MOLECULAR WEIGHT OF THE THYROGLOBULIN MESSENGER RNA OF SHEEP THYROID GLAND

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SUMMARY. Poly(A)+ mRNA from sheep thyroid total or thyroglobulin-specific polysomes obtained by immunological precipitation, was purified by two cycles of chromatography on oligo(dT)-cellulose. Upon electrophoresis in 98 % formamide-polyacrylamide gels, the purified RNA showed a major species of M_r 2.8×10^6. The correlation found between the very high concentration of this species and its thyroglobulin messenger activity in the reticulocyte lysate protein synthesis system demonstrates that the thyroglobulin mRNA contains enough bases to code for the thyroglobulin peptide chain (M_r 300 000).

Thyroglobulin (19 S, M_r 660 000), the major protein component of the thyroid gland, is a dimer of half-sized subunits each formed of likely identical single peptide chains (M_r 330 000) containing about 10 % carbohydrates (1-2). By size analysis of poly(A)+ or polysomal RNA on sucrose gradient, thyroglobulin messenger activity was assigned to a 33 S RNA (3), to 40 S, 30 S, 24 S and 15 S species (4) and in our laboratory to a 33-36 S RNA species (5). The latter was translated into a major component of M_r 300 000 and minor species of smaller size in the reticulocyte cell-free system whereas the poly(A)+ 33 S RNA was translated in Xenopus oocytes into a single species of about 300 000 M_r (3). The synthesis of lighter thyroglobulin peptides promoted by the 33-36 S RNA can be explained by : (a) nicking of some messenger molecules with intact 5'-terminal ends; (b) inadequate composition of the reticulocyte lysate to translate such a large RNA; (c) presence of a mixture of aggregated material in the 33-36 S RNA (6-7).

To test these possibilities and to determine the size of thyroglobulin mRNA, poly(A)+ thyroglobulin mRNA has been purified from immunologically selected thyroid polysomes by oligo(dT)-cellulose chromatography and translated in the reticulocyte lysate into specific thyroglobulin peptide chains. That this mRNA contained enough genetic information to code for a peptide of 300 000 was established by mol.wt. determination in polyacrylamide gel electrophoresis in the presence of formamide.

MATERIALS AND METHODS

Total polysomes were isolated from sheep thyroids as described (5) with one modification. Polysomes were pelleted through 0.5 M instead of 1 M sucrose cushion. This increased the yield of total polysomes and the content of thyroglobulin-specific polysomes which amounted 45 % of total polysomes instead of

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30 %. Antibody amount for indirect immunoprecipitation of thyroglobulin polysomes was increased to take into account the modification of thyroglobulin polysome content. RNA was extracted by SDS-phenol-chloroform treatment (5) from unfractionated polysomes, thyroglobulin-specific polysomes and polysomes remaining in solution after immunoprecipitation.
For messenger activity determination, rabbit reticulocyte lysate (8) was treated with Micrococcus nuclease and incubated as described (9). Assays and control (minus exogenous RNA), were incubated in 50 µl test volumes, for 2 h at 26°C, with [^{3}H]leucine (50 Ci/mmol, 5 µCi/50 µl) unless otherwise indicated and next processed as previously described (5). Total messenger activity of thyroid RNA was calculated as the difference between TCA-insoluble radioactivity in 5 µl assays and control. Direct immunoprecipitation (5, 10) was carried out to determine the thyroglobulin-specific messenger activity, after subtraction of control values. When thyroglobulin peptides were to be analyzed, [^{35}S]methionine (400-700 Ci/mmol; about 15 µCi/50 µl) was used instead of leucine. Thyroglobulin peptides immunoprecipitated from the 145 000 x g supernatant of incubation medium were analyzed in 4 % polyacrylamide gels, 0.1 % SDS and 50 mM Na phosphate, pH 7.2, after full reduction and alkylation (1).

