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A nonsense mutation causes hereditary goitre in the Afrikander cattle and unmasks alternative splicing of thyroglobulin transcripts

(congenital hypothyroidism/thyroxine/thyroid disease)

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ABSTRACT **The hereditary goitre of Afrikander cattle is an autosomal recessive disease characterized in homozygotes by the production of abnormal thyroglobulin (Tg) and the coexistence in the thyroid of normal-sized 8.4-kilobase (kb) Tg mRNA with a misspliced 7.3-kb message having lost exon 9. We have cloned and sequenced the cDNA segment corresponding to the abnormal exon 8–exon 10 junction and the relevant genomic DNA region. The mutation responsible for the disease is a cytosine to thymine transition creating a stop codon at position 697 in exon 9. The original reading frame is maintained in the 7.3-kb mRNA, which, as it lacks the mutated exon, is translatable into a potentially functional protein. This puzzling phenotype in which a mutated exon is apparently removed selectively from transcripts by alternative splicing leads us to suggest that the 7.3-kb transcript could be present in normal animals. Using a sensitive oligonucleotide hybridization assay, we have demonstrated that a 7.3-kb mRNA lacking exon 9 does exist in normal thyroids as a minor mRNA species. As it is fully translatable, the 7.3-kb mRNA is expected to be more stable than the normal-sized 8.4-kb message. This probably accounts for the higher proportion of 7.3-kb transcript found in the goitre.**

Thyroglobulin (Tg), a 660-kDa homodimer, is the biosynthetic precursor of the thyroid hormones (1). The Tg gene is ≈250,000 nucleotide pairs long, of which 8431 nucleotides are represented in the mRNA (2). Congenital goitres with defective Tg production have been described in humans (3) and in animal models (4, 5). They are usually associated with hypothyroidism and may lead, in humans, to the development of the "cretin" phenotype (6). The hereditary goitre of the Afrikander cattle is inherited as an autosomal recessive disease (7). The goitre of homozygotes contains no normal Tg (8) and contains both a shorter and a normal-sized Tg mRNA (9); exon 9 is absent in the shorter Tg mRNA, suggesting that a splicing error is responsible for the disease (9). To identify precisely the mutation, we have cloned and sequenced the cDNA of the affected region of the smaller mRNA and the exon 9–intron 9 region of the genomic DNA from both a normal individual and an individual with goitre. We show that a cytosine to thymine transition in exon 9, changing codon 697 from CGA (arginine) to TGA (stop) is responsible for the disease. The nonsense mutation in exon 9 is apparently associated with and partially cured by removal of the defective exon from a portion of Tg transcripts. An explanation for this curious phenotype was obtained by the identification of a minor mRNA species lacking exon 9 in the thyroids of normal animals. Different stability of the shorter (fully translatable) and larger (containing an early stop codon) messages probably accounts for their relative amounts in goitre tissue.

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METHODS

Isolation of Total and Poly(A) RNA. Total RNA was prepared from normal and goitre tissues according to Auffray and Rougeon (10). Poly(A) RNA and purified Tg mRNA were isolated by poly(U) Sepharose chromatography and sucrose density-gradient fractionation as described (11, 12).

Cloning and Sequencing of Tg cDNA from Goitre. Poly(A) RNA from the thyroid of a homozygous goitrous animal was reverse-transcribed under conditions favoring the obtention of full-length transcripts (13). Double-stranded cDNA was prepared according to standard procedures (14) and cleaved with *Sau*3AI. The resulting fragments were cloned in the *Bam*HI site of pBR322 and an *Escherichia coli* C600 transformant containing the fragment bridging the exon 8–exon 10 junction of 7.3-kilobase (kb) Tg transcripts (see Fig. 1) was identified by hybridization to a ^{32}P-labeled exon 10-specific probe (an *Eco*RI/*Sau*3AI fragment from the pbTg 2.6; see ref. 15) and by restriction analysis of DNA minipreparations. The insert was subcloned in both orientations in M13mp19 and sequenced (16).

