Vol. 93, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1980 Pages 645-653

SYNTHESIS OF A FULL LENGTH DNA COMPLEMENTARY TO THYROGLOBULIN 33 S MESSENGER RNA

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Received January 21,1980

SUMMARY

The conditions allowing the synthesis of long DNA copies of bovine 33 S thyroglobulin mRNA were investigated using Avian Myeloblastosis Virus reverse transcriptase. Under all conditions tested, the size distribution of thyroglobulin cDNA was discontinuous as illustrated by the banding pattern observed following electrophoresis in alkaline agarose gels. Incubations at high temperature (44°C) or inclusion of sodium pyrophosphate in the reaction mixture did not lead to the synthesis of significant amount of cDNA containing more than 3000 nucleotides. However, reverse transcriptions conducted in the presence of a ribonuclease inhibitor prepared from human placenta yielded DNA molecules of about 8500 nucleotides representing most probably the full length copy of thyroglobulin mRNA.

INTRODUCTION

Thyroglobulin is the major protein synthesized by the thyroid gland. This unusually large glycoprotein (MW 660000, Sedimentation constant 19 S) (1), is composed of two equal sized subunits containing each a 300000 dalton polypeptide chain (2,3,4). Thyroid hormones being generated by the iodination of tyrosyl residues of the protein followed by the intrachain coupling of specific iodotyrosines into iodothyronines, the structure of Tg certainly plays a crucial role in the hormonogenesis (5). However, due to the exceptional size of Tg protomeric chains, very little is known about their primary structure (6). It is not even clear whether they are identical or not (7).

The recent development of DNA restriction and sequencing methodologies together with the availability of purified preparation of Tg mRNA (2, 8, 9)

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0006-291X/80/070645-09$01.00/0

opens the attractive possibility to approach the structure of the protein through that of the synthetic double stranded complementary DNA. The first step of such an approach, i.e. the synthesis of a full size Tg cDNA is made challenging by the size of Tg mRNA (33 S, 2.8 10^6 Daltons) (9,10). It was the aim of the present study to optimize the reverse transcription reaction using beef Tg mRNA as template. Our results demonstrate that inclusion of a ribonuclease inhibitor in the reaction allows the synthesis of complete Tg cDNA copy.

MATERIALS and METHODS

Preparation of Tg mRNA

Bovine Tg mRNA was prepared as described previously (9). The last step involved sedimentation through a linear sucrose gradient (5 to 30 % sucrose in 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM EDTA, 150 min. 60000 rev./min., SW65TI Rotor) followed by ethanol precipitation.

Reverse transcription of Tg mRNA

Avian Myeloblastosis virus reverse transcriptase (batch-G 1378) was kindly provided by Dr J. Beard (St Petersbourg, Florida). Unless stated otherwise, the reactions were performed in 50 µl containing 50 mM Tris-HCl pH 8.3, 52 mM KCl, 7.6 mM $MgCl_2$ 4 mM dithiothreithol, 40 µg/ml actinomycin D (PL Biochemicals), 20 µg/ml oligo-dT_{12-18} (Boehringer), 20 µg/ml Tg mRNA, 800 µM each of dATP, dGTP, dTTP, 200 µM dCTP of which 5 to 20 µM were contributed by ^{32}P dCTP (Amersham 400 Ci/mmol) and 200 U/ml reverse transcriptase. Deviation from this protocol as well as duration and temperature of the incubations are given in the legend of the figures. At the end of the incubation the reactions were stopped by the addition of 15 µl of EDTA 0.1 M pH 7.5, SDS 1 M and the samples were filtered through 15 ml Sephadex G50 columns equilibrated with 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA. Material in the exclusion volume was pooled and precipitated with ethanol in the presence of 10 µg tRNA carrier (Bethesda Research Laboratory).

