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Abnormal cellular localization of thyroglobulin mRNA associated with hereditary congenital goiter and thyroglobulin deficiency

(complementary DNA/polysomes/gene expression/DNA·RNA hybridizations)

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ABSTRACT **The goiters in a breed of hypothyroid goats contain only minute amounts of thyroglobulin-related antigens (0.01% of normal value). We have analyzed these goiters for the presence of mRNA coding for thyroglobulin. Using DNA complementary to beef 33S thyroglobulin mRNA as a probe, we found that the mRNA sequence is present in the goat goiter but at a concentration $1/10$–$1/40$ that of normal goat thyroid. Hybrids of cDNA with either goiter or normal thyroid RNA exhibited identical sharp melting curves which suggests that the same RNA sequence is responsible for hybridization in both tissues. Normal goat thyroid contains a population of large membrane-bound polysomes engaged in thyroglobulin synthesis. In contrast, such polysomes are absent in the goiter. In regard to subcellular distribution, the relative amount of thyroglobulin mRNA sequences from the goiter in nuclear RNA was 42% of normal, in cytoplasmic RNA was 7% of normal, and in the membrane fraction was only 1–2% of normal.**

Our results suggest that the lack of thyroglobulin in these goiters is due to a defect in thyroglobulin mRNA which leads to aberrant processing and/or transport of it from its site of synthesis to the endoplasmic reticulum.

Thyroglobulin (TG) is the major protein constituent of the thyroid gland. This large iodoprotein (molecular weight, 660,000; sedimentation coefficient, 19 S) is both the precursor and the storage form of thyroid hormones. A class of congenital goiters has been described in man in which profound hypothyroidism is associated with an almost complete absence of TG (1, 2). As a model system we have studied an inbred strain of goats with a similar disorder (3). Thyroid tissue from these goats is devoid of 19S TG. However, we could detect 7–15 μg of TG related antigens per g of tissue, compared to 100 mg/g in normal thyroids.§ It is unclear whether this extremely low level of antigen is derived from TG or from abnormal iodoproteins. The aim of the present study was to investigate the molecular nature of the defect by direct measurement of TG mRNA sequences (4, 5) in RNA from goiter tissue. A labeled DNA probe (cDNA) complementary to beef TG mRNA was used; this probe has previously been shown to hybridize well to RNA from normal goat thyroid (6). We found that the congenital goiter does contain TG mRNA sequences. However, the sequences have an abnormal intracellular distribution and their concentration is $1/10$–$1/40$ that in normal goat thyroid.

MATERIALS AND METHODS

Thyroid Tissue. Thyroids from normal goats were obtained from the slaughterhouse and transported on ice to the laboratory. The thyroids were cleaned, quickly frozen in liquid nitrogen, and stored at −90°. The normal weight of a goat thyroid is 1–3 g. The two goiters used in this study were from a breed of hypothyroid goats with congenital goiter (3). The goiters were handled in the same manner as normal thyroid tissue. The weights of the goiters were 60 and 120 g. The DNA content as measured by the diphenylamine method was 1.8 mg/g in normal thyroid tissue and 5.1 mg/g in goiter tissue. Histological analysis showed that the goiter was microfollicular and hyperplastic with slitlike, almost empty, colloidal lumina surrounded by hypertrophic cells.§

RNA Isolations. Frozen thyroid tissue was pulverized in dry ice and homogenized, in a lysis mixture containing 2% sodium dodecyl sulfate, 1% sodium triisopropylnaphthalenesulfonate (Eastman), and 3% 2-butanol, with a Potter homogenizer. The homogenate was extracted four times with phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol). The phenol was redistilled and contained 0.1% (wt/wt) 8-hydroxyquinoline and 140 ml of *m*-cresol per kg. After ether extraction the aqueous phase was incubated in proteinase K (Merck) (100 μg/ml) and 0.5% sodium dodecyl sulfate for 30 min at 37°. After precipitation with 0.1 volume of 2 M sodium acetate (pH 5.6) and 2 volumes of ethanol at −20°, the pellet was dissolved and incubated in DNase I (Worthington DPFF, RNase-free) (20 μg/ml)/5 mM $MgCl_2$ for 20 min at 37° and subsequently treated again with proteinase K and phenol extracted. The aqueous phase was chromatographed on Sephadex G-100 (Pharmacia), and the excluded volume fractions were collected and precipitated with ethanol.

