# Messenger RNA–ribonucleoprotein interaction during the initiation of protein synthesis

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The interaction of globin mRNA with proteins during translation has been investigated in order to establish whether and to what extent messenger-ribonucleoprotein complexes are involved in protein synthesis. We present evidence for the functional importance of two minor messenger RNA-associated proteins (55 kDa and 60 kDa) during the initiation of globin mRNA translation in reticulocyte lysates. The formation of an mRNA complex containing the major 78 kDa and 52 kDa messenger-ribonucleoproteins was not detected.

*Initiation* *Protein synthesis, eukaryotic* *mRNA translation* *mRNP formation*

## 1. INTRODUCTION

Since polyribosomal mRNAs can be isolated as complexes with specific proteins which differ from those associated with stored mRNA or mRNA precursors [1,2] it is reasonable to assume that some or all of these ribonucleoproteins may be functionally important in protein synthesis or its control. There are a few reports which indicate that mRNP proteins may be crucial for the translation of mRNA. Lebleu et al. [3] have shown that although both mRNA and mRNP bind equally well to crude ribosomes only mRNP binds to deoxycholate-washed ribosomal subunits. It has also been reported that mRNP complexes bind to salt-washed ribosomes at 0°C under conditions that do not allow the binding of naked mRNA [4]. Furthermore, only mRNP is translated in a cell-free system depleted of RNA-binding proteins [5] and adenovirus-specific mRNA becomes associated with host ribonucleoproteins in HeLa cell cytoplasm [6].

Recently it has been demonstrated that the two major globin mRNP proteins of $M_r$ 52000 and 78000 remain bound to the mRNA throughout initiation and polyribosome formation [7]. Although these mRNP proteins may exchange eventually with free proteins [8] the half-life for this exchange seems to be longer than 2 h and messenger ribonucleoproteins thus remain associated with mRNA during multiple rounds of protein synthesis.

The evidence cited above indicates that some mRNP proteins may be functionally important for the translation of mRNA. However, little is known about the specific role(s) played by any of the individual ribonucleoproteins during protein synthesis and we have therefore investigated the de novo formation of mRNP complexes during the translation of protein-free globin mRNA in the micrococcal nuclease-treated reticulocyte lysate cell-free system. Proteins of $M_r$ 60000 and 55000 were found to bind to mRNA during initiation,

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*Abbreviations:* mRNP, messenger RNA–ribonucleoprotein complex

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forming mRNP complexes stable in 0.5 M KCl. However, we did not detect the formation of an mRNA complex containing the $M_r$ 78000 and 52000 ribonucleoproteins which are isolated normally from cell extracts as the major protein components of the polyribosomal mRNP complex [1,2].

## 2. MATERIALS AND METHODS

T4 RNA-ligase was purchased from Miles Biochemicals, [5′-$^{32}$P]pCp (2000–3000 Ci/mmol) and carrier-free Na$^{125}$I (15.1 mCi/$\mu$g I) from Amersham International, Iodogen from Pierce and Warriner and centrifugation-grade caesium sulphate from BDH. All other chemicals were of Analar grade and were used as supplied by BDH. Glass and plastic ware was washed with chromic acid to remove ribonuclease.

## 3. RESULTS

To analyse polyribosomal mRNP complex formation during protein synthesis, protein-free mRNA was translated in vitro in a messenger-dependent cell-free system containing no endogenous mRNA. The mRNP complexes formed during the translation of the exogenously-added message were isolated and subsequently characterised. The experimental system chosen was the micrococcal-nuclease treated rabbit reticulocyte lysate cell-free system [9] into which rabbit-globin mRNA was introduced. The advantage of using this cell-free system is that it contains those ribonucleoproteins observed normally in the rabbit globin mRNP complex.

