Supporting Information

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SI Results and Discussion

The Presence of 60S Ribosomal Subunits Does Not Affect Initiation Complex Assembly on mRNA with Poly(A) Leader When eIF3 Is Omitted. The multisubunit protein complex eIF3 is known to bind with 40S ribosomal subunit and prevent its reassociation with 60S ribosomal subunit. As a result, the so-called native ribosomal subunit (43S complex) is formed, which participates in binding with mRNA and subsequent searching for the initiation codon (1–3). At the same time, as shown in our experiments (Figs. 1 and 2), eIF3 is not required for binding with mRNA and searching for the initiation codon in the case of an mRNA with poly(A) leader. The question may be raised whether the result reflects the artificial situation of having only 40S subunits present in the initial incubation mixture. Particularly, if 60S subunits were present from the beginning, would one see some stimulation by eIF3 in providing a pool of 40S ribosomal subunits on which to build an initiation complex? Or could an inhibitory effect of 60S subunits on the availability of 40S subunits for the formation of the 48S initiation complex be displayed? To check these possibilities, 60S ribosomal subunits were added to the reaction mixture immediately after the addition of purified initiation factors and before 40S ribosomal subunits, mRNA and Met-$tRNA_i^{Met}$. All of the following experimental procedure was performed as described in *Formation of Ribosomal Initiation Complexes* in *Materials and Methods*. The results are presented in Fig. S1. As seen, the amounts of 48S initiation complex assembled on mRNA with poly(A) leader in the presence and in the absence of eIF3 were the same and did not differ from those in the absence of 60S ribosomal subunits (see Table 1).

SI Materials and Methods

Recombinant eF1, eF1A, eF4A, eF4B, eIF5 and eIF5B. Cells of *Escherichia coli* strain BL21DE3 were transformed with vectors bearing coding sequences of the proteins listed above, grown at 37°C until OD_{600} of ≈0.6 was attained and then induced by 1 mM isopropyl-β-D-thiogalactopyranoside. For cells producing eIF5B the temperature of further cultivation was reduced to 18°C. After induction, cells were grown to OD_{600} of 0.9–1.2, harvested by centrifugation, suspended in a buffer A containing 40 mM Tris-OAc (pH 7.5), 300 mM KCl, and 10% glycerol (vol/vol), with addition of 0.01% Triton X-100 (vol/vol), and disrupted with a Gaulin press. Cell debris was removed by centrifugation at 27,000 × *g* at 4°C for 30 min. The supernatant was loaded onto Ni^{2+}-NTA agarose column (Qiagen) (1 ml of column volume per 1 l of the cell culture), preequilibrated with the buffer A, and washed with 40 column volumes of the same buffer and further with 20 column volumes of a buffer A additionally containing 20 mM imidazole. Elution was carried out with 10 column volumes of the same buffer but containing 300 mM imidazole. DTT was immediately added to the concentration of 4 mM. To purify eIF1, peak fractions eluted from Ni^{2+}-NTA agarose were diluted with 2 volumes of a buffer containing 20 mM Hepes-KOH (pH 7.5), 100 mM KCl, 0.1 mM EDTA, 5% glycerol (vol/vol), and 2 mM DTT and loaded onto mono-S (GE Healthcare) cation exchange column preequilibrated with the same buffer. To purify the other eIFs listed, corresponding peak fractions eluted from Ni^{2+}-NTA agarose were treated as it is described for eIF1 purification, but the buffer was substituted for Tris-OAc of the same pH, and mono-Q (GE Healthcare) anion exchange column was used instead of mono-S. Proteins were eluted with 100–500 mM KCl gradient in the corresponding buffer. A 30–300 mM KCl gradient was used for eIF5B elution. Peak fraction of each protein was divided into aliquots that were frozen in liquid nitrogen and stored at −80°C. Coomassie-stained gels of SDS/PAGE with purified recombinant eF1, eF1A, eF4A, eF4B, eIF5, and IF5B are shown in Fig. S2.