Polyacrylamide gel electrophoresis (2.5% acrylamide/0.13% methylene bisacrylamide/89 mM Tris/89 mM boric acid/2.5 mM EDTA, pH 8.3) showed intact 18S and 28S ribosomal RNA. The recovery of RNA was usually 0.7–1.0 mg/g of normal thyroid and 1.1–1.4 mg/g of goiter tissue.

Subcellular Fractionation. Pulverized frozen tissue was gently homogenized in 250 mM sucrose/50 mM Tris, pH 7.5/25 mM NaCl/5.25 mM $MgCl_2$/5 mM dithiothreitol/0.25 mM EDTA/polyvinylsulfate (Eastman), 50 μg/ml. Fractionation was performed to obtain a nuclear pellet (800 × *g*, 10 min, Sorvall SS34 rotor), a membrane pellet (27,000 × *g*, 10 min, Sorvall SS34 rotor), and a cytoplasmic fraction (27,000 × *g* supernatant). Heparin (100 units/ml) was added to the postnuclear supernatant. Nuclei were further purified by suspending the 800 × *g* pellet in the homogenization buffer containing 0.5% Nonidet P40, adding this suspension to 10 volumes

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Abbreviations: TG, thyroglobulin R_0t, product of RNA concentration in mol of nucleotides per liter and hybridization time in sec; $R_0t_{1/2}$, R_0t value at half-maximal hybridization.

§ deVijlder, J. J. M., Van Voorthuizen, W. F., Van Dijk, J. E., Rijnberk, A. & Tegelaers, W. H. H. (1978) *Endocrinology*, in press.

