IDENTIFICATION OF RNA SPECIES WITH MESSENGER ACTIVITY IN THE THYROID GLAND

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1. Introduction

Thyroid hormones are both formed and stored within the thyroglobulin (Tg) molecule, a glycoprotein of 660 000 daltons with a sedimentation coefficient of 19 S. The size and the number of subunits in this protein is still debated [1–5]. Recent data point to values as high as 300 000 daltons [6], set over against earlier reports of subunits as small as 25 000 daltons [7]. The controversy about the size of Tg-subunits has extended to the size of mRNA species thought to be involved in Tg-synthesis. The values for RNAs ranging between 16 S and 33 S [8–10] are in contrast with a recent report [11] proposing that the size of the precursor of Tg as manufactured by endogenous mRNA in a cell-free system, would be of 15 000 daltons. In this communication, we like to report data showing that in addition to the afore-mentioned values, RNA species of 45 S could be involved in the synthesis of thyroglobulin.

2. Methods

Bovine thyroid glands were homogenized in medium A (Tris–HCl 0.05 M, pH 7.4, KCl 25 mM, $MgCl_2$ 5 mM, DDT 1 mM) supplemented with rat liver RNAase inhibitor (2 U/ml) (Searle, High Wycombe, GB). The homogenate was first centrifuged at 1000 × *g* for 10 min and the supernatant further spun at 27 000 × *g* for 10 min. The pellet was washed with medium A and suspended in Tris 0.1 M, pH 9, containing 2 U/ml RNAase inhibitor, 100 U/ml heparin, 0.5% Na-dodecylsulfate (final concentration). The RNA was extracted according to the procedure described by Brawerman [12].

The RNA was precipitated by addition of 2 vol. ethanol and was allowed to stand at –20°C overnight. The RNA was collected by centrifugation, washed with ethanol, and dissolved in sterile H_2O. This polysomal RNA was further separated either by cellulose chromatography using Sigmacell 38 (Sigma) following the procedure of Schutz et al. [13] and/or gradient centrifugation in sucrose without or with formamide [14]. Details for the latter methods appear in the legend of the figures.

Cell-free protein synthesis assays were performed in the Krebs II ascites cell-lysate (S30) system adapted from Mathews and Korner [15]. Incubation was at 37°C for 60 min. The reaction was stopped by the addition of 1 ml ice-cold trichloroacetic acid. When the products were to be analyzed the reaction was stopped by the addition of bovine pancreatic ribonuclease (100 μg/ml) and EDTA (10 mM). The immunoprecipitation was performed according to a method derived from Schimke et al. [16]. Synthesized products (50 μl) were diluted with 75 μl Na-phosphate buffer (10 mM, pH 7.5) solution containing Triton 1%, deoxycholate 1% and 0.5 M NaCl. Bovine anti-thyroglobulin antiserum raised in rabbits was added and the reaction allowed to occur for 2 h at room temperature, and 15 h at 4°C. The precipitate was collected by centrifugation and washed with the same solution.

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3. Results

As shown on fig.1, 95% of the total RNA applied to the cellulose columns, was eluted with the application buffer (peak I). When the buffer was replaced by H_2O a small but distinctive peak appeared (peak II). An aliquot of each fraction was tested in the Krebs ascites cell-lysate. Stimulation of the protein synthesis activity was mainly located in peak II, which contains poly(A)-rich RNA. In order to have precision about the size of the RNA molecules with mRNA activity,

