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Purification of *Saccharomyces cerevisiae* eIF4E/eIF4G/Pab1p Complex with Capped mRNA

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Abstract

Protein synthesis is one of the most complex cellular processes, involving numerous translation components that interact in multiple sequential steps. The most complex stage in protein synthesis is the initiation process. The basal set of factors required for translation initiation has been determined, and biochemical, genetic, and structural studies are now beginning to reveal details of their individual functions in this process. In *Saccharomyces cerevisiae*, eIF4E is the central component of the eIF4F complex, which binds the 5′ cap structure of the mRNA. This complex is significant in many ways. First of all, it is essential for translational initiation, mediating the initial interactions of ribosomes with the mRNA 5′ end. Secondly, because of its key role in interacting with the 5′ end of the mRNA, and possibly also with proteins such as the poly (A) binding protein (PABP) at the 3′ end of the mRNA, the eIF4F complex is thought to be involved in the process of mRNA degradation. Thirdly, eIF4F is a site of translational regulation, responding to signals communicated along the signal transduction pathway that are induced by stress conditions or hormones. To study about the interactions of eIF4E within the eIF4F complex, we tried to find conditions that would enable us to obtain structural data about *S. cerevisiae* eIF4E/eIF4G/Pab1p interactions. To yield information about the eIF4E/eIF4G/Pab1p complex, affinity chromatography was conducted using synthetic biotinylated capped mRNAs. For this purpose, a capped 55-nucleotide RNA was synthesised and labeled with Biotin-21-UTP at the 3′-end in an *in vitro* transcription reaction. For Biotin labeling of mRNA, rUTP was substituted with Biotin-21-UTP in the reaction. Soft Link Avidin Resin was used for the isolation of biotinylated mRNA, which can bind eIF4E via the capped structure at the 5′-end of the mRNA and Pab1p via the poly (A) tail at 3′ end. These results confirm that a highly pure eIF4E/eIF4G/Pab1p/RNA complex can be generated using the procedures outlined.

Keywords: Translation initiation; eIF4E; eIF4G; Pab1

Introduction

Translation of cellular mRNAs is believed to occur predominantly via a cap-dependent mechanism. The 5′m^{7}GpppN (N; any nucleotide) cap of eukaryotic mRNA is generated by a combination of reactions catalysed by a 5′-triphosphatase, a guanyltransferase and an N^{7}G methyltransferase [1]. The mRNA cap interacts

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with a number of different sets of proteins in the nucleus and cytoplasm and, in doing so, performs a range of functions. In animal cells it has been shown that some cellular mRNAs are also translated cap-independently via internal ribosome entry. In plants, such cellular mRNAs have so far not been described. However, translation of uncapped viral RNAs shows that plant cells contain the machinery to translate mRNAs in a cap-independent manner. A set of proteins termed translation initiation factors (eIF4s) is required for mRNA translation in eukaryotic cells, several of which are involved in the binding of ribosome to mRNA. In the cytoplasm, recognition of the 5′cap structure by the ribosome is a critical step in the initiation of translation of most eukaryotic mRNAs [2]. This step is mediated by the cap-binding initiation complex eIF4F.

Mammalian eIF4F comprises eIF4E, eIF4G and eIF4A [12]. The largest eIF4F component, eIF4G [1], has binding sites for eIF4E, eIF3, eIF4A, eIF5, the poly(A) binding protein PABP and the MAPK-activated protein kinase Mnk1 [2,5,16,11]. More recent work indicates that yeast eIF4G is also capable of binding to the mRNA decapping protein (Dcp1) [17]. The eIF4F complex is tethered to the mRNA cap by the 25 kDa cap-binding protein, eIF4E, which is found in both the nucleus and the cytoplasm [3,4]. The eIF4E-cap interaction can be stabilized by binding of the eIF4E-binding domain of eIF4G to the eIF4E dorsal site [10]. eIF3 is thought to promote interaction between the 40S ribosomal subunit and eIF4F, whereas the binding of eIF4G to PABP may play a role in promoting interaction between the 3′ and 5′ ends of mRNA. The significance of the interaction between eIF4G and eIF4A is not clear, but the latter factor (together with eIF4B) catalyses ATP-dependent RNA helicase activity, which may promote ribosomal scanning along structured mRNA [14].