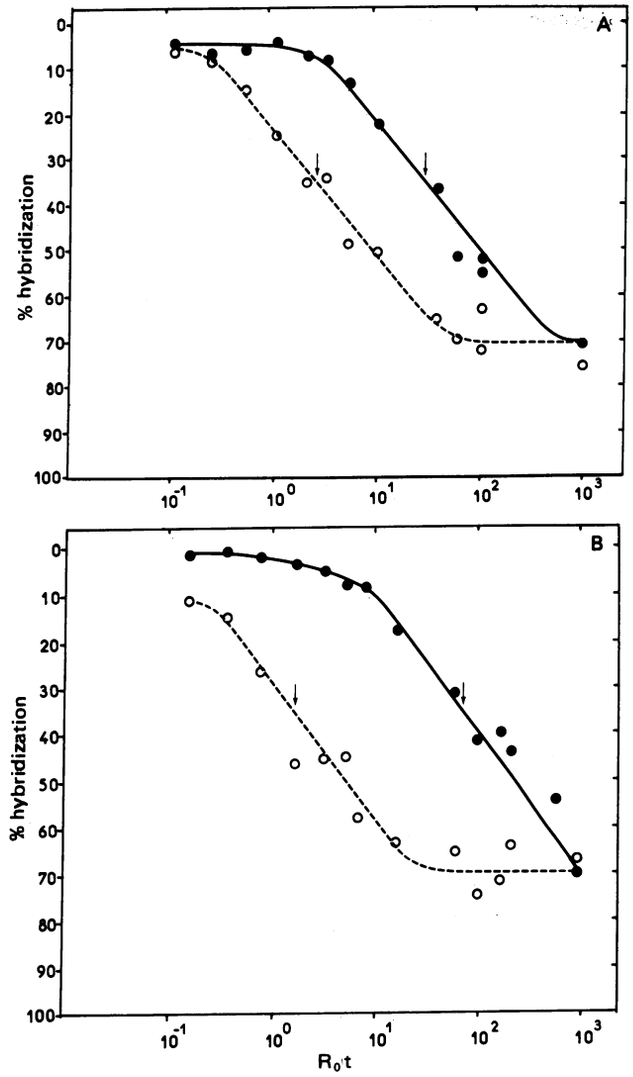

FIG. 1. Hybridization of cDNA with RNA from normal thyroid (○) and from goiters (●). The percentage of cDNA hybridized was assayed with S_1 nuclease. The $R_0t_{1/2}$ values are indicated by arrows. (*A*) Normal thyroid, $R_0t_{1/2}$ = 2.5; goiter, weight 120 g, $R_0t_{1/2}$ = 30. (*B*) Normal thyroid, $R_0t_{1/2}$ = 1.7; goiter, weight 60 g, $R_0t_{1/2}$ = 65.

of 2.4 M sucrose/3.3 mM calcium acetate, and centrifugating at 40,000 × *g* for 1 hr (7). The recovery of nuclei as measured by the diphenylamine reaction was 15–25%. Light microscopic examination showed virtually no cytoplasmic contamination.

The nuclei and the membrane pellets were suspended in 10 mM Tris, pH 7.5/100 mM NaCl/1 mM EDTA/heparin, 100 units/ml/polyvinylsulfate, 50 μg/ml. RNA was isolated from the three fractions as described for RNA isolation, with a 2-fold concentrated lysis mix.

RNA determinations were performed on nuclear preparations after phenol extraction. Colorimetric determinations by the orcinol reaction have been corrected for crossreaction with DNA.

Preparation and Analysis of Polysomes. Membrane-bound polysomes and free polysomes were prepared and analyzed as described (8), by the modified procedure enabling the use of frozen tissue as starting material (9).

cDNA·RNA Hybridizations. The preparation of beef TG cDNA used in this work has been described (6). cDNA·RNA hybridizations in RNA excess were performed in sealed glass

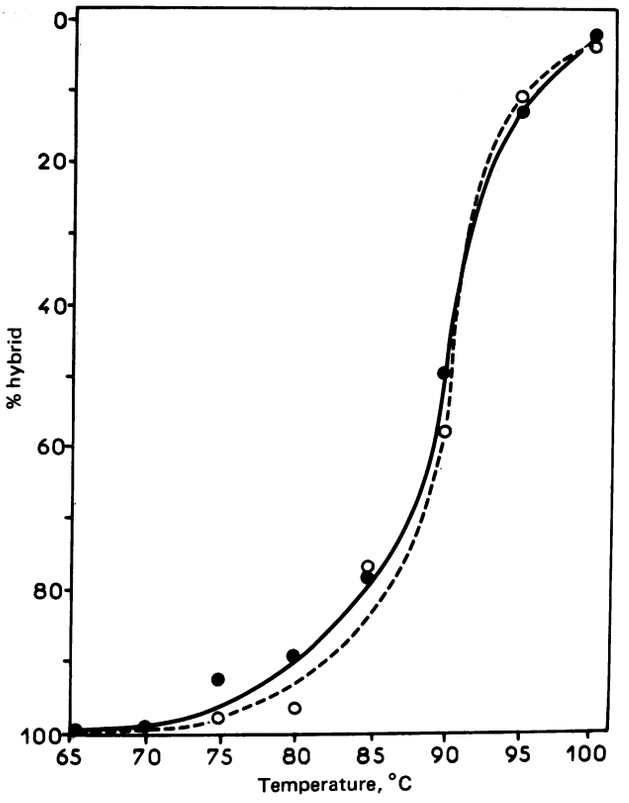

FIG. 2. Melting curves of cDNA·RNA hybrids. Hybrids of cDNA with RNA from normal thyroid (○) and with RNA from goiter (●) were formed at R_0t = 1000. Duplicate samples in hybridization buffer containing 0.3 M NaCl were incubated for 10 min at the chosen temperature and assayed with S_1 nuclease. The percentage hybridization at 65° has been called 100% hybrid.

capillaries in 50 mM Tris, pH 7.4/0.3 M NaCl/1 mM EDTA/0.1% sodium dodecyl sulfate/yeast RNA, 0.1 mg/ml, at 65°; the assay of the hybrids with S_1 nuclease and the melting curves of the hybrids were performed as described (6) except that no correction was made for S_1 nuclease resistance observed at R_0t = 0 (zero time incubation). This value varied between 0 and 4%, except when a very high RNA concentration was used.

