IN VITRO SYNTHESIS OF 300 00 M_r RAT THYROGLOBULIN SUBUNIT

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1. Introduction

Thyroglobulin, one of the largest known glycoproteins (M_r 660 000, sedimentation coefficient 19 S), represents the major biosynthetic product of the thyroid follicular cell and plays a central role in hormone production [1,2]. Thyroglobulin offers several features of special interest:

(i) Its native structure is essential for efficient thyroid hormones synthesis [3];
(ii) It is the major protein secreted by the follicular cell;
(iii) Under denaturating conditions can be dissociated into half molecules (M_r 330 000, $s_{20,w} \sim 12$ S);
(iv) The carbohydrate content comprises ~10% of the thyroglobulin molecule [4,5].

The earlier discussion of the possible existence of smaller thyroglobulin subunits [6–8] had been a matter of controversy. In [9,10] beef thyroglobulin 33 S mRNA, isolated from membrane-bound polysomes [6,11] and injected into *Xenopus* oocytes, was shown to promote the synthesis of a 300 000 M_r polypeptide chain. Tryptic analysis of this polypeptide [10] showed close agreement with the pattern obtained from the native beef thyroglobulin. In [12] thyroglobulin mRNA, purified by immunoprecipitation of sheep thyroid polysomes, directed the in vitro synthesis of a 300 000 M_r polypeptide identified, as thyroglobulin, only by immunoprecipitation.

Here we have used both the wheat germ and the rabbit reticulocyte cell-free systems to identify the products specified by total rat thyroid RNA. The 300 000 M_r polypeptide specified by this RNA has been identified as thyroglobulin on the basis of the following criteria:

(i) Immunoprecipitation by purified, monospecific anti-thyroglobulin antibody;
(ii) Identity of the chymotryptic cleavage products with that derived from authentic thyroglobulin.

Comigration with authentic, in vivo labeled rat thyroglobulin during electrophoresis in SDS–polyacrylamide gels, is achieved only after in vitro glycosylation by segregation into microsomal vesicles (submitted).

This methodological approach represents a very simple and fast assay for thyroglobulin mRNA. Finally, a procedure to obtain total rat thyroid RNA, undegraded and in high yield, is also presented.

Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone

2. Materials and methods

2.1. *Preparation of 19 S thyroglobulin*

Rat 19 S native and in vivo [^{35}S]methionine (1300 Ci/mmol, 1.2 μM) labeled thyroglobulin were obtained according to [13,14].

2.2. *Preparation of rat thyroglobulin mRNA*

Thyroid glands were excised from male Wistar rats (Charles River, Calco/Como) immediately frozen in liquid nitrogen and then stored at –80°C. Total RNA was extracted from the glands using the guanidine–HCl technique in [15] modified as follows: after homogenization of the tissue in 20 vol. guanidine–HCl 8 M, sodium acetate buffer (20 mM, pH 5.0), DTT (1.0 mM) at –10°C (Polytron homogenizer, 2 min, full speed), 2 vol. of chloroformisoamylalcohol (24:1) were added and homogenization continued for

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an additional 2 min (full speed). RNA was then precipitated from the aqueous phase at −20°C for 40 min after addition of 0.5 vol. ice-cold ethanol. The sedimented RNA was resuspended in 1/10th of the original volume of 8 M guanidine–HCl, 20 mM EDTA pH 7.5, DTT (1 mM), made 50 mM in sodium acetate buffer (pH 5.0) and precipitated again with ethanol at −20°C as above. After centrifugation, the pellet was dissolved in 20 mM EDTA (pH 7.5) and extracted with an equal volume of chloroform–butanol (4:1). The aqueous layer was then made 3 M in sodium acetate buffer (pH 6.0) and the RNA allowed to precipitate at −20°C for a minimum of 6 h. The recovered pellet was dissolved in sodium acetate (150 mM, pH 6.0) and precipitated with 2.5 vol. of ethanol at −20°C (6 h). The pellet was twice washed with 75% cold ethanol and the RNA preparations stored at final conc. 1–5 mg/ml in water at −80°C. All centrifugations were carried out in a swinging buckets rotor (Beckman J-6) at 5200 rev./min for 15 min. Total rat liver RNA was isolated accordingly, whereas embryonic chick breast muscle RNA was isolated as in [16].

