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J. H. Ho, G. Kallstrom and A. W. Johnson

RNA 2000 6: 1625-1634

RNA (2000), *6*:1625–1634. Cambridge University Press. Printed in the USA.

Nascent 60S ribosomal subunits enter the free pool bound by Nmd3p

JENNIFER HEI-NGAM HO, GEORGE KALLSTROM, and ARLEN W. JOHNSON

Section of Molecular Genetics and Microbiology and The Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712-1095, USA

ABSTRACT

Nmd3p from yeast is required for the export of the large (60S) ribosomal subunit from the nucleus (Ho et al., 2000). Here, we show that Nmd3p forms a stable complex with free 60S subunits. Using an epitope-tagged Nmd3p, we show that free 60S subunits can be coimmunoprecipitated with Nmd3p. The interaction was specific for 60S subunits; 40S subunits were not coimmunoprecipitated. Using this coprecipitation technique and pulse-chase labeling of ribosomal subunit proteins we showed that Nmd3p bound nascent subunits, consistent with its role in export. However, under conditions in which ribosome biogenesis was inhibited (e.g., inhibition of transcription with thiolutin, inhibition of transcription of ribosomal protein and RNA genes in a *sly1-1* mutant at nonpermissive temperature, and inhibition of translation in a conditional *prt1* mutant), Nmd3p remained associated with 60S subunits. In addition, Nmd3Δ120, a truncated protein that lacked a nuclear localization signal, retained 60S binding. These results suggest that Nmd3p recruits nascent 60S subunits into the pool of free 60S subunits and exchanges on 60S subunits as they recycle during translation.

Keywords: immunoprecipitation; Nmd3p; nuclear export; ribosome; translation

INTRODUCTION

In eukaryotic cells, ribosomal subunits are assembled in the nucleolus (Kressler et al., 1999b; Venema & Tollervey, 1999) and are subsequently exported from the nucleus as preassembled complexes (Warner, 1989; Raué & Planta, 1991; Venema & Tollervey, 1999). Nuclear export requires nuclear export signals (NESs) on the cargo molecules and recognition of these signals by receptor proteins that then mediate export via interaction with the nuclear pore complex (Mattaj & Englmeier, 1998; Stutz & Rosbash, 1998; Görlich & Kutay, 1999; Nakielny & Dreyfuss, 1999; Strässer & Hurt, 1999). Our previous work on Nmd3p from yeast showed that Nmd3p is an essential protein required for a late step in biogenesis of the large (60S) ribosomal subunit. This was most clearly demonstrated in a temperature-sensitive *nmd3-4* mutant, which, at nonpermissive temperature, processed rRNAs with normal kinetics, but the resulting nascent 25S rRNA was extremely unstable ($t_{1/2} = 4$ min). The stability of mature subunits made before the temperature shift did not appear to be affected (Ho & Johnson, 1999). Because the processing and assembly of ribosomal subunits are tightly coordinated (Warner, 1989, 1999), we interpreted the instability of 25S rRNA to indicate a failure in the 60S biogenesis pathway after subunit assembly. In related experiments, the overexpression of a truncated Nmd3p led to inhibition of 60S subunit biogenesis (Belk et al., 1999). Although Nmd3p is predominantly a cytoplasmic protein and associated with free 60S subunits (subunits not associated with 40S subunits) (Ho & Johnson, 1999), we have recently shown that Nmd3p shuttles and that it is required for nuclear export of the 60S subunit (Ho et al., 2000). Indeed, deletion of the NES of Nmd3p traps both Nmd3p and 60S subunits in the nucleus. Thus Nmd3p may provide the NES for export of the 60S subunit.

60S subunits entering the cytoplasm require the loading of several additional ribosomal proteins, including the small acidic proteins P1 and P2 and possibly three other proteins, including the exchangeable protein Rpl10p (Zinker & Warner, 1976; Eisinger et al., 1997). Kinetic analysis in yeast of the appearance of 60S subunits in the cytoplasm and their incorporation into actively translating polysomes indicates that there is a pool of newly formed 60S subunits not yet in polysomes, suggesting the existence of a slow cyto-

Reprint requests to: Arlen Johnson, Section of Molecular Genetics and Microbiology, ESB325, The University of Texas at Austin, Austin, Texas 78712-1095, USA; e-mail: arlen@mail.utexas.edu.

plasmic maturation step (Warner, 1971). Human ribosome biogenesis in HeLa cells also displays a slow cytoplasmic 60S step (Warner, 1966). The nature of this maturation event is not known, but could be a conformational change in the large subunit, a slow protein loading or exchange reaction or a modification of a 60S component.

