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CHARACTERIZATION OF A NATIVE mRNA CONTAINING PREINITIATION COMPLEX FROM RABBIT RETICULOCYTES: RNA AND PROTEIN CONSTITUENTS

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From a rabbit reticulocyte postpolysomal supernatant a fraction has been isolated which is enriched in ribosomal particles sedimenting at 50S. This fraction is efficiently in vitro translated predominantly into α-globin. Besides the RNAs and proteins of the small ribosomal subunit the 50S particle contains α-globin mRNA and additional high molecular weight proteins, most of which correspond to polypeptides of the initiation factors eIF-2 and eIF-3. The 50S particle may represent a native [mRNA·40S·eIF's·Met-tRNA$_f$·GTP] complex which may occur in vivo as a translatable intermediate in the initiation sequence.

The process of eukaryotic peptide initiation has been reported to involve four major steps (1). During this four-step sequence two native complexes involving the 40S ribosomal subunit are found (2-4): preinitiation complex I [40S·eIF's·Met-tRNA$_f$·GTP] and preinitiation complex II [mRNA·40S·eIF's·Met-tRNA$_f$·GTP]. Joining of complex II and the 60S ribosomal subunit results in formation of the 80S initiation complex. Both preinitiation complexes are formed in fractionated systems using purified translational components (2,5,6). Several groups (7-10) succeeded in identifying preinitiation complex I in crude rabbit reticulocyte lysates, while complex II was found only to a low extent in the lysate. Others demonstrated the presence of complex II with the help of inhibitors which interrupt the initiation sequence at the 60S ribosomal subunit joining step (3,11). Still others were able to directly isolate high amounts of mRNA-containing native 40S particles (12,13). Recently we described the isolation of a fraction which can be translated predominantly into α-globin and is enriched by 50S particles (14) and the following study deals with their

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Abbreviations: eIF, eukaryotic initiation factor, see Anderson et al. (1977) FEBS Lett. 76, 1 - 10; SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; DTE, dithioerythritol; Met-tRNA$_f$, initiator methionyl transfer RNA; M_r, molecular weight.

Definition: A_{260} unit, the quantity of material in 1 ml of a solution with an absorbance of 1 at 260 nm (1-cm path length).

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characterization. We suggest that these particles consist mainly of preinitiation complex II isolated without the use of inhibitors of 80S initiation complex formation.

MATERIALS AND METHODS

Materials. Polyribosomes and the 200 000 x g fraction of rabbit reticulocytes were prepared as described (14). Mouse liver pH-5 enzyme isolation was performed according to Falvey (15). Except for slight modifications (14), crude initiation factors and Ehrlich Ascites cell-free extracts were prepared according to the method of Schreier (16), and Morrison (17), respectively. RNAs from polyribosomes, 200 000 x g fraction and RNP particles were extracted following the method of Brawerman (18), and poly(A)mRNAs were enriched according to Aviv (19). Heparin-sepharose 6B was kindly donated by H.O. Voorma (Utrecht, The Netherlands). Purified eIF-2 and eIF-3 from rabbit reticulocytes were generous gifts of T. Staehelin (Basle, Switzerland).

Protein Synthesis in A) the Recombined System and B) the Ehrlich Ascites System.

A) We prefer to use the term 'recombined system' instead of 'fractionated system' to denote the system described formerly (14). Incubation mixtures contained in a final volume of 0.1 ml: 30 mM Hepes/KOH buffer, pH 7.5, 1 mM DTE, 3 mM $Mg(Ac)_2$, 75 mM KCl, 1 mM ATP, 0.4 mM GTP, 20 mM phosphocreatine, 21 µg (3.75 units/ml) creatine phosphokinase, 36 µM each of the common amino acids except lysine, 32 µM unlabelled lysine, and 4 µM [^{14}C]lysine (287 Ci/ mol). Translation was assayed by mixing 5 µl mouse liver pH-5 enzymes, 5 µl polyribosomes (0.5 - 1.0 A_{260} units), 50 µl test solution or buffer, and 40 µl of a 'master mix' containing the ingredients listed above.

