THE POLY A CONTENT AND SECONDARY STRUCTURE OF THE 14S CALF LENS MESSENGER RNA

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SUMMARY

The 14S calf lens messenger RNA (mRNA) coding for the A2 chain of α-crystallin was isolated from calf lens polyribosomes by zonal centrifugation and purified by poly-(dT)-cellulose chromatography and subsequent sucrose gradient centrifugation.
From the purified messenger the poly A content was estimated by measuring the increase of the fluorescence of Ethidium-bromide intercalating the hybrid formed by titration with poly U.*
About 15% of the messenger is composed of poly A. The characteristics of the poly U titration curves indicate that other bases are also present in the poly A track. Further the 14S messenger exhibits a secondary structure comparable with TMV and Globin mRNA.

INTRODUCTION

The presence of poly A in eukaryotic messengers, with exception of histone mRNA is well established (1-7). Also calf lens messengers should contain poly A tracks since they can be purified on oligo(dT)-cellulose and serve as a template for the reverse transcriptase using oligomer of (dT) as primer (8).

As 14S lens messenger has a molecular weight of approximately 450,000

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*A preliminary communication describing these method used here for the study of RNA secondary structure and determination of the poly A content of mRNA was presented by A.F. at the work-shop on mRNA's in eukaryotic cells held in Arolla 11-16 sept. (1972).

dalton (9), more than twice as long as expected, the knowledge of the length and nature of the poly A will contribute to the understanding of the architecture of this messenger.

MATERIAL AND METHODS

Calf lens polyribosomes were isolated as described earlier (10). The lens polyribosomes were dissociated with SDS and the RNA was separated by zonal centrifugation. The different RNA fractions were tested for their coding ability by translation in a cell-free system derived from Ehrlich Ascites cells (EAT cells were a generous gift of Dr. E. Henshaw,Beth Israel Hospital, Boston U.S.) and were grown in suspension (11). A crude cell-free system was prepared as described for the Krebs II ascites cell-free system (12).

The protein synthesis reaction was performed in a final volume of 25 µl and contained the following components: 15 µl Ascites extract, 12 mM HEPES, pH 7.5, 0.14 M KCl, 3 mM Mg acetate, 1 mM ATP, 0.2 mM GTP, 8 mM phosphocreatine, 25 µg kinase, cold amino acids minus methionine as described by Palmiter (13), 1 µCi [^{35}S] methionine (100 Ci/mmol), 3 µl 0.5 M KCl wash from reticulocyte ribosomes (14) and 3 µl messenger RNA (100 µg/ml). Incubation was carried out for 1 hour at 37°. 1 µl 0.25 M EDTA and 1 µl pancreatic RNAase (500 µg/ml) was added and incubation was continued for 15'. After addition of unlabeled carrier lens crystallins, SDS and mercaptoethanol were added to a final concentration of 2 and 5%, respectively. This mixture was incubated for 1 hour at 37° and applied on SDS gels according to Laemmli (15). After staining and destaining longitudinal slices were dried down on filter paper and autoradiographed for 48 hours. The RNA fraction obtained from zonal centrifugation which codes almost exclusively for αA2 was pooled and submitted to fractionation on poly (dT)-cellulose as described elswhere (16). The poly A containing RNA was eluted at 50°

with 0.05% SDS and applied on a 10 - 28% exponential sucrose gradient in 50 mM NaCl and 10 mM Tris-Cl, pH 7.6 and run at 40,000 rpm for 16 hours at 5^{o} in a SW 41 rotor of a Spinco in order to remove slow sedimenting poly A containing 14S mRNA fragments. The material purified on poly (dT)-cellulose migrated as a single component in the 14S position. By this procedure of poly (dT)-cellulose chromatography and sucrose gradient centrifugation the 14S messenger was purified about 20 fold.

Rechromatography on poly (dT)-cellulose did not result in any further purification.

The fluorescence was measured with a Jobin Yvon spectrofluorimeter. The excitation wavelength was 540 nm, the emission wavelength 600 nm. The cuvette containing the RNA in 600 µl of a buffer consisting of 0.05 M sodium cacodylate, pH 7.0 and 0.05 M sodium chloride, was inserted in a cuvette holder thermostated at 25^{o}.

T_1 RNAase digestion was performed by adding 5 µl of 1 mg/ml T_1 RNAase directly to the mixture in the cuvette.

