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Active yeast ribosome preparation using monolithic anion exchange chromatography

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ABSTRACT

In vitro studies of translation provide critical mechanistic details, yet purification of large amounts of highly active eukaryotic ribosomes remains a challenge for biochemists and structural biologists. Here, we present an optimized method for preparation of highly active yeast ribosomes that could easily be adapted for purification of ribosomes from other species. The use of a nitrogen mill for cell lysis coupled with chromatographic purification of the ribosomes results in 10-fold-increased yield and less variability compared with the traditional approach, which relies on sedimentation through sucrose cushions. We demonstrate that these ribosomes are equivalent to those made using the traditional method in a host of *in vitro* assays, and that utilization of this new method will consistently produce high yields of active yeast ribosomes.

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Introduction

Protein synthesis is a critical stage in gene expression and its misregulation is a common theme in a variety of diseases. In vitro studies have been essential to our understanding of the molecular interactions that take place within the prokaryotic translation apparatus, yet progress in understanding eukaryotic translation has been hindered by the difficulty in obtaining sufficient yields of active ribosomal subunits. The ability to study the translation process in a fully reconstituted *in vitro* system rather than in cells or lysates imparts several advantages. Researchers can modulate concentrations or use lethal variants of individual components while monitoring discrete steps in the translation pathway. In addition to biochemical studies of translation, structural studies of the ribosome rely on highly active purified ribosomes.

Eukaryotic ribosomes are intrinsically challenging to purify compared with those from prokaryotes. Lysis of organelles releases cellular nucleases and proteases that require special care be taken to prevent degradation of rRNA and protein components.[1] This has often been addressed by the use of multiple protease inhibitors or the addition of heparin[1–3]; however, these strategies are not always effective, and most protocols expose ribosomes to nucleases in the lysate for many hours. The common technique for obtaining ribosomes from yeast cell lysates via ultracentrifugation has not changed drastically since it was first developed in 1955,[4,5] and relies on ultracentrifugation of the lysate through a series of sucrose cushions and gradients.[2,3,6] These protocols are cumbersome and the pelleting steps introduce high potential for variability and loss of product (Table 1). The small, glassy pellets are difficult to visualize and resuspend, can break into smaller particles that are difficult to see, and incomplete resuspension before running over gradients reduces total yield. Overall, the traditional protocol using sucrose cushion pelleting for ribosome purification warrants improvement.

Alternative protocols for ribosome purification have been used in recent years. These include the use of various chromatographic methods as well as PEG precipitation of ribosomes stabilized in an arrested state following cold shock.[7-10] One such method that reduces the time ribosomes are exposed to degradatory enzymes uses a cysteine-charged resin to produce active ribosomes, but the resin is cost ineffective for large-scale purifications.[9,11] The use of affinity tags is also common[8,12] but the introduction of a tag limits the number of strains from which researchers can purify ribosomes. In addition, introduction of an affinity tag may interfere with the normal functioning of the ribosomes. Ribosomes are uniquely suited for anion exchange purification procedures given their ~67% rRNA content, providing large regions of negative charge density. For this reason, anion exchange chromatography has been recently used for purification of ribosomes and various RNA transcripts.[10,13]

Here we describe a protocol for the rapid purification of active yeast ribosomes using nitrogen mill lysis of cells and a monolithic anion exchange column for 80S separation from lysate. This strategy not only increases yields by 10-fold, but is faster and results in higher consistency in yield and quality among preparations. We used several *in vitro* assays to demonstrate that ribosomal subunits purified by this method retain the same high activity in translation initiation as those obtained through conventional sucrose cushions. Together, these results show that anion exchange monolithic chromatography offers

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Table 1. Ribosomal subunit yields from anion exchange column and sucrose cushion preparations. Mean ribosomal subunits collected per liter of culture using an anion exchange column or sucrose cushion with or without nitrogen mill lysis are reported plus or minus the standard error of the mean.

	40S (pmols /L)	60S (pmols /L)
Column and Nitrogen Mill (n = 3)	1307 ± 61	1540 ± 139
Cushion and Nitrogen Mill (n = 7)	224 ± 52	277 ± 61
Traditional Cushion (n = 17)	103 ± 14	134 ± 15

significant advantages for consistently producing high yields of active yeast ribosomes.

Results

Previous purification strategies for yeast ribosomes by ultracentrifugation of lysates through sucrose cushions produced only a small quantity of active ribosomes, and displayed a high degree of variability in activity of ribosomes obtained (Table 1, unpublished observations).[1,2] We hypothesized that this variability in yield and activity stemmed from 1) differences in the efficiency of lysis of the yeast cells, 2) the long centrifugation steps during which ribosomes were exposed to crude lysate, and/or 3) inconsistent resuspension of ribosomal pellets.

Use of nitrogen mill for lysis

Purification of yeast ribosomes has traditionally relied on glass beads, a blender, or a coffee grinder to lyse cells. Each of these methods has potential for variability, leading to inconsistent temperature control or lysis efficiency when used to extract proteins and ribosomes. To remedy this problem, we tested the efficiency of a Nitrogen mill for lysing yeast cells for ribosome preparation. The Nitrogen mill operates similarly to a blender, in that it pulverizes yeast cells under liquid nitrogen, but offers a greater degree of control of how long and at what speed cells are pulverized, allowing for less variability and avoiding inevitable lysate explosions that occur when pockets of nitrogen gas build up under lysate powder during blender lysis. In addition, because the canister is submerged in liquid nitrogen, the lysate is still kept frozen at all times, preventing nucleases and proteases from degrading the ribosomes. Comparison of the average yield of subunits obtained using blender lysis (103 ± 14 pmols 40S/L and 134 ± 15 pmols 60S/L; n = 17) to that obtained when lysis was performed with a nitrogen mill (224 ± 52 pmols 40S/L and 277 ± 61 pmols 60S/L; n = 7) demonstrated a 2-fold increase in mean ribosome recovery, indicating the nitrogen mill improved lysis efficiency (Table 1).

