EVIDENCE FOR A TRANSLATIONAL INHIBITOR LINKED TO GLOBIN mRNA IN UNTRANSLATED FREE CYTOPLASMIC MESSENGER RIBONUCLEOPROTEIN COMPLEXES

Olivier CIVELLI, Alain VINCENT, Jacques-François BURI and Klaus SCHERRER
Service de Biochemie de la Différenciation, Institut de Recherche en Biologie Moléculaire, Paris, France

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1. Introduction

In eukaryotic cells, mRNA exists in the cytoplasm in the form of ribonucleoprotein complexes [1–3]. In the case of duck immature erythrocytes, the RNA-sequences coding for globin chains have been localized in two sub-cytoplasmic fractions, the '15 S' mRNP bound in polyribosomes and the '20 S' mRNP free in the cytoplasm [4–6]. The proteins bound to globin mRNA from both cytoplasmic sources were previously shown to differ substantially in their composition [5], and it is therefore theoretically possible that the proteins associated with a given mRNA molecule can determine its localization and function within the cytoplasm.

The purpose of this paper is to investigate the possible role of the constituents bound to globin mRNA in the process of translation by analysing, in vitro, the translational activity of the two mRNPs.

Abbreviations: mRNA, messenger ribonucleic acid; mRNP, messenger ribonucleic acid-protein complex; 9 S mRNA, mRNA containing globin sequences; 15 S mRNP, polyribosomal mRNP containing 9 S mRNA; 20 S mRNP, free cytoplasmic mRNP containing 9 S mRNA; 12 S mRNP, particle derived from high-salt washed 15 S mRNP; 13 S mRNP, particle derived from high-salt washed 20 S mRNP; SDS, sodium dodecyl sulfate; EDTA, ethylene diamino tetra acetate disodium salt.

Address correspondence to: Klaus Scherrer, Service de Biochimie de la Différenciation, Institut de Recherche en Biologie Moléculaire, Université de Paris 7 – Tour 43, 2, place Jussieu F-75221 Paris Cedex 05, France

We demonstrate here that the purified globin mRNA from both polyribosomal mRNP and from free cytoplasmic mRNP can be translated in vitro with equal efficiency; that the polyribosomal mRNP particle is also translated with a similar high efficiency; but that in contrast, the free cytoplasmic 20 S mRNP does not stimulate a cell-free protein synthetizing system, and even inhibits the translation of other mRNAs present in the same incubation mixture. We demonstrate further that high-salt wash treatment of the free cytoplasmic mRNP particle, while substantially reducing its sedimentation coefficient, does not destroy its inhibitory action. We therefore suggest that a translational repressor is contained within the salt-resistant globin mRNP-core particle.

2. Materials and methods

Cytoplasm from duck erythroblasts was fractionated into polyribosomes and a polyribosome-free supernatant by ultracentrifugation at 60 000 rev./min for 1 h. 15 S particles were obtained by EDTA dissociation of polyribosomes and free mRNP complexes by exhaustive centrifugation of the supernatant [6].

15 S polyribosome-derived and 20 S free cytoplasmic globin mRNA–protein complexes were purified by two successive sedimentation on low-salt sucrose gradients as described [4,5]. Globin 9 S mRNA was isolated from the polyribosomes by SDS-treatment followed by sucrose gradient purification and phenol extraction [8]. Messenger RNA of the

free cytoplasmic complex was obtained by direct phenol extraction of the purified particle. High-salt washed ribonucleoprotein complexes were obtained by addition of 2 M KCl solution to the purified particles to obtain a final concentration of 0.5 M. Subsequent isolation was performed on isokinetic sucrose gradients (15–32% w/w) containing 0.5 M KCl (Beckman Rotor SW 60, 60 000 rev./min 325 min, 2°C).

In vitro protein biosynthesis directed by the different mRNAs and messenger particles was performed in a wheat germ cell free protein synthetizing system [9]. The incorporation of [^{35}S]-methionine (420 Ci/mmol) into acid-insoluble material was measured by TCA-precipitation of the incubation mixture on Whatman 3MM filters [10]. Electrophoretic analysis of the products of in vitro translation was performed in exponential SDS–acrylamide slab-gels using the buffer system as defined by Laemmli [7]. Acid–acetone precipitated non-radioactive globins were used as markers. The radioactive components separated on these gels were detected by fluorography [11].

