INTRODUCTION

Composed of at least 80 proteins, four ribosomal RNAs (rRNA), messenger RNA (mRNA), and transfer RNA (tRNA), the eucaryotic ribosome and polysome particles are among the most complicated ribonucleoprotein (RNP) complexes. The composition of the eucaryotic 80S ribosome has been analyzed in many studies (reviewed in Warner, 1982; Woolford & Warner, 1991). Most of these have relied on conventional density gradient centrifugation for ribosome purification, followed by one- or two-dimensional gel electrophoresis and sequence analysis of the individual polypeptides (Lee, 1990). These analyses have defined a core set of ribosomal proteins. However, an extensive proteomic and genomic analysis of ribosome-associated factors has been delayed due to the lack of a fast, reliable, and efficient purification method. An example of such an approach using epitope tagging and immuno-affinity purification to study ribosome biogenesis in Saccharomyces cerevisiae has recently been reported (Bassler et al., 2001; Harnpicharnchai et al., 2001).

Immuno-affinity purification of DNA- and RNA-binding proteins, coupled with the use of DNA microarrays, has also been shown to be valuable for genome-wide identification of associated nucleic acid components (Ren et al., 2000; Tenenbaum et al., 2000). For instance, immuno-affinity purification of FMRP, an RNA-binding protein mutated in individuals with Fragile X Syndrome, allowed for the identification of mRNAs enriched in the FMRP-mRNP complex. This complex was found to be associated with about 4% of the mRNAs expressed in the mouse brain, many of which encode proteins involved in synaptic or developmental neurobiology (Brown et al., 2001; Darnell et al., 2001).

Translational control is now recognized as a major mechanism regulating gene expression (Sonenberg et al., 2000). Therefore, to gain a global understanding of how translational control contributes to cellular phenotypes, it has become important to determine both the overall amount and the translation status of each mRNA within a cell. To achieve this goal, high-throughput approaches have recently been developed (Carter et al., 2000; Pradet-Balade et al., 2001). DNA microarrays can accurately quantify the amount of mRNA in a prep-
Affinity purification of the yeast ribosome

Affinity purification of the yeast ribosome

The translational status of an mRNA can be determined by methods that rely on the sedimentation rate difference between heavily translated (many ribosomes bound) and poorly translated (few to no ribosome bound) mRNAs in a sucrose gradient. Although highly informative (Johannes et al., 1999; Zong et al., 1999; Kuhn et al., 2001), this type of analysis requires a large amount of starting biological material, and thus cannot be routinely performed on large numbers of small samples or on samples of limited quantity.

We have created an S. cerevisiae strain expressing a FlagHis6 epitope (FH)-tagged version of the ribosomal protein Rpl25p, and developed a method using extracts from this strain that can purify ribosome-associated proteins and mRNAs with high throughput and small sample requirements. A more detailed analysis of these associated components revealed the presence of abundant proteins bound to the ribosome in a salt-labile manner. In addition, the amount of mRNA associated with the purified ribosomes was found to reflect its translational status within the cell.

RESULTS

Affinity purification of the yeast ribosome

The gene encoding Rpl25p in S. cerevisiae was modified to encode a protein tagged at its C-terminus with an FH epitope. We selected RPL25 for several reasons. First, because a Rpl25p-GFP fusion could partially complement a rpl25::HIS3 mutation (Hurt et al., 1999), it was anticipated that the small FH tag would not seriously affect Rpl25p activity. This proved to be correct, as a yeast strain harboring only RPL25-FH was viable (see Materials and Methods for strain construction details). Second, we chose RPL25 because it is essential in S. cerevisiae, and therefore the activity of a modified RPL25-FH gene could be easily estimated by its ability to support growth of a strain lacking wild-type RPL25. As shown in Figure 1A, the growth rate of the RPL25-FH recombinant strain YIT613 was equivalent to that of the parental strain, indicating that the tag did not seriously compromise Rpl25p function. The last reason Rpl25p was selected as a target for epitope tagging was that its procaryotic homolog, Rpl23p, is located on the surface of the large ribosomal subunit (Ban et al., 2000). Therefore, it seemed likely that the epitope tag would be accessible to affinity-capture reagents.

