

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

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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 obscure³.

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 reconstituted 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 codon⁷.

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

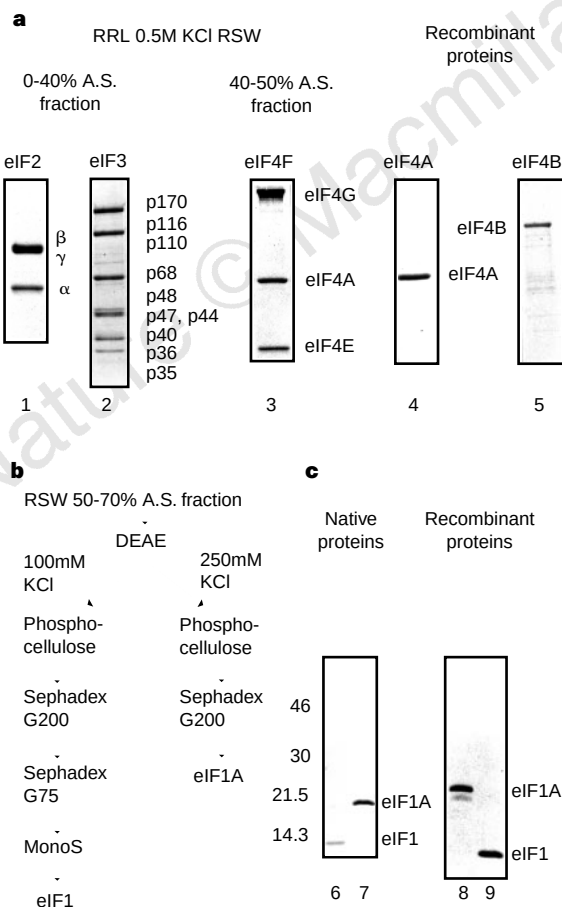


Figure 1 Composition and purification of proteins used in translation initiation. **a, c**, Overview of proteins used in reconstituting translation initiation. **b**, Purification scheme for eIF1 and eIF1A. SDS-PAGE gels were stained with Coomassie blue. The positions of molecular weight markers are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

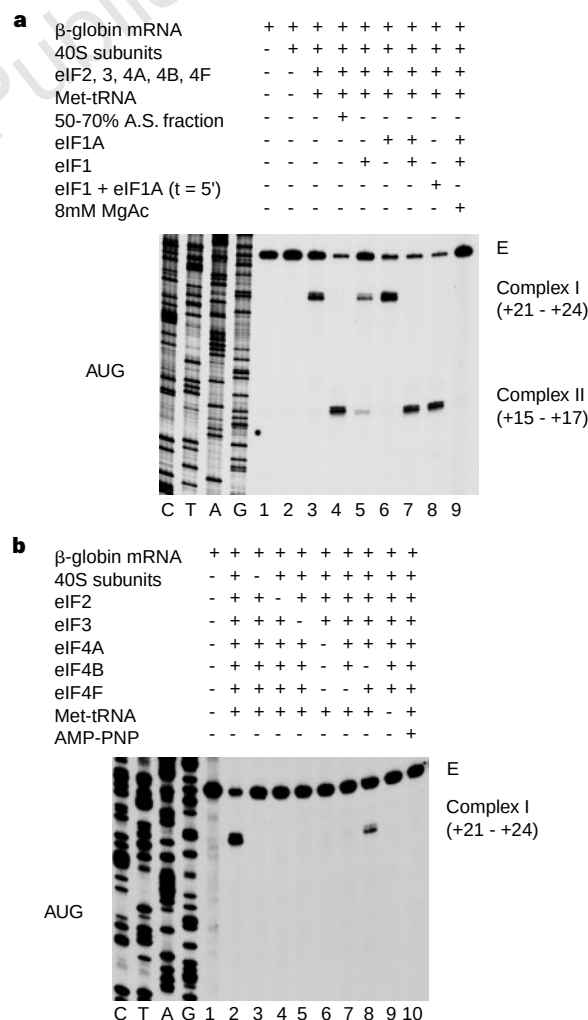


Figure 2 Assembly and toeprint analysis of ribosomal complexes on β -globin mRNA. **a, b**, Reaction mixtures contained ATP and GMP-PNP in addition to translation components, added when indicated (t, time in min). Full-length cDNA is marked E. cDNA products labelled 'Complex I (+21–+24)' and 'Complex 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.

