Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons

Tatyana V. Pestova*†, Sergei I. Borukhov* & Christopher U. T. Hellen*

* *Department of Microbiology and Immunology, State University of New York Health Science Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, New York 11203, USA*
† *A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899 Moscow, Russia*

The scanning model of translation initiation is a coherent description of how eukaryotic ribosomes reach the initiation codon after being recruited to the capped 5′ end of messenger RNA. Five eukaryotic initiation factors (eIF 2, 3, 4A, 4B and 4F) with established functions have been assumed to be sufficient to mediate this process. Here we report that eIF1 and eIF1A are also both essential for translation initiation. In their absence, 43S ribosomal preinitiation complexes incubated with ATP, eIF4A, eIF4B and eIF4F bind exclusively to the cap-proximal region but are unable to reach the initiation codon. Individually, eIF1A enhances formation of this cap-proximal complex, and eIF1 weakly promotes formation of a 48S ribosomal complex at the initiation codon. These proteins act synergistically to mediate assembly of ribosomal initiation complexes at the initiation codon and dissociate aberrant complexes from the mRNA.

The ribosomal scanning model describes the basic steps of translation initiation on most eukaryotic mRNAs[1,2]. In this process, a 43S complex, consisting of a ribosomal 40S subunit, eIF3 and an eIF2–GTP–initiator tRNA complex, binds mRNA at its 5′ end and scans downstream until it locates the initiation codon. First, eIF4F binds the capped 5′ end of the mRNA and, with eIF4A and eIF4B, creates an unstructured cap-proximal binding site for the 43S complex. This complex scans to the first downstream AUG triplet, which acts as the initiation codon. eIF5 stimulates GTP hydrolysis and release of factors from the resulting 48S complex, leaving the initiator tRNA in the P-site of the 40S subunit. The ribosomal 60S subunit then joins the 40S subunit and protein synthesis begins. Other factors, including eIF1 and eIF1A, have been implicated in the initiation of translation but their function remains obscure[3].

Ribosomal binding to the end of an mRNA does not position it at the initiation codon, which is usually 50–100 nucleotides away. The 43S complex is thought to scan downstream, searching for the initiation codon. This model poses three basic questions. (1) Which factors are required for attachment of 43S complexes to capped mRNAs? (2) How does the 43S complex move on the mRNA, and which factors are required for this process? (3) How do components of the 43S complex interact with and inspect mRNA during scanning to recognize and reject mismatched interactions between triplets in the mRNA and the anticodon of initiator tRNA before the correct initiation codon is selected?

We have developed methods to reconstitute initiation from purified components and accurately to map the resulting initiation complexes on mRNAs[4–6]. Here we have reconsituted early stages in initiation on natural capped β-globin mRNA and identified essential, unanticipated activities of eIF1 and eIF1A. eIF1, eIF1A, eIF4A, eIF4B and eIF4F are sufficient for 43S complexes to bind capped mRNAs and to form 48S complexes at the initiation codon. When eIF1 and eIF1A were omitted, 43S complexes bound near the 5′ cap but did not reach the initiation codon. eIF1A enhanced the formation of these 5′-terminal complexes in the presence of the other five factors; in their presence, eIF1 slightly stimulated 48S complex formation and dissociation of aberrant 5′ terminal complexes. These factors have distinct, synergistic activities that are required together for 48S complex assembly at the initiation codon.

Ribosome recruitment of capped mRNA

Ribosomal 48S complexes were assembled *in vitro* on β-globin mRNA using purified factors (Fig. 1a). The position of 40S subunits on the mRNA in these complexes as mapped by toeprinting, which involves extension by reverse transcriptase of a primer annealed to a template RNA to which a ribosome is also bound. Synthesis of complementary DNA is arrested by the bound complex, yielding a toeprint at its leading edge that can be located on a sequencing gel. 48S complexes assembled on β-globin mRNA yield stops 15, 16 and 17 nucleotides downstream of the initiation codon[7].