2.3. *Cell-free protein synthesis*

The methods for the preparation of the wheat germ extracts and of the rabbit reticulocyte lysates were those in [17–19]. Both cell-free systems were made mRNA-dependent by digestion with staphylococcal nuclease as in [19]. For the in vitro translation systems, 12.5 μl incubation contained 4.0 μl of either extract whereas the other components were present as in [20]. Incubations were carried out at 23°C (100 min) and at 29°C (120 min) for the wheat germ and the rabbit reticulocyte lysate, respectively. Synthesized proteins were assayed by hot trichloroacetic acid-precipitable counts on 3 MM filter paper strips (Whatman).

2.4. *Analysis of the cell-free products*

Molecular weight determination: The samples from the cell-free reactions were analyzed on SDS–polyacrylamide slab gels as in [21] and fluorographed [22]. Authentic, rat 19 S thyroglobulin [13,14] and chick breast myosin [16] were used as M_r-markers. Samples were electrophoresed in polyacrylamide slab gels (7.0%).

Immunoprecipitation: The method for the preparation of monospecific, antithyroglobulin antibody was that in [23]. Immunoprecipitation of the in vitro synthesized products was performed by diluting 50 μl aliquots of the translation mixture with 20 vol. buffer A (100 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% Na-deoxycholate) containing PMSF, TPCK and TLCK (50 μg/ml each) and 30 μg/ml of bovine serum albumin. Rabbit monospecific, anti-thyroglobulin antibody (75 μg) was added and the incubation performed for 90 min at room temperature. *Staphylococcus aureus* cell suspension (10% in buffer A, 50 μl) were added and the incubation proceeded at 4°C for 60 min. *Staphylococcus* was sedimentated (3 min in an Eppendorf microfuge), washed 6 times with buffer A, and the immunoprecipitate solubilized by incubating the resuspended pellet 4 min at 100°C in 6 M urea–4% SDS. Aliquots of the immunoprecipitate were subjected to electrophoretic analysis as above.

Chymotryptic cleavage products analysis: In vitro synthesized thyroglobulin bands were excised from preparative slab gels and the protein electrophoretically eluted in a disc-gel apparatus mounted with 5 ml plastic pipettes (10 cm long) connected to dialysis bags. Both [^{35}S]methionine in vivo labeled thyroglobulin (8000 cpm/μg) and the electro-eluted [^{35}S]protein was supplemented with unlabeled, native 19 S rat thyroglobulin and digested with chymotrypsin (68 μg/mg protein) in 50 μl reaction mixture for 45 min at 37°C. The conditions for digestion and product analysis on SDS–polyacrylamide gels were essentially those in [24].

3. Results

3.1. *Translation of total rat thyroid RNA*

We have applied a few RNA extraction procedures to the rat thyroid tissue in order to obtain undegraded RNA in good yield. The integrity of the RNA preparations was assessed by both electrophoresis in agarose gels under denaturating conditions (not shown) and by translation of the RNAs in cell-free systems. The guanidine–HCl extraction procedure (section 2) yielded the most active and the least degraded RNA preparations as judged by both total incorporation and synthesis of high M_r polypeptides (fig.1, lanes 2,6). Other procedures, also reported to give good results for the extraction of high M_r RNA [25,26] yield, in the case of the thyroid tissue, a much less active RNA (fig.1, lanes 3–5). The modification we introduced in the guanidine–HCl extraction procedure [15], i.e., a chloroform extraction of the homogenate, improves