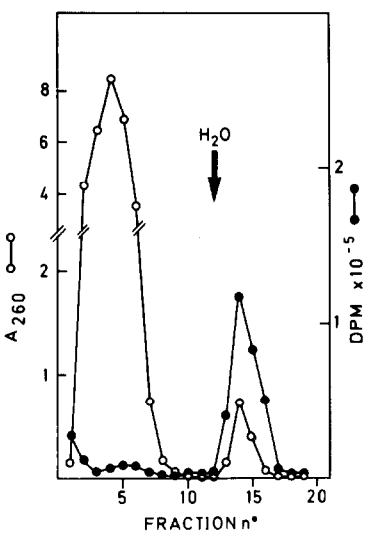

Fig.1. Cellulose column chromatography of bovine thyroid RNA. Thyroid RNA dissolved in 10 mM Tris–HCl, pH 7.5, 500 mM KCl and 0.2 mM $MgCl_2$ was layered on a column of cellulose (Sigmacell 38, Sigma) equilibrated and washed with the same buffer. Elution with water is indicated by the arrow. Fractions of 2.5 ml were collected. Aliquots (25 μl) of each fraction were tested for stimulation of protein synthesis in a reaction mixture (125 μl) with the following composition: Tris–HCl 10 mM, pH 7.5, ATP 1 mM, GTP 0.1 mM, CTP 0.6 mM, KCl 80 mM, Mg-acetate 4 mM, dithio-threitol 2 mM, creatine phosphate 10 mM, creatine phosphokinase 0.1 mg/ml, tRNA (rabbit liver) 120 μg, 40 μM of each amino acid except leucine, which was added either as [^{3}H]- or [^{14}C]leucine. The incubation was for 1 h at 37°C, and the reaction stopped by addition of 1 ml 10% trichloroacetic acid (TCA) containing 20 mM leucine. The precipitate was washed twice with 10% trichloroacetic acid and heated at 90°C for 15 min in 5% trichloroacetic acid. Absorbance at 260 min is depicted with open circles (o–o), radioactivity with black dots (•–•).

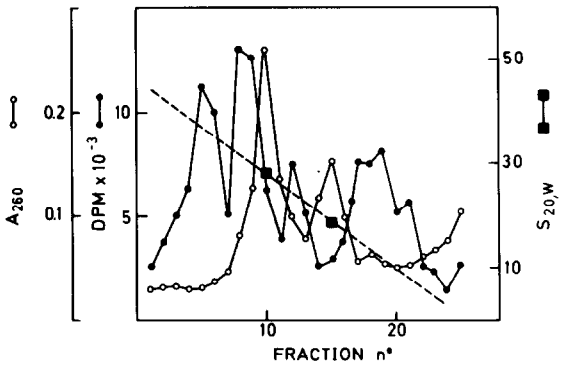

Fig.2. Sucrose density-gradient ultracentrifugation of bovine thyroid RNA. Thyroid RNA was layered on a 7.5–30% sucrose gradient made up in 10 mM Tris–HCl, pH 7.6. Centrifugation was at 42 000 rev/min for 240 min at 4°C in the Spinco SW50 rotor. After centrifugation an aliquot of each fraction was tested for stimulation of protein synthesis in the Krebs ascites cell-lysate (see legend fig.1). The two main absorbance peaks corresponding to 28 S and 18 S ribosomal RNA, were used as internal markers for determining the S-values.

total RNA was separated on sucrose density-gradient. An aliquot of each fraction was tested in the Krebs ascites cell-lysate and the stimulation of protein synthesis assessed (fig.2). The poly(A)-rich RNA fraction, as isolated from a cellulose column, was

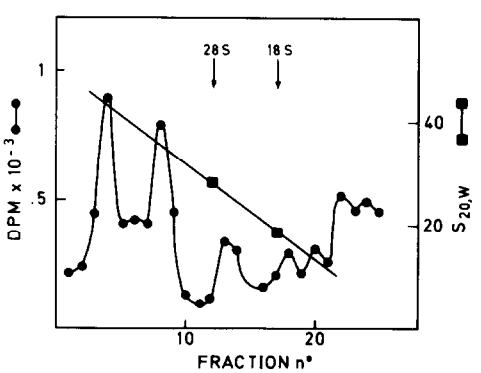

Fig.3. Sucrose density-gradient of poly(A)-rich thyroid RNA. Bovine thyroid RNA was fractionated on a cellulose column. Peak II was recovered and spun on a sucrose-gradient (see fig.2). An aliquot of each fraction was tested for stimulation of protein synthesis in the Krebs ascites cell-lysate (see fig.1). Markers refer to the position of 28 S and 18 S in a control tube in the same run.

separated by sucrose-gradient centrifugation. Stimulation of protein synthesis occured in the presense of RNA with values of 43 S, 34 S and about 24 S (fig.3). To exclude the possibility that the species thus identified were RNA aggregates, the samples were also centrifuged on sucrose-gradient, made up in 50% formamide. In this case also, stimulation of protein synthesis was observed with RNA fractions from the 4 areas of the gradient corresponding to approximately 40 S, 30 S, 24 S and 15 S.

