SYNTHESIS OF DNA COMPLEMENTARY TO 14S CALF LENS
CRYSTALLIN MESSENGER RNA BY REVERSE TRANSCRIPTASE

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SUMMARY

The 14S messenger RNA (mRNA), coding for the A_2 chain of α-crystallin was isolated from calf lens polyribosomes by zonal centrifugation and further purified by oligo(dT)-cellulose chromatography. The purified mRNA was used as a template to synthesize complementary DNA by reverse transcriptase. The DNA product appeared to be smaller in size than the RNA template but a faithful transcript of base sequences in the 14S α-crystallin mRNA.

INTRODUCTION

In vitro synthesis of DNA complementary to several eukaryotic mRNAs by the avian myeloblastosis virus (AMV) DNA polymerase has been recently reported (1-6). Most eukaryotic mRNAs have been shown to contain regions rich in adenylic acid (poly(A)) (7) as an integral part of their structure. If these poly(A) regions are located at or near the 3' end of the RNA, then an oligomer of (dT) can hydrogen bond to it and serve as primer to support the synthesis of complementary DNA by the reverse transcriptase. The 14S lens mRNA has been shown to contain poly(A) containing regions (8) and can thus serve as a template for the reverse transcriptase using oligomer of (dT) as primer.

In this communication we describe the synthesis of DNA complementary to the 14 S calf lens mRNA that codes for the A_2 chain of α-crystallin (9,10,11,12).

MATERIALS AND METHODS

Calf lens polyribosomes were isolated as described earlier (13). RNA was prepared by a chloroform-phenol extraction procedure described by Perry et al (14). The lens mRNA was purified by zonal centrifugation followed by oligo(dT)-cellulose chromatography (15) which resulted in a further 3 to 4 fold purification. The ability of the RNA to direct the synthesis of α-crystallin polypeptides in vitro was tested as described elsewhere (12). The reverse transcriptase from avian myelo-

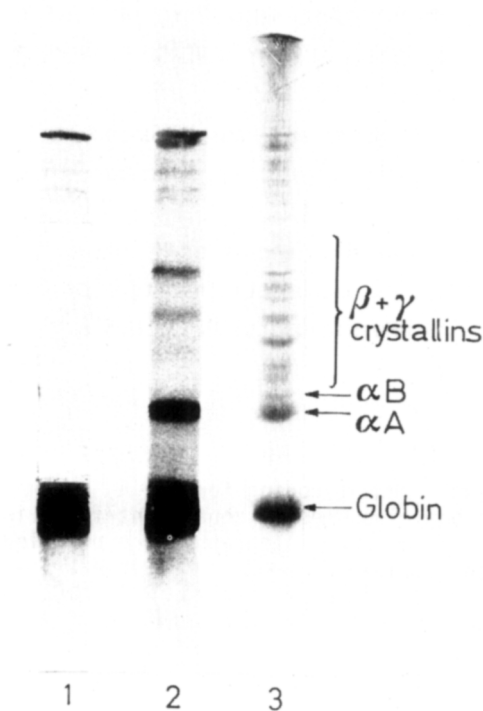[*] to whom requests for reprints should be addressed

Figure 1. Identification of 14S lens mRNA directed translational products.
Calf lens mRNA was added to a reticulocyte lysate with highly labeled
^{35}S-methionine as described in ref. 12. After incubation an acidic acetone
precipitation was carried out. The in vitro products were analyzed by SDS
gel electrophoresis according to Laemmli (19). To each gel about 200 μg of
protein was applied. 20 μg of crystallin polypeptides were added in order
to compare the radioactive pattern with the stained bands. After staining
and destaining longitudinal slices were dried down on filter paper under vacuo
and autoradiographed for 3 weeks. The details of the procedure are described
elsewhere (12).
1) without 14S mRNA 2) with 14S mRNA 3) stained gel

blastosis virus was purified according to Verma and Baltimore (5). The single
strand specific nuclease from Aspergillus oryzae was a generous gift of Drs.
D. Housman and A. Skoultchi.

The reaction mixture consisted of the following components: 50 mM Tris-HCl,
pH 8.3, 20 mM dithiothreiotol, 6 mM magnesium acetate, 60 mM NaCl, 600 μM of
the three unlabeled deoxyribonucleoside triphosphates, 80 μM of the ^{3}H-labeled
deoxyribonucleoside triphosphate, 10 μg/ml oligo(dT)$_{12-18}$ 100 μg/ml Actinomycin
D, 0.2 to 0.5 μg of reverse transcriptase and 6 μg/ml of 14S mRNA. Incubations
were carried out at 37°C for the specified times. The amount of incorporation
was determined by measuring the acid-precipitable radioactivity as described
previously (16).