Preparation of DNA size standards

Commercial λ and ØX174 phage DNA (Bethesda Research Laboratories) were cleaved with restriction enzymes $EcoR_1$ or Hind III and Hae III respectively (Bethesda Research Laboratories) under conditions described by the manufacturer. One to three µg of cleaved DNA were used to calibrate the agarose gels.

Preparation of ribonuclease inhibitor

A ribonuclease inhibitor was prepared from human placenta using a procedure slightly modified from Gribnau et al. (11). In brief, the placenta was homogenized in 0.35 M sucrose, 25 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.8), filtered through gauze and centrifuged at 12000 rev./min for 20 min at 4°C in the Sorvall GSA rotor. The supernatant was centrifuged for 30 min at 40000 rev./min in the Beckman 60 Ti rotor, and applied to a DEAE cellulose column. This column was successively washed

with 0.1 M NaCl and 0.15 M NaCl both in 1 mM EDTA, 0.5 mM dithiothreithol, 20 mM sodium phosphate (pH 6.8) until the A_{280}nm of the eluate was negligible. A NaCl gradient (0.15 to 1.0 M) was then applied. The fractions exhibiting ribonuclease inhibitory activity were pooled, dialyzed extensively against distilled water and freeze dried . The ribonuclease inhibitor was stored at -20°C at a concentration of 10 mg/ml in water. Its specific activity was 220 U/mg (see ref. 11 for the definition of activity). It was shown that the preparation was free of any template or primer activity when tested under reverse transcription conditions.

Electrophoresis of cDNA in alkaline agarose gels

cDNA samples were dissolved in 50 mM NaOH at a radioactive concentration of 20 to 50000 cts/min per 20 µl and subjected to electrophoresis through 1.2 % agarose slab gels prepared in 30 mM NaOH, 2 mM EDTA as described by Mc Donnel et al., 1977 (12). Following electrophoresis (70 volts for 16 hours) the gels were washed in water and soaked for 30 min. in 100 mM Tris-HCl pH 7.5, 100 mM NaCl before staining for 30 min with 1 µg/ml ethidium bromide. They were photographed under U.V. light to allow the recording of the migration of the marker DNA fragments. Thereafter, the gels were dried and subjected to autoradiography (exposure : 1 to 3 days, Kodak Royal X-O-Mat films).

Size determinations

The migration of Tg cDNA was measured on the autoradiograph and compared to that of the restricted DNA fragments as measured on the photograph. Correction for some shrinkage of the gel during drying was made by locating the major marker bands on the dried gel under the U.V. lamp. The size of the restriction fragments were taken from Sanger et al. (13) and Thomas & Davis (14).

RESULTS

Reverse transcription of Tg mRNA under standard conditions has yielded cDNA copies corresponding to 10 to 20 % of the template only (15). We decided to test the influence of a few parameters of the reaction on the size of the product. Since all authors seem to agree that a high deoxynucleotide concentration favors the synthesis of long chains (16,17) we have used throughout a fixed concentration of cold precursors at 800 µM, the labeled nucleotide being at 200 uM. The size of the cDNAs was determined by electrophoresis in alkaline agarose gels, calibrated with phage DNA restriction fragments (12).

Inhibition of RNAase H activity in reverse transcriptase by sodium pyrophosphate has been reported to allow copy of large viral RNAs (18). The results illustrated in fig. 1 demonstrate a definite effect of sodium pyrophosphate when it replaces actinomycin D in the reaction mixture. Although

