Falconer et al. (77, 78)
Serb cattle	Not characterized	Not characterized	Goiter, euthyroidism	Increased 12S Tg monomer	Sinadinovic et al. (80)
Bongo antelope	Not characterized	Not characterized	Goiter, euthyroidism	Abnormal Tg (220 kDa)	Doi et al. (84)

Mutations at the nucleotide level are described denoting the A of the initiator ATG as +1 (27, 28). At the amino acid level the location is described in the mature polypeptide, i.e., after cleavage of the 19 amino acid signal peptide. Figure 2 shows the numbering with and without the signal peptide.

Tg, thyroglobulin; ER, endoplasmic reticulum.

Table 4. List of *Tg* Gene Mutations According to Type of Mutation

Nucleotide	*Amino acid*	*Functional consequence*	*Species*	*Reference*
		Missense mutations		
3790T>C	C1245R	Impaired intracellular transport of Tg	Human	Hishinuma et al. (24,25)
5986T>A	C1977S	Partial retention in ER	Human	Hishinuma et al. (25)
6725G>A	R2223H	No detailed characterization Possibly intracellular retention	Human	Caron et al. (71)
945C>G	Y296X	Impaired intracellular transport of Tg	Dutch goat	Veenboer et al. (85)
6848T>C	L2263P	Impaired intracellular transport of Tg	*cog/cog* mouse	Kim et al. (86)
6958G>C	G2300R	Impaired intracellular transport of Tg	WIC-rdw rat	Hishinuma et al. (31) Kim et al. (30,31)
		Nonsense mutation with documented nonsense-mediated exon skipping		
IVS3-3C>G	Deletion of exon 4	Skipping of exon 4	Human	Gutnisky et al. (62)
4588C>T Nonsense-mediated exon skipping: del4529-4699	R1511X Nonsense-mediated exon skipping of exon 22	Skipping of exon 22	Human	Targovnik et al. Gutnisky et al. (60,62)
2146C>T Nonsense-mediated exon skipping of exon 9	R697X Nonsense-mediated exon skipping of exon 9	Deletion of exon 9	Afrikander cattle	Ricketts et al. (65)
		Nonsense mutation with or without preceding frameshift		
886C>T	R277X	Truncated Tg	Human	Gutnisky et al. (62) Van de Graaf et al., (64) Rivolta et al. (72)
1143delC	FS362>382X	No detailed characterization Possibly truncated Tg	Human	Caron et al. (71)
		Splice site mutations		
IVS34-1G>C	Deletion of exon 35	Skipping of exon 35	Human	Targovnik et al. (60) Gutnisky et al. (62)
IVS34+1G>T	Deletion of exon 30	Skipping of exon 30 Retention in ER	Human	Targovnik et al. (67,68)

Tg, thyroglobulin; ER, endoplasmic reticulum.

into the follicular lumen in a regulated process (45,46). In the follicle, selected tyrosyl residues of the Tg polypeptide are iodinated, giving rise to monoiodotyrosines (MIT) and diiodotyrosines (DIT), a reaction referred to as organification (47,48). Aside from an appropriate substrate, this process, the coupling reaction, requires a properly functioning thyroperoxidase (TPO) and H_2O_2. The next step in thyroid hormone synthesis consists of the coupling of two DIT residues to form T_4, or one DIT and one MIT to form T_3. This process also takes place under oxidative conditions

(47,48). During the coupling reaction, a tyrosyl residue donates its iodinated phenyl group to become the outer ring of the iodothyronine amino acid at an acceptor site, leaving dehydroalanine or its derivative at the donor position. In human Tg, the four main hormonogenic acceptor sites have been localized at positions 5, 1291, 2554, and 2747 in the mature polypeptide lacking the signal peptide (Fig. 1) (32,49). Donor sites include tyrosines 130, 847, and 1448. The most important T_4 forming site is located at tyrosine 5 and there is evidence that tyrosine 130 is the dominant donor site (Fig. 1) (50).

Further processing of Tg requires its reentry into the thyroid cell through vesicular internalization (i.e. micropinocytosis), with subsequent fusion with lysosomes resulting in breakdown of the Tg–iodothyroxine complexes and release of thyroid hormones (51). The micropinocytosis may be initiated by both nonselective fluid phase uptake and by receptor-mediated endocytosis (52,53). Aside from degradation of Tg in lysosomes, Tg can also be recycled back into the follicular lumen (54). Recycling of immature forms of Tg back to the apical membrane after endocytosis is thought to involve an asyaloglycoprotein receptor (55). Intact Tg can also be transported from the apical to the basolateral membrane, where it is released into the bloodstream (56,57). This transepithelial transport or transcytosis is mediated by megalin, a receptor located on the apical membrane of the thyroid follicular cells (52,53).

