CLONING OF HUMAN THYROGLOBULIN COMPLEMENTARY DNA

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1. Introduction

The biosynthetic precursor of thyroid hormones, thyroglobulin (Tg) is a dimer of M_r 300 000 subunits translated from an 8000 base mRNA [1]. Congenital defects of Tg gene expression have been described in animals and in man leading to goiter and hypothyroidism [2]. Characterization of these defects at the molecular level will undoubtedly lead to a better understanding of the mechanisms involved in normal thyroid cell function. While such studies are underway in an animal model [3], the lack of a suitable probe has hindered a similar approach to the human cases. Here, we describe the isolation of human thyroglobulin (h Tg) mRNA and the cloning in *Escherichia coli* of cDNA fragments representing 41% of the structural gene. A preliminary account of this study has been presented [4].

2. Materials and methods

Most methods have been detailed in [5,6].

2.1. Preparation of h Tg mRNA
The mRNA was prepared from thyroid tissue obtained from a single patient undergoing surgery for Grave's disease. The mRNA was purified from a heavy polysome fraction as in [7].

2.2. Translation of mRNA in reticulocyte lysate
Messenger RNA activity was measured in the nuclease-treated reticulocyte lysate (Amersham) using tritiated leucine as precursor (Amersham, 165 Ci/mmol). Immunoprecipitations were performed as in [8] using rabbit anti-h Tg antiserum.

2.3. Synthesis of double-stranded (ds) cDNA
Double stranded cDNA was prepared in the presence of ribonuclease inhibitor from human placenta as in [5,9].

2.4. Preparation of recombinant DNA
About 100 ng of ds cDNA were restricted with *Pst*I. Following extraction with phenol/chloroform and diethyl ether, the resulting fragments were ligated to 200 ng pBR 322 plasmid DNA which had been similarly restricted with *Pst*I. Restriction and ligation reactions were performed under the conditions recommended by the manufacturer (Bethesda Research Labs).

2.5. Bacterial transformation and identification of colonies harboring recombinant plasmids
All methods have been described [5,6]. The probe used for screening by in situ hybridization was a cDNA reverse transcribed from h Tg mRNA (spec. act. ~20 × 10^6 cpm/μg).

2.6. Electron microscopy of nucleic acids
Tg mRNA (20 μg/ml) has been hybridized (3 h at 60°C) as in [10] to plasmid DNA from ph Tg_1 (2 μg/ml) which has been linearized by restriction with *Hind*III and denatured. Following treatment with glyoxal [11], the nucleic acids were spread [12] and examined under a Jeol JEM 100 B electron microscope. Single- and double-stranded ϕX174 DNA were used as length standards for single- and double-stranded molecules, respectively.

3. Results and discussion

In spite of many attempts, isolation of significant amounts of intact h Tg mRNA has been repeatedly unsuccessful in our laboratory. Fig.1 illustrates the sedimentation pattern of the best preparation which could be obtained using a method yielding large
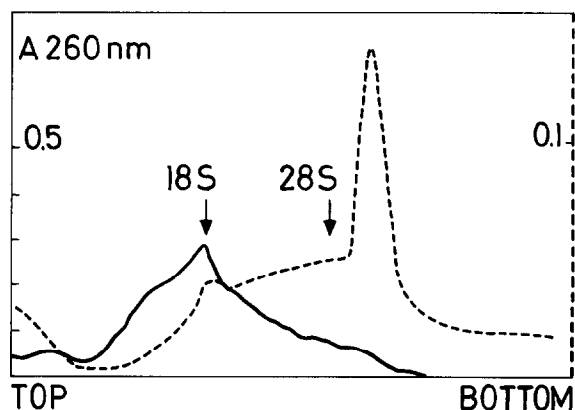
Fig.1. Sedimentation pattern of poly(A)-rich RNA from membrane-bound thyroid polysomes: (——) human sample; (— — —) bovine sample. Poly(A)-rich RNA was centrifuged through a 5–30% linear sucrose gradient and A_{260} was continuously monitored [8]. Sedimentation is represented from left to right. Arrows point to the position reached by 18 S and 28 S rRNA markers sedimented in a separate tube.

