The Role of Acidic Proteins from Cytoplasmic Fractions of Krebs II Ascites Cells for Efficient Translation

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RNA-Binding Proteins

Acidic proteins with affinity to RNA from cytoplasmic fractions of Krebs II Ascites cells were isolated by means of affinity chromatography on RNA-Sepharose CN-Br-columns.

Stepwise elution with 350 mM [K⁺] and 1000 mM [K⁺] removed two fractions of proteins both of which are required for the formation of 40S- or 80S-initiation complexes and for efficient translation.

Introduction

Many previous reports have shown that mRNAs in eukaryotic cells occur always in association with proteins, as messenger ribonucleoprotein particles (mRNPs) [1—9]. Eukaryotic cytoplasm contains two classes of mRNPs: one class consists of "free" mRNPs not bound to ribosomes, often called informosomes [10—14], which may be regarded as masked or inactive form of message [15—20]. The second class is obtained from the polysomal fraction, hence named ps-mRNPs [2, 3, 21—23], and is engaged in polypeptide synthesis. At least some kinetic studies support the view that informosomes are precursors to ps-mRNPs [24, 25]. Since the two classes of particles show different protein composition [26, 27], we have proposed earlier that the protein moieties are exchanged during the conversion of masked and unused messages into ps-mRNPs [26]. If this is true, the proteins of newly engaged ps-mRNPs are most likely supplied from the closest source: the cytosol [28, 29].

One may postulate that all efficient and specific translation in the cell as well as *in vitro* depends firstly on the absence of natural repressors and secondly on the presence of the right kind of stimulating cofactors, *i.e.* initiation factors in the widest sense including such factors which associate with mRNA like the ps-mRNP proteins.

If such components are considered not to be integral and permanent structural part of ribosomes, then they are likely to be found at the same time also elsewhere in the cytoplasm. Since all commonly employed cell-free systems of protein synthesis depend on the addition of more or less purified cytosol fractions in the form of postmitochondrial supernatants (PMS, often called "lysates" or S30) or postribosomal supernatants (PRS, also S100) or as pH 5-enzyme and crude elongation factors, we have investigated these fractions for proteins which possess affinity for mRNA, and which support efficient translation. We have earlier demonstrated that the cytosol contains such proteins by means of affinity chromatography [26, 28]. Recently, similar reports have been published using different approaches [30—32].

There is another rather well-known group of proteins which lead to the attachment of mRNA onto ribosomes yielding 40S- or 80S-initiation complexes for translation, *e.g.* the 20 odd polypeptide chains of the "genuine" initiation factors: eIF-1 through-5 [33]. Although the eIFs have mostly been obtained from the polysome fraction, they are also found in the cytosol [34—37]. Lastly, we wondered how many of the RNA-binding proteins of the cytosol were identical with the "genuine" initiation factors and with ps-mRNP-proteins. It will be shown here by means of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) that some proteins of the two groups have identical mobilities. We shall further demonstrate, that cytoplasmic RNA-binding proteins alone seem to comprise all neces-

sary factors for efficient *in vitro* translation. Under these circumstances efficient translation does not require addition of neither "genuine" initiation factors nor ps-mRNPs nor native 40S subunits, which are indispensable in many systems.

Materials and Methods

1. Cell cultures

HeLa cells were maintained in suspension cultures, harvested and processed further as described earlier [26, 28]; Krebs II Ascites cells were maintained by intraperitoneal injections of 0.1 or 0.2 ml of ascites fluid into BALB-C mice and allowed to multiply for 7 to 9 days. Then, the mice were sacrificed, the cells removed sterilly with a syringe, and transferred into precooled centrifuge tubes. Cells were sedimented by low speed centrifugation and processed further.

2. Cell fractionation procedure

All operations were carried out in the cold ($0°-4°C$). The cell pellet was washed twice with saline (145 mM NaCl, 20 mM TRIS pH 7.5) and the cells were packed by low speed centrifugation for 5 min. Two volumes of hypotonic buffer (10 mM KCl, 20 mM TRIS-HCl pH 7.5, 3 mM $MgCl_2$, 7 mM β-mercaptoethanol) and 20 μmol hemin were added and the suspended cells were transferred into a Dounce homogenizer. They were allowed to swell for 10 min, and were thereafter disrupted by 30 strokes of the tight fitting plunger; tonicity was immediately restored by dropwise addition of 2 M KCl. Nuclei, unbroken cells, membranes and mitochondria were removed by centrifugation ($30,000 \times g$, 20 min). The postmitochondrial supernatant, PMS, was removed carefully, avoiding both pellet and the thin film of lipid at the surface. The PMS was then layered over a discontinuous gradient consisting of 10 ml of 20% over 10 ml of 60% sucrose (w/v) in isotonic buffer (145 mM KCl; 20 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$, 7 mM β-mercapto-ethanol) and centrifuged in a Spinco SW 27 rotor at 25,000 rpm for 24 h. The pellet contains free polysomes and ribosomes. The upper 15 ml of the postribosomal supernatant (PRS) were used as such and for the preparation of pH 5-enzyme or elongation factors.

3. pH 5-enzyme

The PRS fraction was diluted threefold with 7 mM β-mercaptoethanol, and the suspension was slowly adjusted to pH 5 with 1 M acetic acid. The formed precipitate was collected by centrifugation (Sorvall SS 34, 12,000 rpm, 20 min, 4 °C), redissolved in isotonic buffer and dialysed overnight against the same buffer.

4. Crude elongation factors

Crude elongation factors were prepared from the PRS by ammoniumsulphate fractionation: the protein fraction precipitating between 25% and 70% saturation was collected by low speed centrifugation; it was redissolved in isotonic buffer and dialysed overnight against the same buffer.

5. Crude initiation factors

Crude initiation factors were prepared from the polysomal pellets. Polysomes were resuspended in high salt buffer (500 mM KCl, 20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 7 mM β-mercaptoethanol). After 15 min the suspension was centrifuged in a Spinco SW 56 Ti rotor at 45,000 rpm for 4.5 h. The supernatant was diluted twofold with 7 mM β-mercaptoethanol. The protein fraction precipitating at 70% ammoniumsulphate saturation was collected, redissolved in isotonic buffer, and dialysed overnight against the same buffer.