### 3.1. *Translation of mRNA*

Poly(A)$^+$ globin mRNA was prepared as in [10]

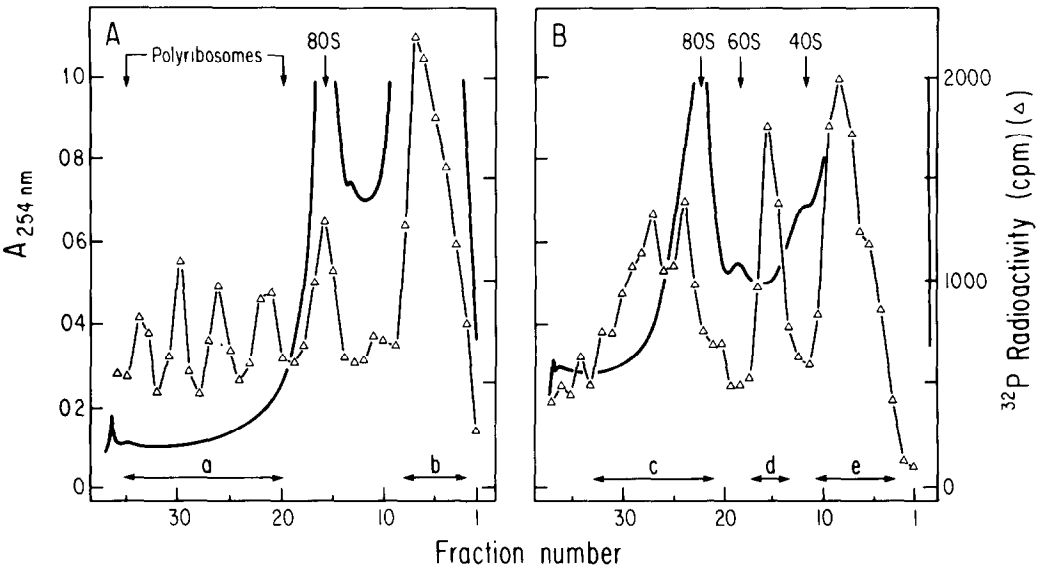


Fig.1. Translation of [$^{32}$P]mRNA in a messenger-dependent cell-free system. [$^{32}$P]mRNA was incubated for 10 min, 30°C at about 1 $\mu$g RNA/ml in 500 $\mu$l of (A) a micrococcal-nuclease-treated reticulocyte lysate cell-free system [9] and (B) a similar lysate preincubated for 5 min at 30°C with 10 mM NaF. Translation was stopped by cooling on ice in the presence of 250 $\mu$g ice-cold cycloheximide/ml, a concentration found to stabilise polyribosomes. The samples were diluted with 250 $\mu$l 25 mM Tris–HCl (pH 7.6), 75 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (TKM-I) and loaded onto 12 ml exponential, 15.0–27.8% (w/v) sucrose gradients in TKM-I. Centrifugation was carried out for 2 h and 4 h, respectively, at 196000 × $g_{av}$, 3°C in a Beckman SW40 Ti rotor. Fractions (about 350 $\mu$l) were collected through an ISCO fractionator with continuous monitoring for absorbance at 254 nm. Cerenkov radiation was determined for each fraction by counting through plastic at 0–5°C [12]. Fractions were pooled as shown: (a) polyribosome translating [$^{32}$P]mRNA; (b) non-translating [$^{32}$P]mRNA; (c) [$^{32}$P]mRNA:ribosome complexes sedimenting at 80 S or larger in a NaF-treated lysate; (d) ([$^{32}$P]mRNA:40 S ribosome subunit:Met$_f^{Met}$) 48 S initiation complex; (e) non-translating [$^{32}$P]mRNA from a NaF-inhibited lysate.

and was subsequently labelled to high specific activity with [5′-$^{32}$P]pCp [11]. Radioactively labelled mRNA was used to ensure unequivocal and accurate identification of the exogenously added mRNA throughout all subsequent manipulations. The mRNA is labelled exclusively at the 3′-poly(A) tail and retains its functional activity as demonstrated by incorporation into high-order polyribosome complexes during translation in either micrococcal-nuclease-treated or untreated reticulocyte lysates [11].