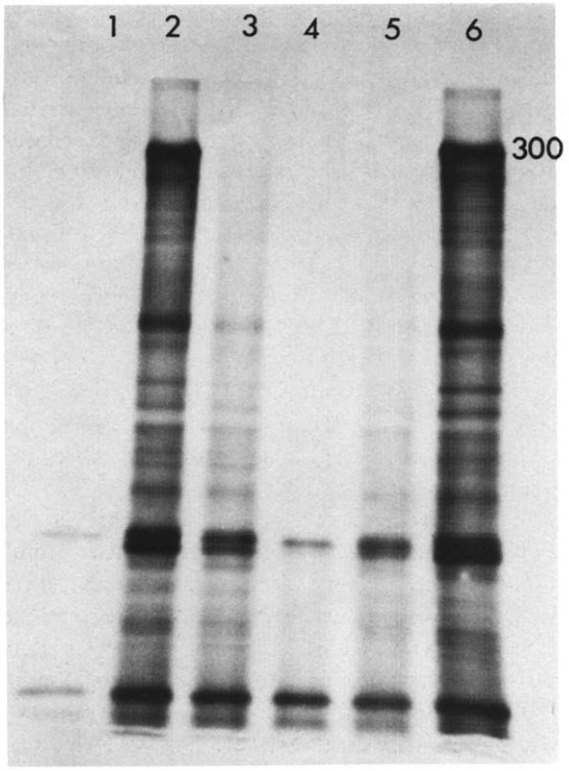

Fig.1. Translation of total rat thyroid RNA isolated employing different extraction procedures. Total RNA from rat thyroid was translated in a nuclease-treated rabbit reticulocyte lysate cell-free system with [^{35}S]methionine (30 μCi, 1300 Ci/mmol) as the labeled amino acid. Polypeptides were separated in a polyacrylamide slab gel (7%) containing SDS (0.1%). The gel was exposed for 12 h. (1) Incubation carried out without added mRNA; (2,6) synthesis in response to 2 different preparations of total rat thyroid RNA obtained by the guanidine–HCl procedure (section 2); (3,5) total rat thyroid RNA extracted by the proteinase K/phenol procedure [25]; (4) by phenol extraction [26]. The same amount (28 μg/100 μl incubation) of total rat thyroid RNA was used in all incubations.

the RNA yield by some 10-fold (up to 2.5 mg/g tissue) probably because of the removal, into the chloroform phase, of substances interfering with the subsequent ethanol precipitation. Furthermore, this modification has been succesfully applied for RNA extraction from other tissues rich in lipids such as brain (B. M. P., unpublished).

Two cell-free systems were used to translate total rat thyroid RNA: the wheat germ [17,18] and the rabbit reticulocyte lysate [19] both made mRNA-dependent by treatment with *Staphylococcus* nuclease [19]. SDS–polyacrylamide slab gel analysis of the translation products obtained with total rat thyroid RNA shows, in both cell-free systems, the synthesis of a very high M_r protein band (fig.2, lanes 1,3, respectively) that also represents one of the major translation products. The M_r-value of these polypeptides migrating slower than chicken myosin (fig.2, lane 2),

