Potential role of mRNP proteins in cytoplasmic control of gene expression in duck erythroblasts

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Abstract

In the present report, we try to summarize recent results concerning our studies on messenger ribonucleoprotein particles isolated from the cytoplasm of duck erythroblasts. A flow scheme of mRNA through the cell, slightly modified from a recent review (10) is proposed as a theoretical framework for analyzing the relationship between the different mRNP characterized.

Introduction

The existence in the cytoplasm of eukaryotic cells of 'masked' inactive messenger RNA in the storage form of messenger ribonucleoprotein complexes was proposed by Spirin as early as 1964. These mRNPs, initially characterized in fish oocytes and early embryos, were designated *informosomes* (1), and may represent 'peripheral memories' of genetic information (2). Experimental evidence for the existence of cytoplasmic non-ribosomal ('free') inactive mRNP has, however, accumulated only within the last few years. The clearest examples of such mRNA containing particles come from studies of embryonic development. In *Artemia salina*, in certain conditions, the development of the brine shrimp is interrupted at the gastrula stage thus inducing a dormant period. In these conditions no polyribosomes are formed, and mRNA is present in the form of stored mRNP (3). During the early development of sea urchin embryos both oogenic and newly transcribed mRNA sequences are utilised. However, the initial rise in protein synthesis following fertilisation is independent of concomitant transcription and results from the mobilisation of mRNA sequences stored in the egg. This mRNA may be isolated from the oocyte as protein-rich free cytoplasmic mRNP (4).

In the case of the mollusc *Spisula solidissima* (the common surf clam), the pattern of protein synthesis in fertilised eggs is modulated by a stage specific utilisation of different subsets of mRNA from a common maternal mRNA population (5). Specificity of expression of mRNA seems to be regulated at the level of phenol-soluble components. In cells cultured *in vitro*, the balance between polysomal active and 'free' inactive mRNA appears to be dependent on growth conditions (6–8) and various proposals have been put forward concerning the role of the untranslated mRNA population in differentiated cells. In the case of the differentiating avian immature erythrocytes the regulation of mRNA expression may be the prominent role of free mRNP. As the cell matures the restriction in gene expression (9) might be explained by the selective gradual and sequential sequestration of some mRNA species into an inactive form.

Flow of mRNA from the nucleus to the cytoplasm

As far as we know, processing of mRNA is confined to the nucleus, i.e. messenger RNA seems

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to appear in the cytoplasm in its final form. The first cytoplasmic mRNA compartment is thus the transfer mRNA or its form of transportation from the nucleus to the cytoplasm, the transfer mRNP; this compartment is not experimentally definable yet. The direction of flow of cytoplasmic mRNA is not evident and has been previously discussed (10). In the absence of any strong evidence for a simple precursor-product relationship between free and polyribosomal mRNA, we may introduce the theoretical notion of pre-translation mRNP complexes constituting the ultimate selection level of mRNA to be translated – or not – in the polyribosomes at a given time. These are likely to correspond to initiation complexes. According to such a scheme, selection would be dependent upon the different affinity for a mRNA type of putative translational activator or repressor molecules, in coordination with recognition factors. The possible nature of such putative regulators will be discussed later. It should however be noted that thus far, no evidence for a requirement of an mRNA-specific set of initiation factors has been demonstrated. Assuming a homogeneous population of polyribosomes the activity of any initiation factor must affect the translation of all cellular mRNA alike. Quantitative regulation of the rate of synthesis of different proteins by competition between different mRNA species for non-specific initiation factors would be a step following the selection of mRNA 'selected' for translation; or, conversely, recognition of mRNA by specific factors for consecutive repression might define the pool of potentially translatable mRNA. At the point of selection at the level of pre-translation mRNP complexes mRNA is either activated, associated in polyribosomes and its information expressed in polypeptides, or enters the inactive free mRNP pool.

Equilibrium labeling and pulse-chase experiments in HeLa cells have shown that according to growth conditions from 40 to 60% of labeled mRNA is in free RNP particles (6). It is interesting to note that only a small fraction of the mRNA in free particles is chasable into polyribosomes; the bulk of free mRNA, like polyribosomal mRNA, decays with an intrinsic half life (11, 12). We have therefore to define two populations of repressed mRNP; one containing mRNA species with a polyribosomal translated counterpart – possibly in equilibrium with each other – and another containing the mRNA fully repressed in a given cell at a given time. Such qualitative definitions of course are expression of quantitative equilibria (10).

