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The screening for mutations in the thyroglobulin cDNA from six patients with congenital hypothyroidism

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Abstract — The six patients described in this study were clinically diagnosed with congenital hypothyroidism. Based on clinical and pathophysiological parameters, the cause of the thyroid dyshormonogenesis was suspected to be a defect in the synthesis of thyroglobulin, the matrix protein for thyroid hormone synthesis in the thyroid gland. After RNA isolation from six goitrous tissues and control thyroid tissues, RT-PCR was used to amplify 20 overlapping thyroglobulin (TG) cDNA fragments. Two alternative splice transcripts were identified: a transcript with a deletion of nucleotides 177–274 and a transcript with a deletion of nucleotides 3430–3736 that result in frame shifts and the introduction of premature stop codons. Two alternatively spliced transcripts not changing the reading frame were also identified: a transcript containing a deletion of nucleotides 4529–4699 and a transcript with a deletion of nucleotides 7301–7561. All these transcripts were expressed in thyroid tissue of both patients and controls. Nucleotide sequence analysis and comparison to the revised TG sequence (1997) revealed one revision and eight polymorphisms that did not result in amino acid changes and four polymorphisms that did change amino acid codons. In three patients a homozygous mutation was present at nucleotide position 229, causing a glycine to serine amino acid substitution.The clinical description 'thyroglobulin synthesis defect' in this population cannot be explained by major mutations in the coding region of the TG gene. Furthermore, the presence and level of expression of the alternatively spliced transcripts do not co-segregate with thyroid dyshormonogenesis, since in normal thyroid tissue the same alternatively spliced transcripts are present. © Société française de biochimie et biologie moléculaire / Elsevier, Paris

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1. Introduction

Primary congenital hypothyroidism (CH) (prevalence 1:3000), formerly one of the diseases most frequently causing mental retardation, is characterized by low thyroid hormone plasma levels and elevated thyrotropin (TSH) values at birth [1]. A neonatal screening program for CH permitting the identification and early treatment of affected children was initiated in Canada in 1974 [2], followed by many industrialized countries. Initiation of L-thyroxine replacement therapy as soon as possible after birth largely prevents the severe neurological, mental, and motor disturbances of CH [3–6].

Agenesis and dysgenesis of the thyroid gland are the most frequent causes of CH. About 20% of CH patients have a normally developed and positioned gland and in this group thyroid dyshormonogenesis occurs. One possible cause of thyroid dyshormonogenesis is the so-called 'thyroglobulin synthesis defect' (TSD) with an estimated prevalence of 1 in 100 000 live births [1].

Thyroglobulin (TG), the predominant glycoprotein in the thyroid gland, is the matrix protein in which thyroid hormone is synthesized and stored. The mature protein can be isolated from thyroid tissue as a homodimer (660 kDa) containing 10% carbohydrate residues [7]. Each monomer contains 2749 amino acids including 66 tyrosine residues [8] of which in vivo 3–17 are iodinated [9]. Within the TG dimer specific diiodotyrosine-residues can be coupled to form thyroid hormone (T_4). The preferential hormonogenic site is Tyr5, which is the first to be iodinated in vivo, but other specific tyrosine residues are also involved in thyroid hormonogenesis [10–12]. A stretch of 19 amino acids at the N-terminus functions as a signal peptide and is considered to play a role in the glycosylation of the protein in that it directs the uptake by the endoplasmatic reticulum [8]. In the amino terminal part of thyroglobulin (aa 1–2187) cysteine rich repeats are found which appear to be important for the stereo structure of the protein [13]. In the carboxy-terminal part (aa 2192–2716) an acetylcholinesterase-like domain is localized of which the function is not clear [14].

The diagnosis of TSD is based on the following clinical and pathophysiological parameters: increased TSH concentration, low to normal T_4 concentration and normal T_3

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Table I. Patient description.