Natural eIF2, eIF3, and eIF3–eIF4F Complex. Rabbit reticulocytes were lysed by addition of equal volume of hypotonic solution containing 10 mM Tris-OAc (pH 7.5), 0.5 mM $Mg(OAc)_2$, 2 mM DTT, and 40 mg/ml hemin. Cell debris was removed by centrifugation at 16,000 × *g* at 4°C for 15 min. Protease inhibitor (Complete EDTA-free; Roche) was immediately added according to manufacturer recommendations. Ribosomes and bound factors were spun down by centrifugation at 250,000 × *g* at 4°C for 4.5 h. The pellet was suspended at 4°C in a buffer containing 40 mM Tris-OAc (pH 7.5), 50 mM KCl, 6 mM $Mg(OAc)_2$, 2 mM DTT, and 0.25 M sucrose. The OD_{260} of the suspension was adjusted to ≈150 AU/ml by dilution with appropriate volume of the same buffer. Then the concentration of KCl was raised to 0.5 M by stepwise addition of 4 M KCl with stirring. The stirring continued at 4°C for 30 min more. The solution was centrifuged at 250,000 × *g* at 4°C for 4.5 h. The pellet was frozen in liquid nitrogen and used for preparation of ribosomal subunits (see below). The supernatant (salt wash fraction containing eIFs) was fractionated by the use of ammonium sulfate precipitation. The fraction precipitated at 50% saturation of ammonium sulfate was collected by centrifugation at 27,000 × *g* at 4°C for 30 min and suspended in buffer B containing 20 mM Tris-OAc (pH 7.5), 100 mM KCl, 10% glycerol (vol/vol), 2 mM DTT, and 0.1 mM EDTA. The solved precipitates were dialyzed overnight against 200 volumes of the same buffer. Aggregates after dialysis were removed by centrifugation at 20,000 × *g* at 4°C for 25 min, and the resulting solution was loaded onto diethylaminoethyl cellulose column (DE52; Whatman) preequilibrated with buffer B; the column was washed with the same buffer. The elution was carried out with buffer B containing 250 mM KCl. The eluate was diluted 2.5-fold with buffer B where KCl was omitted and freed from aggregates by centrifugation at 20,000 × *g* at 4°C for 25 min. The resulting solution was loaded onto cellulose phosphate column (P11; Whatman) preequilibrated with buffer B; the column was washed with the same buffer. The bound material was eluted first with buffer B containing 400 mM KCl ("low-salt fraction") and then with buffer B containing 800 mM KCl ("high-salt fraction").

eIF2 was purified from the high-salt fraction. The high-salt fraction was subjected to overnight dialysis against 200 volumes of buffer B, freed from aggregates by centrifugation at 14,000 × *g* at 4°C for 15 min, and further fractionated on a mono-Q column as it is described for recombinant proteins (see *Recombinant eF1, eF1A, eF4A, eF4B, eIF5, and eIF5B*). eIF2-containing fraction eluted from the mono-Q column at ≈300 mM KCl concentration. The purified protein was frozen in aliquots in liquid nitrogen and stored at −80°C. Purity and protein composition of eIF2 preparation were verified by SDS/PAGE. Coomassie-stained gel of SDS/PAGE with the purified natural eIF2 is shown in Fig. S3.

eIF3 and eIF3–eIF4F complex were purified from the low-salt fraction. To separate free eIF3 from eIF3 bound with eIF4F, cap analog affinity chromatography was used. The low-salt fraction was diluted 2.5-fold with buffer B not containing KCl, clarified by centrifugation at 20,000 × *g* at 4°C for 30 min, and loaded onto 7-methyl-GTP Sepharose 4B column (GE Healthcare). Material that did not bind to the column was collected (7-methyl-GTP

Sepharose column flowthrough). eIF3–eIF4F complex was purified from the 7-methyl-GTP Sepharose bound fraction. The column was washed with 100 column volumes of buffer B. Elution was done with 20 column volumes of the same buffer with the addition of 75 mM 7-methyl-GTP. The eluate was subjected to multiple rounds of concentration and dilution with buffer B to remove 7-methyl-GTP. Concentration was done by ultrafiltration through a Centricon YM30 (Millipore) at 4°C under conditions recommended by the supplier. The resulting solution containing eIF3–eIF4F complex was frozen in aliquots in liquid nitrogen and stored at −80°C. Coomassie-stained gel of SDS/PAGE with the purified natural eIF3–eIF4F complex is shown in Fig. S3.