A ribosomal 'complex I', assembled from 40S subunits, initiator tRNA, eIF2, eIF3, eIF4A, eIF4B and eIF4F, yielded prominent toeprints 21–24 nucleotides from the 5′ end of the mRNA (Fig. 2a, lane 3). Complex I did not form if 40S subunits, initiator tRNA, eIF2, eIF3 or eIF4F individually, or eIF4A, eIF4B and eIF4F together, were omitted, or if ATP was substituted by AMP–PNP (Fig. 2a, lanes 1–3, 2b, lanes 1–7, 10). The formation of complex I was greatly increased by eIF4B (Fig. 2b, lane 8). 43S complexes and eIF4A, eIF4B and eIF4F are therefore unable to form 48S complexes, and instead form ribosomal complexes near the 5′ terminal cap. Parallel experiments using α-globin mRNA led to an identical conclusion. Ribosomal complexes yielded toeprints 16 and 23 nucleotides from the 5′ end of this mRNA, but not at the initiation codon (data not shown).

Assembly of 48S complexes

48S complexes assemble correctly in rabbit reticulocyte lysate (RRL)[7], which we therefore used as a source from which to purify additional factor(s) required for assembly of the 48S complex. The 0.5 M KCl ribosomal salt wash was divided into 0–40%, 40–50% and 50–70% ammonium sulphate precipitation fractions. The 50–70% fraction contained most of the activity that promoted assembly of a 48S complex (complex II) at the initiation codon on addition to reactions that contained 43S complexes and eIF4A, eIF4B and eIF4F (Fig. 2a, lane 4). This fraction was separated by elution from DEAE cellulose into 0.1 M KCl and 0.25 M KCl fractions that together had the same activity as the starting material (Fig. 1b). The 0.25 M KCl fraction doubled complex I formation but did not promote complex II formation; inclusion of the 0.1 M KCl fraction in similar reactions yielded small amounts of complex II without significantly altering formation of complex I, the main product (data not shown). The active constituents in these fractions were purified by chromatography (Fig. 1b) and were assayed after mixing, using toeprinting after each step to identify fractions and proteins that

promoted complex II formation. Apparently homogenous proteins of relative molecular mass (M_r) 13,500 and 19,000 (13.5K and 19K) (Fig. 1c, lanes 6, 7) were necessary, and together (but not individually) were sufficient for 43S complexes and eIF4A, eIF4B and eIF4F to form complex II without any trace of complex I (Fig. 2a, lanes 5–7). The amino-terminal sequence of the 19K protein was PKNKGKG, identical to that of rabbit eIF1A (ref. 8). The sequences of two tryptic peptides derived from the 13.5K protein were GDDLLPAGT and TLTTVQGIA, which correspond exactly to amino acids 18–26 and 45–52 of human eIF1 (refs 9, 10).

Including eIF1A in reactions lacking eIF1 increased the formation of complex I without forming complex II (Fig. 2a, lane 6). Conversely, eIF1 in the absence of eIF1A slightly reduced the prominence of complex I and yielded some complex II (Fig. 2a, lane 5). Without eIF1A, complex II yielded two toeprints 16–17 nucleotides downstream of the initiation codon, whereas complex II assembled with eIF1 and eIF1A yielded a third toeprint at 15 nucleotides downstream (Fig. 2a, lanes 5, 7). eIF1 and eIF1A were also required for complex II assembly on α-globin mRNA (data not shown). When complex I was assembled on β-globin mRNA without eIF1 and eIF1A, and they were added 5 min later, complex I disappeared completely but complex II formed as if eIF1 and eIF1A had been present throughout (Fig. 2a, lane 8). Complex I is therefore not a stable dead-end.

In toeprinting assays, the magnesium concentration was increased to 8 mM after the assembly reaction to optimize reverse transcription. These ionic conditions can stabilize non-enzymatic binding of oligoribonucleotides to 40S subunits. 40S subunits and all factors required for 48S complex formation were incubated in a reaction mixture that contained 8 mM magnesium acetate from the beginning. Neither complex I nor II formed under these conditions (Fig. 2a, lane 9); they are therefore not simply the consequence of the magnesium-induced association of mRNA and the 40S subunit.