The [$^{32}$P]mRNA was translated in a messenger-dependent reticulocyte lysate cell-free system [9] and in a lysate inhibited by pre-incubation with 10 mM NaF. Components of each incubation were separated subsequently by centrifugation through sucrose gradients (fig.1) and fractions were pooled as shown to include actively translating polyribosomal [$^{32}$P]mRNA, the ([$^{32}$P]mRNA:40 S ribosome subunit:Met-tRNA$_f^{Met}$) 48 S initiation complex, non-translating mRNA, and $^{32}$P-labelled mRNA in complexes sedimenting at 80 S and

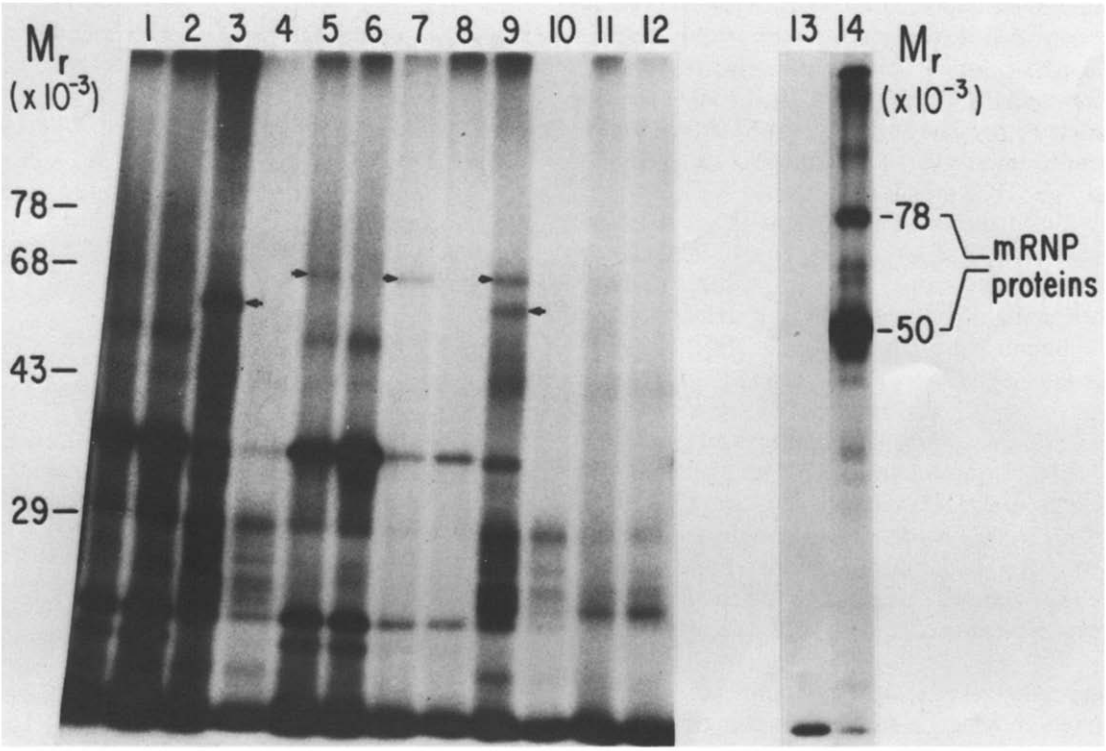


Fig.2. Polyacrylamide gel electrophoresis of ribonucleoproteins extracted with [$^{32}$P]mRNA. Ribonucleoproteins which bound to [$^{32}$P]mRNA during translation in a messenger-dependent cell-free system were isolated as described in the text and fig.1 (fractions a–e) and were iodinated with 50 $\mu$Ci $^{125}$I and 5 $\mu$g Iodogen [7]. Corresponding fractions (a′–e′) from gradients of control incubations containing no [$^{32}$P]mRNA were treated similarly. The iodination reaction was stopped and the labelled protein was prepared for electrophoresis as in [7]. Samples of $^{125}$I-labelled protein (about 37500 dpm as estimated after precipitation with 20% trichloroacetic acid) were analysed by electrophoresis on a 15% polyacrylamide gel [14], followed by autoradiography for 51 days in the presence of intensifying screens. Ribonucleoproteins originated from the following sources: (1) polyribosome translating [$^{32}$P]mRNA (a); (2) control (a′); (3) non-translating [$^{32}$P]mRNA (b); (4) control (b′); (5) [$^{32}$P]mRNA:ribosomal subunit complexes sedimenting at 80 S or larger from NaF-treated lysate (c); (6) control (c′); (7) ([$^{32}$P]mRNA:40 S ribosome subunit:Met-tRNA$_f^{Met}$) 48 S initiation complex (d); (8) control (d′); (9) non-translating [$^{32}$P]mRNA from NaF-treated lysate (e); (10) control (e′); (11) [$^{32}$P]mRNA incubated with post-ribosomal supernatant; (12) control (post-ribosomal supernatant without [$^{32}$P]mRNA); (13) control sample of $^{125}$I-iodinated 9 S poly(A)$^+$ mRNA; (14) a sample of $^{125}$I-labelled globin polyribosomal mRNP, prepared as in [13].