Purification using monolithic QA column

In addition to differences in lysis, yields of active ribosomes could vary due to degradation during the long centrifugation period required to separate ribosomes from crude lysate and variability in the efficiency of ribosome pellets resuspension after the 2 sucrose cushion steps.[1] We therefore tested a chromatographic method for purification, which eliminates the sucrose cushion ultracentrifugation step as well as the potential for variability during pellet resuspension. We chose to use a monolithic anion exchange column for several reasons. Because ribosomes are comprised of roughly 2/3 RNA, they should bind anion exchange columns with high affinity.[13] However, traditional resins generate substantial backpressure when large volumes of cell lysates are applied, so that slow flow rates must be used, which would lengthen the time ribosomes were exposed to degradatory enzymes in the lysate. A monolithic column varies from a traditional resin-packed column, in that it is made of a cross-linked polymer with a consistent, large pore size that reduces backpressure.[14] As there are no interstitial voids, smaller cellular molecules pass quickly through the column. The column has a consistent pore diameter of ~1.5 μm that enables only larger species like ribosomes (25–30 nm diameter) to bind the open structure of the monolith without reducing flow rate and introducing backpressure, thus allowing rapid and selective purification of large charged molecules and complexes.[14] A monolithic anion exchange column was recently used for purification of mycobacterial ribosomes, so we began with a similar strategy for purifying ribosomes from yeast cell lysates.[10] To optimize binding conditions and determine the elution profile, we began by injecting salt-washed 80S ribosomes onto a 0.34 ml analytical monolithic anion exchange column in binding buffer and eluting with a linear salt gradient from 0 to 900 mM KCl (Fig. 1A). The major peak displayed an A_{260}:A_{280} ratio of 1.65 (data not shown), consistent with crude ribosome absorbance properties, and eluted at approximately 600 mM KCl (Fig. 1A; blue, red traces). We reasoned that because 80S ribosomes eluted at such a high salt concentration, we could increase the binding capacity of the column by increasing the salt concentration in the binding buffer, which should prevent other negatively charged cellular components from binding weakly and occupying binding sites. We tested several concentrations of potassium chloride, and found that salt-washed ribosomes still bound the column effectively at 400 mM KCl (data not shown). Yeast lysate was next applied to the column under these optimized binding conditions, but in contrast to the single peak observed with 80S ribosomes, we observed 2 elution peaks with cell lysate (Fig. 1B). Previous work with bacterial ribosomes indicated that an additional peak eluting from anion exchange columns at a higher salt concentration was DNA, so we incorporated a DNase treatment step.[10] Running DNase-treated lysates over the column with 400 mM KCl in the binding buffer resulted in a single elution peak (Fig. 1C), indicating the peak eluting at the highest salt concentration in the first preparation corresponded to DNA and possibly DNA-associated proteins. Gel electrophoresis analysis of peak fractions alongside purified subunits indicated that ribosomal proteins and rRNA were the major components of the peak (data not shown). The elution peak was consistently found at ~600 mM KCl, so a 650 mM KCl step gradient was used in subsequent preparations to elute ribosomes while preventing fractionation of ribosomal proteins.

To purify individual subunits, crude ribosomes are treated with puromycin, followed by separation through a sucrose gradient. Crude ribosomes consistently eluted from the column as a single peak, rather than eluting as separated peaks for subunits, so sucrose gradient centrifugation was necessary for subunit purification. Puromycin treatment deacylates peptidyl tRNAs present within ribosomes, and is necessary to separate ribosomes in eukaryotic species. Ultracentrifugation through a