Cloning and Sequencing of Genomic DNA Segments. An *Eco*RI/*Hin*dIII genomic DNA fragment containing the exon 9–intron 9 region (see Fig. 1 and ref. 9) was partially purified from a homozygous goitrous animal by sequential cleavage of the DNA with *Eco*RI and *Hin*dIII and electroelution of the fragments of the expected size from agarose gels. After ligation in *Eco*RI/*Hin*dIII-cleaved pBR322 and transformation of *E. coli* MC1061, a clone harboring the fragment of interest was identified by hybridization to the ^{32}P-labeled 1.1-kb insert of pbTg 1.1 (15). The *Rsa* I/*Hin*cII fragment containing the exon 9–intron 9 junction (Fig. 1) was subcloned in both orientations in M13mp19 and sequenced (16).

The corresponding *Ras* I/*Hin*cII fragment from a normal animal of European origin was subcloned and sequenced in exactly the same manner, except that the starting material was a cosmid clone harboring the first 16 exons of the Tg gene (9).

Detection of 7.3-kb Tg Transcripts by Hybridization to an Oligonucleotide Probe. Total and purified Tg mRNA were separated by electrophoresis in agarose gels and blotted onto nylon membranes (Biodyne Electronics, Santa Monica, CA) (17). After a 2-hr prehybridization in 6× NET/0.5% NaDodSO$_4$/0.05% sodium pyrophosphate at 50°C (1× NET = 150 mM NaCl/1 mM ethylenediaminetetraacetic acid/15 mM Tris·HCl, pH 7.5), the blots were hybridized in the same medium containing ≈5 × 10^6 cpm of a 20-mer oligonucleotide per ml (see Fig. 2 *Upper*) that had been labeled to ≈ 5 × 10^8 cpm/μg by polynucleotide kinase and [^{32}P]ATP (14). Hybridization was for 2 hr except in one experiment involving total RNA (Fig. 3*A*, lanes 5 and 6), where it was prolonged to 16

Abbreviation: Tg, thyroglobulin.

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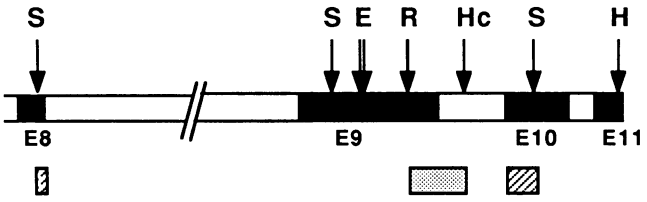

FIG. 1. Schematic representation of the Tg gene from the 3′ end of exon 8 (E8) to exon 11 (E11). Solid bars, exons; open bars, introns. The position of cDNA (hatched bar) and genomic DNA (stippled bar) sequences presented are also indicated. E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; R, *Rsa* I; S, *Sau*3AI.

hr. After three 15-min washes at 0°C, a 1-min wash was performed at 45°C followed by two 15-min washes at room temperature. All washes were in 50 ml of 6× NET/0.5% $NaDodSO_4$/0.05% sodium pyrophosphate. The wet filters were exposed overnight to Kodak XAR-5 films without a screen. Thereafter, the blots were washed for 1 min at 50°C and then autoradiographed again. To obtain a readable autoradiograph with total RNA (see Fig. 3, lanes 5 and 6), it was necessary to rehybridize the filter at 50°C for 16 hr. Thereafter, the washing procedure and autoradiography were as described above; only the results corresponding to the 50°C wash are shown. To display the total Tg mRNA sequences present on the blots, they were washed at 80°C and hybridized to a ^{32}P-labeled Tg cDNA fragment (pbTg 1.9; ref. 15) under standard conditions (14). Exposure was for 10 min without a screen.