amounts of intact mRNA from beef, horse, goat and rat. Higher ribonuclease levels in the human thyroid must be responsible for the poor yield as mixing of thyroid homogenate from human and beef resulted in degradation of the bovine mRNA (not shown). RNA was collected from the region of the gradient where bovine Tg mRNA is known to sediment (—— ——). RNA (6 μg) was obtained exhibiting a definite h Tg mRNA activity when tested in the reticulocyte lysate (table 1).

Double-stranded cDNA was synthetized from 0.5 μg of this mRNA preparation. Considering the very limited amount of mRNA available, we decided to follow a straightforward strategy consisting in the cloning of PstI cDNA fragments. Indeed, the structural genes for beef, goat and rat Tg have been shown
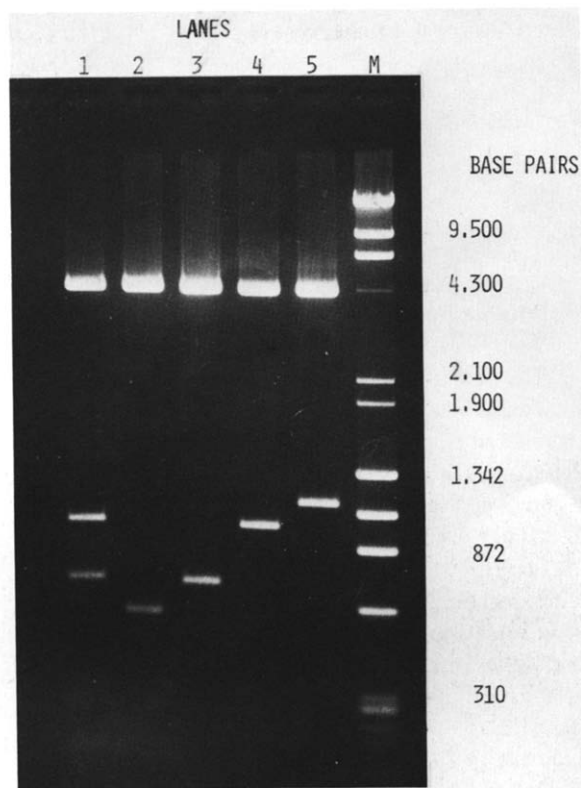

Fig.2. Restriction analysis of selected h Tg cDNA clones. Plasmid DNA (0.5 μg) from each size class (see text) was restricted with 15 units PstI and analyzed by electrophoresis through a 1.2% agarose gel in the Tris/acetate buffer system. The gel was photographed under UV illumination following staining in 1 μg ethidium bromide/ml. Size markers (M) were provided by ϕX174 DNA restricted with HaeIII, and λDNA cleaved with HindIII: (1) ph Tg$_1$; (2) 540 bp clone; (3) 680 bp clone; (4) 980 bp clone; (5) 1150 bp clone.

Table 1
Measurement of h Tg mRNA activity in the reticulocyte lysate

mRNA added to lysate	Radioactivity in trichloroacetic acid precipitate (cpm)	Radioactivity in immunoprecipitate (cpm ± range)
H$_2$O	8672	–
Hb 10 μg/ml	74 780	844 ± 182
'33 S' h Tg 25 μg/ml	48 896	13 395 ± 37

Samples of reticulocyte lysate (10 μl) were programmed with water, rabbit globin mRNA (100 ng) or 33 S poly(A)-rich RNA (250 ng) from human thyroid polysomes. Total mRNA activity and h Tg mRNA activity were measured respectively as the amount of trichloroacetic acid-precipitable radioactivity and radioactivity specifically immunoprecipitable with anti-h Tg antiserum