Finally, the subunit must be recruited to the translation initiation complex waiting at the initiation codon to form an 80S ribosome and initiate translation. Joining of the 60S subunit to the 40S subunit requires the presence of Rpl10p on the 60S subunit (Eisinger et al., 1997) and the release of eIF2 from the initiation complex (reviewed in Merrick, 1992; Voorma et al., 1994; Pain, 1996). This release is stimulated by eIF5 and eIF5B (Pestova et al., 2000). Surprisingly, little is known about recruitment of the 60S subunit to the initiation complex. In addition, the free 60S subunit, which is believed to be the substrate for subunit joining, has not been extensively characterized.

Nmd3p is a highly conserved protein; orthologs are found from archaebacteria and eukaryotes but not eubacteria. All of these proteins share a conserved 43-kDa amino terminal domain that contains four repeats of Cys-x-x-Cys, a putative Zn^{2+}-binding motif similar to that found in some ribosomal proteins (Rivlin et al., 1999). The eukaryotic Nmd3p-like proteins have C-terminal extensions, which in the case of Nmd3p contains a nuclear localization signal (NLS) as well as an NES. These shuttling signals are essential for the ability to export 60S subunits from the nucleus. However, the existence of related proteins in archaebacteria, which lack nuclei, indicates a more ancient and fundamental function for Nmd3p. This could be a catalytic role in maturation of the 60S subunit separate from export, or a role in the recycling of free 60S subunits in the cytoplasm. It is interesting to note similarities between archaebacteria and eukaryotes with respect to rRNA processing and translation initiation. It has recently been shown that archaebacteria have small nucleolar-like RNAs (Gaspin et al., 2000; Omer et al., 2000) previously thought to be restricted to the eukaryotes, implying eukaryote-like rRNA processing mechanisms. In addition, the conservation of translation initiation factors between archaebacteria and eukaryotes suggests a conservation of mechanisms of translation (Dennis, 1997; Kyrpides & Woese, 1998).

In our efforts to understand the role of Nmd3p in 60S subunit biogenesis we epitope tagged Nmd3p to examine its interaction with additional proteins. We show here that Nmd3p forms a stable complex with free 60S subunits and have used this interaction to examine the nature of the free 60S subunit bound by Nmd3p. Our results demonstrate that Nmd3p binds both nascent 60S subunits, consistent with its role in subunit export, as well as mature free 60S subunits, suggesting an additional role for Nmd3p in recycling 60S subunits.

RESULTS

60S subunits are immunoprecipitated with c-mycNmd3p

A DNA cassette expressing 13 tandem copies of the c-myc epitope was integrated into the 3′ end of the genomic locus of *NMD3*. The resulting gene fusion expressed c-mycNmd3p from the genomic locus under control of its native promoter. The fusion protein was expressed at levels similar to wild-type protein (data not shown) and strains bearing this tagged allele of *NMD3* appeared to grow as well as wild-type (data not shown), indicating that the C-terminally tagged protein was functional. The epitope-tagged protein was predominantly cytoplasmic (Ho et al., 2000), consistent with our cell fractionation work (Ho & Johnson, 1999). To gain a greater understanding of the role of Nmd3p in 60S maturation, we used the c-mycNmd3p to look for associated proteins by coimmunoprecipitation. Coomassie-stained SDS-PAGE gels of proteins coimmunoprecipitated with c-mycNmd3p indicated the presence of a very large protein complex (Fig. 1A, c-myc+). The coprecipitating proteins were specific to c-mycNmd3p, as no proteins were immunoprecipitated from extracts prepared from wild-type (untagged) cells

