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Nonsense-Associated Alternative Splicing of the Human Thyroglobulin Gene

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Abstract

Introduction: We have described in previous articles a nonsense mutation (4588C>T, R1511X) in exon 22 of the thyroglobulin (*TG*) gene in a member of a family with a complex history of congenital goiter. In the mutated thyroid gland, full-length thyroglobulin mRNA is almost undetectable. However, a smaller transcript is detected in which the mutated exon 22 is skipped and the reading frame restored. It is conceivable that alternative splicing might be a mechanism involved in the rescue of nonsense mutations.

Methods: To investigate whether the detection of the alternative mRNA is due to an increase in its concentration or its preferential amplification during reverse transcriptase-PCR in the absence of the normal full-length mRNA competitor, we set up an assay in which the competitor mRNA was provided. We also studied the effect of the 4588C>T mutation on exon definition and processing using wild-type and mutated minigenes.

Results: The detection of the alternative mRNA lacking exon 22 is not caused by the absence of the full-length competitor. In contrast, our results demonstrate that the alternative transcript preferentially accumulates in the mutated thyroid at a level similar to the full-length transcript in control tissue. Transient expression experiments with wild-type and mutated minigenes indicate that the mutated exon is as efficiently spliced as the wild-type, suggesting that the 4588C>T mutation does not interfere with exon 22 definition and processing.

Conclusions: The alternative splicing of the *TG* gene described in this article constitutes a new case of nonsense-associated alternative splicing. We have shown that the mutation itself does not interfere with exon definition and processing *in vitro*. Our results support the hypothesis that the alternative splicing of the mutated exon is driven by the interruption of the reading frame.

Thyroglobulin (TG) is a large glycoprotein synthesized by the thyroid gland. It functions as a matrix where thyroid hormones (T_4 and T_3) are produced from the coupling of iodotyrosyl residues in a reaction catalyzed by thyroperoxidase. The complete structure of the human *TG* gene has been determined recently.[1-3] It is coded by a single-copy 270 kb gene that maps to chromosome 8q24 and contains an 8.5 kb coding sequence divided into 48 exons. Thus far, at least 12 alternative splice products have been identified in wild-type TG mRNAs.[4] In addition to these variations in the wild-type phenotype, several mutations in the *TG* gene have been identified in humans resulting in aberrant TG protein expression.[5-16] In previous articles we have described a splicing alteration in the human *TG* gene associated with a nonsense mutation in exon 22 (4588C>T).[6,10,16] This mutation was found in a member of a family with a complex history of congenital goiter. The mutation creates a premature termination codon in exon 22 (R1511X) that should potentially produce a truncated TG protein. Interestingly, this mutation is not present in most of TG mRNA because the detected TG mRNA lacks exon 22. Skipping of the mutated exon in the pre-mRNA restores the reading frame, allowing translation to reach the normal stop codon. This alternative splicing of exon 22 is also present in mRNA obtained from healthy thyroid tissue, although it represents a minor fraction of the total TG transcripts.[17]

Nonsense mutations have been frequently associated with alterations of pre-mRNA splicing. The main explanation for this observation is that alternatively spliced transcripts, normally present in concentrations below the detection limits, become detectable by reverse transcriptase-PCR (RT-PCR) only when there is a reduction in the concentration of the competing normal-spliced mRNA molecule. This reduction in mRNA concentration is known to be achieved by the nonsense-mediated decay (NMD) surveillance pathway. Thus, the detection of an alternative transcript would be due to the loss of the competition normally provided by the full-length transcript during RT-PCR amplification, and would not be a real increase in the steady-state level of the alternative mRNA (see Valentine[18] and references therein). Another explanation is that nonsense mutations occur within cis-acting elements involved in splicing efficiency (i.e. exonic splicing enhancers [ESEs]), interfering with normal splice site selection.[19] A third explanation suggests the existence of a mechanism able to scan the reading frame on nuclear pre-mRNA that is capable of modifying mRNA splicing when the reading frame is interrupted by a premature stop codon.[20]