3. Results

3.1. *In vitro translation of the mRNA–protein complexes and their corresponding mRNA*

3.1.1. Translation of polyribosomal globin mRNA and the mRNA isolated from the free cytoplasmic 20 S mRNP

In the wheat germ protein synthetizing system, the incorporation of labelled methionine into polypeptides (fig.1A) can be efficiently stimulated by polyribosomal 9 S mRNA and by the mRNA isolated from the free cytoplasmic 20 S mRNP (fig.1A). On SDS–polyacrylamide gels, all detectable products of translation directed by these two species of mRNA co-migrate with the two major bands of the purified duck globins (cf. fig.4). This suggests that these two mRNP particles contain predominantly globin-coding mRNA sequences. Dose–response curves indicate that the mRNA prepared from both sources stimulate the wheat germ system with equal efficiency (fig.1B).

3.1.2. In vitro translational activities of the two cytoplasmic globin mRNA-protein particles

Figure 1A shows the time course of in vitro translation of the different mRNAs and their corresponding ribonucleoproteins. From their respective buoyant densities [6], the amount of each particle included in the reaction mixture was calculated so that the same quantity of RNA was added in each case.

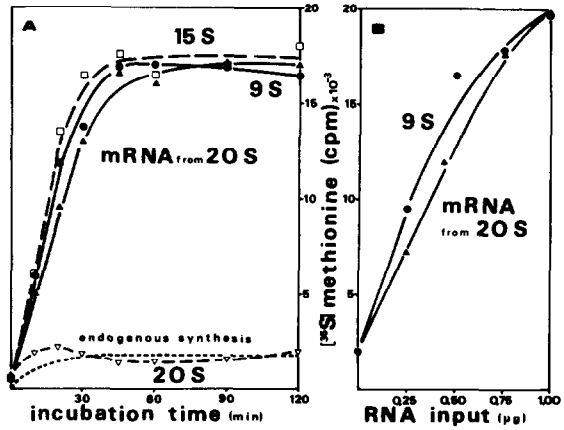

Fig.1. In vitro translation of the globin mRNPs and their corresponding mRNAs. A. Kinetics of translation. In a total volume of 50 µl, the incubation mixture contained 15 µl of wheat germ extract 2.5 µCi [^{35}S] methionine (420 Ci/mmol) and 0.5 µg of mRNA or 2 µg of mRNP (containing 0.5 µg RNA). The final ionic concentrations were 80 mM KCl and 3 mM Mg-acetate. Aliquots of 5 µl were taken at the indicated times, TCA-precipitated on Whatman 3MM filters and the incorporated radioactivity was measured as described [10]. Endogenous synthesis was determined by precipitation of 5 µl samples of the wheat germ reaction mixture in absence of added mRNA of particles. B. Translation as function of mRNA concentration. Increasing amounts of polyribosomal 9 S mRNA and of mRNA isolated from the free 20 S mRNP were incubated in a final volume of 50 µl incubation mixture as described in A. 5 µl samples were precipitated after incubation for 45 min at 30°C. (●——●) Polyribosomal 9 S mRNA (▲——▲) mRNA isolated from 20 S mRNP (□——□) polyribosomal 15 S mRNP (▽——▽) free cytoplasmic 20 S mRNP (- - - - - -) endogenous synthesis.

The data in fig.1A show that the polyribosomal 15 S mRNP particle has a translational efficiency in vitro which corresponds closely to its purified 9 S mRNA. As expected, the 15 S mRNP directs the synthesis of polypeptides which co-migrate in SDS-gels with the duck globins (fig.4). In marked contrast, the free cytoplasmic 20 S mRNP does not stimulate incorporation of labelled amino acids into polypeptides. Furthermore, during early

attemps to translate the 20 S mRNP particle, it became apparent that, in addition to being untranslated itself when incubated in the wheat germ system, the free particle exerts an inhibitory effect on endogenous wheat germ protein synthesis.

3.1.3. Effect of the 20 S mRNP on the translation of 9 S mRNA and 15 S mRNP

Having observed an inhibitory effect of the 20 S mRNP on the endogenous wheat germ protein synthesis, we decided to study its effect on the translation of exogenous mRNA or mRNP. In the experiment shown in fig.2A, 20 S mRNP was incubated in the reaction mixture with 9 S mRNA, or with 15 S mRNP, such that the estimated RNA molar ratio was 1 : 3 in each case. This corresponds to their relative concentrations in vivo. It is evident that under these conditions there is a reduction of 55–65% in the translational activities of both the polyribosomal globin mRNA and the 15 S messenger RNP particle. Furthermore, as shown in fig.2B, the extent of inhibition is related to the molar ratio of 20 S mRNP to 9 S mRNA (or to 15 S mRNP). When stoichiometric amounts of 20 S mRNP and polyribosomal messenger are incubated together the inhibition of translational activity is greater than 90%.