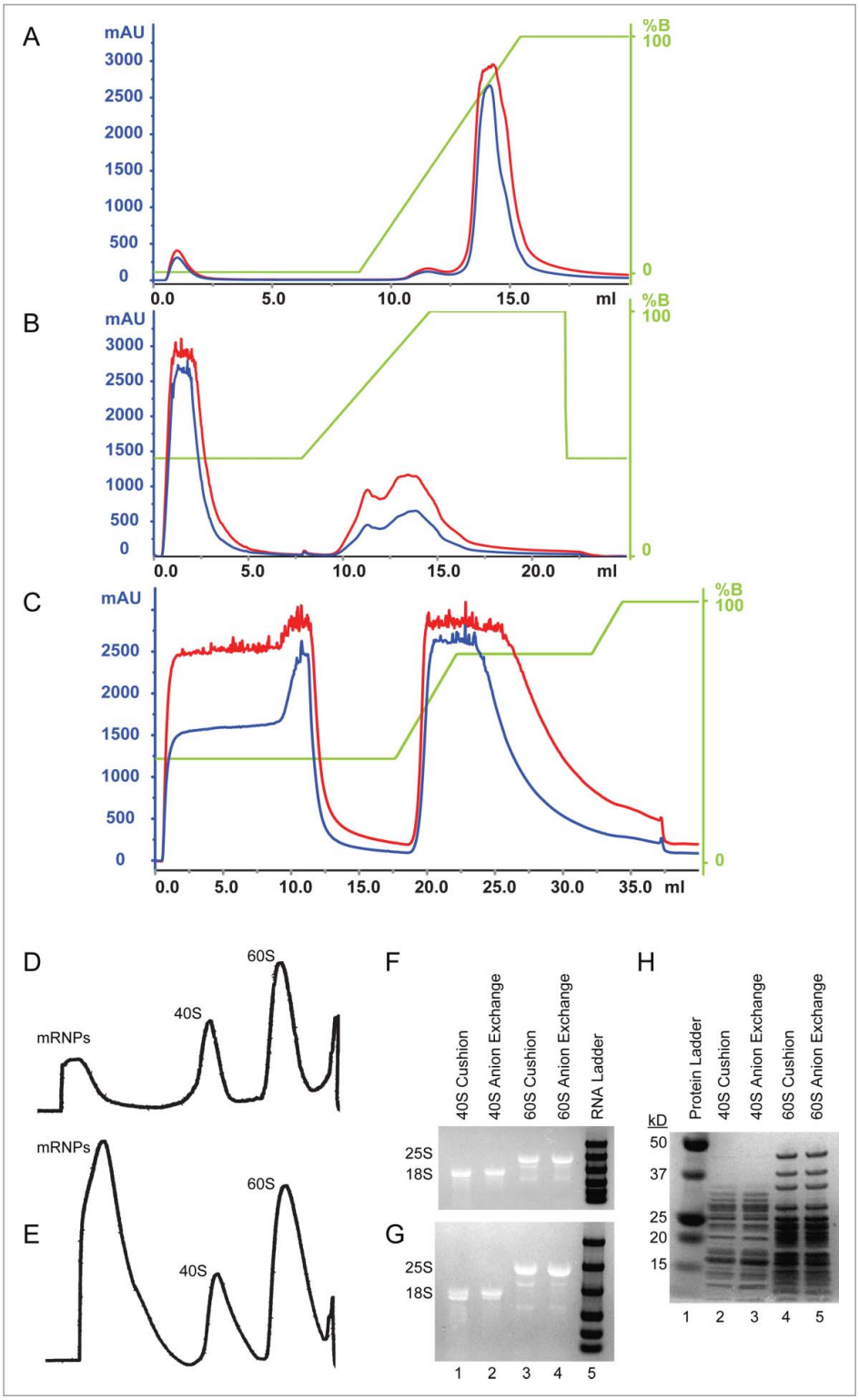

Figure 1. Purification of ribosomes by sucrose cushion and monolithic column method. (A-C) Binding and Elution profiles for ribosomes and lysates from a monolithic anion exchange column, using linear salt gradients and an AKTA Purifier 10 FPLC (GE Healthcare) outfitted with a multi-wavelength UV detector. The X-axis corresponds to mL, the left axis is UV absorbance in mAU at 280 nM (blue curve) and 260 nM (red curve), and the right axis shows the concentration of buffer B (green, 0–100% Buffer A with 900 mM KCl). (A) 100 pmols of crude 80S ribosomes were loaded and eluted from a 0.34 ml QA disk column. (B) 5 ml of clarified yeast lysate was loaded in buffer containing 400 mM KCl before washing with 5 ml of the same buffer and elution with a linear gradient from 400–900 mM KCl. (C) 10 ml of DNase treated yeast lysate was loaded onto the column, followed by washing with 10 ml of the same buffer and elution with buffer B containing 900 mM KCl, as indicated. Saturation of the UV detector by the large quantities of ribosomes produces the fluctuations observed above ~2500 mAU. (D) Sucrose gradient profiles for ribosomes purified using sucrose cushion D, top) and anion exchange (E, bottom) methods showing separation of mRNP peak, 40S and 60S ribosomal subunits. The beginning of the 80S peak is visible at the bottom of the gradient. (F/G) Denaturing agarose gel electrophoresis of rRNA from 40S and 60S subunits from the sucrose cushion (lanes 1 and 3) and anion exchange (lanes 2 and 4) preparations. Bands representing specific rRNAs are labeled. RNA ladder in right-most lane is DynaMarker Prestain Marker for RNA, High. Band sizes are 8, 4, 2, 1, 0.5, and 0.2 kgbases. (G) Agarose gel from panel F was run for 30 additional minutes to resolve nicked 18S band. (H) 4–15% SDS-PAGE of 40S (lanes 2 and 3) or 60S subunits (lanes 4 and 5) alongside Precision Plus prestained marker (lane 1).

high salt sucrose gradient serves to separate 40S and 60S subunits and remove tRNA, mRNA, and translation factors that were present in ribosomal complexes before puromycin treatment.[2] To compare ribosomes from the original sucrose cushion preparation and those obtained from the anion exchange column protocol, we divided the lysate from the same culture and purified crude 80S ribosomes with each strategy. Preparation of crude ribosomes by chromatography was scaled up to use lysate from 1.5 L of culture with an 8 ml anion exchange monolith to generate sufficient ribosomes for purification of subunits. We then treated the resultant crude ribosomes with puromycin, and compared the sucrose gradient traces following ultracentrifugation (Fig. 1D/E). Inactive ribosomes obtained previously by the sucrose cushion purification strategy have either displayed a high apparent 40S:60S peak ratio due to degradation, or have not been effectively dissociated following treatment with puromycin, resulting in a high intensity apparent 80S peak and pelleted polysomes, but fewer separated active subunits (unpublished results). Both the sucrose cushion and monolithic anion exchange methods produced gradient profiles similar to those observed previously for active ribosomes. Moreover, the A_{260}:A_{280} ratio for pooled 40S (1.98 for both preparations) and 60S subunits (1.98 and 1.96 for cushion and anion exchange prepared ribosomes, respectively) were nearly identical. It is worth noting that the anion exchange purification method displays a higher intensity peak for smaller cellular components (e.g. mRNPs), but these components are effectively separated from 40S subunits within the sucrose gradient.