larger from the NaF-inhibited lysate. In addition to control lysates incubated in the absence of added [$^{32}$P]mRNA (not shown), [$^{32}$P]mRNA was also incubated with 500 $\mu$l post-ribosomal supernatant.

### 3.2. *Re-extraction of incubated mRNA and preparation of mRNP*

All gradient samples were diluted with an equal volume of ice-cold TKM-I buffer [25 mM Tris–HCl (pH 7.6), 75 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol] and an equal volume of 15.9% (w/v) sucrose in TKM-I was added to post-ribosomal supernatant control incubations. Each sample was then layered onto a cushion of 38.7% (w/v) sucrose in TKM-I and centrifuged at 350000 × $g_{av}$, 3°C for 20 h in a 10 × 10 Ti (MSE) fixed angle rotor. The sucrose-washed pellets were resuspended gently in 500 $\mu$l TKM-I, adjusted to 50 mM Tris–HCl (pH 7.6), 500 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol (TKM-II) in a final volume of 1 ml and left on ice for 10 min. This treatment does not dissociate the globin polyribosomal mRNP complex but releases less tightly bound protein factors [13]. Crude reticulocyte poly(A)$^+$ RNA (240 $\mu$g, 6 $A_{260}$), prepared as in [10], was then added as an internal standard. Samples were layered onto 3 ml cushions of 38.7% (w/v) sucrose in TKM-II and pelleted by centrifugation at 350000 × $g_{av}$, 3°C for 17 h in a 10 × 10 Ti (MSE) rotor. Haemoglobin sediments half way through the cushion under these conditions. The pelleted, high-salt-washed fractions were resuspended in 200 $\mu$l 10 mM Tris–HCl (pH 7.6).

Resuspended ribosome complexes were dissociated and the $^{32}$P-labelled messenger species were released by incubating for 10 min on ice in the presence of 32 mM EDTA [13]. The components were separated by centrifugation through 12 ml exponential sucrose gradients in 10 mM Tris–HCl (pH 7.6), 1 mM EDTA at 196000 × $g_{av}$, 3°C for 20 h. Gradients were fractionated by upward displacement with continuous monitoring at 254 nm and Cerenkov radiation in each 350 $\mu$l fraction was determined by counting through plastic at 0–5°C [12]. In no case was the sedimentation of $^{32}$P-labelled message observed to be different from the 9 S mRNA internal standard (not shown). The $^{32}$P-labelled peak of each gradient was collected separately, as were corresponding