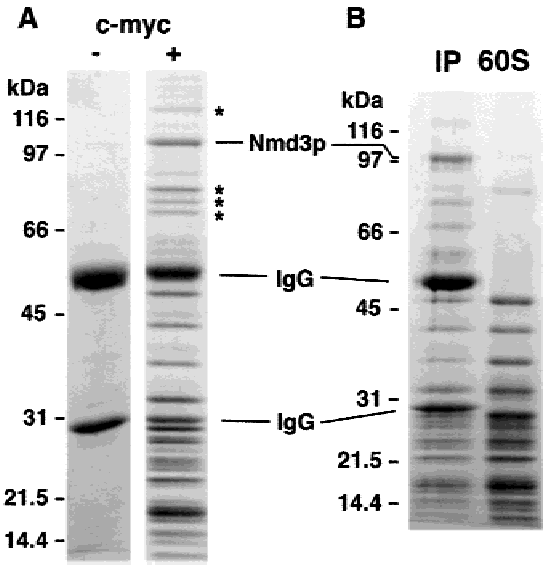

FIGURE 1. Immunoprecipitation of an Nmd3p-60S complex. **A**: Extracts were prepared from wild-type cells (CH1305, c-myc−) or c-mycNmd3p expressing cells (AJY272, c-myc+) and immunoprecipitated with the addition of anti-c-myc antibody and protein A beads as described in Materials and Methods. Proteins were analyzed by SDS-PAGE on 6–20% gels. The positions of Nmd3p, IgG, and molecular weight markers are indicated. Additional proteins that were not observed in standard 60S preparations are indicated by asterisks. **B**: Proteins coimmunoprecipitated with c-mycNmd3p (IP) were compared with proteins from purified 60S subunits (60S) as described in Materials and Methods. The positions of IgG and molecular weight markers are indicated.

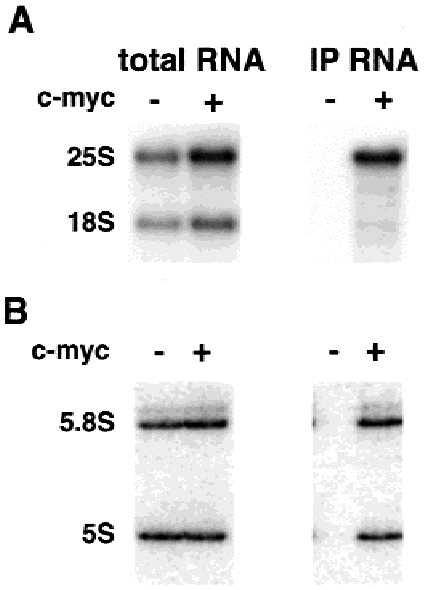

FIGURE 2. RNA analysis of the Nmd3p-60S complex. Cultures of wild-type cells (CH1305, c-myc−) or c-mycNmd3p expressing cells (AJY272, c-myc+) were labeled in vivo with $^{32}PO_4$. RNA was prepared from total cellular extracts (total) or from the material coimmunoprecipitated with c-mycNmd3p (IP). RNAs were separated in 1% agarose formaldehyde gels (**A**) or in 6% polyacrylamide-urea gels (**B**). Gels were dried and autoradiographed.

(Fig. 1A, c-myc−). The pattern of protein bands associated with c-mycNmd3p appeared similar to that of ribosomal 60S subunit proteins. This was confirmed by direct comparison of proteins from purified 60S ribosomal subunits with the proteins coimmunoprecipitated with c-mycNmd3p (Fig. 1B). In addition, several proteins were present in the immunoprecipitate that were not found in the purified 60S subunit preparation (asterisks in Fig. 1A). We are currently characterizing these associated proteins using mass spectrometry. Although we had previously shown that Nmd3p was associated with free 60S subunits on sucrose gradients, such coimmunoprecipitation of 60S subunits with a nonribosomal protein has not been reported previously and indicated a strong interaction between Nmd3p and the 60S subunit. Indeed, the Nmd3p-60S complex was stable to incubation overnight and in buffer containing EDTA or 250 mM NaCl (data not shown).

We examined the RNA composition of the Nmd3p-60S complex coimmunoprecipitated from cells labeled in vivo with [$^{32}PO_4$]. As expected, the complex contained all three 60S subunit rRNAs: 5S, 5.8S, and 25S (Fig. 2). The immunoprecipitation was highly specific for 60S subunits, as the immunoprecipitated fraction was largely free of 18S rRNA (Fig. 2). This specificity for 60S was consistent with our observation that Nmd3p cosediments in sucrose gradients in the position of free 60S subunits (Ho & Johnson, 1999).

