76-kDa poly(A)–protein is involved in the formation of 48 S initiation complexes

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In erythropoietic mouse cells induced by Friend leukemia virus, ~50% of non-polyribosomal globin mRNA is found in 48 S initiation complexes ready to be translated. EDTA releases 15 S globin mRNPs, homologous to polyribosomal globin mRNPs. The 76-kDa poly(A)–protein is one of its main protein components. The other 50% of non-polyribosomal message can be separated as 20 S 'free' mRNPs. Its protein composition is different, especially the 76-kDa protein is lacking. The role of this protein is discussed.

Messenger-ribonucleoprotein *Poly(A)–protein* *48 S initiation complex*

1. INTRODUCTION

Messenger RNA (mRNA) is found throughout the eukaryotic cell cytoplasm including regions other than the polyribosomal fraction; the postribosomal supernatant also harbours a substantial amount of messenger RNA (up to 10%) [1–10]. Many investigations support the concept that messenger RNA molecules are associated with specific proteins as messenger ribonucleoprotein particles (mRNPs) from the time of their synthesis until their involvement in polyribosome formation [11–21].

As first discovered by Spirin in fish oocytes, some non-polyribosomal or free mRNPs occur in a 'masked' state [15]. Later, free mRNPs have been found in many actively translating cells, though their role remained ambiguous [1–7,18,19].

The debate is continuing as to whether in vivo repressed mRNPs are also non-translatable in vitro under conditions where the same protein-free mRNAs are readily translated [1,4,22,23]. Some authors report that they were able to translate non-polyribosomal mRNPs [5,17,24], while others found that even the presence of such mRNPs inhibits in vitro translation of other (e.g., endogenous) messengers [3,25]. These conflicting observations can be reconciled by considering the fact that conventional postribosomal fractions (i.e., $\leqslant$80 S) also contain native small ribosomal subunits (40 S ribosoms). Translation starts with the formation of an initiation complex, when Met-tRNA and mRNA combine with a set of initiation factors to a 40 S ribosome. Indeed, several laboratories showed that 2–10% of the native 40 S ribosomes carry mRNA [26–30]. It is plausible that this class of non-polyribosomal mRNPs is translated.

Sucrose gradient analysis of postribosomal supernatants reveals that mRNPs comprising mRNAs of various sizes, yield a broad heterogeneous population covering the range from about 15 S to 60 S [18,31–34]; some mRNPs may be even larger. In this case, it appears difficult to deal with repressed mRNP fractions which are free of 40 S ribosomes.

Here, initiation complexes are separated as a 48 S fraction from a 20 S fraction of free mRNPs from erythropoietic cells. These cells contain predominantly mRNAs for α- and β-globins, which give rise to homogeneous mRNPs.

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2. MATERIALS AND METHODS

2.1. *Cell fractionation procedure*

The isolation of Friend virus-induced erythropoietic cells from inbred Balb/c mice and the subsequent preparation of the postmitochondrial supernatant has been described [35]. The postmitochondrial supernatant was carefully layered over 2 ml 30% sucrose in the same buffer and then centrifuged to sediment the polyribosomes (rotor SW 40, 34000 rev./min, 3 h). Polyribosomal 15 S globin mRNPs were prepared as in [35]. The postribosomal supernatant was layered over 5 ml 30% sucrose in the same buffer and centrifuged to sediment particles $\geqslant$15 S (rotor Ti 60, 42000 rev./min, 17 h). Pellets were resuspended in the same buffer and analyzed by sucrose gradient centrifugation (rotor SW 27, 20000 rev./min, 16 h). The gradients were then fractionated in 1.7 ml samples, with continuous monitoring of the absorbance at 254 nm; 100 μl aliquots of each sample were hybridized with [^{3}H]poly(U), as in [37]. Fractions containing either 48 S complexes or 20 S free globin mRNP were pooled separately for further purification [38].