B) Incubation mixtures contained in a final volume of 0.1 ml: 10 mM Hepes/KOH buffer, pH 7.5, 5% (w/v) sucrose, 10 mM DTE, 3 mM $Mg(Ac_2)$, 80 - 100 mM KCl, 1 mM ATP, 0.2 mM GTP, 5 mM phosphocreatine, 21 µg (3.75 units/ml) creatine phosphokinase, 120 µM each of the common amino acids except lysine, and 1.75 µM [^{14}C]lysine (287 Ci/mol). Translation was assayed by mixing 5 µl crude initiation factors, 30 µl buffer or test solution, 40 µl Ehrlich Ascites extract, and 25 µl of a 'master mix' including the components listed above.

Globin synthesis was carried out at 34°C for 120 min in A) and at 37°C for 90 min in B). To determine the ratio of α- and β-globin chains produced, the globin products were chromatographied according to the method of Dintzis (20), as modified by Sarre (14), which allows seperation of α- from β-globin as well as seperation of globin from endogenous translational products in the case of system B. To determine the total [^{14}C]lysine incorporation per assay, samples were processed after incubation according to Balkow (21) except that H_2O_2 was omitted and up to 250 µg carrier globin per assay was added prior to application of trichloroacetic acid (final concentration 5%).

Preparative Sucrose Gradient Centrifugation. 10 ml linear sucrose gradients (10 - 30% (w/v)) in gradient buffer (10 mM Tris/HCl, pH 7.5, 50 mM KCl, 5 mM $Mg(Ac_2)$, 1 mM DTE, 0.25 mM EDTA) were formed over a 1.5 ml 30% (w/v) sucrose cushion. The sample to be fractionated was dissolved in gradient buffer without KCl, but containing 250 mM sucrose. 1 ml of this solution (20 A_{260} units) was layered onto each gradient. The tubes were centrifuged at 40 000 rev./min for 180 min in an SW 41 rotor (Beckman) at 4°C. Further treatment of the gradients was performed as already reported (14).

Identification of Individual RNAs.

A) Deproteinization of RNAs. After centrifugation for 30 min and 8 h, respectively, polyribosomal and 200 000 x g pellets were dissolved in the TKM buffer used by Baglioni (7) to give a final concentration of 10 - 20 A_{260} units/ml. The constituents of the 200 000 x g fraction obtained after sucrose gradient centrifugation and precipitation with ethanol were treated in the same

manner. These dissolved materials were disassociated with SDS (18) after endogenous RNases had been distroyed using proteinase K (22). Proteins were removed by adding phenol (redistilled)/chloroform/isoamylalcohol (23). This extraction procedure was repeated three times. The organic phases obtained were pooled and residual RNAs were reextracted with Tris buffer. The RNAs of the final aqueous phase were precipitated with ethanol.

B) Oligo(dT)-Cellulose Chromatography. To enrich for the poly(A)mRNA of polyribosomes and of the 200 000 x g fraction the deprotenized RNAs (at least 20 A_{260} units) were collected, made 0.5 M for KCl, 0.01 M for Tris/HCl, pH 7.5, and loaded onto an oligo(dT)-cellulose column (5 x 2 cm). The chromatography procedure was the same as that described by Aviv (19). After washing out the non-adsorbed material, bound RNAs were eluted with 0.01 M Tris/HCl, pH 7.5. Peak fractions were pooled and precipitated.

C) Polyacrylamide Gel Electrophoresis in 99% Formamide. If found to be active in system B RNA material was collected and dried in vacuo. 40 - 80 A_{260} units were dissolved in 1 ml of 0.02 M barbital buffered formamide, pH 8.0, which was deionized and included 0.05% (w/v) bromphenol blue and 10% (w/v) sucrose. Treatment of formamide, which was adjusted to the indicated pH values using N,N,N',N'-tetramethylethylenediamine (TEMED), was performed according to Pinder (24). The preparation of 0.6 x 10 cm cyclindrical gels (3% (w/v) acrylamide, 0.5% (w/v) bisacrylamide) was carried out as described by Orkin (25) using barbital buffered formamide, pH 9.0. During the polymerization at 4°C the surface was covered with a 70% (w/v) formamide-water layer, pH 9.0, which was replaced with buffered formamide, pH 8.0, before the sample was loaded onto the top of the gel. Electrophoresis buffer was 0.7 mM barbital - 15 mM barbital sodium, pH 9.0. Electrophoresis was performed in the cold room (0.5 mA/gel) until the tracking-dye reached the bottom of the gel. The reservoir contents