In duck erythroblasts and reticulocytes at various stages of maturity we find from 10% to 30% of globin mRNA in the free compartment. Moreover, comparison of the *in vivo* translation products of the polyribosomal and free mRNA populations by two-dimensional gel electrophoresis shows that the majority – and potentially all-mRNA types present in polyribosomes are also present in the free compartment, the distribution of different mRNA sequences between these two classes being subject to large variations. The polyribosomal population contains about 200 mRNA species in duck erythroblasts. In addition about 1,200 different mRNA types are present in the form of long-term repressed mRNP (i.e. only present in free pool) (13). In other cells, the number of mRNA species and their partition may be quite different, in view of the fact that the maturing red cell in an example of high specialisation. Although, a priori, no qualitative difference between the protein composition of transiently and long-term repressed mRNP is theorically required, it was of obvious interest to define which polypeptides interact specifically with mRNA within these two populations of free mRNP. In duck red cells globin mRNP constitute a typical example of mRNP distributed between polyribosomes and free mRNP. Apart from free globin mRNP we may also isolate '35S mRNP' containing nonglobin mRNA sequences which are fully repressed in these cells. Comparative characterization of active and inactive mRNP will be developed in the next section.

Before discussing the components of mRNP particles, a final fraction of mRNA should be mentioned although it may represent an experimental artefact: the so-called 'run off' fraction of mRNA which may arise when the rate of initiation in the cell or the cell lysates is low or blocked as compared to translocation; these may be similar to pre-translation mRNP. To avoid such confusion the polyribosomes should be stabilised at the culture temperature by cycloheximide or preferably by the irreversible emetime treatment before isolation of free mRNP particles. In proposing a schematic representation of the flow of mRNP through the cell (13, 10 and Fig. 1), we have concentrated on the elements which seem necessary on experimental

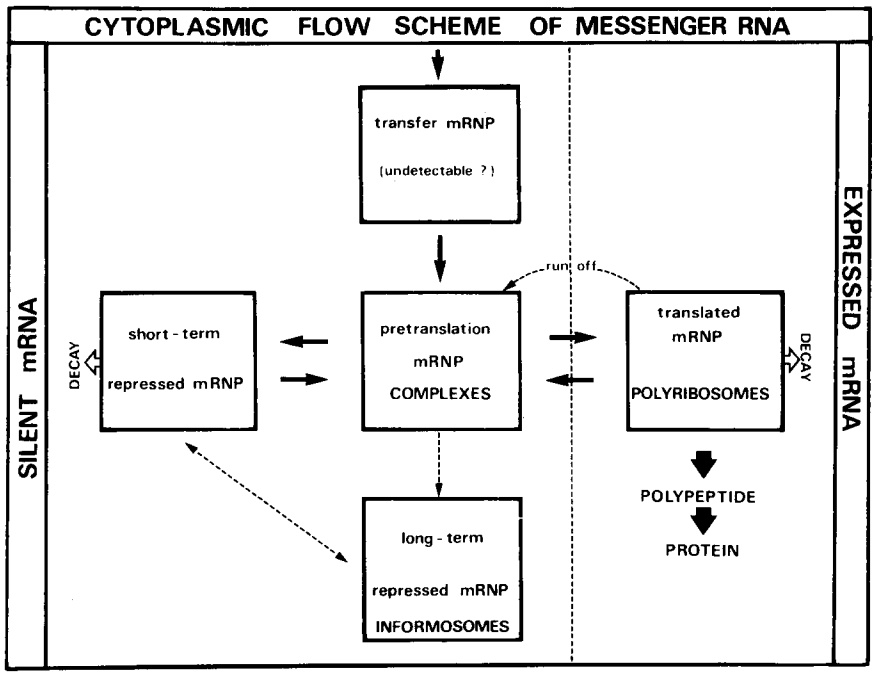

Fig. 1. Tentative flow diagram of messenger RNA in the cytoplasm of duck erythroblasts. Explanations are given in the text; see also refs 10 and 13.

and theoretical grounds. This scheme constitutes only a basic framework for analysing the complex relationship which exists between the mRNA-containing structures in the cell. An initial step in the experimental work necessary to eventually allow the understanding of some of these relationships in duck erythroblasts was the biochemical characterization of translated and silent mRNP isolated from the cytoplasm.

