Requirements for the nuclear export of the small ribosomal subunit

Terence I. Moy\textsuperscript{1} and Pamela A. Silver\textsuperscript{2,*}
\textsuperscript{1}Department of Molecular Biology, Massachusetts General Hospital, 50 Blossum Street, Boston, MA 02114, USA
\textsuperscript{2}The Dana-Farber Cancer Institute, 1 Jimmy Fund Way, Boston, MA 02115, USA
*Author for correspondence (e-mail: pamela_silver@dfci.harvard.edu)

Accepted 7 May 2002

Summary
Eukaryotic ribosome biogenesis requires multiple steps of nuclear transport because ribosomes are assembled in the nucleus while protein synthesis occurs in the cytoplasm. Using an in situ RNA localization assay in the yeast \textit{Saccharomyces cerevisiae}, we determined that efficient nuclear export of the small ribosomal subunit requires Yrb2, a factor involved in Crm1-mediated export. Furthermore, in cells lacking \textit{YRB2}, the stability and abundance of the small ribosomal subunit is decreased in comparison with the large ribosomal subunit. To identify additional factors affecting small subunit export, we performed a large-scale screen of temperature-sensitive mutants. We isolated new alleles of several nucleoporins and Ran-GTPase regulators. Together with further analysis of existing mutants, we show that nucleoporins previously shown to be defective in ribosomal assembly are also defective in export of the small ribosomal subunit.

Key words: Ribosome, Nuclear export, In situ hybridization

Introduction
Eukaryotic ribosome biogenesis requires multiple steps of nuclear import and export. In yeast, the small (40S) ribosomal subunit consists of 32 ribosomal proteins and an 18S rRNA whereas the large (60S) ribosomal subunit consists of 46 ribosomal proteins and the 25S, 5.8S, and 5S rRNAs (Verschoor et al., 1998). Following their synthesis in the cytoplasm, the nascent ribosomal proteins are imported into the nucleolus where they assemble with the rRNA to form the individual subunits. In the nucleolus, RNA pol III transcribes the 5S rRNA, and RNA pol I transcribes the 35S pre-rRNA. The 35S pre-rRNA is processed to the mature 18S, 5.8S and 25S rRNAs. Most of these pre-rRNA processing steps occur in the nucleolus (reviewed by Kressler et al., 1999; Venema and Tollervey, 1995). Once fully assembled, the ribosomal subunits must be translocated through the nuclear pore complex to enter the cytoplasm.

All macromolecular transport into or out of the nucleus is believed to occur through the nuclear pore complex (NPC). In yeast, the nuclear pore is a 60 MDa complex composed of proteins called nucleoporins (reviewed by Davis, 1995; Fabre and Hurt, 1997). The dimensions of the nuclear pore channel are such that only one ribosomal subunit could pass through at any given time. Several models have been put forth for how individual proteins pass through the NPC on their way into the nucleus (Ben-Efraim and Gerace, 2001; Ribbeck et al., 1999; Rout et al., 2000). However, the precise mechanism for nuclear exit of large ribonucleoprotein complexes such as the ribosomal subunits remains unknown.

One key determinant of the directionality of nuclear transport is the nucleotide-bound state of the small GTPase Ran (reviewed by Koepp and Silver, 1996). Directionality is determined by a GTPase-activating protein, in yeast termed Rna1, and by a guanine exchange factor, in yeast termed Prp20 (Rcc1 in mammals). Rna1 is localized in the cytoplasm while Prp20 is inside the nucleus. Consequently, the concentration of Ran-GDP is elevated in the cytoplasm while the concentration of Ran-GTP is elevated in the nucleoplasm. The nucleotide-bound state of Ran affects its interactions with nuclear transport factors containing a Ran-binding domain. One family of yeast Ran-binding proteins includes Yrb1 (RanBP1 in mammals), a protein that resides in the cytoplasm, and Yrb2 (RanBP3 in mammals), a protein that resides in the nucleus (Mueller et al., 1998; Schlenstedt et al., 1995; Taura et al., 1997). The second family of Ran-binding proteins consists of transport receptors termed importins, exportins or karyopherins (reviewed by Görlich and Kutay, 1999).

Importins/karyopherins bind cargo in the cytoplasm and enter the nucleus through the NPC. Once inside the nucleus, the protein cargo dissociates from its importer. This dissociation is driven by Ran-GTP. Conversely, proteins are exported out of the nucleus by binding to exportins in association with Ran-GTP. Once in the cytoplasm the export complex dissociates when the GTP of Ran is hydrolyzed (Görlich and Kutay, 1999). Crm1/Xpo1 (or exportin) is the major karyopherin that exports various cargoes out of the nucleus. Crm1 is perhaps best known for exporting proteins that have the consensus leucine-rich nuclear export sequence (NES) (Fornerod et al., 1997; Fukuda et al., 1997; Neville et al., 1997; Stade et al., 1997). Crm1 also exports some proteins that do not bear the consensus leucine-rich NES. A case in point is snurportin, a protein involved in U snRNP import (Huber et al., 1998; Paraskeva et al., 1999). Additionally, Crm1 exports some of its cargo through adapter proteins. Crm1 exports U snRNAs and the HIV intron-containing RNA through the PHAX (phosphorylated adapter for RNA export) and HIV Rev adapters, respectively (Askjaer et al., 1998; Ohno et al., 2000).
Recently, Crm1 has been shown to be important for the nuclear export of the large ribosomal subunit using the NES-bearing adaptor Nmd3 (Gadal et al., 2001; Ho et al., 2000b). No such adaptor has yet been determined for the small subunit, but analysis of a crm1/spo1-1 mutant suggested that export of the small subunit relies on the NES/Crm1 pathway (Moy and Silver, 1999).