Tg Gene Mutations in Humans

The first individuals with a documented *Tg* gene mutation were reported by Ieiri et al. (58) (Table 2, Fig. 2). The index patient presented with hypothyroidism, congenital goiter, and a marked impairment of Tg synthesis. Her parents were first cousins and two of her five siblings also presented with goiters. Analysis of a restriction length polymorphism in the *Tg* gene demonstrated that the affected individuals were homozygous for this allele and Tg mRNA obtained from the goitrous tissue was slightly reduced in size in comparison to normal individuals. Sequencing of the cDNA revealed that exon 4 was missing from the major Tg transcript in the goiter and analysis of genomic DNA revealed a cytosine to guanine transversion at position −3 in the acceptor splice site of intron 3 (g.IVS3-3C>G) (Fig. 2) (58). Without changing the reading frame, this mutation leads to skipping of exon 4, a region that codes for the donor tyrosine at position 130, an important donor for the outer ring of T_4 to tyrosine 5 (Fig. 1) (50,59).

A nonconsanguineous Brazilian family with two affected siblings and a nephew presenting with congenital goiter, hypothyroidism, and marked impairment of Tg synthesis was

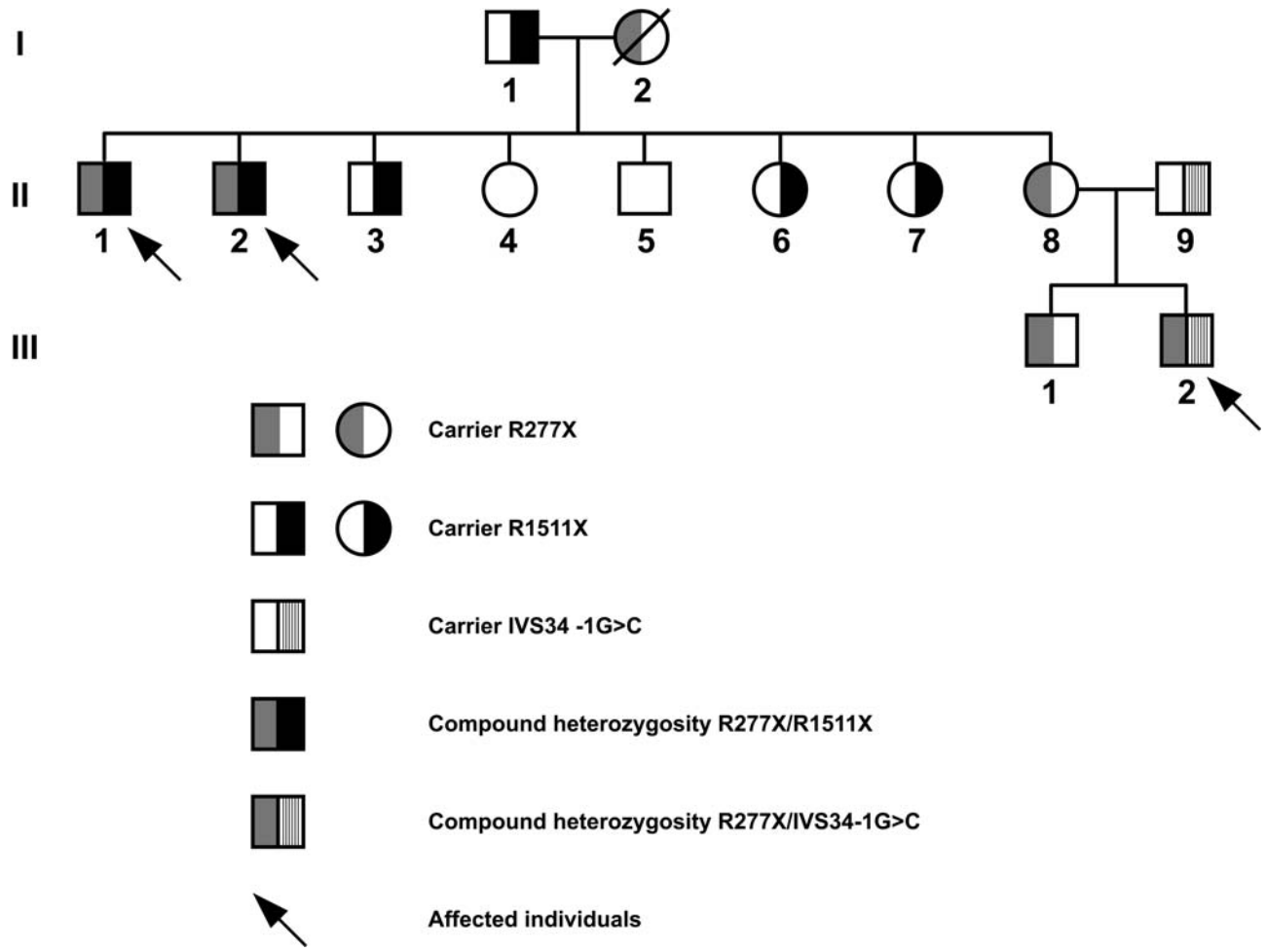

FIG. 3. Pedigree of a Brazilian kindred with two distinct compound heterozygous constellations caused by three distinct thyroglobulin (Tg) mutations. From (62) with permission of the Endocrine Society.

extensively studied by Targovnik et al. (60–62) (Fig. 3). In a first report, the authors reported a diminished level of Tg mRNA resulting in decreased translation of a fully mature Tg (63). Subsequent analyses revealed the presence of an mRNA transcript lacking 171 base pairs corresponding to exon 22 (bp 4529–4699 in the cDNA). The shorter, alternatively spliced Tg predominates in the goiter suggesting that the patients have a mutation resulting in skipping of exon 22. The analysis of the patient's cDNA revealed the presence of a transition c.4588C>T in exon 22 (originally described as c.4626C>T) creating a stop codon at position 1511 in the mature polypeptide (p.R1511X; originally described as p.R1510X) (60). Rather than resulting in the translation of a truncated protein, the nonsense mutation alters splicing pathways that lead to the exclusion of the affected exon in the final mRNA, a phenomenon referred to as nonsense-mediated exon skipping. Genomic DNA of all family members was studied by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) analyses and the results demonstrated that the nonsense cytosine to thymine transition was present in the heterozygous state in the two affected siblings, as well as in four unaffected members of this family. Interestingly, the mutation was not identified in the affected nephew. This finding suggested that at least one, possibly two additional *Tg* gene mutations segregate