Despite yielding a similar gradient profile, and originating from the same cell lysate, the anion exchange column preparation yielded greater than 5-fold more ribosomes (1307 ± 61 pmols 40S/L and 1540 ± 139 pmols 60S/L; n = 3 for anion exchange column 40S and 60S respectively) than sucrose cushions (224 ± 52 pmols 40S/L and 277 ± 61 pmols 60S/L; n = 7) (Table 1) when lysis of cells for both protocols was performed with a nitrogen mill. Together, the combination of the nitrogen mill and anion exchange column provides ~10-fold higher yield than obtained using previously published methods for blender lysis followed by sucrose cushions (103 ± 14 pmols 40S/L; 134 ± 15 pmols 60S/L culture, n = 17). [2]

Denaturing agarose gels were next used to analyze the quality of the rRNA (rRNA) from the ribosomal subunits collected after sucrose gradient centrifugation. The rRNA from the subunits obtained using either the sucrose cushion or chromatography purification protocol displayed similar bands on a 1% denaturing agarose gel (Fig. 1F). A second, lower molecular weight band in the 18S rRNA lanes from the 40S subunits, likely corresponding to nicked but functional rRNA, is observed in both preparations when the gels are run for an extended time (Fig. 1G). The same ribosomal subunits run as a single band on a native TBE gel (data not shown). This lower rRNA band appears more intense in 2 independent preparations generated using sucrose cushions than in anion exchange chromatography preparations. It is possible that the shorter time spent in crude extracts decreases the amount of nicked rRNA in 40S subunits made using the anion exchange column. However, this nicked species has no obvious effect on the activity of the ribosomes in several *in vitro* assays (discussed below).

Subunits from ribosomes purified by either method were next analyzed by SDS-PAGE (Fig. 1H) to determine whether ribosomal protein content was affected by the purification method. Comparison of the protein content of the 40S (lanes 2 and 3) or 60S subunits (lanes 4 and 5), did not reveal major differences between the 2 preparations, suggesting that the major constituents of the subunits are present in both preparations. It is possible that there are minor differences in molar ratios of certain 40S proteins, as visualized by darker or lighter bands. However, any potential changes in molar ratios of these proteins do not appear to affect translation in several *in vitro* assays (discussed below).

Activity assays

To ensure the ribosomes purified by the anion exchange column are as active as the ribosomes purified using sucrose cushions, which have been extensively characterized, we tested ribosomes from each preparation scheme in a variety of *in vitro* assays monitoring individual steps of translation initiation.

We first measured the ability of the 40S subunits to form 43S translation preinitiation complexes (PICs), which consist of the eIF2•GTP•Met-tRNA$_i$ ternary complex (TC) and the 40S ribosomal subunit bound to initiation factors eIF1 and eIF1A.[2] A non-hydrolyzable form of GTP, GDPNP, was used in these experiments to prevent nucleotide hydrolysis and P_i release, and stabilize complexes that are formed. We used an electrophoretic mobility shift assay that monitors incorporation of ^{35}S-Met-tRNA$_i$ into the 43S preinitiation complex to determine the apparent dissociation constants (K_D) for TC binding, in the presence or absence of a model mRNA containing an AUG codon.[1,2] The TC binds to 40S subunits from both preparations in the presence of eIF1, eIF1A and model mRNA with a K_D below the limit of measurement (< 1 nM) (Fig. 2A; sucrose cushion, filled black circles; anion exchange, filled red squares), indicating very high affinity binding. Binding of TC in the absence of mRNA was substantially weaker, as expected for functional 40S subunits (K_D of 13 ± 6 vs 14 ± 2 nM for sucrose cushion and anion exchange subunits, n = 3 respectively).

To test the ability of both ribosomal subunits to support translation initiation, including 60S subunit joining to the PIC after start codon recognition, we performed a similar experiment, but also included eIF5, eIF5B (a second ribosomal GTPase that promotes subunit joining), and 60S subunits (Fig. 2B).[15] Here, GTP was used in the preformed TC instead of GDPNP in order for eIF2 to hydrolyze GTP and dissociate to allow subunit joining. Again, no significant differences were observed between ribosomes prepared by anion exchange chromatography (even-numbered lanes) and ribosomes prepared by traditional sucrose cushions (odd-numbered lanes). Addition of GDPNP along with eIF5B traps a portion of the TC-bound complexes as 43S, but also stabilizes 80S•eIF5B complexes (lanes 1 and 2).[15] In the absence of eIF5B but presence of GDPNP (lanes 3 and 4), all complexes are trapped as 43S. In the presence of eIF5B and the absence of GDPNP all PICs are joined with