5a. Met-tRNA

Met-tRNA was prepared from mouse liver according to Skogerson and Moldave [38].

6. Affinity chromatography

The method has been described recently [26, 28], some minor modifications were employed here.

3 g CN-Br activated Sepharose was pretreated with 1 mM HCl. The swollen gel was then washed thoroughly with 0.2 M MES (Morpholinoethansulfonic acid) pH 6.0. 30 mg polysome derived RNA was dissolved in 0.2 M MES pH 6.0 and allowed to react with the CN-Br activated Sepharose gel; the gel was gently stirred first at room temperature for two hours and thereafter at 4 °C overnight. The binding of RNA was monitored by measuring the absorbance at 260 nm of aliquots taken from the mixture. By this method 6–8 mg RNA could be bound to 1 g of CN-Br activated Sepharose. After

complete coupling, the material was packed into a column (1 cm × 10 cm) and washed extensively first with 0.2 M MES pH 6.0, followed by high salt buffer (500 mM KCl, 20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 7 mM β-mercaptoethanol) and there after 100 mM ethanolamin pH 8.0 and lastly equilibrated with isotonic buffer.

The cytoplasmic fractions containing RNA-binding proteins were circulated through the column thrice. Thereafter followed an extensive washing with isotonic buffer. The bound proteins were eluted stepwise, first using a buffer with 350 mM KCl; 20 mM Tris-HCl pH 7.5; 3 mM $MgCl_2$; 7 mM β-mercaptoethanol; afterwards with 1000 mM KCl, 20 mM Tris-HCl pH 7.5; 3 mM $MgCl_2$; 7 mM β-mercaptoethanol; (flow rate of 60 ml/h).

7. Preparation of polysomal mRNPs (ps-mRNPs) and informosomes through Cs_2SO_4 density gradients centrifugation

a) Polysomal-mRNPs (ps-mRNPs)

Polysomes were resuspended in isotonic buffer and then briefly incubated in a cell-free amino acid incorporation system containing per ml 30 A 260 units polysomes, 0.030 μmol each of 20 amino acids, 0.2 mg pH 5 enzyme proteins, 1 μmol ATP, 0.3 μmol GTP, 10 μmol creatine phosphate, 50 μg creatine phosphokinase, 20 mM Tris-HCl pH 7.5, 70 mM KCl, 4 mM $MgCl_2$ and 7 mM β-mercaptoethanol. The reaction mixture was incubated at 30° for 15 min and then 10 mM puromycin (pH adjusted to 7.5) was added to a final concentration of 0.1 mM; cooled in ice bath and incubated for another 15 min at 30 °C. The pre-incubated polysomes mixture was clarified by low speed centrifugation before layering on the preformed Cs_2SO_4.DMSO gradients [39, 40].

b) Informosomes

Postribosomal supernatants were centrifuged (rotor SW 56, 23,000 rpm 16 h) through a cushion of 30% sucrose in isotonic buffer. The pellet was resuspended in isotonic buffer and analysed by zonal centrifugation (rotor SW 40, 22,000 rpm 17 h) in a 15−30% sucrose gradient in isotonic buffer. Fractions containing 20S−60S particles were pooled, sedimented by centrifugation (rotor Ti 50, 35,000 rpm, 17 h) resuspended in isotonic buffer and then layered over the preformed Cs_2SO_4. DMSO gradients [39, 40].

8. Two-dimensional polyacrylamide gel electrophoresis (=2D-PAGE)

The apparatus and the technique have been described by Mets and Bogorad [41] and Issinger and Beier [42] recently. The protein fractions were spread in the first dimension in 9 cm-tubes with 4% acrylamide in buffered 8 M urea pH 5.0. The upper electrode vessel contained 10 mM BIS/Tris (pH 4.5) and the lower vessel 175 mM K-acetate (pH 6.0). The electrophoresis was run at 2.5 mA per tube for 3 h.

In the second dimension the procedure according to Laemmli [43] was employed; the gel cylinders from the separation in the first dimension were polymerized to gel slabs poured from 12.5% acrylamide in buffered 0.1% sodium dodecylsulphate (SDS).

The electrode vessels contained 24 mM Tris HCl pH 8.8, 0.2 M Glycine and 0.1% SDS; the final pH was 8.6. The electrophoresis was run at 100 V for 6 h. Thereafter the gels were stained with Coomassie Brillant Blue R 250. The protein spots were excised and extracted with 0.5 ml 8 M urea, 10 mM dithiothreitol and 0.5% SDS at room temperature. The extracts were dialyzed against 7.5% propionic acid with 14 mM β-mercaptoethanol, and then lyophilised.

The extracted proteins were analysed on 10% acrylamide gel slabs according to Laemmli [43], using the same buffer systems as indicated above.

9. Cell-free-system from ascites cell lysate for translation

Ascites lysates were prepared as described previously [44] except that 20 μmol hemin were added per ml packed cells before homogenization. The PMS was ready for use after passage through a Sephadex G 25 column, yielding a PMS (+). Ascites lysates from which RNA-binding proteins had been removed by cycling through RNA-Sepharose columns, yielding a PMS (−) were stored as 200 μl aliquots at −80 °C. The assay mixture (100 μl) contained the following components: 0.5 A 260 ascites lysate; 1 nmol ATP; 0.4 nmol GTP; 6 nmol creatine phosphate; 2 I. U. creatine phosphokinase; 30 nmoles of each of the 19 l-amino acids; 20 mM Tris-HCl pH 7.5; 70 mM KCl; 4 mM Mg-acetate; 1 mM DTT and [^{3}H]-leucine with specific activities as indicated in the legend to the figures.

The reaction mixtures were incubated at 30 °C. Aliquots of 50 μl were taken at regular intervals

and pipetted immediately onto 2.5 cm Whatman 3 MM paper disks and dried. They were thereafter placed in ice cold 15% trichloroacetic acid containing 10 mM leucine, boiled for 10 min in 5% trichloroacetic acid, the transferred to cold 5% trichloracetic acid and dried after rinsing in ethanol, ethanol-ether (1:1), and ether [45]. Radioactivity was determined in a Packard liquid scintillation counter.

