METHOD

Affinity purification of eukaryotic 48S initiation complexes

NICOLAS LOCKER, LAURA E. EASTON, and PETER J. LUKAVSKY

MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom

ABSTRACT

In vitro assembly of translation initiation complexes from higher eukaryotes requires purification of ribosomal subunits, eukaryotic initiation factors, and initiator tRNA from natural sources, and therefore yields only limited material for functional and structural studies. Here we describe a robust, affinity chromatography-based purification of eukaryotic 48S initiation complexes from rabbit reticulocyte lysate (RRL), which significantly reduces the number of individual purification steps. Hybrid RNA molecules, consisting of either a canonical 5' UTR or an internal ribosome entry site (IRES) RNA followed by a short open reading frame and a streptomycin aptamer sequence, are incubated in RRL to form 48S complexes. The assembly reaction is then applied to a dihydrostreptomycin-sepharose column; bound complexes are washed and specifically eluted upon addition of streptomycin. The eluted fractions are further purified by centrifugation through a sucrose density gradient to yield pure 48S particles. Using this purification scheme, properly assembled IRES-mediated as well as canonical 48S complexes were purified in milligram quantities.

Keywords: IRES RNA; affinity chromatography; eukaryotic initiation factors; translation initiation; 48S complexes

INTRODUCTION

In higher eukaryotes, at least two distinct pathways lead to the assembly of a functional 80S ribosome containing an initiator tRNA (Met-tRNA_i^{Met}) base-paired to the AUG start codon of mRNA within the ribosomal P (peptidyl) site (Sachs et al. 1997; Kapp and Lorsch 2004; Merrick 2004). The canonical pathway requires a 5'-capped mRNA and the full complement of eukaryotic initiation factors (eIFs) to coordinate 80S assembly. In a first step, eIF4G, eIF4E, eIF4A, and eIF4B mediate binding of the 43S particle—which consists of the small 40S ribosomal subunit, the ternary eIF2, GTP, Met-tRNA_i^{Met} complex, eIF3, eIF1A, and eIF1—to the 5' cap structure. The assembly then scans the 5' UTR to locate the first AUG start codon and to form a stable 48S complex, consisting of the 43S components properly assembled at the initiation codon. Subsequently, eIFs are released in a GTP-dependent manner, assisted by eIF5 and eIF5B, and the large 60S ribosomal subunit joins to form the active 80S ribosome. In contrast, some cellular mRNAs and several viral RNAs

contain structured 5' UTRs, termed IRES elements, which mediate initiation without a 5' cap or scanning and use only a subset of eIFs to form a stable 48S complex.

Both initiation pathways have been studied by reconstituting 48S complexes *in vitro* using ribosomal subunits, Met-tRNA_i^{Met}, and eIFs purified from natural sources (Benne and Hershey 1978; Benne et al. 1979; Pestova et al. 1996; Algire et al. 2002). Using this approach, the roles of several eIFs during initiation have been elucidated, and the eIF requirements of several IRES RNAs have been characterized (for reviews, see Hershey and Merrick 2000; Pestova et al. 2001). Recently, an affinity-tagging method for the isolation of hepatitis C virus (HCV) IRES-mediated 48S initiation complexes from HeLa cell lysates has been presented as an additional tool to study 48S initiation complexes. In brief, RNA hairpin sequences, which form binding sites for the bacteriophage MS2 coat protein, are introduced upstream of the HCV IRES RNA. The hybrid RNA is incubated in the lysate, bound to amylose beads via a maltose-binding protein (MBP)–MS2 fusion protein, eluted with maltose after several washes, and fractionated on sucrose gradients. Using this method, the assembly of HCV IRES-mediated initiation complexes as well as assembly defects of mutant IRES RNAs could be studied (Ji et al. 2004).

Here we present a new affinity purification-based isolation procedure of 48S complexes from rabbit reticulocyte lysate (RRL) using the streptomycin-binding RNA aptamer (Bach-

Reprint requests to: Peter J. Lukavsky, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK; e-mail: pjl@mrc-lmb.cam.ac.uk; fax: +44-1223-213556.

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2227906>.

ler et al. 1999). The purification protocol was established using the HCV IRES RNA tagged at the 3' end. The initial affinity-based isolation of crude 48S complexes is followed by a final cleanup step using sucrose density gradient centrifugation and yields up to 1 mg of pure 48S complex from 10 mL of lysate. Quantitative Western and Northern blot analysis of the 48S particles suggested that eIF2 and eIF3 as well as Met-tRNA_i^{Met} are present in 1:1 stoichiometry with the 40S subunit, and toeprinting analysis showed proper assembly of the components at the AUG start codon. To demonstrate the versatility of the purification protocol, we further isolated 48S complexes using tagged, β -globin mRNA (Dmitriev et al. 2003) and two different IRES RNAs from porcine teschovirus type 1 (PTV-1) (Pisarev et al. 2004) and encephalomyocarditis virus (EMCV) (Borovjagin et al. 1991).

RESULTS AND DISCUSSION

Design of the hybrid RNA for affinity purification of 48S complexes

We designed hybrid RNA oligonucleotides containing either a canonical 5' UTR or an IRES element followed by ~85 nt of open reading frame (ORF), a unique primer-binding site for toeprinting analysis (Pestova et al. 1996), then a short uracil-cytosine linker and the streptomycin aptamer sequence, the so-called StreptoTag (Bachler et al. 1999). We chose this simple affinity tag since it has been used previously to isolate RNA-protein complexes from cellular extracts with high specificity (Bachler et al. 1999). The RNA aptamer comprises <50 nt and binds dihydrostreptomycin coupled to a sepharose column matrix with micromolar affinity in a magnesium-dependent manner. In principle, the aptamer could therefore be eluted from the column upon addition of EDTA, but this would destabilize ribosomal particles. As an alternative elu-

tion method in the presence of magnesium, a buffer that contains streptomycin at a concentration of 10 μ M can be used. Since eukaryotic ribosomes are naturally resistant to the antibiotic streptomycin (Chernoff et al. 1994), this method should allow elution of 48S complexes in the presence of stabilizing amounts of magnesium.

