The EMBO Journal vol.4 no.3 pp.731-737, 1985

Defective splicing of thyroglobulin gene transcripts in the congenital goitre of the Afrikander cattle

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Communicated by S.Lissitzky

The structure of thyroglobulin mRNA was analyzed in an inbred herd of Afrikander cattle with hereditary goitre. Northern transfer of RNA from affected animals revealed both a shorter (~7100 bases) and a normal-sized (~8200 bases) thyroglobulin mRNA when hybridized to bovine thyroglobulin cDNA clones. S1 nuclease mapping experiments established that 1100 bases are deleted in the 5′ region of the smaller mRNA. Electron microscopy of RNA from animals with goitre hybridized to a bovine genomic DNA clone showed that the region deleted corresponds to exon 9 of the thyroglobulin gene. Southern blot analysis of the exon 9 region revealed differences between affected and control animals with the enzymes *Pst*I and *Taq*I. Although they could reflect a linkage disequilibrium between the mutation and restriction fragment length polymorphism, it is noteworthy that these differences map in the region of the exon 9/intron 9 junction. Our results show that a genetic lesion in the thyroglobulin gene causes aberrant splicing of the pre-mRNA, and suggest that the responsible mutation is at the exon 9/intron 9 junction.

Key words: hereditary goitre/thyroglobulin mRNA/splicing defect

Introduction

Thyroglobulin (Tg), a 19S sedimenting dimer consisting of identical 300 000 dalton subunits, is the protein precursor of the thyroid hormones T3 and T4 (Van Herle *et al.*, 1979). Qualitative or quantitative defects of Tg production are responsible for the development of congenital goitre in animals as well as in man (Lever *et al.*, 1983; Lissitzky *et al.*, 1973; De Vijlder *et al.*, 1981; Salvatore *et al.*, 1980). One such situation has been described in the Afrikander cattle, presenting as an autosomal recessive mutation (Ricketts *et al.*, 1985a). It is characterized by the presence of reduced levels of Tg mRNA (Ricketts *et al.*, 1985b), associated with undetectable levels of normal 19S thyroglobulin (Robbins *et al.*, 1966; Van Jaarsveld *et al.*, 1971). Knowledge of the molecular basis of hereditary diseases has been primarily obtained from the analysis of globin genes in thalassemias (Nienhuis *et al.*, 1984). Considerably less is known about the molecular biology of hereditary diseases involving large inducible genes such as thyroglobulin.

The gene for thyroglobulin is interrupted by numerous introns, making it one of the largest genes thus far investigated (Vassart *et al.*, 1983; Van Ommen *et al.*, 1983; Targovnik *et al.*, 1984; Avvedimento *et al.*, 1984). Part of the bovine thyroglobulin gene has been cloned into cosmid vectors (de Martynoff *et al.*, 1983). The normal bovine thyroglobulin mRNA is ~8000 bases long (Vassart *et al.*, 1977) and the cDNA of all but the 5′ extremity of the mRNA has been cloned into pBR322 (Christophe *et al.*, 1982). We report here the results of an analysis of Tg mRNA structure in the hereditary goitre of the Afrikander cattle using as probes the normal bovine Tg cDNA and genomic clones.

Results

Analysis of thyroglobulin mRNA by Northern transfer

RNA from two goitres and from normal bovine thyroid was analyzed by Northern transfer and hybridization with a bovine cDNA probe (pbTg 2.5, see Christophe *et al.*, 1982). In the normal thyroid RNA the expected mRNA of about 8200 bases was detected (Figure 1, lanes 3 and 6). The goitre RNA preparations showed the presence of two thyroglobulin mRNA species; one of the same size as the normal mRNA and one slightly smaller (Figure 1, lanes 1, 2, 4 and 5). Denaturation of the RNA by glyoxal treatment (Figure 1, lanes 1-3) prior to electrophoresis established that the different rates of mobility are due to size and not conformational differences in the RNA. Two thyroglobulin

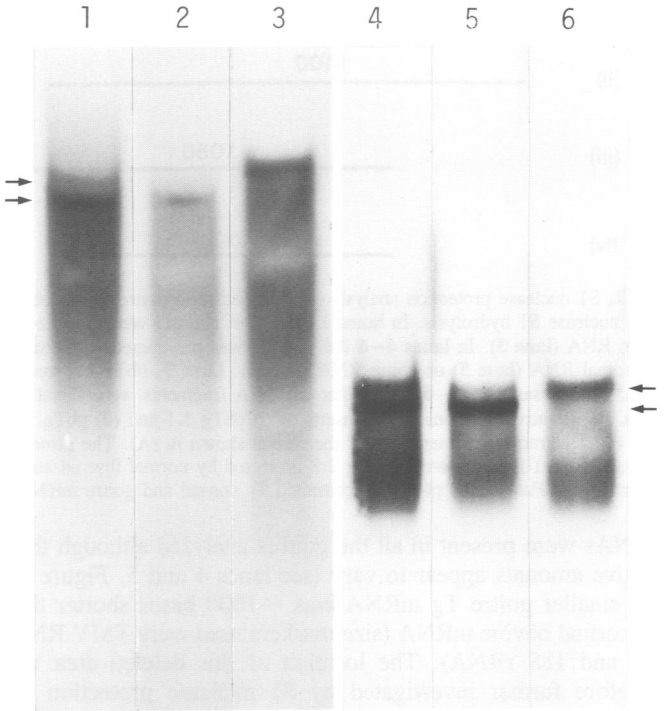


Fig. 1. Northern transfer analysis of RNA from normal and goitre thyroid tissue. **Lanes 1, 2, 4** and **5**: goitre poly(A)$^+$ RNA (7.5 μg). **Lanes 3** and **6**: normal thyroid total RNA (10 μg). In **lanes 1-3** the RNA was pretreated with glyoxal before electrophoresis. Arrows indicate the positions of the two goitre thyroglobulin mRNAs.