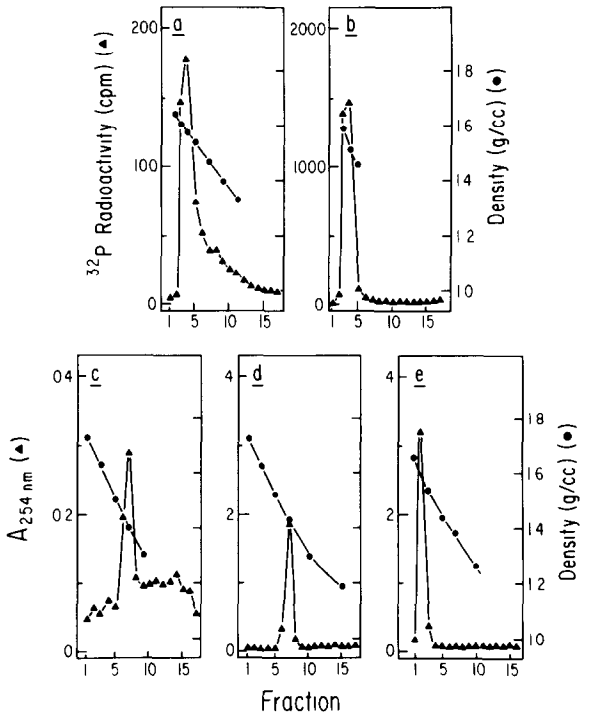


Fig.3. Caesium sulphate equilibrium density gradient centrifugation of $^{32}$P-labelled messenger species after translation. $^{32}$P-Labelled messenger species, fractionated by centrifugation through sucrose gradients (fig.1), were sucrose/salt-washed and isolated as in section 3.2. These samples were adjusted to 25 mM triethanolamine–HCl (pH 7.5), 25 mM KCl, 2.5% glutaraldehyde in a final volume of 2 ml and fixed at 0–5°C for 4 h. A glutaraldehyde stock solution was neutralised with 1 M $NaHCO_3$ and used immediately. Each fixed preparation was adjusted to 3 ml caesium sulphate (1.2 g/ml), 25 mM triethanolamine–HCl (pH 7.5), 5 mM EDTA, 15% (v/v) dimethyl sulphoxide and layered over a 3 ml cushion of 1.5 g/ml caesium sulphate in the same buffer. Samples were equilibrated by centrifugation at 200000 × $g_{av}$, 10°C for 45 h in a Sorvall T-865.1 fixed angle rotor. Gradients were fractionated by puncturing the bottom of the tube and fractions were collected manually. The density across each gradient was determined by weighing 100 $\mu$l of alternate fractions and the position of the $^{32}$P-labelled message was determined by Cerenkov counting. The figures shown are: (a) a gradient representative of glutaraldehyde-fixed samples pooled from any one of the re-extracted, [$^{32}$P]mRNA-containing samples; (b) fixed [$^{32}$P]mRNA; (c) 1 $A_{260}$ fixed, purified mRNP with the protein complement shown in fig.2, lane 14; (d) 4 $A_{260}$ fixed sucrose/salt-washed polyribosomes; (e) 4 $A_{260}$ unfixed polyribosomes.

fractions of each control gradient containing no [$^{32}$P]mRNA.

3.3. *Identification of the ribonucleoproteins*

Protein present in one third of each sample, pooled as in section 3.2, was iodinated with [$^{125}$I]Iodogen [7] without further manipulation. Each preparation was concentrated by trichloroacetic acid precipitation and subjected to polyacrylamide gel electrophoresis [14]. The separated $^{125}$I-labelled proteins were visualised by autoradiography (fig.2). By comparing experimental with control lanes it can be seen that during the incubation proteins of $M_r$ 55000 (arrowed ←) and $M_r$ 60000 (arrowed →) became associated with mRNA in 0.5 M KCl-stable complexes. Neither the $M_r$ 78000 nor the $M_r$ 52000 mRNP protein was detected.

3.4. *$Cs_2SO_4$ gradient analysis of the mRNPs*

The remaining two-thirds of each EDTA-released, $^{32}$P-labelled globin message sample (pooled as in section 3.2) was fixed with glutaraldehyde and analysed by caesium sulphate equilibrium density gradient centrifugation (fig.3). In each case the $^{32}$P-labelled messenger species equilibrated at 1.57–1.60 g/ml (fig.3a), which is similar to or identical with that of protein-free [$^{32}$P]mRNA (fig.3b). Unfixed polyribosomes equilibrated at about 1.6 g/ml (fig.3e) and fixed polyribosomes (fig.3c) and mRNP (fig.3d) at 1.4–1.45 g/ml.

