In vitro translation studies of the cytoplasmic nonpolysomal particles containing messenger RNA

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ABSTRACT

We analyzed the translational capacity of different kinds of free cytoplasmic messenger ribonucleoprotein complexes (free mRNP) in a HeLa cell free system. Native free mRNP are not translated although free mRNP washed with 0.5 M KCl can direct polypeptide synthesis. Furthermore, the 0.5 M KCl wash possesses a factor which inhibits the translation of 0.5 M KCl washed free mRNP as well as globin mRNA naked mRNA from plasmacytoma, or HeLa cells. We also demonstrated that native free mRNP are able to form a complex with ribosomal subunits in the presence of initiation factors. This indicates that inhibition of translation by the 0.5 M KCl wash occurs either at some point after initiation complex formation or at the elongation step.

INTRODUCTION

In prokaryotic cells the control of gene expression occurs mainly at the level of transcription (1). In eukaryotic cells, transcription and translation sites are physically separated by the nuclear membrane, giving rise to the possibility of regulation by post transcriptional mechanisms. Besides the mRNA engaged in protein synthesis there is a class of mRNA sequences found in the cytoplasm free of ribosomes but associated with a specific set of protein (2, 3). However, the proteins bound specifically to the non polysomal mRNA are not very well characterized mainly because purification of these particles is hard to achieve. The question which arises is whether these untranslated non-polysomal mRNA have retained their functional capabilities in the free mRNP. And if so whether their inability to be translated in the cell is due to the conformation of the complex, the structure of the mRNA itself and/or to the presence of some cytoplasmic regulator of translational control. Concerning the ability of free mRNP to be translated, very few works appear in the literature, and there are some discrepancies in the obtained results. In skeletal muscle, the non-polysomal 120S particle is able to program the in vitro synthesis of myosin heavy chain (MHC) in a rabbit reticulocyte

lysate (4). Another study shows that the free cytoplasmic 20S mRNP did not stimulate a cell free protein synthesizing system and even inhibited the translation of other mRNAs (5). The same observation was demonstrated with sea-urchin free mRNP (6, 7).

MATERIAL AND METHODS

Isolation of free mRNP : HeLa cells were grown in suspension as previously described (8) and broken in Buffer A (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 3 mM MgCl₂, 7 mM 2-mercaptoethanol, 0.1 mM hemin) using a Dounce homogenizer. Plasma cells tumours RPC₅ were grown on Balb/c mice. The mice received at 3.5 h and 3 h before sacrifice respectively, 100 µg of actinomycin D and 2 mCi/mouse of [³²P] orthophosphate. As has been shown elsewhere (9) actinomycin completely inhibits rRNA synthesis under these conditions. Post mitochondrial supernatants from both source were layered on discontinuous D₂O sucrose gradient and centrifuged during 20 h at 4° C and at 180.000 g in the Spinco rotor 60 Ti (9). The buffer solutions were adjusted at 150 mM KCl in order to minimize non-specific protein adsorption to particles. The pellet was conserved as the free polysomal fraction and the fraction D₂O-sucrose of a density 1.29 containing free mRNP, was removed diluted for a 0.35 M sucrose concentration and centrifuged 3 h at 200.000 g in a Spinco 50 Ti rotor. Equilibrium sedimentation of free mRNP fraction on a CsCl density gradient showed that the labelled mRNA under actinomycin treatment had a buoyant density of 1.4 with a relatively broad distribution of densities, whereas ribosomal particles were virtually absent (9). The pelleted free mRNP fraction was divided in two parts ; one was conserved as "native free mRNP " ; the other part (free mRNP 200 A 260 nm units/ml) was resuspended in a buffer B (10 mM Tris-HCl pH 7.4, 3 mM MgCl₂, 500 mM KCl, 2 mM 2-mercaptoethanol) stirred 15 min at 4° C and centrifuged at 180.000 g in the Spinco SW 41 rotor for 5 h. The supernatant was precipitated by 70 % SO₄(NH₄)₂ at 0° C. The proteins were dissolved in buffer C (30 mM Hepes, pH 7.4 ; 3 mM magnesium acetate, 120 mM potassium acetate, 7 mM 2-mercaptoethanol) and dialyzed against this buffer. The pelleted 0.5 M KCl washed particles were resuspended and dialyzed against the buffer C. CsCl density gradient of 0.5 M treated free mRNP showed the labelled mRNA with a buoyant density greater than 1.40 ; this was due to the partial elimination of protein from free mRNP (data not shown).