The influence of eIF1 and eIF1A on binding capped ^{32}P -labelled

The stability of complex

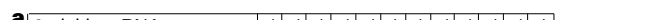


Figure 3 Initiation factor dependence of cap mediated 48S complex formation

Neither complex I nor complex II formed in reactions that

Neither complex I nor complex II formed in reactions that involved $\text{IE1} + \text{IE1}^+$ and $\text{IE1} + \text{M}(\text{M}^{2+})$ (Fig. 2 and 3).



Figure 4 Stability of complex I. **a** Translation components as indicated. ATP

factors might detect that complex I is assembled incorrectly and destabilize it, or complex I might be intrinsically unstable, cycling on and off the mRNA, and could be trapped in the 'off' state by these factors.

The fate of complex I

Complex I can be dissociated from mRNA by eIF1 and eIF1A at Mg^{2+} concentrations that do not allow complex II to form. At normal Mg^{2+} concentrations, addition of these factors to preformed complex I might result in it either migrating to the initiation codon under their influence, or dissociating from the mRNA spontaneously or under their influence, so the 43S complex can restart the initiation process.

To distinguish between these possibilities, sucrose density-gradient centrifugation was used to resolve 48S complexes assembled on labelled globin mRNA with and without unlabelled competitor. If eIF1 and eIF1A promote migration of complex I to the initiation codon on the same mRNA, simultaneous addition of excess unlabelled mRNA and these factors to complex I should yield 48S complexes on the labelled mRNA. Alternatively, if eIF1 and eIF1A promote the formation of 48S complex on another mRNA in a new cycle of initiation then competition should yield 48S complexes on the excess unlabelled mRNA that would not be detected by scintillation counting of sucrose density-gradient fractions. A prominent 48S peak was detected on labelled mRNA in the absence of competitor if eIF1 and eIF1A were present from the beginning (Fig. 4b). Simultaneous addition of 43S complexes, eIF1, eIF1A, eIF4A, eIF4B and eIF4F and a 15-fold excess of unlabelled competitor over labelled globin mRNA to reactions prevented 48S complex assembly on the labelled mRNA. Addition of eIF1, eIF1A and excess competitor to similar reactions 5 min after the start of complex I assembly did not appreciably increase the assembly of 48S complex on the labelled mRNA. This finding suggests that complex I is not an immediate precursor of 48S complexes, and that it dissociates either spontaneously or under the influence of eIF1 and eIF1A, releasing 43S complexes that enter another round of initiation. However, we cannot rule out the possibility that the rate of spontaneous dissociation of complex I is greater than the rate of its fixation by eIF1 and eIF1A in a form that leads to 48S complex assembly. In this circumstance, preassembly of complex I before addition of eIF1, eIF1A and competitor mRNA would not favour 48S complex formation on the same mRNA.

Destabilization of aberrant ribosomal complexes

Initiation on some eukaryotic mRNAs is cap independent, resulting instead from internal ribosomal entry. Encephalomyocarditis virus (EMCV) and classical swine fever virus (CSFV) exemplify different internal initiation mechanisms; neither involves scanning or requires eIF1 or eIF1A for 48S complex assembly⁴⁻⁶.