For purification of poly(A)+ RNA, polysomal RNA, 0.5 mg/ml in binding buffer (0.6 M NaCl, 0.01 M Tris-Cl, pH 7.6, 0.001 M EDTA, and 0.2 % SDS) was applied to a jacketed column filled with oligo(dT)-cellulose (Type 7, PL Biochemicals, Milwaukee, USA), 100 mg/1 mg RNA. The bulk of RNA not retained was precipitated with ethanol and the column was extensively washed with buffer. Elution was performed at 26°C with 0.01 M Tris-Cl, pH 7.6, 0.001 M EDTA, until A_{260} of effluent fell to 0.1. At this time, temperature was raised to 50°C. RNA eluted at both temperatures were pooled and precipitated with ethanol. RNA eluted at the first passage (odT-1 RNA) was heated for 10 min at 65° C in water and submitted to a second chromatographic run (odT-2 RNA). Ethanolic precipitates of odT-1 and odT-2 RNA were extensively washed with 70 % ethanol, 30 % 0.1 M sodium acetate pH 7.0, before use in the protein synthesis cell-free system. Electrophoretic analysis of RNA was performed on polyacrylamide gels in 98 % formamide, according to Duesberg and Vogt (11). Gels (0.7 x 7 cm), were made in 2.7 % acrylamide, 0.6 % bisacrylamide in phosphate buffered formamide, pH 7.0 and allowed to polymerize at least for 16 h before use. RNA ethanolic precipitates dried under nitrogen were dissolved in formamide and heated for 20 min at 45°C before migration for 4 h at 35° C and 4 mA/gel. Gels were stained with stains-all (12). Mengo virus RNA was the kind gift of Dr. E. Falcoff and MS-2 virus RNA was obtained from Boehringer (Mannheim, Germany).

RESULTS AND DISCUSSION

Messenger RNA purification on oligo(dT)-cellulose

As suggested by Bantle et al (13), two successive retention steps on oligo(dT)-cellulose and heat denaturation of odT-1 RNA before the second passage were necessary to purify thyroid mRNA from rRNA and degraded messengers lacking the 3'-poly(A)sequence. Typical results on the fractionation of total polysomal thyroid RNA are illustrated in Table I and Fig. 1 and 2.
[^{3}H]poly(U) binding experiments (5) showed that odT-1 and odT-2 RNA contained 90 % and 70 % of the original binding capacity corresponding to a purification yield of 24- and 45-fold, respectively (Table I). RNA not retained on oligo(dT)-cellulose contained no detectable [^{3}H]poly(U) binding activity.
Total and thyroglobulin-specific messenger activities in oligo(dT)-purified (poly(A)+) fractions were fairly increased (Fig. 1A and 1B) but unretained RNA still contained a small part of the original activity. Thyroglobulin-speci-

Table I. Poly(A)+ RNA content and messenger activity recovery after oligo(dT)-cellulose chromatography of total thyroid polysomal RNA.

RNA	yield	[^{3}H]poly(U) binding		Messenger activity			
				total		thyroglobulin specific	
	%	cpm.µg^{-1}	%	cpm.µg^{-1} x10^{-3}	%	cpm.µg^{-1} x10^{-3}	%
polysomal	100	38	100	120	100	38	100
First step odT-1	3.7	924	90	1763	54	650	64
not retained	96.3	-	-	48	40	12	31
Second step odT-2	1.7	1715	73	3174	44	630	28
not retained	-	-	-	104	-	45	-

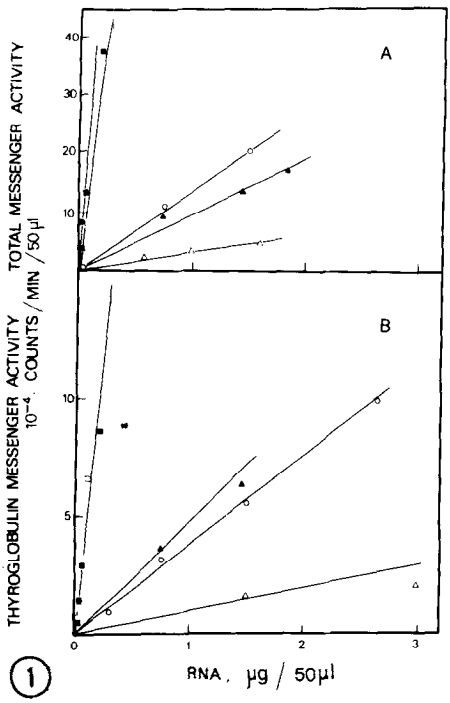
Total polysomal RNA (11 mg) was obtained from about 100 g sheep thyroid glands. At each step of poly(A)+ RNA purification, [^{3}H]poly(U) binding experiments were performed as described (5). Total and thyroglobulin-specific activities were calculated for 50 µl assays (see Methods). -, means not measurable or undetectable.