eIF4F complex formation is regulated, at least in part, through the availability of eIF4E. eIF4G and a repressor of translation, 4E-BP1, can bind eIF4E through a common sequence motif. The binding of eIF4G and 4E-BP1 to eIF4E is mutually exclusive. 4E-BP1 binding to eIF4E is regulated through its phosphorylation state. Hypophosphorylation of 4E-BP1 leads to its binding and sequestration of eIF4E and the subsequent dissociation of the eIF4F complex leading to decrease rates in the initiation of translation. The importance of eIF4E availability in translational regulation is underscored by experiments which show that overexpression of eIF4E leads to increase in translation and cell transformation which can be reversed through overexpression of 4E-BP1. Elevated level of eIF4E is also associated with a number of cancers. One possible strategy in reducing tumorigenicity in these cancers have developed peptides based on the eIF4E binding sequences within 4E-BP1 and eIF4G which can be introduced into cells using penetratin as a carrier.

The structures of murine and *S. cerevisiae* eIF4E bound to 7-methyl GDP have been determined by crystallography [6] and NMR [7]. To study the interactions of eIF4E within the eIF4F complex, we tried to find out the conditions that would enable us to obtain structural data about *S. cerevisiae* eIF4E/ eIF4G/Pab1p interactions.

Material and Methods

Bacterial Strains and Transformation

The *E. coli* strains CAG629, BL21 (DE3) and JM109 (DE3) were used in this study. Bacterial transformation was performed by chemical transformation using calcium chloride.

Protein Purification from E. coli

E. coli strain CAG629 was transfected with pCYTEXP1 containing the *S. cerevisiae* eIF4E gene. For the preparation of recombinant eIF4E, a 500 ml culture of the *E. coli* strain harbouring pCYTEXP1 was induced for 2½ h in LB medium at the non-permissive temperature, 42°C and harvested by centrifugation. The purification of eIF4E expressed as inclusion bodies in *E. coli* was done based on the method described by Lang *et al.* (1994) [3]. After inducing the promoter, the cells were pelleted using centrifugation, and re-suspended in 3.5 ml of sucrose in L-buffer (50 mM Tris pH 8.0, 10 mM EDTA) per gram of cells. About 4 grams of Lysozyme and 1.6 ml of E/T buffer (0.25 M Tris pH 8.0, 10 mM EDTA) per gram of cells were added. The cells were then incubated at 0°C for 1 h. After this incubation period, 7 ml of 8% TritonX-100 in L-buffer per gram of cells was added. The cell lysate was then incubated by sonication for 3 min at 0°C. The lysate was pelleted by centrifugation at 6000 rpm for 20 min at 4°C. The pelleted inclusion bodies were washed using 7 ml of Triton X-100 in L-buffer, resonicated, and centrifuged to reform the cell pellet. The washing and repeated sonication was performed in total three times. The final cell pellet was solubilised in 40 ml guanidine solution (37°C, 2 h). Subsequently, Guanidine was removed by dialysis against buffer A (20 mM HEPES pH 7.5, 0.1 M KCl, 0.2 mM EDTA). The next stage involved the preparation of a Sepharose column for eIF4E purification: 1 ml of m^7GTP-Sepharose was added to a 15 ml purification column. The column was

then washed with 5×1 ml of Buffer A. The eIF4E containing solution was mixed with 1 ml m^7GTP Sepharose and rolled at 4°C for 1 h .The mixture was transferred to the column, and allowed to drain by gravity, with washes of 2×15 ml buffer A. Recycling was incorporated to ensure complete eIF4E binding. Non-specifically bound proteins were removed using 0.1 mM GDP in buffer A and specifically bound eIF4E was eluted in 20 mM GDP in buffer A. The nucleotide was removed from protein-containing fractions by dialysis overnight against 2000 volumes of buffer B (20 mM HEPES, pH 7.5, 1 M KCl) followed by dialysis for 4 h against 2000 volumes of buffer A.