Recombinant eIF1 and eIF1A (Fig. 1c) were used to confirm that these factors are sufficient for 48S complex assembly when used with 43S complexes and eIF4A, eIF4B and eIF4F. Inclusion of recombinant eIF1 with these components yielded complex I and small amounts of complex II; recombinant eIF1A in place of eIF1 slightly increased complex I formation without formation of complex II (Fig. 3a, lanes 3, 4). Inclusion of recombinant eIF1 and eIF1A in similar reactions yielded complex II but not complex I (Fig. 3a, lane 6). Neither complex I nor complex II formed if 40S subunits, eIF2 or eIF3 individually, or eIF4A, eIF4B and eIF4F together, were omitted (Fig. 3a, lanes 7–10). The factors required for complex II formation and its position on mRNA indicate that it is a bona fide 48S complex. eIF1 and eIF1A are not required for recruitment of

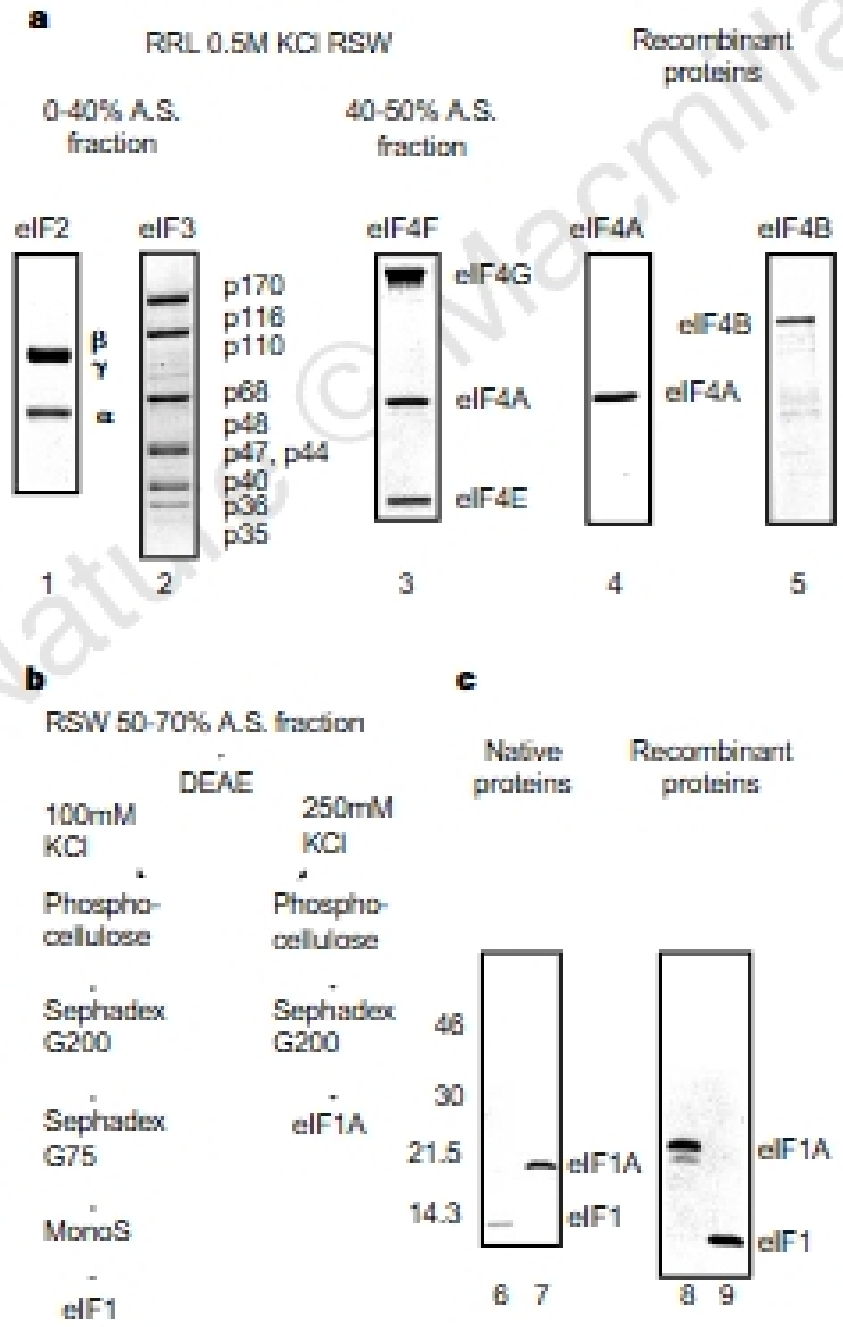

Figure 1 Composition and purification of proteins used in translation initiation. **a**, **c**, Overview of proteins used in reconstituting translation initiation. **b**, Purification scheme for eIF1 and eIF1A. SDS–PAGE gels were stained with Coomassie blue. The positions of molecular weight markers are indicated to the left of lane 6. Subunits of eIF2, eIF3 and eIF4F are indicated to the right of lanes 1–3.