RESULTS

The cDNA copy of beef TG mRNA has previously been shown to hybridize to a level of 70% with excess homologous TG mRNA and to the same extent with total RNA from normal goat thyroid (6). Goat liver RNA does not hybridize, even at a R_0t value of 500 (6). Hybridizations of total RNA from normal thyroids and from goiter with this cDNA in RNA excess resulted in the same final hybridization level, about 70% (Fig. 1). However, the $R_0t_{1/2}$ values in the hybridizations driven with RNA from two goiters were 10 and 40 times higher, respectively, than those in reactions involving RNA from normal tissue. This indicates that the concentration of TG mRNA sequences in the goiters is $1/10$–$1/40$ of normal. Both normal and goiter total RNA preparations were prepared in parallel. On examination by polyacrylamide gel electrophoresis they exhibited intact 28S and 18S rRNA (not shown). With polysome integrity as a sensitive index of RNase activity, no difference could be found between the tissues (see following section). It is therefore unlikely that the difference in $R_0t_{1/2}$ values is due to a differential degradation of the RNAs during isolation. Moreover, partial degradation of the RNA to sizes as small as

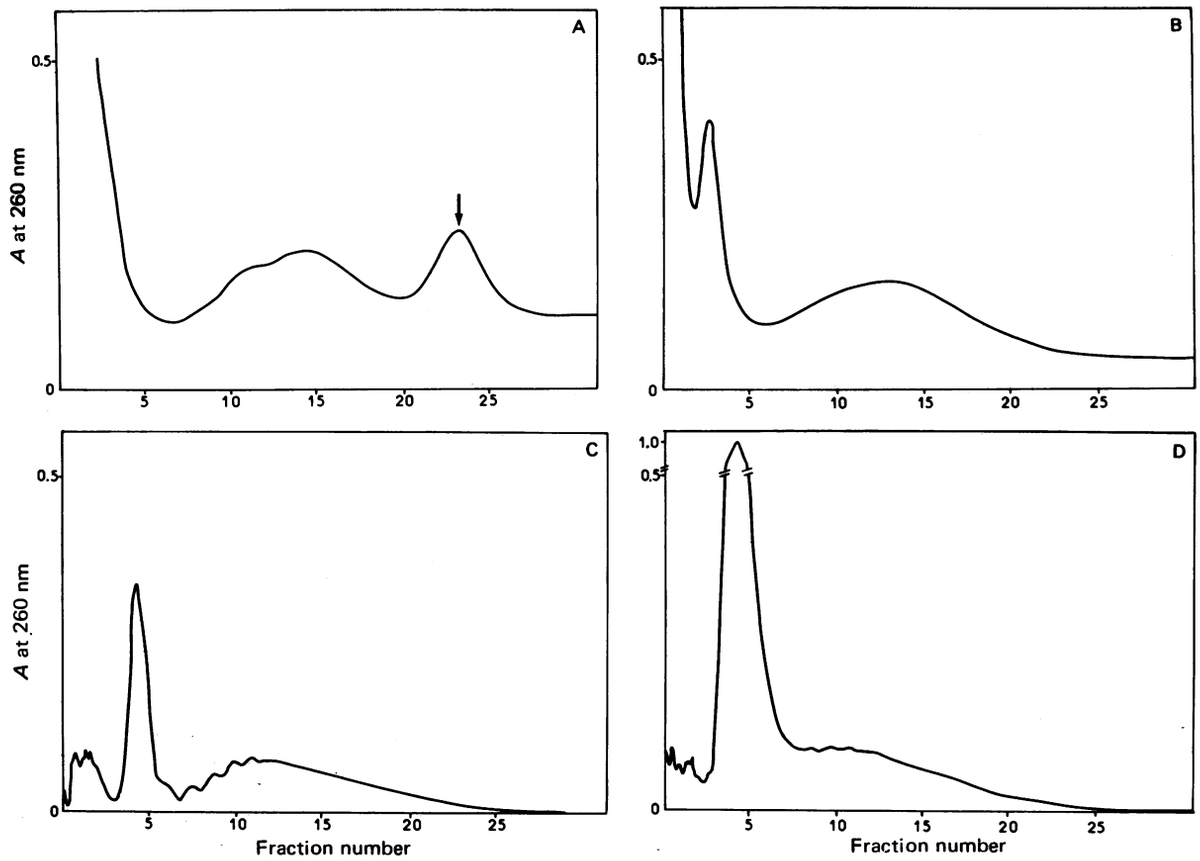

FIG. 3. Sucrose gradient analysis of membrane-bound polysomes and free polysomes in normal thyroid and in goiter. The bottom of the gradient is at the right-hand side in each panel. (*A*) Membrane-bound polysomes in normal thyroid. Arrow indicates TG-synthesizing polysomes. (*B*) Membrane-bound polysomes in goiter. (*C*) Free polysomes in normal thyroid. (*D*) Free polysomes in goiter.

that of the cDNA would not significantly interfere with hybridization (10).

The goiters contained 3 times more DNA per g of tissue than did normal thyroid. This difference is presumably due to the absence of TG and the consequent decrease of the follicular lumen in the goiter tissue. The recovery of RNA per g of tissue was about the same as for normal thyroid. Because both goiter cells and normal cells were diploid (not shown), the RNA content per cell in goitrous thyroid must be about one-third that in normal thyroid. The amount of TG mRNA sequences per cell is therefore even lower than suggested by the R_0t curves.