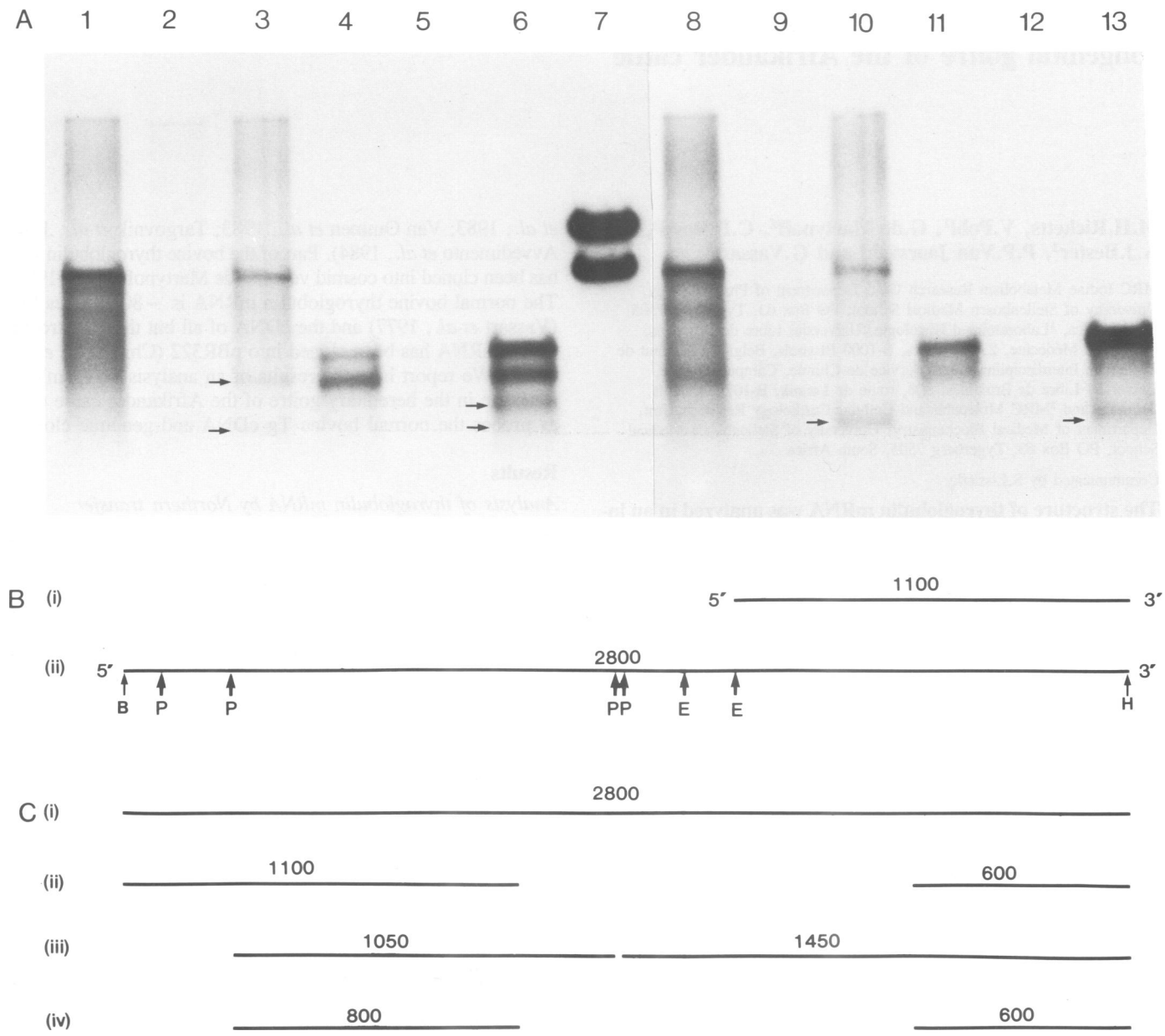


Fig. 2. S1 nuclease protection analysis of the deletion in goitre thyroglobulin mRNA. (**A**) Fragments of the pbTg 2.8 plasmid (see text and under **B**) protected from nuclease S1 hydrolysis. In **lanes 1–3**, uncut plasmid was hybridized with normal thyroid RNA (**lane 1**), rabbit reticulocyte polysomal RNA (**lane 2**) or goitre RNA (**lane 3**). In **lanes 4–6** the plasmid was pre-treated with *Pst*I before hybridization with normal thyroid RNA (**lane 4**), rabbit reticulocyte polysomal RNA (**lane 5**) or goitre RNA (**lane 6**). **Lane 7**, pbTg 2.8 treated with *Bam*HI and *Hind*III to release the 2800 base insert as size marker. **Lanes 8–13** are the same as **1–6** except that the DNA fragments were visualized with pbTg 1.1. The positions of goitre-specific fragments are indicated by arrows. (**B**) Relative positions of the inserts of (**i**) pbTg 1.1 and (**ii**) pbTg 2.8. Restriction endonuclease sites shown are *Pst*I (P), *Eco*RI (E), *Bam*HI (B), *Hind*III (H). (**C**) Diagramatic presentation of the results shown in (**A**). The protected DNA fragments are shown with the same orientation and scale as the cDNA inserts in (**B**). (**i**) Fragments of pbTg 2.8 protected by normal thyroid and goitre mRNA. (**ii**) Fragments of pbTg2.8 protected by goitre mRNA only. (**iii**) Fragments of *Pst*I-treated pbTg 2.8 protected by normal and goitre mRNA. (**iv**) Fragments of *Pst*I treated pbTg 2.8 protected by goitre mRNA only.

mRNAs were present in all the goitres analyzed although their relative amounts appear to vary (see lanes 4 and 5, Figure 1). The smaller goitre Tg mRNA was ~1000 bases shorter than the normal bovine mRNA (size markers used were TMV RNA, 28S and 18S rRNA). The location of this deleted area was therefore further investigated by S1 nuclease protection experiments.

S1 nuclease mapping of the deletion in goitre thyroglobulin mRNA

RNA was denatured and incubated separately with each of the four thyroglobulin cDNA-containing plasmids which span all but the 5′ extremity of the mRNA (Christophe *et al.*, 1982). The size of the DNA fragments protected from S1 nuclease by the RNA were determined by agarose gel electrophoresis. The thyroglobulin mRNAs from both normal and goitre tissue fully protected the inserts from three clones (pbTg 1.0, pbTg 2.5, pbTg 1.9, see Christophe *et al.*, 1982) covering 65% (from the 3′ end) of the mRNA (results not shown). With a 2800 base cDNA containing plasmid, pbTg 2.8 (complementary to the 5′ region of the thyroglobulin mRNA, see Christophe *et al.*, 1982), a 2800 base DNA fragment was fully protected from S1 nuclease by the mRNA of both normal and goitre tissue (Figure 2A, lanes 1 and 3). However, additional fragments of 1100 and 600 bases were also protected by the goitre RNA (Figure 2A, lane 3). The