TABLE 1

REQUIREMENTS FOR DNA SYNTHESIS USING 14S CALF LENS mRNA AS TEMPLATE

Exp. I	pmoles dGMP incorporated
Complete	15
-Actinomycin	25
-dTTP	2.3
-dCTP	2.5
-dATP	2.5
+RNAase	1.7
Exp. II	
Complete	43
-oligo(dT)+oligo(dG)	29
-oligo(dT)+oligo(dA)	3.4
-oligo(dT)-oligo(dC)	4.5
-primer	4.1

Except where indicated, the complete reaction mixture described in the
experimental section was used. The amount of RNA in exp. I and exp. II
was 850 pmoles nucleotides of RNA. Oligomers of dT, dG, dA and dC have
chain length of approximately 12-18 nucleotides long and were obtained from
Collaborative Res. Waltham, Mass. The ribonuclease reagent contained 400 µg
of Pancreatic ribonuclease A/ml (Worthington Biochemical), 80 µg of ribonu-
clease T_1/ml (Calbiochem, 5000 U/mg) and 1 mg bovine serum albumin/ml in
0.01 M Tris-Cl, pH 7.6, and 0.01 M NaCl. The RNAase was added together with
the other reaction constituents. Acid precipitable radioactivity was deter-
mined as described before (16).

RESULTS AND DISCUSSION

A) Purity of mRNA

     Calf lens 14S mRNA used in these experiments was purified by zonal centri-
fugation and oligo(dT)-cellulose chromatography. Identification of the transla-
tional products suggest that the 14S mRNA preparation contained only a minor
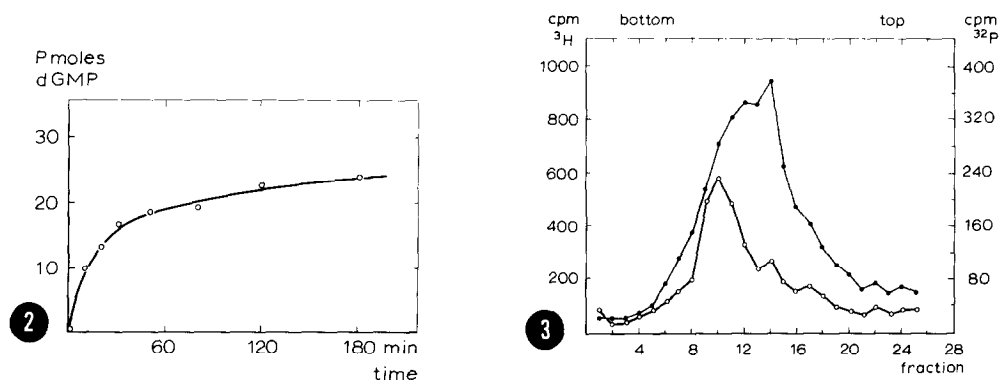contamination of other lens mRNAs (Fig. 1).

Figure 2. Kinetics of DNA synthesis using calf lens 14S mRNA as a template.
    The reaction mixture described in the Method section was used, except
that 2000 pmoles of RNA were added per 200 µl reaction mixture. Aliquots
of 20 µl were withdrawn and the reaction was stopped by addition of 2% sarkosyl.

Figure 3. Alkaline sucrose gradient analysis of the DNA product.
    The complete reaction mixture contained in 0.4 ml was used, except
8000 pmoles of RNA was added along with ^3H dCTP (28 Ci/mmole). Marker globin
DNA was prepared as described before (1). The DNA was purified by G-50 gel
filtration as described before (20). 5 to 20% alkaline sucrose gradients
containing 0.7 M NaCl, 1.0 mM EDTA and 0.3 M NaOH (pH 12.4) were freshly pre-
pared. Centrifugation was performed in a SW 56 rotor of the Beckman ultracentri-
fuge at 56,000 rpm for 14 hours at 2°C. The gradients were fractionated into
25 to 30 fractions and radioactivity counted after the addition of 5 ml of
Instagel (Packard).
●—●—●—●—● ^3H lens DNA
—o—o—o—o—o ^{32}P 8S globin DNA