To understand the relationship between the splicing alteration of TG exon 22 and the 4588C>T mutation that creates the premature stop codon, we studied whether the abnormal detection of the alternative mRNA is a consequence of an increase in its concentration or if it is exclusively detected when the full-length transcript concentration is reduced by NMD. With this aim, we set up an assay in which the full-length transcript was provided as a competitor to the alternatively spliced TG mRNA in an RT-PCR reaction. We also used wild-type and mutated minigenes to evaluate the effect of the 4588C>T mutation on exon 22 definition out of its natural genetic context.

Materials and Methods

Semi-Quantitative and Competitive Reverse Transcriptase-Polymerase Chain Reaction

RT-PCR was performed according to standard conditions, and the cycle number was optimized for semi-quantitative analysis: RT-PCR was performed at cycle number 16, in which the product yield is still at the exponential growing phase (not shown). All PCR products were concentrated by ethanol precipitation and electrophoresed on an agarose gel. The ethidium bromide-stained gel was digitalized and intensity values were obtained from each band. The intensity values corresponding to the smaller molecular weight fragment (alternatively spliced mRNA) were multiplied to a factor obtained from the size ratio between full-length and alternative RT-PCR products (683/512 = 1.33) to compensate for the lower dye incorporation of the smaller fragment. Thus, intensities represent molar and not mass values. TG product intensities were normalized with respect to β-actin intensities and are expressed in relative units.

The competitive reaction was performed by mixing together equal amounts (0.5μg) of total RNA from wild-type and mutated thyroid gland. This RNA mix allowed the evaluation of the relative amounts of full-length and alternative transcripts in wild-type thyroid tissue and in the thyroid tissue carrying the mutation in exon 22.

Human TG primers: forward (1.3F), 5′ ggcctgtgtcccatgtcctg 3′; reverse (2.3R), 5′ tggctcctgaggctgagaac 3′.

Human β-actin primers: forward, 5′ gggacgacatggagaaaa 3′; reverse, 5′ ttcatgaggtagtcagtcaggt 3′.

Construction and Expression of the Minigenes

Minigenes were constructed using the exon trapping vector pSPL3[21] that contains a minimal gene organization: the SV40 promoter followed by an exon-intron-exon structure with a multiple cloning site (MCS) located inside the intron. When a fragment cloned in the MCS contains functional exons with their corresponding splicing sites, they will be included in the mature mRNA.

- **Minigene TG22-WT.** A 699 bp Hind III-EcoR V fragment containing exon 22 and intronic flanking sequences (288 bases upstream from the 3′ splice site and 240 bases downstream from the 5′ splice site) was obtained from a recombinant phage containing the *TG* gene region encompassing exon 22[2] and cloned into Bluescript plasmid (pBsc; Stratagene, La Jolla, CA, USA) [figure 1]. Next, the insert was excised from pBsc with Xho I and BamH I and subcloned into pSPL3 using Xho I and BamH I restriction sites.
- **Minigene TG22-Mut.** The genomic region encompassing the 699 bp Hind III-EcoR V fragment was PCR amplified (figure 1). The 5′ end of the forward primer (*i22F*: 5′ aaagactgggg-gagctgt 3′) was located upstream of the Hind III site while the 5′ end of the reverse primer (*i22R*: 5′ ctagattagtgaagtcaagctgcc 3′), was located downstream of the EcoR V site. The PCR product was double digested with Hind III and EcoR V and cloned into pBsc. Since the individual from which the genomic DNA was obtained is heterozygous for the 4588C>T mutation, the wild-type and the mutated alleles were cloned. A mutated clone was selected and digested with Xho I and BamH I to excise the insert. This insert was subcloned into pSPL3 as described for minigene TG22-WT construction.