Compared with the previously published MBP-MS2 fusion protein-based HCV IRES 48S affinity purification, which used an RNA tag at the 5' end of the HCV IRES RNA, we attached the StreptoTag at the 3' end of the RNA. This should allow both the internal ribosome entry and scanning mode of initiation to occur on the tagged RNA oligonucleotides, since they contain a 5' end sequence that is not blocked by RNA hairpin sequences. In addition, placing the tag at the 3' end ensures that only full-length RNAs are bound to the affinity column. To minimize interference from the different coding regions with aptamer binding, we placed three UC repeats upstream of the aptamer as a short, presumably flexible linker. The length of the ORF was kept at ~85 nt to ensure proper spacing between the 48S particle and the StreptoTag. In addition, this length is ideal to detect inhibition of primer extension by reverse transcriptase (toeprinting) (Pestova et al. 1996), a method commonly used to test proper assembly of ribosomal initiation complexes (Hartz et al. 1988). To perform toeprinting, a primer needs to anneal to the ORF under the native condition within an intact 48S complex. Since strong secondary structure within the ORF could interfere with native binding, we engineered a unique, previously described primer site downstream of the individual ORF sequences (Pestova et al. 1996). To facilitate subcloning of several different IRES RNAs, we introduced an XbaI restriction site upstream of the toeprint primer site into the plasmid DNA template, so that individual IRES RNA elements could be subcloned between the HindIII and the XbaI sites (Fig. 1A). For tagged IRES RNAs, the full-length

A

5' Hind III — T7 Promoter — 5' UTR — AUG — ORF (~85 nt) — Xba I — Toeprint primer binding site — (UC)₃ — linker — Streptomycin aptamer — EcoR I 3'

B

FIGURE 1. Ribosomal 48S complex purification. (A) Design of the tagged RNA for affinity purification and analysis of initiation complexes. (B) Strategy for affinity purification of HCV IRES 48S complexes from RRL.

5' UTR sequence was introduced together with the authentic ORF, since the first nucleotides of the coding sequence can be required for efficient IRES function (Reynolds et al. 1995). The tagged β -globin mRNA comprised the full-length 5' UTR with a 5'-GGGAG sequence to allow efficient *in vitro* transcription using T7 polymerase and 54 nt of the coding sequence (Dmitriev et al. 2003). To introduce a 5' cap structure, transcription reactions were supplemented with $m^7G(5')ppp(5')GTP$ (Nielsen and Shapiro 1986).

Affinity purification of 48S complexes

Our affinity purification protocol for the isolation of eukaryotic initiation complexes was developed and tested using a tagged HCV IRES RNA (Fig. 1B), which contained the first 426 nt of genomic HCV RNA and was constructed as shown in Figure 1A. RRL was used as source of ribosomal subunits, initiation factors, and Met-tRNA_i^{Met} to assemble ribosomal complexes as previously described (Wilson et al. 2000). Briefly, RRL was treated with puromycin to part the ribosomal subunits and then was incubated in the presence of the non-hydrolyzable GTP analog, GMPPNP, to inhibit ribosomal subunit joining and to ensure a blockage of translation initiation events at the 48S stage (Merrick 1979). Then, tagged HCV IRES RNA was introduced to allow initiation complexes to form. After incubation, tagged HCV IRES RNA was bound to dihydrostreptomycin-coupled sepharose resin, washed, and released from the column by competitive elution with streptomycin (Fig. 2A). The elution fraction was then analyzed by native agarose gel electrophoresis and compared with purified ribosomal subunits. As shown in Figure 2B, the elution fraction contains the excess of tagged HCV IRES RNA. In agreement with the HCV IRES-mediated initiation pathway, the presence of binary complex between the small ribosomal subunit and the tagged HCV IRES RNA was also detected (Otto and Puglisi 2004). As expected, the eluted 48S particle migrated as a single band between the references of the large and the small ribosomal subunits. To further purify 48S complexes, HCV IRES-bound translation initiation complexes were pelleted by centrifugation and then fractionated using sucrose density gradient centrifugation, yielding separation between the free RNA, binary complexes, and 48S particles (Fig. 2C). As detected on native agarose gel electrophoresis (Fig. 2D), pure 48S complexes were obtained. Following this procedure, an input of 2 mg of tagged HCV IRES RNA and 10 mL of RRL yields up to 1 mg of 48S particle.

A control experiment was run in a similar way using an RNA containing the StreptoTag, the linker, and part of the HCV IRES ORF without a start codon. Only the initial RNA could be eluted from the column, indicating that no unspecific binding occurs to the linker or StreptoTag sequence (data not shown).