EMCV translation initiates at AUG₈₃₄ and infrequently at AUG₈₂₆ (ref. 11). 43S complexes and eIF4A, eIF4B and eIF4F form 48S complexes at AUG₈₂₆ and predominantly at AUG₈₃₄ (refs 4, 5) (Fig. 4a, lane 2). The proportion of complexes assembled at AUG₈₂₆ exceeds the relative frequency of initiation at this codon during translation. eIF1 strongly reduced 48S complex formation at AUG₈₂₆ and increased 48S complex formation at AUG₈₃₄ commensurately; eIF1A enhanced the stop 15 nucleotides downstream of AUG₈₃₄ but had no effect on the relative proportion of complexes formed at AUG₈₂₆ and AUG₈₃₄. Together these factors had an additive effect (Fig. 5a, lanes 3–5). eIF1 and eIF1A were present throughout these reactions so these results do not discriminate between the possibilities that these factors prevent the assembly of 48S complex at AUG₈₂₆ and that they destabilize complexes that have already assembled at this triplet. Addition of both factors to reactions after 5 min incubation reduced 48S complex formation at AUG₈₂₆ as much as when they were present from the beginning (Fig. 5a, lanes 5, 6). We do not know which features of the complex

assembled at AUG₈₂₆ target it for destabilization. eIF1 and eIF1A can therefore destabilize incorrectly assembled ribosomal complexes and increase 48S complex assembly at the initiation codon during IRES- and cap-mediated modes of initiation. 48S complexes assembled equally well at EMCV AUG₈₃₄ at 2 mM and at 8 mM Mg^{2+} , with or without eIF1 and eIF1A, in otherwise identical conditions (Fig. 5a). This difference between IRES- and cap-mediated initiation suggests that scanning may be inhibited at high magnesium concentrations.

48S complex formation on the CSFV IRES requires only eIF2 and eIF3 (ref. 6). This IRES promotes direct binding of 43S complexes to the initiation codon, yielding toeprints at UGA₃₉₀₋₃₉₂. eIF1 and eIF1A did not affect the yield of 48S complexes but slightly altered the toeprint pattern at UGA₃₉₀₋₃₉₂ (Fig. 5b, lanes 2, 3). 48S complexes assembled equally well on the CSFV IRES at 2 and 8 mM Mg^{2+} (data not shown), as described for EMCV.

Discussion

We have found that eIF1 and eIF1A are essential for cap-mediated initiation of translation in higher eukaryotes. They have distinct, synergistic activities that together promote 48S complex assembly at the initiation codon and dissociation of aberrant ribosome-mRNA complexes. Without them, 43S complexes bound exclusively to the cap-proximal 5' non-translated region in a reaction that required ATP, eIF4A, eIF4B and eIF4F. These ribosomal complexes did not reach the initiation codon. When eIF1 and eIF1A were added, the bound ribosomal complex dissociated from the mRNA and entered