Yrb2 is required for the efficient nuclear export of proteins containing a leucine-rich NES (Taura et al., 1998). In vitro studies show that Yrb2 can bind to Ran-GTP but not Ran-GDP (Noguchi et al., 1997). Yrb2 contains the phenylalanine-glycine repeat motifs that are also found on many nucleoporins, but Yrb2 is not a stable component of the NPC (Taura et al., 1998). YRB2 is an essential gene in yeast, but cells lacking YRB2 are slower growing at 15°C and accumulate the NES reporter protein in the nucleus (Taura et al., 1998). The mammalian Yrb2 homologue, RanBP3, has been shown to promote the interaction of Crm1 with its substrates and thus affect export (Englemeier et al., 2001; Lindsay et al., 2001).

In order to study the export of the small ribosomal subunit in yeast, we previously reported the implementation of a novel assay to monitor the distribution of nascent small ribosomal subunits by in situ analysis. The small ribosomal subunit is exported as a 43S particle containing 20S pre-rRNA. In the cytoplasm, the 20S pre-rRNA is cleaved to produce the mature 18S rRNA and a 209 base fragment, the 5′ ITS1 rRNA (Moy and Silver, 1999). With this assay, we previously reported that export of the small ribosomal subunit depended on the nucleotide-bound state of Ran and certain nucleoporins. We have now further refined the assay and extended its use to a number of export factor and nucleoporin mutants. In addition, we have used the assay to screen a large collection of temperature-sensitive mutants for small subunit export defects.

Materials and Methods

Small ribosomal subunit export assay

Yeast strains used in this study are listed in Table 1. Crm1 (PSY1968, MNY7) and crm1 (T539C) (PSY1969, MNY8) were grown to a density of 2×10^7 cells/ml in YPD at 30°C (Burke et al., 2000; Neville and Rosbash, 1999) and leptomycin B was added to a final concentration of 200 nM (kind gift of M. Rosbash, Howard Hughes Medical Institute, Brandeis University, Waltham, MA). Cells were fixed with formaldehyde at time points of 5, 15 or 60 minutes as previously described (Moy and Silver, 1999). PSY580 (wild-type, FY23) (Winston et al., 1995) and PSY2070 (yrb2Δ) were grown a density of 1×10^7 cells/ml in met- dropout media at 30°C (Burke et al., 2000). The cultures were diluted by 1:10 with fresh media and shifted to 15°C. Cells were fixed at time points 0, 3, 6, 12, 18 or 24 hours after the temperature shift. FY23 with pPS293 (vector) or pPS1082 (GAL-pro-YRB2 URA2 2μ) was grown in ura- dropout with 2% raffinose to 1×10^7 cells/ml at 25°C. Galactose was added to 2% and cells were fixed after 1, 2 or 3 hours. For ts- strains, cells were grown to a density of 1×10^7 cells/ml in YPD at 25°C before the temperature shift to 37°C and treated as previously described (Moy and Silver, 1999). 5′ ITS1 rRNA was localized by fluorescence in situ hybridization as described (Amberg et al., 1992) with the following modification. An oligonucleotide complementary to the first 50 bases of ITS1 RNA accumulates in the nucleoplasm in these cells. Furthermore, in cells defective in small ribosomal subunit export, the 20S pre-rRNA does not mature to the 18S rRNA (Moy and Silver, 1999). With this assay, we previously reported that export of the small ribosomal subunit depended on the nucleotide-bound state of Ran and certain nucleoporins. We have now further refined the...
shifted to 15°C for 24 hours. 5x10^8 cells were concentrated to a volume of 3 ml and pulse labeled with 250 μCi of [methyl-^3]Hmethionine for 5 minutes to methylate rRNA (70-85 Ci/mmol, 1 mCi/ml; NEN, Boston, MA). 500 μl of culture was transferred to a new tube, 1 ml of ice-cold media was added, the tube was centrifuged, supernatants were removed, and the cell pellets were frozen on dry ice. Chase was initiated by adding 150 μl unlabeled methionine at a concentration of 20 mg/ml to the 2.75 ml of culture. At time points of 5, 10, 20, 40 and 60 minutes of chase, 500 μl of culture were removed and processed as described above. RNA was isolated by the hot acid phenol method (Ausubel et al., 1997). 10,000 cpm of radioactivity was loaded per lane onto a 1.2% agarose-formaldehyde gel. RNA was transferred to Hybond-N* membranes by vacuum blotting (Amersham-Pharmacia), UV-crosslinked, and sprayed with Enhance (NEN). The membrane was exposed to film for 4 days at -80°C.

PSY1968 and PSY1969 were grown at 30°C, and 10^8 cells were concentrated to a volume of 1 ml in met media and treated with leptomycin B at a final concentration of 200 nM for 15 minutes. PSY580, PSY2090, PSY2092, and PSY2460 were grown at 25°C and shifted to 37°C for 1 hour. Cells were pulse labeled with 50 μCi of [methyl-^3]Hmethionine for 1 minute and chased with 45 μl unlabeled methionine at a concentration of 20 mg/ml for 2, 4 or 10 minutes. Samples were processed as described above.