RESULTS

Cell-free translation of goitre RNA gave Tg-related peptides of relative molecular mass 250 and 75 kDa (18). As the shorter (7300-nucleotide) mRNA could encode a 250-kDa peptide, the presence of a 75-kDa peptide was compatible with a mutation in the vicinity of the exon 9–exon 10 junction, which directly or indirectly caused an in-frame stop codon in the 8400-nucleotide mRNA. This, together with the presence of genomic restriction fragment length differences in the vicinity of the exon 9–intron 9 junction and the absence of exon 9 from the smaller Tg mRNA (9), strongly suggested that the responsible mutation was localized at or near the exon 9–intron 9 junction. This region of the goitre Tg cDNA and of both the normal and goitre genomic DNA was therefore selectively cloned and sequenced (Fig. 2). The sequence across the exon 8–exon 10 junction of the smaller mRNA establishes that exon 9 is precisely spliced out with its two flanking introns. There is no change in the reading frame, although a new amino acid (glycine) is generated at the junction (Fig. 2 *Upper*). The most notable difference between the genomic DNA sequences of normal and goitre animals is a cytosine to thymine transition, which changes codon 697 from CGA (arginine) to TGA (stop) (Fig. 2 *Lower*). This results in loss of a *Taq* I restriction site as reported (9). Translation of the goitre 8400-nucleotide mRNA therefore terminates at codon 697 to give a protein of a size consistent with the 75-kDa peptide found in the cell-free translation studies (18). Other minor differences do exist between the normal and the goitre sequences: there is an adenine to guanine transition in codon 699 resulting in a conservative amino acid change (alanine to glycine) 21 nucleotides from the exon 9–intron 9 junction, and an intronic adenine to guanine point mutation 25 nucleotides 3′ of the same junction, generating a *Pst* I recognition site. Although these polymorphisms are linked to the goitre phenotype (9), they most probably reflect differences between the genetic background of Afrikander and European cattle from which the normal sequences were obtained.

Considering together the cDNA and genomic sequencing data, the results would indicate that the occurrence of a nonsense mutation in exon 9 of goitrous animals results in the removal of the defective exon from a fraction of Tg transcripts by alternative splicing. As no causal relationship is expected to relate splicing with translatability, we reasoned that the splicing out of exon 9 might preexist in normal animals at a level too low to be detected in our previous S1 nuclease mapping experiments (9). To test this hypothesis, a 20-mer oligonucleotide complementary to a segment bridging the exon 8–exon 10 region (see Fig. 2 *Upper*) was used to probe normal Tg mRNA for the presence of 7.3-kb transcripts. Hybridization of RNA blots at 50°C followed by stepwise washing at increasing temperatures demonstrated clearly the presence of a 7.3-kb RNA in Tg mRNA isolated from two normal individuals (Fig. 3*A*). A total RNA preparation from the goitre also showed the expected 7.3-kb band, while its amount was apparently too low to be detected in total RNA from normal thyroids. Hybridization of the same blot with a Tg cDNA probe failed to detect the 7.3-kb mRNA, except in the goitre where it coexists with similar amounts of normal-sized 8.4-kb mRNA, as demonstrated (Fig. 3*B*) (9).

DISCUSSION

The existence of a minor 7.3-kb mRNA in Tg transcripts from normal animals leads to a coherent explanation of the goitrous phenotype. As demonstrated in other cases (19, 20), the nonsense mutation at codon 697 is expected to cause a dramatic destabilization of the 8.4-kb mRNA as a consequence of its limited translatability. The 7.3-kb mRNA, preexisting as a minor mRNA species, is actively translated into a 250-kDa Tg polypeptide and escapes destabilization as it does not contain the mutation. Even though no normal 300-kDa Tg can be made by the goitrous animals, the production of the 74-kDa and the 250-kDa Tg polypeptides is compatible with thyroid hormone production. Indeed, the amino-terminal hormonogenic domain of Tg responsible for the bulk of thyroxine synthesis (2) is present in both peptides, which probably accounts for the euthyroid status of the animals (7).