In order to block the translation initiation pathway at the 48S stage, we used a nonhydrolyzable GTP analog, GMPPNP, which inhibits the ribosomal subunit joining by preventing the

FIGURE 2. HCV IRES ribosomal 48S complex purification. (A) Profile of streptomycin affinity column. Plots of absorbance at 280 nm vs. elution time are shown. (B) Analysis of elution fraction composition by 1% native agarose gel. Lanes are loaded as noted on the gel. Bands corresponding to free RNA, RNA/40S binary, and 48S complex are indicated. (C) Sucrose density gradient analysis of translation initiation complexes bound to HCV IRES RNA. Plots of absorbance at 260 nm vs. sucrose density gradient fractions are shown; peaks corresponding to free RNA, RNA/40S binary, and 48S complex are indicated. (D) Analysis of the pooled and concentrated 48S fractions for all the assembled particles is shown on 1% native agarose gel. Lanes are loaded as indicated on the gel.

hydrolysis of eIF2-bound GTP. This blocks the release of eIF2 from 48S complexes and therefore the subsequent joining of the 60S subunit to form 80S ribosomes (Merrick 1979). The purification of 48S complexes was performed either with or without GMPPNP. The resulting 48S particles were then assayed for the presence of eIF2 using Western blot analysis (Fig. 3A). When GMPPNP was omitted in the 48S assembly reaction, only a small amount of eIF2 α could be detected within the 48S complexes, because GTP-dependent eIF2 release from the complex was not blocked during the assembly. The same Western blot performed on 48S complexes purified in the presence of GMPPNP shows a strong signal for eIF2 α , demonstrating efficient blockage of GTP-dependent eIF2 release and thereby translation initiation at the 48S stage.

Analysis of the eluted 48S particles

First, we tested the efficiency of the purification protocol by immunoblotting all fractions of the individual purification steps for the presence of eIF2 and eIF3 with antibodies against the eIF2 α or eIF3d subunits, respectively. About 20% of eIF2 and 35% of eIF3 present in the RRL were not incorporated into 48S particles and therefore were detected in the flow-through of the affinity column (Fig. 3B, lanes 1,2). Prolonged incubation time of the tagged HCV IRES RNA in the RRL did not alter the result (data not shown). No eIF2 and eIF3 was detected during the wash of the affinity column, indicating

FIGURE 3. Analysis of the composition of HCV IRES 48S complexes. (A) Immunoblotting analysis of HCV IRES 48S complexes assembled in the absence and the presence of GMPPNP using anti-eIF2 α antibodies. (B) Efficiency of the purification protocol. The presence of eIF2 and eIF3 is followed by immunoblotting through the entire purification. Lanes are loaded as follows: (1) application fraction, (2) flowthrough of the application fraction, (3) wash fractions, (4) elution, (5) supernatant of the centrifugation step, (6) 48S peak from the sucrose density gradient, (7) 40S/IRES peak from the gradient, (8) pooled top fractions from the gradient. (C) Toeprinting assay of both purified and assembled 48S HCV initiation complexes. Arrows denote positions of the initiation AUG codon and toeprinting stops. A dideoxynucleotide sequence generated with the same primer (shown on the left) was run in parallel. The upper part of the gel, which contains additional, previously observed toeprints (Pestova et al. 1998) caused by the processivity of the reverse transcriptase in combination with the particle stability under the assay condition (see Materials and Methods), is omitted for clarity. (D) Detection of Met-tRNA_i^{Met} within initiation complexes. Northern blot analysis of HCV IRES 48S compared with a transcribed tRNA_i^{Met}.

that the particle stayed intact, and ~80% of the amount of eIF2 and 65% of the amount of eIF3 initially present in the RRL could be detected in the elution fraction of the affinity column containing the 48S complexes (Fig. 3B, lanes 3,4). Also, during the next purification steps the particle stayed intact, since no eIF2 and eIF3 could be detected in the supernatant of the subsequent centrifugation step or in the top fractions of the final sucrose density gradient (Fig. 3B, lanes 5,7,8); only the 48S fractions contained the same amount of eIF2 and eIF3 as the initial elution fraction (Fig. 3B, cf. lanes 4 and 6). The presence of both eIF2 and eIF3 in the 48S particle, no losses of eIF2 or eIF3 during the individual purification steps, and the migration characteristics of the complex as a single peak in sucrose density gradients as well as a single band on native agarose gels (Fig. 2C,D) therefore strongly suggest that our purification protocol yields pure HCV IRES 48S particles containing the expected initiation factors (Pestova et al. 1998).

Assembly of 48S complexes at the initiation codon of an mRNA can be assessed using a so-called toeprinting assay, which directly identifies the position of the 40S subunit on the mRNA chain (Hartz et al. 1988). In a 48S complex, this arrest of reverse transcription occurs always at the same position 16–18 nt downstream of the adenine of the AUG start codon. Such a toeprint indicates that the start codon is placed in the P site of the small ribosomal subunit and is base-paired to charged Met-tRNA_i^{Met} (Hartz et al. 1988). 48S complexes assembled onto HCV IRES RNA using native Met-tRNA_i^{Met} and purified eIF2, eIF3, and 40S sub-

unit yielded stops 16–18 nt downstream the AUG codon (Pestova et al. 1998). Toeprinting analysis of 48S complexes assembled onto HCV IRES RNA in GMPPNP-treated RRL yielded stops +16 and +17 nt downstream of the AUG codon (Otto and Puglisi 2004).

Toeprinting was used to assess the proper assembly of the isolated initiation complexes. First, 48S complexes were assembled onto tagged HCV IRES RNA in RRL in the presence of GMPPNP. Toeprints characteristic of a properly assembled 48S complex could be detected (Fig. 3C). The same toeprinting reaction was also carried out on the affinity-purified particles. The detection of the same stops at positions +16 and +17 nt downstream of the AUG demonstrated that our purification protocol yielded authentic 48S complexes with correct positioning of the AUG start codon within the ribosomal P-site (Fig. 3C).