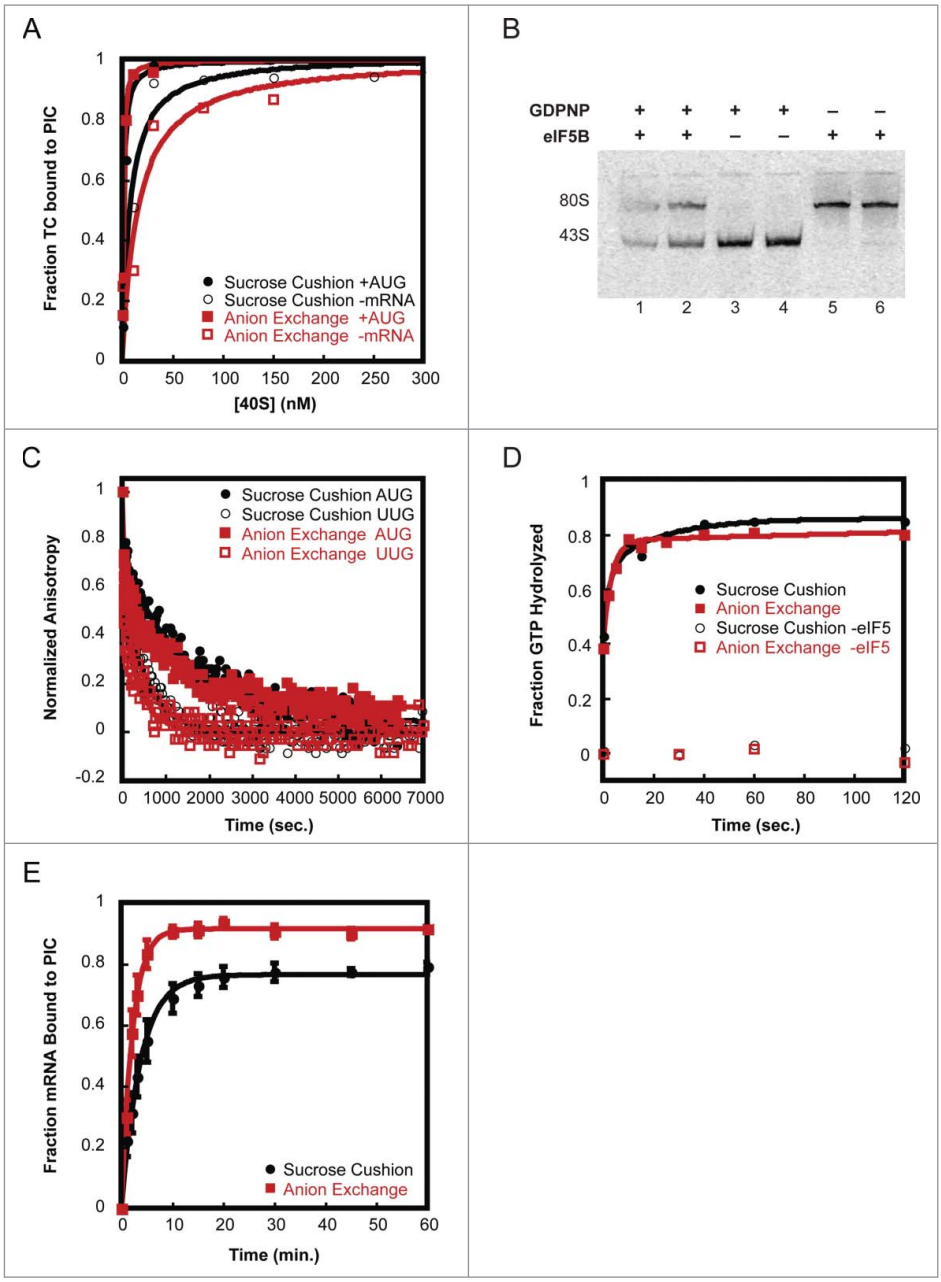

Figure 2. *In vitro* measurements of translation activity are consistent between the sucrose cushion (black circles) and anion exchange column (red squares) preparations. (A) PICs were formed with increasing concentrations of 40S subunits in the presence (closed points) or absence (open points) of an AUG-containing model mRNA and saturating eIF1 and eIF1A and reactions were run on a 4% polyacrylamide 1X THEM native gel. The fraction of ^{35}S-Met-tRNA$_i$ bound to the PIC was measured and plotted versus 40S concentration, and data were fit to either quadratic (+mRNA AUG) or hyperbolic (-mRNA) equations. (B) The fraction of ^{35}S-Met-tRNA$_i$ bound to the 43S and 80S complexes was measured in the presence or absence of eIF5B and GDPNP (denoted with + or -) for subunits purified using the sucrose cushion (lanes 1, 3 and 5) and anion exchange column (lanes 2, 4, and 6) methods as in A. (C) Dissociation of eIF1A followed by the decrease in fluorescence anisotropy of eIF1A-fluorescein. A 10-fold excess of unlabeled eIF1A was added to induce dissociation of fluorescently-labeled eIF1A from either sucrose cushion prepared or anion exchange column prepared 40S subunits as indicated, and AUG (closed) and UUG (open) containing model mRNAs. (D) The fraction of GTP hydrolyzed by eIF2 was assayed by PEI cellulose thin-layer chromatography for complexes formed with sucrose cushion (black) and anion exchange column (red) preparations of 40S in the presence or absence of eIF5. Data were plotted vs. time, and fit to a double exponential equation to obtain rates for the first phase of 1.04 sec^{-1} and 0.86 sec^{-1} and for the second phase of 0.056 sec^{-1} and 0.11 sec^{-1} for sucrose cushion and anion exchange column preparations, respectively. When an equal volume of storage buffer was added in place of eIF5 (open marks), neither ribosome preparation produced measureable GTP hydrolysis. (E) The fraction of radiolabeled capped *RPL41A* mRNA (15 nM) bound to the PIC was monitored over time following addition of 2 mM ATP, in the presence of 300 nM eIF3, 2 μM eIF4A, 300 nM eIF4B, and 50 nM eIF4G•eIF4E copurified complex. Fitting to a single exponential rate equation demonstrated that a similar fraction of mRNA was recruited to the PIC (amplitude = 0.8, sucrose cushion; amplitude = 0.9, anion exchange) at comparable apparent rates ($k_{app} = 0.27 \pm 0.07$ min^{-1}, sucrose cushion; $k_{app} = 0.48 \pm 0.10$ min^{-1}, anion exchange, n = 3).