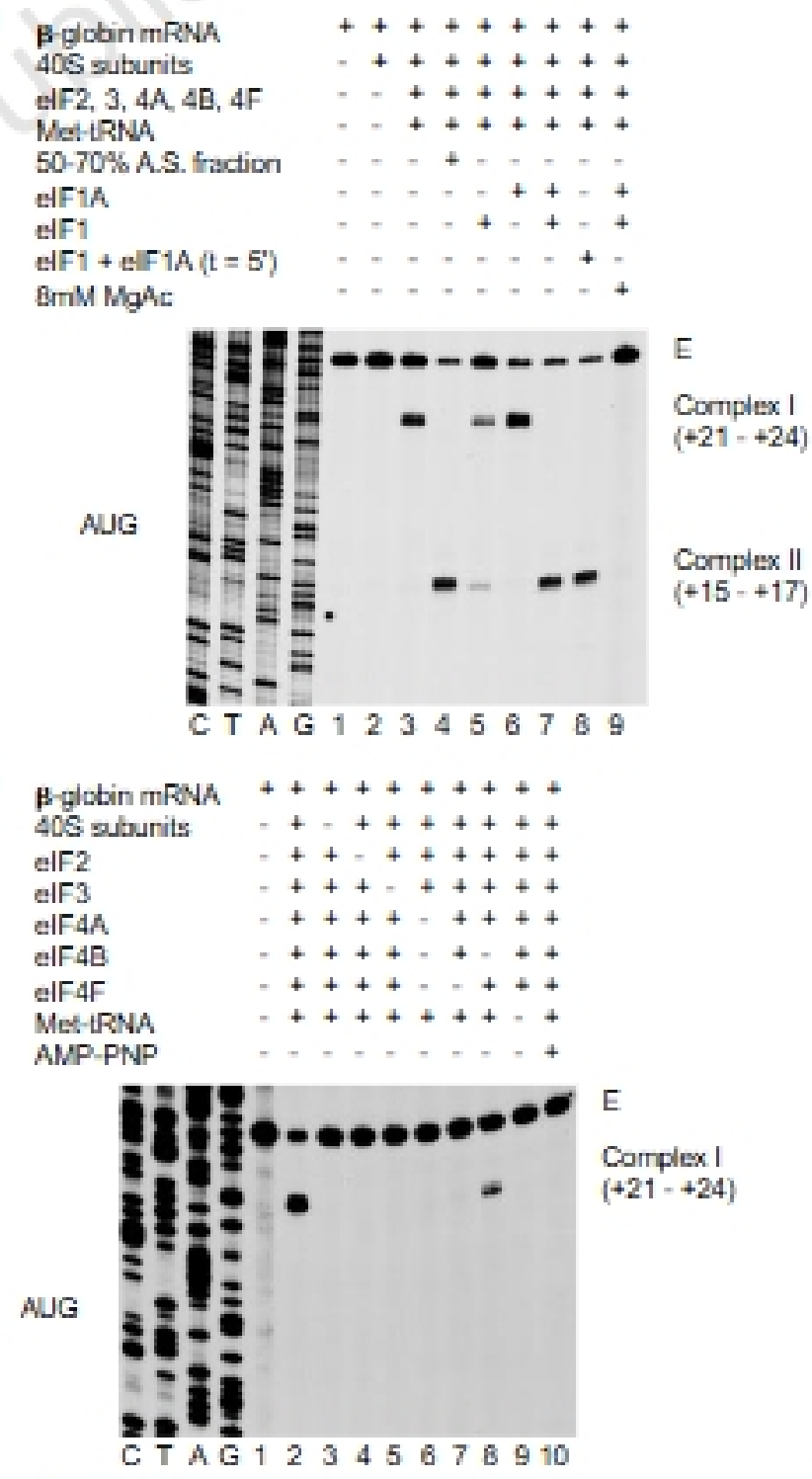

Figure 2 Assembly and toeprint analysis of ribosomal complexes on β-globin mRNA. **a**, **b**, Reaction mixtures contained ATP and GMP–PNP in addition to translation components, added when indicated (t, time in min). Full-length cDNA is marked E. cDNA products labelled 'Complex I (+21–+24)' and 'Complex II (+15–+17)' terminated 21–24 nucleotides from the 5′ end and 15–17 nucleotides downstream of the initiation codon of β-globin mRNA, respectively. The position of the initiation codon is shown to the left of the reference lanes, which show the β-globin sequence derived using the same primer.