latter two DNA fragments therefore span the position of a deletion in the smaller goitre thyroglobulin mRNA. Cleavage of the plasmid DNA with *Pst*I prior to hybridization with the RNA protected the expected fragments of 1050 and 1450 bases as well as fragments of 800 and 600 bases in the goitre (Figure 2A, lane 6). In these experiments reannealing of the DNA was minimal; only with prolonged exposure were fragments of 6800 bases (for the entire plasmid reannealing) and 3400, 2150 and 1100 bases (the *Pst*I fragments used in lanes 4–6) faintly visible in the controls (lanes 2 and 5).

The orientation of the two protected fragments flanking the deletion was determined by using a 1100-bp cDNA probe corresponding to the 3′ segment of the 2800-bp clone (see Figure 2B). In addition to the 2800 and 1450 base normal-size fragments, the 600 base fragment in the goitre was detected with this probe (Figure 2A, lanes 10 and 13). The 600 base fragment therefore flanks the 3′ side of the deletion. The interpretation of these results is summarized in Figure 2C, which shows the existence, in the smaller goitre Tg mRNA, of a 1100 base deletion situated approximately between position 1100 and 2200 from the 5′ end of the normal mRNA.

Analysis of RNA-DNA hybrids by electron microscopy

To correlate the deletion in the goitre Tg mRNA with respect to the normal intron/exon organization of the gene (Targovnik *et al.*, 1984; de Martynoff *et al.*, in preparation), RNA enriched for thyroglobulin mRNA was prepared from goitre tissue, hybidized with a cosmid containing the 5′ region of the bovine Tg gene (de Martynoff *et al.*, 1983) and the hybrids were examined by electron microscopy. The 8200 base goitre thyroglobulin mRNA hybridized with the bovine genomic DNA and gave hybrids resembling the normal pattern (Figure 3A). The smaller mRNA gave an abnormal hybridization pattern (Figure 3B) in that exon 9 (1100 bases) and intron 9 (520 bases) were not seen, while the size of intron 8 was increased by 1240 ± 500 bases. The enlarged intron 8 therefore contains the sequences normally found as exon 9 and intron 9, indicating that exon 9 is deleted from the smaller goitre thyroglobulin mRNA. The evidence thus indicates the presence of a mutation in the thyroglobulin gene of affected animals which leads to partial defective splicing of the pre-mRNA with excision of exon 9 (Figure 5).