Patients	*1*	*2*	*3*	*4*	*5*	*6*	*N*[a]
Personal information							
Sex	F	F	M	F	F	F	
Age at operation	13	14	27	8	12	31	
Plasma levels							
TSH mU/l	7	2.8	11.4	>50	28	9.6	<5
T4 nM	65	115	37	58	50		70–160
fT4 pM		12.3			5.5	2.7	10.9–26.9
T3 nM	2.6	2.5	2.4	1.8	3.8		1.1–3.1
TG pM	12	60	61.5		86	6678	22.5–45
TG/TSH	1.7	21.4	5.4		3.1	696	>4.5

[a]N gives normal ranges as indicated in AMC manual. For additional features see *Materials and methods.*

concentration in plasma. If not or suboptimally treated, goiter will develop which excludes an inactivating TSH receptor defect in these cases. A rapid and high radioactive iodide uptake, without significant discharge after ClO_4^- administration excludes a iodide organification defect. Thyroglobulin concentrations are low in thyroid tissue and are variable in plasma. Iodinated proteins, mostly iodoalbumin, are present in thyroid tissue and plasma, and urine contains breakdown products from aberrantly iodinated proteins (LOw Molecular Weight IOdinated Material, LOMWIOM) [15, 16].

The human TG gene is located on chromosome 8q24.2-8q24.3 [17, 18], covers over 300 kb and contains more than 40 exons separated by introns of up to 64 kb [19]. The 8.7 kb mRNA of thyroglobulin (TG) contains 8307 nucleotides coding sequence [8, 13]. Mutations in the TG gene might result in aberrant TG protein expression, and subsequent impaired thyroid hormone synthesis, giving rise to CH with goiter [20]. Only a few thyroglobulin mutations have so far been identified in animal and human pathology. In Dutch goats a homozygous nonsense mutation, Tyr296AMB [21], results in a truncated thyroglobulin product (35 kDa) [22] and causes hypothyroidism with goiter. When iodine intake is increased in the diet of these goats, goiter remains but the animals become euthyroid indicating that thyroid hormone synthesis occurs in the truncated TG protein [23]. In Afrikander cattle a homozygous nonsense mutation results in the introduction of a premature stop codon at amino acid residue 697, giving rise to a truncated TG protein of 75 kDa. This mutation is localized in exon 9 of the gene and in this case also an alternatively spliced mRNA lacking exon 9 sequence is observed that encodes a TG protein of 250 kDa [24, 25].

In humans three variants of a 'thyroglobulin synthesis defect' have been elucidated at the molecular level. A homozygous mutation at the acceptor splice site of intron 3 results in the in frame deletion of exon 4 sequences (nc 275–478) from the mRNA [26]. A homozygous 138 base pairs in frame deletion of nt 5552–5789 from the mRNA, and the preferential accumulation of a TG mRNA with an 171 bp in frame deletion of nt 4529–4699, as a result of a homozygous premature stop codon at position 1510 have also been reported [27, 28].

In the present study, we screened thyroglobulin mRNA isolated from goitrous thyroid tissue of six congenital hypothyroid children, with a defect in thyroglobulin synthesis as a suspected cause for their thyroid dyshormonogenesis.

2. Materials and methods

2.1. Patients

Patients 1 to 5 originate from the Netherlands and patient 6 from France. All subjects except patient 2 had hypothyroid family members. In all cases, (sub)total thyroidectomy was performed for local compressive reasons. Clinical and laboratory determinations were done prior to surgery, after cessation of thyroid hormone substitution therapy for several weeks. Data are summarized in *table I.* Additional features are indicated hereafter. In patient 6 a free T_3 concentration in plasma of 2.8 nM was determined (normal range: 1 to 2.5 nM). Patients 1 and 4 were screened for thyroid peroxidase antibodies, that were negative. Uptake of radioactive iodide by the gland was rapid and high in all patients and no discharge was observed after perchlorate administration.

After surgery thyroid tissue was frozen rapidly in liquid nitrogen and stored at –70 °C for RNA isolation and part of the tissue was fixed in 10% formalin for histological examination. Most thyroid glands showed cylindrical and cubical epithelium cells indicating prolonged TSH stimulation of the thyroid follicles. In thyroid tissue of patients 1, 2, 3 and 4, dimer thyroglobulin concentration (19S) was low.

In all patients abnormal iodoproteins were detected in either the thyroid or in blood plasma, and/or LOMWIOM

Table II. Sequence of PCR oligonucleotides.