While this picture sounds coherent from a qualitative point of view, some quantitative aspects are worth discussing. Our results demonstrate significantly more 7.3-kb transcripts in goitre as compared to normal (compare lanes 5 and 6 in Fig. 3*A*). It is conceivable that the chronic hyperstimulation leading to goitre (21) would result in a significant increase in transcription or would affect the precision of the splicing mechanisms. Against this hypothesis is the observation that under basal conditions, transcription of the Tg gene is already close to its maximum (at least in rats; see ref. 22), and that normal thyroid stimulating hormone levels have been reported in the plasma of goitrous animals as measured by a heterologous bioassay (7). Despite the latter data, it is hard to escape the idea that some kind of hyperstimulation is responsible for the presence of a large goitre in these animals. Alternatively, the nonsense mutaton near the end of exon 9 or the polymorphic variations found in this region in the Afrikander animals (Fig. 2) might influence the choice of the splice site, thus favoring generation of the 7.3-kb mRNA. Examples of exonic mutations affecting the choice of splice sites have been documented (23, 24).

The significance of 7.3-kb transcripts in normal animals is obscure. While we cannot exclude that they may play a specific role, the evidence for this is lacking. We consider it more likely that they represent a low level of "noise" in the efficiency of the splicing machinery. The recent sequencing

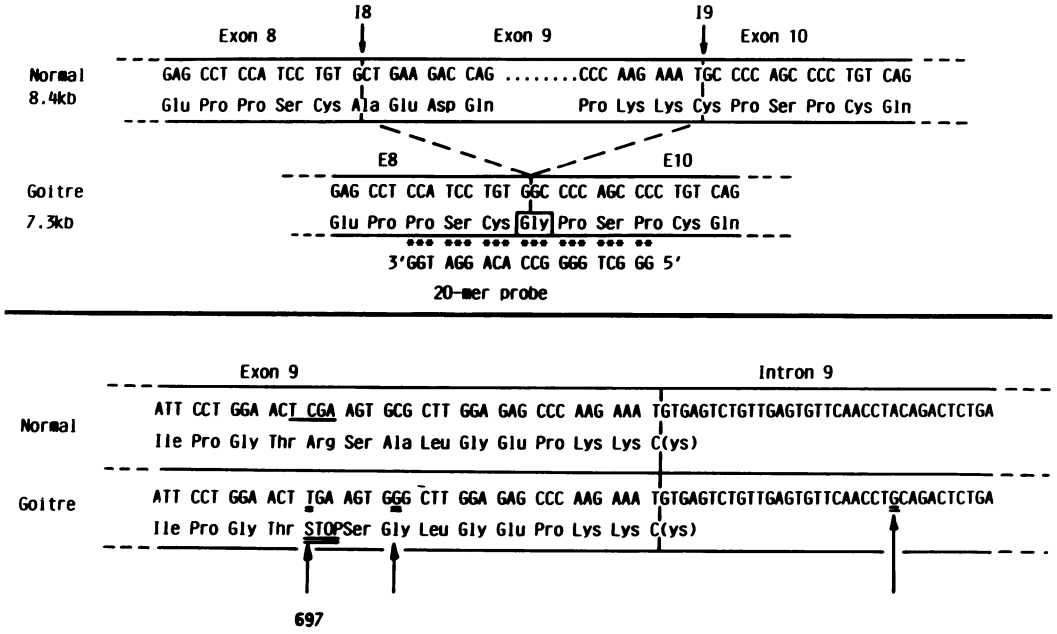

FIG. 2. (*Upper*) Nucleotide (and deduced protein) sequences of bovine Tg cDNA and genomic DNA from normal individuals and individuals with goitre. Normal sequences are from beef of European origin. Exon 8–exon 9 and exon 9–exon 10 borders as they appear in normal 8.4-kb transcripts (27). The exon 8–exon 10 border generated by alternative splicing in the 7.3-kb transcript of the goitre is represented below, together with the 20-mer oligonucleotide probe used in the RNA blotting experiment described in Fig. 3. (*Lower*) Exon 9–intron 9 junction in normal and goitre genomic DNA. Sequence differences are indicated by arrows. The *Taq* I site in the normal sequence is underlined.

of a bovine Tg cDNA variant lacking two exons in the 3′ region of the mRNA strengthens this contention (L.M., unpublished data). Similar splicing "errors" operating at a low rate have been reported (see ref. 25 and refs. therein). They may be more frequent than generally believed since, as in the present case, special techniques more sensitive than S1 nuclease mapping or chance observations might be required to detect them.