Blot hybridization analysis of the thyroglobulin gene of normal and goitrous animals

The genomic DNA of normal and goitrous animals was compared in the vicinity of exon 9 by blot hybridization using a number of restriction enzymes. A representative Southern blot experiment which included double digests with the enzymes *Pst*I, *Taq*I and *Eco*RI is shown in Figure 4A. The DNA fragments were orientated for the construction of the restriction map (Figure 4B) by double digests, differential hybridization with the cloned *Eco*RI/*Eco*RI and *Eco*RI/*Hind*III cDNA fragments (see Figure 2B) and by reference to known sites in the cloned cDNA (Mercken *et al.*, 1984). Differences between goitrous and control animals were detected only with the enzymes *Pst*I and *Taq*I. No difference was detected with many enzymes including *Pvu*II, *Hinc*II, *Rsa*I, *Eco*RI and *Hph*I. With *Pst*I a fragment of 1000 bp in normal, bearing sequences complementary to both the *Eco*RI/*Eco*RI and *Eco*RI/*Hind*III cDNA clones, was reduced to 800 bp in the goitre. This is compatible with either a deletion of ~200 bp or the presence of an additional *Pst*I site in the goitre. The absence of any difference in Tg genomic fragments generated in normal and affected animals with eight other restriction enzymes argues for the latter possibility. In double digests with *Pst*I and *Eco*RI the 1000- and 800-bp fragments are both shortened by 300 bp, as expected from the known cDNA restriction map. The 1250-bp *Pst*I fragment is unchanged in the goitre. The additonal *Pst*I site maps in the 5′ donor region of intron 9. *Taq*I digests of the DNA showed the goitrous animals to be homozygous for two changes. There is an additional *Taq*I site in exon 10 and the loss of a *Taq*I site at the 3′ end of exon 9. The proximity of both the lost *Taq*I site and the additional *Pst*I site on the exon 9/intron 9 junction is compatible with the existence of a mutation at or near this junction which would be reponsible for the incorrect processing of the pre-mRNA. As no size difference was found with short DNA fragments which span this region (as produced with *Hinc*II, *Pvu*II and *Rsa*I) the mutation must be restricted to a very small insertion, deletion or rearrangement. Alternatively, the observed difference in restriction patterns could simply reflect linkage disequilibrium between a restriction fragment length polymorphism and the mutation responsible for the disease.