2.2. *Preparation of globin mRNP from 48 S initiation complexes*

Pooled fractions containing 48 S complexes were sedimented by centrifugation in a Beckman Ti 60 rotor at 48000 rev./min for 18 h. The pellets were resuspended in EDTA containing buffer [20 mM Tris–HCl (pH 7.4); 100 mM KCl; 10 mM EDTA; 7 mM 2-mercaptoethanol] and held in an ice bath for 10 min. The suspension was then layered over 10–25% sucrose gradients [in 20 mM Tris–HCl (pH 7.4); 100 mM KCl; 7 mM 2-mercaptoethanol] and centrifuged at 36000 rev./min in a Beckman SW 40 rotor for 18 h. Fractions of 0.5 ml were collected and 100 μl aliquots removed to be hybridized with [^{3}H]poly(U) [37]. Fractions which hybridized were pooled, dialyzed against 20 mM Tris–HCl (pH 7.4); 300 mM KCl; 2 mM EDTA; 7 mM 2-mercaptoethanol, and passed through an oligo(dT)–cellulose column equilibrated in the same buffer. Bound material was eluted with 50% formamide [2,38] to be further analyzed by 10% Laemmli polyacrylamide gel electrophoresis [40]. Likewise polyribosomal globin mRNPs and free globin mRNPs were prepared.

2.3. *Protease digestion of mRNP proteins*

Lyophilized proteins of mRNPs from polyribosomes and 48 S complexes were dansylated according to [39]. The protein mixture was then analyzed by gel electrophoresis according to Laemmli. The 76-kDa protein bands were cut out from the gels; the protein was eluted from the gel by electrophoresis (80 V, 4°C, overnight). After dialysis against 125 mM Tris–HCl (pH 6.8); 10% glycerol; 0.5% SDS, the protein was digested with 2.5 g *Staphylococcus aureus* V8 protease or with 2.5 g chymotrypsin at 37°C for 30 min. The digestion was terminated by the addition of SDS, and 2-mercaptoethanol to give final concentrations of 2% each. The digest was heated to 95°C for 2 min and oligopeptides were analyzed on 10–20% polyacrylamide gradient gels containing 1% SDS.

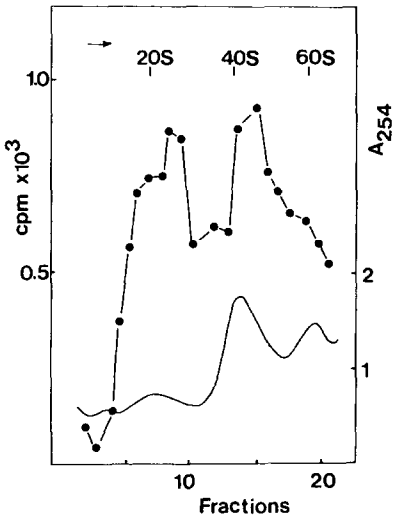

Fig.1. Sedimentation profile of the postribosomal supernatant. The postribosomal supernatant was centrifuged to concentrate particles larger than 10 S as in section 2. The sediment was resuspended in 20 mM Tris–HCl (pH 7.4); 100 mM KCl, 3 mM Mg-acetate; and 7 mM 2-mercaptoethanol and subjected to centrifugation in a 10–25% sucrose gradient (rotor SW 27, 20000 rev./min, 16 h). Individual fractions of 100 μl were hybridized with [^{3}H]poly(U) to detect poly(A) containing sequences according to [37]; (——) absorbance at 254 nm; (●——●) [^{3}H]poly(U) hybridization.

3. RESULTS

Postribosomal supernatants of FLV-cells contain two different mRNP fractions which hybridize with [^{3}H]poly(U) indicating the presence of mRNA with poly(A) sequences (fig.1). One of these fractions sediments at 48 S, slightly faster than the 40 S ribosomal subunits, as shown by the profile of 254 nm absorbancy. The other fraction corresponds to about 20 S and consists of free globin mRNPs, which will be the subject of a subsequent paper. To see whether as in the case of polyribosomes, globin messenger is also a component of 48 S particles, we extracted them with chloroform/phenol and compared the RNA pattern by polyacrylamide gel electrophoresis (fig.2). While polyribosomes yield 28 S and 18 S ribosomal RNAs, the 48 S particles (fraction 14–17) contained only 18 S rRNA, but both show two bands corresponding to sedimentation rates of about 9 S migrating along with rabbit globin mRNA. No substantial amount of any other mRNA could be detected. The other RNA species with a size of 5.6 S belongs to the family of small cytoplasmic RNAs [38].