Immunoprecipitation with antiserum against bovine thyroglobulin showed that specific immunoreactive material was present amongst the products synthesized in the incubation media containing RNA fractions of these different sizes (fig.4).

4. Discussion

The data presented indicate that two or possibly more RNA species could be involved in thyroglobulin synthesis. The presence of large sized RNA species is unaffected by formamide treatment excluding therefore the eventuality that the larger RNA molecules are aggregates of smaller ones. The size

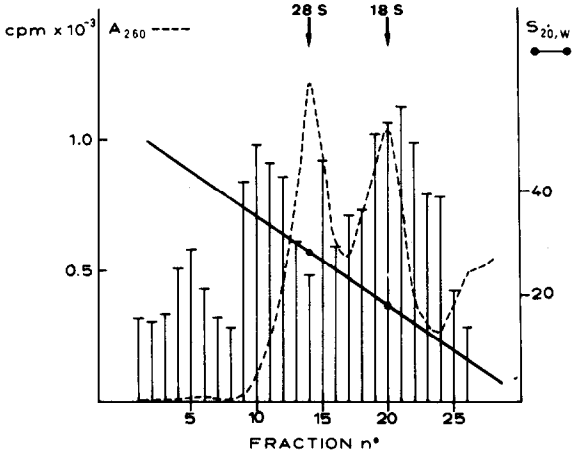

Fig.4. Immunoprecipitation with rabbit anti-bovine thyroglobulin antiserum of peptides made in cell-free system in the presence of different sized RNA. Bovine thyroid RNA was fractionated by sucrose-gradient ultracentrifugation (see fig.2). An aliquot of each fraction was incubated in the cell-free system and immunoprecipitation performed on the synthesized products. The vertical bars correspond to the radioactivity detected in the precipitate. A_{260} (– – – –).

of the largest RNA (45 S) is in the range of values corresponding to nuclear mRNA precursors [17]. Contamination with such material seems unlikely. Indeed we verified the presence of the 45 S mRNA in the RNA extracted from polysomes prepared according to Palacios et al. [18].

Earlier data from Seed and Goldberg indicate that after in vitro short-time labeling of thyroid slices with [^{3}H]uridine, the label was predominantly incorporated in 44 S RNA [19].

An alternate interpretation of the identification of several RNAs could be that smaller RNAs are due to degradation of larger ones. The constant finding of different sized RNA species with stimulating activity indicates that the 5′-end is maintained. Evidence that the 3′-end is preserved is based on the method itself and may be derived from hybridisation experiments showing poly(A) streches in 33 S, 16 S and 13 S thyroid RNA [9].

It is difficult to assign a role for these different RNAs in Tg-synthesis. Although the immunoprecipitation data shows that these RNAs are able to direct the synthesis of proteins which are related to Tg, the determination of the size of these polypeptides is hampered by the fact that the systems available for translation – with the possible exception of the oocytes – are not likely to achieve the complete reading of the largest RNAs. An indirect estimate however of the size of the peptides can be obtained on the basis of the M_r of the RNAs as estimated by gel electrophoresis. Using this method in preliminary experiments, values of 2.9×10^6, 2.1×10^6 and 0.9×10^6 were obtained indicating that the RNAs could have a coding ability for peptides with a M_r in the range of respectively 3×10^5, 2×10^5 and 1×10^5. The M_r of these polypeptides is compatible with the values reported by Haeberli et al. [6] for guinea pig Tg, and also agree with the values reported for translation of thyroid mRNA in oocytes [8,10,20]. The coding ability for the largest size mRNA (45 S) would be close to that of fibroin mRNA (47 S), which has be shown to code for a protein of 375 000 daltons [21].

Acknowledgement

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