3.2. *In vitro translation of high-salt washed ribonucleoprotein complexes*

3.2.1. Isolation of ribonucleoprotein complexes at high ionic strength

In order to exclude the possible presence of an inhibitory contaminant resulting from isolation of the 20 S mRNP in low-salt buffer, experiments were carried out to determine whether this effect remains associated with the globin mRNP particle after treatment with buffer of high-salt concentration.

Polyribosomal and free cytoplasmic mRNP, purified by centrifugation on low-salt sucrose gradients (Materials and methods) were exposed to 0.5 M KCl and recentrifuged on sucrose gradients containing 0.5 M KCl buffer. The results of such treatment are shown in fig.3. Control preparations not exposed to KCl were analysed simultaneously on low salt gradients of the same sucrose concentrations.

Under conditions of high ionic strength, the 15 S polyribosomal mRNP migrates predominantly as a 12 S particle; no released material absorbing at 260 nm can be observed in the lighter fractions of the gradient.

To our surprise, under the same conditions, the free cytoplasmic 20 S mRNP proved to be a more complex structure. The 20 S material, which migrates as a homogenous peak on low-salt gradients is separated into two major components on high-salt gradients with sedimentation coefficients of 13 S

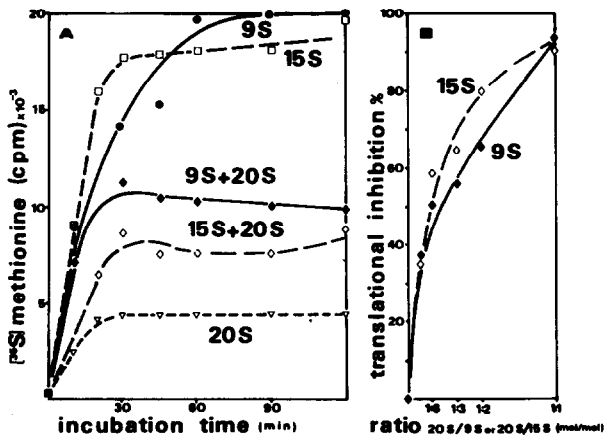

Fig.2. Inhibition of the translation of 9 S mRNA and 15 S mRNP in the presence of 20 S mRNP. A. Kinetics of inhibition. 9 S mRNA and 15 S mRNP either alone or in presence of 20 S mRNP in a ratio 3 : 1 (mol/mol) respectively were incubated in a final volume of 50 μl of wheat germ incubation mixture. Aliquots of 5 μl were removed at various time points, as indicated on the graph. For comparison 20 S mRNP (same amount as used in the presence of 9 S mRNA or 15 S mRNP) was incubated alone in the cell-free mixture. Conditions were the same as in fig.1A. B. Percentage of inhibition as a function of the concentration of 20 S mRNP. In a final incubation volume of 50 μl containing 15 μl of wheat germ extract, 9 S mRNA or 15 S mRNP were incubated with increasing amounts of 20 S mRNP. After 60 min at 30°C, aliquots were removed, precipitated on 3 MM Whatman filters and the incorporation of [^{35}S] methionine into polypeptides was determined. Incorporation of isotope due to the presence of 20 S mRNP alone was substracted. Inhibition for different ratios (mol/mol) of 20 S mRNP to 9 S mRNA or 15 S mRNP in expressed as a percentage of the translation of 9 S mRNA of 15 S mRNP alone. Zero inhibition represents the incorporation obtained in absence of 20 S mRNP. (●——●) 9 S mRNA alone (□——□) 15 S mRNP alone (▽——▽) 20 S mRNP alone (◆——◆) 9 S mRNA + 20 S mRNP (◇——◇) 15 S mRNP + 20 S mRNP.