Cos-7 cells were transfected with 1μg of TG22-WT and TG22-Mut plasmid DNA with Transfectam Reagent (Promega,

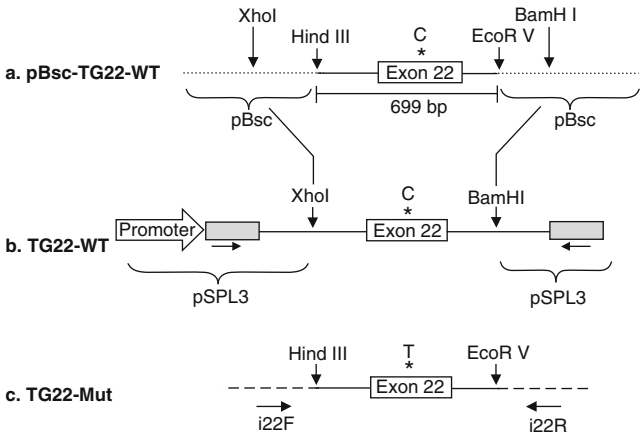

Fig. 1. Representation of the minigene construction. (**a**) Bluescript plasmid (pBsc)-TG22-WT: Hind III-EcoR V fragment cloned in pBsc. (**b**) TG22-WT: the Hind III-EcoR V fragment cloned in pBsc was excised with Xho I and BamH I and directionally cloned into pSPL3. (**c**) TG22-Mut: the region encompassing exon 22 was polymerase chain reaction (PCR) amplified with i22F and i22R primers using 4588C>T-mutated genomic DNA as a template. The PCR product was digested with Hind III-EcoR V and cloned with the same strategy used for TG22-WT. The asterisks indicate the position of the 4588C>T mutation in *TG* exon 22. Horizontal arrows represent primers (pSPL3F and pSPL3R) located at pSPL3 exons used to amplify minigene transcripts on mRNA from transfected cells. **WT** = wild-type.

Madison, WI, USA). Forty-eight hours after transfection, total RNA was purified (Trizol Reagent, Invitrogen, Life Technologies, Carlsbad, CA, USA). 400ng of each RNA were reverse transcribed and PCR amplified with SuperScript One Step RT-PCR System (Invitrogen, Life Technologies, Carlsbad, CA, USA) using vector-specific primers (*pSPL3F*: 5′ tctgagtcacctggacaacc 3′; *pSPL3*R: 5′ atctcagtggtatttgtgagc 3′). The reaction products were analyzed by Southern blot with a TG exon 22 probe. The RT-PCR products were excised from the gel, cloned into pGemT-easy vector (Promega, Madison, WI, USA) and sequenced.

Results

The Alternative Thyroglobulin (TG) Messenger RNA (mRNA) Lacking Exon 22 Preferentially Accumulates in the Mutated Thyroid Tissue

We first attempted to elucidate whether the detection of the alternative product in the mutated thyroid is due to a real increase in the concentration of the alternative TG mRNA lacking exon 22 or if its amplification is simply favored by the reduction in the concentration of the full-length competitor. The observation that the alternative mRNA lacking exon 22 exists in low concentration in normal thyroid tissue suggests that this minor mRNA could be preferentially amplified when the concentration of the full-length mRNA is reduced by the NMD surveillance pathway.

To address this possibility, we set up a competition assay between the alternative and full-length TG transcripts. RT-PCR was performed at the exponential growth-phase cycle number of the reaction where the product yield is proportional to the initial template concentration.[22] We used the following as a template: (i) RNA purified from the thyroid gland that carries the nonsense mutation in exon 22 (4588C>T), where the alternative transcript was mainly detected; (ii) a control thyroid RNA as a source of the normal full-length transcript; and (iii) a mix of equal amounts of (i) and (ii) RNAs. In (iii), the control RNA provides full-length TG transcripts to compete with alternatively spliced mRNA in the mix. If the proposal is true (i.e. the alternative transcript is detected only when the full-length transcript is reduced or absent), the band pattern detected in the 1 **:** 1 mixture should be similar to the one observed in the control tissue, where the alternative transcript is almost undetectable. In contrast, in the competition assay we observed that the normal band pattern was not restored (figure 2). Moreover, the full-length and the alternative RT-PCR products were equally amplified when the 1 **:** 1 mix was used as a template. Band intensities indicate that the alternative transcript is present in the mutated thyroid in a concentration similar to the one observed for the full-length mRNA in the control tissue (figure 2). Thus, these results show that the concentration of the alternative mRNA lacking exon 22 is increased in the thyroid tissue that carries the nonsense mutation (4588C>T) in the skipped exon.