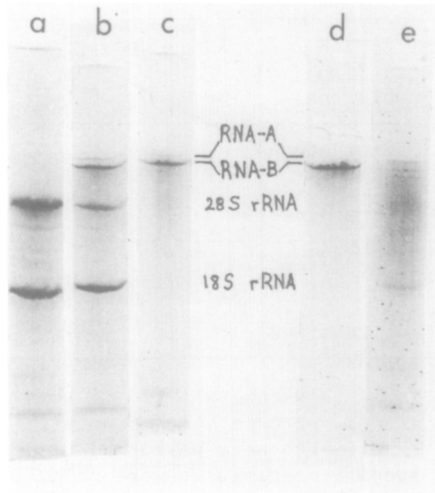
fic messenger activities of odT-1 and odT-2 were almost identical (Fig. 1B). As illustrated in Table I, the RNA not retained on oligo(dT)-cellulose after the first passage contained about 30 % of the original messenger activity which might represent either mRNA devoid of poly(A) sequences or translatable 5'-segments of degraded messengers.

Analysis of odT-2 RNA by polyacrylamide gel electrophoresis in formamide (Fig. 2c) showed two bands (RNA-A and -B) migrating more slowly than 28 S rRNA. The faster migrating band (RNA-B) was always by far the more abundant. Fig. 2 (b, c) also illustrates the efficiency of the second filtration on oligo(dT)-cellulose to eliminate residual rRNA from odT-1 RNA. Assignment of thyroglobulin mRNA activity to one of these species or both is deduced from our previous finding that 65 % or more of thyroglobulin mRNA activity of polysomal RNA is supported by a 33-36 S species in sucrose gradient (5).

Thyroglobulin mRNA characterization

Previous studies (5) demonstrated that the immunological selection of thyroglobulin-specific polysomes allowed a 3-fold purification of thyroglobulin-specific mRNA activity. In the present experiments, poly(A)+ RNA from thyroglobulin-specific polysomes were separated on oligo(dT)-cellulose and compared to RNA obtained from polysomes not precipitated after antibody treatment. As illus-





Left : Fig. 1. Total (A) and thyroglobulin-specific (B) messenger activity of thyroid RNA fractions. o, total polysomal RNA : □ , odT-1 RNA; ■ , odT-2 RNA; Δ, ▲, RNA not retained at the first and at the second filtration, respectively. Total polysomal RNA was submitted to two cycles of chromatography on oligo(dT)-cellulose as described in Methods. Measured activities correspond to 50 µl assays.

Right : Fig. 2. Polyacrylamide gel electrophoresis in the presence of formamide of several thyroid RNA fractions. Unfractionated polysomal RNA at different stages of purification on oligo(dT)-cellulose : (a), total polysomal RNA, 5 µg; (b) odT-1 RNA, 5 µg and (c), odT-2 RNA, 4 µg; thyroglobulin-specific and odT-2 RNA were obtained from immunologically precipitated polysomes. (d), odT-2 RNA (5 µg) from thyroglobulin-specific polysomes; (e), odT-2 RNA (5 µg) from supernatant polysomes.

trated in Table II, odT-2 RNA from thyroglobulin-specific polysomes exhibited 15-times more thyroglobulin mRNA activity than odT-2 RNA from supernatant polysomes. Gel electrophoresis in formamide of odT-2 RNA from thyroglobulin-specific polysomes revealed a predominent RNA-B species (Fig. 2d). odT-2 RNA from supernatant polysomes was heterogeneous and contained only a very low amount of RNA-B. The latter is likely thyroglobulin mRNA.

