Volume 8 Number 21 1980 Nucleic Acids Research

Ribonucleotide sequences non-adjacent to poly(A) participate in the poly(A)-protein complex in 15S duck globin mRNP particles

Samuel Goldenberg, Alain Vincent and Klaus Scherrer*

Laboratoire de Biochimie de la Différenciation, Institut de Recherche en Biologie Moleculaire, Universite Paris VII - Tour 43, 2 Place Jussieu, 75221 Paris Cedex 05, France

Received 30 June 1980

ABSTRACT

The study of the interaction between mRNA and proteins in the polyribosomal 15 S duck globin messenger ribonucleoprotein complex showed that proteins protect specific mRNA sequences against digestion by the non-specific micrococcal nuclease (Nucleic Acids Research 6 (8) 2787, 1979). Here we report the isolation of the poly(A)-protein RNP complex from nuclease digested 15 S mRNP by two different methods : sucrose gradient sedimentation and oligo(dT)-cellulose chromatography. We show by fingerprint analysis, that apart from the periodically fragmented poly(A) segment, mRNA sequences adjacent and non-adjacent to the poly(A) segment are protected by the poly(A) binding proteins against nuclease digestion. The duck globin poly(A)-protein RNP complex, with a sedimentation coefficient between 7 S and 10 S, shows a characteristic protein composition, with a major 73,000 MW polypeptide and some minor components. The results are discussed in view of a dynamic ribonucleoprotein structure.

INTRODUCTION

In eukaryotic cells, messenger RNA sequences are found associated with proteins in nuclear pre-mRNP (1-3) and in cytoplasmic mRNP particles (4-7).

In the cytoplasm of duck reticulocytes, globin mRNA is found in two distinct forms of mRNP complexes : polyribosomal 15 S mRNP (active in translation) (8-10) and cytoplasmic-free 20 S mRNP (repressed for translation)(10-11).

The protein composition of polyribosomal mRNP was demonstrated to be quite similar in a great variety of cells, including always two major polypeptides with molecular weights of about 75,000 and 50,000 MW, and some minor polypeptides between 25,000 and 120,000 MW, varying with the cellular system studied (12-16). It has been shown that a major polypeptide about 75,000 MW is associated with the poly(A) segment of all polyribosomal mRNPs studied thus far (17), and that some minor polypeptides can also be found associated with the poly(A) sequence located at the 3'-end of mRNA (14,18-19).

On the other hand, a 25,000 MW polypeptide has been found interacting with the 5'-end of mRNA (20-21).

We have recently shown that in 15 S duck globin mRNP, 25 % of the mRNA sequences are protected by proteins against staphylococcal nuclease digestion, and that proteins interact non-randomly with the mRNA sequences, protecting specific mRNA fragments (22). Similar results were obtained for nuclear pre-mRNP particles (23).

Evidence was provided showing that the 73,000 MW poly(A) binding protein interacts with RNA sequences other than poly(A) (14,19,24). In the study presented here we investigated whether only poly(A) adjacent sequences are protected by the poly(A) binding proteins in the 15 S duck-globin mRNP, or whether mRNA sequences non-adjacent to the poly(A) tail participate in the complex, being protected by the same set of proteins against nuclease digestion. Our results show that mRNA sequences adjacent and non-adjacent to the 3'-terminal poly(A) interact with the poly(A)-binding protein complex in 15 S mRNP. It is therefore likely that the mRNA folds back to interact with the poly(A) complex in the mRNP native particle devoid of ribosomes.

MATERIALS AND METHODS

Materials : Acrylamide and Bis-acrylamide were from Bio-Rad, formamide from Fluka and RNase-free sucrose from Merck. γ-(^{32}P)ATP (3,000 Ci/mmol) was from the Radiochemical Center (AMERSHAM) and ^{3}H-polyuridylate was from Miles Laboratories (361 µCi/µmole P). Ribonuclease T1 was from Sankyo (via Calbiochem), RNase A from Sigma, micrococcal nuclease from Worthington and T4-polynucleotide kinase from P.L. Biochemicals. Oligo-(dT)-cellulose was purchased from Collaborative Research. Cellulose acetate strips were from Schleicher-Schüll and DEAE cellulose HR 2-15 thin-layer plates were from Machery-Nagel. For autoradiographs Fuji RX and Du-Pont Cronex Light-Plus intensifying screens were used.

