TRANSLATION OF 33S mRNA, PREPARED FROM BOVINE THYROID GLANDS, INTO THYROGLOBULIN-LIKE PROTEIN IN A RETICULOCYTE LYSATE CELL-FREE SYSTEM

Andre J. Bester, Martin Pammenter [1], Wieland Gevers, Pieter van Jaarsveld [2]

MRC Research Units for Iodine Metabolism and for Molecular and Cellular Cardiology, Medical School, University of Stellenbosch, South Africa.

Received June 24, 1977

SUMMARY: 33S mRNA was isolated from bovine thyroid glands and translated in a heterologous cell-free amino acid incorporating system into a 12-19S protein judged to be thyroglobulin-like by immunoprecipitation, sodium dodecyl sulfate polyacrylamide gel electrophoresis and ion exchange chromatography.

The major protein synthesized by the thyroid is thyroglobulin (660,000 molecular weight) which is composed of 12S half-molecule subunits associated both non-covalently and by interchain disulfide bonds (1,2).

Vassart et al (3,4) have recently purified a 33S mRNA from bovine thyroid glands, which was translated into a 19S thyroglobulin-like protein in Xenopus oöcytes.

We are studying the nature of the molecular lesion which causes severely impaired thyroglobulin synthesis in a genetically determined goiter of Afrikander cattle (5). For this purpose we required a heterologous protein-synthesizing system which would allow the addition of several components of the translation machinery from normal or goitrous tissue in one incubation mixture. We used the reticulocyte lysate cell-free system to show that 33S mRNA, obtained from normal bovine thyroid glands, is translated under given experimental conditions into half-sized and fully-assembled 19S thyroglobulin-like protein products with a high degree of fidelity.

MATERIALS AND METHODS: *Extraction and Purification of mRNA.* Batches of bovine thyroid glands (200g) were homogenized gently in a Waring Blendor (about one hour after death of the animals) with 2 ml extraction buffer per g of tissue (0.25 M NH_4Cl, 0.01 M $MgCl_2$, 6 mM mercaptoethanol, 0.2 mM EDTA

(1) Ph.D. candidate, University of Stellenbosch.
(2) Reprint requests to: Department of Pharmacology, P. O. Box 63, Tygerberg 7505, South Africa.

ISSN 0006-291X

and 0.2 M Tris-HCl (pH 7.4) plus 60-100 µg heparin per ml buffer). The post-mitochondrial supernatant was treated for 10 min with 0.5% Triton X-100 and 0.5% sodium deoxycholate and subsequently centrifuged through 2.0 M sucrose in the extraction buffer (SW 27 Spinco rotor, 4 hr). The total polyribosomal pellets were suspended in 0.02 M Tris-HCl (pH 7.4), 0.1 M NaCl, 0.01 M EDTA, 1% SDS(3), and the high molecular weight RNA precipitated overnight at 4° with an equal volume of 4 M LiCl (6). The RNA was extracted from the pellet with phenol (7) and the 33S sedimenting material collected by sucrose gradient centrifugation (see legend to Fig 1). To establish the poly (A) content of the 33S material, the sucrose gradient fractions were chromatographed on oligo-(dT) cellulose (7,8).

Reticulocyte lysate cell-free system. Rabbit reticulocyte lysates were prepared as described (9). Cell-free synthesis was performed in 0.1 ml of reticulocyte lysate as described by Bester et al (10). About 15 µg of thyroid IF 3, extracted according to Heywood et al (11), and about 15 µg 33S mRNA per 0.1 ml lysate were used. After incubation, several aliquots of the reaction mixture were pooled, the polyribosomes removed by centrifugation and carrier thyroglobulin added to the supernatant, followed by dialysis against 0.1 M KCl, 0.05 M phosphate buffer (pH 7.4).

^{14}C labelled thyroglobulin was prepared by incubating 10g thyroid slices with 2 mCi ^{14}C-protein hydrolysate (New England Nuclear) according to Vassart et al (4). The labelled as well as cold 19S thyroglobulin was prepared by homoginizing every g of the unfrozen slices or glands in 2 ml 0.1 M KCl, 0.05 M phosphate buffer (pH 7.4). Purification was obtained by centrifugation in sucrose gradients (10-40% in the SW 27 Spinco Rotor at 25,000 rpm for 18 hours).

Rabbit antithyroglobulin antiserum was prepared as previously described (5). Goat anti-rabbit immunoglobulin antiserum was purchased from Miles.