Forward primer	*Nucleotide sequence (5′→ 3′)*	*Reverse primer*	*Nucleotide sequence (5′→ 3′)*
1aF	TCCTCCTTCCTCCCAGGAAGG(-30)	1R	AAAGGCCTCCCCAGCTGGCG(473)
1bF	AAGCAAGCAGACTACGTGCCC(130)		
2F	CAGCAGGTCCAGTGCTGGTGT(400)	2R	TTTGTGGGGCATCGGAATCTGC(900)
3F	ACCGGATACTGCAGAGACGGT(830)	3R	GGAGGGAAAAATTGCTCGGATG(1350)
4F	GCTTCTCCGCCCAATGGTGGA(1260)	4R	CAGAGATAGCATGTTGCAAGAAG(1759)
5F	GCCCTCAAATTCCTTGCTTCTC(1690)	5R	ACAGGGCGTGGGGCATTTCTT(2190)
6F	ATGCTGAGGGTCAGGCCATTC(2120)	6R	CATTTAAGATCTGCCAGAAGAGG(2620)
7F	TGGAAGGGAAACGGCCCCAG(2549)	7R	CAGCCGAGTCGTCCGGGGAA(3049)
8F	ATTCGCCTGGCGGCTCAGTCT(2980)	8R	TCTCCTAGAGAGGACTCCACTC(3480)
9F	CTGGCTCGAGCAGCAGTGCC(3410)	9R	TGCCCGGCGGGAGCTGGAG(3910)
10F	CTGCCAACGGCCCCAGCTGT(3840)	10R	ACTTGGTAGAATCCTTCCGAGC(4340)
11F	TTCCTCCAAGGGGACCACTTTG(4270)	11R	TCTGACAGCCACAGGAGCATTG(4770)
12F	GATGCAGAAGTTTGAGAAGGTTC(4701)	12R	AACACAGATCACGGTCGCATGC(5200)
13F	CTCAGCCTCAGGAGCCAATCT(5130)	13R	TGCTTCTGGCTGGATAGTGATTG(5630)
14F	GAACTTTTCTCCCCTGTGGACC(5560)	14R	TCTCCTCCTTTTAACTGGGAAAC(6062)
15F	ACGACGGTGCGATGCGGACC(5991)	15R	GTGCAGCTTTGAGTATCAGCATA(6491)
16F	TCTCATCACCACTCTGCAAACC(6420)	16R	GTCCATGGTGTTGTGGAAGAAC(6921)
17F	CTCAATGTGTTCATCCCTCAGAA(6850)	17R	CACTTCTTGGCTGGATGACATG(7350)
18F	AGAGGGCTCAGCAGCAGGCAA(7280)	18R	ATAGGGCAGATGATAAAGTAGTCC(7778)
19F	GGATGACTATGCCTCCTTCTCC(7710)	19R	ACTGCCCGCCCTTGGCTCCA(8209)
20F	ATCCCAACTACCCTTATGAGTTC(8000)	20R	AAAAGATGACCATAGTGGGCAGC(+57)

Forward (F) oligo's are linked to M13 forward tag 5′TGTAAAACGACGGCCAGT3′.
Reverse (R) oligo's are linked to M13 reverse tag 5′CAGGAAACAGCTATGACC3′.
Most 5'cDNA nucleotide number in parentheses.

was present in the urine [15, 16]. In the family of patient 1, CH has been reported as an autosomal dominant trait.

Informed consent from patients and/or parents to use the thyroid tissue for research purposes was obtained in all cases.

2.2. RNA isolation and complementary DNA preparation

Total RNA was isolated from goitrous and normal thyroid gland tissue using TRIzol®Reagent (Life Technologies BV). cDNA was synthesized using random hexamers and reverse transcriptase according to standard procedures.

2.3. DNA amplification

PCR amplification [29] was performed using 100 ng cDNA as template in a total reaction volume of 25 μL. For nucleotide sequencing, fragments of 500 bp (with 20–70 bp overlap) were amplified with 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer) using the protocol: 2 min 94 °C; 35 cycles of 15 s 94 °C, 1 min 60 °C, 1 min 72 °C; 10 min 72 °C.