Fingerprint analysis : Electrophoresis in first dimension was on cellulose acetate strips at 4,000 volts for 60 minutes using a buffer containing 5 % acetic acid, 2 mM EDTA, 7 M urea adjusted to pH 3.5 with pyridine. Second dimension analysis was homochromatography on DEAE-cellulose thin layer plates using homomixture C, 65° C, 6 hours, as described previously (22), according to Brownlee's procedure (25).

Affinity chromatography : mRNA was chromatographed on oligo-(dT)-cellulose as described by others (26-27), with the difference that binding of poly(A^+) RNA was performed at 0.2 M ionic strength, the polyadenylated material was

eluted with water. For mRNP, oligo-(dT)-cellulose chromatography was as described previously (15,18). Fractions eluted from the column were precipitated at - 70° C after addition of three volumes ethanol.

Titration of poly(A) with radioactive poly(U) was carried out in an excess of radioactive probe (28), with the modification that both the hybridization and RNase A digestion, were performed at 37° C.

Sucrose gradient sedimentation was on 5-21 % sucrose isokinetic gradients (16 hours, 2° C, 41,000 rpm, BECKMAN SW-41 rotor).

SDS-polyacrylamide gel electrophoresis were performed on 13 % uniform gels (29).

In vitro labelling of RNA with ^{32}P at the 5'-end was carried out with γ-(^{32}P)ATP in the reaction catalysed by the polynucleotide kinase (30).

Enzymatic digestions and phenol extraction were performed as described previously (22), and autoradiographs were performed at - 70° C.

RESULTS

In order to determine whether proteins isolated with the poly(A) segment of mRNA from polyribosomal mRNP are associated with the poly(A) sequence exclusively or as well with a sequence adjacent to poly(A), we digested native duck globin 15 S mRNP with the calcium dependant non-specific nuclease from Staphylococcus aureus, in conditions where protein protected sequences are not digested. The RNA extracted from the nuclease digested complex was chromatographed on oligo-(dT)-cellulose and the poly-adenylated fraction was subsequently labelled in vitro with γ-(^{32}P)ATP-polynucleotide kinase. Half of the sample was directly analysed by fingerprinting (Fig. 1.A) and the other half was exhaustively digested with RNase T1 prior to fingerprint analysis, in order to identify the 5'-oligonucleotides of the RNA fragments (Fig. 1.B). In analysing this experiment it has to be kept in mind that the poly-adenylated part of mRNA can be non-pure poly(A) and might therefore not be fully resistant to T1 digestion. Furthermore, RNA sequences might co-purify on oligo-(dT) columns associated with mRNA by hydrogen bonding rather than in covalent linkage ; indeed, no denaturation steps were included in our protocole.

Figure 1.A shows essentially the poly(A) smear at the origin of the second dimension. According to its position in the fingerprint, the faint oligonucleotide at the top of the fingerprint, can be either an uridine-rich oligonucleotide co-purifying with poly(A) during affinity chromatography (31) or a poly(A) degradation product. In the RNase T1