10. Cell-free system from ascites cell lysates for translation of exogenous mRNA

mRNA from HeLa cells was obtained by the procedures described by Perry *et al.* [46] and Lindberg and Persson [47]. Cowpea mosaic virus RNA was a gift from Dr. H. Beier.

The PMS (+) or PMS (−) lysates were supplemented with ATP, GTP, creatine phosphate, creatine phosphokinase and all 20 l-amino acids in quantities as indicated in the chapter above and were incubated for 30 min at 30 °C. After cooling in ice the gelatinous material, which often formed, was removed; the "preincubated" PMS (+) or preincubated PMS (−) were kept frozen in aliquots at −80 °C until use. The preincubated PMS and mRNA were added to the assay mixture as indicated above.

11. Sucrose gradient centrifugations

The formation of 40S- and 80S initiation complexes were studied by layering the various mixtures (as indicated in the legends to Figs 4, 5 and 6) on linear 5% −20% sucrose gradients in 100 mM KCl, 20 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, and 3.5 mM β-mercaptoethanol.

12. Materials

CN-Br activated Sepharose purchased from Pharmacia, Upsala, Sweden. [14]C- and ^{3}H-labeled amino acids: leucine, methionine from New England Nuclear. ^{3}H-labeled adenosine (36.6 Ci/mM from Amersham-Buchler).

Results

1. Efficient translation of endogenous and exogenous mRNA depends on RNA-binding proteins

A cell-free system from Krebs II ascites cells currently employed in our laboratory was investigated previously for the conditions of optimal efficiency of translation. When translational inhibitors were removed by passing a PMS fraction through a Sephadex G 25 column, the rate of amino acid incorporation into polypeptide could be increased five times in the presence of endogenous mRNA (Fig. 1a). After preincubation, the system is dependent on added exogenous messages (Fig. 1b and c) [44]. This system is, therefore, ideal for the investigation of natural cofactors and initiation factors of translation. We expected to find such stimulating factors among proteins which possess a pronounced affinity to mRNA. For this reason a PMS (+) with high translational efficiency was passed through Sepharose with covalently linked polysomal RNA (containing mRNA and rRNA). A portion of the proteins was adsorbed to the RNA-Sepharose column and was thus removed from the PMS (+) rendering the latter rather inefficient for translation [PMS (−) compare curve 1 in Fig. 2a with curve 1 in Fig. 2b]. Further experiments showed that full efficiency of translation was restored to starting conditions when the RNA-binding proteins were eluted from the column by raising the KCl concentration and were thereafter added back to the PMS (−) system in optimal amounts. Elution of bound proteins was performed in two steps: a first fraction containing moderately tightly bound proteins was eluted with 350 mM KCl (BP-350). A second fraction of more tenaciously bound proteins was detached by 1 M KCl (BP-1000). The eluate contains no measurable amounts of nucleic acids. As seen from Figs 2 neither the BP-350 fraction nor the BP-1000 fraction alone sufficed to restore the initial activity (curve 1 in Fig. 2a and curve 4 in Fig. 2b) although each fraction stimulated the translation of endogenous and exogenous messengers slightly (Fig. 2a and b, curves 2 and 3). Only optimal concentrations (*i.e.* 5 μg per 100 μl assay mixture) of both fractions led to full efficiency of translation (Fig. 2b, curve 4).

Likewise, purified mRNA from HeLa cells was only translated when both fractions of stimulating proteins were present of added back (Fig. 3a and b, curves 2). The controls clearly showed that no exogenous mRNA was translated by a PMS (−) fraction (*i.e.*, from which RNA-binding protein had been removed) (Fig. 3b, curve 1).

2. The requirement of RNA-binding proteins for the binding of Met-tRNA and mRNA to the small ribosomal subunits

The functional activities of the two RNA-binding protein fractions BP-350 and BP-1000 were analy-
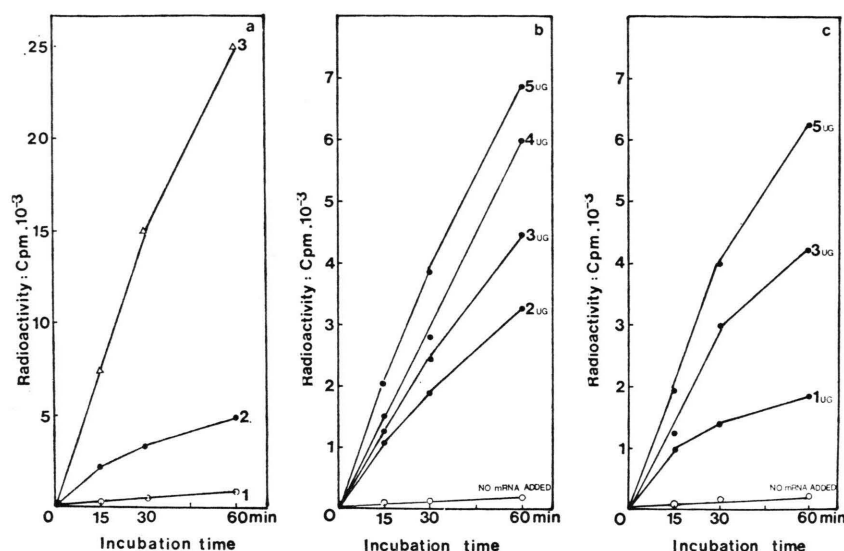
Fig. 1. The effect of various pretreatments on a PMS fraction from Krebs II ascites cells and translation of exogenous mRNA. 1000 cpm correspond to approximately 1 pmol leucine. a) The PMS was obtained and incubated as indicated in "Materials and Methods". Samples were taken after 15, 30, and 60 min incubation. Curve 1 ○—○ PMS after passage through Sephadex G 25 and 30 min preincubation; curve 2 ●—● untreated control; curve 3 △—△ PMS after passage through Sephadex G 25. b) Translation of poly A+mRNA from HeLa cells. The PMS was prepared and preincubated for 30 min, passed through Sephadex G 25. 300 cpm correspond to 1 pmol leucine. The amount of RNA added to the system is given for each curve. ○—○ Control: no RNA added. c) Translation of cowpea mosaic virus RNA. 300 cpm correspond to 1 pmol leucine. ○—○ Control: no RNA added.