His_6-tagged eIF4G (N-terminal) was expressed in BL21 (DE3) *(hsdS gal* (λcIts857 *ind1 S*am7 *nin5 lacUV5*-T7 gene 1). Cells were harvested by centrifugation at 4000 rpm, 15 min, 4°C and the pellet was then resuspended in 20 ml of lysing buffer (20 mM HEPES pH 8.0, 200 mM KCl, 20 mM Imidazole) with two complete ™, Mini, EDTA-free, protease Inhibitor Cocktail tablets (Roche). The solution was sonicated for 2 min and cell debris were separated by centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was mixed with 1 ml loaded resin (Chelating Sepharose, Pharmacia), which was activated with nickel. The column material was then rolled for 1 h in a cold room. Then unbound protein material was collected as the flow through. All weakly bound proteins were washed off three times with ten times the column volume of wash buffer (20 mM HEPES pH 8.0, 200 mM KCl, 50 mM Imidazole). Then the His-tagged proteins were eluted in a continuous gradient of Imidazol with a maximum concentration 500 mM. After desalting the column, the materials were loaded on an anion exchange chromatography column (BioCAD 700E). Finally, eIF4G was eluted with 20 mM HEPES pH 7.5, 400 mM KCl. Fractions rich in protein were pooled and dialysed in 8 L dialysis buffer for 6 h at 4°C.

The *E. coli* strain JM109 (DE3) was transformed with pET2a containing the N-terminally $(His)_6$-tagged yeast Pab1p gene (*PAB1*). Maximum expression was observed after 4 h. In the first step of purification of His-tagged Pab1p, nickel affinity chromatography was again used, but before loading the supernatant to the column, precipitation with 75% ammonium sulphate was performed. In the next step to reduce the salt concentration of the buffer, a Desalting column (AP Biotech) a BioCAD 700E (PerSeptive Biosystem Inc., USA) was used and high salt buffer substituted with 20 mM HEPES pH 7.5, 30 mM KCl. In the final step, heparin column on the BioCAD 700E was used and elution was performed with 20 mM HEPES pH 7.5, 350 mM KCl and followed by multiple dialysis with buffer containing 20 mM HEPES pH 7.5, 100 mM KCl, 2 mM $MgCl_2$ and 5% glycerol (v/v).

RNA Preparation

A synthetic biotinylated capped 55-nucleotide RNA was synthesised and labeled with Biotin-21-UTP at the 3′-end in an *in vitro* transcription reaction by using two oligonucleotides: Oligo A: 5′ GAA TTG TAA TAC GAC TCA CTA TAG 3′and Oligo B: 5′ AAG GGG GGT TTT TTT TTT TTG GTT GTG GTG GTT GTG GTG GTT GTG GTG GTC TAT AGT GAG TCG TAT TAC AA TTC 3′ For Biotin labeling of mRNA, rUTP was substituted with Biotin-21-UTP (Clontech) *in vitro* transcription reaction.

UV Cross-linking

Cross-linking reactions generally contained 0.2 µg of eIF4E, eIF4G and Pab1 in 20 µl of binding buffer (20 mM HEPES pH 7.5, 100 mM KCl, 5% glycerol (v/v), 2 mM $MgCl_2$, 1 mM DTT) containing 40 U of Rnasin and 1 µg/µl wheat germ tRNA (Sigma). After incubation at 0°C for 10 min, an equimolar amount of α^{32} P-labeled mRNA was added and the reaction continued for another 20 min at 0°C. After incubation, the reaction mixtures were transferred to ice and UV-irradiated for 10 min in a stratalinker 1800. Samples were digested with 40 µg of RNase A for 30 min at 37°C and analysed by SDS-10% polyacrylamide gel.