To test this last hypothesis, crude extracts from YIT613 cells (see Table 1 for strain details) were incubated with an anti-Flag antibody affinity resin. After extensive washing, bound proteins were eluted with high concentrations of the Flag peptide. By a variety of criteria, the single-step affinity method yielded highly purified ribosomes. First, a subset of the polypeptides found in the affinity-purified ribosomes (Fig. 2A, lanes 2, 3) were similar in mass to those observed in 80S ribosome purified by a multistep conventional centrifugation method (Fig. 2A, lane 5). A significant number of proteins associated with the ribosome in the low salt IXA-100 buffer (asterisks in Fig. 2A, lane 2) were eluted from the immobilized particles with the higher ionic strength IXA-500 buffer (Fig. 2A, lane 3). This suggested that ribosome-associated proteins could also be purified by this affinity technique (see below for more detail).

A second indication that the affinity method yielded intact ribosomes was derived from an examination of the rRNA in the purified material. Both the 18S and 25S rRNAs within the purified preparation were intact, whereas little tRNA was detectable (Fig. 2B). Both the integrity of the rRNA and the near absence of tRNA suggested a significant purification of intact ribosomes had occurred.

A third indication of the success of the purification was the near absence of free 40S subunits in the eluate from the affinity resin (Fig. 3A, lower panel). This suggested a specific purification of 60S and 80S particles. In addition, polyribosomes were also present in the eluted material (Fig. 3A, lower panel). This suggested that intact mRNA would also be present in the purified material, as intact mRNA tethers the individual ribosomes in the polyribosome particle (see below).

We note that larger polyribosomes were missing from this purified material. As the unbound fraction still con-
tain large polyribosomes (Fig. 3A, middle vs. lower panel), we exclude the possibility that these were lost during the procedure due to RNase activity. Instead, we hypothesize that these large polysomes have a unique feature that prevents their purification. One possibility is that their large size prevents them from entering into the matrix of the antibody-coated agarose affinity media. In support of this hypothesis, preliminary results suggest that the use of impermeable superparamagnetic uniform monodisperse polymer beads (Dynabeads, Dynal) allow for a more representative purification of the polyribosomes in the extract (E. Winstall & A.B. Sachs, unpubl. observations).

We also created a yeast strain containing GAL1: RPL25-FH, which expresses the tagged protein in a galactose-inducible and glucose-repressible manner. This strain allows for several additional experimental features to be added to a study design. First, it permits the high level, inducible synthesis of a tagged ribosome in a strain containing the endogenous RPL25 by having galactose in the growth medium. This synthesis can be rapidly inhibited by the addition of glucose. One example of the utility of this inducible/repressible system is in the study of ribosomes synthesized prior to some cellular challenge, such as the addition of a nutrient or a radiolabeled compound. By repressing the synthesis of the tagged subunit by the addition of glucose to the growth medium at the time of the challenge, a stable pool of presynthesized ribosomes can be experimentally distinguished from the newly synthesized ribosomes. Alternatively, the system could allow for the study of ribosomes synthesized after a challenge by inducing the synthesis of the tagged protein with galactose at the time of the challenge.

Several simple experiments were performed to validate the utility of this inducible RPL25-FH strain (YIT623), whose growth rate in galactose was indistinguishable from its wild-type parent. First, the expression level of ribosomes containing Rpl25p-FH produced by the GAL1:RPL25-FH gene in a RPL25\(^+\) background was examined by immunoblot analysis. The level of FH-tagged Rpl25p in YIT623 cells (RPL25\(^-\) [pGAL1-RPL25-FH]) grown on galactose medium was nearly half of that in YIT613 cells (rpl25::LEU2 [pRPL25-FH]; Fig. 4A, lanes 1, 3, 4). This indicates that high level expression could be obtained. Furthermore, the expression of GAL1:RPL25-FH was efficiently repressed in cells grown in glucose medium (Fig. 4A, lanes 1, 2). The rapid repression of RPL25-FH synthesis after transfer from galactose medium to glucose medium was confirmed by northern blot analysis (Fig. 4B, lanes 3, 4).