Isolation of cytoplasmic mRNP

Initially, isolation of polyribosomal mRNP and free cytoplasmic mRNP has essentially been performed by sucrose gradient sedimentation; later on, electrophoresis of mRNP and affinity chromatography on oligo(dT)cellulose was adopted. In the case of polyribosomal mRNP it was established that two major proteins of approximately 50 000 and 75 000 molecular weight were associated with most of the mRNA species investigated. However, the presence and number of additional proteins was variable (14). Such variations may arise from the different methods employed for the isolation of mRNP and/or from the specificity of the proteins interacting with different mRNA depending upon the cellular system. In order to define more rigorously the mRNP protein composition in relation to mRNA function, it was necessary to investigate the influence of the isolation procedures on the quantitative and qualitative recovery of proteins of mRNP. Procedures for preparing anaemic blood and subsequent fractionation of polyribosomes and the post-polyribosomal supernatant of duck erythroblasts have been previously described (15). Duck globin mRNP first isolated by sucrose gradient sedimentation was further purified using different methods including affinity chromatography on oligo(dT)cellulose, centrifugation in Cs_2SO_4 density gradients and polyacrylamide gel electrophoresis. Moreover, different ionic strength buffers were used during purification allowing on the one hand the definition at low ionic strength of physiological complexes, and of high ionic strength minimal 'core' complexes on the other hand.

The polyribosomal globin mRNP complex in duck erythroblasts

Following EDTA dissociation of polyribosomes, and subsequent fractionation by sucrose gradient sedimentation, a major mRNP particle can be isolated with a nominal coefficient of sedimentation of 15S (16). The mRNA contained in these particles codes (exclusively) for α- and β-globin chains (17). The protein composition of the 15S mRNP subsequently purified by sedimentation in different ionic strength conditions, or by oligo (dT)cellulose chromatography, was consistently characterized by a major 73 000 Mr polypeptide, 7 minor basic polypeptides with molecular weights ranging from 45 000 to 68 000, and 5 acidic components in the 80 000 to 130 000 molecular weight range (18). All these are retained in the core particle isolated at 0.5 M KCl. At 0.05 M KCl, i.e. in more physiological conditions, in addition, a specific group of acidic polypeptides with molecular weight ranging from 35 000 to 150 000 was found associated with globin mRNA. When dissociated from the core complexes at 0.5 M KCl, this group of polypeptides does not sediment as free protein but rather as an aggregate particle with a coefficient of sedimentation of 12S. This particle is essentially proteic in nature, since it is not modified by RNAse or staphyloccocal nuclease treatment. During recent *in vivo* phosphorylation studies we found that the 105 000 and 67 000 Mr components of this particle are phosphorylated. In view of similar molecular weights and the state of phosphorylation it is tempting to propose a correlation of this loosely bound mRNP protein complex with some initiation factors, in particular with EIF_3 which is known to assume pleiotropic functions and is required for the binding of mRNA to the ternary complex 40S ribosomal subunit Met~tRNA~EIF_2~GTP (19). According to Shafritz *et al.* (20). EIF_3 may bind mRNA independently of the presence of the 40S ribosomal subunit. It is therefore possible that the 15S globin mRNP is loosely but functionally associated with this initiation factor.

The binding of mRNP proteins to the 3′ polyadenylated region of mRNA was also investigated. Electrophoretic analysis of the residual complexes resistant to digestion by ribonucleases A and T_1, i.e. of the poly(A)-protein complex derived from 15S mRNP, isolated by binding on oligo(dT)cellulose, indicated that both the prominent 73 000 molecular weight and a 47 000 molecular weight polypeptide interact specifically with the poly(A) segment of globin mRNA. Identical results have been observed for the poly(A) protein complex isolated following digestion of 15S mRNP with micrococcal nuclease (21). Our results suggest that protein(s) in addition to the so called poly(A) binding protein of about 75 000 molecular weight described in most mRNP analysed so far, interact with the poly(A) segment of globin mRNA. Results from studies of the RNA composition of the poly(A) protein particle derived from 15S mRNP are given in the next section and suggest a complex but organized structure of this particle. It is interesting to note that the poly(A)-binding proteins isolated by poly(A)-sepharose chromatography of the post-ribosomal soluble cytoplasmic fraction of duck erythroblasts are identical in two-dimensional gel electrophoretic analysis to the 73 000 and 47 000 molecular weight major polypeptides of 15S globin mRNP (unpublished). Thus, as is the case in rabbit reticulocytes (22) and rat liver (23), duck erythroblasts contain a free cytoplasmic pool of these proteins. Intriguingly, it was observed that in duck erythroblasts the mRNA coding for the 73 000 molecular weight polypeptide is not found in polyribosomes but is present in the form of repressed mRNP. This was first suggested by comparison of the *in vitro* translation products of mRNA from free mRNP and of the polyribosomal globin mRNP mRNA, using two-dimensional gel electrophoresis. Indeed, a major product of translation detected exclusively among the polypeptides encoded by free mRNA displayed pI and R.F. identical to those of the 73 000 molecular weight poly(A) binding protein. The identity of these two polypeptides was confirmed by partial proteolytic digestion according to Cleveland *et al.* (24). That the mRNA coding for the major polypeptide tightly bound to translated mRNA is no longer expressed in duck erythroblasts cells raises the possibility of the role of this expression in the control of protein synthesis during terminal differentiation of these cells. A general role of this protein in mRNA metabolism and transport from nucleus to the cytoplasm is suggested by its ubiquitous distribution in mRNP isolated from different cellular systems (14) as well as its detection in nuclear RNP particles (25). Such a hypothesis is supported by its reduction in