Nmd3p binds directly to the 60S subunit

To determine if Nmd3p alone was sufficient for binding to the 60S subunit we asked if the Nmd3p-60S complex could be reconstituted in vitro using purified Nmd3p and 60S subunits. Glutathione S transferase (GST) fused to the amino terminus of Nmd3p (GST-Nmd3p) or GST alone was expressed at high levels and purified from yeast (Fig. 3, lanes 2 (GST-Nmd3) and 1 (GST)). The fusion protein was functional, indicated by complementation of an *nmd3::TRP1* disruption mutant (data not shown). Wild-type 60S ribosomal subunits were prepared from the total pool of 60S subunits as described in Materials and Methods (Fig. 3, lane 3). We then incubated GST-Nmd3p or GST alone with purified 60S subunits under conditions established for subunit joining (see Materials and Methods). Proteins were repurified on glutathione-sepharose beads and bound proteins were eluted with glutathione. As seen in Figure 3, 60S subunits were efficiently retained by GST-Nmd3p (lane 4) but not by GST alone (lane 5). The binding of 60S subunits to GST-Nmd3p in this highly purified system indicated that Nmd3p binds directly to the 60S subunit without the need of additional nonribosomal proteins.

Nascent 60S subunits are bound by Nmd3p

The requirement for Nmd3p in the nuclear export of 60S subunits together with its physical association with

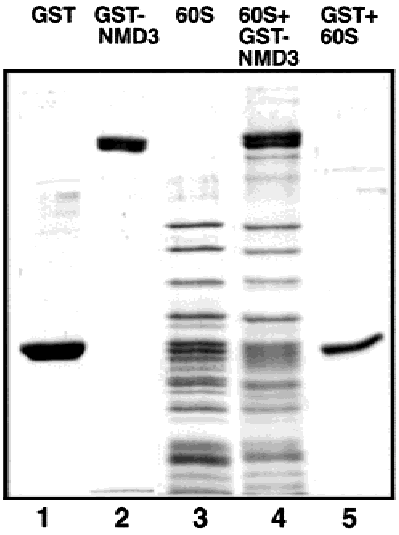

FIGURE 3. Nmd3p binding to 60S is direct. Coomassie blue-stained SDS-(12%) polyacrylamide gel of purified proteins and binding products. GST and a GST-Nmd3p fusion protein were overexpressed and purified from yeast (lanes 1 and 2, respectively). 60S subunits were also purified from yeast (lane 3). GST-Nmd3p or GST alone were incubated with 60S subunits, repurified by binding to glutathione sepharose beads, and eluted with glutathione (lanes 4 and 5, respectively) (see Materials and Methods for details).

60S subunits suggested that Nmd3p binds to nascent subunits. We tested this idea by pulse-chase labeling ribosomal proteins with [^{35}S]methionine and immunoprecipitation of the Nmd3p-60S complex at various times before and after labeling. Immunoprecipitated proteins were analyzed by western blotting for c-mycNmd3p and the 60S subunit protein L12 and then by autoradiography for labeled proteins. The amount of [^{35}S] label in the immunoprecipitated proteins was greatest immediately following the labeling and had decreased to a minimum by 20 min after the addition of unlabeled methionine (Fig. 4). The signal for L12 and for c-mycNmd3p was relatively constant throughout the time course of this experiment. Quantitation of the amount of [^{35}S] in a given ribosomal protein band relative to the total amount of ribosomal proteins deduced from the L12 western blot indicated that the specific activity decreased 11-fold from 0 to 20 min. These results demonstrate that the nascent subunits chase through the Nmd3p-60S complex.

Mature 60S subunits are bound by Nmd3p

If Nmd3p bound only nascent 60S subunits, we would have expected a greater decrease in the amount of signal in the ribosomal proteins bound by Nmd3p reflecting the 5,000-fold drop in specific activity of [^{35}S]methionine upon the addition of unlabeled methionine. A drop of only 11-fold could be explained by Nmd3p binding to mature subunits as well as nascent subunits. To test this, we asked if Nmd3p bound to 60S subunits under conditions in which ribosome biogenesis was blocked. This was done three ways: with a conditional mutation in *SLY1*, a gene required for the secretory pathway that in turn is required for ribosome biogenesis (Mizuta & Warner, 1994); using the transcription inhibitor thiolutin; and inhibiting translation initiation with a conditional mutation in *PRT1*, encoding a subunit of eIF3.