The oligonucleotides *(table II)* (synthesized on Expedite Nucleic Acid Synthesis System; Millipore) were human TG specific (for sequence see GenBank locus U93033) and coupled to M13 tags.

Reactions were electrophoresed on 0.8% agarose gel and purified using the Quiaquick DNA gel extraction kit (Quiagen).

2.4. Southern blotting

Southern blotting of all PCR amplified fragments was done according to standard procedures, using a ^{32}P-labeled 8.3 kb PCR amplified human TG cDNA fragment as probe.

2.5. Nucleotide sequencing

Both the sense and antisense strand were sequenced using the M13 tags linked to the PCR fragments. Reactions were performed using the Big Dye Primer Cycle Sequencing Kit (M13 forward and M13 reverse) and the Big Dyedeoxyterminator Cycle Sequencing Kit (in combination with PCR primers) depending on the GC content of the fragment (both kits from Applied Biosystems/ Perkin Elmer). After electrophoresis on a sequencing gel the samples were analyzed on the ABI Prism 377 DNA sequencer and aligned to the TG cDNA sequence [8, 13] using AutoAssembler software (Applied Biosystems/ Perkin Elmer, Foster City, USA).

2.6. Mutation analysis

The determined mutated nucleotides in the patients that after translation would lead to an amino acid alteration

were validated by either restriction fragment length polymorphism (RFLP) analysis or by sequencing of 12 patients' and 22–24 normal alleles. For RFLP analysis cDNA PCR fragments were digested according to the manufacturer's protocol with either *Nde*I (Gibco BRL) or *Taq*I (Boehringer Mannheim). The proposed revision on nt position 3753 was validated by RFLP analysis using *Sty*I (Boehringer Mannheim).

3. Results

Table I summarizes the clinical and laboratory parameters of the six patients diagnosed with CH due to a proposed 'thyroglobulin synthesis defect'. Matching the general features, all patients had goiter, with moderate hypothyroidism and showed rapid and high uptake patterns with a negative perchlorate test. Plasma levels of T_4 varied from low (patient 3) to normal (patient 2). Plasma T_3 levels were normal in patients 1–4 and relatively high in patient 5. Thyroglobulin plasma concentrations in relation to TSH were low to normal in patient 1, 3 and 5; high in patient 2 and extremely high in patient 6. The presence of iodoproteins, mostly iodoalbumin, was measured in all patients either directly in plasma, as protein bound iodine (PBI minus iodine- bound to iodothyronines) or as iodinated fragments excreted in urine (LOMWIOM).

After amplification of 20 overlapping RT-PCR fragments of approximately 500 bp from the TG cDNA, all products were screened by agarose gel electrophoresis and visualization by ethidium bromide staining. Several alternative spliced TG RT-PCR fragments were present. Southern blotting using a ^{32}P labeled human TG cDNA probe (data not shown) was done, in order to detect low abundant alternatively spliced TG fragments, but this did not yield more data. Direct sequencing of all amplified fragments was performed using M13 primer sequences which annealed to the M13 tags linked to the PCR fragments.

Alternatively spliced amplification products were present in fragment 1 (nt 130–473), fragment 9 (nt 3410–3910), fragment 11 (nt 4270–4770) and fragment 18 (nt 7280–7778). The relative amounts of both wild type and aberrant spliced mRNA products were evaluated using RT-PCR amplification on patients' and normal cDNA, and subsequent visualization of the ethidium bromide stained bands *(figure 1)*. Alternatively spliced products of fragments 1, 9, 11 and 18 were present in both patients' and normal mRNA. Expression of the alternative splice products compared to wild type was high for fragment 9 and low for fragments 1, 11 and 18. For the alternatively spliced products of fragments 9, 11 and 18 there was no substantial difference between patients and normal controls with respect to relative abundance of wild type and alternatively spliced products.

Sequencing of the alternatively spliced products established their nucleotide sequence: in fragment 1 an out-frame deletion of nucleotides 177–274 (exon 3 sequence) was present, in fragment 9 an out-frame deletion of nucleotides 3430–3736 was present, fragment 11 contains an in-frame deletion of nucleotides 4529–4699 (exon 22 sequence) and fragment 18 shows an in-frame alternative transcript with a deletion of nucleotides 7301–7565. *Figure 2* schematically shows the alternatively spliced transcripts in fragments 9 and 18 that have not been described yet.