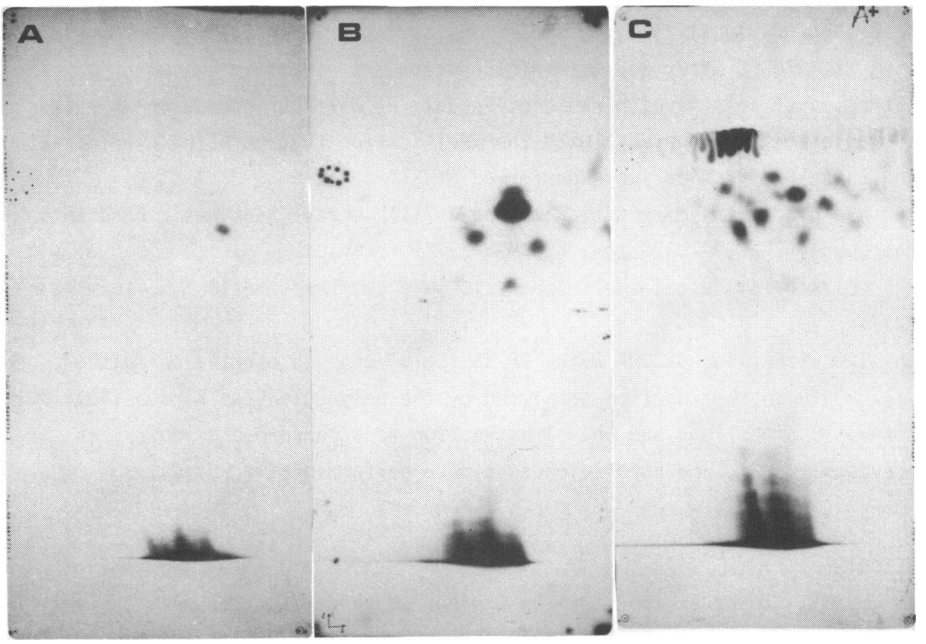

Figure 1 - 15 S mRNP was digested with staphylococcal nuclease and the phenol extracted RNA was chromatographed on oligo-(dT)-cellulose. The polyadenylated fraction was labelled with ^{32}P and directly analysed by fingerprint (A) ; digested with RNase T1 prior to fingerprint analysis (B) ; or digested with RNase T1 before ^{32}P labelling and fingerprinting (C). First dimension is from the left to the right and second dimension is upwards.

digested poly-adenylated RNA (Fig. 1.B), several other oligonucleotides can be seen ; furthermore, the poly(A) tail seems to be cut in a periodical fashion.

It has to be kept in mind that the mRNP complex contains the mRNA for all three adult globins, namely α^A, α^D and β, in a ratio of 3:1:4 (32) ; the difference in the ratio of the globin chains explains the difference in intensity among different oligoribonucleotides. A priori, one should expect only three oligonucleotides in the fingerprint of the T1 digested material (Fig. 1.B) since three distinct mRNA populations are present in the polyribosomal mRNP. The fact that additional oligonucleotides are present and that poly(A) chains themselves are labelled might be due to the dynamic nature of the RNA-protein association in the complex, as will be discussed below.

The poly(A) adjacent sequence does not seem to be very large, in contrast to the poly(A) sequence itself. Figure 1.C shows the fingerprint of the total T1 oligoribonucleotides produced by RNase T1 as in Fig. 1.B, but labelled with ^{32}P after T1 digestion. As expected, additional oligoribonucleotides can be seen, but all of them are in the range of 3 to 5 nucleotides. As discussed later, we calculate that the poly(A) adjacent sequences are about 10 nucleotides long. Their position in the fingerprint indicates that they are not pure oligo(A) split from inside the poly(A) chain. The results presented in Figure 1 indicate , thus, that in addition to the tract of pure poly(A), oligoribonucleotide sequences sensitive to RNase T1 and adjacent to the poly(A) sequence, are protected against micrococcal nuclease digestion in the poly(A)-protein RNP complex. However, they did not provide any insight concerning the possible interaction of mRNA sequences non-adjacent to the poly(A) segment within the poly(A)-protein RNP complex.

In order to investigate the possible participation of mRNA sequences non-adjacent to the poly(A) tract in the poly(A)-protein RNP complex, we digested 15 S duck globin mRNP with staphylococcal nuclease as described above, and we isolated by affinity chromatography on oligo-(dT)-cellulose, the poly(A) containing residual complex rather than the deproteinised RNA fragments analysed in Fig. 1. The RNA extracted from the formamide eluted polyadenylated fraction was labelled in vitro with γ-ATP^{32} and analysed by fingerprint as above, prior to, or following RNase T1 digestion (Fig. 2.A and 2.B respectively).