Analysis of the Genomic Regions Involved in Exon 22 Definition and Processing

Exon inclusion in a mature mRNA depends on the integrity of basic cis-acting elements in the pre-mRNA. These elements are the 3′ and 5′ splice sites and the branch point sequence, located between positions −40 and −20 from the 3′ splice site. We searched for alterations in these elements that could be accompanying the nonsense mutation. We amplified and cloned the region that encompasses exon 22 and its flanking sequences from the genomic DNA of the individual who carries the nonsense mutation. All basic splicing cis-acting elements accompanying the nonsense mutation are conserved (figure 3) [GenBank Accession Number AF105687]. Furthermore, no difference was found (except for the exonic 4588C>T mutation) along a region of 422 bases analyzed.

TG Wild-Type Exon 22 is Spliced in the Minigene Context as in the Native mRNA

We constructed a minigene with the TG wild-type genomic region encompassing the alternatively spliced exon 22

(TG22-WT). It was transfected in Cos-7 cells to evaluate RNA processing of the TG exon in the minigene genetic context. TG22-WT produced two mRNA molecules: a larger and more abundant one that corresponds to exon 22 inclusion and a smaller and less abundant one that corresponds to exon 22 exclusion (figure 4). Sequencing of the RT-PCR products proved that the splice sites recognized in the minigene context are the same as the ones used in the native mRNA in the thyroid tissue.

These results showed that exon 22 is excluded in a minority of the minigene mRNAs with an inclusion/exclusion ratio similar to the one previously observed in native TG mRNA[17] (figure 2 and figure 4). Therefore, the minigenes and the Cos-7 cells are an adequate genetic and cellular environment to recreate and study TG exon 22 splicing.