Analysis of proteins associated with the affinity-purified ribosomes

Tagged ribosomes were purified using extracts of YIT623 cells (RPL25\(^+\) [pGAL1-RPL25-FH]) grown on galactose medium. Several of the ribosomal proteins and ribosome-associated proteins were identified in this mixture by tryptic digestion and mass spectrometry (Link et al., 1999). We initially focused on two proteins bound to the ribosome in near stoichiometric quantities (Fig. 2C). One of these abundant proteins was Asc1p (Fig. 2C; see Materials and Methods for protein identification). Asc1p has previously been reported to be a novel ribosomal component (Link et al., 1999). We also found Asc1p to be purified with 40S subunits using a more conventional purification (Fig. 2A,

**TABLE 1.** Yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strain(^a)</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB012</td>
<td>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 pep4Δ::His3 prbΔ::His3 prc1Δ::HisG</td>
<td>Sachs strain collection</td>
</tr>
<tr>
<td>YAV129</td>
<td>trp1 leu2 ura3 his4 lys2 cup1:::LEU2PGKMA2 rex1:::TRP1 rex2::LYS2 rex3::HIS4 rex4:::NEO</td>
<td>van Hoof et al. (2000)</td>
</tr>
<tr>
<td>YIT613</td>
<td>CB012 rpl25::LEU2 [pRPL25-FH-URA3CEN] (BIT700)</td>
<td>This study</td>
</tr>
<tr>
<td>YIT623</td>
<td>CB012 [pGAL1-RPL25-FH-LEU2CEN] (BIT751)</td>
<td>This study</td>
</tr>
<tr>
<td>YIT722</td>
<td>YIT613 [pX3-LUC-TRP1CEN] (BIT757)</td>
<td>This study</td>
</tr>
<tr>
<td>YIT723</td>
<td>YIT613 [pB3-LUC-TRP1CEN] (BIT758)</td>
<td>This study</td>
</tr>
<tr>
<td>YIT724</td>
<td>YIT613 [pLUC-TRP1CEN] (BIT759)</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a\) Strain requests should be sent to Toshi Inada (p47294a@nucc.cc.nagoya-u.ac.jp).

\(^b\) Strain number to be used when making requests.

\(^c\) Bacterial strain containing indicated plasmid and number to be used when making plasmid requests.
Affinity purification of the yeast ribosome

FIGURE 2. Purification of yeast ribosomes. A: Proteins associated with purified ribosomes. Tagged ribosomes were purified as described in Materials and Methods. Lane 1: purification from strain CB012 in IXA-100 buffer. Lane 2: purification from strain YIT613 in IXA-100 buffer. Lane 3: purification from strain YIT613 in 500 mM KCl buffer. Lane 4: purified 40S subunits. Lane 5: purified 80S ribosomes. Material shown in lanes 4 and 5 was derived from a conventional purification method (see Materials and Methods). Proteins were resolved on 12% SDS-PAGE and visualized by silver staining. Asterisks indicate high molecular weight ribosome-associated proteins within the sample shown in lane 2. B: RNA associated with purified ribosomes. RNA samples were prepared from YIT613 crude extracts (lane 1) or affinity-purified ribosomes (lane 2), resolved on a 1% agarose gel, and visualized by ethidium bromide staining. C: Identification of Asc1p and Mpt4p. Lane 1: purification from strain CB012 in IXA-100 buffer. Lane 2: purification from strain YIT613 in IXA-100 buffer. The positions of Asc1p and Mpt4p are indicated. Proteins were resolved on 12% SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

FIGURE 3. Analysis of mRNAs associated with affinity purified ribosomes. A: Polysome profiles of affinity-purified material. Tagged ribosomes were purified from 15 A260 units of YIT613 crude extract (upper panel). This and the unbound fraction (middle panel) and the elution fraction (lower panel) were resolved by velocity sedimentation on 15–50% sucrose gradients. The positions of 40S and 60S subunits, 80S subunits, and polysomes are indicated. The top of each gradient is on the right. B: Poly(A) RNAs are copurified with the tagged ribosomes. Lane 1: in vitro transcribed poly(A) control RNA. Lanes 2, 4, 5: total RNA (5 µg) isolated from the indicated sample. Lane 3: total RNA isolated from the eluate of the untagged RPL25 strain. Lane 6: yeast tRNA (5 µg). RNA samples were hybridized to oligo-dT, and then reverse transcribed in the presence of [α-32P]-dCTP. Following electrophoresis of the samples along with DNA size markers on a formaldehyde-1.5% agarose gel, radioactive signals were obtained and analyzed by phosphorimaging. The arrow indicates the position of the 700-bp cDNA fragment generated by a control poly(A) RNA. The positions of fragments of the DNA ladder within the gel are indicated to the right.
Analysis of the mRNAs copurifying with the ribosome