Observation of Fig. 2.A and comparison with Fig. 1.A allow us to conclude that additional large oligoribonucleotides are present in the RNA population of the polyadenylated residual complex, indicating that RNA sequences other than poly(A), or adjacent to it, are present in the poly(A)-protein RNP complex. Figure 2.B shows the fingerprint pattern of the oligoribonucleotides generated by digesting the nuclease resistant sequences shown in Fig. 2.A with RNase T1. Comparison with Fig. 1.B shows a more complex pattern ; in addition to the adjacent sequences (the spots forming a kind of cross in the middle of the fingerprint) other oligoribonucleotide sequences are visible ; hence, new oligoribonucleotides were generated by the RNase T1 digestion due to the presence, in the poly(A)-protein RNP complex, of globin mRNA sequences non adjacent to poly(A). Again a certain length periodicity of the large resistant poly(A) fragments is observed.

Proteins extracted from both the unbound and the bound (poly-

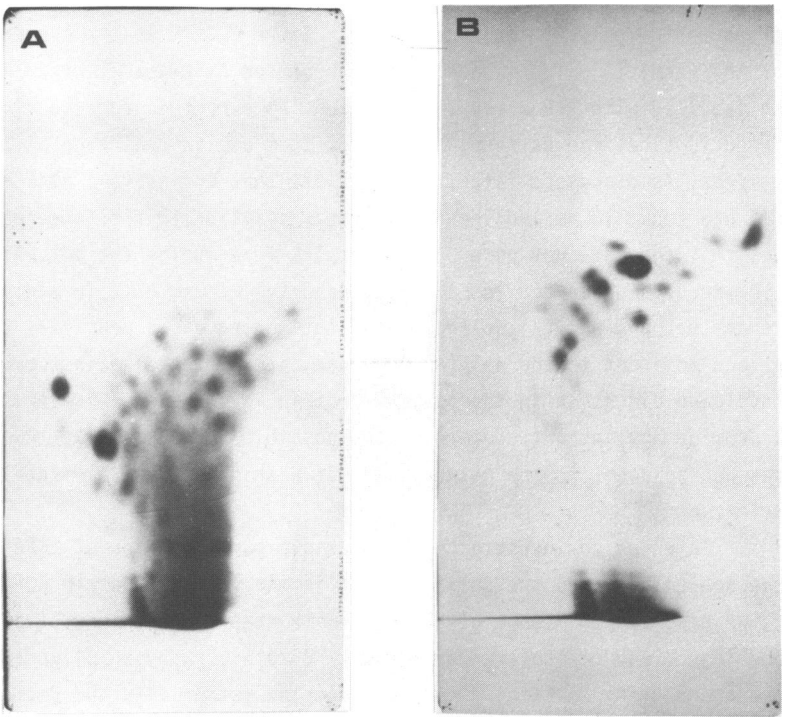

Figure 2 - 15 S mRNP was digested with staphylococcal nuclease and directly chromatographed on oligo-(dT)-cellulose. The RNA extracted from the formamide eluted fraction (poly(A)$^+$ RNP) was labelled with ^{32}P. Half of the sample was directly analysed by fingerprint (2-A), and half was digested with RNase T1 prior to fingerprint analysis (2-B). First dimension is from the left to the right and second dimension is from the bottom to the top.

adenylated) fractions eluted from the oligo-(dT)-cellulose column upon chromatography of the 15 S mRNP digested with staphylococcal nuclease, are shown in Fig. 4.B (lanes X and Y, respectively). The polyadenylated fraction is characterized by the presence of a major 73,000 MW polypeptide, a band around 47,000 MW and some minor polypeptides, as observed previously in our and other laboratories (14,18-19,34).