To purify eIF3, the 7-methyl-GTP Sepharose column flowthrough was fractionated on a mono-Q column as it is described for recombinant proteins (see *Recombinant eF1, eF1A, eF4A, eF4B, eIF5, and eIF5B*). eIF3-containing fraction eluted from the mono-Q column at ≈450 mM KCl concentration. Purity and protein composition of eIF3 preparation were verified by SDS/PAGE. The purified protein was frozen in aliquots in liquid nitrogen and stored at −80°C. Coomassie-stained gel of SDS/PAGE with the purified natural eIF3 is shown in Fig. S3.

***E. coli* Met-tRNA Synthetase and Aminoacylation of Mammalian $tRNA_i^{Met}$.** *E. coli* Met-tRNA synthetase was prepared and stored as it is described for recombinant proteins eF1A, eF4A, eF4B, and eIF5 (see *Recombinant eF1, eF1A, eF4A, eF4B, eIF5, and eIF5B*). Coomassie-stained gel of SDS/PAGE with purified *E. coli* Met-tRNA synthetase is shown in Fig. S2.

The reaction mixture for aminoacylation of mammalian $tRNA_i^{Met}$ contained 40 mM Tris-OAc (pH 7.5), 10 mM $Mg(OAc)_2$, 4 mM ATP, 0.2 mM L-methionine, 14 KBq/ml [^{35}S] L-methionine (GE Healthcare) 0.25 $mAU_{280\ nm}$/ml purified *E. coli* Met-tRNA synthetase, 0.8 uits/ml RNase inhibitor (RiboLock; Fermentas), and either 10–15 pmol/ml $tRNA_i^{Met}$ transcript or 8 mg/ml total calf liver tRNA (Novagen) purified with Superdex 200 HR column (GE Healthcare) (Fig. S4). Mixtures were kept at 37°C for 25 min and deproteinized with phenol extraction. The fraction containing transfer RNA was gel-filtered using ProbeQuant G-50 micro columns. The yield of aminoacylation reaction was calculated from ^{35}S specific activity of the sample.

Purification of Ribosomal Subunits. The pellet of ribosomes washed with 0.5 M KCl (see *Natural eIF2, eIF3, and eIF3–eIF4F Complex*) was resuspended in buffer C containing 40 mM Tris-OAc (pH 7.5), 50 mM KCl, 2 mM DTT, and 4 mM $Mg(OAc)_2$ with a magnetic stirrer; the stirring was continued for 30 min more in ice. The suspension was clarified by centrifugation at 14,000 × *g* at 4°C for 15 min. The final OD_{260} was adjusted with buffer C to 150–200 AU/ml. To release the nascent peptide chain from ribosomes and facilitate ribosomal subunit dissociation, puromycin was added to the ribosome suspension to the concentration of 1 mM, and the reaction mixture was incubated at 37°C for 10 min, followed by the addition of 4 M KCl to the final concentration of 0.5 M on ice. The resulting solution was loaded onto 10–30% wt/wt sucrose gradient containing buffer C with 500 mM KCl. Dissociated 40S and 60S ribosomal subunits were separated by centrifugation in a swinging-bucket rotor at 100,000 × *g* at 4°C for 11 h. The 40S and 60S ribosomal subunits from combined corresponding fractions of the sucrose density gradients were sedimented by centrifugation at 450,000 × *g* at 4°C for 120 min. The pellets were suspended in buffer C containing 250 mM sucrose and 2.6 mM $Mg(OAc)_2$ instead of 4 mM. The suspensions were freed from aggregates by centrifugation at 14,000 × *g* at 4°C for 15 min, and 20-ml aliquots were frozen in liquid nitrogen. The protein patterns of isolated 40S and 60S ribosomal subunits were analyzed by SDS/PAGE (Fig. S3).