Table 1

Protein Synthesis in A) the Recombined System and B) the Ehrlich Ascites System

	A) Recombined System			B) Ehrlich Ascites System		
additions	amount added [A_{260} units]	[^{14}C]-lysine incorporated [cpm/assay]	ratio of globin chains [α/β]	amount added [A_{260} units]	[^{14}C]-lysine incorporated [cpm/assay]	ratio of globin chains [α/β]
none	0	650	1.0	0	1250	no globin
200 000 x g fraction	0.5	3500	2.1	0.05	2850	2.8
50S	0.11	1800	not measurable	0.05	2100	2.5
top	0.12	2700	1.0	0.05	800 - 2500	no globin

The specific assay conditions are described in Materials and Methods. In the case of the recombined system, 50S and top correspond to fractions 12 and 17 shown in Fig.1. In the case of the Ehrlich Ascites system a phenolic extraction of the proteins preceded the assays of the 200 000 x g fraction and of the pooled fractions from the top and the 50S region of the sucrose gradient depicted in Fig.1. In addition, the RNAs of the 200 000 x g fraction were submitted to an oligo(dT)-cellulose chromatography.

circulated between the buffer chambers. Staining and scanning procedures were adopted from Orkin (25). Using precisely standardized conditions the optical density of bands resulting from defined amounts of tRNA was measured (A_{660}) and plotted as a function of the applied quantity (µg). If the same amounts of different kinds of RNA yield the same optical densities, it is possible to calculate the molar ratio between different RNA species of known molecular weights.

Other Methods. To demonstrate the existence of eIFs, RNP particles were dissolved in buffer A (at least 0.5 A_{595} units, measured by the method of Bradford (26)), and layered onto a 1 ml Heparin-sepharose column. Buffer A and chromatography conditions were adapted from van der Mast (27). Column derived and other protein fractions were analysed in SDS/polyacrylamide gel slabs using the buffer system described by Laemmli (28). The gels were stained according to Ernst (29), and single tracks were cut out for scanning at 550 nm.

RESULTS

When a postpolysomal fraction from rabbit reticulocytes, pelleted by centri fugation at 200 000 x g for 8 h, is added to a recombined protein synthesizing system or to an Ehrlich Ascites system a strong stimulation of globin synthesis is observed. Preferentially α-globin is synthesized (Table 1; 14).

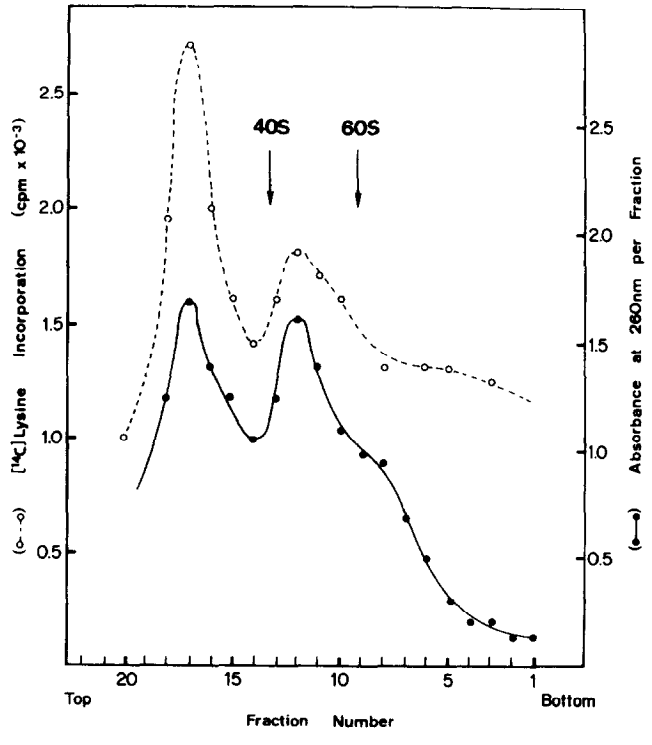

Fig.1. Density Gradient Analysis of the 200 000 x g Fraction
For procedural details see Materials and Methods. Arrows indicate the positions of 40S and 60S ribosomal subunits from rabbit reticulocytes determined in parallel gradients. The isolation of the subunits was according to Brown (30). 0.7 ml fractions were collected, the optical density at 260 nm was measured (—●———●—), and aliquots were assayed for globin synthesis in the recombined system (--O------O--).