One could argue that artefacts might be generated during the oligo-(dT)-cellulose chromatography, due to non-specific binding of proteins and RNA, leading to the appearance of the oligoribonucleotides seen in Fig. 2. To rule out such a hypothesis, and in order to confirm the result described above, we decided to investigate by a different approach the

involvement of RNA sequences non-adjacent to poly(A) in the poly(A)-protein RNP complex.

We isolated the poly(A)-protein RNP complex by sedimentating nuclease digested 15 S mRNP through a 5 %-21 % isokinetic sucrose gradient. Aliquots of each fraction were titrated with ^{3}H-poly(U) in conditions of poly(U) excess. The result obtained is presented in Fig. 3, showing a peak of radioactivity centered around fractions 17 to 21 with a sedimentation coefficient of about 7 S. The arrows indicate the respective positions of 15 S mRNP, 9 S mRNA and the 73,000 MW poly(A) binding protein analysed on parallel sucrose gradients.

To characterize proteins specifically present in the poly(A) containing residual mRNP, we subjected the material present in different pooled sucrose gradient fractions to systematic SDS-polyacrylamide gel electrophoresis (Fig. 4.A). Proteins present in the poly(A) peak fractions 17 to 21 coincide with the proteins that we found associated with the formamide eluted fraction from the oligo-(dT)-cellulose chromatography of nuclease digested 15 S mRNP (lane Y of Fig. 4). Again, one can see the major 73,000 MW poly(A) binding protein, the 47,000 MW polypeptide and some minor components. Once more, the protein composition of the poly(A) residual mRNP differs markedly from that of intact 15 S mRNP which is given by the sum of lanes X and Y (Fig. 4) (8,15-19,34).

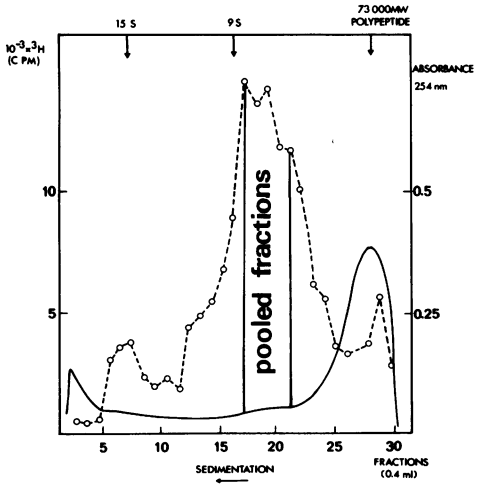

Figure 3 - 15 S mRNP was digested with staphylococcal nuclease and loaded on a 5 %-21 % isokinetic sucrose gradient containing 50 mM KCl, 10 mM TEA pH 7.4, 5 mM EGTA and was centrifuged (16 hours, 41,000 rpm, 4° C, BECKMAN SW-41 rotor). 3.5 µl aliquots were taken from each fraction of the gradient, adjusted to 250 mM Na^{+}, hybridized with ^{3}H-poly(U) in excess, for 30 minutes at 37° C, digested with RNase A (0.25 mg/ml) for 30 minutes at 37° C, precipitated with cold TCA and counted. The broken line shows the profile of the TCA precipitable counts and full line shows the optical density profile. Arrows indicate the respective positions of 15 S mRNP, 9 S mRNA and the 73,000 MW poly(A) binding protein sedimentations on parallel gradients.