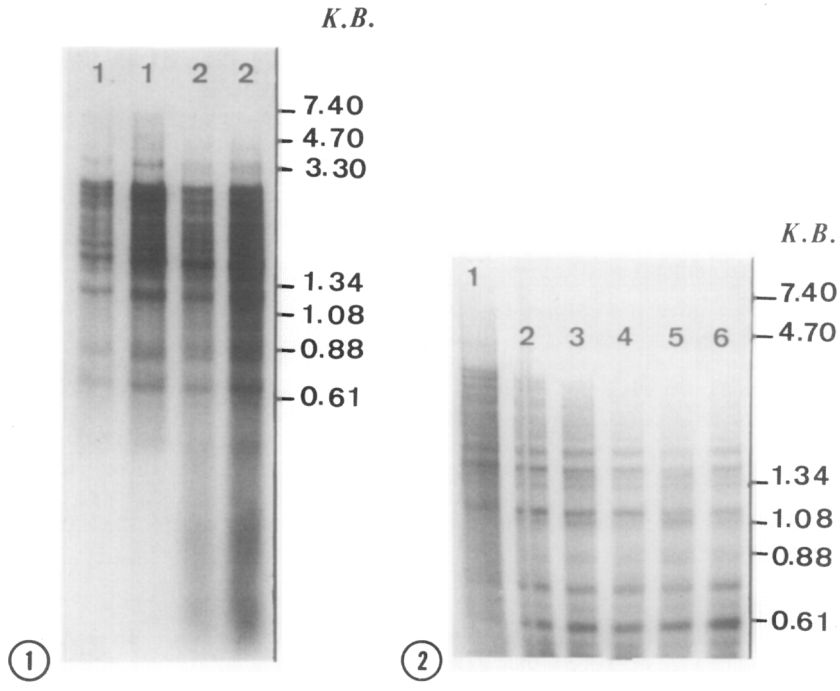

Figure 1. Influence of sodium pyrophosphate on the electrophoretic pattern of Tg cDNA. Tg mRNA (1 µg) was transcribed at 44°C in the standard reaction mixture containing actinomycin D (lane 1) or in the presence of 4 mM sodium pyrophosphate (lane 2). Electrophoresis was performed in an agarose slab gel (1.2 %).

Figure 2. Influence of temperature, duration of incubation and tracer deoxynucleotide on the electrophoretic pattern of Tg cDNA. Reactions were performed under the standard conditions where actinomycin D had been replaced by sodium pyrophosphate (4 mM). The deoxynucleotides were present at 800 µM except for the tracer which was at 200 µM. The following specific conditions were tested :

Lane 1 : mRNA 40 µg/ml, tracer $dCTP^{32}$, t° 45°C, 60 min;
Lane 2 : mRNA 4 µg/ml, $dATP^{32}$, 45°C, 30 min;
Lane 3 : mRNA 4 µg/ml, $dATP^{32}$, 45°C, 10 min;
Lane 4 : mRNA 4 µg/ml, $dCTP^{32}$, 45°C, 60 min;
Lane 5 : mRNA 4 µg/ml, $dATP^{32}$, 45°C, 60 min;
Lane 6 : mRNA 4 µg/ml, $dATP^{32}$, 35°C, 60 min;

Electrophoresis was performed in an alkaline agarose gel (1.2 %).

some very large molecules were synthesized under this conditions (up to 7000 bases), the more obvious effect was the drastic reduction of the amount of small chains (< 600 bases). The size distribution obtained under both conditions exhibit a clearcut banding pattern corresponding to the synthesis of cDNA molecules of discrete sizes.

This very strong pattern prompted us to check whether it could originate from the imbalance in concentration between the labeled (200 µM) and

cold (800 µM) deoxynucleotides in the reaction mixture. As shown on figure 2 only very little differences in the pattern were observed when dATP replaced dCTP (compare lanes 5 and 4) as the limiting nucleotide. While under standard conditions, incubation at 44°C yielded cDNA slightly larger than those obtained at 37°C (results not shown), in the presence of pyrophosphate, the temperature of incubation had virtually no effect on both the pattern and the maximal size of cDNA distribution (compares lanes 5 - 45° and 6-35°). On the contrary, shortening of the incubation time resulted in a relative increase in the proportion of longer cDNAs (compare lanes 2-30 min and 5-60 min)as did the increase in mRNA/transcriptase ratio (compare lanes 1 and 4,5).