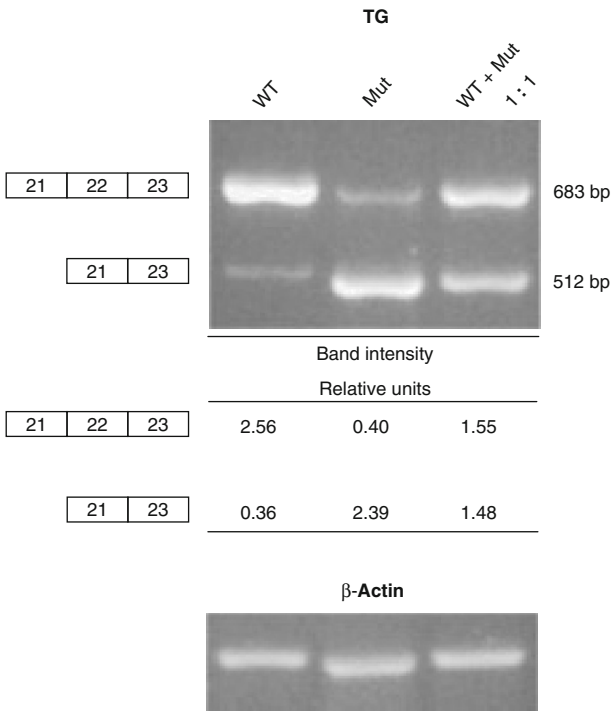

Fig. 2. Competitive reverse transcriptase-polymerase chain reaction (RT-PCR). Wild-type (WT) and 4588C>T (Mut) thyroglobulin (TG) mRNA were analyzed by RT-PCR either individually or in a 1 : 1 mixture of both RNAs. Primers (1.3F and 2.3R) define fragments of 683 bp and 512 bp in the full-length and alternative TG complementary DNAs, respectively. The RT-PCR products were concentrated by ethanol precipitation, electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The images were digitalized and intensity values were obtained for each fragment. The intensity values were corrected to compensate for the lower dye incorporation of the small fragment, normalized with respect to β-actin intensity, and expressed in relative units. At the left, the exonic structure of both mRNAs is represented.

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gccttattgatcagaacagtggggacactgaagagttttaagctggaatgtct
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ctag  GT GTC ACT GAC TGT CAG AGG AAC GAA GCA GGC CTG
     Cys Val Thr Asp Cys Gln Arg Asn Glu Ala Gly Leu

CAA TGT GAC CAG AAT GGC CAG TAT CGA GCC AGC CAG AAG
Gln Cys Asp Gln Asn Gly Gln Tyr Arg Ala Ser Gln Lys

GAC AGG GGC AGT GGG AAG GCC TTC TGT GTG GAC GGC GAG
Asp Arg Gly Ser Gly Lys Ala Phe Cys Val Asp Gly Glu

GGG CGG AGG CTG CCA TGG TGG GAA ACA GAG GCC CCT CTT
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Fig. 3. DNA sequence of the genomic region encompassing exon 22 and the basic splicing cis-acting elements. Exon and intron sequences are indicated in uppercase and lowercase letters, respectively. 5′ and 3′ splicing consensus sequences are underlined. Amino acids encoded in exon 22 are indicated in three-letter code. The 4588C>T mutation that creates the premature termination codon is indicated with an asterisk.

The 4588C>T Mutation is not the Effector of Exon 22 Skipping

To address whether the 4588C>T mutation directly influences exon 22 definition and its inclusion in mature mRNA, we constructed a minigene with the mutated exon 22. As exon 22 is not inserted in any open reading frame, this construct enables the testing of the splicing phenotype independently of the coding function. We found that in the TG22-Mut minigene, exon 22 is as efficiently processed as its wild-type counterpart (figure 4). In consequence, the 4588C>T mutation does not interfere with exon 22 definition and inclusion and is not sufficient by itself to recreate the splicing alteration observed in the mutated TG mRNA.

Discussion

In this article we address the study of an alternative splicing of the *TG* gene associated with a nonsense mutation. The notion that nonsense codons can interfere with mRNA splicing has been explained in at least three different ways. The first considers that mRNAs bearing nonsense mutations often become degraded by the NMD pathway. It has been proposed that the reduction in the concentration of the mutated transcripts by NMD favors the detection by RT-PCR of less abundant mRNA molecules, naturally produced by splicing inefficiency, that are usually undetected (see Valentine[18] and references therein). Hence, the detection of alter-

natively or incompletely spliced mRNAs might be a consequence of a preferential amplification by RT-PCR of minority mRNA when the natural full-length competitor is reduced or absent and not a reflection of an increase in their cellular concentration. In the case of TG mRNA, this possibility acquires special importance because the alternative transcript, in which exon 22 is skipped, is detected even when the concentration of the full-length competitor is normal.

When competition between the full-length and alternative transcripts was re-established *in vitro* (in the 1 **:** 1 mixture of wild-type and mutated RNA), the normal splicing pattern was not restored and the alternative transcript was detected to approximately the same extent as the full-length transcript. Consequently, the detection of the alternative TG mRNA (in which the mutated exon is skipped) is not caused by the reduction in the concentration of the full-length competitor. Furthermore, this alternative TG transcript preferentially accumulates in the mutated thyroid tissue to a level similar to the full-length transcript in control RNA.