quantity in cordycepin treated HeLa cells (26) as well as by our finding that this 73 000 molecular weight protein is an integral component of the nuclear matrix. Since, as already reported, a free cytoplasmic pool of this protein exists in the duck lysate, it probably has not yet become a limiting factor of protein synthesis. The relationship between the absence of the production of this protein and the overall pattern of protein synthesis in terminally differentiating erythropoietic cells will shortly be investigated.

The structure of RNP and in particular of the poly(A)-protein complex derived from 15S polyribosomal globin mRNP

Previous electron microscopic studies carried out in collaboration with our laboratory have shown that mRNP proteins in the 15S complex are distributed along the whole length of the mRNA molecules (27). The first question to be answered was that of the specificity of binding of the proteins to the mRNA molecule. We have recently demonstrated that proteins protect mRNA in duck 15S polyribosomal globin mRNP in a nonrandom fashion (28), which may correlate with the results obtained from ethidium bromide intercalation studies suggesting that free and protein-bound mRNA may have a very similar degree of secondary structure but with distinct detailed conformations in bihelical regions (29). In fact, the first insight into the specificity of binding of some mRNP proteins to particular mRNA segments came from the observation by several groups that a specific set of polypeptides including a major 75 000 molecular weight component was bound to the 3′ terminal poly(A) sequence of polyadenylated polyribosomal mRNA (see discussion in (30)); it was however not established whether the poly (A) binding proteins were bound only to the poly(A) sequence or to additional mRNA sequences as well. In order to answer this question, 15S mRNPs were digested with the calcium-dependent staphylococcus aureus nuclease in conditions where RNA regions protected by protein are not digested. The resulting poly(A)$^+$ residual complex was isolated by oligo(dT)cellulose chromatography or by sucrose gradient sedimentation. The poly(A)-protein complex sedimented between 7S and 10S as determined by [^{3}H]poly(U) titration – and displayed a protein composition including a major 73 000 molecular weight polypeptide, a polypeptide of 47 000 molecular weight and some minor polypeptides of intermediate molecular weight. Thereafter, we investigated whether the poly(A) binding proteins protected exclusively the poly(A) segment of globin mRNA or additional poly(A)-adjacent and nonadjacent mRNA sequences. RNA fingerprinting analysis was used to compare two RNA populations obtained from the nuclease digested 15S mRNP:

1) The total phenol extracted RNA from the residual poly(A)-protein complex isolated on oligot(dT) cellulose columns and 2), the poly(A) containing RNA fragments isolated by oligo(dT) cellulose chromatography of the already phenol extracted RNA. The results obtained (21) show that: 1) Poly(A) binding proteins do not interact only with the poly(A) sequence of globin mRNA but also with poly(A) adjacent and nonadjacent mRNA sequences, 2), the poly(A) segment is not completely protected by the proteins against nuclease and is cut at constant intervals along its length; 3), the poly(A) protein complex is a dynamic structure, in the sense that protein-RNA interactions are formed and broken according to dynamic equilibria between the components. The fact that the poly(A) binding proteins interact with mRNA sequences nonadjacent to the poly(A) suggests potential interactions resulting in the folding back of the messenger molecule on itself. Such secondary structures at the 3′-end of the mRNA molecule could play an important role in the termination of mRNA translation (31), diminishing the possibility of read-through as recently described for globin mRNA (32).

The interaction of mRNP proteins with regions of secondary structure observed by comparative high resolution melting of naked globin mRNA and globin mRNA in a RNP form (unpublished) could mean either that these structures are stabilized by proteins and/or that these regions serve as recognition sites for mRNP proteins. The maintenance of a well defined level of secondary structure in the mRNA molecule seems to be crucial for the efficiency and specificity of mRNA translation, as recently described for rabbit globin and reovirus mRNAs (33, 34).