In separate experiments, PSY580 and PSY635 were grown at 25°C and shifted to 37°C for 1 hour. PSY580 and PSY1772 were grown at 25°C, shifted to 37°C for 3 hours, and then shifted back to 25°C for 0.5 hours. 10^8 cells were concentrated to a volume of 3 ml in met media, pulse labeled with 250 μCi of [methyl-^3]Hmethionine for 3 minutes, and chased with unlabeled methionine at a final concentration of 1 mg/ml for 0, 3 or 10 minutes. Samples were processed as previously described (Moy and Silver, 1999).

**Polysome profiles**

Polysome profiles and ribosomal subunit profiles were performed as described (Kressler et al., 1997) with the following modifications. PSY2070 was covered by pPS327 (TRP CEN) and grown in trp drop out media at 30°C to a density of 2x10^7 cells/ml. The culture was diluted and shifted to 15°C for 24 hours. For the polysome profiles, lysate (4 A260 units) was layered onto 10 ml linear 7-49% sucrose gradients. Samples were centrifuged in a Beckman SW41Ti rotor for 2 hours at 261,000 g at 4°C (Beckman Instruments, Fullerton, CA). For the ribosomal subunit profiles, lysate (2 A260 units) was layered onto 10 ml linear 10-35% sucrose gradients and centrifuged in a Beckman SW41Ti rotor for 4 hours at 261,000 g at 4°C. A Beckman fraction recovery system was used to pass the gradients through a Pharmacia UV-1 monitor to measure A254.

**ts** mutant screening

The yeast strain FY23 was disrupted in XRN1 (Moy and Silver, 1999). The xrn1Δ strain was mutagenized with ethyl methanesulfonate (EMS) as described (Ausubel et al., 1997) to produce 50% cell death. 160 temperature-sensitive mutants were isolated, essentially as described (Amberg et al., 1992). Approximately 800 XRN1ΔΔ ts mutants, previously used in the screen for mRNA trafficking mutants (RAT), were kindly provided by C. N. Cole (Dartmouth Medical School, Hanover, NH) (Amberg et al., 1992). Yeast strains were grown on YPD plates at 25°C for 3-4 days. The strains were transferred to 2 ml YPD at a density of 10^7 cells/ml and cultured for 1 hour at 25°C to allow cells to resume growth and stimulate ribosome biogenesis. The cultures were shifted to 37°C for 1 hour. Half of the culture was fixed with 100 μl 37% formaldehyde for 2 hours at 25°C. The other half of the culture was shifted back to 25°C for 1 hour before fixation. 5’ ITS RNA was localized as previously described (Moy and Silver, 1999). Complementation group analysis was used to determine which mutants are defective in known nuclear transport factors. Mutants that were not identified by complementation grouping were backcrossed to the FY23 or FY86 strains three times (Winston et al., 1995). Then, the mutations were cloned by complementation of their temperature-sensitivity with a genomic library (Rose et al., 1987). The mutations were verified by complementation group analysis. The mutated gene was PCR amplified, and the PCR product was subjected to DNA sequencing.

**Results**

**Assay for localization of the small ribosomal subunit**

We previously reported the first cell-based assay for the localization of the nascent small ribosomal subunit. The assay relies on the localization of 5’ ITS1 RNA using fluorescent in situ hybridization (Moy and Silver, 1999). We originally performed this assay in strains lacking the Xrm1 exonuclease, which degrades the 5’ ITS1 fragment. In xrn1Δ cells, the 5’ ITS1 RNA is distributed throughout the cytoplasm because, in these cells, the small ribosomal subunit is exported, the 5’ ITS1 RNA is cleaved off, and the 5’ ITS1 fragment accumulates in the cytoplasm (Fig. 1a). We were able to assay various temperature-sensitive mutants in the xrn1Δ strain background to detect both ribosomal assembly defects and nuclear export defects. In the case of a ts ribosomal assembly mutant in the xrn1Δ strain background, the 5’ ITS1 RNA accumulates in the crescent-shaped nucleolus at the restrictive temperature (Fig. 1c) after sufficient time elapses to allow for degradation of the cytoplasmic 5’ ITS1 fragment. In the case of a xrn1Δ mutant blocked in export, the 5’ ITS1 accumulates in the nucleolus and the rest of the nucleoplasm after shift to the nonpermissive temperature (Fig. 1b).
out in XRNI+ wild-type yeast cells. Although the loss of cytoplasmic 5' ITS1 fluorescent signal is an easily identifiable change in xrn1Δ mutants, we determined that the excess 5' ITS1 RNA in xrn1Δ strains can obscure a mild nuclear export defect. In addition, we wished to use the assay to screen existing mutant collections, which are XRNI-positive. Consequently, we have used XRNI+ strains to examine small ribosomal subunit nuclear export. Wild-type XRNI+ strains primarily localize 5' ITS1 RNA to the nucleolus and a very small amount to the cytoplasm (Fig. 1d). However, in a mutant blocked in export, accumulation of 5' ITS1 can be observed in both the nucleolus and nucleoplasm (Fig. 1e).

Crm1 and its co-factor, Yrb2, affect the export of the small ribosomal subunit

Cells bearing a leptomycin-B-sensitive (LMB) allele of crml display a block in the export of the small ribosomal subunit dependent on drug treatment. Wild-type S. cerevisiae are insensitive to LMB because LMB does not interact with wild-type Crm1 protein. However, the crml(T539C) mutant is inhibited by LMB, resulting in the nuclear export defect of leucine-rich NES proteins (Neville and Rosbash, 1999). We performed the small ribosomal subunit export assay on strains treated with LMB. LMB does not affect the localization of 5' ITS1 RNA in wild-type (XRNI+) cells (Fig. 2Aa-c); 5' ITS1 remains localized to the nucleolus as indicated by arrowheads, and it does not significantly accumulate in the regions of the nucleus occupied by DAPI-stained chromatin as indicated by arrows. However, LMB treatment causes 5' ITS1 to accumulate in the entire nucleoplasm in crml(T539C) cells as indicated by arrows and arrowheads (Fig. 2Ad-f). Mislocalization of 5' ITS1 in these cells occurs 5-15 minutes after addition of LMB.