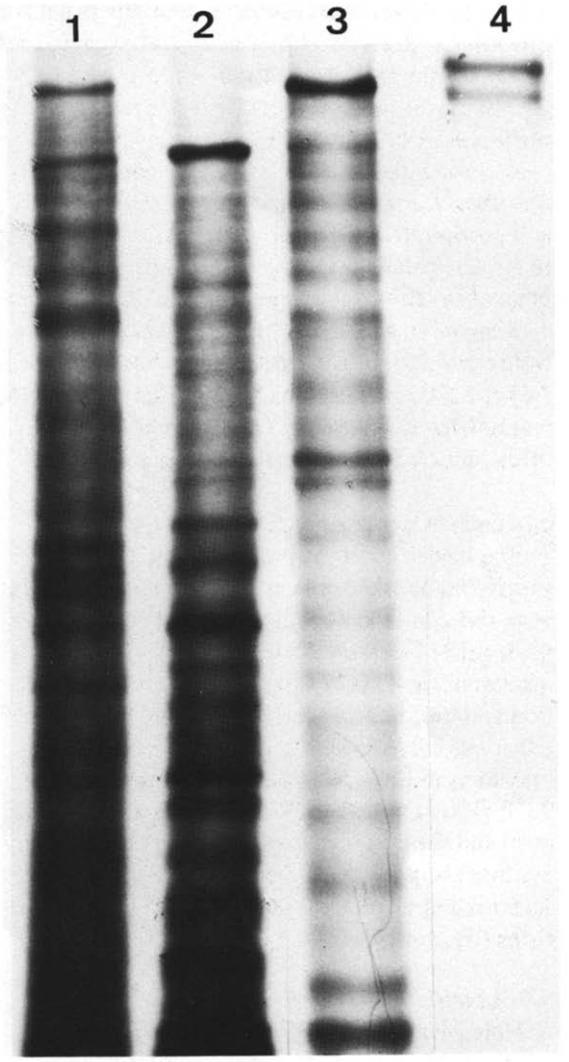

Fig.2. mRNA-dependence of the nuclease-treated wheat germ and rabbit reticulocyte lysate cell-free systems. Total rat thyroid RNA isolated as in section 2, was added to both cell-free translation systems: (1) polypeptides synthesized using the wheat germ cell-free system; (3) polypeptides synthesized using the rabbit reticulocyte lysate cell-free system. The positions of the markers are: (2) polypeptides synthesis in the wheat germ system in response to chicken skeleton muscle total RNA [16]; (4) authentic, [^{35}S]methionine-labeled 19 S rat thyroglobulin. Conditions are those in fig.1.

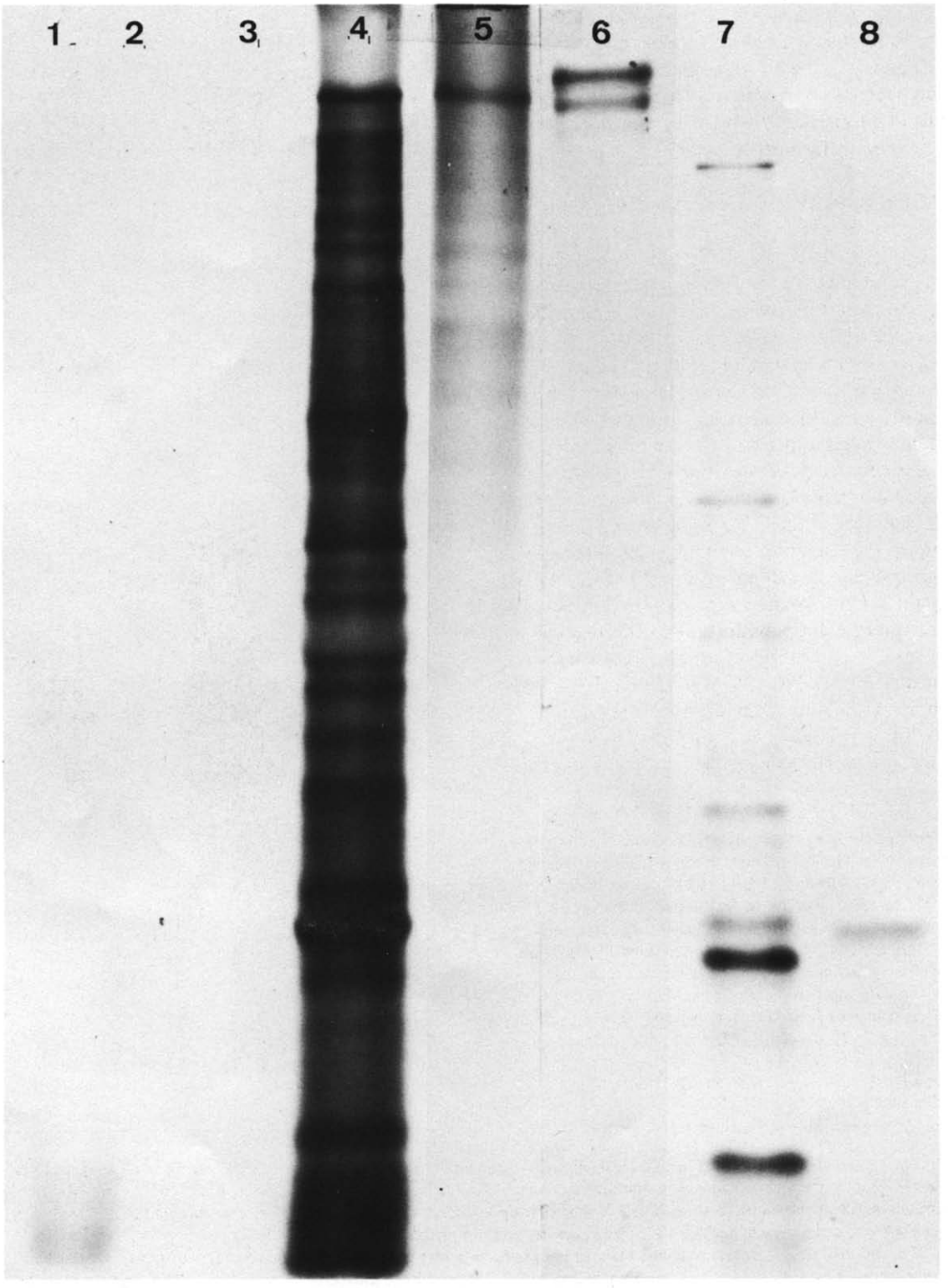

Fig.3.

but close to authentic 19 S rat thyroglobulin (fig.2, lane 4), was estimated to be ~300 000 (see section 4). The efficiency of the 2 systems in translating high M_r polypeptides was comparable though more reproducible in the reticulocyte lysate system which was the one selected for further studies.