One of the interesting features of the poly(A)

structure is the periodic protection of the poly(A) sequence by poly(A) binding proteins. Baer and Kornberg (35) as well have shown that RNase T2 digestion fo total cytoplasmic RNA results in poly(A) fragments with a periodic size distribution in multiples of 27 nucleotides. An interesting observation possibly correlating with the periodic size of the protein protected poly(A) fragments stems from Marbaix's group: they have shown by microinjection of globin mRNA in *Xenopus* oocytes, that the minimum size of the poly(A) necessary to ensure the stability of the mRNA (i.e. translatability after 36 hours of injection) is 20 residues of adenosine (36). Hence, one possible role of the 73 000 molecular weight polypeptide might be the maintenance of a minimum poly(A) size insuring mRNA stability and function.

The repressed 'free' cytoplasmic mRNP complexes

As mentioned previously, in duck erythroblasts and reticulocytes 10 to 30% of cytoplasmic globin mRNA is not present in polyribosomes but is found in the postpolyribosomal supernatant. This mRNA was previously isolated in an mRNP complex of '20S' nominal sedimentation coefficient at low ionic strength (15). Centrifugation of this complex on sucrose gradients containing 0.5 M KCl, or fractionation by polyacrylamide gel electrophoresis in nondissociating conditions resulted in the separation of two distinct free globin mRNP particles sedimenting at 16S and 13S (37). A characteristic set of about 14 acidic polypeptides are common to both these mRNPs; the 13S mRNP contains however 5 additional major polypeptides (see Fig. 2). Comparison by SDS gel electrophoresis performed in a single slab gel of the proteins of 15S polyribosomal and of free globin mRNP demonstrated that they were essentially unrelated (18). In particular, as noted in a preliminary report and recently confirmed by two-dimensional electrophoresis (38) neither form of free globin mRNP is associated with the polyribosomal 73 000 molecular weight poly(A) binding protein. This absence appears very interesting in view of the possible roles of this protein as discussed above. Globin mRNA from free 20S mRNP is polyadenylated and translatable *in vitro* with an efficiency identical to that of polyribosomal globin mRNA (17). Unlike the case

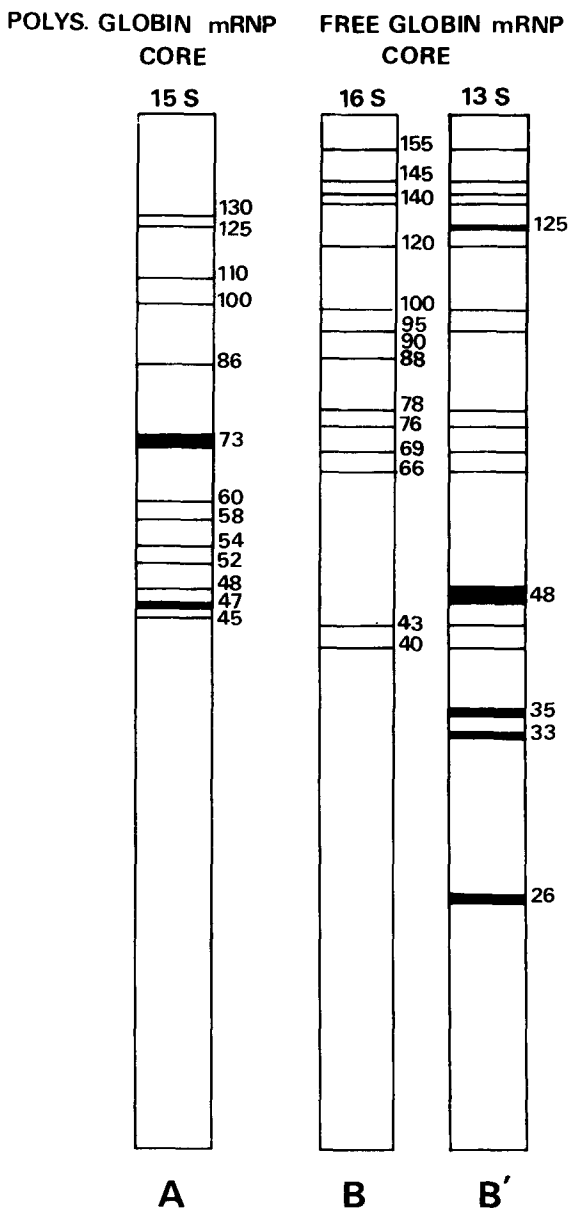

Fig. 2. Diagrammatic representation of polypeptides from purified polyribosomal and free globin mRNP analyzed by electrophoresis on SDS polyacrylamide gels.
Data from a compilation of results presented in refs 18 and 37.
A) polypeptides of core 15S polyribosomal globin mRNP (i.e. isolated in high ionic strength conditions (0.5 M KCl))
B, B') polypeptides of core (0.5 M salt resistant) free globin mRNP
B) polypeptides from 16S mRNP
B') polypeptides from 13S mRNP (same as in (B) + 5 major polypeptides)

in the mammalian reticulocyte, the mRNAs for both α and β globin chains are present in similar concentrations in duck free mRNP.