Ethidium-bromide was a gift of Dr. Cobb of the Boots and Pure Drug Co. (Nottingham, England). Poly (U) and poly (A) were purchased from Miles Laboratories and T_1 RNAase from Sigma Chemical Company. Tobacco Mosaic Virus RNA (TMV RNA) was a kind gift of Dr. H. Guilley.

RESULTS AND DISCUSSION

A. Purity of the 14S messenger

Calf lens messenger, used in these experiments, was purified by zonal centrifugation and poly (dT)-cellulose chromatography followed by an additional sucrose gradient centrifugation step. The zonal centrifugation step separates the 14S messenger almost completely from the other lens messengers; as can be seen from the in vitro products synthesized under the direction of the different RNA fractions (Fig. 1).

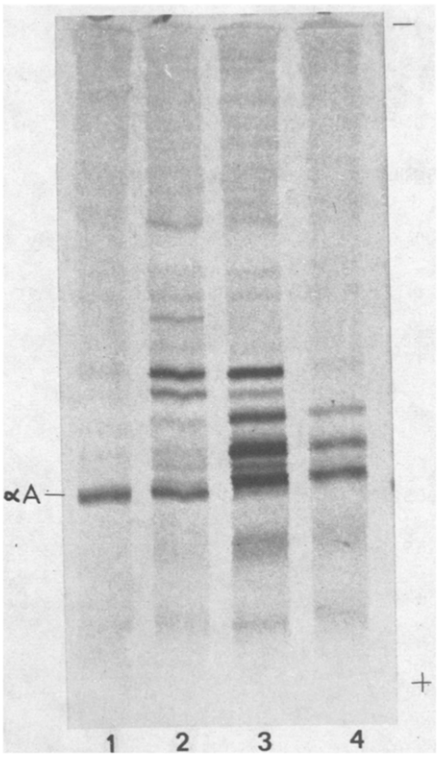

Figure 1. Autoradiographs of lens mRNA directed translational products. 1. with 14S messenger; 2.,3.,4.: with different 10S messenger fractions.

The poly (dT)-cellulose chromatography results in a further purification; only 5% of the crude 14S messenger preparation, which contained also some 18S ribosomal RNA, was eluted at high temperature. As rechromatography on poly (dT)-cellulose resulted in no further purification all the RNA obtained by this procedure should contain poly A sequences. In order to purify intact RNA from possibly degrated messenger products the material eluted at high temperature was recentrifuged on an exponential sucrose gradient. The material migrated in the 14S position as a single component without any detectable degradation.

B. Secondary structure of the 14S messenger

It is well known (17) that at low concentrations of ethidium-bromide and

nucleic acid, in a medium of moderate ionic strength, the dye specifically intercalates in the double-stranded regions of the nucleic acid. The intercalation process is accompanied by a considerable increase in ethidium-bromide fluorescence and can thus be easily monitored. We have already used this technique to study RNA-protein interactions (18).

The determination of the number of intercalation sites in a given RNA molecule can be related to the extent of secondary structure. In Fig. 2 the uptake of dye by lens mRNA is shown. Addition of T_1 RNAase resulted in a

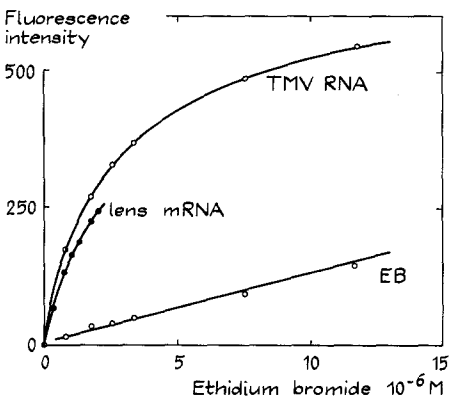

Figure 2. Secondary structure of lens 14S mRNA.
Fluorimetric titration of lens mRNA - 0.0980 A_{260} unit/ml- and Tobacco Mosaïc Virus RNA - 0.0975 A_{260} unit/ml- by ethidium-bromide at 25° in 600 µl of 0.05 M NaCl, 0.05 M sodium cacodylate pH 7 buffer. The fluorescence signal was read (λ exc 540 nm - λ em 600 nm) after each addition of 3 (or 5 µl) of a 10^{-4} M dye solution.

drastic decrease of the fluorescence intensity indicating extensive degradation of the RNA structure (Fig. 3). The extent of base pairing was less in 14S mRNA than in Tobacco Mosaïc Virus RNA and was close to that observed in duck and rabbit globin mRNA in which bihelical regions account for 45 to 60% of the total RNA length (19).