When a *sly1-1* strain is shifted to nonpermissive temperature, there is an immediate and coordinated inhibition of transcription of genes for ribosomal RNA and protein (Mizuta & Warner, 1994). We introduced a centromeric plasmid expressing c-mycNmd3p into a *sly1-1* strain. After shift to nonpermissive temperature for 2 h, 60S proteins were efficiently coimmunoprecipitated with Nmd3p and were stoichiometric with Nmd3p as seen with wild-type strains (Fig. 5A). We confirmed that rRNA transcription was blocked under these conditions by labeling with [$^{32}PO_4$] after shift to nonpermissive temperature (Fig. 5B). We also examined the position of Nmd3p in sucrose gradients after shifting the *sly1-1* mutant to nonpermissive temperature (Fig. 6). Under these conditions Nmd3p remained in the position of free 60S subunits. Cosedimentation of Nmd3p with free 60S was also observed in the *sly1-1* mutant at permissive temperature and in wild-type cells at all temperatures (data not shown). Nmd3p was also present in the position of 80S and polysomes but at a much lower level with respect to the ribosomal protein L3. A similar distribution of Nmd3p was observed in wild-type cells (data not shown). The dramatic increase in 80S and reduction in polysomes in the *sly1-1* mutant at nonpermissive temperature mutant (Fig. 6) was probably due to growth arrest of the *sly1-1* mutant at nonpermissive temperature arrest and unrelated to *NMD3* function.

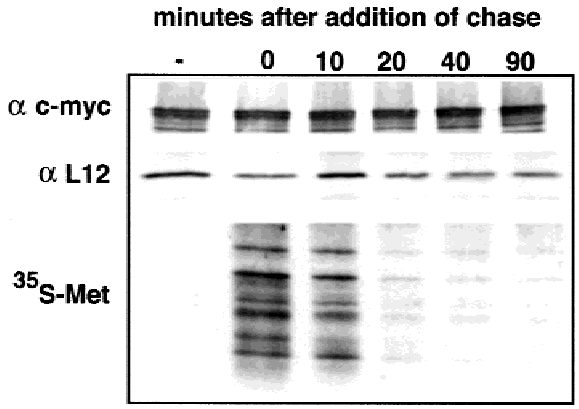

FIGURE 4. Nascent subunits chase through the Nmd3p-60S complex. Cells were labeled for 5 min with [^{35}S]methionine followed by the addition of excess unlabeled methionine as described in Materials and Methods. Samples were removed before labeling (−), before the addition of chase (0), and at the indicated times after the addition of chase. Extracts were prepared and proteins immunoprecipitated with anti-c-myc antibody. Immunoprecipitated proteins were analyzed by western blotting for Nmd3p (α c-myc) and L12 (α L12) and visualized by autoradiography (^{35}S-Met).

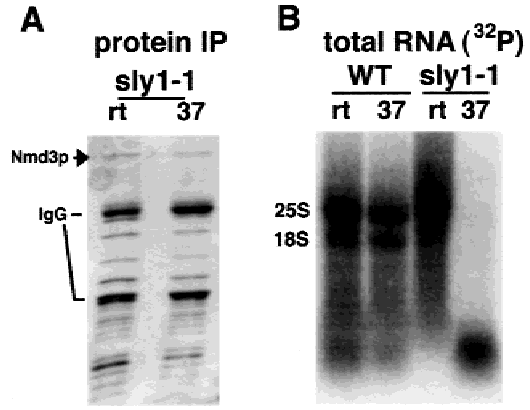

FIGURE 5. Coimmunoprecipitation of 60S with Nmd3p in a *sly1-1* mutant at nonpermissive temperature. Cultures of W303 (WT) and 312XX (*sly1-1*) containing pAJ401 (c-mycNmd3p) were grown at room temperature or at 37 °C for 2 h. Extracts were prepared, immunoprecipitated with anti-c-myc antibody, and the immunoprecipitated proteins analyzed by SDS-PAGE and staining with Coomassie blue. The position of Nmd3p and immunoglobulin bands are indicated. The same results were obtained with W303 (data not shown). **B**: Cultures as described in **A** were labeled for 30 min with $^{32}PO_4$ and total RNA was prepared. The RNA was visualized by autoradiography after electrophoresis through a 1% agarose gel and transfer to a nylon membrane.