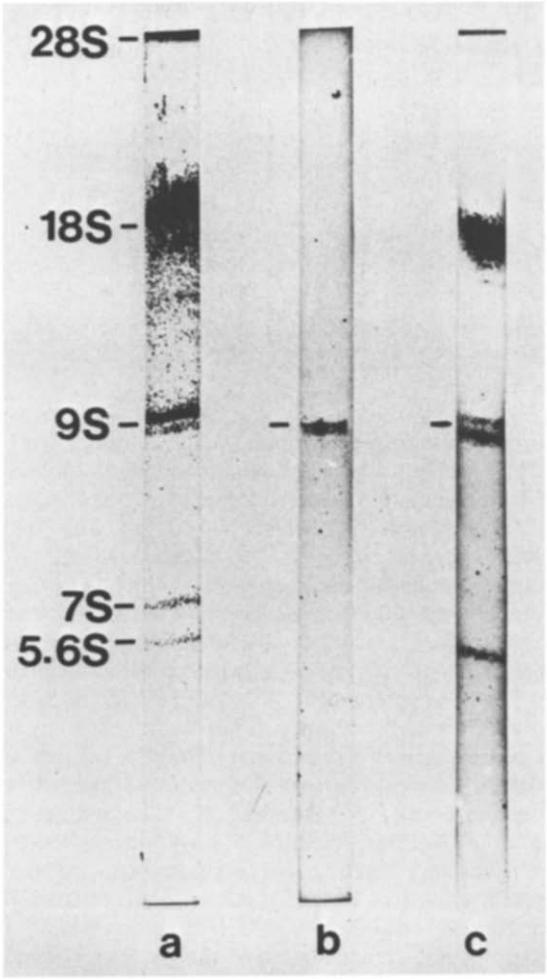

Fig.2. Electrophoretic analysis of the RNAs isolated from 48 S complexes and polyribosomes. Deproteinized RNA [49] was analyzed in 4–15% polyacrylamide gel gradients with 90% formamide (pH 9) [50]. Electrophoresis was run at 120 V for 6 h. The gels were stained with ethidium bromide (24 μg/ml): (a) 20 μg RNA extracted from polyribosomes; (b) 2 μg of 9 S rabbit globin mRNA (Sigma Chemical Co., St Louis MO); (c) 20 μg RNA extracted from 48 S complexes (fractions 14–18, fig.1).

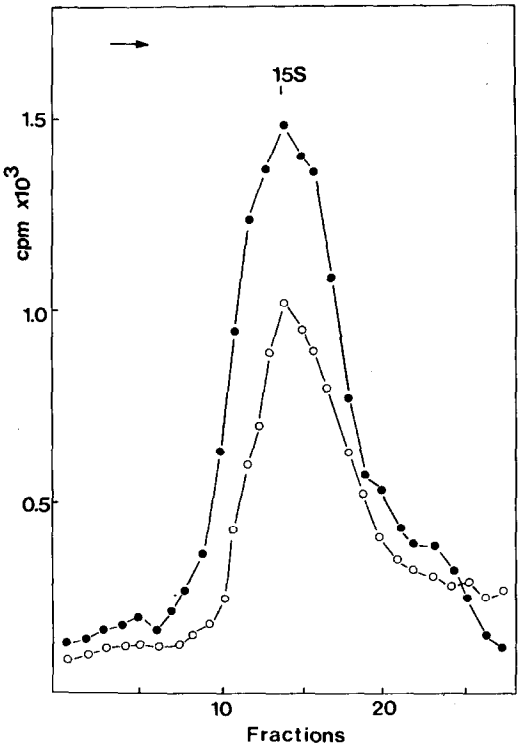

Fig.3. Sucrose gradient analysis of globin mRNP from EDTA dissociated 48 S complexes. EDTA-treated 48 S complexes were centrifuged through a 10–25% sucrose gradient (rotor SW 40, 36000 rev./min, 18 h); 100 μl of each fraction were hybridized with [^{3}H]poly(U) to detect poly(A) containing sequences [37]. (●—●) [^{3}H]Poly(U) hybridization of 15 S mRNPs of 48 S complexes; (○—○) [^{3}H]poly(U) hybridization of 15 S mRNPs of polyribosomes.

When the 48 S fraction was treated with buffered EDTA, a 15 S particle was released (fig.3). This particle cosediments with 15 S globin mRNPs obtained from polyribosomes by the same procedure. This indicates that globin messengers of 48 S particles are homologous to 15 S polyribosomal mRNPs. They are associated with proteins (fig.4a) yielding mRNPs with a buoyant density of $\varrho = 1.38$ g/cm^3, when analyzed by Cs_2SO_4–DMSO gradient centrifugation (not shown).