To further confirm the suggested codon–anticodon base-pairing, we investigated whether Met-tRNA_i^{Met} is present in the purified 48S particle. Extraction of total RNA from the 48S complex was performed and then compared with an uncharged tRNA_i^{Met} transcript by Northern blot analysis using a probe complementary to the 3' end of tRNA_i^{Met}. As shown in Figure 3D, this experiment confirmed the presence of the tRNA_i^{Met} in our purified 48S complexes, consistent with the toeprinting results.

Our data demonstrate that eIF2, eIF3, and Met-tRNA_i^{Met} are present in the purified 48S complexes, and that Met-tRNA_i^{Met} is base-paired to the AUG start codon in the ribosomal P-site. To further assess the quality of the isolated particles, we performed quantitative Western and Northern blot analysis to also estimate the stoichiometry of the particle components. Serial dilutions of uncharged tRNA_i^{Met} ranging from 5–25 pMol and total RNA extracted from 15 pMol of HCV 48S particle were separated by denaturing PAGE, followed by Northern blot analysis and quantification of band intensities. As shown in Figure 4A, our measurements confirm that Met-tRNA_i^{Met} is present in the 48S particle in a 1:1 stoichiometry compared with 40S subunits. Since Met-tRNA_i^{Met} association with the 40S subunit withstands sucrose density gradient centrifugation, which was used as the final purification step, only in the presence of eIF2 (Unbehauen et al. 2004), the quantitative Northern analysis also strongly suggests that equimolar amounts of eIF2 are present in the particle. Quantitative Western blot analysis using serial dilutions of recombinant eIF2 α and eIF3d over a linear range of 90–450 fMol and

FIGURE 4. Quantitative analysis of 48S complexes. (A) Quantitative Northern blot analysis of purified 48S complexes using a probe specific to tRNA_i^{Met}. Dilutions of transcribed tRNA_i^{Met} (5–25 pMol) and 15 pMol of purified 48S complexes were loaded as indicated and resolved by 8% PAGE. Band intensities were quantified using ImageQuant software, and relative levels of tRNA_i^{Met} were normalized to that of the 15-pMol tRNA_i^{Met} intensity. A graphical representation of the relative intensities is displayed below. All error bars are standard error of the mean. Using a response curve analysis, the following tRNA_i^{Met} concentrations within the 48S complexes have been determined: 17.3 ± 1.5 pMol for HCV, 14.7 ± 1.3 pMol for PTV-1, 15.6 ± 1.5 pMol for β-globin, and 16.8 ± 2.1 pMol for EMCV. (B) Quantitative immunoblotting analysis of purified 48S complexes using antibodies specific to eIF2α. Dilutions of recombinant eIF2α (90–450 fMol) and 225 fMol of purified 48S complexes were loaded as indicated and resolved by 12% NuPAGE gel. Band intensities were quantified using ImageQuant software, and relative levels of eIF2α were normalized to that of the 225-fMol eIF2α intensity. A graphical representation of the relative intensities is displayed below. All error bars are standard error of the mean. Using a response curve analysis, the following eIF2α concentrations within the 48S complexes have been determined: 224.8 ± 16 fMol for HCV, 210.6 ± 14 fMol for PTV-1, 225.4 ± 3 fMol for β-globin, and 213.4 ± 21 fMol for EMCV. (C) Quantitative immunoblotting analysis of purified 48S complexes using antibodies specific to eIF3d. Dilutions of recombinant eIF3d (6–90 pMol) and 30 pMol of purified 48S were loaded as indicated and resolved by 12% SDS-polyacrylamide gels. Band intensities were quantified using ImageQuant software, and relative levels of eIF3d were normalized to that of the 30-pMol eIF3d intensity. A graphical representation of the relative intensities is displayed below. All error bars are standard error of the mean. Using a response curve analysis, the following eIF3d concentrations within the 48S complexes have been determined: 38.7 ± 4.2 pMol for HCV, 33.1 ± 4.0 pMol for PTV-1, 32.7 ± 1.5 pMol for β-globin, and 26.7 ± 3.6 pMol for EMCV.

6–90 pMol, respectively, indeed suggested the equimolarity of both eIF2 and eIF3 and the 40S subunits (Fig. 4B,C).

General applicability of the purification protocol

To further probe the versatility of the purification protocol, we also purified 48S complexes assembled onto a tagged PTV-1 IRES RNA. The PTV-1 IRES is one of the shortest picornavirus IRES elements. Unlike other picornavirus IRES elements, there is no polypyrimidine tract 20 nt upstream of the AUG codon, and its activity is not affected by eIF4G cleavage (Kaku et al. 2002), reminiscent of the HCV IRES element. Toeprinting experiments performed on in vitro assembled initiation complexes suggested that PTV-1 IRES-mediated 48S complexes already form in the presence of only 40S subunits, eIF2, and Met-tRNA_i^{Met} (Pisarev et al. 2004). The presence of eIF3 in the complex only seemed to enhance the formation of the 48S-specific toeprint, suggesting that eIF3 might be an essential component of the PTV-1 IRES-mediated 48S particle. In order to test whether eIF3 is a component of the PTV-1 IRES 48S complex, we designed a tagged PTV-1 IRES RNA and assembled and purified initiation complexes using the protocol described above. Quantitative analysis again showed that, as for the tagged HCV IRES

RNA, the 48S particle contains Met-tRNA_i^{Met}, eIF2α, and eIF3d in equimolar amounts (Figs. 2D, 4). Our purification protocol is also applicable for IRES RNAs with different eIF-requirements, such as the IRES from the picornavirus EMCV (Jang et al. 1988). In contrast to PTV-1- and HCV IRES-mediated initiation, the EMCV IRES also requires the eIF4F complex and ATP-hydrolysis to assemble 48S complexes (Pestova et al. 1996). The assembly reaction in RRL using tagged EMCV IRES was therefore supplemented with ATP and the complex was purified as before, yielding homogeneous 48S particles (Figs. 2D, 4).