60S to form 80S (lanes 5 and 6). Together these data demonstrate the ability of subunits purified using anion exchange chromatography to provide a similar level of function as those subunits purified in the traditional purification scheme in generating full 80S initiation complexes.

In addition to determining whether ribosomes bind various components and form initiation complexes, we tested their ability to undergo a conformational change from the

open scanning competent state to the closed scanning arrested state upon start codon recognition.[5] Formation of the

closed state is achieved after start codon recognition, upon release of eIF1 with concomitant conformational changes in eIF1A, eIF5 and ribosomal components, and the irreversible release of P_i from eIF2•GDP•P_i. We tested this activity in 2 ways: by monitoring the affinity of eIF1A for the PIC with mRNAs that had an AUG start codon or no start codon, and by monitoring the GTPase activity of eIF2.

Addition of a large amount of unlabeled eIF1A to PICs preformed with labeled eIF1A yields a 2-phase decrease in fluorescence anisotropy of the labeled eIF1A over time.[2] This change provides both the rate of eIF1A dissociation from the preformed PICs, and the relative amplitudes of the fast and slow phases, which together report on the propensity of complexes to properly adopt a conformational change upon start codon recognition. Dissociation of eIF1A from the PIC occurs more slowly in the presence of an mRNA with an AUG start codon than with mRNA containing a near-cognate start codon (e.g., UUG), indicating eIF1A binds more tightly to the closed, scanning-arrested complex. We measured eIF1A dissociation from 40S subunits from both preparations (Fig. 2C). Preinitiation complexes using subunits from both traditional sucrose cushion (black circles) and the anion exchange preparation (red squares) behave identically in this assay, giving slower rates and larger amplitudes for the slow phase of eIF1A dissociation with AUG (closed points) than with UUG (open points) containing mRNAs, indicating the anion exchange column prep yields subunits that are capable of adopting a closed state of the PIC upon encountering a cognate start codon.

eIF2 is a GTPase that delivers tRNA to the complex, and its ribosome-associated hydrolysis activity is critical for gating downstream steps in translation initiation following start codon recognition by the PIC. Rapid GTP hydrolysis by eIF2 requires productive incorporation of the TC into a 43S PIC and the presence of the GTPase-activating protein eIF5.[16] We measured the ability of 43S•mRNA complexes made with each preparation of 40S subunits to hydrolyze ^{32}P-γ-GTP incorporated into TCs, following initiation of the reaction by addition of eIF5. Products of the reaction were separated by thin-layer chromatography to quantify the fraction of GTP hydrolyzed. Again, we did not observe a difference between the stocks of ribosomes prepared using sucrose cushions vs. those purified from the monolithic anion exchange column. Both samples gave comparable rate constants for the slow P_i-release-limited step ($k_2 = 0.056$ sec^{-1} and 0.11 sec^{-1} for sucrose cushion and anion exchange respectively) with more than 80% GTP hydrolysis by eIF2 (Fig. 2D). This indicates both pools of ribosomes rapidly adopt the closed conformation to promote irreversible GTP hydrolysis upon recognition of the start codon. Additionally, active hydrolysis was not detectable in the absence of eIF5 for both sucrose cushion and anion exchange preparations (open circles and open squares respectively), indicating no contamination of either pool of ribosomes with the GTPase-activating protein eIF5.

The final assay used to examine the activity of the ribosomes assessed the ability of 43S PICs formed with ribosomes from either preparation to recruit a capped natural mRNA (*RPL41A*). Rapid loading and scanning of a ribosome along a natural mRNA requires the productive interaction of eIF3, eIF4A, eIF4B, eIF4G, and eIF4E within the PIC. We found that PICs made with each preparation of 40S subunits gave similar endpoints of mRNA recruitment (0.8, sucrose cushion; 0.9, anion exchange; Fig. 2E), while the observed rate constants were comparable, yet slightly faster for the anion exchange preparation ($k_{app} = 0.27 \pm 0.07$ min^{-1}, sucrose cushion; $k_{app} = 0.48 \pm 0.10$ min^{-1}, anion exchange; Fig. 2E). The rates and endpoints for both preparations are consistent with values reported in previous studies.[17,18] Together, these results demonstrate that both strategies yield functional ribosomes, with minor improvements to the activity of the preparation by using anion exchange purification.

Summary

In vitro studies provide critical knowledge of the mechanics of translation that cannot be obtained *in vivo* or in fractionated cellular extract systems. To ensure *in vitro* results of translation accurately report cellular function, it is essential to have fully active purified ribosomes. Here we demonstrate sources of variability in yield and quality of yeast ribosomes stemming from traditional protocols, and introduce an alternative method that diminishes these sources of variability. This protocol eliminates lengthy steps that decrease active ribosome yield, and when coupled to the use of a nitrogen mill for cell lysis, yields greater than 10-fold higher ribosome recovery per liter of highly active ribosomes.