Analytical m^7GTP-Sepharose Chromatography

These experiments were performed as described earlier. Eluted proteins were separated on an SDS-polyacrylamide gel electrophoresis gel, visualized using silver staining, and quantified with a Bio-Rad GS-700 imaging densitometer and Bio-Rad molecular analyst software. eIF4E-binding proteins were added as indicated to an amount equimolar to the original 10 µg of eIF4E.

eIF4E/eIF4G/Pab1/RNA Complex Formation

Soft Link™ Soft Release Avidin Resin was used for the isolation of biotinylated mRNA, which can bind eIF4E via the poly (A) tail at 3′ end. About 400 µl of resin was poured into a 10 ml BIO-RAD column. After equilibration with 0.1 M $NaPO_4$ pH 7.0, the resin was washed with 5 mM biotin in phosphate buffer to preabsorb non-reversible binding sites on fresh resin. Finally the column was regenerated to purify biotinylated RNA using the following protocol:

The column was washed with 8 column volumes of 10% acetic acid and 8 column volumes of 100 mM

$NaPO_4$ pH 7.0. When the pH of eluate reached pH 6.8, the flow rate was stopped for a minimum of 30 min to allow the avidin to refold. Finally, the column was equilibrated in 50 mM Tris-HCl pH 8.0, 5% glycerol, 1 mM DTT and 100 mM KCl. Before loading the RNA/complex to the column, equimolar amounts of Pab1p and mRNA were mixed and allowed to bind for 30 min at 4°C; then eIF4E/eIF4G (N-terminal) were added to the Pab1p/RNA in 50 mM Tris-HCl pH 8.0, 100 mM KCl, 5% glycerol, 1 mM DTT and loaded on the column for 1 h at 4°C. The column was washed with about 5 column volumes of Tris buffer and finally, the biotinylated RNA and protein complex was eluted with 10 mM biotin in Tris buffer and was loaded on a SDS-PAGE and urea-acrylamide gel.

Results

Interaction of eIF4E and Pab1 with eIF4G

Having established the specificity of the individual interactions, it was decided to determine whether eIF4E and Pab1p could simultaneously interact with eIF4G (N-terminal) by using eIF4E bound to cap analogue resin m^7GTP-Sepharose. Figure 1 is a model showing the reconstitution of eIF4E/eIF4G/Pab1p with immobilization of eIF4E on the cap-analogue resin.

Following extensive washing and then elution of the bound proteins from the resin; the proteins were detected by silver-stained SDS-PAGE. Figure 2 is an analysis of these fractions on a SDS-10% polyacrylamide gel. It was found that Pab1p co-associated with eIF4E when eIF4G was used. Similarly, Pab1p co-associated with eIF4E/m^7GTP when eIF4G (N-terminal) was used.

eIF4E/eIF4G (N-terminal) Pab1 Complex with Capped mRNA

For preparation of reconstitution of the eIF4E/ eIF4G/Pab1p/capped RNA complex, using Soft Link Soft Release Avidin resin, a biotinylated capped 55-nucleotide RNA was synthesised, which can bind eIF4E via the capped structure at the 5′-end of the mRNA and Pab1p via the poly (A) tail at 3′ end. Figure 3 shows an ethidium bromide-stained 12% denaturing PAGE with the synthesis of single stranded mRNA (55 nucleotides) by an *in vitro* transcription.

The ability of eIF4E and Pab1p to interact with capped polyadenylated mRNA was tested using UV cross-linking analysis (Fig. 4). Cross-linking experiments were performed using a (capped polyadenylated) radioactively labeled mRNA. No cross-linking of eIF4G (N-terminal) was observed but as expected, eIF4E and Pab1p were able to cross-link to the mRNA.