Preparation of cell free protein synthesis system : The cell free protein synthesizing system consisted of HeLa cell extracts supplemented with reticulocyte initiation factors as previously described (10). HeLa cells were broken using Dounce homogenisation in buffer A. The post-mitochondrial supernatant (S 30) was passed through a G-25 Sephadex column equilibrated with buffer C, for elimination of amino-acid and small molecules. The flow through fraction containing cytoplasmic extract was stored at -90°C for 2 weeks. Globin mRNA and crude initiation factors from rabbit reticulocytes were prepared according to the SCHREIER and STAHELIN procedure (11). In order to eliminate endogeneous protein synthesis, stored cytoplasmic extracts (S 30) were treated with 50 U/ml micrococcal nuclease in the presence of 1 mM CaCl_2 . After 15 min at 20°C , 2 mM EGTA was added to inhibit the enzyme (10). The incubation system contained 10 $\mu\text{l/ml}$ of initiation factors, 10 μl of master MIX containing 1.2 μCi [^3H] lysine (20 Ci/nmole, CEA, Saclay) and 30 μl of other components e.g. mRNA, free mRNP ... in buffer C. Incubation time was 1 h at 30°C . Aliquots of the sample were spotted on Whatman filters and then precipitated according to the technique of MANS and NOVELLI (12). RNA from mRNPs were extracted by the phenol/chloroform method (13).

Sodium dodecylsulfate gel electrophoresis : To analyse proteins synthesized by the cell free system, 45 μl of translation mixture were treated with 1 μg of RNase A (Sigma) 10 min at 30°C , then precipitated with 10 % trichloroacetic acid. The pellet washed with ether-ethanol was dissolved by heating 2 min at 90°C in 100 mM Tris-HCl buffer pH 7.4 containing 1% 2-mercaptoethanol and 2 % SDS. The proteins were run on a SDS acrylamide slab gel (15 %) and fluorography was carried out according to BONNER and LASKEY (14).

RESULTS

Translational efficiency of free mRNP in HeLa cell cell free system : "Native free mRNP", 0.5 M KCl treated free mRNP and mRNA extracted from free mRNP from HeLa cells or from plasmacytomes were assayed for their translational capacity in the HeLa cell-free system made dependent upon exogenous message by nuclease treatment. When native free mRNP were used as messenger in the HeLa cell free system, very low incorporation of [^3H] lysine was found in the neo-synthesized polypeptides (Fig. 1). This is in contrast to the result obtained with mRNA extracted from the free mRNP particles which is an active template (Fig. 1) as active as polysomal mRNA (data not shown). These results confirm that the difference in the translation ability cannot be

Fig. 1 : Comparison of the in vitro translational activity of free mRNA and their RNA :

Free mRNA were purified from HeLa cells by centrifugation on D₂O sucrose gradients and one third of the particles were washed by 0.5 M KCl as described in Material and Methods. Another third was used for mRNA extraction. Free mRNPs, salt washed mRNA and free mRNA /RNA were used as messenger in a HeLa cell in vitro proteins synthesizing system containing [³H] lysine. Different amount of RNP expressed by their RNA content were used as messenger to synthesize proteins during 1 h at 30° C. 5 µl were spotted on Whatman paper and hot TCA precipitated material counted.

- ▲ ——— ▲ : Naked RNA extracted from free mRNA
- ——— ○ : Salt washed free mRNA
- ——— ● : Native free mRNA

explained only by some messenger RNA modification. As compounds eliminated during the chloroform-phenol extraction could be responsible for the absence of translation of free mRNA, factors associated with mRNA in the mRNA complex which might alter the translation efficiency were looked for. In order to test this hypothesis we treated native free mRNA under relatively mild conditions, using 0.5 M KCl. After this treatment, the particles called "0.5 M washed free mRNA", displayed translational activity. As it could be deduced from Fig. 1, for 2.6 µg of RNA content, 0.5 M washed free mRNA, shows a considerable increase in their mRNA template activity compared to naked free mRNA-RNA (around 5.000 cpm of tritiated lysine) in the same experimental conditions (See Material and