In order to determine the components responsible for this stimulatory activity, we tested the constituents of the 200 000 x g fraction distributed on top and in the 50S region of a sucrose gradient (Fig. 1). Using the described conditions, not only fractions from the 50S peak were demonstrated to be active in the recombined system, but also fractions derived from the top of the gradient (Table 1; Fig. 1). To test the material from both peaks for stimulatory activity in the Ehrlich Ascites system, the corresponding fractions were pooled and the proteins were extracted. While the presence of RNAs from the 50S region resulted in an increased [^{14}C]lysine incorporation the RNAs from the top of the gradient produced ambiguous results in different experiments (Table 1).

For a more detailed characterization the RNAs of the two different fractions were analysed by gel electrophoresis (Fig. 2). The top of the gradient contained exclusively low molecular weight RNAs (4-5.8S; not shown). Since the translation of the endogenous template could be stimulated in the recombined system by addition of this fraction, in the absence of globin mRNA, we decided to investigate the material from the top more thoroughly. The data will be published elsewhere. The 50S constituent of the 200 000 x g fraction contains 18S ribosomal RNA (18S rRNA), 4-5.8S small RNAs, and 9-10S RNA. The latter could be characterized as mainly α-globin mRNA due to the comparison with the migration of α- and β-globin mRNAs in a parallel gel run (Fig. 2). The observation that the product of protein synthesis testing 50S material in the Ehrlich Ascites system is predominantly α-globin (Table 1) is in accordance with this result.

Having measured the optical density of stained RNA bands, it was possible to calculate the quantities of applied RNAs with a calibration curve (see Materials and Methods). Assuming molecular weights of 200 000 for globin mRNA (31) and 700 000 for 18S rRNA (32), the molar ratio of mRNA to 18S rRNA in the 50S component was estimated. In three consecutive experiments it ranged from 0.6 to 1.0, and it was concluded that more than two thirds of the analysed material corresponds to 50S particles composed of 40S ribosomal subunits and globin mRNA possibly associated with proteins. The ability of these particles to bind [^{3}H]Met-tRNA$_f$ specifically (unpublished) supports the interpretation that they represent predominantly preinitiation complex II as defined above.

An analysis of the proteins of the 50S particle by SDS gel electrophoresis also supports its identification as preinitiation complex II. Fig. 3 (tracks A, B) shows the migration of the polypeptides of salt-washed 40S ribosomal subunits and of gradient-derived 50S material. Both preparations contain low molecular weight proteins ($M_r \leq 50.000$); presumably most of these are structural ribosomal proteins. Noteworthy are the additional high molecular weight pro-