SDS polyacrylamide gel electrophoresis of thyroglobulin was performed in 5% gels (12), while RNA was electrophoresed in 2.6% gels (13). Optical density profiles (550 nm for Coomassie blue bands and 260 nm for RNA) were obtained by analysing the gels in a Unicam SP 1700 spectrophotometer equipped with a scanning attachment. Gel slices were made with a Joyce-Loebl gel cutter, then oxidized in an automatic Packard oxidizer, and the radioactivity determined in a Packard Tricarb liquid scintillation counter.

Tryptic peptides were prepared and analyzed as described by Hayashi et al (14) according to the legend of Fig 5. ^{3}H and ^{14}C were counted simultaneously using the external standard and channels-ratio method to correct for the distribution of ^{14}C in the ^{3}H channel and for quenching by pyridine.

RESULTS : *Isolation and purification of mRNA:* Fig 1 shows a sucrose gradient profile of the total RNA extract. The presence of material sedimenting at approximately 33S in the "leading edge" of the 28S ribosomal RNA peak is evident. Further purification of this fraction was achieved by chromatography on oligo-(dT) cellulose. The RNA which hybridized with the oligo-(dT) cellulose was precipitated with ethanol together with 15 µg tRNA, and analyzed by SDS polyacrylamide gel electrophoresis (Fig 2). Apart from 4S tRNA, the major component migrated in the region where a 33S species was expected.

Translation, isolation and characterization of the products: Incubations were performed as described in the methods. Fig 3 shows the elution profile

(3) Abbreviation: SDS, sodium dodecyl sulfate

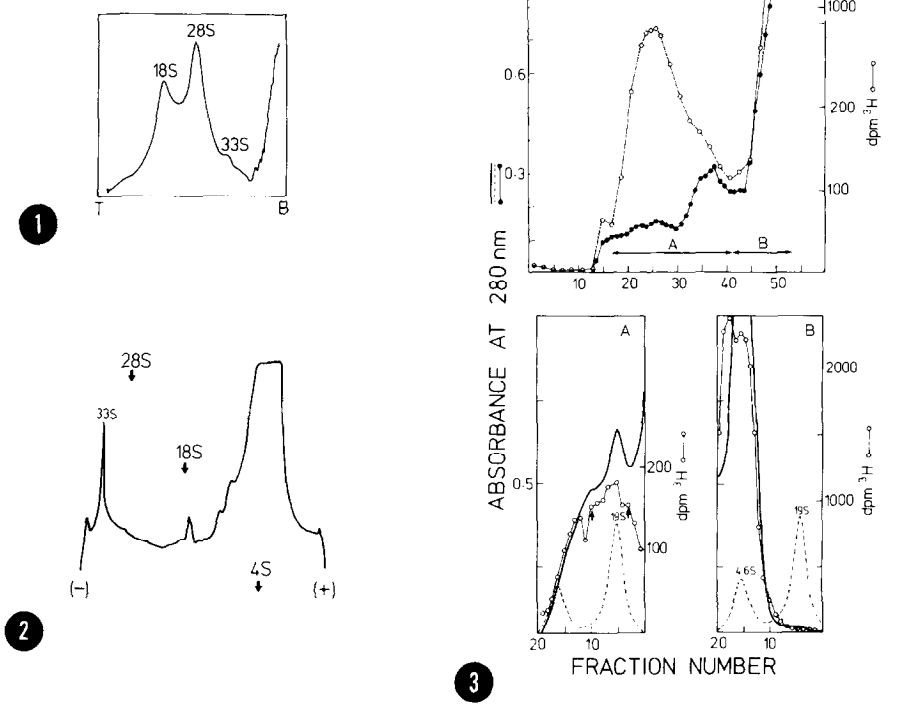

Figure 1 Sedimentation profile of total RNA extract from bovine thyroid polysomes in a 10-30% sucrose gradient (Spinco SW 65 rotor for 3 hours at 60,000 rpm). For preparative purposes the 33S material was obtained by collecting it together with the fast sedimenting limb of the 28S peak present in 35 ml 10-30% sucrose gradients (SW 27 Spinco rotor centrifuged for 13 hours at 25,000 rpm). The sucrose gradients contained 0.02 M Tris-HCl (pH 7.4), 0.1 M NaCl, 0.01 M EDTA and 1% sodium dodecyl sulfate.

Figure 2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of 33S mRNA purified by oligo-(dT) cellulose chromatography and precipitated together with tRNA (4S). The positions of marker 28S and 18S ribosomal RNA are indicated by arrows.