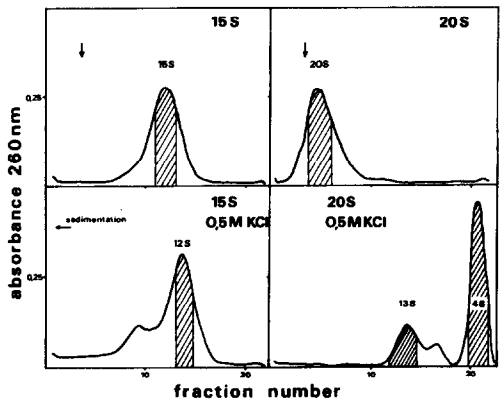

Fig.3. Sedimentation on sucrose gradients of the mRNPs obtained after high-salt treatment. To 0.5 A_{260} units of purified 15 S mRNP or 20 S mRNP, suspended in a buffer contained 10 mM KCl and 10 mM triethanolamine (TEK) pH 7.4, 2 M KCl solution was added to obtain a final concentration of 0.5 M KCl. These two high-salt preparations were loaded onto separate 15–32% (w/w) isokinetic sucrose gradients containing 0.5 M KCl, 10 mM triethanolamine, pH 7.4. In parallel 0.5 A_{260} units of purified 15 S mRNP and 20 S mRNP, suspended in 10 mM TEK, were loaded onto isokinetic sucrose gradients of 15–32% (w/w) contained 10 mM TEK, pH 7.4. Centrifugation was for 325 min at 2°C (Beckman Rotor SW 60, 60 000 rev./min). Absorbance at 260 nm was monitored. The arrows indicate the position of EDTA-treated small ribosomal subunits (26 S) according to [19], in a low salt gradient. The peaks were pooled as indicated, and concentrated by dialysis under vacuum. (——) absorbance at 260 nm.

and 4 S. Analysis of the ultraviolet absorption characteristics on the two particles liberated by high-salt treatment of the 20 S mRNP indicates that both fractions contain a mixture of nucleic acid and protein.

3.2.2. Translation of the particles obtained by high-salt treatment

The high-salt treated ribonucleoprotein complexes, isolated as described above, were tested in the wheat germ translation system for their ability to stimulate incorporation of labelled amino acid into acid-insoluble products.

The 12 S particles, liberated by salt treatment of the polyribosomal 15 S mRNP, stimulates protein synthesis in vitro with the same efficiency as the intact 15 S mRNP (data not shown). The products of translation of the high-salt washed particle are the same as those of untreated 15 S mRNP, and co-migrate on SDS–polyacrylamide gels with duck globin markers (fig.4).

In contrast, the two fractions derived from the

Fig.4. Analysis of products of in vitro protein synthesis. The wheat germ cell-free mixtures, containing different mRNAs or particles (9 S mRNA, mRNA isolated from 20 S mRNP, 15 S mRNP, and 14 S mRNP obtained by salt wash of the 15 S mRNP) were incubated for 2 H at 30°C and immediately chilled at 0°C. Conditions of incubation were the same as in fig.1A. Aliquots of about 8 000 TCA-precipitable cpm were removed and loaded onto 10 cm exponential (10–15% w/v) SDS–acrylamyde slab-gels. Non-radioactive globin chains were used as markers; electrophoresis was for 5 h at 25 mA. After staining in Coomassie blue, the gel was impregnated with PPO (2,5-Diphenyloxazole) and processed for fluorography using the technique of Laskey and Mills [11]. Slots show the products of translation after exposure to Kodak RP Royal X-Omat for 24 h at −70°C. (A) 9 S mRNA (B) mRNA isolated from 20 S mRNP (C) 12 S mRNP isolated by salt wash of 15 S mRNP. (D) 15 S mRNP. The arrows indicate the position of the duck globin chains.

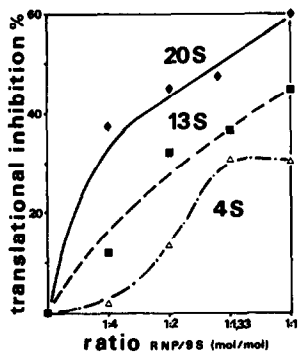

Fig.5. Inhibitory effect of the components obtained by high-salt treatment of the 20 S mRNP particle. In 50 μl incubation mixture, 9 S mRNA was mixed with increasing amounts of 20 S mRNP or with either the 13 S or 4 S RNP complexes, obtained by salt wash of the 20 S mRNP as described in fig.4. The (mol/mol) ratio, indicated in the abscissa, was measured by absorbance at 260 nm, assuming a buoyant density of 1.4 g/ml for the different RNP complexes. Incubation was for 60 min at 30°C. The percentage of inhibition was calculated as in fig.2B. (♦——♦) 9 S mRNA + 20 S mRNP (■——■) 9 S mRNA + 13 S RNP complex (△——△) 9 S mRNA + 4 S RNP complex.