The fact that polysomal particles were efficiently purified using the anti-FLAG affinity matrix (see Fig. 3A) strongly suggested that intact or near intact ribosome-associated mRNAs were recovered upon elution from the resin. To test this hypothesis, we performed oligo-dT-primed reverse transcription reactions on total RNA isolated from affinity purified samples. Specifically, total RNA prepared from crude extracts or the eluate obtained after FLAG affinity purification was used for reverse transcription in the presence of 32P-labeled dCTP. As specificity controls, yeast tRNA or RNA isolated from a strain lacking the FLAG-tagged RPL25p were also used in this analysis. Following primer extension, equal volumes of reaction were subjected to electrophoresis in a denaturing agarose gel. As expected, the material from the crude extract RNA preparation appeared as a smear ranging from 0.3 kb to about 2 kb in size, consistent with the average range of length of yeast mRNAs (Fig. 3B, lanes 2, 4). Discrete bands of about 1 kb most probably reflect the high abundance of specific pol II transcripts in yeast, such as TDH2/TDH3 or the TEF1/TEF2 genes (Veclulescu et al., 1997). A similar reverse transcription profile was found for RNA prepared from the affinity purified material derived from an extract containing Rpl25p-FH tagged ribosomes (Fig. 3B, lane 5). In contrast, no signal could be detected in the eluate obtained from lysates lacking tagged ribosomes or from tRNA (Fig. 3B, lanes 3, 6). From these results, we conclude that poly(A)+ mRNAs are specifically copurified with the tagged ribosomes.

We next wanted to test whether the amount of a specific mRNA copurifying with the ribosomes changed according to its translational status. To test this, we used a set of very well characterized in vivo reporter mRNAs encoding firefly luciferase (LUC) with or without stable stem-loop structures in their 5′ untranslated region (5′ UTR; Oliveira et al., 1993). It has been shown that a stem-loop in the 5′ UTR inhibits translation, and that the extent of inhibition depends upon the thermodynamic stability of the stem (Oliveira et al., 1993). Figure 5A illustrates the structure of the three reporter mRNAs that were tested. Plasmids expressing each of these LUC mRNAs were transformed into yeast strain YIT613, which expresses Rpl25p-FH protein as its sole source of Rpl25p.

To confirm the inhibitory effect of the 5′ UTR stem-loop structure on translation initiation of the reporter mRNAs, we first examined the sedimentation profile of each of the LUC transcripts. Extracts were sedimented on 15% to 50% sucrose gradients that were then fractionated into 12 fractions of equal volume. Polysome profiles were generated during fractionation by continuous measurement of the absorbance at 254 nm. A
A typical polysome profile is shown in Figure 5B. Total RNA was isolated from each fraction, and hybridized simultaneously to radiolabeled RNA probes complementary to the LUC reporter and ACT1 transcripts. Following digestion with RNases A and T1, the protected fragments were resolved by gel electrophoresis and detected by phosphorimaging. As shown in Figure 5C, LUC mRNAs without any stem-loop in their 5' UTR (right panel) were mostly found (>70%) in fractions cosedimenting with polysomes (Fig. 5B). This sedimentation profile and the one observed for the control actin mRNA (Fig. 5C, all three panels) are expected for transcripts that are efficiently recruited to the ribosomes. In contrast, both LUC mRNAs with stable stem-loop structures (~17 kcal/mol) in their 5' UTR were diffusely distributed across the gradient, with about 60% of the transcripts being present in fractions lighter than those with a single 80S ribosome.
Luciferase activity was then assayed in extracts expressing the different reporter mRNAs. As expected, LUC mRNAs bearing a stem-loop near the 5' cap structure (B3-LUC) or close to the first AUG codon (X3-LUC) produced 5% to 10% of the luciferase activity detected in the extract expressing a LUC mRNA with an unstructured leader sequence (Fig. 6A). This decrease in LUC activity is thought to be mostly the result of a decrease in the efficiency of translation initiation, as the steady-state levels of each reporter transcript have been found to be highly comparable (Oliveira et al., 1993; Fig. 6B).

Given the above results, we hypothesized that following affinity purification of the tagged ribosome, the amount of LUC mRNAs detected in the eluate (bound fraction) should reflect the translational status of the mRNA. To test this hypothesis, extracts were incubated with the anti-FLAG affinity resin under conditions that were designed to avoid mRNA degradation (see Materials and Methods). Following the washing steps, tagged ribosomal particles were eluted using the FLAG peptide. Total RNA was purified from the eluted fractions and then quantitated. Equal amounts of total RNA isolated either from the crude extract or from the anti-FLAG resin eluate were then hybridized in solution to radiolabeled RNA probes complementary to the 5' end of the LUC reporter mRNA or to the ACT1 mRNA. The amount of probe used in the assay was in large excess over the target sequence in the RNA samples, as these assays were found to respond linearly to the amount of input RNA (data not shown).