In addition to IRES-driven 48S assembly, we also tested whether our protocol is suitable for the assembly and isolation of canonical initiation complexes. Canonical initiation requires scanning of the 5′ UTR, which should be possible with the affinity tag being placed at the 3′ end of the mRNAs. In contrast to IRES-mediated initiation, cap-dependent initiation requires the full complement of initiation factors (Sachs et al. 1997; Kapp and Lorsch 2004; Merrick 2004). In order to validate our method for mRNAs, which initiate translation through the canonical pathway, we used a tagged β-globin mRNA to isolate canonical 48S complexes. Once again, the 48S particle migrates as a single band on native agarose gels and con-

tains equimolar amounts of Met-tRNA^{Met}, eIF3d, and eIF2 α , demonstrating that our protocol is suitable for purifying 48S complexes that require scanning during initiation.

Conclusion and outlook

We presented a novel, affinity chromatography-based method for the purification of eukaryotic 48S initiation complexes from RRL. Both canonical and diverse IRES-mediated 48S complexes could be purified in a quick, two-step procedure. The proper composition and stoichiometry of the components within the isolated particles was assessed by quantitative Northern and Western blot analysis, respectively. Our method can be used as a biochemical tool to study the initiation factor composition of 48S complexes assembled onto different cellular or viral IRES RNAs, as we demonstrated with the HCV, PTV-1, and EMCV IRES RNAs. This could complement approaches that use reconstitution of 48S particles from purified components. Isolation of 48S particles assembled onto mutant IRES RNAs could be used to study the effect of mutations on proper 48S assembly, initiation factor recruitment, or release. In addition, our purification scheme provides an efficient way to isolate milligram quantities of 48S complexes, which should benefit structural studies of eukaryotic initiation complexes.

MATERIALS AND METHODS

Preparation of tagged mRNAs

Oligodeoxynucleotides were purchased from Operon or Sigma Genosys, desalted and lyophilized in 40-nMol synthesis scale. Standard PCR and cloning techniques were used to create DNA inserts from overlapping primers (Lukavsky and Puglisi 2004). First, the Streptomycin aptamer with an upstream toeprint primer region was prepared using two primers (5' primer, GAGCTCTAG ACGCCGAGATCAGAAATCCCTCTCTCGGATCGCATTTGGAT TCTGCC; 3' primer, GCTCGAATTCGGATCCGACCGTGGTGC CCGAAGGCAGAAGTCCAATGCGATCC). The resulting PCR product was restriction digested with XbaI and EcoRI and ligated into pUC18 digested with the same enzymes. The purified vector (Strepto-vector) containing the StreptoTag and toeprint primer sequence was then used to subclone DNA inserts (containing a HindIII site, a T7 RNA polymerase promoter, the appropriate 5' UTR and coding sequence, and an XbaI site) between the HindIII and XbaI sites. The HCV IRES (GenBank database no. AF271632) insert corresponding to nucleotides 1–426, the PTV-1 IRES (GenBank database no. AB038528) insert corresponding to nucleotides 3–479, and the β -globin mRNA (Dmitriev et al. 2003) corresponding to nucleotides 1–107 with an additional 5' GGGAG sequence were prepared from overlapping primers. The resulting PCR products were restriction digested with HindIII and XbaI and ligated into the Strepto-vector digested with the same enzymes. The EMCV IRES insert corresponding to nucleotides 406–930 was amplified by PCR from a plasmid containing nucleotides 1–1740 (a kind gift from Richard Jackson, Cambridge University) using

two primers (5' primer 5'-GAGCAAGCTTAATACGACTCACTAT AGCATTTCCTAGGGGTCTTTCC-3', 3' primer 5'-CTCGGCGTC TAGAGGTAATAATCCATTACGGTATTGTAGAGC-3'). The PCR product was restriction digested with HindIII and XbaI, yielding two fragments that were ligated into the Strepto-vector digested with the same enzymes. RNA oligonucleotides were transcribed from the above plasmids linearized with EcoRI (HCV IRES, EMCV IRES) or BamHI (PTV-1 IRES) using established protocols and purified by size-exclusion chromatography as described in Lukavsky and Puglisi (2004). The final RNA oligonucleotides were equilibrated into 10 mM Tris-HCl (pH 7.4) buffer using Centriprep centrifugal devices, concentrated to 0.5–1.0 A₂₆₀ units per microliter, and stored at –20°C. β -globin RNA was transcribed from the above plasmid linearized with EcoRI; a commercial cap structure analog, m⁷G(5')ppp(5')G (New England Biolabs) was included in the transcription reaction according to a published protocol (Nielsen and Shapiro 1986) to produce a 5'-capped β -globin mRNA. The RNA was purified using the RNeasy Mini purification kit (Qiagen), equilibrated into 10 mM Tris-HCl (pH 7.4) buffer using Centriprep centrifugal devices, concentrated to 0.5–1.0 A₂₆₀ units per microliter, and stored at –20°C.