free cytoplasmic 20 S mRNP by high-salt treatment do not stimulate incorporation of labelled amino acids in vitro; in addition, as was found for the intact 20 S mRNP particle, both 13 S and 4 S components possess an inhibitory effect on the translation of exogenous 9 S mRNA. In fig.5, the extent of inhibition of translation of the 9 S mRNA by these particles is expressed as a function of increasing particle concentration. This analysis demonstrates that the strongest inhibiting capicity is found associated with the 13 S fraction, and thus that even under high-salt conditions in which non-specific RNA–protein interactions are eliminated [18], a factor capable of inhibiting mRNA translation in vitro remains bound to the globin mRNP core particle.

4. Discussion

In this report we demonstrate that the mRNA extracted from the free 'informosomal' [2] cytoplasmic 20 S mRNP directs in the wheat germ translation system, the synthesis of duck globin chains with the same efficiency as the 9 S mRNA extracted from the polyribosomes. As judged by the fluorogram of fig.4, it appears that at least the majority, and possibly all of the translatable sequences in these two classes of mRNA code for globin chains. These results are in agreement with our previous reports [5,12].

In the wheat germ protein synthetizing system, the polyribosomal 15 S mRNP also stimulates synthesis of duck globins with the same efficiency as does the purified 9 S mRNA.

It seems therefore that, in vitro, the proteins associated with the globin mRNA in polyribosomes exert neither positive nor negative control on the process of translation. This observation is in agreement with results obtained for other polyribosomal mRNPs in different cell-free systems [13–16].

In contrast, the 20 S mRNP is not translated in vitro and, in its presence, the translation of globin mRNA or mRNP is clearly inhibited. Moreover, the extent of inhibition of translation is very similar when 9 S mRNA or 15 S mRNP are translated. This indicates that the proteins bound to the polyribosomal mRNA do not, in vitro, prevent the inhibitory effect of the free cytoplasmic particle, at least in the case when the two mRNPs are present in approximately equimolar amounts.

The experiments using high-salt washed 20 S mRNP were performed in order to determine whether the inhibitory capacity was located in the loosely bound components of free globin mRNP, or whether it was retained in the salt-resistant core particle. At high ionic strength, the 20 S mRNP is separated into two major fractions which migrate in sucrose gradients as 13 S and 4 S components. Control experiments with labelled RNA show that 9 S mRNA sediments with the 13 S particle.

Experiments comparing the effects of these two components of the 20 S mRNP on translation has shown first that neither component alone stimulates in vitro translation (data not shown) and, also, that both components possess inhibitory action on the translation of exogenous 9 S mRNA. Their inhibition curves however are not identical. The 13 S fraction possesses an inhibitory capacity which is stronger than that of the 4 S component and which, as judged by the dose–response curve (fig.5),

resembles more closely that of the intact 20 S mRNP.

The mechanism of translational inhibition by the free cytoplasmic particle is not yet known. Control experiments in which 20 S mRNP and polyribosomal mRNA were incubated together prior to translation in the wheat germ system, indicate that ribonuclease activity is not present in the 20 S mRNP, and thus, that the observed effect of translational inhibition is not a consequence of mRNA degradation (data not shown). Further, the 20 S mRNP was also shown to possess similar inhibitory effect on translation of mRNAs isolated from other sources (chicken liver, rabbit reticulocytes, TYMV), indicating that the observed inhibition due to the free cytoplasmic mRNP is a general effect on the translation of mRNA in vitro. Experiments are in progress to determine the nature of the inhibitory factor.

The non-translatability of the globin mRNA sequences in the 20 S mRNP represents, to our knowledge, the first direct evidence for the existence of a cytoplasmic form of 'masked messenger RNA' as originally proposed by Spirin in his 'Informosome' theory [2]. The presence of a translational repressor in the free cytoplasmic particle could extend this concept to include a general role of a factor controlling the balance of translated and non-translated globin mRNA in the cytoplasm, as suggested within the framework of the 'Cascade Regulation Hypothesis' [20].

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