Cells deleted for YRB2, which is involved in Crm1-mediated export, also show a defect in export of the small subunit. yrb2Δ cells are cold-sensitive for growth, and export of NES-containing proteins is blocked more so at the non-permissive temperature. While 5' ITS1 RNA is confined to the nucleolus of wild-type
subunit in subunits is decreased by 10-20% relative to the 60S ribosomal profile). Therefore, the concentration of 40S ribosomal A254 because the 60S subunit contains twice as much RNA as and the ratio of 60S:40S subunits is 2.1:1 when measured by 
contain an equal molar ratio of 60S to 40S ribosomal subunits by sucrose gradient centrifugation. Wild-type cells 
subunits by disassociating the ribosome and separating the polysomes in 
the empty vector (Fig. 4Aa-b). While expression of 
A254nm was monitored continuously and represents the 
vertical axis of the profiles. The top of the gradient is on the left-hand side of the profile. The positions of free 40S and 60S subunits, 80S ribosomes, and polysomes are indicated. (B) YRB2* and yrb2Δ cells were shifted to 15°C for 24 hours, and ribosomal subunit profiles were resolved in 10-35% sucrose gradients.

Fig. 3. yrb2Δ cells are deficient in the levels of the small ribosomal subunit. (A) YRB2* and yrb2Δ cells were shifted to 15°C for 24 hours, and polysome profiles were resolved in 7-49% sucrose gradients. Abs254nm was monitored continuously and represents the vectorized axis of the profiles. The top of the gradient is on the left-hand side of the profile. The positions of free 40S and 60S subunits, 80S ribosomes, and polysomes are indicated. Therefore, in cells lacking YRB2, the nascent small ribosomal subunit appears to be less stable and its nuclear export is delayed.

To determine the effect of the yrb2Δ mutation on ribosome activity, we examined pre-rRNA processing. Strains were shifted to 15°C, pulse-labeled with [3H-methyl]-metionine for five minutes, and then chased with excess unlabeled methionine for up to 60 minutes. In wild-type cells, 35S pre-rRNA is quickly converted to 27S and 20S pre-rRNAs, and then these pre-rRNAs are matured into the 25S and 18S rRNAs (Fig. 2C, lanes 1-6) (Kressler et al., 1999). At 15°C, wild-type cells convert 20S pre-rRNA to 18S rRNA after 10-20 minutes of chase (Fig. 2C, lanes 1-6). However, in yrb2Δ cells, this conversion is delayed and occurs after 20-40 minutes of chase (Fig. 2C, lanes 7-12). Furthermore, the levels of 18S rRNA in yrb2Δ cells at the 40-60 minute timepoints are significantly lower than the levels of 25S rRNA. In contrast, wild-type cells produce equimolar quantities of the 18S and 25S rRNAs since both rRNA species are transcribed together in the 35S pre-rRNA. Therefore, in cells lacking YRB2, the nascent small ribosomal subunit appears to be less stable and its nuclear export is delayed.

To determine the effect of the yrb2Δ mutation on ribosome activity, we examined polysome profiles. When cell lysates were subjected to sucrose gradient centrifugation, the ribosome migrates as an 80S particle and as polysome peaks, which contain multiple 80S ribosomes. In wild-type cells, approximately 10% of the ribosomal subunits are not assembled into ribosomes, and these subunits migrate as 40S or 60S peaks (Fig. 3, left profile). In yrb2Δ cells, the amount of free 40S subunit is decreased while the amount of free 60S ribosomal subunit is dramatically increased (Fig. 3A, right profile). The amount of polysomes in yrb2Δ is marginally decreased, and these results suggest that the deficiency of the small ribosomal subunits is limiting mRNA translation. We examined the relative concentration of 60S to 40S ribosomal subunits by disassociating the ribosome and separating the subunits by sucrose gradient centrifugation. Wild-type cells contain an equal molar ratio of 60S to 40S ribosomal subunits and the ratio of 60S:40S subunits is 2.1:1 when measured by A254 because the 60S subunit contains twice as much RNA as the 40S subunit (Fig. 3B, left profile). In yrb2Δ, the ratio of 60S:40S is increased to 2.5:1 (t-test P<0.05, Fig. 3B, right profile). Therefore, the concentration of 40S ribosomal subunits is decreased by 10-20% relative to the 60S ribosomal subunit in yrb2Δ cells.

Overexpression of YRB2 also causes a defect in small subunit export similar to the effect on NES-containing proteins (Taura et al., 1998). YRB2 was expressed at high levels from a galactose-inducible promoter in wild-type yeast cells. After a 1 hour induction with galactose, 5′ ITS1 accumulates in the nucleoplasm of cells expressing YRB2 (Fig. 4Ac-d). Overexpression of YRB2 also causes the accumulation of 20S pre-rRNA (data not shown). 5′ ITS1 localization and pre-rRNA processing was not affected in galactose-treated cells containing the empty vector (Fig. 4Aa-b). While expression of YRB2 from its own promoter on a high copy plasmid is not toxic to wild-type cells, high copy expression of YRB2 impairs the growth of rat2Δ/nup120, a nucleoporin mutant defective in small ribosomal subunit export (see below), at the permissive temperature of 25°C (Fig. 4B). Only full-length YRB2 had this effect as N terminal, C terminal, or FG repeat YRB2 truncation mutants were not toxic to rat2-1 cells (Fig. 4B) (Taura et al., 1997). The percentage of rat2Δ cells mislocalizing ITS1 at the permissive temperature was mildly increased in the presence of high copy YRB2. The synergistic alteration in ribosome export in these cells may explain their impaired growth rate. In any case, these data indicate that proper levels of Yrb2 are required for the efficient nuclear export of the small ribosomal subunit.