Materials and methods

Reagent preparation

Eukaryotic initiation factors used in these studies, including eIFs 1, 1A, 2, and 5, ribosomes purified via sucrose cushions, as well as mRNA and [^{35}S]-Met-tRNA$_i$ were prepared as described previously.[2,19] Ribosome concentrations and purity were analyzed by measuring absorbance at 260 nm with molar extinction coefficients of 40S = 2×10^7 M^{-1}cm^{-1} and 60S = 4×10^7 M^{-1}cm^{-1}.

Growth and lysis of yeast cells

One.5 L of YPD media in baffled Fernbach flasks were inoculated with strain YAS2488 and grown overnight to an OD_{600} of ~0.9–1 before being placed on ice and harvested by centrifugation at 4500 × g for 12 min. in a fixed angle rotor. Cells were washed once with water or lysis buffer to combine all pellets, repelleted using the same centrifugation conditions, and resuspended in 1/3 volume of the pellet weight of lysis buffer (20 mM HEPES•KOH [pH 7.4], 100 mM KOAc [pH 7.6], 2.5 mM $Mg(OAc)_2$, 2 mM DTT). The resultant slurry was then frozen dropwise into liquid nitrogen and stored at −80°C. [2]

Lysis of frozen cell droplets was performed using a large canister, filled no more than half-way in a nitrogen mill (Spex Sample Prep 6870), with the following parameters: Cycles = 10, Precool = 15 min, Run = 1 min, Cool = 2 min, Rate = 15 cps. Lysates were stored in prechilled bottles at -80°C.

Liquid nitrogen blender lysis of cell droplets was performed as described, in a waring blender. [2]

Anion exchange chromatography

Prior to loading on the column, lysate powder was resuspended in lysis buffer to which one Roche Complete EDTA-fee tablet

had been dissolved per 50 ml, at a ratio of 15 mL buffer/L of yeast culture. Suspended lysates were DNase treated by incubating for 30 minutes on ice with 1.25 μL of Turbo DNase (Thermo AM2239) per 10 mL of resuspended lysate. This DNase is not active at salt concentrations above 350 mM. Lysate was clarified by centrifugation for 30 min at 30,000 × g, KCl was added to 400 mM, and the mixture was filtered through glass fiber filters (Whatman) or 5 μm syringe filters, then 0.8 μm bottle top filters or 0.22 μm syringe filters, depending on volume. Filtered lysate was loaded onto a pre-equilibrated 8 ml CIMmultus QA-8 column (BIA Separations #412–5113, quaternary amine advanced composite column) at a flow rate of 10 mL/min, and the column washed with 25 column volumes (cv, column volume = 8ml) of low salt buffer (Lysis Buffer + 400 mM KCl). Ribosomes were then collected in 5 mL fractions using a step gradient to 50% buffer B (Lysis Buffer + 900 mM KCl) for 5–10 cv, followed by 100% B for 5 cv. This 8 ml column yielded ~2000 OD_{260} units of crude ribosomes from lysate of 1.5–3L of culture. For initial optimization of the protocol in Fig. 1A-C, a smaller 0.34 ml CIM-QA disk was used at a flowrate of 1–2 ml/min, and generated ~80 OD_{260} units from lysate of less than 200 ml of culture. Fig. 1C shows an example elution trace, with the ribosomes eluting at 50% B. Prior to reequilibration in water and storage in 20% ethanol, the column was regenerated by washing with 20 cv of 2M NaCl. Omitting this wash resulted in loss of binding capacity that could be corrected by cleaning-in-place with 1M NaCl and 1M NaOH for several hours. Ribosome fractions above 100 A_{260} Units/mL were pooled, and in some cases, additional fractions were concentrated at 3500–4000 × g as directed by the manufacturer using Amicon Ultra 15 100kDa MWCO concentrators (Millipore UFC910024). Note, overconcentration of fractions at this step lead to ribosome loss on the filter, so only dilute fractions (<~80 A_{260} U/mL) were subjected to concentration before puromycin treatment and gradient separation. The concentration of all pooled fractions was then adjusted to ~100 A_{260} Units/mL, and the pooled fractions were treated with puromycin and subjected to gradient centrifugation as described.[2] A 100 mM stock of puromycin was added to a final concentration of 1 mM and incubated for 15 minutes on ice followed by 10 minutes at 37°C. 1 mL of puromycin-treated ribosomes was carefully layered onto prepared 5–20% sucrose gradients and spun at 27K rpm in an SW32 rotor (Beckman) for 8 hours 45 min. Gradients were pumped immediately following the spin to avoid diffusion, and subunit peaks were collected, avoiding the edges of the peak and potential contaminants. Peak fractions were then pooled and concentrated as above, and buffer exchanged using ribosome sucrose storage buffer, until the concentration of KCl was less than 20 μM. Subunits were flash-frozen and stored at −80°C in small aliquots that were capable of at least 5 freezing and thawing cycles with no observable decrease in activity.

rRNA quality gels

rRNA was extracted from 40S and 60S subunits using the RNeasy kit (Qiagen). 2 μg of purified RNA was separated on a 1% denaturing agarose gel in MOPS buffer (20 mM MOPS, 5 mM NaOAc, 1 mM EDTA, 1 mM EGTA) with 37% v/v formaldehyde, and visualized with the ethidium bromide substitute Greenglo (Denville).

SDS-PAGE

Roughly 5 pmols of 40S or 60S subunits were loaded onto a 4–15% TGX gel (Bio-rad) in 2X Laemmli loading buffer (Bio-rad) containing 5% 2-mercaptoethanol. Gels were run in 1X TGS Buffer (Bio-rad) at 200 V until the lower molecular weight markers of the Precision Plus prestained marker were visibly separated, then stained with Bio-Safe Coomassie stain (Bio-rad).