To test whether TG mRNA sequences from both tissues hybridize equally well to the cDNA probe, hybrids were made at high R_0t values ($R_0t = 1000$) and a melting curve analysis was performed on the hybrids (Fig. 2). Both RNAs formed stable hybrids with identical 50% melting temperatures, 90°, in 0.3 M NaCl. This value is 4° lower than that observed for the beef TG mRNA·cDNA hybrid (6). From this we conclude that the RNA sequences from goitrous and normal goat thyroids that hybridize with the cDNA probe are closely related and probably identical.

TG-Synthesizing Polysomes. Because the goitrous tissue contains a measurable amount of TG mRNA sequences, it was of interest to search for the presence of TG-synthesizing polysomes among membrane-bound polysomes. Polysome profiles from normal tissue exhibited a prominent peak (Fig. 3*A*) which has been shown to be characteristic for polysomes synthesizing TG (8). No such peak could be detected in polysomes isolated from goiter (Fig. 3*B*). To exclude the possibility that the absence of the large polysomes was due to a higher RNase activity in these goiters, portions of normal and goitrous tissue were mixed and homogenized and the mixture was processed and analyzed for polysomes. The result was that of a normal superposition of the profiles of the individual tissues with a clear peak of large TG-synthesizing polysomes (not shown). It is noteworthy that the monosome content in the goitrous tissue was much higher than that in the normal thyroid, especially in free polysomes (Fig. 3 *C* and *D*). These monosomes could represent an inactive fraction of the ribosomes normally engaged in TG synthesis. An excess of nontranslating ribosomes is indeed unusual in highly stimulated thyroid tissue (11). Free polysomes from normal thyroid and from goiter do not show large TG-synthesizing polysomes because TG is synthesized only on membrane-bound polysomes (12).

Subcellular Localization of TG mRNA Sequences. Because no TG-synthesizing polysomes were observed in the membrane-bound polysome fraction of goitrous tissue, we investigated the subcellular distribution of the TG mRNA sequences detected in total RNA. Homogenates of the goiter used in the experiments shown in Fig. 1*B* were fractionated into nuclei, cytoplasm (27,000 × *g* supernatant), and membrane (27,000 × *g* pellet) fractions, and RNA was extracted from each fraction. There was no significant difference between the recovery of normal and goiter RNAs in any of these fractions (Table 1).