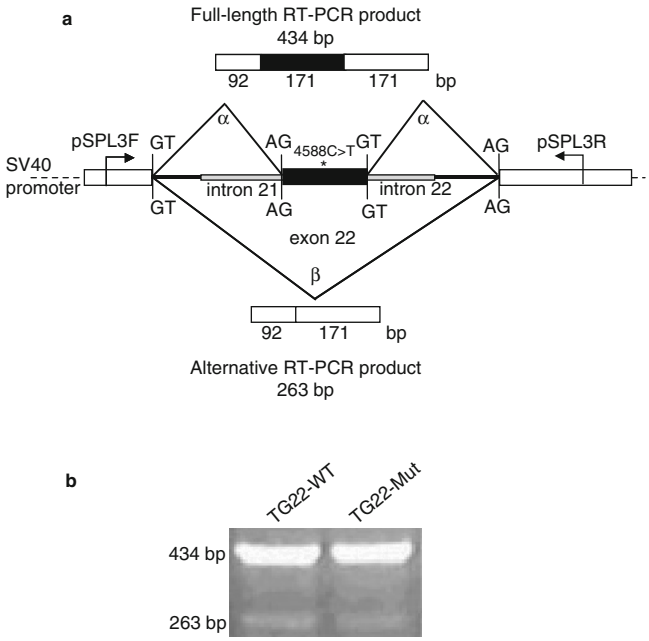

Fig. 4. *In vitro* expression of the wild-type and mutant minigenes. (**a**) Schematic representation of exon 22 inclusion (α) and exclusion (β) events in the context of TG22-WT and TG22-Mut minigenes. Complementary DNA was synthesized from mRNA obtained from Cos-7 cells transfected with the minigenes and amplified with pSPL3F and pSPL3R primers located at flanking vector sequences. Reverse transcriptase-polymerase chain reaction (RT-PCR) products corresponding to full exon 22 inclusion and exclusion are represented. Minigene and genomic DNA splice donor (GT) and acceptor (AG) sites are indicated. * indicates the position of the 4588C>T mutation in thyroglobulin (TG) exon 22. (**b**) Agarose gel electrophoresis of the RT-PCR products obtained in the amplification on mRNA from Cos-7 cells transfected with TG22-WT and TG22-Mut minigenes. The RT-PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

The second explanation to nonsense-associated alternative splicing is based on the observation that exonic mutations, in addition to their intrinsic coding effect, can reduce the activity of ESEs. ESEs are sequence tracts present in constitutive and alternative exons that are required for correct exon definition and splicing. They act as binding sites for essential splicing factors on pre-mRNA. It has been reported that not only nonsense[23] but also missense[19] and silent[24,25] mutations induce splicing alterations through the disruption of these elements. However, evidence of ESE disruption is uncommon because most splicing alterations associated with nonsense mutations have been described in clinical papers where the splicing mechanism was not directly tackled. A database-based approach to ESE prediction has been reported.[26] Fifty exon sequences in which single-base substitutions (missense, nonsense or silent mutations) cause exon skipping were analyzed for the presence of serine-arginine-rich (SR) protein-binding sites with SR protein-motif scoring matrices. More than half of those mutations reduce or eliminate high-scoring SR protein-binding motifs present in the normal sequences. Thus, it becomes apparent that the interruption of ESEs should be considered as a possible explanation for the association of splicing alterations with nonsense mutations.

To investigate whether the 4588C>T mutation induces TG exon 22 skipping by interfering with exon definition or splicing efficiency, we constructed minigenes in which wild-type and mutated exon 22 were located in a simple genetic context. Minigenes allowed us to evaluate mutated exon 22 splicing efficiency independently of the coding effect and independently of the natural genetic and cellular context. In this experimental situation, mutated exon 22 was efficiently spliced as the wild-type exon. This observation indicates that the mutation by itself is not sufficient to produce exon 22 skipping and suggests that the mechanism underlying alternative processing of TG mRNA in the mutated thyroid is not the disruption of any cis-acting element implicated in exon definition and inclusion.