Although resulting in some improvement over our previous results, the conditions described here above did not allow the synthesis of significant

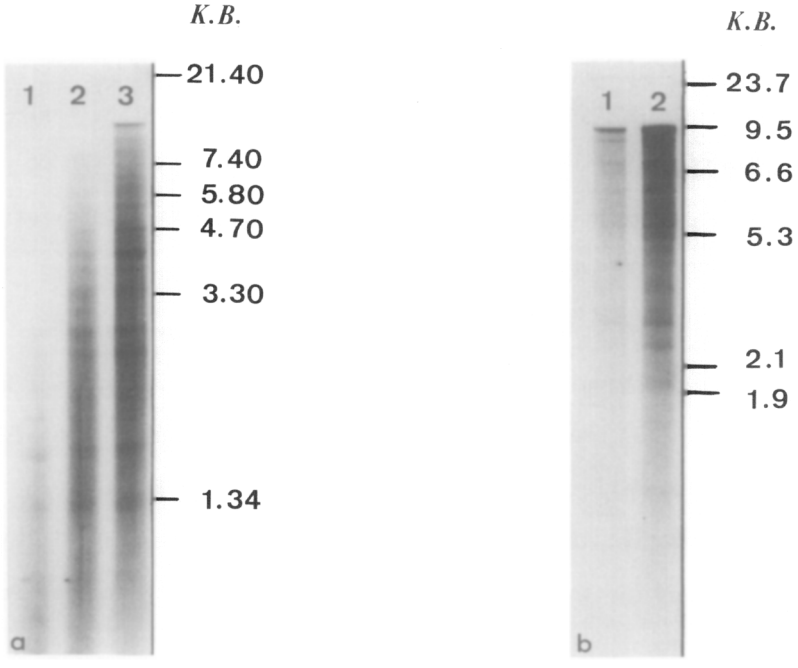

Figure 3. Electrophoretic pattern of Tg cDNA synthesized in the presence of ribonuclease inhibitor.
(a) Tg mRNA (1 µg) was transcribed in the standard reaction mixture (50 µl) containing actinomycin D. Ribonuclease inhibitor was added to the incubation medium at 0 (lane 1), 0.2 (lane 2) and 1.0 mg/ml (lane 3).
(b) Tg mRNA (10 µg) was transcribed similarly in the presence of 1.0 mg/ml ribonuclease inhibitor.

amount of cDNA larger than 3000 bases. As it is known that traces of RNAase contaminate reverse transcriptase preparations (19,20) it seemed logical to test the effect of a ribonuclease inhibitor as first suggested by Emtage et al (21). Figure 3a illustrates the results obtained when variable amounts of a ribonuclease inhibitor extracted from human placenta (11) were included in a reaction mixture containing actinomycin D. At a concentration of 0.2 mg/ml of the inhibitor the size distribution of the cDNA was shifted towards longer molecules. It exhibited a banding pattern qualitatively identical to that of the control cDNA prepared in the absence of inhibitor. Addition of the inhibitor at 1mg/ml yielded a pattern presenting a strong band at its upper size limit. The proportion of the cDNA in this band could be further increased by using a higher mRNA/enzyme ratio (fig. 3b). Estimation of the size of this cDNA gave 8500 bases (average of 9 determinations).

DISCUSSION

Our attempts to synthesize a full size Tg cDNA yielded results, the main characteristics of which are : i) under all conditions tested, the majority of the cDNA we obtained were incomplete; ii) the incomplete transcripts exhibited a discontinuous size distribution.