As shown in Figure 6B, lanes 1–3, similar levels of the different LUC reporter mRNAs were found in the total extract, confirming that the different leader sequences do not substantially affect the steady-state level of the transcript (Oliveira et al., 1993). LUC mRNA can also be detected in the ribosome-associated fraction (Fig. 6B, lanes 4–6). However, the amount of LUC mRNA with a stem-loop in its leader region that was bound to ribosomes was at least four times less than that found for the LUC mRNA with an unstructured leader sequence (Fig. 6B, compare lanes 4 and 5 to lane 6). These results show that the level of LUC mRNA that is copurified with the tagged ribosome reflects its translational efficiency within the cell.

We note that because very large polysomes (>5 ribosomes) are not effectively purified using the affinity resin (see Fig. 3A), those mRNAs associated with more than five ribosomes (such as longer mRNAs) would be underrepresented in the ribosome-bound fraction. Consequently, the purification method reported here does not allow for the direct prediction of the translational status of an mRNA, and could significantly underestimate the translatability of mRNAs bound to large numbers of ribosomes. To overcome this limitation, the technical difficulties of purifying very large polyribosomes will have to be solved.

DISCUSSION

We report here the successful development of a simple affinity purification strategy for yeast ribosomes. Material purified with this procedure contains 60S subunits, 80S monosomes, and small polyribosomes. Associated with these are many proteins, including Asc1p and...
Mpt4p, as well as intact mRNAs. The degree to which the mRNA is purified correlates well with its translational activity within the cell.

Our results suggest that the affinity purification of ribosome-bound mRNA could be used as a fast and easy high-throughput screening procedure to identify subsets of mRNAs that change their translational activity in different yeast mutants or under different growth conditions. Preliminary experiments in which mRNAs copurifying with the tagged ribosome were hybridized to yeast cDNA microarray were, in fact, able to identify transcripts that have been previously shown by sedimentation analysis to be translationally regulated following changes in the nutritional status of the yeast (E. Winstall & A.B. Sachs, unpubl. observation; Hinnenbusch, 1984; Kuhn et al., 2001).

There are several advantages of using affinity-purified ribosomes over centrifugation techniques to analyze ribosome-associated proteins and mRNAs. Co-purification on an immobilized resin represents a more direct means than cosedimentation for assessing ribosome association. The purification procedure is simple and rapid, so that a large number of different samples can be processed at the same time. The amount of starting biological material needed to analyze associated proteins and mRNAs can be scaled down, thereby allowing experiments designed to assess the consequences of multiple conditions on ribosome association to be performed. Finally, coupled to mRNA amplification methods, the procedure described here can theoretically allow for the determination of the translation status of mRNAs at the single- or near single-cell level.

For now, affinity purification of the ribosome is restricted to small polyribosomes carrying a tagged ribosomal protein. Future development milestones will include optimizing methods to purify the larger polyribosomes and obtaining monoclonal antibodies directed against epitopes on the ribosome in higher cells. With these advances, expression profiling of mRNAs in translationally active and inactive pools in mammalian cells should be greatly simplified. In addition, efficient scaling of the preparation is possible (T. Aragon & A.B. Sachs, unpubl. observations), and this should allow for the very rapid purification of large amounts of intact ribosomes for structural studies. Many other applications of this technology are certainly imaginable, and each of these should further advance studies on the ribosome and on translational control processes.

### MATERIALS AND METHODS

#### Strains and growth conditions

*S. cerevisiae* strains and oligonucleotides used in this study are listed in Tables 1 and 2. Strains were grown in rich medium (YP) or synthetic complete (SC) yeast medium supplemented with 2% carbon source, as described (Guthrie & Fink, 1991).

YIT613 was created from parental strain CB012. CB012 was transformed with pRPL25-FH (see below), and then the chromosomal RPL25 was disrupted by transformation with the NotI-XhoI fragment used to construct the rpl25::HIS3 (pL25-GFP) strain (Hurt et al., 1999), with the LEU2 gene replacing the HIS3 gene. Recombinants containing rpl25::LEU2 were identified by standard methods.