If the mutation itself is not the only factor necessary to recreate the splicing alteration, what is the additional factor? Considering the obvious differences between our experimental system and the *in vivo* scenario, two main possibilities merit discussion.

The Splice Sites Environment and Cellular Context

It could be postulated that efficiency in splice site selection may be influenced by the strength of the neighboring splice sites. The observation that the wild-type TG exon 22 is as efficiently spliced in the minigene context as in the native TG mRNA suggests that the splice sites present in the minigene do not interfere with normal TG exon 22 definition and inclusion.

Since the *TG* gene is only expressed in thyroid cells, we could argue that the mutation in exon 22 might interfere with a tissue-specific exonic splicing element not functional in the Cos-7 cells. It has been reported that splicing alterations induced by nonsense mutations can depend on cell type.[27] In our experimental conditions, exon 22 was not only correctly recognized (exon definition) but also alternatively skipped in a minority of transcripts as observed in normal thyroid tissue. These observations suggest that correct exon 22 splicing does not depend on cell type or on neighboring cis-acting elements.

The Reading Frame

It has been shown that splice site selection is influenced by the maintenance of the reading frame.[28] Nonsense-associated alternative splicing implies a relationship between nonsense codon identification and splice site selection. These two processes have some features that make it difficult to find a reliable connection: first, they take place at different cellular compartments (in the cytoplasm and nucleus, respectively); second, nonsense codon recognition occurs on mature mRNA, after introns have been removed, whereas splice site selection occurs on immature pre-mRNA.

How can we understand that nonsense codon influences splicing if our cellular common sense dictates that nonsense codon recognition occurs after splicing? Different models propose an interactive connection between splicing and translation and they constitute the third way in which the association between nonsense mutations and splicing alterations have been explained. One of the models proposes that mRNA translation initiates during transport from nucleus to cytoplasm before splicing has been completed. Accordingly, at the cytoplasmic side of the nuclear membrane, mRNA is being translated while, at the nuclear side, the same mRNA molecule is being spliced. This model can explain nuclear mRNA NMD and the retention of downstream introns. However, it cannot explain how, in some cases, upstream introns are retained as a consequence of a nonsense codon. Another model postulates the existence of a feedback mechanism between cytoplasm and nucleus but there is no experimental evidence of such a phenomenon. The third model postulates the existence of a nuclear ribosomal-like particle capable of recognizing the reading frame on pre-mRNA or mature mRNA when it is still in the nucleus, with the ability to inhibit splicing or to promote alternative pathways.[20,29,30]

Two recent reports have contributed to an understanding of the relationship between nonsense mutations and splicing. One of them showed that translation can occur in the nucleus of mammalian cells near sites of active transcription.[31] The second article directly addressed the problem of nonsense-associated splicing alterations. The authors showed by *in situ* fluorescence hybridization that unspliced transcripts accumulate at or near the site of transcription of alleles carrying nonsense mutations.[32] Taken together, these two observations aim at bridging translation and splicing and, therefore, new perspectives become available.

Conclusions

In the present study we showed that an alternative TG transcript lacking exon 22 preferentially accumulates in thyroid tissue when exon 22 harbors a nonsense mutation. Exclusion of this mutated exon restores the reading frame, potentially allowing the mRNA to be completely translated. We also demonstrated that the mutation itself is not sufficient to induce exon 22 exclusion, suggesting that the disruption of an ESE is not the mechanism underlying the accumulation of the alternative mRNA. Our data support the notion that the alternative splicing of the mutated exon is induced by the interruption of the reading frame.

These results open up new perspectives in the knowledge of the mechanism of alternative splicing for the TG pre-mRNA and could be useful in the clinical molecular diagnosis of patients with hereditary thyroid diseases involving defective TG synthesis.

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Dr Targovnik is an established investigator of the CONICET.

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The authors have no conflicts of interest directly relevant to the content of this study.

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