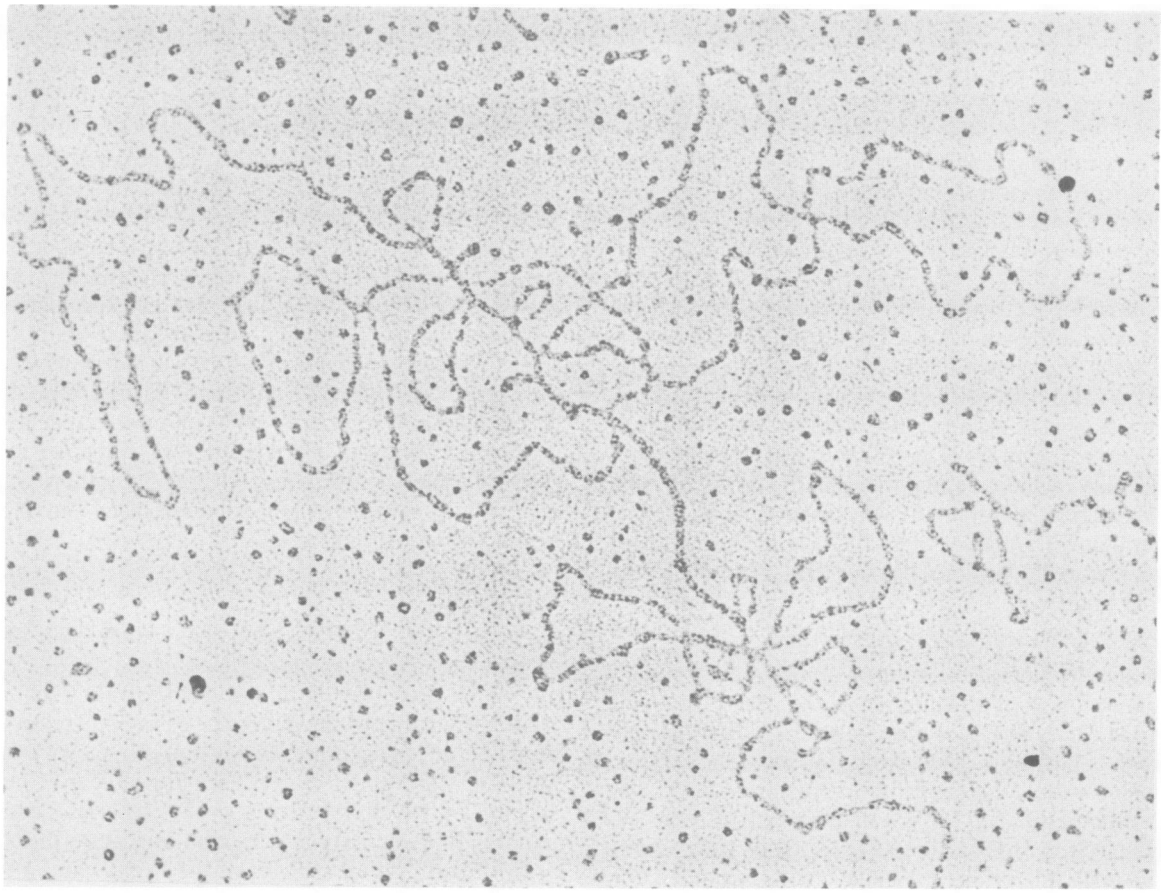
Discussion

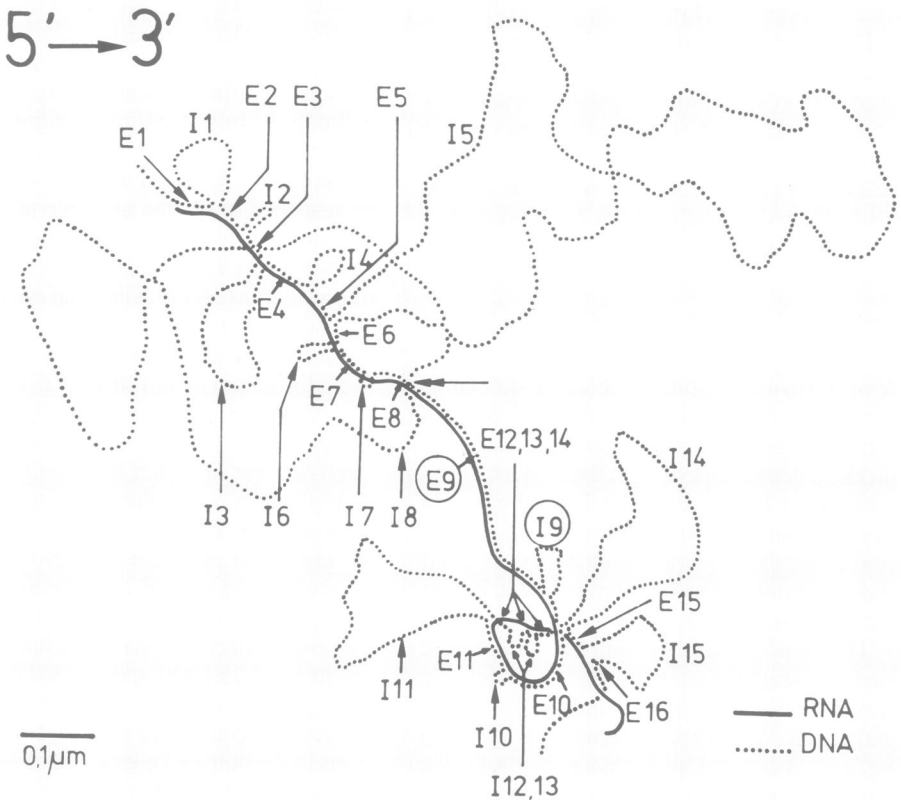
Hereditary defects of thyroglobulin synthesis have been observed in both man and animals (Lever *et al.*, 1983; De Vijlder *et al.*, 1981; Lissitzky *et al.*, 1973; Salvatore *et al.*, 1980). However, until now, the molecular basis of these defects has not been defined. In the present study we have shown that the thyroglobulin synthesis defect in Afrikander cattle involves defective splicing of the thyroglobulin gene transcript, leading to the removal of exon 9 from a fraction of mature mRNAs.

Blot hybridization analysis of the exon 9 area of the thyroglobulin gene shows a series of changes in the region of the donor sequence of intron 9 (Figure 4). In the absence of data concerning the prevalence of restriction fragment length polymorphism in this region of the bovine gene, it is difficult to correlate this finding with the mutation responsible for the disease. Indeed, polymorphism of the *Taq*I recognition sequence is known to be particularly frequent in higher eukaryotes (Barker *et al.*, 1984). Nevertheless, a mutation in the donor region of intron 9 could be responsible for the anomalous splicing out of exon 9. In a human thalassemia, a defect in the donor sequence of intron 2 gave two mRNAs when the β-globin gene was transfected and transcribed in HeLa cells: one was 47 nucleotides longer than normal, due to the utilisation of a cryptic donor sequence within intron 2, and the other was shortened by 223 nucleotides due to the ligation of exon 1 directly to exon 3 (Treisman *et al.*, 1982). The defective synthesis of thyroglobulin in Afrikander cattle is analogous to this β°-globin mutation in that both give rise to two mRNAs, one of which has neatly lost one exon. In the bovine thyroglobulin model the result of this aberrant splicing has been demonstrated within the tissue.

Translation of goitre thyroglobulin mRNA in both *Xenopus* oocytes (Ricketts *et al.*, 1985b) and in a cell-free system (Tassi *et al.*, 1984) produced no normal thyroglobulin, indicating that the normal-sized mRNA is malfunctional. Interestingly, cell-free products of 250 000 and 75 000 dalton antigenically related to thyroglobulin were detected. These findings can be rationalised in view of recent data relating to the precise localisation of introns in the human Tg gene (Malthiery and Lissitzky, 1984; Parma *et al.*, 1984 and in preparation). The clean splicing out of exon 9 would not break the reading frame, giving rise to a 250 000 dalton protein, while any frame shift in the exon 9/exon 10 junction would generate a peptide of ~80 000 dalton. According to this interpretation, the smaller mRNA would encode