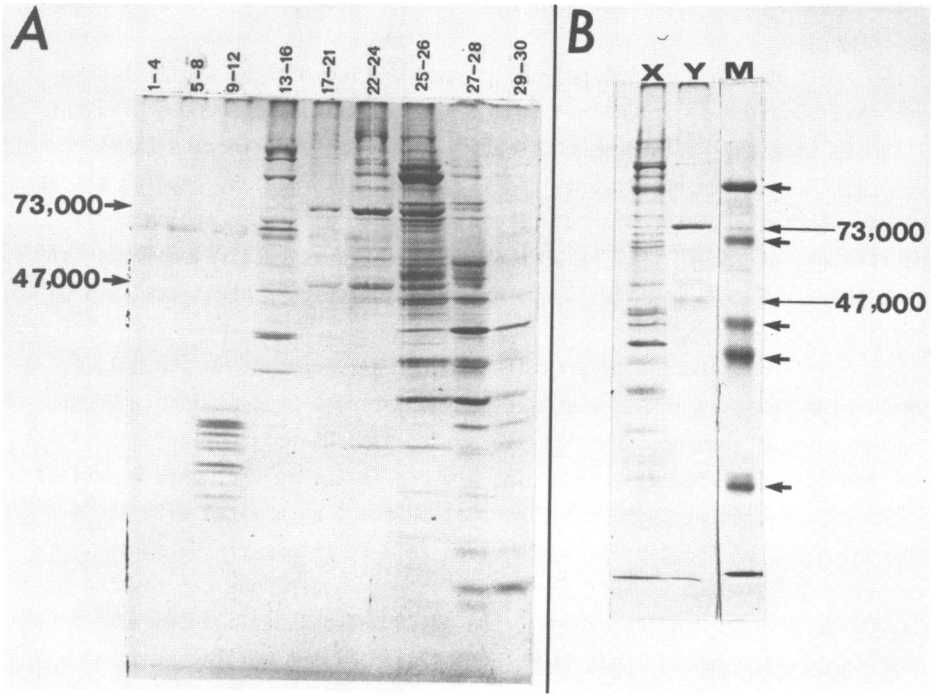

Figure 4 - A) Fractions from the gradient shown in Fig. 3 were pooled as indicated ; each pool was precipitated with ethanol and the proteins were analysed on 13 % SDS-polyacrylamide gel. B) Proteins extracted from the nuclease digested 15 S mRNP chromatographed on oligo-(dT)-cellulose as described in Fig. 2 ; X is the unbound fraction, Y is the polyadenylated formamide eluted fraction and M indicates protein markers, 90K, 68K, 45K, 38K and 25K, respectively, as indicated by the small arrows.

The RNA extracted from the pooled fractions 17 to 21 was labelled in vitro with γ-(^{32}P)-ATP ; half was analysed directly by fingerprint (Fig. 5.A) and half was digested with RNase T1 prior to fingerprint analysis (Fig. 5.B). Comparison with Fig. 2.A and 2.B, respectively, shows that our second approach led to essentially the same result.

Figure 5.A shows that large oligoribonucleotides are present in the poly(A)-protein RNP complex, giving a strong background in the second dimension of the fingerprint. Comparison with Fig. 2.A shows, in both cases, the same large smear and some distinct oligoribonucleotides in the fingerprint. This smear is due to the large size of heterogeneous oligoribonucleotides obtained. Digestion of the oligoribonucleotides shown in Fig. 5.A with RNase T1 led to a fingerprint (Fig. 5.B) identical to that shown in