Apart from explaining the cattle disease in molecular terms, our results illustrate a curious phenotype in which a nonsense mutation portrays a splicing error. In view of the high mutability of CG sequences (26) and considering that the segment involved is perfectly conserved in human Tg (26), an analogous mutation may well be responsible for some hereditary goitres in humans.

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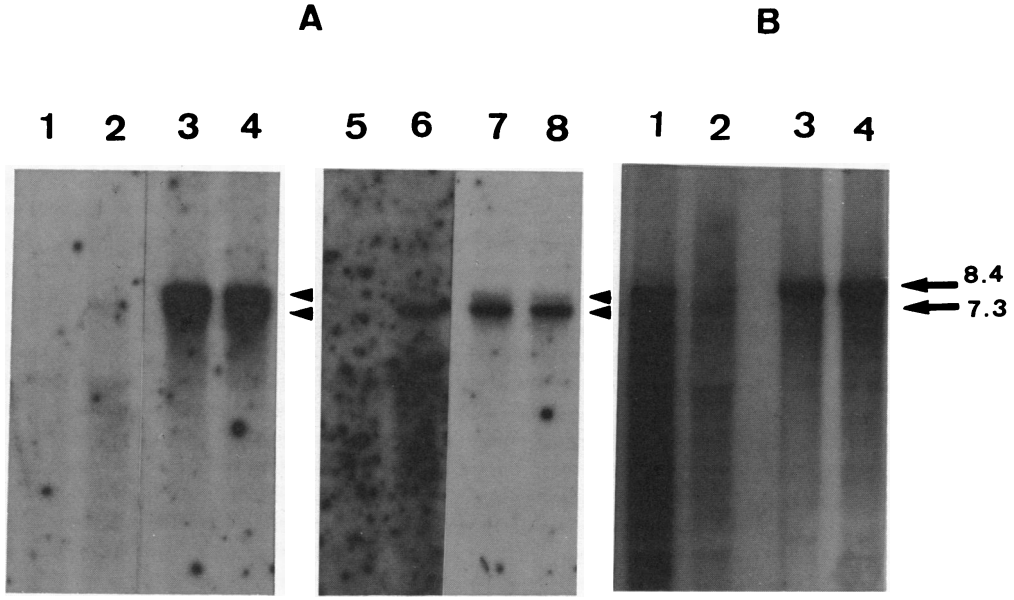

FIG. 3. Detection of 7.3-kb transcripts lacking exon 9 in Tg mRNA from normal thyroids. (*A*) A 20-mer oligonucleotide probe complementary to the exon 8–exon 10 border of 7.3-kb goitre transcripts (see Fig. 2 *Upper*) was hybridized to an RNA blot containing total thyroid RNA (10 μg) from normal (lanes 1 and 5) and goitre (lanes 2 and 6) South African animals, and Tg mRNA (0.5 μg) purified from two normal Belgian cattle (lanes 3, 4, 7, and 8). Hybridization was for 2 hr (lanes 1–4, 7, and 8) or 16 hr (lanes 5 and 6; see *Methods*). Washing at different temperatures (lanes 1–4, 45°C; lanes 5–8, 50°C) demonstrated the presence of 7.3-kb Tg transcripts in total goitre RNA (lane 6) and in normal Tg mRNA (lanes 7 and 8). (*B*) Hybridization of the same blot with Tg cDNA displaying the size distribution of the bulk of Tg mRNA sequences.