Methods). The fact that 0.5 M washed free mRNP directed protein synthesis with a lower template activity than naked mRNA (Fig. 1) suggests two major possibilities : either the template activity of washed free mRNP is less efficient due to some compounds still remained on the mRNA and/or to mRNA conformation itself or only a certain population of washed free mRNP express their template activity after the 0.5 M KCl treatment. To discriminate between these two possibilities, we analysed by gel electrophoresis the [^3H] lysine labelled polypeptide products from naked mRNA and salt washed free mRNAs (Fig. 2). Roughly

Fig. 2 : Gel electrophoresis of polypeptide synthesis directed by differently treated Hela free mRNP :

40 μl of the incubation mixture corresponding to 4 μg RNA were treated 15 min at 30° C with 1 μg RNase A, then precipitated by 10 % TCA. The proteins were analysed by SDS gel electrophoresis on 15 % acrylamide slabs (Material and Methods). GL = globin

Track a : Blank without RNA

Track b : Salt washed free mRNP

Track c : Native free mRNP

Track d : Naked free mRNP -RNA

Track M : Markers (Bovin serum albumin, ovalbumin, chymotrypsogen, cytochrome C)

the same protein patterns were obtained when 0.5 M KCl washed free mRNP (track b) and naked mRNA from Hela cells (tracked) were used as templates. The intensity of protein labelling reflects the difference of template activity between samples. Incorporation per μg RNA is less with 0.5 M KCl treated particles (track b) than with "naked mRNP-mRNA". However, at first approximation it appears that the messenger RNA population are translated in both cases. However, we have to notice in each autoradiography of gel the presence of globin band. This band is the translation product of globin mRNA present in the reticulocytes initiation factors, which were added in the Hela cell-free system ; the initiation factor fraction was not treated by micrococcal RNase (11).

Action of 0.5 M KCl extract from free mRNP : As it could be deduced from preceding results, the material extracted from the particles by 0.5 M KCl should have some inhibitory effect on the translation efficiency of free mRNP-mRNA. The results are shown in Table 1. The 0.5 M KCl extract dialyzed in buffer C was tested in the Hela cell free system using different mRNA as templates. The 0.5 M KCl extracts inhibit the translational capacity of free mRNP-mRNA as well as exogeneous globin mRNA. Moreover, once a sufficient quantity of 0.5 M KCl extract is added to the translatable 0.5 M washed free mRNP (Fig. 1),

Table 1 : Inhibition of translation by 0.5 M KCl extract removed from free mRNP .

PROTEIN ADDED		TEMPLATE		
0.5 M KCl extract	Bovin serum albumin	Globin mRNA (1 μg)	Free mRNP (1 μg)	Washed free mRNP (3 μg)
-	-	38 920	18 950	6 230
5 μg	-	4 540	4 400	3 290
-	5 μg	37 950	18 800	6 050
10 μg	-	4 190	4 170	3 020
-	10 μg	38 200	18 150	5 850

In vitro proteins synthesizing system was carried out with washed free mRNP or mRNA prepared as described in Material and Methods. An aliquot of 0.5 M KCl extract expressed in protein concentration and prepared as in Material and Methods was added to the cell free system and incubated 1 h at 30° C. The values determined the amount of [^3H] lysine (cpm) incorporated in synthesized polypeptide.

the inhibitory effect is completely restored (Table 1). The amount of added material (expressed in protein concentration) to 0.5 M KCl washed free mRNP was calculated on the basis of the 1.40 density in CsCl gradient which is one of the physico-chemical characteristic of free mRNP (2). Addition of bovine serum albumin 5 to 20 μ g in the cell free system or greater amount of post mitochondrial supernatant S 20, has no effect on the translation efficiency of either globin mRNA or free mRNP-mRNA. From control levels, expressed as [3 H] lysine incorporation into polypeptide (See table 1), addition of serum albumin has no effect on the translational capacity of the mRNA (less than 5 % of reduction) whereas addition of 5 μ g of 0.5 M KCl extract gave 85 % inhibition. It is possible that an RNase activity is extracted from the particles in 0.5 M KCl salt and is responsible for the inhibition of free mRNP-mRNA upon addition to the cell free system. Such an inhibitory activity has been found associated with reticulocytes ribosomes (15) and shown to be the consequence of an unusual endonuclease. Results presented in Fig. 2 demonstrate that RNA extracted from free mRNP directed the production of proteins with molecular weight about 10.000 to more than 60.000 which represent translation of mRNA from 8 S to 22 S. Furthermore, native plasmocytoma cell free mRNP incubated with homologous cytoplasmic [3 H] RNA show no remarkable endogeneous RNase action : less than 5 % of the RNA was degraded after 1 h incubation time in Tris-HCl 10 mM pH 7.6 at 37° C (results not shown). Consequently the non-translatibility of free mRNPs may be due to material present in the free mRNP itself, and not as a result of either non specific association e.g. soluble cytoplasmic material or RNase action.