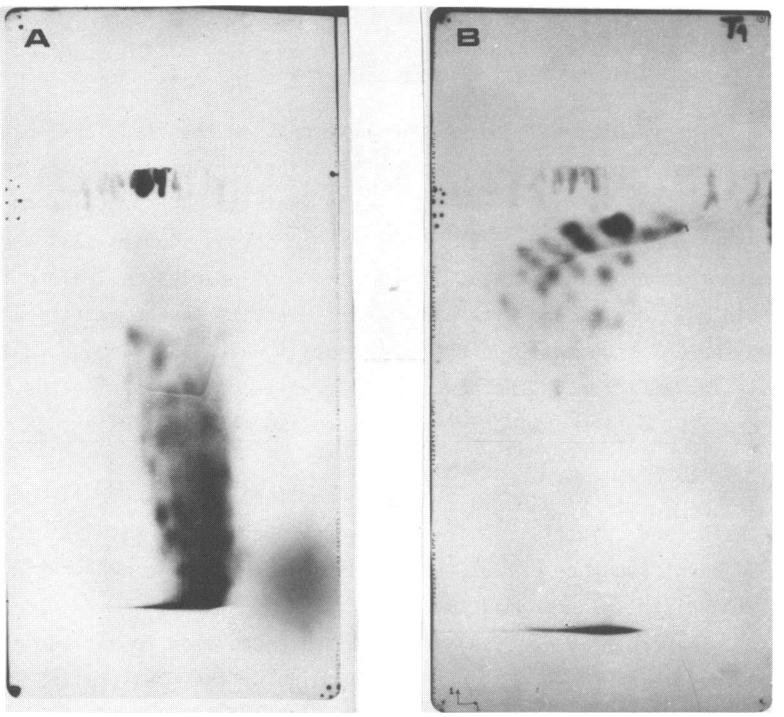

Figure 5 - The RNA extracted from the pooled fractions 17-21 of the sucrose gradient shown in Fig. 3, was labelled with γ-^{32}P-ATP. Half of the sample was directly analysed by fingerprint (A) and half was digested with RNase T1 prior to fingerprint analysis (B). First dimension is from the left to the right and second dimension is from the bottom to the top.

Fig. 2.B when compared spot by spot.

The difference in intensity of spots seen in Fig. 2.B and 5.B is due to the fact that in order to avoid contamination by other oligoribonucleotide sequences, we have taken advantage of the gradient fractionation in pooling just the peak region of the poly(U) titration curve (Fig. 3). The apparent differences are hence explained by the fact that we dit not include fractions 22-24 (despite the abundance of the poly(A) binding protein) which contained additional polypeptides as found in polyacrylamide gel electrophoresis (Fig. 4.A).

In conclusion, two different methods allowed us to identify the poly(A)-protein RNP complex, and corroborate our observation that mRNA sequences non-adjacent to the poly(A) stretch interact with poly(A) and

poly(A) adjacent sequences within the poly(A)-protein RNP complex.

DISCUSSION

The results to be discussed here bear on the structural organisation of the polyribosomal 15 S globin mRNP, liberated off ribosomes by EDTA dissociation. We have shown previously that the mRNP proteins do not interact at random with mRNA (22). Electron microscopy studies (33) showed furthermore that proteins attach, like a string of pearles, all along the duck globin mRNA chain, and with particular intensity to the extremities including the CAP structure and the poly(A) tail which, both, have functions in translation efficiency. The new results presented here bear on the structure of the 3'-terminal poly(A) complex. Two main conclusions can be drawn :

1) The 73,000 MW poly(A) binding proteins are lined up periodically on the poly(A) chain, and 2) this complex interacts in a dynamic fashion with the poly(A) adjacent sequence as well as, bending back upon the mRNA chain, with poly(A) distal mRNA segments.

The protected poly(A)-adjacent sequences seem to include about 10 nucleotides ; this figure is based on summing up the ^{32}P labelled T1 fragments (Fig. 1.C), ranging from 3 to 5 nucleotides in length, which correspond to the three adult globin mRNA chains. The difference in the intensity of some oligonucleotide spots can hence be explained by the ratio of the different globin mRNA molecules present in polyribosomes (32).

Sequences non-adjacent to the poly(A) tail are protected as well ; indeed, the RNA extracted from the poly(A)-protein complex, isolated by two different methods (affinity chromatography and sucrose gradient sedimentation) showsan identical fingerprint pattern, including some oligonucleotides in addition to poly(A) and those of the adjacent regions (e.g. Fig. 2.B and 5.B).

The protein composition of the poly(A)-protein RNP complex isolated by both methods (Fig. 4.A lane 17-21 and 4.B lane Y) is identical and agrees with the results obtained previously by our and other laboratories, showing the major 73,000 MW poly(A) binding polypeptide, a polypeptide around 47,000 MW along with some minor components (14,18,19,34).