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1. Van Herle, A. J., Vassart, G. & Dumont, J. E. (1979) *N. Engl. J. Med.* **301,** 239–249 & 307–314.
2. Mercken, L., Simons, M. J., Swillens, S., Massaer, M. & Vassart, G. (1985) *Nature (London)* **316,** 647–651.
3. Salvatore, G., Stanbury, J. B. & Rall, J. E. (1980) in *The Thyroid Gland*, ed. De Visscher, M. (Raven, New York), pp. 443–488.
4. De Vijlder, J., Baas, F., Kok, K., Van Dijk, J. E., Geurts, A., Van Ommen, G. J. B. & Tegelaers, W. H. (1985) in *Progress in Endocrine Research and Therapy*, ed. Burrow, G. N. (Raven, New York), Vol. 2, pp. 69–76.
5. De Vijlder, J., Van Voorthuizen, W. F., Van Dijk, J. E. & Tegelaers, W. H. (1978) *Endocrinology* **103,** 2105–2111.
6. Stanbury, J. B. & Dumont, J. E. (1983) in *The Metabolic Basis of Inherited Diseases*, ed. Stanbury, J. B. (McGraw-Hill, New York), p. 231.
7. Ricketts, M. H., Schulz, K., Van Zyl, A., Bester, A. J., Boyd, C. D., Meinhold, W. & Van Jaarsveld, P. P. (1985) *J. Hered.* **76,** 12–16.
8. Pammenter, M., Albrecht, N., Liedenberg, W. & Van Jaarsveld, P. (1978) *Endocrinology* **102,** 954–965.
9. Ricketts, M. H., Pohl, V., de Martynoff, G., Boyd, C. D., Bester, A. J., Van Jaarsveld, P. P. & Vassart, G. (1985) *EMBO J.* **4,** 731–737.
10. Auffray, C. & Rougeon, F. (1980) *Eur. J. Biochem.* **107,**303–314.
11. Vassart, G., Brocas, H., Lecocq, R. & Dumont, J. E. (1975) *Eur. J. Biochem.* **55,** 15–22.
12. Vassart, G., Verstreken, L. & Dinsart, C. (1977) *FEBS Lett.* **79,** 15–18.
13. de Martynoff, G., Pays, E. & Vassart, G. (1980) *Biochem. Biophys. Res. Commun.* **93,** 645–653.
14. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
15. Christophe, D., Mercken, L., Brocas, H., Pohl, V. & Vassart, G. (1982) *Eur. J. Biochem.* **122,** 461–469.
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
17. McMaster, G. K. & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 4835–4838.
18. Tassi, V. P. N., Di Lauro, R., Van Jaarsveld, P. & Alvino, C. G. (1984) *J. Biol. Chem.* **259,** 10507–10510.
19. Moschonas, N., de Boer, E., Grosveld, F. G., Dahl, H. H., Wright, S., Shewmaker, C. K. & Flavell, R. A. (1981) *Nucleic Acids Res.* **9,** 4391–4401.
20. Chang, J. C. & Kan, Y. W. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2886–2889.
21. Van Zijl, A., Schulz, B., Wilson, B. & Pansegrouw, D. (1965) *Endocrinology* **76,** 353–361.
22. Van Heuverswyn, B., Streydio, C., Brocas, H., Refetoff, S., Dumont, J. E. & Vassart, G. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5941–5945.
23. Somasekhar, M. B. & Mertz, J. E. (1985) *Nucleic Acids Res.* **13,** 5591–5609.
24. Reed, R. & Maniatis, T. (1986) *Cell* **46,** 681–690.
25. Amara, S. G. (1985) *Mol. Cell. Endocrinol.* **42,** 191–205.
26. Malthiery, Y. & Lissitzky, S. (1985) *Eur. J. Biochem.* **147,** 53–58.
27. Parma, J., Christophe, D., Pohl, V. & Vassart, G. (1987) *J. Mol. Biol.*, in press.