## 4. DISCUSSION

Previous work from our laboratory [7] has demonstrated that the major mRNP proteins remain associated closely with globin mRNA throughout translation. The results indicate that mRNP is translated as an intact particle, as suggested in [2], with little or no exchange of proteins during each round of translation. However, no difference in the translational efficiency of mRNA and mRNP has been observed in vitro [13]. The experiments described here were therefore designed to determine whether protein-free mRNA forms an mRNP complex before or during translation.

Protein-free globin mRNA was found to bind certain proteins during translation (fig.2). Thus, a protein of $M_r$ 60000 was associated with [$^{32}$P]mRNA isolated from the ([$^{32}$P]mRNA:40 S ribosome subunit:Met-tRNA$_f^{Met}$) 48 S initiation complex (fig.2, lane 7) as well as from ribosome complexes sedimenting at 80 S or larger (fig.2, lane 5). It has been reported previously that several 40 S subunits bind to globin mRNA in the presence of NaF [15,16] and these large messenger-containing complexes, whose $^{32}$P-profile does not coincide with the $A_{254}$ peaks of mono-, di- and tri-ribosomes, probably represent multiple 40 S subunits bound to a single mRNA molecule. The presence of the $M_r$ 60000 protein in such complexes is therefore not unexpected. This protein was not detected on actively translating polyribosomal [$^{32}$P]mRNA (fig.2, lane 1) which suggests that it is recycled after initiation.

Non-translating [$^{32}$P]mRNA was isolated as a complex with a protein of $M_r$ 55000 (fig.2, lane 3). Binding of this polypeptide to mRNA is probably dependent on the interaction between mRNA and some ribosome-associated component during the 10 min incubation since very little protein is complexed with mRNA when incubated in post-ribosomal supernatant (fig.2, lane 11). Non-translating [$^{32}$P]mRNA from the NaF-inhibited lysate was isolated as a complex not only with the $M_r$ 55000 protein but also with a protein of $M_r$ 60000 (fig.2, lane 9). This observation may be explained if the $M_r$ 60000 protein is the same as that bound to mRNA in the 48 S initiation complex and is released subsequently with mRNA from abortive complexes formed in the presence of NaF. Indeed, it is known that Met-tRNA$_f^{Met}$ deacylation and complex destabilisation occur when the 60 S ribosomal subunit joining reaction is inhibited by NaF [17]. This would not normally occur in the absence of inhibitor because of the rapid formation of the 80 S initiation complex and subsequent polypeptide chain elongation.

It is important to note that the conditions of sucrose/salt washing were chosen so as to allow the results of this experiment to be interpreted. In early experiments gels similar to that shown in fig.2 were uninterpretable due to excessive contamination caused by pelleted protein complexes. However, the $M_r$ 60000 protein was always observable in samples re-extracted from the 48 S initiation complex, even in these preliminary studies, and therefore must be greatly enriched in this complex. The identity of both the $M_r$ 60000 and 55000 proteins remains to be determined, although pro-

teins of similar $M_r$-value are frequently observed as minor components of globin polyribosomal mRNP (fig.2, lane 14).

None of the messenger ribonucleoprotein complexes formed in these incubations is separable from protein-free mRNA by sedimentation in sucrose gradients (not shown) or by isopycnic centrifugation in caesium sulphate (fig.3). Although we have not determined the exact protein:mRNA ratio, it is evident that the re-extracted [$^{32}$P]mRNP complexes contain significantly less protein than purified preparations of native mRNP. Thus the typical 0.5 M KCl-stable polyribosomal mRNP complex, containing proteins of $M_r$ 78000 and 52000, is not formed during the translation of protein-free globin mRNA in the reticulocyte lysate.

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