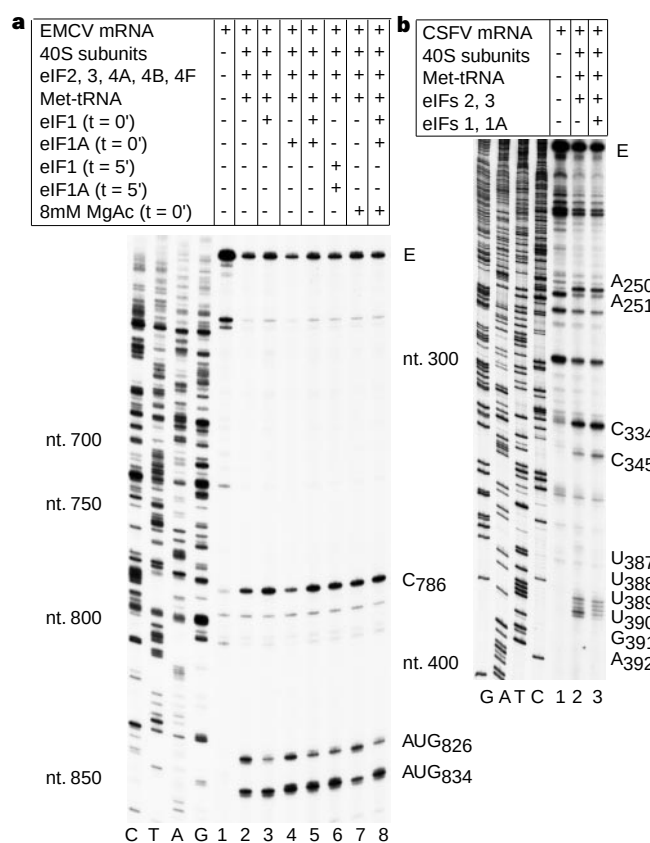


Figure 5 Assembly and toeprint analysis of ribosomal complexes on EMCV and CSFV mRNAs. **a**, EMCV; **b**, CSFV. Reaction mixtures contained ATP and GMP-PNP, and translation components were added when indicated (time t, in min). Full-length cDNAs are marked E. cDNA products labelled AUG₈₃₄ and AUG₈₂₆ terminated 15–17 nucleotides (nt.) from the stated EMCV codon. The cDNA product labelled C₇₈₆ terminated at this nucleotide⁴. cDNA products labelled A₂₅₀, C₃₃₄, G₃₄₅ and C₃₈₇–A₃₉₂ terminated at these nucleotides⁵. The positions of some EMCV and CSFV nucleotides are indicated on the left.

a new round of initiation that led to 48S complex formation. The roles in initiation of eIF1 and eIF1A have remained obscure since their identification^{12,13}. They have been described as having pleiotropic and usually minor effects on various stages in initiation^{14–18}, but homologous factors are essential for the viability of yeast *Saccharomyces cerevisiae*^{19,20}. By using toeprinting to map ribosomal complexes on mRNAs (rather than sucrose density-gradient centrifugation merely to monitor their association), we have now identified essential, unanticipated activities of eIF1 and eIF1A.

Ribosomal scanning is the most coherent model for cap-mediated initiation of translation, and we shall discuss our data in terms of it. Complex I stopped primer extension at identical 5'-proximal positions on mRNA regardless whether or not it was assembled under ionic conditions that allowed scanning. It probably did not move on the mRNA after attachment. eIFs 2, 3, 4A, 4B and 4F are therefore sufficient for attachment of a ribosome to capped mRNA but not for it to begin scanning. We do not know how the 43S complex interacts with mRNA during scanning, but conclude that these five factors do not provide the 43S complex with the necessary 'active centre'. Inhibition of scanning by raised concentrations of Mg²⁺ may be due to alterations in the conformation of this active centre. Little complex II was formed in the presence of eIF1 unless eIF1A was also present. eIF1A thus increases the competence of 43S complexes to scan, possibly by increasing the affinity of eIF1 for the 43S-mRNA complex or by enhancing its stability on an mRNA during scanning. eIF1A is a strong RNA-binding protein²¹. The mRNA-binding site on eukaryotic 40S subunits appears to be an open cleft that can bind internal sequences of an mRNA in the absence of a free 5' end during IRES-mediated initiation²². This cleft probably also binds an internal sequence downstream of the eIF4F-bound 5'-cap during cap-mediated initiation, but may be clamped shut during scanning so the mRNA can be inspected base by base without bypassing hairpins that might harbour the initiation codon²³. eIF1 and eIF1A are candidates to close this cleft.

eIF1A increased the formation of complex I in reactions lacking eIF1. We have not distinguished between the possibilities that eIF1A stabilizes 43S complexes in solution and that it stabilizes their interaction with mRNA^{17–20}. Gel filtration analysis showed that eIF1 stabilizes binding of eIF1A to 43S complexes¹⁶. eIF1A strongly enhanced eIF1-mediated assembly of 48S complexes and caused a striking qualitative difference in the resulting complex from that produced in its absence. 48S complexes formed in the presence of eIF1 and eIF1A yielded prominent toeprints 15–17 nucleotides, downstream of the initiation codon, whereas in the presence of eIF1 alone they yielded only the stops 16–17 nucleotides downstream. Similarly, a third toeprint 15 nucleotides downstream of EMCV AUG₈₃₄ was apparent only when eIF1A was present. We have not yet determined whether this difference is indicative of a conformational change in the 48S complex induced by eIF1A that is necessary for subunit joining to form an 80S complex.