The large ribosomal subunit appears to be exported out of the nucleus via a Crm1-dependent mechanism (Gadal et al., 2001; Ho et al., 2000b; Stage-Zimmermann et al., 2000). Therefore, we expected that the decreased efficiency of NES export in yrb2Δ would also cause defects in 60S ribosomal subunit export. However, we do not see mislocalization of the 60S nuclear export reporter proteins Rpl11b-GFP, Rpl25-GFP, or GALpro-Rpl25-GFP in yrb2Δ cells (data not shown). It may be that the 60S export assays are not sensitive enough to detect subtle export defects in yrb2Δ cells. Alternatively, Yrb2 is not involved in 60S export.
Identification of nucleoporin and Ran regulator mutants that are defective in small ribosomal subunit nuclear export

In order to further characterize small ribosomal subunit export, we performed the small ribosomal subunit export assay on libraries of ts– mutants, and we examined the involvement of known nuclear transport factors by analyzing the corresponding mutants in greater detail. In addition to examining ts– mutants at the restrictive temperature, our screens also used a shift-back protocol in which cells at 37°C were transferred back to 25°C. This shift-back step is useful when examining mutants that are defective in ribosome assembly at restrictive temperatures. Ribosome assembly defects prevent the detection of ribosome export defects. The shift-back protocol allows cells to resume ribosome assembly, and ribosome export defects become detectable if the rate of ribosome assembly exceeds the rate of ribosome export (Hurt et al., 1999).

The utility of the shift-back protocol is illustrated with the nucleoporin mutant nic96-1 that lacks the Xrn1 exonuclease (Zabel et al., 1996). At permissive temperatures, 5¢ ITS1 RNA is localized throughout the cytoplasm in these cells similar to NIC96 + xrn1D cells (data not shown). At the restrictive temperature, 5¢ ITS1 accumulates in the nucleolus in the nic96-1 xrn1D strain, indicating a defect in ribosome assembly (Fig. 5Ae-f).

Upon shift-back to permissive temperatures, the localization of 5¢ ITS1 RNA expands to fill the entire nucleus (Fig. 5Ag,h). In contrast, the localization of 5¢ ITS1 does not change in NIC96+ xrn1Δ cells at any temperature (Fig. 5Aa-d). Upon shifting back to permissive temperatures, the nic96-1 XRN1+ strain is less efficient than wildtype at converting 20S pre-rRNA to 18S rRNA (Fig. 5B, lanes 10-12). nic96-1 cells are still able to export the small ribosomal subunit export, at a reduced rate, since 18S rRNA is produced in these cells, but these results demonstrate that mild nuclear export defects can be detected using this shift-back protocol.

Fig. 4. Overexpression of YRB2 is toxic. (A) Overexpression of YRB2 causes the mislocalization of 5¢ ITS1. 5¢ ITS1 was localized in wild-type cells containing pGAL vector (b) or pGAL-YRB2 (d) after 1 hour induction with galactose. Chromosomal DNA was labeled with DAPI (a,c). Arrows point to DAPI-stained chromatins while arrowheads point to the nucleus. Magnified views of two cells are shown below each panel. (B) High-copy expression of YRB2 inhibits the growth of rat2-1/nup120 cells. rat2-1 (PSY209) or wild-type (PSY580) cells containing 2μ vector (pPS701), full-length YRB2 (pPS2096), N-terminal truncation (pPS2104), deletion of the Nup FG motifs (pPS2105), or the C-terminal/Ran-binding domain truncation (pPS2106) were grown on trp– dropout plates at 25°C.

Fig. 5. A shift-back protocol facilitates the detection of ribosome biogenesis defects. (A) 5¢ ITS1 was localized in xrn1Δ, nic96-1 xrn1Δ, and kap104-16 xrn1Δ after shifting to 37°C for 3 hours (b,f,j, respectively) or shifting back from 37°C to 25°C for 30 minutes (d,h,l). Chromosomal DNA was labeled with DAPI (a,c,e,g,i,k). (B) pre-rRNA processing in wild-type (lanes 1-6) and nic96-1 (lanes 7-12) cells shifted to 37°C or shifted-back to 25°C. Cells were pulse labeled for 3 minutes and chased for 0, 3 or 10 minutes. RNA was extracted and run on a formaldehyde-agarose gel. Sizes of the rRNAs are indicated.
Table 2. Classification of yeast mutants affecting the assembly and nuclear export of the small ribosomal subunit