A




5'→3'
E1
I1
E2
E3
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I2
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E12,13,14
I14
E9
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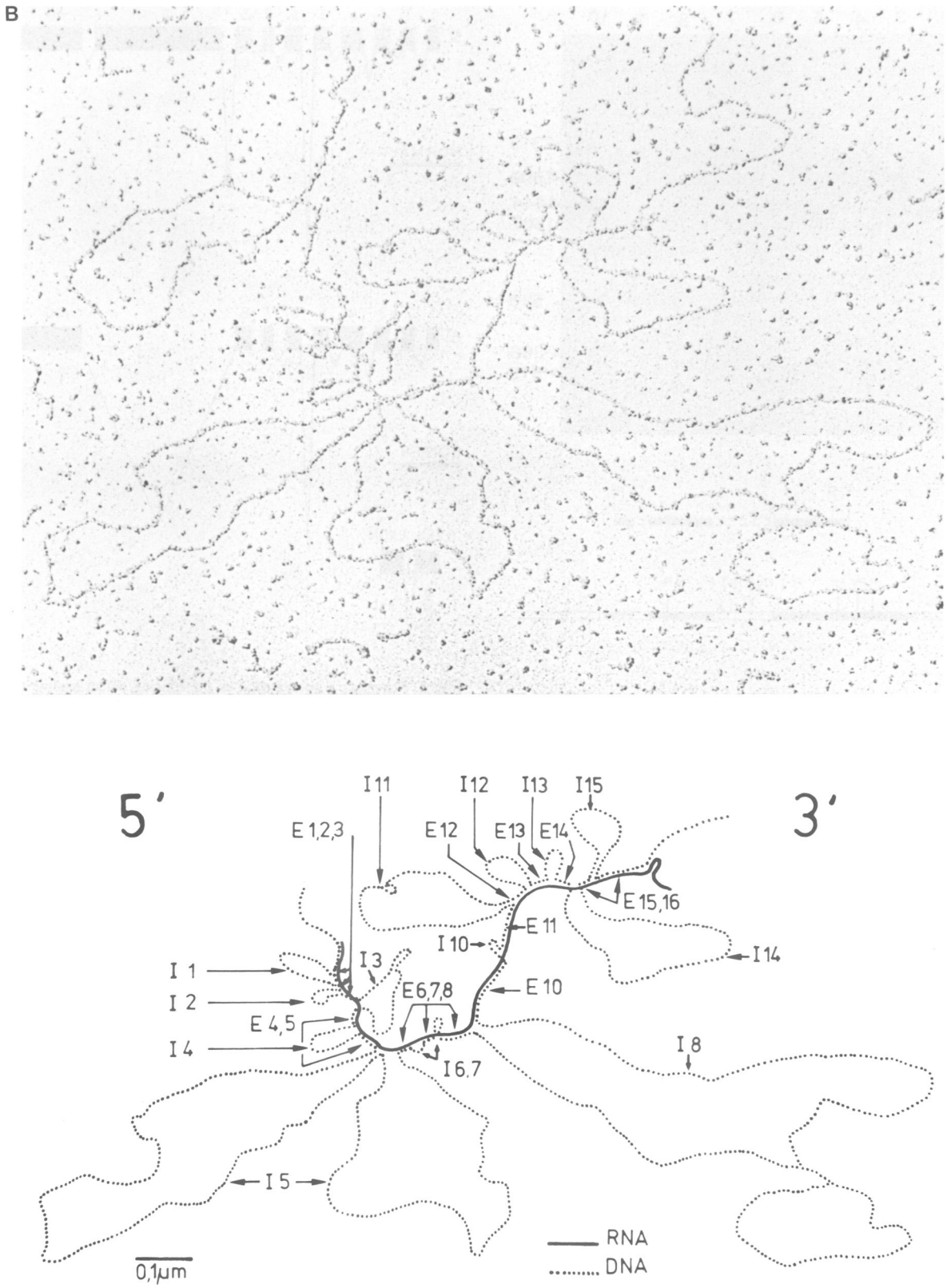

Fig. 3. Electron microscopy of hybrids between goitre thyroglobulin mRNA and cloned normal bovine genomic DNA. (**A**) Hybrid with 8200 base goitre RNA. (**B**) Hybrid with smaller goitre RNA. The DNA/RNA hybrids have been oriented with their 5′ side on the left. Arrows E_1 to E_{16} identify the first 16 exons (E) of the Tg gene. Arrows I_1 to I_{15} identify the corresponding 15 introns (I). Length measurements: in **A**: I_8 (6428 ± 389 bp); E_9 (1091 ± 79 bp); I_9 (517 ± 68 bp) and E_{10} (540 ± 44 bp); in **B**: I_8 (7668 ± 295 bp), absence of E_9 and I_9, E_{10} (539 ± 66 bp). Hybrids were prepared as described in Materials and methods.