Thyroglobulin peptides synthesized in the reticulocyte lysate under the control of polysomal and poly(A)+ RNA were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). A 300 000 M_r peptide, migrating slightly faster than the mature

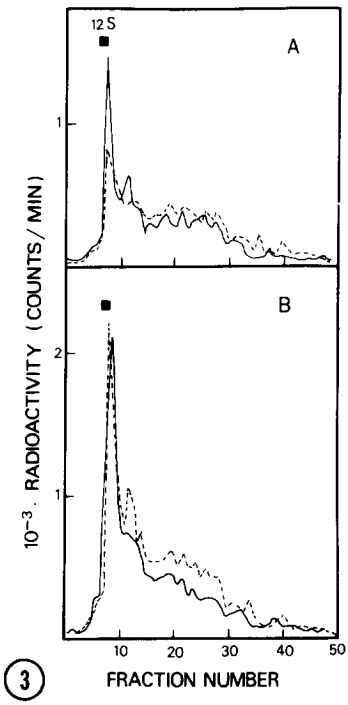
Table II. Recovery of thyroglobulin mRNA activity after immunological selection of thyroglobulin-specific polysomes.

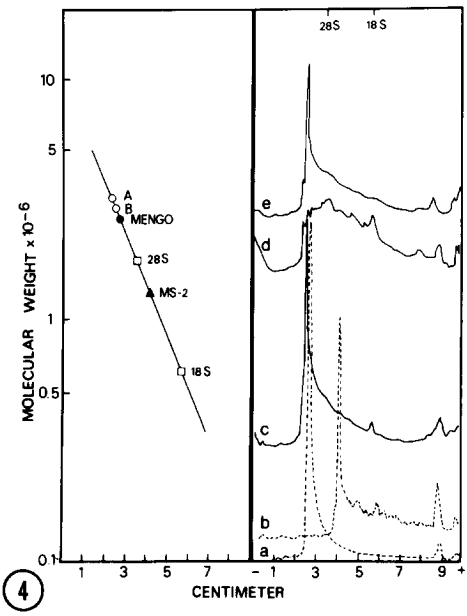
RNA	yield	Thyroglobulin messenger activity	
	%	10^{-3}.cpm.μg^{-1}	%
polysomal	100	22	100
thyroglobulin-specific	45	43	90
odT-1	1.7	574	44
odT-2	0.7	621	21
supernatant	55	4	9
odT-1	3.1	41	6
odT-2	1.8	44	4

Total thyroid polysomes were incubated with anti-thyroglobulin antibodies. One tenth of the incubation mixture was removed for immediate RNA extraction (polysomal RNA) and 9/10 were centrifuged to yield thyroglobulin-specific polysomes (pellet) and unreacted polysomes (supernatant). After extraction from fractionated polysomes, RNA underwent 2 cycles of chromatography on oligo(dT)-cellulose.

12 S thyroglobulin chain (M_r 330 000) represented about 50 % of the synthesized peptides. Faster migrating material was always present but was never associated with discrete peaks of similar importance which should be found if the studied RNA contained RNA coding for thyroglobulin chains of different sizes. The use of odT-2 RNA, theoretically devoid of translatable 5'-extremities of degraded messenger, did not eliminate the presence of lighter peptides. This strengthens the idea that premature release depending on the cell-free system composition (9) might explain the results.

It thus appears that synthesis of the large thyroglobulin peptide must be assigned to the high M_r RNA (RNA-B and possibly RNA-A). Mol.wt. estimation of this material was performed by polyacrylamide gel electrophoresis in the presence of formamide which was shown to overcome the tendency of RNA to aggregate and to minimize the influence of secondary structure on mol.wt. determinations (14). Thyroid odt-2 RNA and external markers (Mengo virus and MS-2 RNA, 28 S and 18 S thyroid rRNA) were run in 2.7 % gels (Fig. 4). Estimations of M_r for Mengo-RNA (2.7×10^6) and MS-2 RNA (1.25×10^6) relative to 28 S (1.75×10^6) and 18 S rRNA (6.05×10^5) (14) were similar to those reported in the literature (14-15). In 5 independent experiments, the average apparent





Left : Fig. 3. SDS-polyacrylamide gel electrophoresis of immunologically related ^{35}S methionine-labeled thyroglobulin peptides synthesized in mRNA-dependent reticulocyte lysate. A, odT-2 RNA (1.3 µg/ml incubation medium) from unfractionated (____) and thyroglobulin-specific (---) polysomes; B, polysomal RNA (20 µg/ml) from unfractionated (____) and thyroglobulin-specific (---) polysomes. ■ , position of migration of native thyroglobulin 12 S subunit.