<table>
<thead>
<tr>
<th>Class I: Mutations that affect the export of the small ribosomal subunit</th>
<th>Small ribosomal subunit nuclear export</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation</td>
<td>Permissive temp.</td>
</tr>
<tr>
<td>yrb2Δ</td>
<td>–*</td>
</tr>
<tr>
<td>ccm1Δ(T539C)</td>
<td>+</td>
</tr>
<tr>
<td>nic96-1</td>
<td>+</td>
</tr>
<tr>
<td>gle2/hup40 (N273K, D290N)</td>
<td>+</td>
</tr>
<tr>
<td>gle-1(W334Stop)</td>
<td>+</td>
</tr>
<tr>
<td>nup49-313</td>
<td>+</td>
</tr>
<tr>
<td>nup116-5</td>
<td>+</td>
</tr>
<tr>
<td>nup120Δ/rat2-1</td>
<td>–*</td>
</tr>
<tr>
<td>nup133Δ/rat3-1</td>
<td>–*</td>
</tr>
<tr>
<td>nup159Δ/rat7-1</td>
<td>–*</td>
</tr>
<tr>
<td>nsp1(10A)</td>
<td>–*</td>
</tr>
<tr>
<td>nsp1(L697P)</td>
<td>–*</td>
</tr>
<tr>
<td>prp20Δ(S297N)</td>
<td>+</td>
</tr>
<tr>
<td>yrb1Δ(F191S)</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Class II: Mutations that affect small ribosomal subunit assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>kap104-16</td>
</tr>
<tr>
<td>nmd5Δ</td>
</tr>
<tr>
<td>nup82Δ/108</td>
</tr>
<tr>
<td>rps31Δab</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Class III: Mutations that do not show defects in small ribosomal subunit assembly or export</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>nup85Δ/rat9-1</td>
</tr>
<tr>
<td>xrn1Δ</td>
</tr>
</tbody>
</table>

Class I mutants accumulated 5’ ITS1 RNA to the nucleoplasm. The conditions of nucleoplasmic accumulation are indicated as occurring under permissive conditions, restrictive conditions/temperatures, or upon shift back from 37°C to 25°C. + indicates wild-type export and – indicates a defect; nd, not done. Class II mutants affect the assembly of ribosomal subunits and accumulate 5’ ITS1 to the nucleolus as an xrn1Δ strain or to the cytoplasm as an XRN1+ strain. Class III mutants do not mislocalize 5’ ITS1 and do not have detectable defects in ribosome assembly or export. This table partially updates Table 1 described in Moy and Silver, 1999.

*Mislocalization of 5’ ITS1 is only detectable in XRN1+ strains.

1Mutants are defective in ribosome assembly; they accumulate 5’ ITS1 to the nucleolus as a xrn1Δ strain.

1Mutant is also defective in small ribosomal subunit nuclear export.

The shift-back protocol is also useful in identifying mutants that affect ribosome assembly. We previously reported that kap104-16 xrn1Δ and nmd5Δ xrn1Δ strains do not mislocalize 5’ ITS1 RNA at 25°C or at 37°C (Moy and Silver, 1999). Using the shift-back protocol, we show that a small amount of 5’ ITS1 accumulates in the nucleolus of these cells (Fig. 5AΔ1). Mammalian orthologues of KAP104 and NMD5 have been shown to be importers of ribosomal proteins (Jakel and Görlich, 1998). We interpret the defect of kap104-16 and nmd5Δ to be a result of decreased import of ribosomal proteins.

In an attempt to identify additional factors important for export of the small ribosomal subunit, we screened 960 ts- mutants with the small ribosomal subunit export assay. Twelve mutants were found to mislocalize 5’ ITS1 RNA. We determined that nine of these mutants are defective in the RAT2/NUP120, RAT3/NUP133, RAT7/NUP159, NSP1, GLE2/NUP40, MTR4/DDB1, PRP20, and YRB1 genes (Table 2). Two independent rat3Δts- mutants were identified by this screen.

The RAT2, RAT3, and RAT7 (mRNA trafficking) genes encode nucleoporins that are required for mRNA export (Gorsch et al., 1995; Heath et al., 1995; Li et al., 1995). Both the rat2-1 and the rat2-2 alleles display strong small ribosomal subunit assembly and export defects. 5’ ITS1 accumulated in the nucleolus in rat2-1 xrn1Δ cells shifted to the restrictive temperature (Moy and Silver, 1999), and 5’ ITS1 accumulated to the entire nucleoplasm after shifting back to the permissive temperature (data not shown). In rat2-1 XRN1+ cells, 5’ ITS1 accumulated in the nucleoplasm at both permissive temperatures (Fig. 6Aβ,ι) and upon shift back. These data suggest that RAT2/NUP120 is involved in both ribosome assembly and nuclear export.

The rat3-1 and rat7-1 mutants have milder defects in small ribosomal subunit nuclear export; the conditions in which these mutants mislocalize 5’ ITS1 are more limited. In rat3-1 cells, nucleoplasmic accumulation of 5’ ITS1 is most noticeable when these cells are grown to stationary phase and then transferred to fresh media. These conditions allow cells to
resume growth which stimulates ribosome biogenesis and facilitates the detection of ribosome export defects (Hurt et al., 1999). In rat7-1 cells, nucleoplasmic accumulation of 5’ ITS1 was detectable only when the mutant contained the Xrn1 exonuclease; rat7-1 xrn1D cells did not mislocalize 5’ ITS1 at restrictive temperatures or upon shift-back (Moy and Silver, 1999).

rat7-1 XRN1 + cells accumulate nucleoplasmic 5’ ITS1 at all temperatures (Fig. 6Ac,j; Table 2). Furthermore, the conversion of 20S pre-rRNA to 18S rRNA was defective in rat7-1 cells (Fig. 6B, lanes 4-6). Similar to the yrb2D mutant, the rat7-1 mutant appears to be less efficient in the export of the small ribosomal subunit.