capped mRNAs by 43S complexes, but are required together for them to reach the initiation codon. Adding recombinant eIF1 and eIF1A to preformed complex I resulted in its disappearance and in the formation of complex II (Fig. 3a, lane 11). Native eIF1 and eIF1A and their recombinant counterparts therefore have identical activities.

The influence of eIF1 and eIF1A on binding capped, ^{32}P-labelled globin mRNA to 40S subunits was assessed by sucrose density-gradient centrifugation. The incorporation of mRNA into ribosomal complexes was not influenced by eIF1A alone, but was greatly increased by eIF1 and even more by both factors in reactions that contained 43S complexes and eIF4A, eIF4B and eIF4F (Fig. 3b). Toeprinting indicated that the amounts of complex I formed without eIF1 and eIF1A, and of complex II formed in their presence, are similar, whereas much less complex I than complex II was detected by sucrose density-gradient centrifugation. Complex I is therefore unstable under conditions of sucrose density-gradient centrifugation.

Stability of complex I

The stability of complex I was assessed by toeprinting after the addition of competitor β-globin mRNAs that lacked the primer binding site. Inclusion of a 15-fold excess of competitor in reactions at the beginning of incubation abolished detectable formation of complex I; complex I was barely detectable when the same competitor excess was added after 5 min incubation (Fig. 4a, lanes 7, 8). In parallel experiments, complex II formation in the presence of eIF1 and eIF1A was unaffected by competitor added after 5 min incubation (data not shown). Complex I is therefore intrinsically unstable, whereas complex II is resistant to challenge.

Neither complex I nor complex II formed in reactions that contained eIF1, eIF1A and 8 mM Mg^{2+} (Figs 2a, lane 9 and 3a, lane 12). Unlike complex II, complex I can assemble at normal (2 mM) and elevated (8 mM) Mg^{2+} concentrations in the absence of eIF1 and eIF1A (Fig. 4a, lanes 2, 3). The failure to form complex I in the presence of 8 mM Mg^{2+}, eIF1 and eIF1A could be because these factors prevent its formation under these conditions, or because they destabilize it immediately. We investigated the effect of these factors on preformed complex I. Neither factor individually affected its prominence if added to reactions when the Mg^{2+} concentration was increased from 2 to 8 mM but, if these factors were added together, complex I was not detectable (Fig. 4a, lanes 4–6). eIF1 and eIF1A can therefore identify and promote the dissociation of incorrectly assembled complex I under conditions that do not allow the formation of complex II (that is, 8 mM Mg^{2+}). These