in this family and that the affected individuals are compound heterozygous for *Tg* gene mutations (61). Recently, genomic DNA sequencing revealed that the affected nephew was indeed heterozygous for a previously described c.886C>T transition in exon 7 resulting in a premature stop codon at amino acid 277 (p.R277X) (64), and for a novel guanine to cytosine transversion at position −1 of the splice acceptor site in intron 34 (g.IVS34-1G>C) (62). The two affected siblings inherited the p.R277X mutation from their mother and the p.R1511X mutation from their father. *In vitro* transcription analysis revealed that exon 35 is skipped entirely in the presence of the IVS34-1G>C mutation (62).

The c.4588C>T/p.R1511X mutation, together with the mutation documented in the Afrikander cattle (see below) (65), illustrates the phenomenon of nonsense-mediated altered splicing, in which an exon harboring a premature stop codon is removed from the mature transcript (66). Several mechanisms have been proposed to explain nonsense-mediated altered splicing. They include removal of the altered exon by nuclear scanning, nonsense-mediated mRNA decay of the mutant transcript in combination with translation of small amounts of exon-skipped isoforms, disruption of the secondary structure leading to exon excision, or disruption of exonic splicing enhancers by the premature stop codon (66).

In a further consanguineous family with two affected individuals studied by Targovnik et al. (67), the patient's Tg mRNA was missing 138 nucleotides corresponding to exon 30. Analysis of genomic DNA showed a guanine to thymine transversion at position +1 in the splice donor site of intron 30 (g.IVS30+1G>T) resulting in subsequent skipping of exon 30. Remarkably, the deletion does not affect the reading frame of the resulting mRNA and generates a Tg polypeptide chain that is shortened by 46 residues. The deletion of 46 residues in the central region of Tg molecule might affect its tertiary and quaternary structure resulting in impaired thyroid hormone synthesis (68). Immunopositivity for Tg was found in thyroid cells, but not in the follicular lumina, and electron microscopy indicated abnormal distention of the ER (69). The retention of the misfolded Tg induces the expression of ER chaperones such as GRP94 and BiP suggesting that the Tg mutations leads to a defective folding and/or assembly, which results in a markedly reduced ability to export the protein from the ER (69). In spite of the fact that the two siblings harbor the same mutation in the *Tg* gene, their phenotype was quite different. The affected boy was severely hypothyroid and has mental retardation, while his sister was mildly hypothyroid with normal mental development. The phenotypic differences may be related to the onset of treatment with levothyroxine and/or, since this family moved within Brazil, to variable nutritional iodide intake.