After preparation of capped and polyadenylated mRNA, equimolar amounts of eIF4E, eIF4G, Pab1p and mRNA were mixed in a buffer and allowed to bind to avidin resin for at least 1 h at 4°C. Unbound proteins and mRNA were removed by washing with buffer. Finally, RNA-protein complexes were eluted from the column with 7 mM biotin in buffer. Figure 5.A shows a silver stained SDS-10% polyacrylamide gel with elution from the avidin column (Lanes 3-7). To confirm the presence of mRNA in the eluate complex, the eluate material was loaded on a 12% urea/polyacrylamide gel (Fig. 5.B).

Discussion

In order to investigate the interactions of *S. cerevisiae* eIF4E within eIF4F complex, eIF4E/eIF4G/ Pab1p was reconstituted. It has been shown that eIF4E could be easily purified from cell extract by affinity chromatography with m^7GTP-agarose [15]. It has been reported that the interaction of wheat germ PABP with eIF4F increases the affinity of eIF4E for a cap analogue by some 40-fold and similarly, that the affinity of eIF4F-complexed plant PABP for Poly (A) is greater than that of free PABP [18].

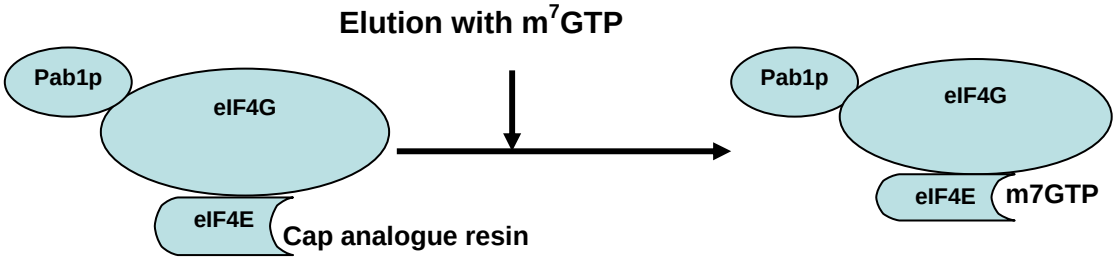

Figure 1. A model showing the reconstitution of eIF4E/eIF4G/Pab1p complex, using cap-affinity chromatography.

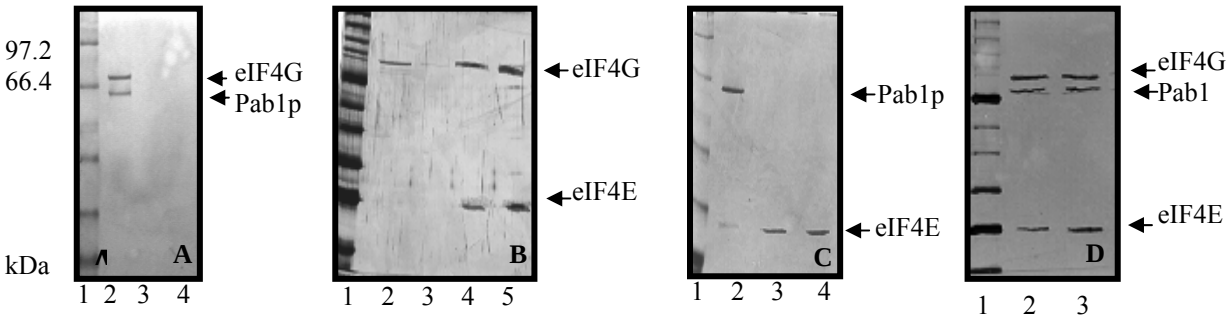

Figure 2. Reconstitution of the eIF4E/eIF4G/Pab1p complex using cap analogue resin. Purified eIF4G (N-terminal) /Pab1p (A), eIF4E/eIF4G (B), eIF4E/Pab1p (C) and eIF4E/eIF4G/Pab1p (D) were incubated with m^7GTP Sepharose at 4°C for 90 min. The proteins, which remained bound to the m^7GTP ligands, were eluted with excess m^7GTP. Bound (Lanes 3 and 4) and unbound materials (Lane 2) were analysed by SDS-10% polyacrylamide gel electrophoresis followed by silver staining. The molecular weight marker in the gel is indicated. The arrows indicate the positions of individual proteins.