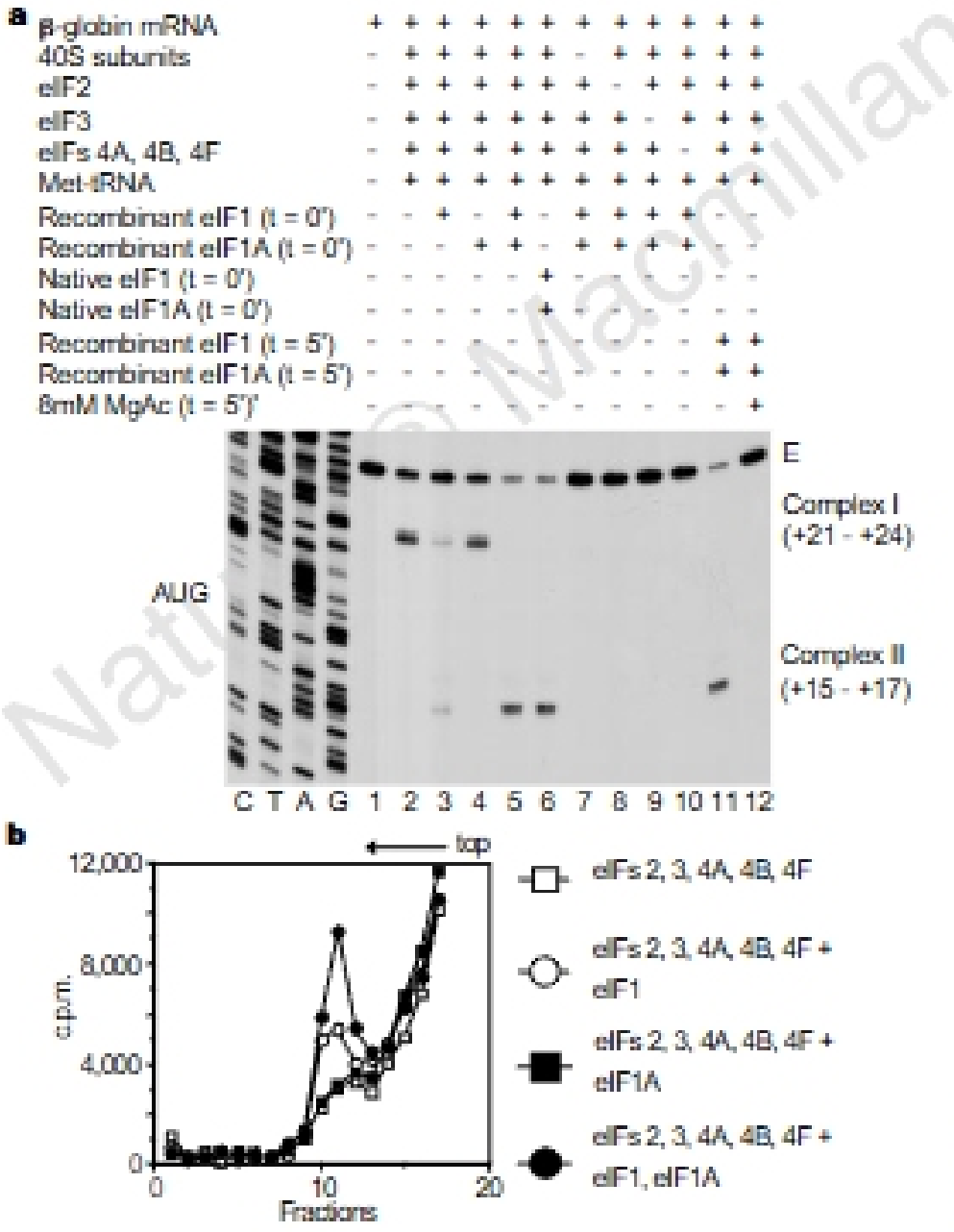

Figure 3 Initiation-factor dependence of cap-mediated 48S complex formation. **a**, Translation components (added when indicated; *t*, time in min), ATP, GMP-PNP and natural capped globin mRNA were incubated together. Ribosomal complexes were then mapped by toeprinting. cDNA products are labelled as in Fig. 2. Reference lanes depict the β-globin sequence. **b**, Ribosomal complexes were assembled using factors as indicated, ATP, GMP-PNP, aminoacylated initiator tRNA, 40S subunits and [^{32}P]-end-labelled α- and β-globin mRNAs, and were analysed by sucrose density-gradient centrifugation. Sedimentation was from right to left. Upper fractions from the gradient have been omitted for clarity.