C. The poly A track of 14S mRNA

The experimental principle used here is the following: the hybridization of

a homopolyribonucleotide (poly U) to a complementary polymer (poly A) results in the formation of a bihelical structure that can be visualized and quantitated by following the increase in fluorescence of ethidium-bromide present in excess in the mixture. As soon as the hybridization process is going to completion, the fluorescence intensity will reach a plateau. As illustrated in Fig. 3, the amount of poly U needed to saturate synthetic poly A is equal to the amount of poly A initially present, and is given by the intersection of the initial and plateau segments of the titration curve (saturation point). When applied to the 14S mRNA, this technique revealed the presence of poly A tracks. As in the case of synthetic poly A and poly U, a sharp saturation point is obtained (Fig. 3) but the slope of the initial part of the titration

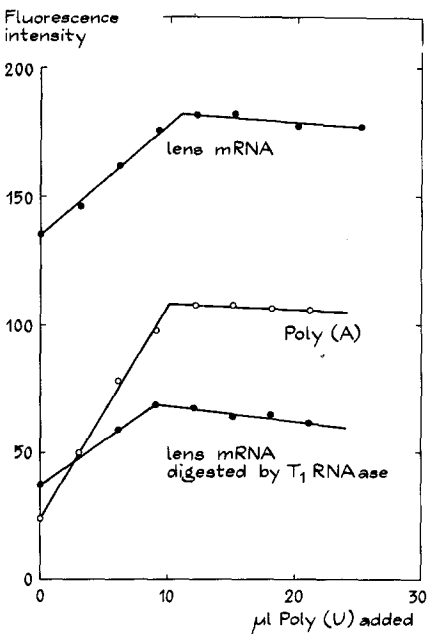

Figure 3. Detection of poly A tracks in lens mRNA.
Solution, 600 µl, of ethidium-bromide 2.10^{-6} M containing respectively 0.049 A_{260} unit/ml lens mRNA or 0.0087 A_{260} unit/ml of poly A were prepared in the solvent conditions of Fig. 1. The T_1 RNAase digested mRNA sample was obtained by incubation for 1 hour with 10 µl of a 1 mg/ml enzyme solution. Titration of each sample was performed at 25° by stepwise addition of 3 µl aliquots of a 5.10^{-5} M poly U solution and the fluorescence signal read 2 min after each addition.

curve is two times smaller than those obtained with synthetic poly A, duck and rabbit globin mRNA (19) and with α casein messenger from sheep mammary gland, respectively (20). A possible explanation to this finding could be that the association constant of poly U for the poly A tracks of 14S mRNA is much lower than for synthetic poly A; this would, however, be consistent with a convex titration curve instead of the straight segments and sharp saturation point obtained here. The slope of the titration curve of synthetic poly A by poly U decreases as ionic strength increases: In order to avoid this artefact the 14S mRNA was precipitated and washed with ethanol before performing the fluorescence experiments. The fact that the slope of the titration curves remains unchanged after T_1 RNase degradation strongly indicates that the conformational freedom of the poly A segment is not limited by the mRNA secondary and tertiary structure.

Therefore the only consistent interpretation of our results is that the poly A track is interspersed by other bases which prevent a perfect duplex formation leaving short unmatched regions. Further evidence was obtained by titrating with poly U the RNase T_1 digest of the 14S mRNA (Fig. 3). The extent of hybridization decreased by 20%, suggesting that small poly A segments, shorter than 10 residues in length, were formed and were unable to hybridize poly U in our conditions.

Assuming an extinction coefficient (P) of 8000 for mRNA, 3.6 nmoles of mRNA were initially present in the titration experiment. From the amount of poly U added to reach the saturation point the adenine plus the interspersed bases of the poly A track account for 0.55 nmoles, that is to approximately 15% of the total base content of the mRNA.

CONCLUSION

The secondary structure found in the 14S calf lens mRNA resembles that of other messengers from viral as well as mammalian origin.

The total poly A track is estimated to contain approximately 200 residues. However the poly A is probably interspersed by other nucleotides. Therefore the application of other techniques to determine the length of the poly A in the 14S messenger (e.g. T_1 digestion and determination of the molecular weight of the undigested fragment) may lead to underestimation of the actual size.

Since our findings show that the discrepancy between the length of the 14S messenger (approximately 1300 nucleotides) and the size of the encoded protein (173 residues) (21) cannot be explained by the presence of an extremely long poly A track, it cannot be excluded that the 14S mRNA is bicistronic or codes for a much longer precursor molecule, although so far no evidence could be provided for either of these possibilities.

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