Van de Graaf et al. (64) studied three offspring of a consanguineous marriage presenting with mild hypothyroidism associated with defective Tg synthesis. The *Tg* cDNA was analyzed by direct sequencing and revealed a cytosine to thymine transition at nucleotide position 886 (c.886C>T) in exon 7 that results in a stop codon at amino acid position 277 in the mature polypeptide (64). This mutation was expressed in a construct containing the first 2110 bp of the coding sequence. *In vitro* expression of this truncated Tg cDNA showed that the mutated Tg protein can be glycosylated. Moreover, this truncated form of Tg still harbors both the acceptor tyrosine 5 and the donor tyrosine 130. The presence of the glycosylation sites may permit migration of the Tg mutant from the ER to the Golgi. The partially retained hormone synthesis of this mutant is in agreement with a study using a 26 kd Tg peptide consisting of the aminoterminus with the hormonogenic site A (i.e., tyrosine 5) that showed that this small peptide is sufficient for T_4 synthesis (Fig. 1 and 2) (70).

Hishinuma et al. (24,25) identified a homozygous mutation at position 3790 (c.3790T>C; originally described as c.3787T>C) in the *Tg* cDNA from two unrelated patients with congenital goiter. One of them was euthyroid, the other individual had mild hypothyroidism, but both had undetectable Tg levels. The mutation results in a substitution from cysteine to arginine at codon 1245 in the mature peptide, respectively 1264 in the uncleaved precursor (p.C1245R; originally described as C1263R). The mutated protein is retained in the ER as demonstrated by sensitivity to digestion with endoglycosidase H and formation of high molecular aggregates (24,25).

In two sisters presenting with euthyroid adenomatous goiter and increased serum Tg levels, Hishinuma et al. (25) identified a thymine to adenine substitution at nucleotide 5986 of the Tg cDNA (c.5983T>A; originally described as 5983T>A) resulting in an amino acid substitution from cysteine to serine at codon 1977 in the cleaved peptide, respectively 1996 in the precursor (C1996S; originally described as C1995S) (25). The C1996S Tg is only partially resistant to endoglycosidase H treatment, and a fraction of the protein is transported to the Golgi and, as reflected by the slightly increased serum levels, secreted into the circulation (25).

Coincidental intrauterine detection of a fetal goiter by ultrasound led to the detection of compound heterozygous *Tg* gene mutations by Caron et al. (71) (Table 2). The goiter was first observed at 6-months of gestation and cordocentesis revealed severe hypothyroidism of the fetus. Repeated intraamniotic injection of 200 μg levothyroxine was not sufficient to suppress the fetal TSH and at birth the neonate had a TSH of 284 mU/L. Moreover, a very low serum Tg led to the suspicion that a defect in its synthesis could be the cause of the goitrous hypothyroidism. Similar findings were ob-

served in a subsequent pregnancy. During the second gestation, intra-amniotic injection of 500 μg levothyroxine at 32 and 36 weeks led to a significant reduction of the TSH, which was 472 mU/L at 29 weeks and 39 mU/L at birth. Sequence analysis of genomic DNA obtained from the two siblings, a girl and a boy, revealed a paternal *Tg* gene deletion 1143delC in exon 9 resulting in a frameshift beginning at residue 362 that generates a stop codon at position 382 (382×) in the mature Tg protein, and a 6725G>A transition in the maternal allele that leads to a substitution of arginine 2223 by histidine (R2223H) (Fig. 2) (71).

In a patient of Argentinean origin with congenital hypothyroidism, goiter and Tg deficiency, Rivolta et al. (72) identified biallelic c.886C → T (p.R277X) mutations in exon 7. This mutation has been reported previously in two Brazilian kindreds (60,62,64). Comparison of intragenic polymorphisms excluded a common ancestor of this patient and one of the Brazilian individuals reported by Gutnisky et al. (62), a finding indicating that this mutation is independently recurrent. RT-PCR with RNA obtained from thyroid tissue excluded that this nonsense mutation would lead to nonsense-mediated exon skipping. The patient's sister presented with the same phenotype, but was not available for molecular analyses (72,73).