sed more closely. Previous results of many laboratories indicated that, due to a series of initiation factors (eIF, -1, -2, -3, -4A, -4B, and -4C), Met-tRNA and mRNA can be bound to the small ribosomal subunit forming a 40S-initiation complex [48, 49]. Our experiments, documented in Fig. 4 and 5, demonstrated that 40S-initiation complexes formed readily upon addition of BP-350 and BP-1000, whereas 40S ribosomes did not bind neither Met-tRNA nor mRNA alone or both together spontaneously (Fig. 4a, b and Fig. 5a). The addition of BP-350 led to formation of some 40S+Met-tRNA complexes, but GTP increased the reaction strongly. This supports the idea that initiation of translation begins with a ternary complex which includes GTP (Fig. 4c, d). ATP was dispensible for the fixation of Met-tRNA, but was required for the attachment of mRNA ultimately. BP-350 did, however, not yield more than 50% of the possible initiation complexes in the system, even when ATP and GTP were present (Fig. 5b). The addition of BP-1000 finally led ot the attachment of all Met-tRNA and mRNA to the 40S subunits (Fig. 5d). Control experiments showed that BP-1000 alone (without BP-350) likewise resulted

in a limited 40S-initiation complex formation (Fig. 5c and other experiments not shown).

3. The requirement of RNA-binding proteins for the formation of 80S-initiation complexes

According to Staehelin's concept a further initiation factor eIF-5 leads to the attachment of the large subunit (60S) to the 40S-initiation complex [48]. When we repeated our experiments with a system which contained both ribosomal subunits we demonstrated that the 80S-initiation complex also required components of both RNA-binding proteins. As seen in Fig. 6a, mRNA and Met-tRNA did not attach spontaneously. Upon addition of either BP-350 or BP-1000 (plus ATP and GTP) some complex formation occurred (Fig. 6b and c). When both fractions were given, the complex formation was strongly enhanced (Fig. 6d). A similar result was obtained when crude initiation factors (Fig. 6e) were added instead of RNA-binding proteins.

From these results we conclude that certain RNA-binding proteins in the cytosol are either identical with or may substitute for certain proteins in crude initiation factor preparations.
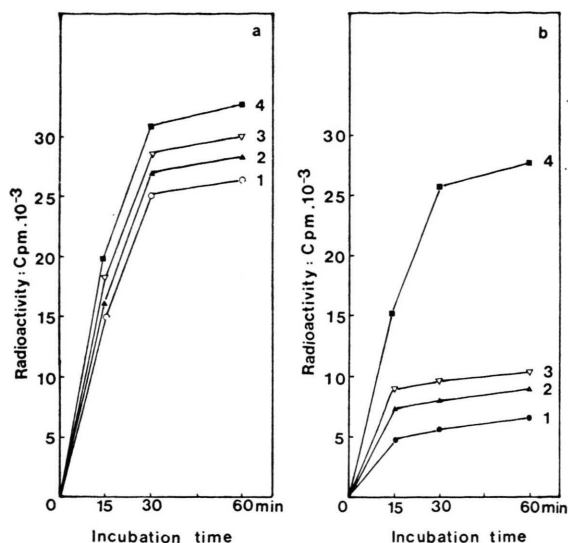
Fig. 2. Translation of endogenous mRNA by the PMS from Krebs II ascites cells .The PMS was prepared and passed through Sephadex G 25 as indicated in "Materials and Methods" and a cell-free translation system was set up. 1000 cpm correspond to approximately 1 pmol leucine. a) Curve 1 ○—○: no additions; curve 2 ▲—▲: PMS(+) +5 μg BP-350 added; curve 3 ▽—▽: PMS(+)+5 μg BP-1000 added; curve 4 ■—■: PMS(+)+5 μg BP-350 and 5 μg BP-1000 added. b) The prepared PMS(+) was passed 3 times through a RNA-Sepharose column to remove RNA-binding proteins. Curve 1 ●—●: PMS(−); no additions; curve 2 ▲—▲: PMS(−)+5 μg BP-350 added; curve 3 ▽—▽: PMS(−)+5 μg BP-1000 added; curve 4 ■—■: PMS(−)+5 μg BP-350 and 5 μg BP-1000 added.
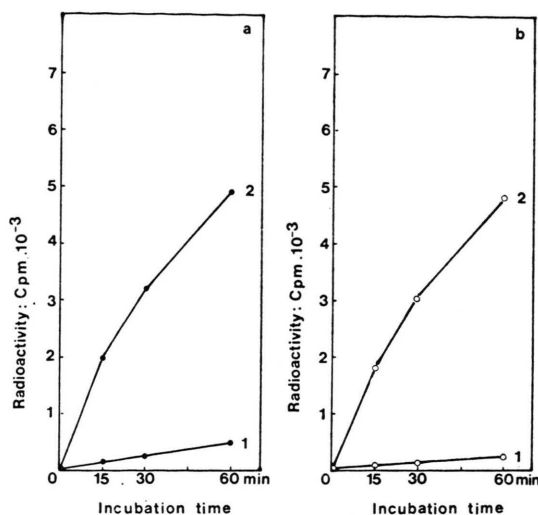
Fig. 3. Translation of exogenous mRNA by the PMS from Krebs II ascites cells. A cell-free system was set up as indicated in "Materials and Methods". mRNA was prepared from HeLa cells according to Lindberg *et al.* using a poly U-Sepharose column [47]. The PMS was preincubated for 30 min. 300 cpm correspond to 1 pmol leucine. a) Curve 1: preincubated PMS(+), no mRNA added; curve 2: preincubated PMS(+), and 2 μg HeLa mRNA added. b) From the preincubated PMS the RNA-binding proteins were removed by passing three times through a RNA-Sepharose column PMS(−). Curve 1: preincubated PMS(−), and 2 μg HeLa mRNA added; curve 2: preincubated PMS(−), 2 μg HeLa mRNA, 5 μg BP-350, and 5 μg BP-1000 added.