In fragment 19 an alternatively spliced product was detected at low abundance and could therefore not be sequenced. Due to the relatively high expression of the alternatively spliced products compared to the wild type in fragment 9, the wild type fragment could not be sequenced in patients 1 and 5.

Sequencing of all other RT-PCR products resulted in the identification of 14 nucleotide substitutions *(table III)*. A revision is needed for position 3753; the previously reported A should be a G, not changing amino acid codon 1232. This change was observed in 12 patient alleles and 22 control alleles. The nucleotide substitutions at positions 192 (G/A), 261 (A/G), 3474 (T/C), 3906 (G/A), 5769 (A/G), 6501 (G/A), 6846 (G/A) and 7920 (C/T) were silent.

Nucleotide substitutions at four positions will result in an amino acid change in the protein, but these changes occur both in patients and controls. For all nucleotide substitutions that imply an amino acid change, alleles were screened using either RFLP or sequencing analysis. At position 229 the substitution G to A was homozygously present only in patients 1, 2 and 5. The G→A substitution results in the replacement of glycine 58 by serine. The G→A change at position 3935 results in the substitution of the glycine residue 1293 for an aspartic acid residue. The G nucleotide was scored in 20/34 and the A nucleotide in 14/34 alleles. At position 5995 a C→T mutation is present in 10/36 alleles. Translation of this codon will result in tryptophan at position 1980 instead of arginine. The C→T substitution at nucleotide 6695 in 11 out of 34 alleles result in a leucine instead of a proline at amino acid residue 2213. The previously reported revision of position 7589 from an A to an G, resulting in the amino acid change from residue 2511 from glutamine to arginine is on hindsight also a polymorphism. The G nucleotide was scored in 15 out of 34 alleles.

4. Discussion

In this paper we describe the screening of the TG mRNA of six patients who are clinically diagnosed to have congenital hypothyroidism (CH) due to a proposed 'thyroglobulin synthesis defect' (TSD). This disorder has such a heterogeneous appearance that the defect is not