Natural β-Globin mRNA. The RNA was isolated from S100 fraction of rabbit reticulocyte lysate by phenol deproteinization and purified by two successive steps of chromatography on oligo(dT) cellulose (Sigma–Aldrich) column. In both steps, columns were equilibrated with buffer containing 20 mM Tris-OAc (pH 7.5), 700 mM KCl, 2 mM EDTA, and 0.1% SDS. The elution buffer contained 10 mM Tris-OAc (pH 7.5) and 1 mM EDTA. The eluate was precipitated in 70% ethanol and 0.7 M NH_4OAc, and the precipitate was dissolved in a buffer containing 20 mM Tris-OAc (pH 7.5), 3 mM $Mg(OAc)_2$, 100 mM KCl, and 1 mM EDTA. The solution was freed from particles sedimenting faster than 12S by centrifugation. The supernatant fraction was analyzed by denaturing urea PAGE; the gel stained with toluidine blue O is shown in Fig. S4.

1. Sadnik I, Herrera F, McCuiston J, Thompson HA, Moldave K (1975) Studies on native ribosomal subunits from rat liver. Evidence for activities associated with native 40S subunits that affect the interaction with acetylphenylalanyl-tRNA, methionyl-tRNAf, and 60S subunits. *Biochemistry* 14:5328–5335.
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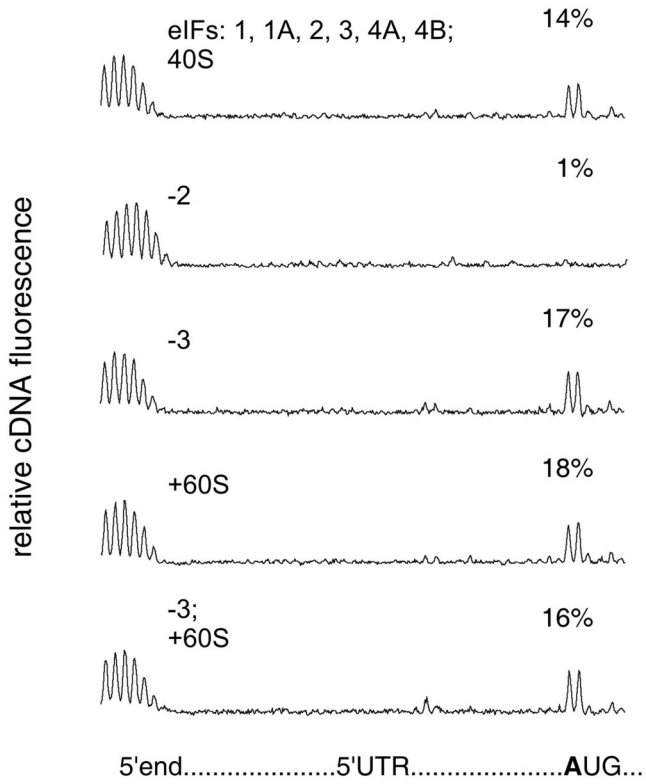

Fig. S1. Formation of initiation 48S ribosomal complexes on noncapped firefly luciferase mRNA with poly(A) leader sequence in the presence of purified 60S ribosomal subunits. The uppermost plot shows the formation of 48S complex in the presence of initiation factors eIF1, eIF1A, eIF2, eIF3, eIF4A, and eIF4B. The lower plots represent the results of omission of some eIFs and addition of 60S ribosomal subunits to the complex formation mixtures at the initial stage of initiation complex assembly (see *SI Text*): plot −2 is the result of the control reaction where eIF2 was omitted; plot −3 is the omission of eIF3; plot +60S is the full set mixture with added 60S ribosomal subunits; plot −3; +60S is the mixture with omitted eIF3 and added 60S subunits. Relative fluorescence intensities of cDNA products generated by reverse transcriptase are plotted versus leader sequence of mRNA. The integral fluorescence of the left major peak reflects the amount of the full-length product when mRNA was read out by reverse transcriptase up to the 5′ end without stop. The integral fluorescence of the three major peaks (''trident'' with the third peak reduced) at the initiation site, when it appeared, corresponds to the product of the reversed transcription stopped by initiation 48S ribosomal complex formed at the initiation AUG codon.