43S formation K_D measurements

Gel shift assays were performed as described previously using 4% polyacrylamide gels in 1X THEM buffer (34 mM Tris Base, 57 mM HEPES free acid, 1 mM EDTA, 1.5 mM $MgCl_2$).[2] TC was formed by incubating GDPNP with eIF2 for 10 minutes, followed by a 5 minute incubation upon addition of ^{35}S-Met-$tRNA_i$. Final reaction concentrations were 1X Recon buffer (30 mM HEPES•KOH [pH 7.4], 100 mM KOAc [pH 7.6], 3 mM $Mg(OAc)_2$, 2 mM DTT), 300 μM GDPNP, 0.2 μM eIF2, 0.5 nM ^{35}S-Met-tRNA, 1 μM eIF1, 1 μM eIF1A, 1 μM mRNA, and 40S concentrations as indicated. Complexes were formed by combining all components, and incubating those containing mRNA(AUG) for no less than 30 minutes, whereas complexes with mRNA(UUG) or lacking mRNA were incubated 90 minutes or more to reach equilibrium. The fraction of ^{35}S-Met-$tRNA_i$ in the 43S was quantified in ImageQuant, plotted against 40S concentration, and fit with either hyperbolic or quadratic (in the case of apparent K_D within 3-fold of tRNA concentration) equations.

80S Formation

Formation of 80S complexes was monitored as described previously.[20] Reactions were performed in Recon buffer and final concentrations of components were 67 μM GTP, 800 nM eIF2, 0.5 nM Met-$tRNA_i$, 800 nM eIF1, 800 nM eIF1A, 40 nM 40S, 800 nM mRNA, 800 nM eIF5, 400 nM 60S, 500 nM eIF5B and 2 μM GDPNP. TC was preformed as described above for 15 minutes at 3 times the final concentration. 40S subunits were combined with eIF1, eIF1A, and mRNA at 3 times the final reaction concentrations and then incubated with 3X TC for 10 minutes to form 43S complexes. Finally, 3 × 60S complexes, containing eIF5 in the presence and absence of a combination of eIF5B and GDPNP, were incubated with the preformed 43S complexes for 5 minutes and run on a 4% polyacrylamide gel in 1X THEM buffer for 45 minutes to achieve separation of the 80S and 43S.

1A dissociation kinetics

C-terminally fluorescein-labeled eIF1A (eIF1A-Fl) was incubated with 40S, eIF1, eIF1A, mRNA and TC for 30 minutes at 26°C. Reactions were performed in a Tecan microplate reader, with component concentrations as follows: 1X Recon buffer, 0.03 μM eIF1A-Fl, 1 μM eIF1, 0.12 μM 40S,

1 μM eIF5, 0.3 μM eIF2, 0.15 μM Met-tRNA$_i$, 0.25 mM GDPNP, and 10 μM mRNA. Reactions were performed in a flat black 384 well plate (Corning) with 30 μL of the above components, to which the chase was added. Excitation and emission were 470 and 520 nm, respectively. Z-position, gain, and g factor were all calculated using the preincubated sample without chase and then set manually. Fluorescence anisotropy was monitored before, and upon injection of 15 μl of unlabeled eIF1A (for 3 μM final, 10X over labeled) via the automated injector system. Plots were fit with a double exponential equation.

GTPase activity kinetics

GTP hydrolysis from 43S complexes was monitored as described previously.[16] 4X TC (3.2 μM eIF2, 3.2 μM Met-tRNA$_i$, 250 pM [γ-^{32}P]-GTP) was formed by incubating for 15 min. at 26°C before mixing with 4X Ribosome complexes (1.2 μM 40S, 3.2 μM eIF1 and 3.2 μM eIF1A). 2 μM eIF5 and 20 μM mRNA(AUG) were added to the PIC and quenched with 100 mM EDTA at various times. Samples were run on polyethyleneimine-cellulose thin layer chromatography (TLC) plates in 0.4 M KOAc (pH 3.4) buffer and the fraction of GTP hydrolyzed was quantitated using PhosphoImager analysis and ImageJ. [21]

mRNA Recruitment

mRNA recruitment was measured as described previously, with the following small modifications.[17] 45 μL reactions containing 1X Recon Buffer, 0.5 mM GDPNP, 300 nM eIF2, 200 nM Met-tRNA$_i$, 1 μM eIF1, 1 μM eIF1A, 30 nM 40S, 300 nM eIF3, 300 nM eIF5, 2 μM eIF4A, 50 nM eIF4G•eIF4E copurified complex, 300 nM eIF4B, 1 U/μl RNAseOUT Ribonuclease inhibitor (Invitrogen), and 15 nM ^{32}P-m^{7}G-*RPL41A* mRNA were incubated at 26°C for 10 minutes. Reactions were initiated by addition of 2 mM ATP and aliquots were quenched at desired timepoints by loading onto a running 4% native polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) buffered in 1X THEM. Complexes were separated by running the gel at 200 V for 1 hr., cooled by a circulating water bath set at 22°C. Gels were visualized and quantified using a phosphorimager and ImageQuant software. Fraction of ^{32}P-m^{7}G-*RPL41A* mRNA recruited to the PIC was plotted against time and fit with a single exponential equation using KaleidaGraph software. The assay was repeated 3 times.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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