Figure 3 (Upper panel) Gel chromatography on Sepharose 6B of the dialyzed soluble proteins of reticulocyte lysate cell-free system incubated with 33S mRNA and initiation factor extract plus 2 mg added carrier thyroglobulin.
(Lower panel) Sedimentation profiles of fractions A and B respectively in 10-40% sucrose gradients.
The gel column was 1.6 x 80 cm while the gradients were centrifuged for 18 hours at 20° in the SW 27 Spinco rotor at 25,000 rpm (marker bovine serum albumin (4.6S) and thyroglobulin (19S) were run in separate tubes).

from Sepharose 6 B of the lysate incubated with mRNA and extract containing initiation factors. When the latter two additions were not made, no radio= activity was detected under the area marked A in Fig 3, which represented the elution position of carrier thyroglobulin.

The fractions marked A and B in Fig 3 were pooled separately, concen= trated by vacuum dialysis against 0.1 M KCl, 0.05 M phosphate buffer (pH 7.4) and analyzed by sucrose gradient centrifugation. The sedimentation profiles thus obtained, together with those of marker thyroglobulin and bovine serum albumin, run in separate tubes, are shown in Fig 3. The radioactivity in pooled fraction A, but not that in pooled fraction B, overlapped the 19S thyroglobulin peak. The separate fractions sedimenting from 12-19S in fraction A (indicated by arrows in Fig 3) were pooled and concentrated by vacuum dialysis to a final volume of about 100 µl. A slight excess of antithyroglobulin antiserum was added to one half of this and it was incubated overnight. An equivalent volume of anti-rabbit immunoglobulin antiserum was then added. The precipitate was collected by centrifugation after 16 hours and dissolved in 1% SDS, 0.05 M sodium phosphate (pH 7.4). The second half of the sucrose gradient concentrate was also dialyzed against the same buffer. Subsequently both samples were reduced with 1% mercaptoethanol with heating for 2 min in a 100° water bath, and analyzed by polyacrylamide gel electrophoresis.

Fig 4 shows the control optical density and radioactivity scan of reduced ^{14}C-labelled thyroglobulin; the radioactivity of the 12-19S sucrose gradient concentrate is also shown. The profile for the immunoprecipitate of the 12-19S fraction was identical, indicating that practically all the radioactivity was precipitated. It is further evident from Fig 4 that the mobility of the translation product coincided with that of the reduced 12S half-molecule of the control ^{14}C-labelled thyroglobulin. No radioactivity of any significance was detected in gel slices apart from the characteristic half-molecule bands of thyroglobulin.

In order to compare the tryptic peptides of ^{14}C-labelled control thyro=

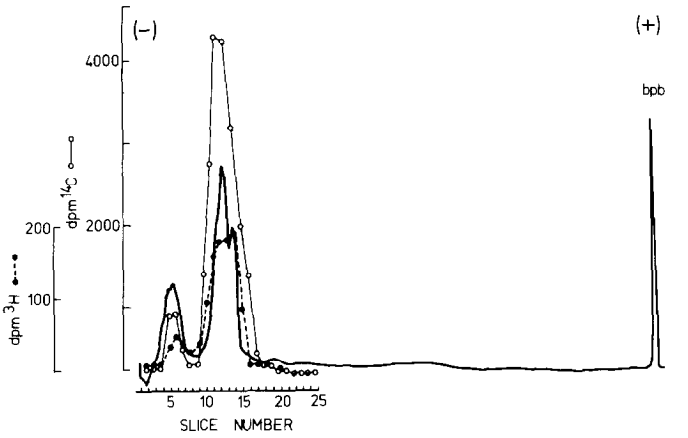

Figure 4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of reduced ^{14}C-labelled thyroglobulin and the reduced 12-19S fraction of the ^{3}H translation products of 33S mRNA (Fig 3). The solid line represents the optical density scan of the gel stained with Coomassie blue.

globulin with those given by the translation products, fractions from the Sepharose 6B column were used (pooled material A, Fig 3). About equal counts of ^{14}C and ^{3}H labelled material were mixed, trypsinized and chromatographed. Fig 5 shows that the same elution profile was obtained in both instances.

DISCUSSION: The reticulocyte lysate system requires the presence of 6 mM mercaptoethanol which obviously affects disulfide bond formation. Most of the cysteine side-chains of thyroglobulin are in the disulfide form and very few are present as free -SH groups (1). Reduction of the disulfides not only causes unfolding, but also changes the equilibrium of 19S $\rightleftharpoons$ 12S in the direction of dissociation. We added carrier thyroglobulin to the incubation mixture after translation was terminated, and subsequently dialyzed the mixture in order to allow reoxidation. It is known that the latter occurs almost quantitively under these conditions (15). Nevertheless, not all the thyroglobulin was present as homogeneously sedimenting 19S protein after dialysis. Some radioactivity was, however, present in the 19S zone which indicates that, apart from *de novo* synthesis and polymerization, limited hybridization between unlabelled and radioactive half-molecules had occurred. It is further known that