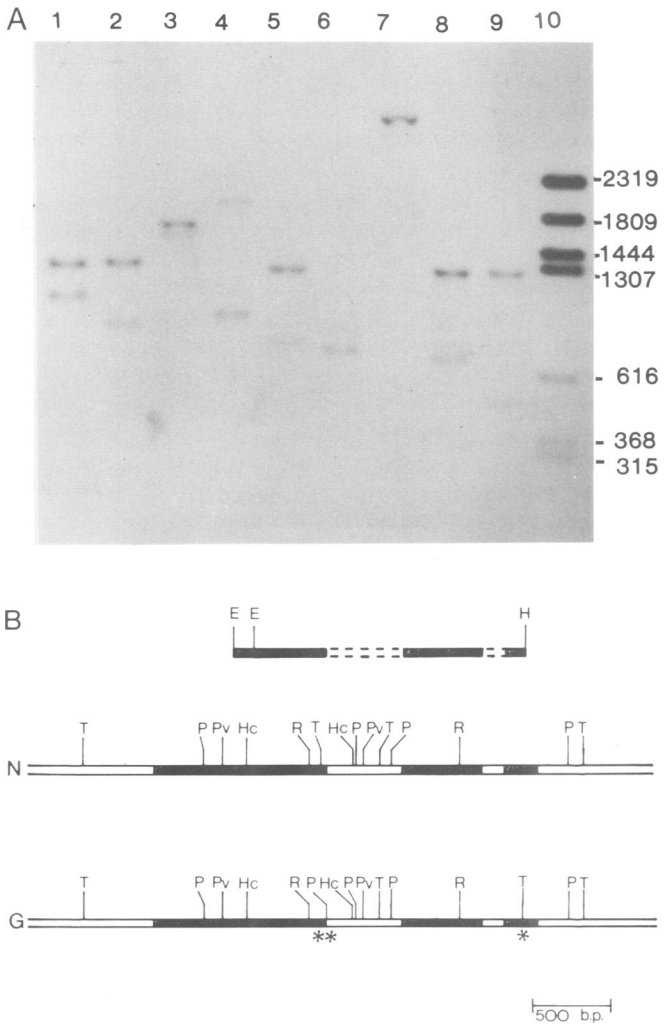

Fig. 4. Blot hybridization analysis of genomic DNA from normal and goitrous cattle. **(A)** Autoradiograph of genomic DNA hybridized with pbTg 1.1: normal DNA cut with *PstI* **(lane 1)**, goitre DNA cut with *PstI* **(lane 2)**, normal DNA cut with *TaqI* **(lane 3)**, goitre DNA cut with *TaqI* **(lane 4)**, normal DNA cut with *PstI* and *TaqI* **(lane 5)**, goitre DNA cut with *PstI* and *TaqI* **(lane 6)**, normal DNA cut with *EcoRI* **(lane 7)**, normal DNA cut with *EcoRI* and *PstI* **(lane 8)**, goitre DNA cut with *EcoRI* and *PstI* **(lane 9)** and pBR322 cut with either *BglI* or *TaqI* as size markers **(lane 10)**. **(B)** Restriction enzyme sites in normal bovine genomic DNA (N) and goitre bovine genomic DNA (G). Exons are represented by the solid boxes (from left to right, exons 9, 10 and 11 of the thyroglobulin gene) and introns by the open boxes. T = *TaqI*, P = *PstI*, Pv = *PvuII*, Hc = *HincII*, R = *RsaI*, E = *EcoRI* and H = *HindIII*. Asterisks indicate the positions where the goitre DNA differs from the control.

the 250 000 dalton Tg peptide, while a frame shift would exist in the normal-sized message leading to the synthesis of a truncated 75 000 dalton peptide. Direct sequencing of the goitre Tg gene in the exon 9/intron 9 region will be required to substantiate this hypothesis.

In addition to providing a rationale to explain the molecular basis of hereditary goitre in Afrikander cattle, these results represent the first known molecular biological data accounting for an hereditary goitre. Because the thyroglobulin gene is ~250 000 bp long (Van Ommen *et al.*, 1984), the localisation of the molecular lesion to a defined region of this extremely large gene makes cloning and sequencing of the relevant part feasible.

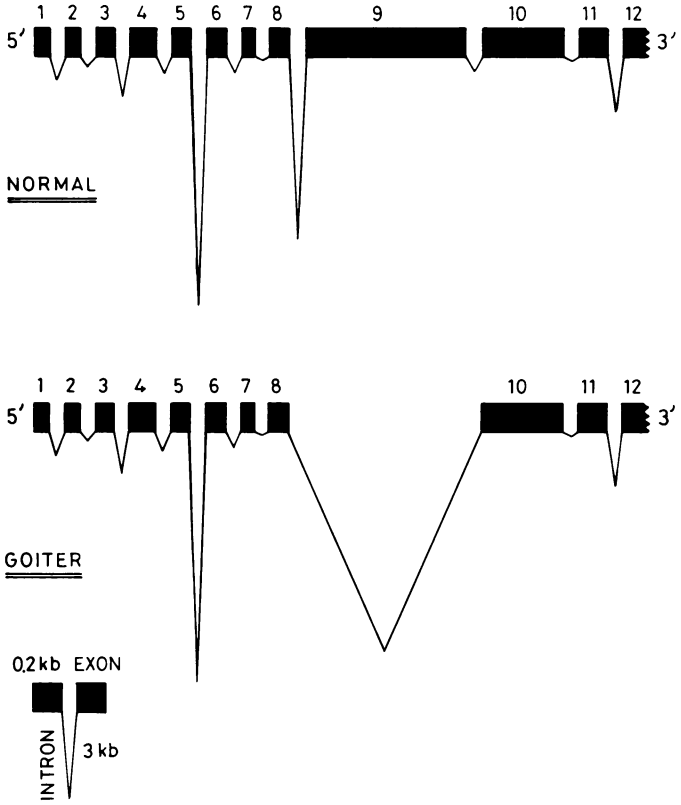

Fig. 5. Schematic representation of the splicing defect leading to the production of a 7.1-kb Tg mRNA in the goitre. The general outlay of the 5' region of the gene is taken from de Martynoff *et al.* (1983).