#### Plasmids construction

pRPL25-FH (BIT751) was constructed as follows. Two complementary oligonucleotides, OIT39 and OIT40, encoding the FLAG-(His)$_6$ epitope were annealed and inserted into pBlueScript KS+ using EcoRV-HindIII sites to create pBluecriptFH. The BamHI-EcoRV fragment of pSB32::L25-TAG (Hurt et al., 1999) containing RPL25 was inserted into the corresponding sites of pBluecriptFH to create pRPL25-FH. Both the BamHI-HindIII fragment containing the 3’ UTR region of RPL25 and the XbaI-BamHI fragment containing 5’ UTR region of RPL25 (Hurt et al., 1999) were inserted into the corresponding sites of the yeast centromeric vector YCpLac33 (Gietz & Sugino, 1988) to create pRPL25/5’+3’UTR. The BamHI-BamHI fragment of pRPL25-FH was inserted into BamHI site of pRPL25/5’+3’UTR plasmid. The resulting plasmid pRPL25-FH contains the RPL25 promoter and the open reading frame, fused in frame to sequences that encode the FLAG-(His)$_6$ epitope followed by the transcriptional terminator from the RPL25 gene.

To construct pGAL1-RPL25-FH (BIT757), the full-length C-terminal FH-tagged form of RPL25 was first obtained by amplifying RPL25 DNA fragments from pRPL25-FH with primers OIT57 and OIT58. PCR product was cloned into p415GAL1 (Mumberg et al., 1994) using XbaI and EcoRI sites, and the DNA sequence of the resulting plasmid pGAL1-RPL25-FH was checked by standard sequencing procedures. The resulting plasmid contains the RPL25 open reading frame transcribed by the GAL1 promoter, fused in frame to sequences that encode the FH tag, followed by the transcriptional terminator from the CYC1 gene.

The plasmids YCpLUCEX1, YCp3LUCEX1, and YCp3LUCEX1 used to generate luciferase reporter mRNA are a kind gift of John E.G. McCarthy, and have been described in details by Oliveira et al. (1993). For the purpose of our study, the LUC expression modules had to be excised.
from YCPX3LUCEX1, YCPB3LUCEX1, and YCpLUCEX1 (all URA3CEN vectors) as HindIII fragments and were then introduced into HindIII-digested Ycplac22 (Gietz & Sugino, 1988) to produce plasmids BIT757, BIT758, and BIT759, respectively (TRP1CEN vectors). The plasmid BIT760, used to generate the LUC antisense RNA probe, was constructed by introducing a 225-bp BamH1-BsaAI fragment from BIT758 into BamH1-EcoRV-digested pBluescript KS+

The pellets were dissolved in buffer E (50 mM Tris-HCl pH 7) and 153 nt of the LUC ORF fragment encompassing the ACT1 gene (see Fig. 3A) and transferred to a 50-mL conical tube in which they were pelleted for 3 min at room temperature in a clinical centrifuge. The cell pellets were snap-frozen in liquid nitrogen and then ground in liquid nitrogen using a mortar and a pestle. The resulting cell powder was then resuspended on ice in 4 mL of lysis buffer (20 mM HEPES-KOH, pH 7.4, 2 mM Mg(OAc)2, 100 mM KOAc, 2% glucose or galactose, 1× amino acids) equilibrated at 30°C, and transferred to a 50-mL conical tube in which they were pelleted for 3 min at room temperature in a clinical centrifuge. The cell pellets were dissolved in buffer E and analyzed for protein and RNA content as described.

Purification of ribosomes by conventional centrifugation methods

Yeast strain yAV129 (van Hoof et al., 2000) was grown in 500 mL YPD to an OD600 of 2.0. The 80S ribosomes were purified on discontinuous sucrose gradients. The pelleted ribosome complexes were washed twice with IXA-500 buffer (50 mM Tris-HCl, pH 7.5, 500 mM KCl, 12 mM Mg(OAc)2, 1 mM DTT, 1 mM PMSF) and then resuspended for 15 min at 37°C in IXA-800 containing 10 mM pyruvmycin. After recentrifugation, pellets were dissolved in buffer E (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol) and the resulting solution was sedimented on a 15-25% sucrose gradient to separate and fractionate the 60S and 40S ribosomal subunits. Each of the fractions was mixed with the equal volume of IXA dilution buffer (50 mM Tris-HCl, pH 7.5, 12 mM Mg(OAc)2, 12 mM β-mercaptoethanol), and pelleted at 40,000 rpm for 12 h. The pellets were dissolved in buffer E and analyzed for protein and RNA content as described.