4. Analysis of RNA-binding proteins by 2D-PAGE

The various cell-free systems reported in the literature all require certain crude cytoplasmic fractions to supply necessary additives for efficient translation. We have investigated more closely the following 4 fractions: the PMS, once freed from ribosomes by high speed centrifugation, yields in the first place a PRS from which secondly pH 5-enzyme or thirdly elongation factors were prepared (see: section Material and Methods). Fourthly, crude initiation factors (CIF) were extracted with 500 mM KCl from thoroughly washed ribosomal pellets. These four cytoplasmic extracts were loaded on four RNA-Sepharose columns in order to remove RNA-binding proteins which were eluted with 350 mM KCl and with 1 M KCl subsequently (Fig. 7). Of the four extracts CIF is rich in RNA-binding proteins: 50−70% its constituents were retained by the column and eluted with 350 mM KCl; 1000 mM KCl removed another 5% of more tenaciously bound proteins. The other three extracts

(S100, pH 5-enzyme, crude elongation factors) contained only less than 2% of RNA-binding proteins (Table I and Fig. 7).

The protein composition of the four extracts and the eluted fractions, BP-350 and BP-1000, was analysed by 2D-PAGE. In addition, we prepared ps-mRNPs and free cytoplasmic mRNPs (informosomes) and also investigated their protein moieties by 2D-PAGE. The results are documented in Figs. 8, 9 and 10). All preparations comprise many proteins, mostly more than 20. Almost all proteins are acidic or neutral, and they are readily distinguishable from the structural proteins of the small and large ribosomal subunits (Fig. 10c and d). It is also evident that the majority of the RNA-binding proteins have higher molecular weights than most ribosomal proteins. All spots were tentatively numbered. Several congruent spots were punched out and further analysed by 1D-PAGE on slab gels. It is evident that many of the numerous spots analysed so far contain proteins of the same molecular
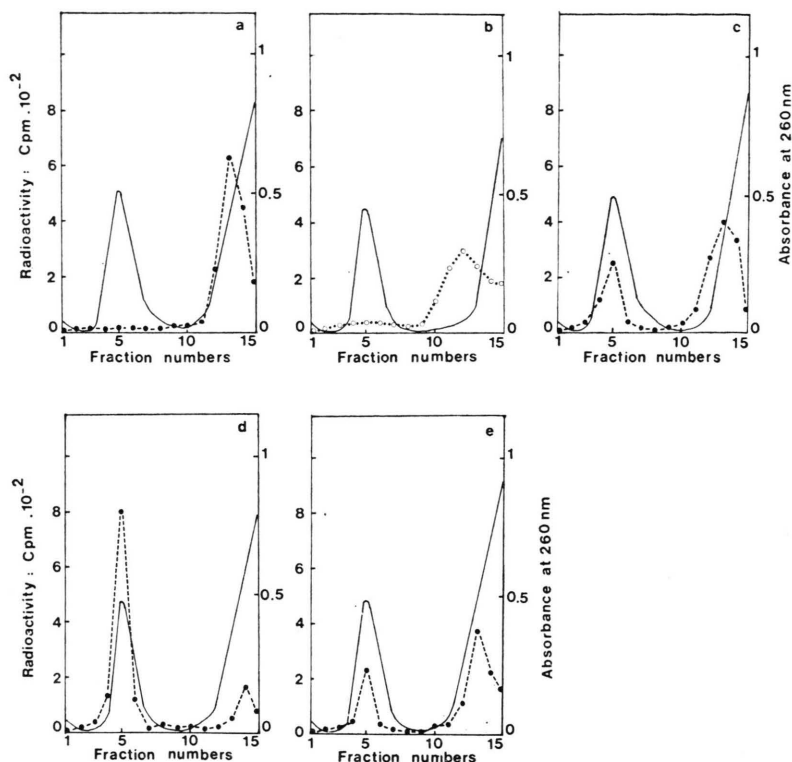
Fig. 4. Influence of BP-350 on the information of 40S-initiation complexes. Standard reaction mixture of 0.1 ml contained one A_{260} 40S ascites ribosomes subunits prepared as described by Sundkvist *et al.* [50]. 0.05 A_{260} units HeLa [³H]mRNA, 20 pmol [¹⁴C]Met-tRNA (mouse liver), 5 μg BP-350 and 5 μg BP-1000, and the following reagents: 3.5 mM β-mercapto-ethanol, 0.008 mM GTP, and 0.5 mM ATP. The mixture was incubated for 10 min at 30 °C analysed on 5—20% sucrose gradient in 100 mM KCl, 8 mM $MgCl_2$, 3.5 mM β-mercaptoethanol, 20 mM TRIS-HCl pH 7.5. Centrifuged in a Spinco SW 56 rotor at 45 000 rpm for 2 h at 4 °C. The gradients were fractionated through a LKB-UV monitoring flow-cell system, and to each fraction, 1 ml 10% trichloroacetic acid and 1 ml BSA 100 mg/l solution was added, let stayed in ice bath for 20 min, filtered through glass filterpaper, dried and counted in Packard liquid szintillation counter. ●----● [¹⁴C]Met-tRNA (300 cpm/pmol); ○----○ [³H]mRNA (450 cpm/pmol). a) 40S and Met-tRNA; b) 40S, and mRNA from HeLa cells; c) 40S, Met-tRNA, and BP-350; d) 40S, Met-tRNA, BP-350 and GTP; e) 40S, Met-tRNA, BP-350 and ATP.

weights and the same charges, and thus seem to be identical. This is best illustrated by the spots marked by circles in Figs. 9 a, b, d and 10 a, b which were subjected to further PAGE. Four of the five samples seem to have two proteins in common. Further research is required to identify and characterize the proteins detected by 2D-PAGE and to define their role in mRNA translation.