Materials and methods

Thyroid material

Goitre tissue was obtained from freshly slaughtered cattle inbred for this hereditary defect at the agricultural farm of the University of Stellenbosch, Mariendahl. Normal bovine thyroid glands were obtained from freshly slaughtered cattle at a nearby abbatoir. All material was transported to the laboratory on ice, frozen in liquid nitrogen and stored at −80°C.

RNA isolation

Total RNA was isolated from normal and goitre thyroid tissue by guanidinium chloride extraction (Strohman *et al.*, 1977). Poly(A)$^+$ RNA was prepared from total RNA by affinity chormatography on oligo(dT)-cellulose (Aviv and Leder, 1972). For electron microscopy the poly(A)$^+$RNA was enriched for thyroglobulin mRNA sequences by sucrose gradient centrifugation and a second passage on oligo(dT)-cellulose.

Northern transfer analysis of RNA

RNA was treated with glyoxal as described by McMaster and Carmichael (1977). Electrophoresis (in 3 mm thick agarose gels), transfer to nitrocellulose and hybridization (with [^{32}P]dCTP-labelled pbTg 2.5) was according to Thomas (1980), except that the final stringency washes were in 0.5 x SSC at 68°C.

S1 nuclease protection experiments

S1 nuclease protection experiments were performed according to Favaloro *et al.* (1980). The chimeric plasmid DNA was denatured together with 25 μg RNA (20 μg total RNA from normal thyroid or 20 μg poly(A)$^+$ RNA from goitre with 5 μg rabbit reticulocyte polysomal RNA, or 25 μg rabbit reticulocyte polysomal RNA alone) in 10 μl at 85°C for 15 min. The incubations were transferred to and maintained at 61°C for 3 h. The DNA fragments protected from S1 nuclease digestion by RNA were analyzed by electrophoresis in 1.4% alkaline agarose gels, transfer to nitrocellulose and hybridization with the original recombinant plasmid (labelled with ^{32}P).

Blot-hybridization analysis of DNA

Genomic DNA was isolated from normal thyroid and goitre tissue, digested with

the relevant restriction enzymes according to the suppliers specifications and analyzed by transfer to nitrocellulose and hybridization as described (Southern, 1975).

Electron microscopy

2.5 μl of genomic CBT1 clone (i.e., the 5′ region of the bovine Tg gene, 100 μg/ml in 10 mM Tris, 1 mM EDTA, pH 8.5) were diluted in hybridization buffer [70% formamide in 0.1 M Pipes (piperazin-N,N′-bis-2-ethanolsulfonic acid), 23 mM Tris-HCl (pH 7.8), 5 mM Na_2 EDTA, 0.5 M NaCl and 200 mM KCl]. 10 μl of the mixture was heated at 67°C for 10 min, added to lyophilized poly(A)$^+$RNA enriched for Tg mRNA sequences (200 ng), and incubated for 24 h at temperatures ranging from 58°C to 60°C. Deionized glyoxal (Aldrich, 3.3% final concentration) and sodium phosphate buffer (0.01 M, pH 8.0) were added to the hybridization mixture for 30 min at 30°C in order to allow RNA spreading. Spreading and contrast enhancement for electron microcopy were carried out as described elsewhere (Davis *et al.*, 1971). The hybrids were observed under a JEOL JEM 100 B (accelerating voltage: 60 kV) electron microscope. Length measurements were established with a Hipad TM digitizer (Bausch and Lomb) connected with a Panasonic JD-850 M computer. Double-stranded and single-stranded φX174 phage DNA (5.39 kb) were used as length standards.

Recombinant DNA probes

Construction of the cloned bovine thyroglobulin cDNA probes used has been described (Christophe *et al.*, 1982). The 148-bp *EcoRI/Eco*RI fragment of pbTg 2.8 (formerly named pbTg 2.6) was subcloned into the *Eco*RI site of pBR322. The isolation of the bovine genomic clones from a cosmid library has been described (de Martynoff *et al.*, 1983 and in preparation). Radioactive labelling of recombinant cDNA probes was by nick-translation (Rigby *et al.*, 1977).

Acknowledgements

We thank Professors A.van Zyl and J.E.Dumont for their support and interest, Mr. D.Carsenti for assistance with the DNA isolations and Mr. H.Bequet for the development of the computer program allowing the easy measurements of hybrids with a digitizer. This work was supported by the South African Medical Research Council, an NIH grant (21732) to G.Vassart and by a grant 'Action Concertée' of the 'Ministère de la Politique Scientifique' to J.E.Dumont.

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Received on 10 December 1984; revised on 15 January 1985