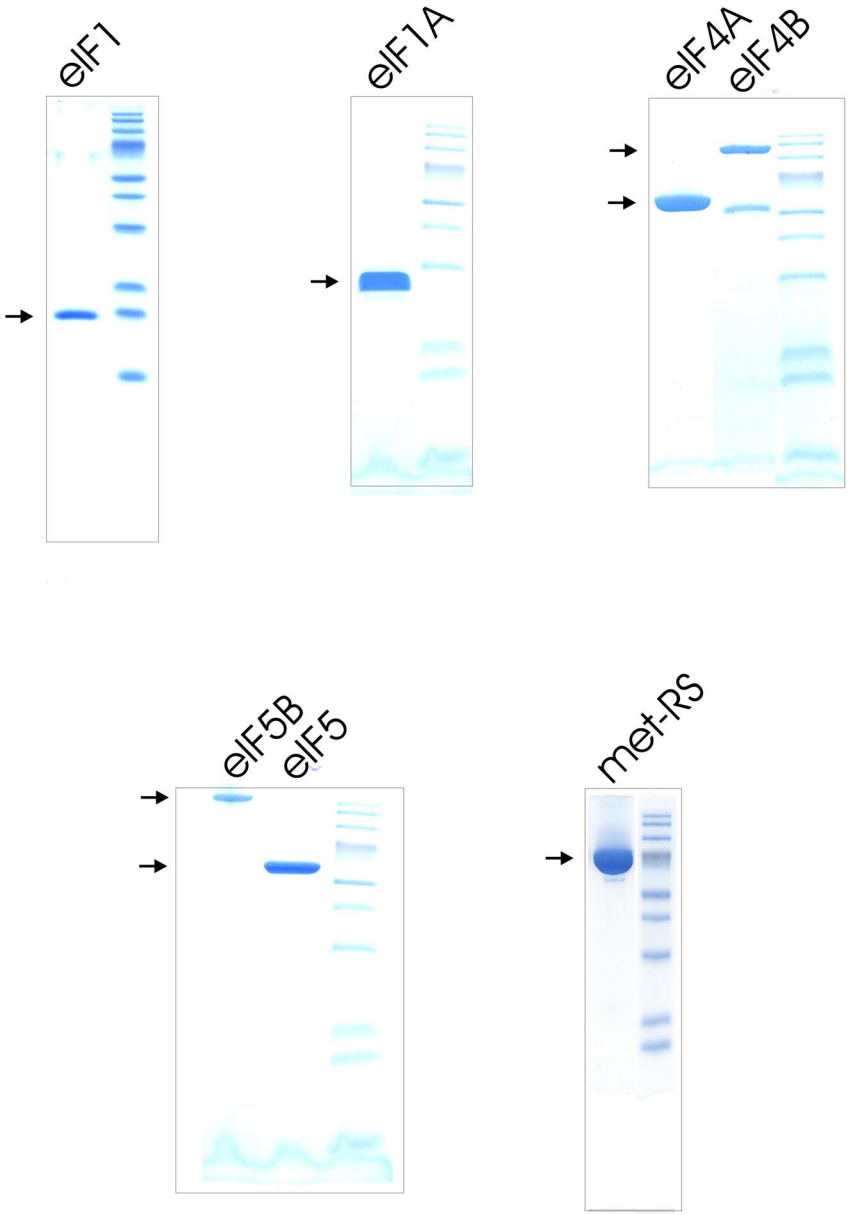

Fig. S2. Electrophoretic analysis of recombinant proteins in 12.5% SDS-gels according to the procedure described in ref. 7. Shown are Coomassie-stained gels.