Discussion

Our results indicate that the postribosomal supernatant of Krebs II ascites cells contains a fraction of proteins which are capable of forming complexes with immobilized mRNA in isotonic buffer (*i.e.* at a salt concentration similar to the one in the living cell). This fraction of RNA-binding proteins can substitute for the genuine initiation factors in cell-free translation. Similar protein fractions have recently been investigated in other laboratories using *E. coli* RNA- and heparin affinity chromatography [30—32, 52, 53]. This way of approach will hopefully shed more light on the interaction of mRNA with proteins before and during translation, since the onset of messenger translation is more elaborate in eukaryotes and requires more than the mere three initiation factor as does *E. coli* [54—56]. The assembly of the eukaryotic 80S-initiation complex from ribosomal subunits, Met-tRNA, and mRNA is mediated by a host of protein factors. One may assume that such proteins have variety of functions (for review see ref. 57):

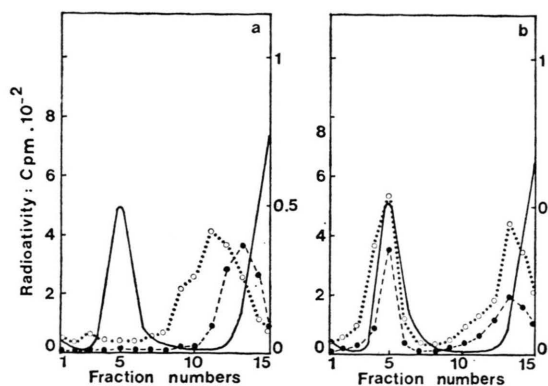1. they may serve for the fixation of mRNA to cytoskeleton [58];

Fig. 5. Influence of BP-1000 on the formation of 40S-initiation complexes. Conditions were the same as in Fig. 4. ●----● [14C]Met-tRNA (300 cpm/pmol); ○----○ [3H]mRNA (450 cpm/pmol). a) 40S, Met-tRNA and mRNA; b) 40S, Met-tRNA, mRNA, BP-350, GTP, ATP, *no* BP-1000 added; c) 40S, Met-tRNA, mRNA, GTP, ATP, BP-1000, no BP-350 added; d) BP-350 *and* BP-1000 added to the above system.

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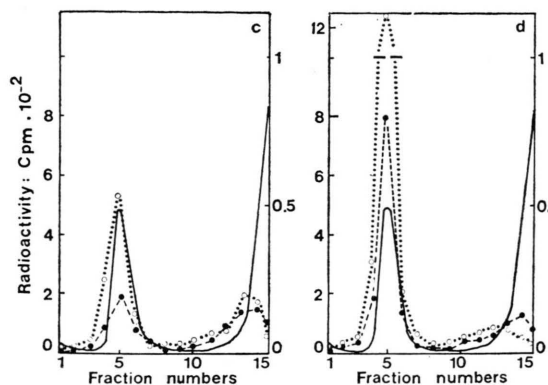
2. they may be responsable for its selective and specific activation from a dormant state;
3. they constitute the "genuine" eIFs;
4. they are firmly bound in the form of ps-mRNP-proteins.

The highly efficient translation in Krebs II ascites extracts indicates that its PRS fraction probably contains the full complement of such factors.

Some of the proteins which influence the structural or functional properties of mRNA have been previously analysed: *First*, there are the *"genuine" initiation factors* which mostly have been obtained from polysomes [59]; they have also been found in the cytosol [34–37], and on native small ribosomal subunits [60–62]. Seven factors, the "genuine" eIF-1 through -5, have been purified and characterized [63–72]. The addition of these seven factors restores translational activity in a refined assay
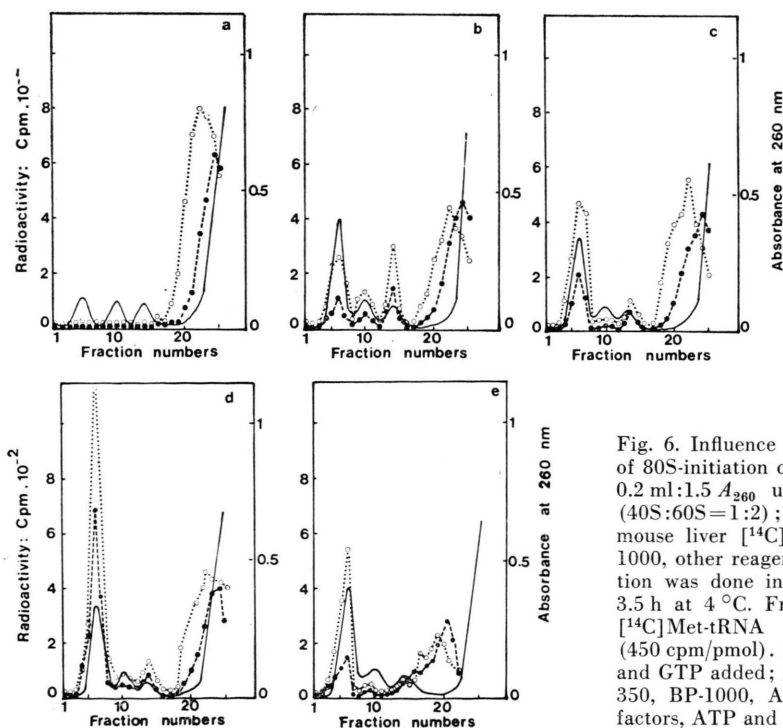

Fig. 6. Influence of RNA-binding proteins on the formation of 80S-initiation complexes. The complete assay contained in 0.2 ml:1.5 A_{260} units of mixed ascites ribosome subunits (40S:60S=1:2); 0.1 A_{260} units HeLa [3H]mRNA, 30 pmol mouse liver [14C]Met-tRNA, 15 μg BP-350 and 15 μg BP-1000, other reagents were as described in Fig. 4. Centrifugation was done in a Spinco SW 40 rotor at 36 000 rpm for 3.5 h at 4 °C. Fractionated as described above. ●----● [14C]Met-tRNA (300 cpm/pmol); ○----○ [3H]mRNA (450 cpm/pmol). a) no further additions; b) BP-350, ATP, and GTP added; c) BP-1000, ATP, and GTP added; d) BP-350, BP-1000, ATP and GTP added; e) crude initiation factors, ATP and GTP added.
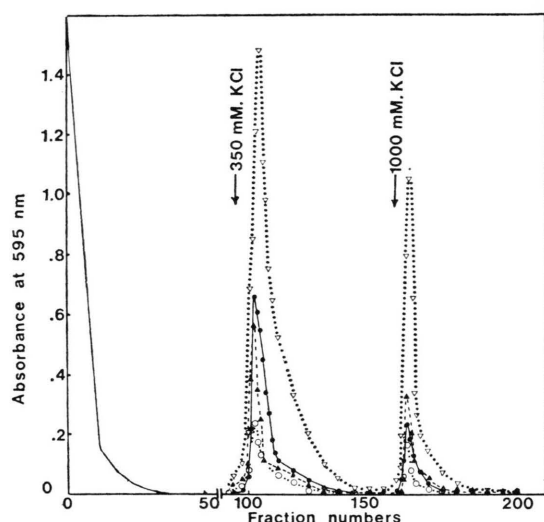
Fig. 7. RNA-binding proteins of four cytoplasmic extracts eluted from RNA-Sepharose columns. 20 mg of polysomal RNA was covalently linked to Sepharose (bed volume: 10 ml). The columns were extensively rinsed with isotonic buffer. The protein fractions were charged three times, and the columns were rinsed again with isotonic buffer. The bound proteins were eluted with buffered 350 mM KCl and subsequently with buffered 1 M KCl. The elution was monitored by a LKB uvicord and one ml fractions were collected. Protein was determined according to Bradford [51]. ●—● Proteins eluted from column loaded with PRS; ▲—▲ proteins eluted from column loaded with pH 5-enzyme; ○----○ proteins eluted from column loaded with crude elongation factors; ▽----▽ proteins eluted from column loaded with crude initiation factors.