B) Kinetics of transcription

    When the 14S mRNA was used as a template in the standard reaction mixture,
extensive polymerization of deoxyribonucleoside triphosphates was observed in the
presence of oligo(dT) primer. The requirements of the reaction are summarized in
Table 1. In the presence of Actinomycin D incorporation was inhibited by about
40%. Actinomycin D prevents the synthesis of double stranded DNA (1,17). All
4 deoxyribonucleoside triphosphates are required for the synthesis of complementa-
ry DNA. Ribonuclease almost completely inhibits incorporation of deoxyribonucleo-
side triphosphates. Although oligo(dT) is the most efficient primer it can be
replaced by an oligo(dG) primer. This has also been observed in the synthesis of
DNA complementary to globin mRNA (1,18). The extent of DNA synthesis varied from
one experiment to another even when the same RNA preparation was used.
If the four deoxyribonucleoside monophosphates are present in approximately equi-
molar amounts in the DNA transcript then the amount of dGMP incorporation multi-
plied by four will give the extent of transcription. In most experiments the
transcription amounted to 20-60% of the input RNA. Fig. 2 shows the kinetics of

synthesis of complementary DNA. The maximal synthesis was achieved between 120 to 180 min. of incubation. The amount of incorporation was proportional to the amount of RNA added. However at elevated RNA concentrations the incorporation is no longer linear (above 15,000 pmoles of nucleotide/ml).

C) Size of the complementary DNA

Figure 3 depicts the sedimentation pattern of the DNA on an alkaline sucrose gradient. The complementary 8S rabbit globin DNA was included as marker. The DNA complementary to the 14S lens mRNA appears slightly smaller and more heterogenous than globin DNA. Since the 14S mRNA appears larger than the 10S globin mRNA (although it directs the synthesis of a polypeptide with a molecular weight of only 20,000) this indicates that the mRNA is only partially transcribed or has been transcribed in several pieces.
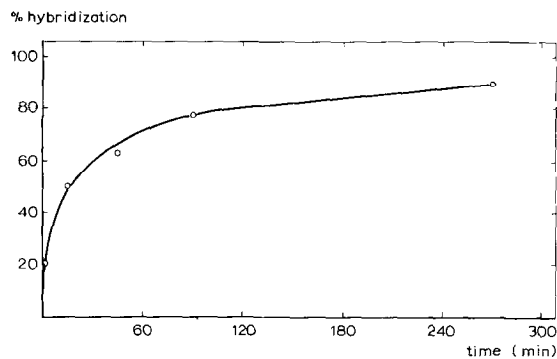

Figure 4. Hybridization of the DNA product with the 14S mRNA.
    High specific activity labeled DNA transcript (2400 cpm of ^{3}H dGMP/pmole) was made using the complete reaction mixture described in the Method section. The DNA was purified as described before (20). DNA-RNA hybridization was performed in 10 μl capilliary tubes in a medium containing 0.2 M phosphate buffer, pH 7.0 and 0.5% SDS. After heating the mixture for 5 min. at 95°C the ca-pilliary tubes were transferred immediately to a 65°C waterbath. After hybridi-zation the capilliary tubes were cooled in ice, broken and the solution expelled into 1.0 ml of digestion buffer (1mM ZnSO$_4$, 0.1 M sodium acetate, pH 4.5 and 10 μg/ml calf thymus DNA). 6 μl of Aspergillus single strand nuclease was added and the solution was incubated at 45°C for 30 min. Acid-precipitable radioactivity was determined. The hybridization mixture in each capilliary contained 1 pmole of complementary DNA and 100 pmol 14S mRNA.

The same situation has been encountered with the transcription of the AMV RNA where only DNA pieces of 5 to 7 were synthesized (17). It is possible that the secondary structure of the 14S crystallin mRNA and the AMV-RNA prevent their com-plete transcription.

(D) Nature of the product

To determine if a faithful transcript of DNA complementary to the 14S mRNA was synthesized we studied the ability of the DNA to hybridize with the RNA

template. The kinetics of hybridization are shown in Figure 4. About 90%
of the DNA annealed to the RNA, indicating that the DNA product is a faithful
transcript of the lens mRNA. Some self annealing could be detected in the absen-
ce of RNA suggesting that either the synthesis of double stranded DNA is not com-
pletely inhibited by actinomycin D or the single stranded DNA formed "hairpins".
The extent of self annealing was substracted from the RNA-DNA annealing values.
When the DNA was hybridized to tRNA or 10S globin mRNA it remained completely
sensitive to single strand specific nuclease.

CONCLUSION

The 14S calf lens mRNA can be transcribed into complementary DNA using purified
DNA polymerase from AMV. The product though smaller in size than the template
is a faithful transcript of the template. Attempts are being made to determine
if there are any homologies in the base sequences of various fractions of calf
lens mRNAs using the labeled complementary DNA as a probe. We are also trying
to transcribe the complementary DNA into RNA by E. coli RNA polymerase in an
attempt to synthesize labeled RNA for nucleotide sequences analysis. The availa-
bility of labeled complementary DNA will also enable us to determine the number
of gene copies of α-crystallin 14S mRNA in the calf lens.

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