Purification of factors and ribosomal subunits

DNA fragments encoding human eIF2 α and eIF3d were prepared by PCR from cDNA libraries (Clontech) using primers 5'-CCA GGGTAGCCATATGCCGGGTCTAAGTTGTAGATTTTATC-3' and 5'-GGTGGTGCTCGAGTCATTAATCTTCAGCTTTGGCTTCCATT TCTTC-3' for eIF2 α and 5'-CCAGGGTAGCCATATGGCAAAGTT CATGACACCCG-3' and 5'-GGTGGTGCTCGAGTCATTAAGTTT CTTCTCTTCTTCTTCTC for eIF3d, respectively. PCR products were digested with NdeI and XhoI and inserted into pET28a (Novagen) digested with the same enzymes to yield plasmids encoding His₆-eIF2 α and His₆-eIF3d. Recombinant His₆-eIF2 α was expressed in Rosetta (DE3) cells (Novagen) and purified using HiTrap columns (Amersham) as previously described (Ito and Wagner 2004). Recombinant eIF3d was expressed in Rosetta (DE3) cells (Novagen) and purified under denaturing conditions using HIS-SELECT spin columns (Sigma) according to manufacturer instructions. The purity of both factors was assayed by Western blot analysis using specific antibodies and protein gel analysis, showing no apparent contamination.

Ribosomal subunits were purified according to published procedures (Pestova et al. 1996).

Affinity purification of initiation complexes

One milliliter (1 mL) of untreated RRL (Green Hectares) was incubated in 3 mL of binding buffer (10 mM MgCl₂, 120 mM KCl, 8% sucrose, 20 mM Tris-HCl at pH 7.6) in the presence of 2.5 μ L of ribonuclease inhibitor (Promega) and half a tablet of protease cocktail inhibitor (EDTA-free, Roche) for 10 min at 37°C. After incubation, puromycin was added (final concentration 1 mM), followed by 10 min incubation on ice and 10 min at 37°C. GMPPNP (Sigma; final concentration 2 mM) was added for an additional 5 min at 37°C. Then, tagged mRNA (final concentration 1 μ M) was added, and incubation continued for another 10 min at 37°C. In addition, for the assembly of 48S complexes onto EMCV and β -globin mRNA, 1 mM ATP was included to stimulate

the scanning process. One gram (1 g) of dihydrostreptomycin-coupled sepharose 6B (Amersham), prepared as described in Bachler et al. (1999), was equilibrated with binding buffer at 4°C. Assembly reactions were then slowly loaded onto the column using a peristaltic pump while monitoring absorbance at 280 nm with a UV Monitor (UV-1, Amersham). A stable baseline was usually reached after washing with 10 column volumes of binding buffer at 4°C, and then the initiation complexes were eluted at room temperature with binding buffer containing streptomycin (final concentration 10 μ M). Complexes were pelleted by centrifugation of the eluted fraction for 16 h at 45,000 rpm in a Ti90 rotor (Beckman). Ribosomal pellets were resuspended in 20 mM Tris-HCl (pH 7.6), 100 mM KOAc, 200 mM KCl, 2.5 mM MgCl₂, 2 mM DTT, layered onto a 15%–40% sucrose density gradient in the same buffer, and centrifuged at 22,000 rpm in a Beckman SW28 rotor for 16 h at 4°C. 48S complexes were recovered by fractionating the gradient while monitoring absorbance at 260 nm. Each 1-mL fraction was analyzed on a 1% agarose gel using Tris-borate as running buffer at 4°C; the clean fractions were pooled, concentrated to a final concentration of 10 A₂₆₀ units/mL using a YM-50 centricon (Millipore), and stored at –20°C. The same protocol was used to purify 48S complexes assembled onto PTV-1, EMCV, and β -globin RNAs, except that β -globin RNA ribosomal complexes were resuspended in 20 mM Tris-HCl (pH 7.6), 80 mM KCl, 2.5 mM MgCl₂, 2 mM DTT, and purified through a sucrose gradient prepared in the same buffer conditions. All purified 48S complexes were finally analyzed on 1% native agarose gel.

Western blot analysis

Proteins from 0.05 A₂₆₀ units of purified 48S particle were resolved by electrophoresis on 4%–12% NuPAGE gels (Invitrogen), transferred to nitrocellulose membranes, and blocked with 5% dry milk in PBS-Tween 0.2%. Membranes were then probed for eIF2 α with monoclonal antibodies (Abcam, ab5369, dilution 1:2000) and eIF3d with polyclonal antibodies (PTGlab, 10219–1-AP, dilution 1:1000). Primary antibody binding was detected using appropriate HRP-coupled secondary antibodies (Abcam, ab6728 and ab6721, dilution 1:2000) and enhanced chemiluminescence (ECL reagent, Amersham) followed by PhosphorImager analysis.

To follow the presence of eIF2 α and eIF3d through the purification process, equivalent volumes of each fraction (taking the different volumes of fractions into account compared with the initial volume of the assembly reaction) were loaded on a 12% NuPAGE gel.

Quantitative immunoblotting

Immunoblotting and detection by chemiluminescence were employed to quantify the level of eIF2 α and eIF3d within 48S complexes assembled onto HCV, PTV-1, EMCV, and β -globin mRNA. The concentrations of purified eIF2 α and eIF3d were determined using extinction coefficients predicted from primary sequence and absorbance measurements at 280 nm of both proteins denatured in 6 M guanidinium chloride. Serial dilutions of purified eIF2 α and eIF3d were loaded together with a fixed amount of 48S complexes with the extinction coefficient of the particle calculated from the three RNA components, the 18S rRNA, tRNA^{Met}_i, and tagged mRNA (1 A₂₆₀ unit = 43 pMol).