In our screen of the ts– mutant libraries, we also identified a novel allele of the nucleoporin NSP1. Previously, we have shown that the nsp1(10A) xrn1Δ strain is defective in ribosome assembly in that it accumulated 5’ ITS1 to the nucleolus at the restrictive temperature (Moy and Silver, 1999). Here, we identify a nsp1 mutant that encodes a protein with a single amino acid change in which leucine 697 is altered to a proline. The nsp1(L697P) mutant accumulates 5’ ITS1 to the entire nucleoplasm at all temperatures (Fig. 6Ad,k; Table 2). Addition of a plasmid containing wild-type NSP1 rescues the mislocalization defect of the nsp1(L697P) mutant (data not shown). At 37°C, nsp1(L697P) is delayed in processing the 35S, 32S and 20S pre-rRNAs (Fig. 6B, lanes 10-12). We conclude that Nsp1 affects both assembly and nuclear export of the small ribosomal subunit.

We identified a gle2 mutant that accumulates 5’ ITS to the nucleoplasm (Fig. 6Af,m) and has a mild delay in 20S pre-rRNA processing at the restrictive temperature (Fig. 6B, lanes 13-15). This gle2 mutant encodes a protein containing two amino acid changes in which asparagine 273 is altered to a lysine and aspartate 290 is altered to an asparagine. Previously, we reported that the gle2-1 xrn1D mutant does not mislocalize 5’ ITS1 at permissive or restrictive temperatures (Moy and Silver, 1999; Murphy et al., 1999). The xrn1D mutation appears to have obscured the ribosome export defect because the gle2-1 XRN1 + mutant accumulates 5’ ITS1 at the restrictive temperature (data not shown).

In addition to the nucleoporin mutants identified by our ts– screen, we examined previously characterized nucleoporin mutants to determine whether they affect small ribosomal subunit export. Similar to the nic96-1 xrn1Δ mutant, nup49-313 xrn1Δ and nup116-5 xrn1Δ mutants accumulate 5’ ITS1 to the nucleus after shifting the cells back to their permissive temperature. Furthermore, the nup49-313 mutant has a delay in 20S pre-rRNA processing after the shift back in temperature (data not shown; Table 2).

Finally, we identified two novel alleles of genes encoding regulators of the Ran GTPase: the RanGEF, PRP20, and the RanGAP accessory factor, YRB1. The yrb1 mutant encodes a protein in which phenyalanine 191 is mutated to a serine. This allele is similar to the previously identified yrb1-1 allele in that the majority of cells, when shifted to the restrictive temperature, accumulate nucleoplasmic 5’ ITS1 (Fig. 6Af,m) and 20S pre-rRNA (Fig. 6B, lanes 16-18).
The novel \textit{prp20} allele is different from the much-studied \textit{prp20-1} allele. The \textit{prp20} allele that we identified encodes a protein in which serine 297 is converted into an asparagine. This results in a weak allele of \textit{PRP20} with regard to nuclear transport defects. Although this \textit{prp20(S297N)} strain does not grow at 37°C, at this restrictive temperature only a small fraction of cells (10-20%) exhibit nucleoplasmic accumulation of 5’ ITS1 (Fig. 6Ag,n; data not shown). In contrast, greater than 95% of \textit{prp20-1} cells accumulate 5’ ITS1 at restrictive temperatures (Moy and Silver, 1999). Furthermore, we cannot detect an mRNA export defect in \textit{prp20(S297N)} while the \textit{prp20-1} mutant has a strong mRNA export defect (Amberg et al., 1993). Interestingly, the \textit{prp20(S297N)} mutant was previously identified from the same \textit{ts}− mutant library in a screen for mutants that mislocalize Npl3 (Corbett and Silver, 1996).

**Discussion**

In this paper, we used a novel assay in combination with yeast mutants to further define the requirements for proper export of the small ribosomal subunit out of the nucleus. \textit{YRB2} is required for both the stability of the nascent small ribosomal subunit and the efficient nuclear export of this subunit. In addition, a large-scale screen of temperature-sensitive mutants yielded additional evidence for the involvement of Ran and the nuclear pore complex in small ribosomal subunit export. These findings are discussed in the context of what we know about the role of these factors in nuclear export. In previous studies, we showed that small subunit export is inhibited in the temperature-sensitive mutant \textit{xpo1-1}. However, it remained a formal possibility that this was an indirect effect since the \textit{xpo1-1} mutant mislocalizes the RanGAP Rna1 to the nucleus at the restrictive temperature resulting in the disruption of the Ran gradient (Feng et al., 1999). Consequently, when \textit{xpo1-1} cells are shifted to the restrictive temperature, they immediately show a defect in mRNA export (Stade et al., 1997), which can be suppressed by overexpression of \textit{DBP5}, an RNA helicase essential for mRNA export (Hodge et al., 1999). In contrast, upon leptomycin B addition, the \textit{crm1(T539C)} mutant accumulates the NES reporter in the nucleus within 5 minutes, but the \textit{crm1(T539C)} strain does not accumulate mRNA in the nucleus until 1 hour after treatment, suggesting that the primary defect is indeed in NES-dependent export (Neville and Roshash, 1999). We now show that LMB-treated \textit{crm1(T539C)} cells accumulate the small ribosomal subunit in the nucleus with timing similar to that of NES accumulation. Therefore, the involvement of Crm1 in small ribosomal subunit export is likely to be direct.

Yrb2 is a member of the Ran binding protein family and is required for the efficient export of Crm1-mediated cargo (Taura et al., 1998). Here, we show that the export of the small ribosomal subunit is delayed in \textit{yrb2Δ} cells. Initially, we could not detect mislocalization of 5’ ITS1 in \textit{yrb2Δ xrn1Δ} cells because the accumulation of cytoplasmic 5’ ITS1 fragment conceals the small ribosomal subunit export defect. However, in \textit{yrb2Δ XRN1}+ cells we could detect nuclear accumulation of 5’ ITS1 and the delay in processing 20S pre-rRNA.