In addition to globin mRNA, two discrete small MW RNA species are present in free globin mRNP isolated at low ionic strength. One species, (4S RNA) is fully dissociated from both 16S and 13S globin mRNP during sedimentation in 0.5 M KCl sucrose gradients, while the second species (7S RNA) remains associated with the 13S mRNP, being absent in 16S. This 7S RNA comigrates in highly resolutive gel electrophoretic systems with a major snRNA extracted from duck snRNP particles (39): the possible identity of the cytoplasmic '7S RNA' associated with free globin mRNP and of a small nuclear RNA is now under investigation by RNA sequence analysis.

Digestion of the free globin mRNP complex with RNAse A and T_1 was used to isolate and characterise the residual poly(A) protein complex. This subcomplex displayed a sedimentation coefficient of 10S, similar to that derived from polyribosomal globin mRNP as determined by [^{3}H]poly(U) titration. However, protein analysis showed that a characteristic set of polypeptides interacts with poly(A) in free globin mRNP, different to that of polyribosomal mRNP. Hence not only the overall protein compositions of polyribosomal and free globin mRNP appear to be very different but also the polypeptides interacting specifically with the poly(A) segment of globin mRNA (18).

In addition to globin mRNP we started to investigate free mRNP particles containing mRNA other than globin mRNA which can also be isolated from the duck erythroblast cytoplasm. These (35S mRNPs) contain mRNA coding predominantly for two polypeptides of 22 000 and 26 000 Mr. Their protein composition has previously been shown to differ largely from that of free globin mRNP suggesting a selective recognition of specific mRNA sequences by specific sets of proteins in the free mRNP compartment (38). This observation which is at present unique in giving a hint to the possible mechanism of differential recognition for expression or repression of specific mRNA's in the cytoplasm, is under further investigation in view of its obvious interest. Careful reinvestigation of the protein complement of free mRNP led us to the conclusion that whereas some differences may be quantitative (i.e. the relative amount of some of the free mRNP proteins varied from one type of particle to another) others were definitely qualitative. In particular, the clearest difference between the protein composition of globin and nonglobin mRNP containing the two specific mRNA's previously reported consists of the presence in the latter of a major polypeptide of 19 000 molecular weight (38). This was confirmed by the characterization of high ionic strength 'core' mRNP. An interesting observation concerning this protein is based on *in vivo* labeling of polypeptides synthetized by duck erythroblast cells incubated in the presence of $_{35}$S-methionine. Whereas no substantial radioactivity could be incorporated into polyribosomal or free globin mRNP proteins during a 3 hour incubation, the 19 000 Mr polypeptide was consistently labeled indicating a fast turn over of this protein (unpublished). Whether this could be functionally correlated with the binding to long-term repressed mRNA in duck erythroblasts is now under investigation.

The in vitro translational activity of cytoplasmic globin mRNP

Characterization of the translational activities of the 15S and 20S globin mRNP particles initially performed in the wheat germ cell-free system (17) revealed that, while the 15S polyribosomal mRNP is translated as efficiently as the purified globin 9S, the 20S free globin mRNP was untranslatable prior to deproteinization. These results suggested a direct or indirect role of the free mRNP proteins in the translational repression of globin mRNA.

In more recent experiments, carried out in the reticulocyte lysate treated with micrococcal nuclease, we have confirmed the translational repression of globin mRNA in 16S and 13S 'core' mRNP; these contain 9S globin mRNA exclusively (40). Complementary studies using protease K digested mRNP particles indicate that the protein complement of free mRNP is required for maintaining the free globin mRNA in an *in vitro* translationally inactive state and moreover that the repression activity is tightly bound to mRNA. These results do not exclude, however, the involvement in conjunction with the proteins of a low molecular weight RNA in this translational repression. Dissociation and isolation of the molecule(s) respon-

sible for repression is now in progress.

The *in vitro* translational repression of mRNA in free cytoplasmic mRNP has also been reported in cryptobiotic g astrulae of *Artemia salina* (41), in sea urchin embryos (42), in HeLa cells (43) and mouse sarcoma ascites cells (8). On the other hand, the nonpolyribosomal '120S' mRNP isolated from chicken embryonal skeletal muscles was able to program the *in vitro* synthesis of myosin heavy chain in a rabbit reticulocyte lysate as effectively as deproteinized myosin heavy chain mRNA (44). However in that case, due to the size of the mRNP particle, it is not possible to totally exclude contamination by some mRNA material of polyribosomal origin. An additional source of error in the interpretation of *in vitro* translation of free mRNP is pointed out by the work of Brawerman showing that mRNP released from polyribosomes (in that particular case in cells subject to starvation) are effective in promoting polypeptide synthesis (8). Hence conditions of cell lysis and mRNP isolation must be carefully controlled, especially in rapidly dividing cells in which free mRNP generally represent a minor fraction of total cytoplasmic mRNA.