Extract preparation for ribosomal protein and ribosome-associated protein analysis

Yeast strain YIT613 was grown in 200 mL YPD at 30°C to an OD600 of 0.8. Cells were pelleted for 3 min with the use of a clinical centrifuge, resuspended in 400 μL of lysis buffer (20 mM HEPES, pH 7.4, 2 mM Mg(OAc)2, 100 mM KOAc, 100 μg/mL cycloheximide, 0.5 mM DTT) on ice, and transferred to 1.5-mL microcentrifuge tubes. After pelleting for 5 min at 7,000 rpm in 1.5-mL microcentrifuge tubes, cells were resuspended in an equal volume of lysis buffer containing a protease inhibitor cocktail (complete mini-EDTA free; Roche), and lysed in the presence of 1 vol of glass beads by vortexing six times for 20 s at 40-s intervals. Lysates were cleared briefly at 10,000 rpm for 5 min, followed by a 20-min 10,000-rpm centrifugation to give the final lysate.

Extract preparation for mRNA analysis

Yeast strains expressing the Rpl25p-FH fusion protein were grown in 500 mL of rich (YPD) or synthetic complete (SC) medium containing 2% (w/v) of the appropriate carbon source to an OD600 of 0.8. Cells were pelleted at 30°C for 10 min at 6,000 rpm in a GSA rotor (Sorvall) prewarmed at 30°C. Cells were then resuspended in 50 mL of washing buffer (20 mM HEPES, pH 7.4, 2 mM Mg(OAc)2, 100 mM KOAc, 2% glucose or galactose, 1× amino acids) equilibrated at 30°C, and transferred to a 50-mL conical tube in which they were pelleted for 3 min at room temperature in a clinical centrifuge. The cell pellet was snap-frozen in liquid nitrogen and then ground in liquid nitrogen using a mortar and a pestle. The resulting cell powder was then resuspended on ice in 4 mL of lysis buffer (20 mM HEPES-KOH, pH 7.4, 2 mM Mg(OAc)2, 100 mM KOAc, 1 mM DTT, 1 mM PMSF) supplemented with a protease inhibitor cocktail (Complete tablets; Roche Biochemicals) and an RNase inhibitor (50 U/mL RNasin; Promega). Extracts were then transferred to 1.5-mL Eppendorf tubes and centrifuged by centrifugation at 4°C for 5 min at 5,000 × g. Supernatant was recovered and centrifuged at 4°C for 10 min at 9,000 × g to remove membranes and insoluble material. Extracts were kept in aliquots at −80°C until ready to use.

Affinity purification of the ribosome

For ribosome purification, about 15 A280 units of extract were mixed with an equal volume of ice-cold 2× binding buffer (100 mM Tris-HCl, pH 7.5, 24 mM Mg(OAc)2, 1 mM DTT, 1 mM PMSF, 50 μg/mL RNasin) and with 400 μL (bead volume) of anti-FLAG M2-agarose affinity resin (Sigma) in a 2-mL Eppendorf tube. Binding was allowed for 2 h at 4°C with constant and gentle rocking. Resin was then washed five times with 0.8 mL of ice-cold IXA-100 buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 12 mM Mg(OAc)2, 1 mM DTT, 1 mM PMSF). Elution was achieved by incubating the resin in 0.4 mL of IXA-100 buffer containing the FLAG peptide at 100 μg/mL for 20 min at 4°C with gentle rocking. Eluate was either frozen in liquid nitrogen and stored at −80°C for future use or processed immediately for total RNA purification or protein analysis.

Total RNA preparation

Total extracts, eluates, or sucrose gradient fractions were mixed with an equal volume of 2× proteinase K buffer (0.2 M Tris-HCl, pH 7.5, 0.3 M NaCl, 2% SDS, 25 mM EDTA) and digested with 0.1 mg/mL proteinase K for 45 min at 37°C. Samples were then extracted twice with 1 vol of phenol:chloroform:isoamyl alcohol (25:24:1) and extracted once with 1 vol of chloroform:isoamyl alcohol (24:1). RNA in the aqueous phase was then precipitated with 1 vol of isopropanol and resuspended in DEPC-treated water after washing the RNA pellet with 70% ethanol.