Table 2
Functional characterization of h Tg cDNA clones

mRNA added to lysate eluted from clone	Radioactivity in trichloroacetic acid precipitate (cpm)	Radioactivity in immunoprecipitate (cpm ± range)
PBR 322	8478	250 ± 30
540 bp	50 238	3323 ± 58
680 bp	53 280	4008 ± 753
980 bp	63 702	4026 ± 180
1150 bp	96 102	6620 ± 330

Plasmid DNA (25 μg) from each size class (see text) and wild-type pBR 322 DNA were immobilized on 1 cm^2 DBM paper according to a protocol modified from [15]. The 5 paper fragments were hybridized for 5 h at 40°C to 4.5 μg h Tg mRNA [6]. Following extensive washing, the RNA was eluted from the paper fragments and translated in the reticulocyte lysate [6]. h Tg mRNA activity was measured as in the legend to table 1
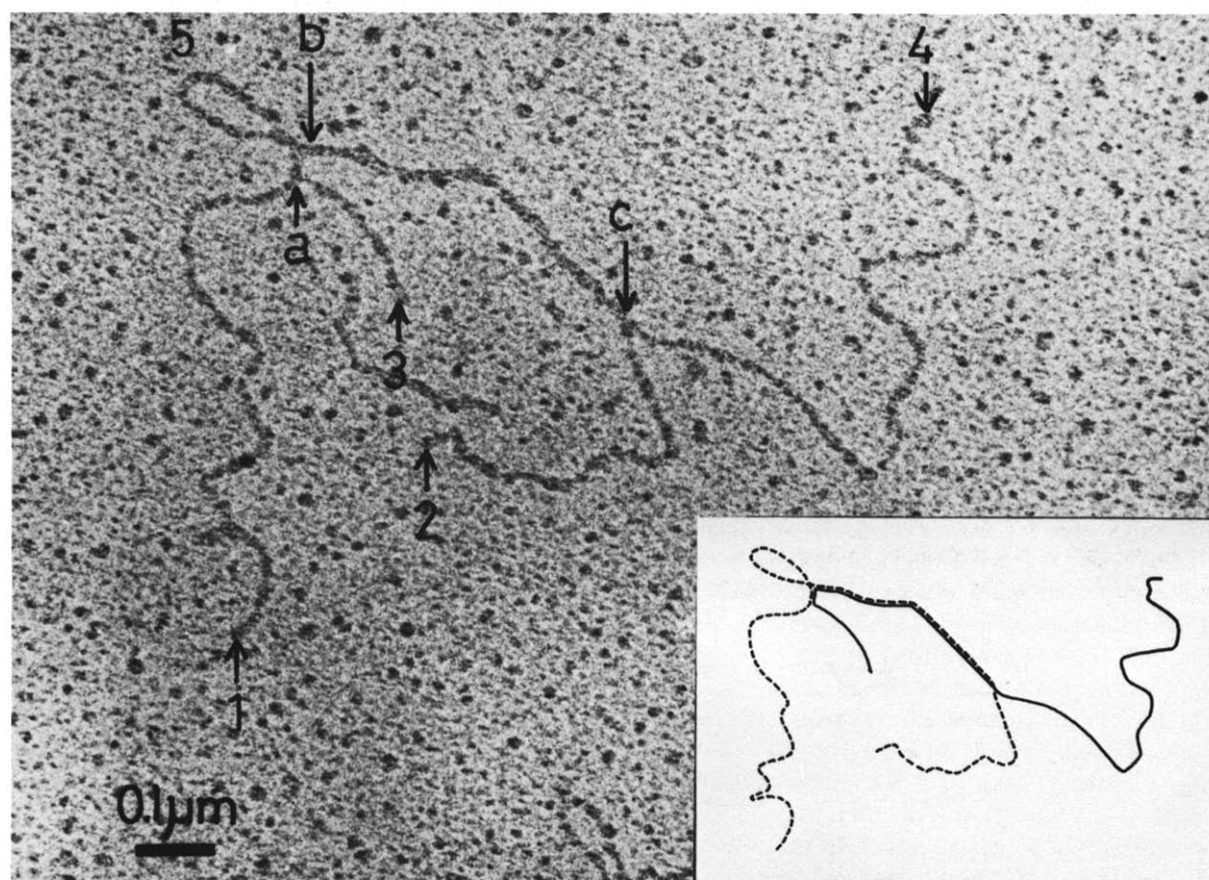