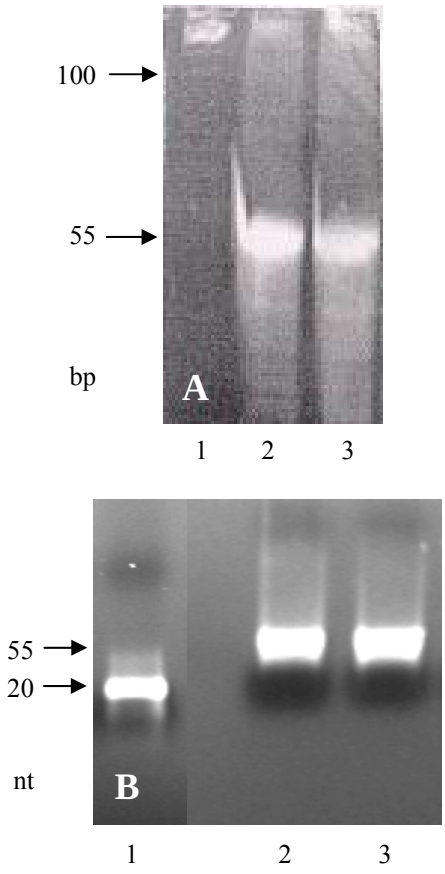

Figure 3. Synthesis of capped and biotinylated RNA by *in vitro* transcription. The 55 nucleotide RNA contains a cap structure and a 15 nucleotides poly (A) tail. (A) Lane 1 is a DNA marker, lane 2 and 3 are the products of *in vitro* transcription after phenol extraction and salt precipitation on a 12% urea/polyacrylamide gel. After gel extraction, the purified RNA was loaded on an agarose gel (B, lane 2, 3) with a 20 nucleotide oligo as a marker (lane 1).

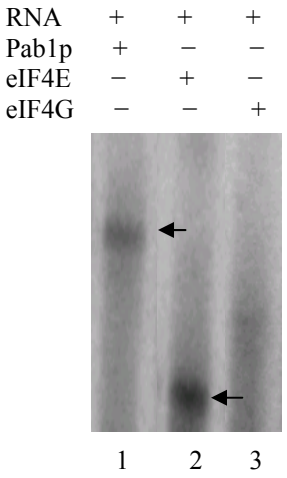

Figure 4. eIF4E and Pab1p interact with capped, polyadenylated mRNA. Radiolabelled, capped RNA was incubated with eIF4E, eIF4G and Pab1p. RNase treatment was used to remove non-cross linked RNA after UV irradiation, thus generating a labelled Pab1p-RNA and eIF4E-RNA complex visible in lanes 1 and 2.

Two other major proteins were found to be co-purified with eIF4E; the 220-kDa protein was named eIF4G, and 46-kDa protein was the already-characterised initiation factor eIF4A. The structures of murine and yeast eIF4E bound to 7-methyl GTP have been determined by crystallography and NMR [6,7]. It has been shown by atomic force microscopy that it is possible to make a circular, capped and poly adenylated RNA in the presence of recombinant eIF4E, eIF4G and Pab1p, thus demonstrating that there can be a physical interaction between the two ends of mRNA [19].