Association of free mRNP with cell-free protein synthesizing system component : CIVELLI et al (5) have shown that cytoplasmic 20 S mRNP containing globin mRNA from duck erythroblasts do not stimulate a wheat germ cell-free system. They found that the presence of 20 S mRNP inhibits the translation of purified 9 S globin mRNA, 15 S globin polysomal mRNA (translatable particles) as well as naked mRNA . We have performed similar experiments to determine if free mRNP per se have some inhibitory effect on the in vitro translation. Various amount (expressed by their RNA content) of salt washed free mRNP , or "native free mRNP" were mixed with purified mRNP-RNA, and this mixture was added to the HeLa cell cell free system. Fig. 3 shows that a greater amount of salt washed mRNP or "native free mRNP" than naked globin mRNA or HeLa free mRNP-mRNA was necessary to obtain 50 % inhibition. Inhibition of excess of mRNA was observed by ANDERSON group (30). This implies first that

Fig. 3 : Inhibition of globin mRNA translation

Activity of the HeLa cell free proteins synthesizing system was analysed using different amount of gl. mRNA (○—○) and HeLa mRNA (△—△). Competitions experiments were performed including in the cell free system 1 µg globin mRNA and various amount of salt washed free mRNP (●—●) or native free mRNP (▲—▲) from HeLa cells. Protein synthesized were monitored by hot trichloroacetic acid precipitation as described in Material and Methods.

free mRNP per se does not act as an inhibitor of other mRNA ; if it did, the inhibition would be immediate ; secondly it seems that the components required for translation expression are shared between the two entities : naked mRNA and free mRNP. From the results shown Fig. 3, it could be deduced that initiation factors and ribosomal subunits have a greater affinity (possibility to be associated) for naked mRNA than for free mRNP as it was equally observed elsewhere (31) ; it could also be noticed that a greater amount of "native" free mRNP than 0.5 M salt washed mRNP was necessary to add to 1 µg of naked globin mRNA to see 50 % inhibition. This could be correlated with the fact that 0.5 M KCl washed free mRNP are able to direct the synthesis of some polypeptides (Fig. 2). Also an excess of washed or native free mRNP inhibits translation mechanism as well as naked mRNA which

suggest that they can interfere with protein synthesis components (31). To confirm this, *in vivo* labelled native free mRNP, prepared as described above, were added to an incubation mixture containing all the required components for protein translation (initiation factors, ribosomal subunits, but no naked endogeneous messenger RNA). After incubation, the samples were centrifuged on 15-30 % sucrose gradient as described in the legend of Fig. 4. The gradients show that once free mRNP are in presence of components required for protein synthesis, some association occurs. This could be noticed by the fact that the mRNP-mRNA which was labelled under actinomycin treatment (See Material and Methods) were bound to both the 40 S small ribosomal subunit and to 80 S ribosome, in order to form complexes as it is evidenced on the figure 3, respectively at 50 S and 105-110 S. This association is relatively tight ;

Fig. 4 : Binding of free mRNP to ribosomes after incubation in the cell free system.

³²P *in vivo* labelled plasmocytes free mRNP were purified as described in Material and Methods. Half of the labelled material was incubated in the Hela cell free system for 20 min at 30° C. The different fractions were layered on a 15-30 % sucrose gradient in buffer A, and centrifuged in a Beckman SW 41 rotor for 15 h at 20 000 rpm and 4° C.