Even in the case of relatively short mRNA templates the synthesis of incomplete transcripts has been the rule until studies by Efstriatiadis et al. (16) and Monahan et al. (17) showed that full length copies could be obtained under certain reaction conditions involving the use of high nucleotide concentration (16) or incubation at high temperature in the absence of KCl (17). Addition of sodium pyrophosphate to the reaction has been reported to permit the complete reverse transcription of poliovirus RNA probably by its virtue to inhibit RNAase H (18). By combining sodium pyrophosphate and incubation at 44°C, Wahli et al. obtained some 6.300 base cDNAs representing the full length copy of Xenopus vitellogenin mRNA (22). While sodium pyrophosphate allowed the synthesis of longer Tg cDNAs (fig. 1), the incubation tempera-

ture in its presence was without noticeable effect in the 35-45°C range tested (fig. 2).

The addition of a ribonuclease inhibitor to the reaction mixture increased dramatically the average size of Tg cDNA. The size distribution of transcripts became asymmetrical with a strong band at its upper limit (fig.3). The cDNA in this band most probably represents a full length copy of Tg mRNA. Its size measured from the alkaline agarose gel electrophoresis was about 8500 bases, which is in good agreement with estimations of Tg mRNA size (about 8000 bases) (9,10) obtained from electrophoresis in polyacrylamide gels in the presence of formamid.

Under all the conditions tested, the Tg cDNA exhibited a discontinuous size distribution yielding a reproducible banding pattern on the autoradiographs (fig. 1-3). Similar results have been obtained by various authors dealing with very different mRNA templates (16, 22-24). Amongst the causes which could account for the generation of incomplete transcripts of discrete sizes, it has been suggested : i) that specific regions of the mRNA template could be difficult for the enzyme to traverse because of their extensive secondary structure (16,23) or ii) because of a particular sequence which would be recognized as a partial stop signal by the transcriptase (16,23); iii) that the discrete transcripts could originate from different starting points on a mRNA presenting internal oligoadenylic sequences (22) ; iv) that the presence of a deoxynucleotide precursor in limiting concentration (usually the tracer) could be responsible for some slowdown of the enzyme while transcribing regions of the mRNA particularly rich in the complementary ribonucleotide (24). In addition, cleavages of the mRNA at specific sites either before or during the transcription reaction could account for the cDNA pattern.

The observation that identical patterns of incomplete transcripts were obtained when the reaction was performed at 35° or 45° suggests

that if regions of secondary structure are responsible for the pattern, they have to be relatively stable. The reproducibility of the pattern obtained from reactions involving different nucleotide tracers indicates that under our conditions at least the limiting nucleotide is not responsible for the size distribution. At present, we cannot exclude the possibility that multiple starting point for cDNA synthesis exist along Tg mRNA. In order to account for the complexity of the patter one would have to postulate the existence of innumerable oligo-adenylic stretches in the mRNA.

The integrity of the mRNA template used in these experiments has been checked by electrophoresis in agarose gels in the presence of methylmercuric hydroxyde (25) under which conditions it migrates as a single band (not shown). However, the presence of a ribonuclease contaminant in reverse transcriptase preparations has been documented (19,20) and is certainly responsible for Tg mRNA degradation during the reaction since addition of a ribonuclease inhibitor to the medium allows the synthesis of a putative full length transcript (fig. 3). It is tempting to speculate that a single cause, namely the ribonuclease contaminant, would be responsible for the incomplete transcription as well as for the banding pattern. This would imply that degradation of Tg mRNA is non random, which would in turn pose the problem of the nature of the specificity involved in the cleavage process.

The 8500 base Tg cDNA represents one of the largest non viral cDNA synthesized up to now. The reaction conditions described in the present study may be of value in the reverse transcription of other large mRNA and represents the first step in the cloning of their complete sequence.

ACKNOWLEDGMENTS

We want to thank Dr J.E. Dumont for his continuous support and interest. We are indebted to Dr J. Beard for his generous supply of reverse transcriptase. We thank Mrs Guy-Lafabrègue for expert technical assistance and Mrs D. Leemans for the preparation of the manuscript.
This work was supported by NIH grant n° AM 21732-02 and FRSM grant n° 3.4534.79 to G.V.

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