These results show that the 48 S fraction contains initiation complexes consisting of small ribosomal subunits and globin mRNPs. Further important constituents are the initiation factors [26–30], which are temporarily bound during the initiation phase and which can be detached under various in vitro conditions. EDTA treatment releases a part of the initiation factors which sedimented with 15 S mRNPs in sucrose gradients [2]. Therefore all globin mRNPs from sucrose gradients were purified by oligo(dT)–cellulose chromatography under high salt conditions. After this procedure only tightly bound proteins remained on globin mRNA. Unspecifically or weakly

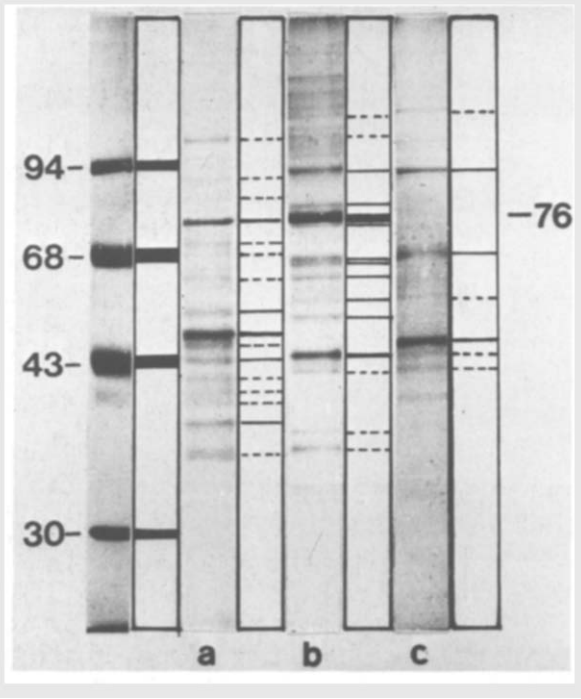

Fig.4. Protein composition of mRNPs from various cytoplasmic regions. Messenger ribonucleoprotein particles, purified by oligo(dT)–cellulose chromatography (section 2) were dissolved in SDS buffer and subsequently analyzed in 10% polyacrylamide SDS gels according to [40]. Protein bands were stained with Coomassie blue: (a) proteins from mRNPs of 48 S complexes; (b) proteins from polyribosomal mRNPs; (c) proteins from free-mRNPs. M_r standards: phosphorylase *b* (94 kDa); bovine serum albumin (68 kDa); ovalbumin (43 kDa); and carboanhydrase (30 kDa).