A second critical function of eIF1 and eIF1A in initiation is to maintain the accuracy of this process by recognizing and destabilizing aberrant preinitiation complexes. eIF1 alone can recognize and destabilize ribosomal complexes incorrectly assembled at EMCV AUG₈₂₆. This editing function is also active on capped mRNAs but depends on the presence of eIF1A. Together, these factors destabilize complex I. We do not know which properties of this complex and of the complex at EMCV AUG₈₂₆ target them for destabilization by eIF1. However, identification of this activity in mammalian eIF1 is consistent with properties of its yeast homologue Sui1, which is a monitor of translational accuracy. Mutations in Sui1 allow aberrant initiation at a UUG triplet^{19,24}. The specific role of Sui1 in maintaining translation accuracy is not known. Our finding that eIF1 destabilizes aberrant ribosomal complexes suggest that these Sui1 mutants may be unable to discriminate against mismatched codon-anticodon interactions. The importance of these activities of eIF1

and eIF1A is to monitor the accuracy of the start-site selection throughout the scanning process to prevent spurious initiation events.

Taken together, our results suggest a model for cap-mediated initiation in which interactions between eIF3, mRNA and the eIF4G subunit of cap-bound eIF4F promote attachment of 43S complexes to unstructured, cap-proximal regions of an mRNA. eIF1 and eIF1A must interact with 43S complexes when or immediately after they bind mRNA for them to be competent to begin scanning. These factors may contribute to the correct interactions of the 43S complex with mRNA that enable it to enter a processive mode, for example by forming part of the channel through which mRNA moves during ribosomal scanning, by contributing to the ribosomal translocation process, or by both of these. Indeed, these activities could be integrated as part of a mechanism for base-by-base inspection of an mRNA during scanning. □

Methods

Plasmids. pCSFV (1–442).NS' (ref. 6), pTE1 (ref. 25), pBS⁺(β-globin) (ref. 26), pT7-7-1A (ref. 21), pET (His₆-eIF4A) and pET (His₆-eIF4B) (refs 4, 5) have been described. cDNA corresponding to the eIF1 coding region⁹ was amplified by the polymerase chain reaction (PCR) from phagemid ATCC 156441 from the American Type Culture Collection (Rockville, MD) with primers 5'-CGACGGATCCTATGTCGCTATCCAG-3' and 5'-CTAGAAGCTTAAACCATGAACC-3' and inserted into pQE31 (Qiagen Santa Clarita, CA) to yield pQE(His₆-eIF1). cDNA corresponding to the eIF1A coding region was amplified by PCR from pT7-7-1A (ref. 21) with primers 5'-ATATGGATCGATGCCCAAGAATAAAGGAGG-3' and 5'-CAACAAGCTTGATGATGT-CATCAATATCTTC-3' and inserted into pET28 (Novagen, Madison, WI) to yield pET (His₆-eIF1A).

Purification of factors and 40S ribosomal subunits. 40S ribosomal subunits and eIF2, eIF3 and eIF4F were purified from RRL (Green Hectares, Oregon, WI) as described^{4,6}. No cross-contamination was detected by western blotting using specific antibodies and functional assays dependent on each factor^{4–6}. eIF1 and eIF1A were purified from the 50–70% ammonium sulphate ribosomal salt wash precipitation fraction from 2 litres of RRL. This fraction was resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM DTT, 0.1 mM EDTA) containing 100 mM KCl, applied to a DEAE cellulose (Whatman DE52) column and eluted first with buffer A containing 100 mM KCl and then with buffer A containing 250 mM KCl. These fractions were the starting materials for purification of eIF1 and eIF1A, respectively. eIF1 in the 400–600 mM KCl fraction eluted from a phosphocellulose (Whatman P11) column was applied in buffer A containing 200 mM KCl to FPLC Superdex G200 and then G75 gel filtration columns (Pharmacia). The eIF1-containing fraction in buffer B (20 mM HEPES, pH 7.5, 10% glycerol, 1 mM DTT, 0.1 mM EDTA) containing 100 mM KCl was applied to an FPLC MonoS HR 5/5 column (Pharmacia). Fractions were collected across a 100–500 mM KCl gradient; apparently homogeneous eIF1 eluted with 250 mM KCl. eIF1A in the 700–900 mM KCl elution fraction from a phosphocellulose column was purified to apparent homogeneity by gel filtration in buffer A containing 200 mM KCl on an FPLC Superdex G200 column. Recombinant eIF4A and eIF4B were purified as described⁴. Recombinant eIF1 and eIF1A were expressed in *Escherichia coli* BL21 (DE3) and purified using Ni²⁺-NTA (Qiagen) and heparin-Sepharose (Pharmacia) matrices.