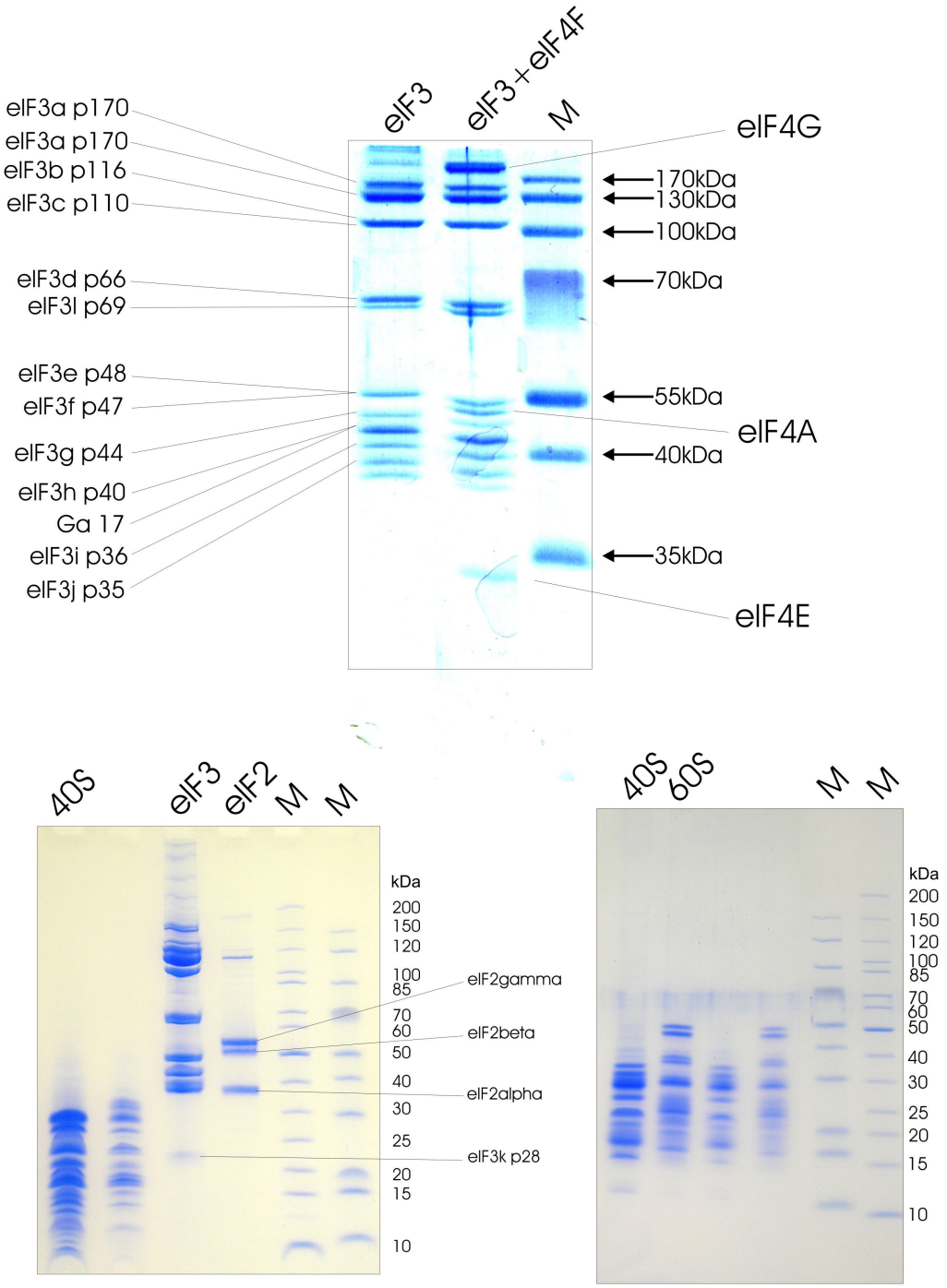

Fig. S3. Electrophoretic analysis of isolated natural initiation factors and ribosomal proteins extracted from 40S and 60S subunits by SDS-EDTA mixture. The PAGE gels used were 12.5% (*Upper*) and gradient 4–20% (*Lower*); all were prepared according to procedures described in ref. 7. Shown are Coomassie-stained gels.