Tg alterations associated with simple goiter

A monoallelic Tg mutation has been associated with nonendemic simple goiter. Analyzing 56 individuals, a 2610G>T transversion in exon 10 substituting glutamine 851 by histidine (Q851H) in the mature polypeptide was found in 14 individuals with simple goiter from three different families and the authors proposed an autosomal dominant inheritance of the defect (26). However, 11 unaffected individuals also carried the same allele (26). Subsequently, the same authors reported the Q851H mutation in 1 of 36 patients with endemic goiter and suggested an association of this allele with goiter development (74). Given that the *Tg* gene contains multiple polymorphisms, and in the absence of functional data, it remains unclear whether this alteration is indeed causally involved in the development of the abnormal phenotype. Although the role of this alteration remains uncertain, it is conceivable that a subset of mutated and misfolded Tg polypeptides could lead to retention of the wild type allele in intracellular compartments. This would, for example, be analogous to the situation observed with aquaporin 2 (AQP2) mutations causing nephrogenic diabetes insipidus (NDI). While the majority of patients with NDI caused by AQP2 mutations are homozygous or compound heterozygous for inactivating mutations that lead to ER retention, a few substitutions exert a dominant effect by retaining the wild-type allele in the Golgi (75).

In another series of 50 patients with simple euthyroid goiter a monoallelic Tg deletion encompassing the promoter and the first 11 exons was found in a single patient (76). Given that heterozygous individuals with inactivating Tg mutations do not display an abnormal phenotype, it remains questionable whether this alteration has any significance for the development of the goiter (76).

T_g Gene Mutations in Animals

Important insights into the consequences of Tg alterations have been obtained from studies of various animal strains such as sheep (77,78), cattle (79,80), goats (81,82), mice (83), the Bongo antelope (84), and rats (30,31). In some of these animal species, the molecular defect in the *Tg* gene has been identified (Table 3, Fig. 2) (30,31,65,85,86).

The Afrikander cattle have a phenotype characterized by euthyroid congenital goiter with Tg deficiency (65). The goitrous tissue of these animals harbors a minor Tg mRNA of 7.3 kb besides the normally sized Tg mRNA. A cytosine to thymine transition (c.2081C>T) in exon 9 of the *Tg* gene creates a stop codon (R697X in the mature polypeptide). Rather than generating a truncated protein, alternative splicing removes the exon harboring the premature stop codon by nonsense-mediated altered splicing (65). This truncated Tg protein is shorter (approximately 75 kd), but contains the amino-terminal hormonogenic site and appears to be sufficient for hormone synthesis at the expense of a large, compensatory goiter.

De Vijlder et al. (81) identified an inbred strain of Dutch goats with congenital goiter and hypothyroidism. Analysis of the proteins expressed in the goiter of these animals showed that the 19s Tg was not detectable, but a smaller 7s protein with a molecular weight of a 35 kd could be identified. The amino-terminal hormonogenic site was maintained in this Tg fragment and these shorter proteins were shown to be able to produce thyroid hormone in circumstances of high iodine intake (87). Under these nutritional conditions, the animals are euthyroid but their goiters do not shrink (88). At the molecular level, a transition of cytosine to thymine at position 945 of the Tg cDNA results in a stop codon in exon 8 (945C>T, Y296X in the cleaved polypeptide) and translation of a truncated aminoterminal Tg polypeptide (85,89).

Beamer et al. (83) identified a mouse strain (*cog/cog*) with autosomal recessive hypothyroidism and congenital goiter (83). The *cog/cog* mouse has a Tg with abnormal immunologic and sedimentation properties, rather than an absent Tg protein (90). The molecular basis of this abnormal Tg is explained by a thymine to cytosine substitution at position 6848 of the *Tg* cDNA and creates a missense mutation of leucine to proline at position 2263 (6848T>C, L2263P in the mature polypeptide) (86). This mutation is localized in a region that is strictly conserved in the Tg of all species and that displays remarkable homology with *Torpedo californica* acetylcholinesterase. This domain appears to be essential for the structural properties of the Tg molecule such as dimer formation and transport through the secretory pathway (36). The *cog/cog* Tg protein is unable to exit the ER and, analogous to Tg mutations identified in humans, it is resulting in an ER storage disease (ERSD) and thus thyroid dyshormonogenesis (86).