To measure the concentration of TG mRNA sequences in each of these fractions, a R_0t curve analysis was carried out (Fig. 4). The calculated $R_0t_{1/2}$ values are shown in Table 1 together with the estimates of the relative concentrations and relative distribution of TG mRNA sequences in both tissues. The con-

Table 1. $R_0t_{1/2}$ values from hybridization of cDNA with RNA from subcellular fractions and consequent distribution of TG mRNA sequences

RNA fraction	RNA recovered, mg/10 g tissue		$R_0t_{1/2}$		Relative amt. of TG mRNA sequences in goiter, % of normal	Distribution of TG mRNA sequences, % of recovered sequences	
	Normal	Goiter	Normal	Goiter		Normal	Goiter
Nuclear	1.4	1.1	15	36	42	5	35
Cytoplasmic	3.8	3.4	7	100	7	25	38
Membrane-bound	1.7	1.7	1.2	70	1–2	70	27

$R_0t_{1/2}$ values were computed from the hybridization experiments shown in Fig. 4. The relative concentration of TG mRNA sequences is estimated from the quotient of two corresponding $R_0t_{1/2}$ values. The distribution is estimated from $(1/R_0t_{1/2}) \times$ amount RNA recovered.

centration of TG mRNA sequences in the nuclear RNA fraction of goiters was not much lower (42%) than in normal thyroid. However, the concentration in the cytoplasmic fraction was about 7% of the normal value and in the membrane fraction from goiter, only 1–2% of normal. The RNA/DNA ratio was 0.27 and 0.29 in the nuclear preparations of goiters and normal thyroids, respectively. This suggests that the actual amount of TG mRNA sequences in the nuclei from goiter tissue is about 40% of the normal value. In the absence of a precise knowledge of the losses involved in RNA extractions from each subcellular fraction, it is impossible to determine the exact distribution of TG mRNA sequences. However, the results in Table 1 show that there is a striking difference between normal and goiter tissue. Although 5% of the recovered TG mRNA sequences were in the nuclear fraction of normal thyroid (and this may be an overestimate due to some contamination with the membrane fraction), in the goiters 35% of the sequences measured were found in the nuclear fraction. Similar results were obtained with two independent RNA preparations from two subcellular fractionations of the 60-g goiter.

DISCUSSION

The significance of our data on TG mRNA sequences in congenital goiter in goats depends on how faithfully the hybridization of beef cDNA with goat RNA reflects the presence of goat TG mRNA sequences. The beef cDNA is only 10% of the length of complete beef TG mRNA and may only contain this proportion of the total TG mRNA sequence (6). Our experiments can therefore only refer to these sequences, which are presumably restricted to the 3′ end of the TG mRNA. Also, very small mutations, even in this region, would not be detected. Several lines of evidence, however, suggest that the probe hybridizes to goat TG mRNA both in goiter and in normal thyroid. (*i*) Excess beef TG mRNA and RNA from goat thyroid tissue converts 70% of the cDNA probe into hybrid. (*ii*) The melting curves of the goat RNA·cDNA hybrids show that stable DNA·RNA hybrids are formed with both normal thyroid RNA and goiter RNA. This would be expected if goat TG mRNA is responsible for the hybridization in both cases. (*iii*) Hybridization only occurs with thyroid-specific RNA; goat liver RNA does not hybridize to beef TG cDNA. This makes it unlikely that the heterologous hybridization is due to cross-annealing of related sequences at the 3′ end of mRNAs other than TG mRNA.