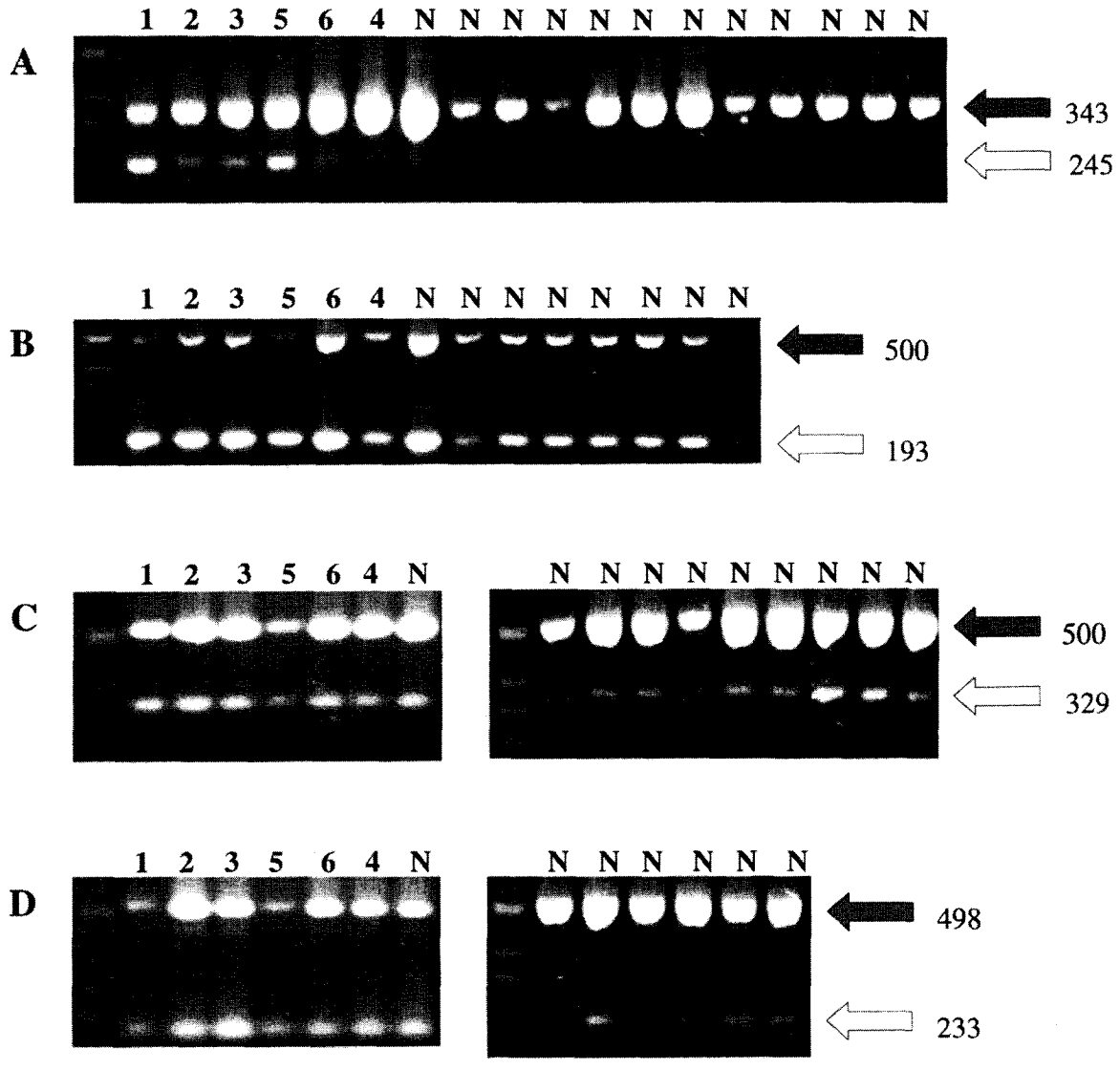

Figure 1. RT-PCR amplification of TG fragments from thyroid tissue of patients and controls. Primer combinations used were 1bF and 1R (**A**), 9F and 9R (**B**), 11F and 11R (**C**), and 18F and 18R (**D**). Expected wild type amplification products are indicated by a closed arrow, alternatively spliced products by an open arrow and basepair length is given. Numbers indicate patients (see *table I*). Normal control thyroid tissue is represented by N.

always what the name strictly implies [16]. Illustrative for this is the detection of low concentration of thyroid transcription factor 1, an important factor for thyroglobulin transcription in goitrous thyroid tissue of a TSD patient [30]. In our study thyroglobulin levels in plasma were very high in patient 6 in relation to TSH and low to normal in patients 1, 3 and 5. In accordance with the presence of thyroglobulin, TG mRNA could be isolated from thyroid tissue of all patients. For sequencing purposes overlapping 500 bp fragments were RT-PCR amplified from thyroid RNA. Although not performed quantitatively, the RT-PCR amplification resulted in comparable yields of 500 bp products making a defect at the transcription level unlikely.

Defects in post-translational modification may also be involved in the molecular cause of TSD, as shown for the *cog/cog* mouse and two human kindreds. In these cases it has been suggested that TSD causing CH can be an