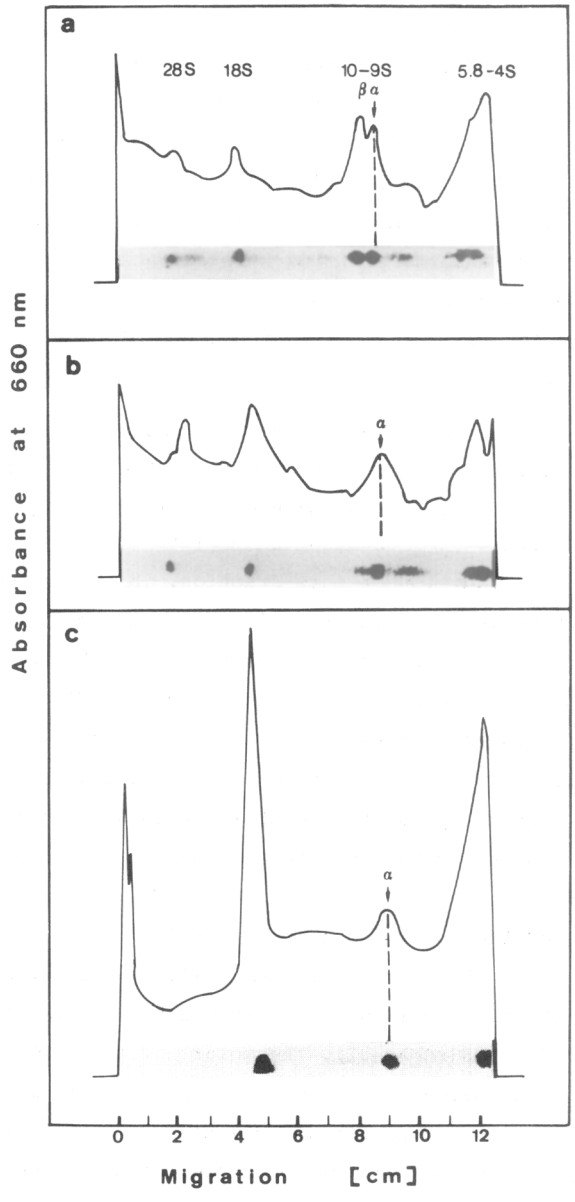

Fig.2. Electrophoretic Analysis of RNA Fractions in Polyacrylamide/Formamide Gels
Electrophoresis and interpretation of the gels were performed as described in Materials and Methods. The Methylene blue stained gels were continuously scanned at 660 nm.

Panel a shows the analysis of polyribosomal RNAs (0.4 A_{260} units) used as markers to indicate the positions of single RNA species with characteristic sedimentation values. Polyribosomes were isolated as described recently (14), except that the final centrifugation was carried out at 45 000 rev./min for 180 min. After deproteinization, RNAs were submitted to oligo(dT)-cellulose chromatography. Panel b shows the pattern obtained from 0.4 A_{260} units RNA of the 200 000 x g fraction treated as described in Panel a. Panel c shows the RNAs of gradient-derived 50S material; 0.6 A_{260} units were analysed.

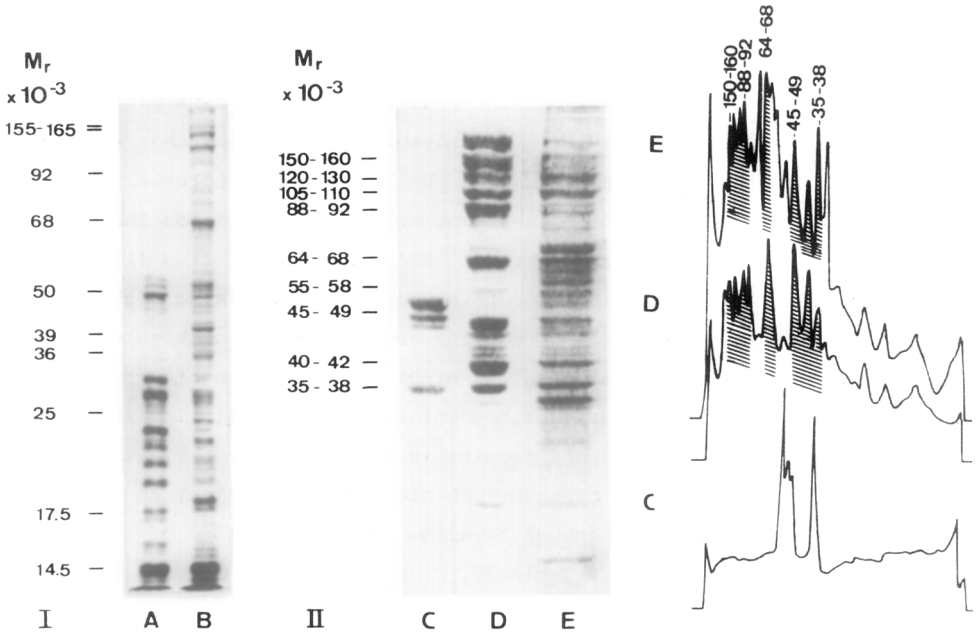

Fig.3. Electrophoretic Analysis of Protein Fractions in SDS/Polyacrylamide Gels

SDS gel electrophoresis and interpretation of the gels were performed as described in Materials and Methods. The Coomassie blue stained gels were continuously scanned at 550 nm.