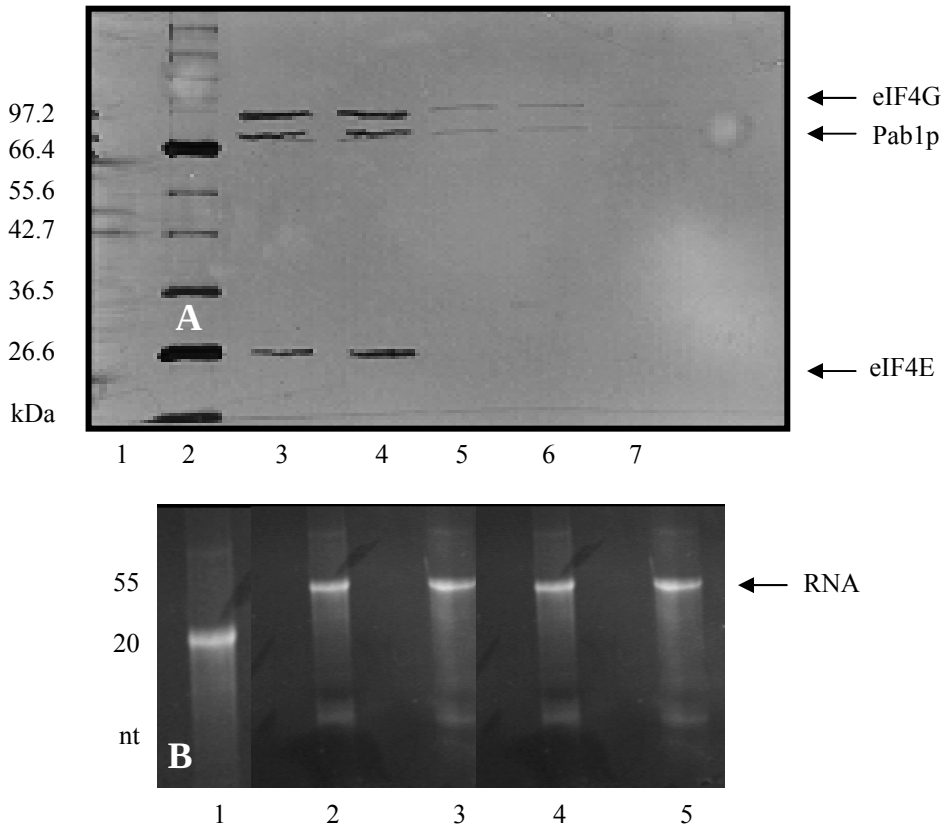

Figure 5. Reconstitution of the eIF4E/eIF4G/Pab1p and capped mRNA. The biotinylated mRNA bound to avidin resin was incubated with eIF4E, eIF4G and Pab1p. Bound protein/RNA complexes were eluted with buffer containing 7mM biotin, resolved by SDS-PAGE (A) and denaturing PAGE (B). (A) Lane 1, material not retained by the column; lanes 3, 4, 5, 6 and 7 material eluted with 20 mM HEPES pH 7.5, 100 mM KCl and 7 mM biotin. To confirm the presence of RNA, unbound (lane 2), bound materials (lanes 3-5) and 20 nucleotide oligo (lane 1) as a marker were loaded on the 12% denaturing gel.

In the first step, the proteins were purified separately. Since eIF4E binds to eIF4G and eIF4G binds to Pab1p, cap affinity chromatography was used to reconstitute the eIF4E/eIF4G/pab1p. It was found that Pab1p co-associated with eIF4E when eIF4G was used. Similarly, Pab1p co-associated with eIF4E/m^7 GTP when eIF4G (N-terminal) was used.

In an alternative approach, eIF4E/eIF4G/Pab1p/RNA complex was reconstituted, using biotinylated and capped RNA. For this purpose, a capped 55-nucleotide RNA was synthesised and labeled with Biotin-21-UTP at the 3′-end in an *in vitro* transcription reaction. Soft Link Avidin Resin was used for the isolation of biotinylated mRNA, which can binds eIF4E via the capped structure at the 5′-end of mRNA and pab1p via the poly (A) tail at 3′ end. The results confirm that a highly pure, eIF4E/eIF4G/Pab1p/RNA complex can be generated using the procedures outlined. After scale-up, this preparation should serve as the basis for a range of crystallisation trials in the future.

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