The phenotype of the *rdw* rat is defined by dwarfism and hypothyroidism (91). Initially, the phenotype was thought to be caused by pituitary dysfunction associated with hypoplasia of the hypophysis. However, subsequent studies suggested that a dysfunction of the thyroid gland is causing the abnormal dwarfed phenotype (92). The *rdw* rat has no goiter and low levels of Tg in the follicular lumen. However, these animals have detectable Tg in the dilated ER, suggesting that the export of Tg is impaired (93). Concomitantly, there is an increased expression of the molecular chaperones GRP94, GRP78, and (hsp70), proteins that are involved in protein quality control (94). The molecular defect has been unraveled independently by two groups. Genetic analyses established linkage to rat chromosome 7 and the Tg locus,

and subsequent sequencing of the rdw Tg cDNA revealed a transversion of guanine to cytosine at position 6958. This transversion results in the substitution of glycine by arginine at position 2320 (6958G>C, G2301R in the mature polypeptide, respectively G2320 in the precursor protein) (30). This finding has been independently confirmed by Hishinuma et al. (31), who, after cloning of the rat wild-type and rdw cDNA, identified the same mutation. Prediction of the protein structure by computer analysis suggests that the mutation might give rise to a subtle structural change in the Tg protein causing an extended helix, while maintaining normal disulfide bonds, and allowing partial monomer formation (31). Alternatively, the substitution of the neutral amino acid glycine by the charged residue arginine in a highly conserved domain in the Tg molecule may cause an important conformational modification in the carboxyterminal region of the Tg protein preventing proper folding and causing retention in the ER (30). The amino acid glycine contains only a hydrogen atom as side chain thus permitting to adopt a much wider range of conformations than other residues. Therefore, it could play a structurally important role allowing unusual main chain conformations at this position of Tg.

Recently, Baryshev et al. demonstrated that the rdw mutation, as well as the C1245R and C1977S mutations found in humans, induce the so-called unfolded protein response (UPR) (95). The UPR is activated by excessive accumulation of mutant secretory proteins that are unable to attain their correct three-dimensional structure and are thus retained in the ER. The UPR includes the induction of ER chaperones such as ERp29, ERp72, calreticulin, protein disulfide isomerase (PDI), cytoplasmic (heat shock protein [HSP] 70, HSP90) and mitochondrial (mtHSP70) upregulated chaperones and folding enzymes, as well as the appearance of the active form of the X-box binding protein (XBP1) and the transcription factor 6 (ATF6). The processing of ATF6 was observed in both human and rat tissues with Tg mutations (95).

Summary and Perspective

Naturally occurring mutations continue to provide unique insights into pathophysiologic mechanisms. In the case of Tg, it has become apparent that missense mutations can be associated with a classic ERSD (39). Mutations that cause alterations in the protein structure give rise to intracellular retention of the altered proteins, emphasizing that a correct conformation is essential for protein transport and biologic activity. The goitrous phenotype can be explained by long-term TSH stimulation and the accumulation of misfolded proteins in the cells affected by ERSD that result in expansion and dilatation of the ER (39). This contrasts, however, with the mutation identified in the rdw rat, which does not develop a goiter (30,31).

Some of the nonsense mutations in the *Tg* gene are of particular interest because of the plasticity generated by the mechanism of nonsense-mediated exon skipping (R697X resulting in skipping of exon 9 in the Afrikander cattle; C1510X resulting in skipping of exon 22 in humans) (60,65). Exon skipping can also be caused by mutations in splice acceptor and donor sites (IVS3-3C>G; IVS30+1G>T) (58,67,68).

Remarkably, several very short, truncated Tg molecules can be secreted and are sufficient for partial thyroid hormone synthesis (945C>T, Y296X in the Dutch goat; 886C>T, R277X in humans (62,64,82,88).

It can be anticipated that the study of further pedigrees and sporadic cases with Tg deficiencies will provide further insights into structure–function relationships of this unusual protein. These endeavors are greatly facilitated by the thorough sequence information that is now available (13,17,18, 22,23).

Recent studies established linkage of the Tg locus with autoimmune thyroid disease suggesting that alterations in the *Tg* gene, together with variations in other genes and environmental factors, may play a role in the pathogenesis of these multifactorial disorders (96,97).

There are currently no data on the three-dimensional structure of the protein. Given the size of Tg, this remains a challenging task. A better understanding would, however, be of great interest for the understanding of the structural requirements underlying thyroid hormone synthesis.

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