The function of the proteins loosely or tightly associated with the polyribosomal mRNP is still subject to speculation. It is known that initiation factors are required during the process of entry of mRNA into polyribosomes. Though these specific polypeptides or multiprotein complexes have been fully characterized (45), no direct relation between mRNA associated proteins and initiation factors has been established. The major difficulty in approaching this problem is the lack of a direct biological assay for testing the possible different requirements for initiation factors of mRNP versus mRNA during translation. One possible approach is to use highly fractionated systems such as that developed by Schreier and Staehelin (46) which however have the disadvantage of having a very low efficiency compared to the rate of initiation and protein synthesis in intact cells. Crude lysates are more active but in these cases, too, addition of 0.5 M KCl wash of polysomes is necessary to improve the efficiency of translation. The 0.5 M KCl treatment of polyribosomes was chosen initially to dissociate initiation factors which may be then added back to a homologous or heterologous cell-free system. However, one cannot be sure that in these conditions some loosely associated component of mRNP are not released which may have a role during the initiation or elongation process.

Phosphorylation of mRNP proteins

One experimental approach to extend the comparison between mRNP proteins and initiation factors, was to determine which mRNP polypeptides are phosphorylated *in vivo,* since it is well established that some of the initiation factors or initiation factor subunits are phosphorylated. In the case of EIF_2, phosphorylation of the 38 000 molecular weight subunit is correlated with a drastic decrease in the rate of initiation of protein synthesis in the reticulocyte lysate subjected to heme deprivation, addition of low concentrations of dsRNA, and other metabolic changes (47). Although it had been proposed that the 52 000 molecular weight subunit of EIF_2 was identical to the prominent 52 000 molecular weight polypeptide of mRNP in *Ehrlich* ascites cells (48), this conclusion was not supported by further investigation (49).

Another initiation factor, EIF_3, is a multipolypeptide complex required for mRNA binding to the 40 S-Met tRNA-GTP ternary complex and consists of 9 to 12 polypeptides, two of which of 67 000 and 110 000 molecular weight, respectively, are phosphorylated in intact reticulocytes (50). As discussed previously, the complex released from the 15S mRNP by 0.5 M KCl treatment includes proteins of similar molecular weights.

In order to compare initiation factors and mRNP proteins we isolated mRNP in different ionic strength conditions from cells incubated for 3 hours in the presence of $H_3[^{32}P]O_4$ (1mCi/ml of cells), before characterization of their phosphorylated protein components. In the case of 15S polyribosomal globin mRNP, three polypeptides with 130 000, 105 000 and 67 000 molecular weight were reproducibly phosphorylated; these components were washed off the mRNP by treatment with 0.5 MKCl. Whether these polypeptides are identical to some of the EIF_3 subunits as suggested above is now under investigation. In the case of free globin mRNP the only polypeptides phosphorylated *in vivo* were some of the major polypeptides

characteristic of the 13S core globin mRNP (see Fig. 2); none of the polypeptides common to both 16S and 13S globin mRNP were labeled in this experiment. The significance of the phosphorylation of this particular group of major proteins (with molecular weights of 48 000, 35 000, 33 000, and 26 000, respectively) and its possible role in a translation regulation mechanism is now under investigation. The tightly mRNA-bound 47 000 molecular weight polypeptide resistant to 0.5 M KCl was also phosphorylated *in vivo* as already reported in the case of mRNP from HeLa cells (51). Careful reinvestigation by two-dimensional gel analysis of 15S mRNP polypeptides demonstrated that the 73 000 Poly(A) binding protein is not phosphorylated (unpublished). Unfortunately, although abundant published data are concerned with phosphorylated proteins of mRNP or mRNP associated kinase activities (52–54), evidence for a functional role of such phosphorylation has not been clearly established yet.