3.2. *Identification and characterization of thyroglobulin synthesized in vitro*

The products synthesized in response to total rat thyroid RNA in the rabbit reticulocyte lysate were challenged against monospecific, anti-thyroglobulin antibody [23]. As shown in fig.3 (lanes 4,5), only the highest M_r band is immunoprecipitated in high yield. Unrelated antigens did not show any cross-reactivity with purified anti-thyroglobulin antibody (lanes 7,8). The immunological specificity of the reaction was further established by competition with an excess cold thyroglobulin in the immunoprecipitation reaction: in this case none of the in vitro synthesized proteins is found in the immunoprecipitate (lane 3). Monodimensional peptide mapping as in [24] of the largest polypeptide synthesized in vitro provided conclusive evidence on its identity with authentic thyroglobulin. In fact, under the cleavage conditions employed, we obtained an identical pattern of proteolytic fragments with both the in vitro synthesized thyroglobulin (~300 000 M_r) eluted from the gel and the native, [^{35}S]methionine labeled thyroglobulin (fig.4, lanes B,C).

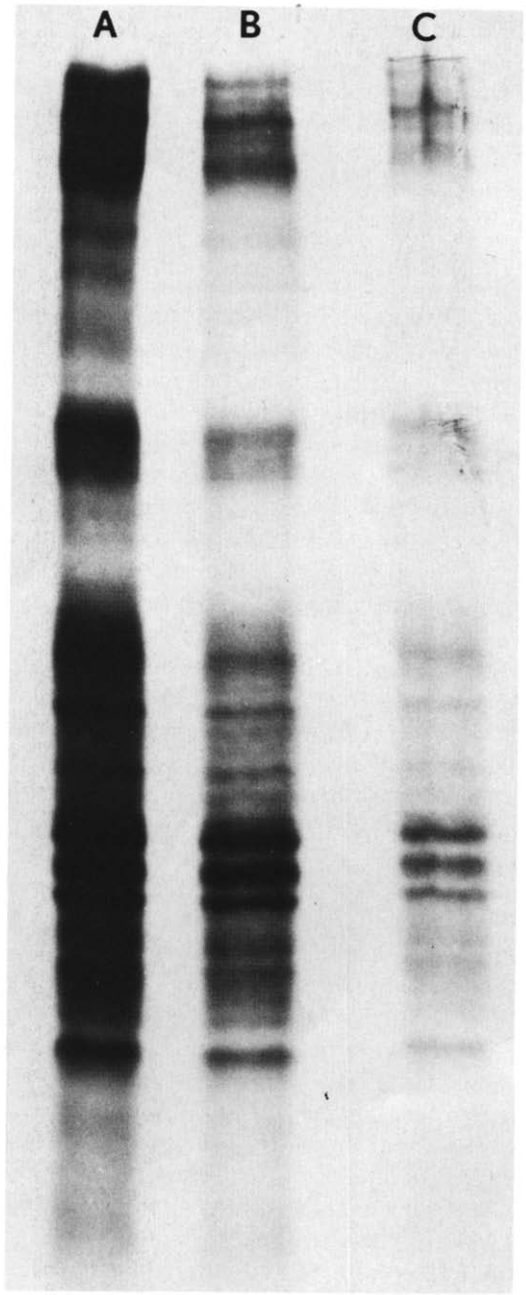

Fig.4. Peptide mapping of thyroglobulin synthesized in vitro. The figure shows the fluorograph of the peptide mapping of authentic, [^{35}S]methionine labeled 19 S rat thyroglobulin and of the cell-free synthesized 300 000 M_r polypeptide (section 2) on a polyacrylamide slab gel (15%) containing SDS (0.1%), exposed to X-ray film for 25 days. (A,B) Chymotryptic cleavage products using two different concentrations (2.5 μg and 1.25 μg, respectively) of authentic, [^{35}S]thyroglobulin supplemented with cold, native thyroglobulin (35 μg, final conc.), as in section 2. (C) Chymotryptic digest of the cell-free synthesized 300 000 M_r polypeptide also supplemented with the same amount of cold rat thyroglobulin.