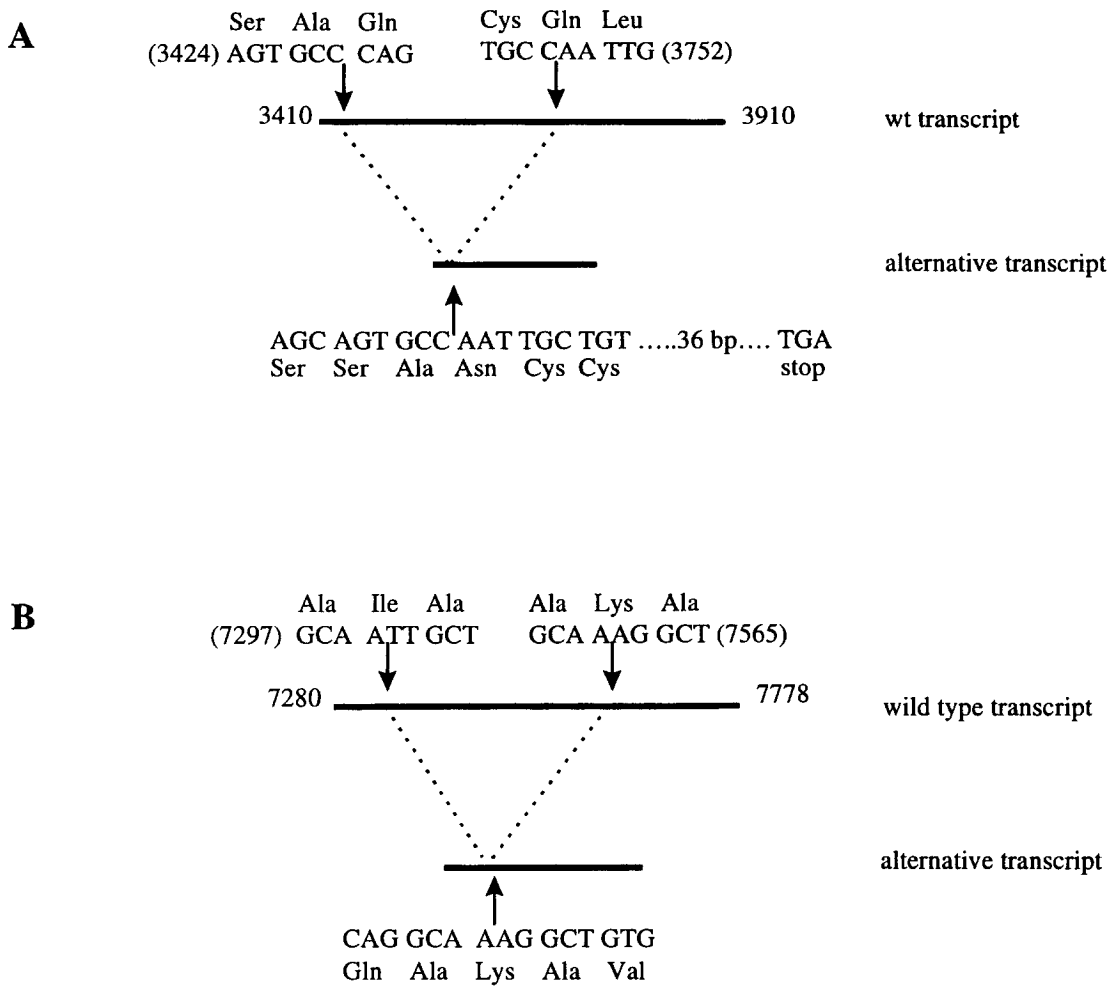

Figure 2. Schematic illustration of the TG wild type amplification product, and the differentially spliced products. The 5′ and 3′ nucleotide positions of fragments 9 (**A**) and 18 (**B**) are indicated beside the solid lines representing TG cDNA. Dotted lines indicate the deletion. The nucleotide and amino acid sequence around the breakpoint in the wild type and the alternative transcript are shown.

endoplasmatic reticulum storage disease (ERSD). In one kindred most intrathyroidal TG fails to reach the Golgi compartment probably as a result from a deletion of nucleotides 5552–5789 from the TG mRNA [31].

We have not observed this deletion in our population even though the histological description of the goitrous thyroid tissue of patient 5 (foamy cytoplasm in the epithelial cells around small follicles) resembles the description of thyroid tissue from the kindred with the ERSD.

All RT-PCR reactions were blotted and hybridized with a TG cDNA probe in order to detect products with deletions or insertions and alternative transcripts that are low abundant, but this provided no extra information compared to the ethidium bromide stained gels.