mixture, but only with a low efficiency, which indicates that some important ingredients are still missing (*e.g.* ref. 63). In contrast, we have shown above that the addition of the two fractions BP-350 and BP-1000 to a PMS (−) system fully restores its amino acid incorporation activity (Fig. 3). Correspondingly, the formation of 40S-initiation complexes only occurs in the presence of these same fractions. The BP-350 have characteristic initiation

factor activities; they stimulate Met-tRNA binding to 40S complexes in the presence of GTP. Experiments of other laboratories indicate that 40S-initiation complexes include the three "genuine" initiation factors eIF-2, eIF-3, and eIF-4C (summarized in ref. 49). The first two of these are reported to be only transiently attached to the complex and then to be released from it, when the 80S-initiation complex is formed. One may assume that they are less tightly bound.

The BP-1000 is indispensable for the efficient binding of deproteinized mRNA to 40S-Met-tRNA-GTP complexes (Fig. 5d). This reaction is stimulated by ATP and according to Benne and Hershey [49], it is carried out by eIF-1, eIF-4A, and eIF-4B. We have demonstrated above that BP-1000 include polypeptides with molecular weights of 15 kd, 50 kd and 75 kd respectively.

The *second* group of proteins more firmly associated with mRNA is *the protein moiety of the ps-mRNPs*. As we have shown earlier in HeLa cells, at least two BP-1000 proteins are identical with ps-mRNP proteins [28]. The analysis of the circled areas in Fig. 9a, b, d and Fig. 10a suggests that the same is true for Krebs II Ascites cells, and this is demonstrated in Fig. 11, gels 1 and 4. This is in accordance with recent results reported for rat liver [29], and for rabbit reticulocytes [74]. The absence of ps-mRNP proteins from the above mentioned translation systems may account for their reduced efficiency.

Postribosomal fractions, whether obtained by serial centrifugation of homogenates or by stepwise extraction of cells [58] are likely to form a rich pool of factors indispensable for translation. It is of interest that eIF-2 and eIF-3 are released after 80S-initiation complex formation [49] Polysomal pellets are therefore not the richest source for ob-

Table I. RNA binding proteins eluted from RNA-Sepharose column with 350 mM and 1 M KCl. (See: legend to Fig. 8). All values are mean of 5-determination.

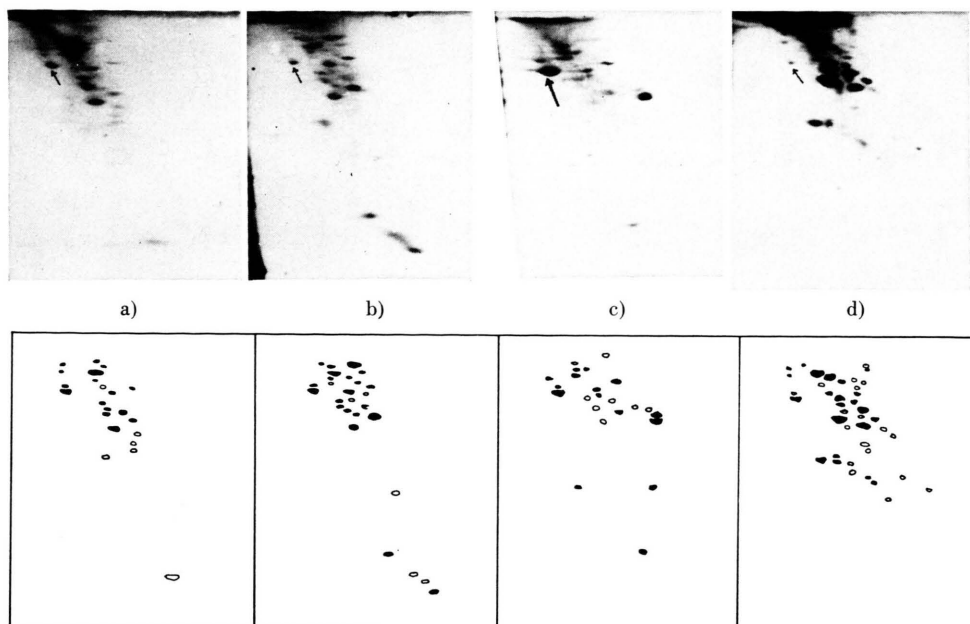| Column No.	Protein loaded 600 mg of	Proteins eluted at 350 mM KCl		Proteins eluted at 1000 mM KCl	
		mg	%	mg	%
I	PRS (S100)	7.5 ± 1.5	1.25	2 ± 1	0.3
II	pH 5-enzyme	7 ± 1.3	1.17	2.5 ± 1	0.4
III	Curde elongation factors 1+2	4 ± 1	0.66	1 ± 0.5	0.16
IV	Crude initiation factors	350 ± 50	58	25 ± 5	4.1