Quantitation of eIF2 α and eIF3d over a linear range, 90–450 fMol and 6–90 pMol, was achieved using a standard dilution response curve constructed for each immunoblot. Quantitation of eIF2 α and eIF3d was performed by comparing the band intensities on Hyperfilm ECL (Amersham). The photographic negatives of the immunoblots were scanned to a resolution of 600 dpi in grayscale mode. The scanned images were converted to histograms with ImageQuant software to plot the optical density as a response profile. The total optical density of individual bands was obtained by defining a rectangular area around each band and summing the total OD. To account for irregular baselines, sections of the same sizes as the integrated area were defined under the bands, and their intensity was subtracted. The intensity values were then normalized to that corresponding to a 1:1 stoichiometry within the 48S complexes and displayed as a percentage referred to this value. The values displayed correspond to the average of three independent experiments.

Primer extension analysis of ribosomal complexes

For toeprinting analysis of purified 48S particles, 0.2 A₂₆₀ units of complexes were first exchanged into buffer A containing 5% sucrose, 2 mM DTT, 100 mM KOAc, 20 mM Tris (pH 7.6), 2.5 mM Mg(OAc)₂, 0.1 mM GMPPNP, and 0.25 mM spermidine. Extension reactions were then carried out as follows: Ribosomal complexes were incubated for 3 min at 30°C and then 10 min on ice after adding 5 pmol of primer 5'-GGGATTTCTGATCTCGGCG-3' (Pestova et al. 1996). Then, final concentrations of 1 mM dNTPs, 5 mM Mg(OAc)₂, 1 μ L α -³²P ATP (3000 Ci/mmol; Amersham), and 0.7 U of avian myeloblastosis virus reverse transcriptase (Promega, 24 U/mL) were added and extension was performed for 45 min at 30°C. The mixtures were then carefully extracted with phenol to remove proteins. cDNA products were precipitated with ethanol overnight and analyzed on a 6% sequencing gel. Toeprinting analysis of translation initiation complexes assembled in RRL was performed as described previously (Wilson et al. 2000). Briefly, a master mix containing 15 μ L RRL and 0.15 μ L of ribonuclease inhibitor (Promega) was prepared. GMPPNP was added to a final concentration of 1 mM, and the mixtures were incubated for 5 min at 30°C, followed by the addition of 0.5 μ g of mRNA and incubation for another 5 min at the same temperature. Mixtures were diluted to 40 μ L in buffer A, and extension reactions were carried out and purified as above. cDNA products of the toeprinting reactions were compared with appropriate dideoxynucleotide sequence ladders performed on the unbound mRNA using the same primer.

Northern blot analysis

Total RNA was extracted from 0.25 A₂₆₀ unit of purified 48S particle using the acetic acid/MgCl₂ method (Hardy et al. 1969). The extracted RNAs were then separated using a 4% denaturing polyacrylamide gel together with in vitro transcribed tRNA^{Met}_i as a positive control (Pestova and Hellen 2001), then transferred to nylon membrane and blotted with ³²P-labeled probe against the 3' end of tRNA^{Met}_i (5'-GGTAGCAGAGGATGGTTTCGATCC-3') using ExpressHyb solution (BD Biosciences). The results were visualized using a PhosphorImager.

To perform quantitative Northern blot analysis, serial dilutions of transcribed and purified tRNA^{Met} from 25 to 5 pMol were loaded on an 8% denaturing polyacrylamide gel together with 15 pMol of 48S complexes assembled onto HCV, PTV-1, β -globin, and EMCV RNAs. The intensity of each band was quantified by PhosphorImager analysis using ImageQuant software. To account for irregular baselines, sections of the same sizes as the integrated area were defined under the bands and their intensity subtracted. The intensity values were then normalized to that corresponding to a 1:1 stoichiometry within the 48S complexes and displayed as percentage referred to this value. The values displayed correspond to the average of three independent experiments.

ACKNOWLEDGMENTS

We thank Kiyoshi Nagai for helpful discussions and comments on the manuscript. N.L. is supported through a career development fellowship from MRC.

Received September 13, 2005; accepted December 21, 2005.