Right : Fig. 4. A, polyacrylamide gel electrophoresis in the presence of formamide of : a, Mengo virus RNA (4 µg); b, MS-2 RNA (3 µg); c, odT-2 RNA from thyroglobulin-specific polysomes (5 µg); d, odT-2 RNA from supernatant polysomes (5 µg); e, odT-2 RNA from unfractionated polysomes (4 µg).
B, molecular weight determination. The following M_r for marker RNA have been used: Mengo, 2.7 x 10^6; MS-2, 1.25 x 10^6; 28 S, 1.75 x 10^6; 18 S, 6.05 x 10^6.

M_r of RNA-B and RNA-A were 2.8 x 10^6 and 3.2 x 10^6, respectively.
Since a peptide of 300 000d will be coded for by a mRNA of M_r 2.50 to 2.78 x 10^6 (according to the mean M_r, 900 or 1020, assigned to a codon triplet) mRNA-B is large enough to contain the genetic information required for the synthesis of the thyroglobulin chain together with the 3'-poly(A) segment and other possible not translated sequences. The significance of the largest mRNA-A always present in minor amount in poly(A)+ thyroglobulin mRNA preparations is unclear. Experiments are in progress to test whether A is a precursor of B, an artefact due to the association of heterogenous species (16) or a contaminant of nuclear origin.

Nota. While this manuscript was in preparation, a similar report on the mol.wt. of thyroglobulin mRNA was published by Vassart et al. (FEBS Letters, 1977, 79, 15-18).

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REFERENCES

1. Lissitzky, S., Mauchamp, J., Reynaud, J., Rolland, M., (1975) FEBS Letters, 60, 359-363.
2. Marriq, C., Rolland, M. and Lissitzky, S. (1977) Eur. J. Biochem. in press.
3. Vassart, G., Refetoff, S., Brocas, H., Dinsart, C. and Dumont, J.E., (1975) Proc. Nat. Acad. Sci. USA, 72, 3839-3843.
4. De Nayer, P., Caucheteux, D., and Luypaert, B. (1977) FEBS Letters, 76, 316-319.
5. Chebath, J., Chabaud, O., Becarevic, A., Cartouzou, G. and Lissitzky, S. (1977) Eur. J. Biochem. 77, 243-252.
6. Palmiter, R.D. (1974) Biochemistry, 13, 3606-3615.
7. Schechter, I. (1974) Biochem. Biophys. Res. Commun. 57, 857-864.
8. Schimke, R.I., Rhoads, R.E. and Mac Knight, G.S. (1974) Methods in Enzymology, 30, 694-701.
9. Pelham, H.R. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256.
10. Rhoads, R.E. (1975) J. Biol. Chem. 250, 8088-8097.
11. Duesberg, P.H. and Vogt, P.K. (1973) J. Virol. 12, 594-599.
12. Dahlberg, A.E., Dingman, C.W. and Peacock, A.C. (1969) J. Mol. Biol. 41, 139-147.
13. Bantle, J.A., Maxwell, I.H. and Hahn, W.E. (1976) Anal. Biochem. 72, 413-427.
14. Spohr, G., Mirault, M.E., Imaizumi, T. and Scherrer, K. (1976) Eur. J. Biochem. 62, 313-322.
15. Marshall, M.V. and Arlinghaus, R.B. (1975) J. Virol. 19, 743-745.
16. Boedtker, H., and Lehrach, H. (1976) Progress in Nucleic Acid Research and Molecular Biology, 19, 253-260.