Several alternative TG mRNA transcripts were present in fragments 1, 9, 11 and 18. The alternatively spliced products of TG fragment 1 and 11 correspond to the deletion of exon 3 and exon 22 sequences from the TG mRNA respectively that have been reported before [27, 32]. The alternatively spliced product without exon 22 was more abundantly expressed compared to control thyroid tissue, due to a nonsense mutation located within the deletion [28]. This phenomenon does not occur in our

Table III. Sequence substitutions after aligning with published Tg sequence [8].

nt position	*nt / nt*	*aa position*	*aa change*	*Allele frequency*	
				Patients	Control
192	G /A	45	–	A: 1/12	n.d.
229[a]	G / A	58	Gly → Ser	A: 6/12	A: 0/22
261	A / G	68	–	G: 1/12	n.d.
3474	T / C	1139	–	C: 2/12	n.d.
3753[b]	A → G	1232	–	G: 12/12	G:22/22
3906	G / A	1283	–	A: 2/12	n.d.
3935	G / A	1293	Gly → Asp	A: 9/12	A: 5/22
5769	A / G	1904	–	G: 1/12	n.d.
5995	C / T	1980	Arg → Trp	T: 3/12	T: 7/24
6501	G / A	2148	–	A: 1/12	n.d.
6695	C / T	2213	Pro → Leu	T: 2/12	T: 9/22
6846	G / A	2263	–	A: 1/12	n.d.
7589	A / G	2511	Gln → Arg	G: 3/12	G: 12/22
7920	C / T	2621	–	T: 9/12	n.d.

[a]Mutation.
[b]Revision.
nt, nucleotide; aa, amino acid; –, no change; n.d., not done.

patients meaning that this particular nonsense mutation is not present in these patients. Sequencing of fragment 11 confirmed this. The alternatively spliced product present in fragment 9 did not correspond to the earlier reported deletion of exon 17 sequence from the TG mRNA [33]. Sequencing of this product showed a deletion of nucleotides 3430–3736, encoded by exon 16 and part of exon 17 from the TG gene. Due to the low abundance, the alternatively spliced product of fragment 19 could not be sequenced, but it is likely that it corresponds to the 134 bp deletion already shown to be present in the TG mRNA of two patients with Pendred's syndrome [34]. We observed this alternative splicing also in normal thyroid tissue. The 2 bp deletion of nc 7873 and 7874 observed in that study is not present in thyroid tissue of our six patients.

Direct sequencing of the complete TG cDNA in six patients suspected of thyroid dyshormonogenesis caused by a TSD resulted in the detection of one revision and 13 nucleotide substitutions *(table III)* compared to the published sequence [8]. Of these 13 substitutions, eight were silent and five would result in an amino acid change when translated. The homozygous substitution of nucleotide 229 was exclusively present in patients 1, 2 and 5, and not in 22 normal alleles. When translated this mutation will result in a substitution of glycine 58 to serine. Whether this substitution is causally related to the thyroid dyshormonogenesis has to be investigated using an in vitro expression system. We speculate that the incorporation of an extra OH group is not so drastic that it will interfere with the functionality of the thyroglobulin protein. The biochemical evaluation of patients 1, 2 and 5 is dissimilar and therefore it does not seem likely that the thyroid dyshormonogenesis in these patients has the same molecular background. All other substitutions occur both in the patient population and in normal control alleles and are considered to be polymorphisms. They are unlikely to be causally related to thyroid dyshormonogenesis.

The absence of major mutations in the TG cDNA in the six patients with thyroid dyshormonogenesis is illustrative for the inadequacy of the description 'thyroglobulin synthesis defect' on clinical and pathophysiological features only. The remaining potential of the thyroid gland to synthesize thyroid hormone, in case of impaired thyroglobulin synthesis, will be dependent on the iodine intake. It has been shown for the Dutch goats with a homozygous premature stopcodon, resulting in the expression of a small N-terminal truncated TG protein of 35 kDa, that high iodine intake completely restores euthyroidism. Furthermore, it is still possible that other yet unidentified genes involved in thyroid hormonogenesis are impaired in patients who are now erroneously thought to suffer from a thyroglobulin synthesis defect.

The TG cDNA revision and polymorphisms are submitted to GenBank locus U93033.

Note added in proof

The polymorphism at nucleotide position 7589 (amino acid position 2511) in this report is also recently described in Mendive F.M., Rosetti L.C., Vassart G., Targovnik H.M., Identification of a new thyroglobulin variant: a guanine-to-adenine transition resulting in the substitution of arginine 2510 by glutamine, Thyroid 7 (1997) 587–591.

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