Sequencing of eIFs 1 and 1A. Purified 13.5K and 19K proteins were resolved by gel electrophoresis. The 19K protein was transferred to PVDF membrane for N-terminal sequencing done using an Applied Biosystems Procise sequencer. Gel bands containing the 13.5K protein were excised, washed with 0.2 M ammonium bicarbonate/50% acetonitrile, dried and rehydrated in 0.2 M ammonium bicarbonate. Overnight digestion by sequencing grade modified trypsin (Promega, Madison, WI) was stopped by addition of 10% trifluoroacetic acid (TFA) to a final concentration of 1%. The supernatant obtained after washing the gel piece with 0.1% TFA/60% acetonitrile was reduced by rotary evaporation. Peptides were isolated from this extract by reversed-phase liquid chromatography, and two well-resolved peaks were sequenced.

Assembly and analysis of ribosomal complexes. For analysis of 48S complexes by sucrose density-gradient centrifugation, 0.3 μg native α- and β-

globin mRNAs (Life Technologies, Grand Island, NY) that had been 3'-end-labelled with [32 P]pCp using T4 RNA ligase was incubated for 5 min at 30 °C in a 100- μ l reaction volume that contained buffer C (2 mM DTT, 100 mM potassium acetate, 20 mM Tris, pH 7.6) with 2 mM magnesium acetate, 1 mM ATP, 0.1 mM guanylimidodiphosphate (GMP-PNP), 0.25 mM spermidine, eIF2 (6 μ g), eIF3 (14 μ g), eIF4A (4 μ g), eIF4B (1 μ g), eIF4F (4 μ g), 12 pmol Met-tRNA Met and 12 pmol 40S subunits, with or without eIF1 (1 μ g), eIF1A (1 μ g) and unlabelled competitor mRNA, as indicated in the text. 48S and ribonucleoprotein complexes were resolved by centrifugation through sucrose density gradients as described^{4,6}. For toeprint analysis, ribosomal complexes were assembled essentially as described above by incubating 1 μ g CSFV or EMCV mRNA⁴⁻⁶ or 0.3 μ g native α - and β -globin mRNAs for 5 min at 30 °C in a 40- μ l reaction volume. In some experiments, reaction mixtures were supplemented with 4–8 μ g of different RRL subfractions as indicated in the text. Incubation was continued for 3 min at 30 °C following addition of 4 pmol primer 5'-GCATTTCAGAGGACAGG-3' (complementary to β -globin nucleotides 177–194), 5'-GTCAATAACTCTCTGG-3' (complementary to EMCV nucleotides 957–974) or 5'-CTCGTTTGGCGACATGCC-3' (complementary to part of the NS' coding sequence in CSFV-NS' mRNA) as appropriate. Ribosomal and RNP complexes were analysed by primer extension^{4,5} using avian myeloblastosis virus reverse transcriptase (Promega) in the presence of [α - 32 P]dATP (~6,000 Ci mmol $^{-1}$; ICN Radiochemicals). cDNA products were analysed by electrophoresis through 6% polyacrylamide sequencing gel. cDNA products were compared with appropriate dideoxynucleotide sequence ladders.

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