³²P labelled and cold trichloroacetic precipitated free mRNP (●—●) incubated in a cell free system (○—○) were run in parallel.

the incubation medium and the gradient contain 150 mM KCl and 6 mM Magnesium acetate. Control experiments were performed and indicated that incorporation of [3 H] lysine plateaus after 1 h and no significant degradation of synthesized polypeptides and messenger RNA occurs. In HeLa cell free as in reticulocyte system it seemed that the majority of messenger RNA were initiated after 10 min incubation time at 37° C. Also, to maximize elongation, termination and release, samples were routinely incubated for 20 min. In these conditions, we consider that degradation action is negligible.

DISCUSSION

In this study, the free mRNP from HeLa cells and from plasmocytes when prepared under isotonic buffer conditions were found to be untranslatable in a cell free system. On the contrary, both the 0.5 M KCl washed mRNP and the extracted mRNP-mRNA were able to direct protein synthesis. Though the synthesized polypeptides profiles are identical for both entities (Fig. 2), the translational efficiencies of the messenger RNA differ. This observation is in agreement with those obtained from free mRNP from chick embryos (4), sea urchin eggs (7) ; those particles were prepared using high salt concentration.

That free mRNP are not translated in a cell free synthesizing system is not the result of inhibition of translation by some non specific adsorption of proteins to the free mRNP. Neither the addition of soluble cytoplasmic proteins from a post mitochondrial supernatant (S 20), nor the addition of bovin serum albumin inhibit polypeptide formation by salt washed mRNP. Also polysomal mRNP when prepared in the same isotonic buffer than free mRNP are translated with the same efficiency as naked messenger RNA (data not shown and 5). It is clear from our results (Fig. 1 ; Table 1) that the removal by 0.5 M KCl of some components is an important step in the conversion of some mRNP particles from a nontranslatable form to the translatable one usually associated with ribosomes. In the cell with rapid growth rates, e.g. cultured cells or tumors, the kinetic studies (17, 18) have shown that at least a part of the free mRNP-mRNA behave as precursor for the polysomal mRNA. Thus, some mRNA species must be maintained in an inactive state in HeLa or plasmocytoma cells. Obviously, those mRNP do not contain a special class of mRNA which may code for some proteins (Fig. 2) that are required by the cells at a particular event of their life cycle as it is known for histone mRNA (19). The inhibitor factor removed by 0.5 M KCl has some effect on other purified mRNA as messenger globin RNA ; this does not occur with associated sea urchin eggs mRNP proteins (7). The nature of inhibitor is not yet determined ; this

may be due to some RNA species as translational control RNA (21, 22) or small poly-U rich RNA (23, 24), or to some proteins as it was suggested elsewhere (5, 20). Previous results have shown that 0.5 M KCl wash from free mRNP contain protein as well as RNA (25). Works are in progress in our laboratory to isolate and study this factor in protein synthesizing machinery.

The existence of free mRNP themselves and their ability to be associated in vitro with 40 S subunit and 80 S ribosome, without being translated is rather intriguing (Fig. 1 and Fig. 4). First it must be pointed that 10 fold excess mRNP are necessary to add to naked mRNA to obtain 50 % inhibition (Fig. 3). Consequently it may be emphasized that the engagement of mRNP is probably reversible because free mRNP per se do not inhibit the translation of mRNA in a cell free system. Furthermore LEE and ENGELHART (26) have found that cytoplasmic mRNA representing a specific set of mRNA could be included in the polysomes in the presence of cycloheximide. This may imply that free mRNP represent a class of messenger which although not very active in protein synthesis can be translated with low efficiency when the competition for the initiation is not too great. Perhaps it is during the first translation that inhibitor bound to mRNA is exchanged and/or modified. Secondly the existence of non translated mRNP-80 S ribosome complexe was observed in reticulocyte system deprived of hemin. In sucrose gradient only 80 S and 120 S ribosomes-mRNP complexe were observed ; no polysomes were found. The addition of hemin restores the polysomal profil (27). It was equally evidenced in vivo, in rabbit reticulocytes a mRNP-40 S complexe which possess eIF₂ and eIF₃ initiation factors, this complex sediment at 50 S in sucrose gradient (16, 28). These complexes could represent an emphasis for regulatory control at the level of protein synthesis initiation or in the beginning of elongation (23). Such inhibitor associated to free mRNP may prevent the formation of 80 S active initiation complexes as the hemin-controlled translational repressor (29). Once free mRNP inhibitor is removed and/or modified the translational capacity of the native particles which are or not associated with ribosomal subunit may be restored.

FOOTNOTES

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