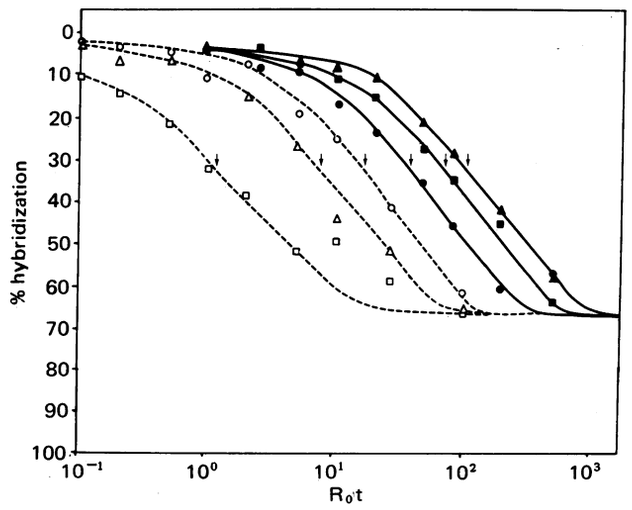

FIG. 4. Hybridization of cDNA with RNA from subcellular fractions from normal thyroid and goiter. Hybridization was with nuclear RNA (○, ●), with cytoplasmic RNA (△, ▲), and with membrane-bound RNA (□, ■). Open symbols, normal thyroid; solid symbols, goiter. The percentage of cDNA hybridized was assayed with S_1 nuclease. The $R_0t_{1/2}$ values, indicated with an arrow, are shown in Table 1.

Our results suggest that this form of congenital goiter, characterized by the virtual absence of TG in the thyroid gland, is the result of a defect in the metabolism of TG mRNA. A crucial question that remains to be answered is whether the TG mRNA sequences present in goiter tissue are capable of being translated to give TG at all. No TG-synthesizing polysomes were observed, and the concentration of TG mRNA sequences in the membrane fraction was greatly decreased. Although no 19S TG could be detected in these goiters, the possibility remains that a small amount of either normal or abnormal TG is made and immediately degraded to provide some thyroid hormone. However, if the TG mRNA from goiter could be translated, we would be confronted with the following paradox. Although the concentration of TG mRNA sequences in goiter is $1/10$–$1/40$ that in normal thyroid, the goiters were 60 and 30 times heavier, respectively, than normal thyroids. This suggests that the synthetic potential of the goiter is greater than that of the normal gland. However, the clear-cut difference in the subcellular distribution of TG mRNA sequences between normal and goiter thyroid (Table 1, Fig. 4), argues against the possibility that the goiter would be composed of a sample of normal thyroid cells surrounded by a large excess of cells completely lacking TG mRNA. For the above reasons we consider it likely that the goiter TG mRNA sequences are defective in translation. The decreased level could be a consequence of this defect.

A priori, the lower level of TG mRNA sequences in goiter could be caused by a defect in any of the following steps: (*i*) transcription of TG (pre)mRNA; (*ii*) processing of TG (pre)-mRNA in the nucleus; (*iii*) transport and/or processing of TG mRNA from nucleus to the endoplasmic reticulum membrane; and (*iv*) degradation of TG mRNA in the cytoplasm or at the endoplasmic reticulum membrane.

TG mRNA sequences have an abnormal subcellular distribution in goiter tissue, with about 35% of the recovered se-

quences confined to the nucleus compared to 5% in normal thyroid (Table 1). This observation argues against transcription as the primary defect. In turn, this suggests that the lower level of TG mRNA sequences in goiter may be due to enhanced mRNA degradation. Such abnormally fast turnover could occur in the cytoplasm although a nuclear site cannot be excluded.

The congenital goiter described in this study presents certain similarities with some forms of β^0 thalassaemia in which β-globin mRNA was shown to be present but translationally inactive (13). In that study, two Chinese patients had normal levels of inactive β-globin mRNA, and one Italian patient had decreased levels of both α- and β-globin mRNAs. Our findings present an instance of a genetic defect that results in an abnormal intracellular distribution of a mRNA. Further study of this genetic defect may provide fundamental knowledge about the metabolism of mRNA in higher organisms. Ultimately we hope that this system will allow better understanding of similar forms of congenital goiter in humans (2, 14, 15).

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