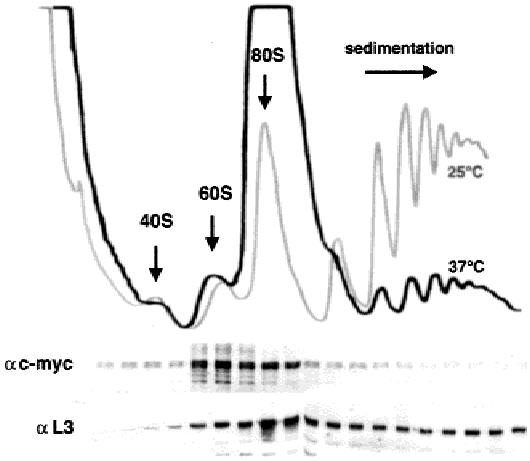

FIGURE 6. Cosedimentation of Nmd3p with free 60S in a *sly1-1* mutant at nonpermissive temperature. Extracts were prepared from cultures as described in Figure 5 and fractionated by sedimentation through sucrose gradients. The gradients were analyzed by continuously monitoring the UV absorbance at 254 nm (black trace: *sly1-1* strain at 37 °C; gray trace: *sly1-1* strain at 26 °C). The polysome profile of the WT strain at both temperatures was similar to that of the *sly1-1* mutant at 26 °C (data not shown). Fractions from the gradient were collected and analyzed by western blotting for c-mycNmd3p and L3. The western blots for the *sly1-1* strain grown at 37 °C are shown. Similar western blotting results were obtained for the *sly1-1* strain at 26 °C and for the WT strain at both temperatures (data not shown).

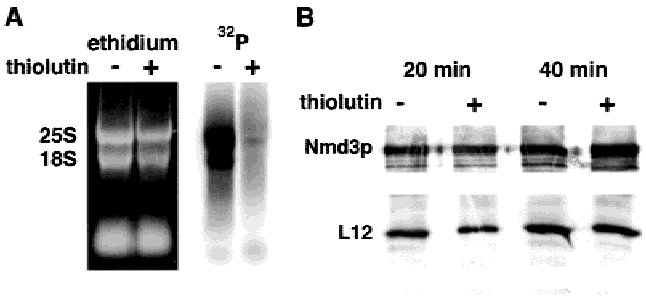

FIGURE 7. Nmd3p remains associated with 60S subunits in the absence of rRNA transcription. Thiolutin was added at 20 μg/mL to a culture of strain AJY272. As a control, samples were prepared in parallel from a culture not treated with thiolutin. After 10 min, $^{32}PO_4$ was added and cells were labeled for 50 min. **A**: Total RNA was prepared, separated on an agarose gel, and analyzed by ethidium bromide staining followed by autoradiography after transfer to nylon membrane. **B**: Alternatively, cells were harvested after 20 or 40 min of incubation in thiolutin, and extracts were prepared and immunoprecipitated with anti-c-myc antibody. The immunoprecipitated proteins were analyzed by SDS-PAGE and western blotting for c-mycNmd3p and for the 60S protein L12 as an indicator of coprecipitating 60S subunits. Similar results were obtained with an antibody specific for L3 (data not shown).

Because a *sly1-1* mutation inhibits ribosome biogenesis in an indirect way, we examined Nmd3p-60S interaction after rRNA transcription was inhibited by thiolutin, an inhibitor of fungal RNA polymerases (Jimenez et al., 1973). We found that 20 μg/mL thiolutin effectively blocked RNA pol I transcription. As in the case of a *sly1-1* mutant at nonpermissive temperature, 60S subunits were efficiently coimmunoprecipitated with c-mycNmd3p when rRNA transcription was inhibited with thiolutin (Fig. 7A,B). Similar results were obtained using a temperature-sensitive mutation in *PRT1*, *prt1-63*, which encodes a subunit of translation initiation factor 3 (data not shown). The inhibition of translation in a *prt1* mutant at nonpermissive temperature blocks the production of ribosomal proteins thereby inhibiting ribosome biogenesis. Thus under all conditions tested in which ribosome biogenesis was blocked, Nmd3p continued to bind 60S subunits.