Except for the initiation factors (tracks C,D), all samples were concentrated after their isolation (for 40S ribosomal subunit isolation see Fig.1). 20 µl were heated at 90°C for 2 min and subsequently electrophoresed in 10%(tracks A,B) and 10 - 18% (tracks C,D,E) SDS containing gel slabs, respectively.

The individual tracks show the proteins of A: 40S ribosomal subunits (50 µg), B: 50S particles prior to Heparin-sepharose chromatography (80 µg), C: eIF-2 (3 µg), D: eIF-3 (9 µg), E: 50S particles after Heparin-sepharose chromatography (50 µg). Scans of the tracks C, D and E are given in a smaller scale in the right margin. Similarities in the densitometric patterns are hatched.

The molecular weights indicated in I are those of reference proteins run in parallel tracks (not shown) and in II of eIF-2 and eIF-3. Reference proteins were RNA polymerase (E.coli), phosphorylase b, bovine serum albumine, γ-globulin (H chain), lactate dehydrogenase, chymotrypsinogen A, myoglobin, lysozyme. The molecular weights of the polypeptides of the initiation factors are those determined by Schreier (33).

teins of the 50S particle. We supposed that some of these could be important translational factors. As most of the eukaryotic initiation factors have an affinity to heparin (27), the 50S complexes were submitted to a Heparin-sepharose chromatography. Subsequently the bound material and, in comparison, eIF-2 and eIF-3 were analysed on SDS gels. Tracks C and D and the corresponding densitometric scans in Fig. 3 show the three polypeptides of eIF-2 (33; one band split) and the nine polypeptides of eIF-3 (33; besides some additional bands due to contamination). With regard to their molecular weights some of the Heparin-sepharose adhered proteins of the 50S material (track E) correspond well to the polypeptides of both initiation factors. Some of the residual bound proteins might be identical with other initiation factors not compared in this study.

DISCUSSION

A number of characteristics of the 50S particles described here agree well with those of similar complexes (40S ribosomal subunit plus mRNA plus Met-tRNA$_f$) which were identified by others (12, 13). Moreover we can show that such complexes contain additional proteins besides the ribosomal structural proteins (Fig.3). It is likely that some of these correspond to initiation factors like eIF-2 and eIF-3, both indipensible for Met-tRNA$_f$ and mRNA binding (1). We also suppose that some of the additional proteins could be associated with the globin mRNA. The presence of non-ribosomal proteins and of mRNA, probably in the form of an mRNP particle (unpublished result), might result in the higher sedimentation value of 50S versus 40S of the ribosomal subunit (6). Similar mRNA-containing complexes are formed if subunit joining is inhibited (3, 6, 11). During our isolation procedure it was not necessary to block 80S formation, and we obtained 50S particles in amounts great enough to allow characterization of their mRNAs as mainly α-globin mRNAs (Fig.2, Table 1).

The possibility that we have isolated free cytoplasmic mRNP particles (20S) which contain predominantly α-globin mRNA (7) and comigrate with 40S ribosomal subunits can be ruled out because the sedimentation value of the particle isolated as described is shifted to 50S. Two further observations support our assumption that the 50S particle is not an artefact, but a true intermediate in the initiation sequence:

(1) The 200 000 x g fraction can be efficiently translated in both protein synthesizing systems used in this study (Table 1; 14). The reason for the decrease in translational activity when the 50S constituent is tested in the recombined system is uncertain. As we find stimulatory activity in fractions derived from the top of the gradient, we assume that the observed loss is due to the dissociation of factor(s) from the 50S particle during preparation, rather than to degradation of mRNA.

(2) In the recombined system as well as in the Ehrlich Ascites system the specific inhibition of globin synthesis by the hemin-controlled translational repressor (HCR; 34) can be overcome by the addition of the 200 000 x g fraction (14).

This study, together with results published recently (3, 14), draws attention to preinitiation complex II as a target of the HCR mediating the regulation of globin synthesis. As we are able to isolate the presumed preinitiation complex II in a translatable form, one possibility for studying its significance as an intermediate in the initiation sequence is to investigate the influence of HCR on 60S ribosomal subunit joining.

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