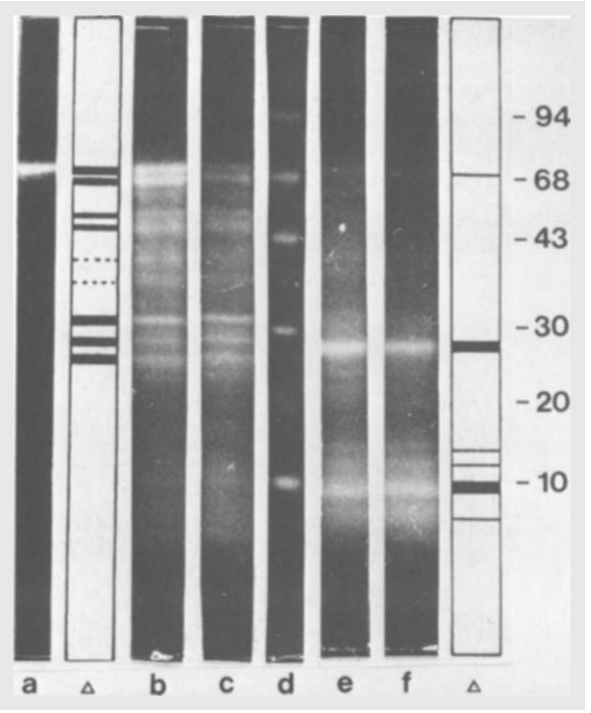

Fig.5. Partial peptide mapping. The lyophilized proteins of 15 S globin mRNPs obtained from polyribosomes or 48 S complexes were dansylated as in [39] and analyzed by polyacrylamide gel electrophoresis [40]. The 76-kDa protein bands were cut out and eluted from polyacrylamide by electrophoresis. The eluted proteins were digested with *Staphylococcus aureus* V8 protease or chymotrypsin (section 2). The digestion products were analyzed by 10–20% SDS–polyacrylamide gradient gels: (a) 76-kDa protein of 15 S mRNPs derived from polyribosomes, non-digested; (b) as (a), digested with Staphylococcus V8 protease; (c) 76-kDa protein 15 S mRNPs derived from 48 S complexes digested with Staphylococcus V8 protease; (d) M_r standards (see fig.4); (e) 76-kDa protein of 15 S mRNPs derived from polyribosomes digested with chymotrypsin; (f) 76-kDa protein associated with 15 S mRNPs derived from 48 S complexes digested with chymotrypsin; (Δ) diagrammatic representation of the generated peptides.

bound proteins and cosedimenting higher molecular mass protein complexes were washed from the column [2,41]. 15 S mRNPs of 48 S initiation complexes contain only a few proteins, which gave quite different patterns from the protein of polyribosomal and free mRNPs. We found two major bands, one at 50 kDa and the other 76 kDa, besides some minor bands (fig.4a). It is important that the 76-kDa protein can be aligned with the major 76-kDa band of 15 S in mRNPs from polyribosomes. This protein is regularly found at the 3′-poly(A) sequence of many messengers and in many different cells [31,42–44]. It is highly conserved in evolution [45]. From many gels we excised 76-kDa bands of 15 S mRNPs, derived from 48 S initiation complexes and from polyribosomes, respectively. Partial digests of either sample were prepared with two different proteases (fig.5). Analysis by Laemmli gel electrophoresis indicates that the 76-kDa protein of 48 S initiation complex is identical to the one of polyribosomes.

It has to be pointed out that 20 S free mRNPs of the postribosomal supernatants of FLV cells do not contain this protein in fig.4c.

4. DISCUSSION

From these results, we conclude that the ribosomal supernatant contains two different functional classes of mRNPs, one of them ready to be translated as initiation complex and a second that is not translated. Erythropoietic cells proved to be an excellent source for obtaining the two mRNP classes as separate homogeneous fractions, both containing globin mRNA. In FLV-cells, the two classes are present in roughly equal amounts (fig.1).

It is generally accepted that untranslated message of free mRNPs can be recruited onto ribosomes. This happens when a cell requires its translation; e.g., after insemination of sea urchin eggs or in stage-specific changes in the course of embryonic development. Young et al. [46] showed that untranslated mRNP can also be converted in vitro by ionic changes into the translated form. How this functions in the intact cells is not clear. We propose that the conversion includes exchanges of proteins associated with the mRNA. The initiation complex clearly constitutes the intermediate stage. All three classes of globin mRNPs in the cytoplasm show rather different protein compositions. There is, however, potent evidence that the presence of the 76-kDa protein (first discovered by Blobel [43]) on the 3′-poly(A) sequences plays an important role in translation:

1. The 76-kDa protein has been found in polyribosomal mRNPs as well as in mRNPs of 48 S initiation complexes but not in repressed globin free mRNPs.
2. It is also found free in the cytosol. When it is removed from extracts used in cell-free protein synthesizing systems, a deproteinized mRNA can no longer be translated. In contrast, the same systems readily translate polyribosomal mRNPs [25,35].
3. The 76-kDa protein from the cytosol binds with a high specificity to poly(A)$^+$ mRNA and poly(A) in vitro, hence named poly(A) protein, but not to any other homopolyribonucleotides (unpublished).
4. Globin mRNA translation in vitro is inhibited by the addition of poly(A); this is likely to be due to sequestration of free 76-kDa proteins from the cytosol. But globin mRNP stimulates protein synthesis in the presence of poly(A) (N. Standart, personal communication). Other homopolyribonucleotides were ineffective.
5. The poly(A) protein is not released during translation; it remains closely associated with the mRNA throughout the ribosome cycle [47].
6. The poly(A) protein is thought to be the candidate which links polyribosomes to cytoskeletal elements [48]. One could assume that the presence of the 76-kDa protein at the 3′-end of poly(A)$^+$ mRNA is the main criterion for distinguishing polysomal mRNPs and mRNPs of 48 S initiation complexes from genuine free and untranslated mRNPs in cell extracts.

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