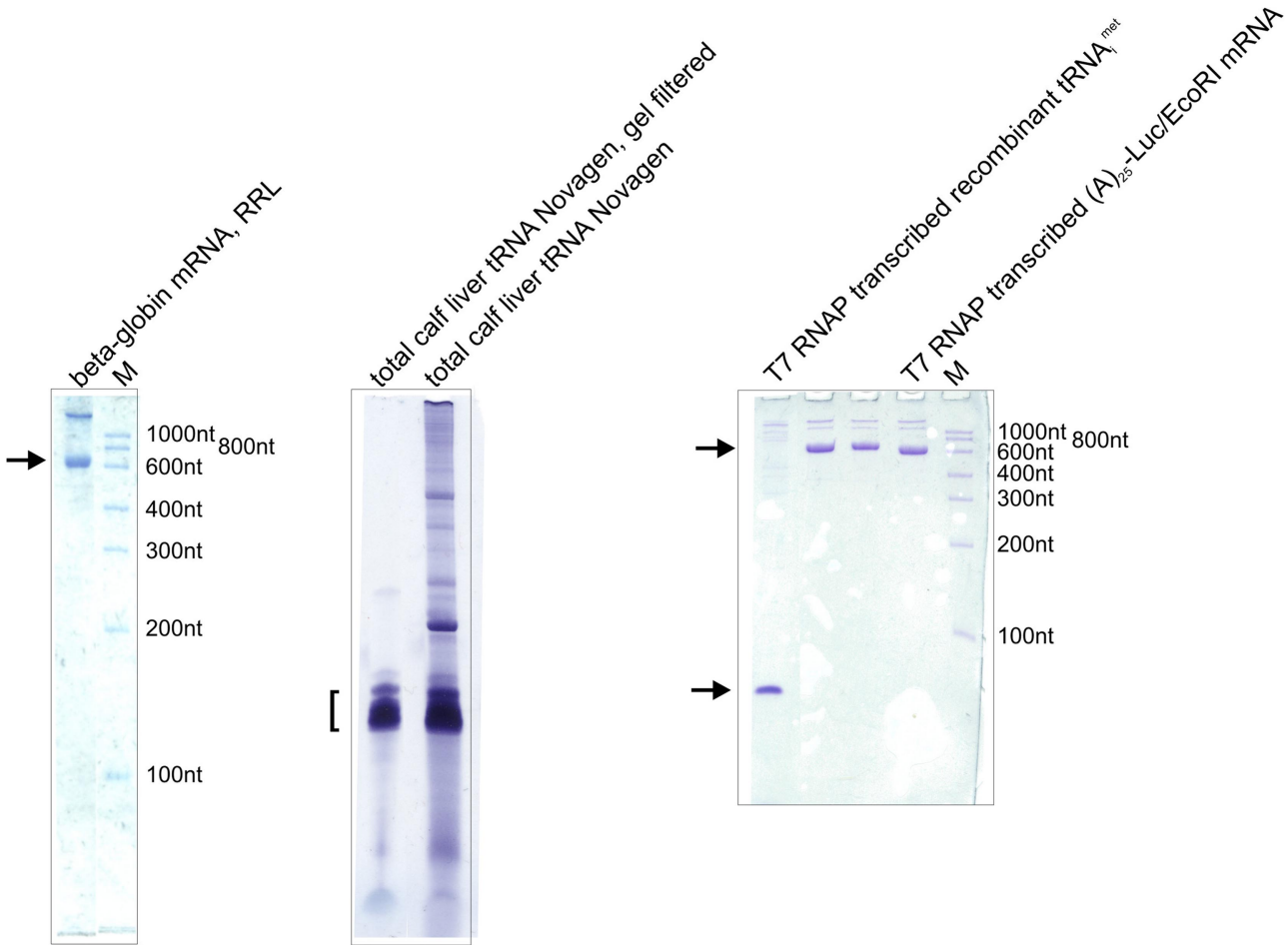

Fig. S4. Electrophoretic analysis of RNAs in denaturing 6% polyacrylamide gels with 7 M urea (stained with Toluidine blue O).