Fig.3. Immunoprecipitation of thyroglobulin synthesized in vitro. Total rat thyroid RNA was added to the nuclease-treated rabbit reticulocyte lysate supplemented with [^{35}S]methionine. After 120 min incubation, immunoprecipitation with monospecific, anti-thyroglobulin antibody was done as in section 2. The immunoprecipitate was solubilized and electrophoresed in a 7% polyacrylamide slab gel in the presence of SDS (0.1%). The figure of the fluorograph shows: (1) ^{35}S-labeled polypeptides obtained in absence of added mRNA; (2) immunoprecipitation of the products shown in (1); (4) ^{35}S-labeled polypeptides synthesized in presence of total rat thyroid RNA; (3,5) immunoprecipitation of the products shown in (4) in presence (lane 3) or in absence (lane 5) of an excess of cold thyroglobulin as in section 3; (6) authentic, [^{35}S]thyroglobulin labeled in vivo [13,14]; (7) polypeptides synthesized from total rat liver RNA; (8) immunoprecipitation of the products shown in (7).

4. Discussion

In vitro synthesis of thyroglobulin from different sources has already been investigated in several laboratories. Preparations enriched for thyroglobulin mRNA have been obtained either by isolation from membrane-bound polysomes [6,11] or by immunoprecipitation of polysomes with anti-thyroglobulin antibody [12]. Because of the availability in our laboratory of several rat thyroid cell lines with different thyroglobulin synthesizing capability [27] we attempted to extract from rat thyroid biologically active RNA in high yield adopting a faster procedure. However, 2 major problems had to be overcome:

(i) The yield, considering the size of the rat thyroid gland;

(ii) The extreme sensitivity of the expected large (2.5×10^6) [28] thyroglobulin mRNA to both chemical and enzymatic degradations.

The guanidine–HCl procedure, as modified by us, fulfills both needs as judged from the RNA yield (~2.5 mg/g tissue) and, from the in vitro results obtained employing 2 heterologous cell-free systems. Immunoprecipitation of the peptides synthesized in vitro by total rat thyroid RNA shows that only the largest (~300 000 M_r) polypeptide is immunologically related to native, 12 S thyroglobulin subunit. The amount of minor immunoprecipitable peptides seems to be inversely related to the activity of the cell-free extracts and/or to the presence, in trace amounts, of RNA fragments (not shown).

The identity of the in vitro synthesized major peptide was further confirmed by comparison of the proteolytic fragments generated from authentic, 19 S rat thyroglobulin and the 300 000 M_r polypeptide extracted from the gel. The identity of the 2 chymotryptic patterns suggests, very strongly, that authentic rat thyroglobulin (660 000 M_r) is constituted of two 330 000 M_r subunits. The slightly different migration pattern observed on SDS–polyacrylamide gel between the in vitro and the in vivo synthesized thyroglobulins (cf. fig.3, lanes 4–6) is due to the absence of glycosylation activity into the cell-free extracts we used. In fact, in vitro glycosylation by cotranslational segregation into microsomal vesicles (submitted) of the synthetic polypeptide, shows comigration with the glycosylated (>300 000 M_r) polypeptide made by rat thyroid epithelial cells cultured in vitro [27].

Beside the information that our experimental approach can generate on the structure of rat thyroglobulin, it represents an extremely simple assay for thyroglobulin mRNA. The cell-free systems we adopted are much more simple, as compared to the *Xenopus* oocytes injection technique. Furthermore, the combination of the low background of the cell-free systems, the large size of the thyroglobulin and the apparent high efficiency of its synthesis under these conditions, allows the detection of newly synthesized thyroglobulin by simple visual inspection of the autoradiograms. We found no peculiar requirements for efficient in vitro thyroglobulin synthesis besides a reliable RNA preparation which is reproducibly obtained using this modification of the guanidine–HCl procedure [15].

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