For the experiments described in Figures 5 and 6, yeast strains YIT722-724 (containing the galactose-inducible LUC mRNAs described in Table 1) were first grown to log phase in selective medium with glucose as the carbon source, and then diluted into medium with galactose to induce expression of the LUC reporter mRNAs. Cells were then allowed to grow to mid-log phase, and extracts were prepared as described above.
cDNA synthesis
Total RNA isolated from the eluate of the control, untagged RPL25 strain and 5 μg of total RNA isolated from each of the other fractions were allowed to hybridize to oligo-dT, and were then reverse transcribed in the presence of [α-32P]-dCTP according to standard protocols (Sambrook et al., 1989). Unincorporated nucleotides and oligo-dT were removed using Qiagen microspin column (Qiagen). Equal volumes of each sample were then loaded on a 1.5% denaturing agarose gel. Following electrophoresis, the gel was dried and analyzed using a Typhoon 9400 phosphorimager (Amersham Pharmacia Biotech). An OD600 of 0.1. Cells were pelleted in a clinical centrifuge for 3 min and resuspended immediately in fresh chase medium containing 0.1 mg/ml methionine and 2% glucose (SCD-leu). Samples (50 ml) were withdrawn at the indicated time points, and the pellets were frozen. Total RNA samples were extracted from the frozen pellets and used for northern blot analysis as described (Ashe et al., 2000). The BamHI–EcoRV fragment of pSB32-L25::TAG (Tollervey et al., 1993) containing RPL25 gene was used as a probe.

Luciferase assays
Protein concentration was determined in each extract by the method of Bradford (1976) using the Bio-Rad Protein Assay reagent (Bio-Rad). Extracts were then serially diluted from 1:100 to 1:1,000 in Buffer A (30 mM HEPES-KOH, pH 7.4, 100 mM KOAc, 2 mM Mg(OAc)2) supplemented with 1 mg/ml bovine serum albumin. Ten microliters of the diluted extract were then mixed with 50 μl of the reconstituted luciferase assay substrate (Promega) and luminescence was immediately counted for 15 s on a TD-20/20 luminometer (Turner Designs). Each sample was analyzed in triplicate and the signal obtained was normalized using the protein concentration in the extract.

Northern blot analysis
Yeast strain YIT623 was grown in 200 mL SC-Galactose to an OD600 of 0.1. Cells were pelleted in a clinical centrifuge for 3 min and resuspended immediately in fresh chase medium containing 0.1 mg/ml methionine and 2% glucose (SCD-leu). Samples (50 ml) were withdrawn at the indicated time points, and the pellets were frozen. Total RNA samples were extracted from the frozen pellets and used for northern blot analysis as described (Ashe et al., 2000). The BamHI–EcoRV fragment of pSB32-L25::TAG (Tollervey et al., 1993) containing RPL25 gene was used as a probe.

Protein analysis
Mass spectrometry was performed as described (Link et al., 1999).
Tryptic peptides identified from Mpt4p were (frequency of peptide): EAQADAIAEAIAEDADGKPK (1), EAYVATPK, EYLEFDATVESNTR (1), KADVPPPSADPSK (1), TAAQLQDYLNQANNQFKN (1), VNOQGWDQKK (1), KGNN TANATNSNVTQV (2), NIDVSNLPSLA, PSGNEAIR, SKDVDSTTAK (2).
Tryptic peptides identified from Asc1p were: AAEPHA VLSAWSADGQTFL (1), ADDDSVTIIAASDK (1), DGEIMLWNAAK, SDVMSVDIYD (1), VFSLDPOYLVDDLRFEPFAGYSK (1), GTLEGHNWVTLSATSGQNP (1), LWDVTGATETYQR (1), YWLAATATGIK (1), AMYTLSAODEVFSLAFSPN (2), AAEPHAVLSAWSADGQTLFYTDNVR (3), VFSLDPOYLVDDLRFEPFAGYS (3), AWLNQFQIEADFIHGNSNINTLTA SG (8).

Acknowledgments
We thank Ed Hurt for pSB32::L25-TAG and the plasmid to construct rpl25::LEU2. Roy Parker for the strain yAV129, and John E.G. McCarthy for the YCpLUC plasmids. This work was supported by the Japan Society for the Promotion of Science to T.I., National Institutes of Health grant GM 50308 to A.B.S., and grant RR11823 from the Yeast Resource Center to J.R.Y. E.W. was supported by a postdoctoral fellowship from The Natural Sciences and Engineering Research Council of Canada (NSERC).

Received March 26, 2002; returned for revision April 5, 2002; revised manuscript received April 26, 2002.
REFERENCES


One-step affinity purification of the yeast ribosome and its associated proteins and mRNAs.

Toshifumi Inada, Eric Winstall, Salvador Z Tarun, Jr, et al.

RNA 2002 8: 948-958