REFERENCES

- Algire, M.A., Maag, D., Savio, P., Acker, M.G., Tarun Jr., S.Z., Sachs, A.B., Asano, K., Nielsen, K.H., Olsen, D.S., Phan, L., et al. 2002. Development and characterization of a reconstituted yeast translation initiation system. *RNA* **8**: 382–397.
- Bachler, M., Schroeder, R., and von Ahsen, U. 1999. StreptoTag: A novel method for the isolation of RNA-binding proteins. *RNA* **5**: 1509–1516.
- Benne, R. and Hershey, J.W. 1978. The mechanism of action of protein synthesis initiation factors from rabbit reticulocytes. *J. Biol. Chem.* **253**: 3078–3087.
- Benne, R., Brown-Luedi, M.L., and Hershey, J.W. 1979. Protein synthesis initiation factors from rabbit reticulocytes: Purification, characterization, and radiochemical labeling. *Methods Enzymol.* **60**: 15–35.
- Borovjagin, A.V., Ezrokhi, M.V., Rostapshov, V.M., Ugarova, T., Bystrova, T.F., and Shatsky, I.N. 1991. RNA–protein interactions within the internal translation initiation region of encephalomyocarditis virus RNA. *Nucleic Acids Res.* **19**: 4999–5005.
- Chernoff, Y.O., Vincent, A., and Liebman, S.W. 1994. Mutations in eukaryotic 18S ribosomal RNA affect translational fidelity and resistance to aminoglycoside antibiotics. *EMBO J.* **13**: 906–913.
- Dmitriev, S.E., Pisarev, A.V., Rubtsova, M.P., Dunaevsky, Y.E., and Shatsky, I.N. 2003. Conversion of 48S translation preinitiation complexes into 80S initiation complexes as revealed by toeprinting. *FEBS Lett.* **533**: 99–104.
- Hardy, S.J., Kurland, C.G., Voynow, P., and Mora, G. 1969. The ribosomal proteins of *Escherichia coli*. I. Purification of the 30S ribosomal proteins. *Biochemistry* **8**: 2897–2905.
- Hartz, D., McPheeters, D.S., Traut, R., and Gold, L. 1988. Extension inhibition analysis of translation initiation complexes. *Methods Enzymol.* **164**: 419–425.
- Hershey, J.W.B. and Merrick, W.C. 2000. The pathway and mechanism of initiation of protein synthesis. In *Translation control of gene expression* (eds. N. Sonenberg et al.), pp. 33–88. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Ito, T. and Wagner, G. 2004. Using codon optimization, chaperone co-expression, and rational mutagenesis for production and NMR assignments of human eIF2 α . *J. Biomol. NMR* **28**: 357–367.
- Jang, S.K., Krausslich, H.G., Nicklin, M.J., Duke, G.M., Palmenberg, A.C., and Wimmer, E. 1988. A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J. Virol.* **62**: 2636–2643.
- Ji, H., Fraser, C.S., Yu, Y., Leary, J., and Doudna, J.A. 2004. Coordinated assembly of human translation initiation complexes by the hepatitis C virus internal ribosome entry site RNA. *Proc. Nat. Acad. Sci.* **101**: 16990–16995.
- Kaku, Y., Chard, L.S., Inoue, T., and Belsham, G.J. 2002. Unique characteristics of a picornavirus internal ribosome entry site from the porcine teschovirus-1 talfan. *J. Virol.* **76**: 11721–11728.
- Kapp, L.D. and Lorsch, J.R. 2004. The molecular mechanics of eukaryotic translation. *Annu. Rev. Biochem.* **73**: 657–704.
- Lukavsky, P.J. and Puglisi, J.D. 2004. Large-scale preparation and purification of polyacrylamide-free RNA oligonucleotides. *RNA* **10**: 889–893.
- Merrick, W.C. 1979. Evidence that a single GTP is used in the formation of 80 S initiation complexes. *J. Biol. Chem.* **254**: 3708–3711.
- . 2004. Cap-dependent and cap-independent translation in eukaryotic systems. *Gene* **332**: 1–11.
- Nielsen, D.A. and Shapiro, D.J. 1986. Preparation of capped RNA transcripts using T7 RNA polymerase. *Nucleic Acids Res.* **14**: 5936.
- Otto, G.A. and Puglisi, J.D. 2004. The pathway of HCV IRES-mediated translation initiation. *Cell* **119**: 369–380.
- Pestova, T.V. and Hellen, C.U. 2001. Preparation and activity of synthetic unmodified mammalian tRNAⁱ(Met) in initiation of translation in vitro. *RNA* **7**: 1496–1505.
- Pestova, T.V., Hellen, C.U., and Shatsky, I.N. 1996. Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. *Mol. Cell. Biol.* **16**: 6859–6869.
- Pestova, T.V., Shatsky, I.N., Fletcher, S.P., Jackson, R.J., and Hellen, C.U. 1998. A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. *Genes & Dev.* **12**: 67–83.
- Pestova, T.V., Kolupaeva, V.G., Lomakin, I.B., Pilipenko, E.V., Shatsky, I.N., Agol, V.I., and Hellen, C.U. 2001. Molecular mechanisms of translation initiation in eukaryotes. *Proc. Nat. Acad. Sci.* **98**: 7029–7036.
- Pisarev, A.V., Chard, L.S., Kaku, Y., Johns, H.L., Shatsky, I.N., and Belsham, G.J. 2004. Functional and structural similarities between the internal ribosome entry sites of hepatitis C virus and porcine teschovirus, a picornavirus. *J. Virol.* **78**: 4487–4497.
- Reynolds, J.E., Kaminski, A., Kettinen, H.J., Grace, K., Clarke, B.E., Carroll, A.R., Rowlands, D.J., and Jackson, R.J. 1995. Unique features of internal initiation of hepatitis C virus RNA translation. *EMBO J.* **14**: 6010–6020.
- Sachs, A.B., Sarnow, P., and Hentze, M.W. 1997. Starting at the beginning, middle, and end: Translation initiation in eukaryotes. *Cell* **89**: 831–838.
- Unbehaun, A., Borukhov, S.I., Hellen, C.U., and Pestova, T.V. 2004. Release of initiation factors from 48S complexes during ribosomal subunit joining and the link between establishment of codon-anticodon base-pairing and hydrolysis of eIF2-bound GTP. *Genes & Dev.* **18**: 3078–3093.
- Wilson, J.E., Pestova, T.V., Hellen, C.U., and Sarnow, P. 2000. Initiation of protein synthesis from the A site of the ribosome. *Cell* **102**: 511–520.

RNA

A PUBLICATION OF THE RNA SOCIETY

Affinity purification of eukaryotic 48S initiation complexes

NICOLAS LOCKER, LAURA E. EASTON and PETER J. LUKAVSKY

RNA 2006 12: 683-690

References

This article cites 28 articles, 14 of which can be accessed free at:
<http://rnajournal.cshlp.org/content/12/4/683.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

To subscribe to *RNA* go to:
<http://rnajournal.cshlp.org/subscriptions>