Fig. 8. 2D-PAGE of RNA-binding protein fractions eluted with buffered 350 mM KCl. Top row: photographs of gels stained with Coomassie Brilliant Blue, only the most prominent spots are reproduced; bottom row: diagram with all spots found constantly in all good gels. a) PRS; b) pH 5-enzyme; c) crude elongation factors; d) crude initiation factors.
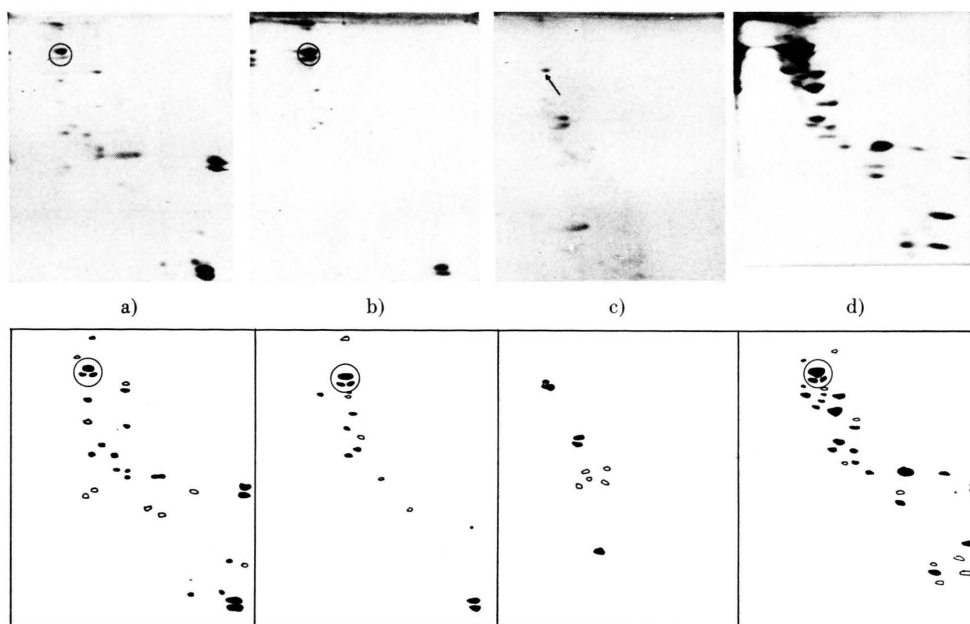

Fig. 9. 2D-PAGE of binding proteins eluted with buffered 1M KCl. Top row: photographs of gels stained with Coomassie Brilliant Blue, only the most prominent spots are reproduced. Bottom row: diagram with all spots found constantly in all good gels. a) PRS; b) pH 5-enzyme; c) crude elongation factors; d) crude initiation factors.
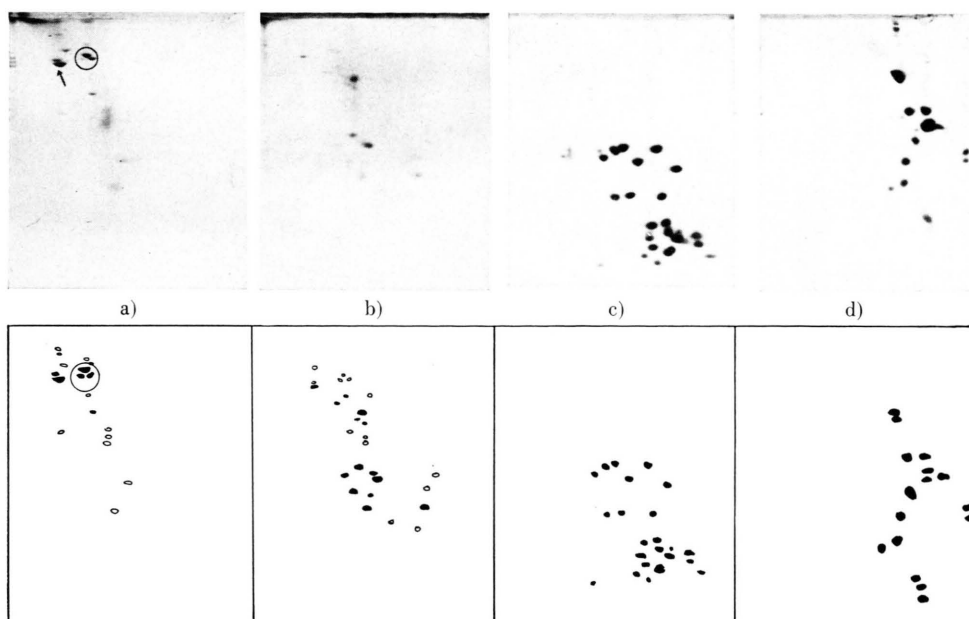
Fig. 10. 2D-PAGE of proteins from mRNPs and ribosomes (see "Materials and Methods"). Top row: photographs of gels stained with Coomassie Brilliant Blue, only the most prominent spots are reproduced. Bottom row: diagram with all spots found constantly in all good gels. a) Proteins of ps-mRNPs; b) informosomes proteins; c) structural proteins of the 40S subunit; d) structural proteins of the 60S subunit.

Fig. 11. Slab gels of the region circumscribed by a circle in 2D-gels of Figs 9 and 10. The triple spots were excised, extracted, lyophilised, and analysed by a 10% PAGE according to Laemmli [43]. Gel 1, from ps-mRNPs (Fig. 10 a); gel 2, from the 1000 mM KCl eluate from column loaded with crude initiation factors (Fig. 9 d); gel 4 from the 1000 mM KCl eluate from column loaded with PRS (Fig. 9 a); gel 5 from the 1000 mM KCl eluate from column loaded with pH 5-enzyme (Fig. 9 b); gel 3, marker proteins: ribonuclease, carboanhydrase, ovalbumin, katalase, bovine serum albumine and galactosidase.

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taining initiation factors. The fact that at least some of them may be obtained from three different cell fractions indicates that they are apparently recycled. Thus, the PRS is likely to yield any of such recycling constituents.

In the past, 2D-PAGE techniques have been important for the elucidation of multicomponent structures like *E. coli* ribosomes [73]. In this investigation we apply the same principals to the analysis of proteins with affinity to mRNA. We are aware that mere affinity to any RNA (or heparin) is not conclusive, but hope that future investigation will lead to exact location of proteins relative to mRNA sequence sections, like cap, leader sequence, and polyA-tails.

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