Concluding remarks

Strong evidence for a role of mRNP in selective cytoplasmic control of gene expression comes from the determination of the number and types of mRNA sequences expressed or silent in duck erythroblasts. The results obtained both by sequence complexity studies and *in vitro* translation analysis demonstrate that specific mRNA sequences are maintained in a translationally repressed form (13). The search for the factor(s) responsible for such mRNA-specific controls led us to characterize in detail the proteins interacting with mRNA in different functional forms and to study their phosphorylation. As discussed in the previous sections, our results support the hypothesis of a direct or indirect role of mRNP proteins in some translational control mechanism. Technically, some important points have to be considered when isolating and characterizing mRNP. First, carefully controlled cell lysis conditions have to be established in order to minimize disaggregation of polyribosomes and artefactual production of 'free' mRNP by 'run off'. Secondly, the application of several experimental conditions and different methods of purification of mRNP are required in order to rigorously define the protein and RNA composition of mRNP. In particular, the binding to mRNA at low ionic strength of specific RNA and protein components must be considered as well as the tightly associated components since they may actually carry out an important biological role. Finally, it became obvious during our investigations on the interactions between specific proteins and specific mRNA sequences involved in the mRNP structure (21, 28) that new methods have now to be developed which allow the isolation of mRNP containing a single mRNA coding sequence. For example, although we have extensively purified globin mRNP, sequence analysis of the protein protected fragments are rendered very difficult to interpret due to the presence in these mRNP of three (α_A, α_D and β) globin mRNAs in different stochiometric ratios. Conditions of isolation of structurally unmodified pure sequence-specific globin mRNP are now being established in our laboratory using hybridization to cloned cDNA sequences. Another approach of analysis of both the structural and functional roles of mRNP proteins is obviously the use of antibodies directed against some of these proteins. The difficulty of this approach is the purification of individual mRNP proteins in sufficient amount for preparation of high-titer sera. That difficulty should be overcome by a postimmunisation selection of desired antibody producing cells using hybridoma cell cloning techniques (55).

The preparation of resolutive probes for specific subcomponents of mRNP will allow us to further investigate the metabolism and function of these components in erythropoietic cells at different stages of differentiation and hopefully increase our understanding of the gene expression regulatory mechanisms potentially played by the mRNA (or pre-mRNA)associated components. Theoretical considerations (2) lead us to some still vague but nevertheless discernible patterns of mechanisms of post-transcriptional cytoplasmic regulation. The evidence for repressed mRNA shows that mechanisms of negative control exist in the cytoplasm which differentially regulate the expression of mRNA. In contrast, positive control mechanisms promoting specific mRNA for translation have not yet been found; initiation factors do not select specific mRNAs. This notion correlates well with the fact that polyribosomal mRNP proteins do not modify in either sense the translation of messenger

RNA *in vitro,* whereas we have good evidence that proteins in repressed free mRNP condition directly or indirectly the inactive state of mRNA. In turn these facts relate well *1)* to the general finding that all polyribosomal mRNP analyzed thus far in many systems contain closely similar proteins; no message-specific protein patterns were reported at this level, and *2)* to our finding that such specific, although overlapping, patterns of proteins can be observed associated with mRNA in the repressed state.

A system of control emerges in which the mRNA in the pre-translational state is competed for simultaneously or consecutively by non-message-specific initiation factors on one hand and by specific sets of recognition proteins on the other. The former would indiscriminately draw all mRNA into polyribosomes and hence lead to expression; the latter would condition directly or indirectly, in combination with (possibly nonspecific) repressing factors the inactive state of specific mRNA. Furthermore it is not excluded that some factors condition reversible versus long term or permanent repression; our observation of a rapidly labeled, 19 000 molecular weight major protein very strongly bound to fully repressed mRNP – but not to the partially repressed *globin* mRNP – might point towards such a possibility. Such a system of control seems quite satisfactory on theoretical grounds since it satisfies several conditions at both the levels of biochemistry and information processing. On the latter level control must be exerted by relatively small – as compared to the number of controlled components – sets or signals operating in combination (and possibly permutation) along a sequence of discriminating thresholds (2) recognizing families of and eventually individual mRNAs. Thus, relatively small sets of recognition proteins would associate with families of signal sequences in mRNA populations and condition them differentially for repression. Biochemically, individual concentrations of the control proteins given by their rates of synthesis and turnover, and individual dissociation constants, different intrinsically or modulated by chemical or allosteric modification would suffice to regulate differentially the activity of some tens or thousands of different mRNA encountered in the cytoplasm of most animal cells.

Although all the evidence thus far gained in our laboratory and others points to the importance of mRNA associated proteins as factors of control, the system we have in mind needs not solely rely on mRNP proteins as controlling factors. As developed elsewhere (2), control proteins could act in conjunction with RNA type or combined RNA-protein type factors. If confirmed, the tcRNA proposal of Heywood (56), or the observation of the influence of small cytoplasmic RNA (Sc RNA?) on *in vitro* translation as reported by Sarkar (57) are liable to be built into the mechanisms of post-transcriptional control proposed earlier within the frame of the Cascade Regulation Scheme (2).

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