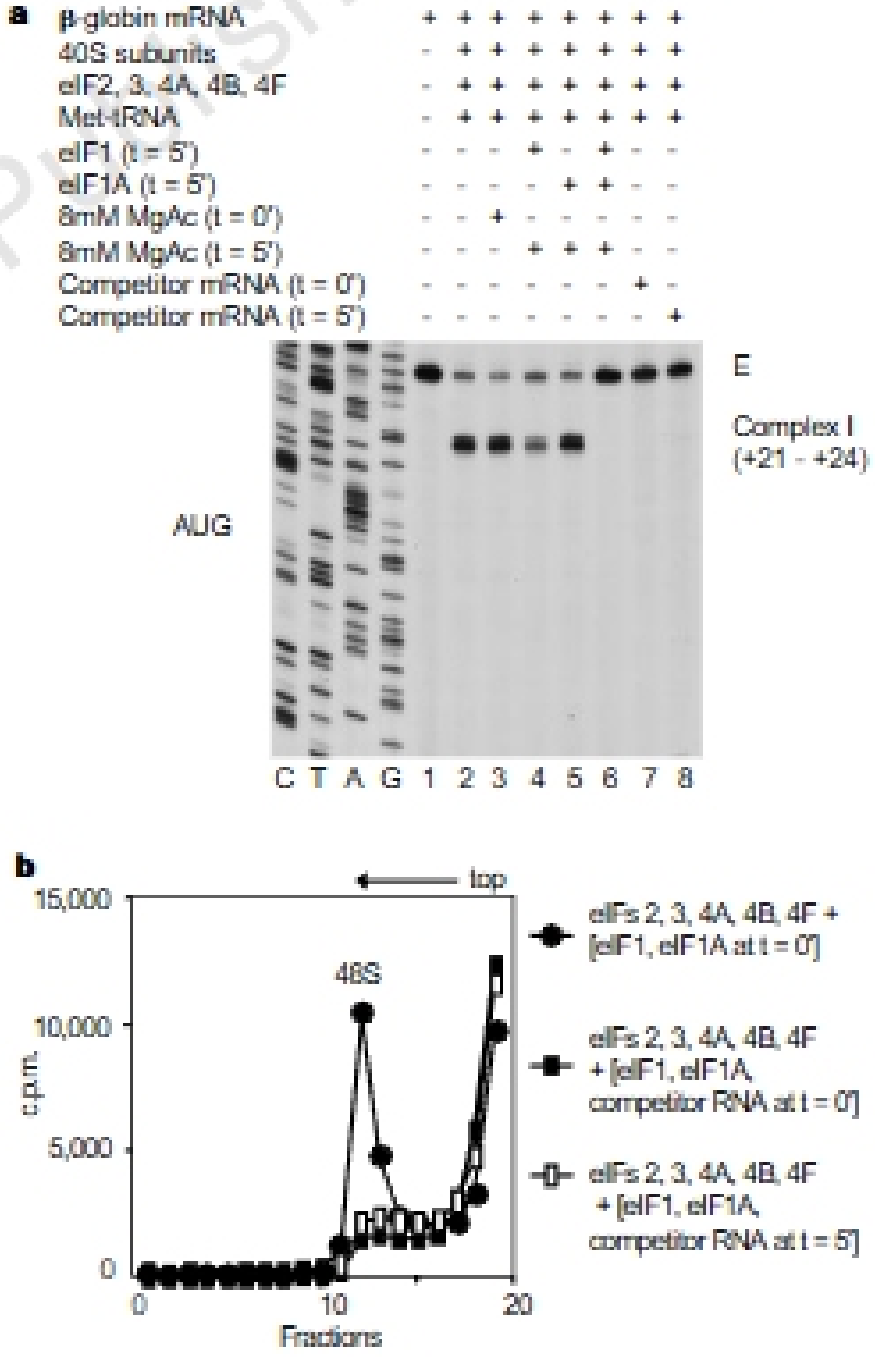

Figure 4 Stability of complex I. **a**, Translation components as indicated, ATP, GMP–PNP and natural globin mRNA were incubated under standard conditions except that a 15-fold excess of competitor β-globin mRNA transcript truncated 34 nucleotides past the initiation codon, eIF1, eIF1A and additional magnesium acetate (MgAc) were added at times (*t*, in min) indicated. Ribosomal complexes were mapped by toeprinting. cDNAs are labelled as in Fig. 2. Reference lanes depict the β-globin sequence. **b**, Ribosomal complexes were assembled using aminoacylated initiator tRNA, ATP, GMP-PNP, 40S subunits, [^{32}P]-end-labelled globin mRNA and eIFs 2, 3, 4A, 4B and 4F, followed by addition of eIFs 1 and 1A and a 15-fold excess of unlabelled full-length globin mRNA transcript as indicated and incubation for a total of 5 min. Ribosomal complexes were analysed by sucrose density-gradient centrifugation. Sedimentation was from right to left. Upper fractions of gradients have been omitted for clarity.