Fig.3. Hybrid between ph Tg$_1$ and h Tg mRNA. The hybrid regions appear thicker and are defined by arrows a–c: These hybrids measure respectively 159 ± 28 bp (between a and b) and 1839 ± 171 bp (between b and c). Arrows 1 and 2 define the ends of single stranded mRNA (1995 ± 29 bp at the 2 side; 3236 ± 129 bp at the 1 side). (5) a RNA loop (1400 ± 204 bp) which separates the 2 hybrid regions. Total length of mRNA corresponds to 8649 ± 561 bp. Arrows 3 and 4 define the ends of the 2 single-stranded arms of the vector pBR 322 (710 ± 95 bp at the 3 side; 3723 ± 236 bp at the 4 side): (– – –) RNA; (——) DNA.

to present an unusually large number of *Pst*I sites (16 for the 8 kilobase bovine cDNA) [14]. Therefore, *Pst*I restricted double stranded cDNA was ligated in the *Pst*I site of pBR 322 and the resulting recombinant plasmids were used to transform *E. coli* C 600 to terramycin resistance. Out of the many hundreds of colonies which were obtained (absence of phosphatase treatment of the vector explains this high background), 18 were shown to harbor a plasmid hybridizing to the h Tg cDNA. Small scale plasmid DNA preparations were obtained from each colony and restricted with *Pst*I. All plasmids except one fell into 4 classes characterized by the size of their insert: 1160, 980, 680 and 540 basepairs (bp), respectively (fig.2). One colony from each class was grown in bulk quantity and the plasmid DNA was purified. Definite identification of each plasmid as a h Tg cDNA recombinant was performed by demonstrating its ability to hybridize to functionally active h Tg mRNA in a 'hybridization, elution, translation' experiment (table 2).

One additional plasmid (ph Tg_1) was found to release 3 fragments after restriction with *Pst*I (fig.2, lane 1). Two of these fragments (980 bp and 680 bp) were shown to hybridize with the inserts of the corresponding size described here above, in Southern blotting experiments (not shown). The third fragment (170 bp) was not represented in the 4 plasmids already described. The presence of multiple *Pst*I fragments in a single plasmid could originate either from the cloning of a fragment resulting from a partial *Pst*I digestion of the ds cDNA, or from the ligation of >1 fragment in the *Pst*I site of pBR 322. To clarify this point, electron microscopic examination of hybrids between h Tg mRNA and the plasmid was performed. It clearly demonstrated that the 980 bp and 680 bp fragments were contiguous and similarly oriented while the 170 bp fragment hybridized to a region of the mRNA located 1400 bp further (fig.3).

Together, the 5 clones described hereabove cover ~41% of the structural gene for h Tg. Cross hybridizations of Southern blots prepared from each clone with selected ^{32}P-labelled cDNA probes of bovine origin [14] demonstrated that they are mainly representative of the 3'-half of the mRNA (not shown). The availability of cloned h Tg cDNA fragments will permit the easy isolation and characterization of the corresponding genomic DNA segments from existing human gene libraries and provides the tool allowing the study of congenital defect of thyroglobulin production in molecular terms.

Since this study was completed, it has come to our knowledge that a similar study has been performed resulting in the cloning of 63% of the h Tg cDNA [13].

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