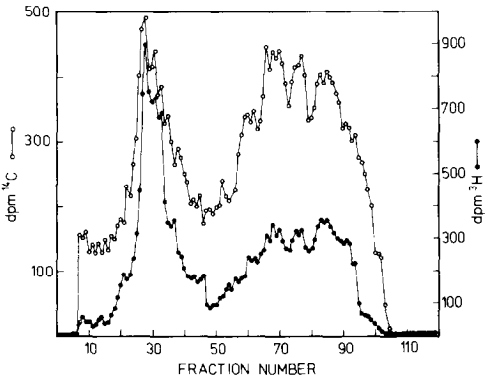

Figure 5 Tryptic peptide analysis of ^{14}C labelled thyroglobulin and ^{3}H translation product of 33S mRNA (fraction A, Fig 3). Equal counts of the ^{14}C and ^{3}H labelled proteins were mixed, reduced in 6 M guanidinium chloride with 1% mercaptoethanol followed by alkylation with iodoacetic acid, dialysis against NH_4HCO_3 (pH 8.1) and overnight trypsinization at 37°. After lyophylization the tryptic peptides were dissolved in the application buffer of a pyridine-acetate gradient and then applied and eluted linearly from pH 2.5 to pH 6.0 (14). A 0.8 x 30 cm SP Sephadex G-25 column and 400 ml gradient were used while 2 ml fractions were collected.

newly-synthesized thyroglobulin, i.e. non-iodinated protein, has a sedimentation rate about 1-2 Svedberg units less than 19S (1). By collecting the zones sedimenting at 12-19S in the sucrose gradients, we demonstrated the immunological relationship with, and similar polypeptide composition of, the translation product and authentic thyroglobulin prepared from tissue slices. Furthermore, the tryptic peptides of the translation products also co-incided with those of thyroglobulin.

We are using these techniques to study the nature of the molecular lesion in the genetically-determined Afrikander cattle goiter, where there is impaired thyroglobulin synthesis, and have isolated 33S mRNA from the goiter. This was translated into a product similar to that formed on addition of control mRNA described in this paper. This is remarkable since thyroglobulin is virtually absent in goiter extracts.(4)

ACKNOWLEDGEMENT : We are indebted to Prof. A. van Zyl, director of the Iodine Metabolism Research Unit, for his continued interest and encouragement.

(4) To be published elsewhere.

REFERENCES

1. Salvatore, G., and Edelhoch, H. (1973) In Hormonal Proteins and Peptides, C.H. Li, ed. Academic Press, New York, Vol. 1, 201 - 241.
2. Lissitzky, S., Mauchamp, J., Reynaud, J., and Rolland, M. (1975) FEBS Lett., 60, 359 - 363.
3. Vassart, G., Brocas, H., Lecocq, R., and Dumont, J.E. (1975) Eur. J. Biochem., 55, 15 - 22.
4. Vassart, G., Refetoff, S., Brocas, H., Dinsart, C., and Dumont, J.E. (1975) Proc. Nat. Acad. Sci. USA, 72, 3839 - 3843.
5. Van Jaarsveld, P., Van der Walt, B., and Theron, C. (1972) Endocrinology, 91, 470 - 482.
6. Shapiro, D.J., Taylor, J.M., McKnight, G.S., Palacois, R., Gonzalez, C., Kiely, M.L., and Schimke, R.T. (1974) J. Biol. Chem. 249, 3665 - 3671.
7. Aviv, H., and Leder, P. (1972) Proc. Nat. Acad. Sci., U.S. 69, 1408 - 1412.
8. Heywood, S.M., and Kennedy, D.S. (1976) Biochemistry, 15, 3314 - 3319.
9. Evans, M.J. and Lingrel, J.B. (1969), Biochemiestry 8, 829 - 831.
10. Bester, A.J., Kennedy, D.S., and Heywood, S.M. (1975) Proc. Nat. Acad. Sci., U.S.A. 72, 1523 - 1527.
11. Heywood, S.M., Kennedy, D.S. and Bester, A.J. (1974) Proc. Nat Acad. Sci., U.S.A. 71, 2428 - 2431.
12. Weber, K., and Osborn, M. (1969) J. Biol. Chem., 244, 4406 - 4412.
13. Loening, U.E. (1967) Biochem J. 102, 251 - 257.
14. Hayashi, T., Iwai, K., and Ui, N. (1971) Biochim. Biophys. Acta. 251, 208 - 216.
15. De Crombrugghe, B., and Edelhoch, H. (1966) Biochemistry, 5, 2238 - 2245.