The exact function of Yrb2 in Crm1-mediated export is still not clear. In vitro, Yrb2 disassociates the Crm1/NES/Ran-GTP export complex so Yrb2 may function in the terminal release step in Crm1-mediated export (Maurer et al., 2001). In contrast, the mammalian orthologue of Yrb2, RanBP3, stimulates formation of the export complex (Lindsay et al., 2001). These contrasting activities of Yrb2 and RanBP3 may stem from different experimental conditions. When RanBP3 is at sub-stoichiometric concentrations, the Crm1/NES/Ran-GTP complex formation is increased while higher concentrations of RanBP3 inhibit complex formation (Englmeier et al., 2001). Importantly, RanBP3 has variable effects on Crm1 export complex formation depending on the cargo substrate. When Snurportin and the leucine-rich NES cargos are mixed with Crm1 and RanGTP, the Crm1-Snurportin-Ran-GTP complex is favored over the Crm1-NES-Ran-GTP complex because Snurportin forms a higher affinity complex (Englmeier et al., 2001; Paraskeva et al., 1999). However, when RanBP3 is added to this mixture, the Crm1/NES/RanGTP complex is efficiently formed and the complex formation of Crm1/Snurportin/RanGTP is decreased (Englmeier et al., 2001).

The differential effects of RanBP3 on Crm1 function may help to explain why loss of Yrb2 in yeast affects the biogenesis of the small ribosomal subunit more than the biogenesis of the large ribosomal subunit. Both ribosomal subunits appear to use a Crm1-dependent export pathway (Galal et al., 2001; Ho et al., 2000b). If Yrb2 functions similarly to RanBP3, Yrb2 may favor the nuclear export of the small ribosomal subunit over the export of the large ribosomal subunit. The decreased abundance of the small subunit in \textit{yrb2Δ} cells could be a result of the degradation of unexported small subunits. Nuclear export of 60S subunits is required for their stability (Ho and Johnson, 1999; Ho et al., 2000a).

The question remains as to how Crm1 and Yrb2 promote export. One possibility is that Crm1 binds directly to the small subunit. However, despite extensive attempts we could not detect Crm1 bound to small subunits and could not reconstitute Crm1 binding to purified ribosomal subunits. An attractive alternative is that there is an adaptor protein that promotes binding of Crm1 to the small subunit. Such an adaptor would be analogous to the role of Nmd3 in promoting binding of Crm1 to the large subunit. Yrb2 could be such an adaptor. However, \textit{YRB2} is not essential for normal growth while one might expect such an adaptor to be essential if it is a critical part of the small subunit export pathway. Further experiments will be required to identify such an adaptor.

In order to identify additional \textit{trans}-acting factors involved in small ribosomal subunit export, we screened 960 \textit{ts}− mutants with the 5’ ITS1 localization assay. We identified 5 nucleoporin mutants and 2 Ran regulator mutants that are defective in small subunit export. Interestingly, no novel factors were identified. This could indicate that it may be difficult to generate conditional alleles of such factors. Alternatively, there may be inherent limitations to the assay presented here as screens of the same collection have yielded large numbers of mutants defective in assembly and export of the large ribosomal subunit (Bassler et al., 2001; Galal et al., 2001; Milkreit et al., 2001).

From our \textit{ts}− screen, we identified three mutants in which the \textit{ts}− mutation was not linked to the 5’ ITS1 mislocalization phenotype. After separation from the \textit{ts}− mutation, the 5’ ITS1 mislocalizing mutants grew at normal rates at all temperatures (data not shown). Since the characteristics of these mutants do not match the phenotypes of known nuclear transport factors,
these mutants may correspond to novel factors involved in small ribosomal subunit export.

In total, we examined 17 nucleoporin mutants with the 5′ ITS1 localization assay. Twelve of these mutants are defective in the assembly of the small ribosomal subunit (Moy and Silver, 1999). These assembly defects could be caused by alterations in the nuclear export of mRNAs encoding ribosomal proteins or by defects in the nuclear import of ribosomal proteins, assembly factors, or ribosome biogenesis regulators. Seven of these nucleoporin mutants are also defective in the nuclear export of the small ribosomal subunit (Table 2). Two nucleoporin mutants are defective in small ribosomal subunit export, but do not have detectable defects in ribosome assembly. Overall, these results emphasize the multiple transport functions of nucleoporins and the importance of the NPC in ribosome biogenesis.

It is interesting to speculate on the role of certain nucleoporins in ribosome export. It could be that certain nucleoporins define docking sites on either side of the NPC that are critical for binding and/or release. They could also define contact sites within the NPC that the ribosome makes as it passes through the channel. The manner in which a large particle such as a ribosomal subunit passes through the NPC remains one of the outstanding questions in cell biology and further analysis of these and other transport mutants in combination with biochemical assays will help to further elucidate the process.

We thank all of the investigators who generously shared yeast strains and reagents. We thank Elissa Lei and Tetsuya Taura for critical reading of this manuscript and for their support. This work was funded by grants from the National Institutes of Health to P.A.S. and an Institute for T.I.M. reading of this manuscript and for their support. This work was funded by grants from the National Institutes of Health to P.A.S. and an National Cancer Institute training grant to the Dana-Farber Cancer Institute for T.I.M.

References