Since both of our experimental procedures led to the identification of the same RNA and protein components, we may consider them as reflecting the physiological composition of the poly(A) complex. The 73,000 MW protein is thus the major component of the poly(A) complex, and

of the 15 S mRNP as a whole, present in a stochiometry of 5-10 fold excess relative to minor proteins. Correspondingly, the protected poly(A) segment is quite long as indicated by the "smear" at the origin of the second dimension of the fingerprint. Indeed, this smear disappeared when deproteinized globin mRNA was digested by staphylococcal nuclease (22).

We can therefore conclude that a string of at least 4-5 (likely in view of the CsCl density analysis) (34,35) 73,000 MW proteins are ligned up along the poly(A) tail, which in the case of duck globin mRNA is about 80 nucleotides long (36). This alignment is not haphasard but seems to obey some rule of organisation. Indeed, upon closer inspection the "smear" of poly(A) observed in our fingerprints (cf. e.g. Fig. 1.C) shows a pronounced periodicity in size most likely due to an alternative pattern of regions protected from and exposed to the nuclease attack. Similar observations and conclusions are the subject of recent papers (37,38) published after completion of this investigation.

This string of poly(A) binding proteins cannot be considered as a rigid structure. Electron micrographs (33) already showed large balls at the extremities of the globin mRNP pointing to a curling up of the poly(A) chain. Further evidence for a dynamic structure stems from the very observation of the poly(A) smear in fingerprints of RNA, labelled after T1 digestion. Since poly(A) adjacent sequences are equally protected, the poly(A) chain itself would not be labelled was there not some "breathing" i.e. temporal dissociation of the protecting proteins.

This dynamic process is particularly illustrated by the folding-back of the poly(A)-protein chain which protects mRNA segments not immediately adjacent to the poly(A). Since ribosomes have to move in translation, this fold-back is a priori only a facultative structure. On the other hand, such a tendency of the globin mRNA to form internal secondary structure may be of importance in the control of translation (6,39-40).

We have previously demonstrated the extensive bi-helical structure of duck globin mRNA (36), and evidence is presented here and elsewhere (22) for the interaction of mRNP proteins with regions of secondary structure in mRNA. Secondary structure could be either simply stabilized by proteins, or could provide the recognition sites for the binding of proteins factors. In both cases, a functional role would be carried by mRNP proteins in influencing the control of translation.

It has been proposed that mRNA sequences could hybridize to the 3'-end of mRNA (24), and a computer-generated model of the secondary

structure of rabbit globin mRNA (41) shows that nucleotide sequences from the 5'-end non-coding region of the messenger molecule interact with oligoribonucleotide sequences from the 3'-end, forming several internal hairpin-loops. Such a structure might be possible in the case of duck globin mRNA as well. In the computer model mentioned above, four to five hydrogen bonded structures are involved in the interaction of the 3'-5' non-coding regions. Between 50 and 100 nucleotides are hence protected ; this number fits well with the population of oligoribonucleotides that we observe in the poly(A)-protein RNP complex (e.g. Fig. 2).

Enhancement of secondary structure could play an important role in the termination of translation by destabilizing the mRNA-ribosome interaction diminishing the possibility of protein read-through, as described recently for β-globin (42) and viral systems (43). In such a case, the complex we have studied in this article might have a fundamental role.

More generally we think that ribonucleoproteins play a role in the translation or repression of cytoplasmic mRNPs since, furthermore, different sets of proteins are associated with the same mRNA molecule in different functional states (11,34). Secondary structures might provide recognition sites for repressor protein(s), or assume the stabilization (or de-stabilization) of specific secondary structures which may control mRNA translation or repression.

ACKNOWLEDGEMENTS

We thank N.M. Standart and Cl-A. Reynaud for helpful suggestions and the critical reading of this manuscript ; C. Sagot for help in its preparation and Mr. R. Schwartzmann for the photographs. This research was supported by grants from the French C.N.R.S., D.G.R.S.T., INSERM and the Fondation pour la Recherche Médicale. S.G. was supported by a fellowship from the Brazilian Conselho Nacional de Deşenvolvimento Cientifico e Tecnologico (CNPq).

*To whom correspondence should be addressed

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