The experiments described above in which ribosome biogenesis was inhibited did not rule out the possibility that subunits in the biogenesis pathway become stalled and remain associated with maturation factors when biogenesis is inhibited. Thus the association with Nmd3p could represent such stalled maturation intermediates. Although the pulse-chase labeling experiment argued against this possibility, we sought another means of addressing this issue. Nmd3Δ120 is a C-terminal truncation of Nmd3p that lacks both the NLS and NES and is consequently restricted to the cytoplasm. Because these shuttling signals are essential for 60S export and biogenesis, Nmd3Δ120 cannot support ribosome biogenesis (Ho et al., 2000). We asked if 60S subunits could be coimmunoprecipitated with Nmd3Δ120. Low-copy centromeric plasmids expressing c-myc-tagged Nmd3Δ120, c-myc-tagged full-length Nmd3p, and full-length Nmd3p without a c-myc tag were introduced into a wild-type strain. Extracts were prepared and proteins were immunoprecipitated by the addition of anti-c-myc antibody and protein A beads. The immunoprecipitated proteins were then analyzed by SDS-PAGE and western blotting for Nmd3p and the 60S subunit protein L12. As seen in Figure 8, L12 was coimmunoprecipitated with c-mycNmd3Δ120. No L12 was detected in the control lacking a c-myc-tagged Nmd3p. Because

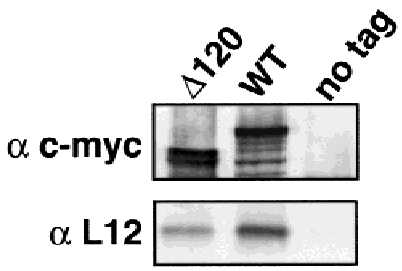

FIGURE 8. Nmd3p lacking a nuclear localization signal binds 60S subunits. C-myc tagged Nmd3Δ120 (Δ120), c-myc tagged full-length Nmd3p (WT), or untagged full-length Nmd3p (no tag) were expressed from centromeric vectors (pAJ536, pAJ538, and pAJ123, respectively) in the wild-type strain CH1305. Extracts were prepared and similar amounts of each extract was fractionated by SDS-PAGE on a 12% acrylamide gel. Proteins were transferred to nitrocellulose membrane and western blotting was carried out using anti-c-myc (α c-myc) antibody or anti-L12 (α L12) antibody.

Nmd3Δ120 lacks an NLS and therefore cannot participate in export of nascent 60S subunits, subunits bound by Nmd3Δ120 must already have been exported from the nucleus. This result taken together with the pulse chase analysis and the binding of Nmd3p to 60S subunits in the absence of ribosome biogenesis supports the conclusion that Nmd3p binds mature 60S subunits in the cytoplasm.

Quantitation of Nmd3p compared with free 60S subunits

To examine the relative abundance of the Nmd3p-60S complex with respect to the total pool of free 60S subunits, we quantitated the amount of Nmd3p and 60S subunit proteins in the free 60S peak obtained by sucrose-gradient sedimentation. Extracts were prepared from wild-type cells and fractionated by ultracentrifugation on 15–35% sucrose gradients. Fractions across the gradient were assayed by western blotting for the relative amounts of Nmd3p and the large subunit protein L12 (Fig. 9). The amounts of Nmd3p and L12 in the 60S peak were then quantitated by western blotting, comparing the signal from Nmd3p and L12 in fraction 17 to the signal obtained from known quantities of purified Nmd3p and 60S subunits determined by Bradford assay. From this analysis, we estimated that there was a 1:9 ratio of Nmd3p to L12 in the 60S peak. To confirm this, we directly quantitated the amount of Nmd3p to 60S ribosomal proteins in Coomassie blue-stained gels of 60S peak fractions obtained by sucrose-gradient sedimentation. Extracts were prepared from wild-type cells and from cells expressing c-mycNmd3p from the genomic *NMD3* locus and under control of the *NMD3* promoter. These extracts were fractionated on 15–35% sucrose gradients and peak 60S fractions were analyzed by SDS-PAGE followed by staining with Coomassie blue (data not shown). We identified Nmd3p from the shift in mobility of the epitope-tagged protein. Quantitation of the Coomassie blue-stained bands of Nmd3p and L3, the largest of the 60S subunit proteins, in these fractions yielded a ratio of 1:6 (Nmd3p:L3), similar to the ratio obtained by quantitation of western blots. Thus, only 11%–16% of the free pool of 60S subunits is bound by Nmd3p.

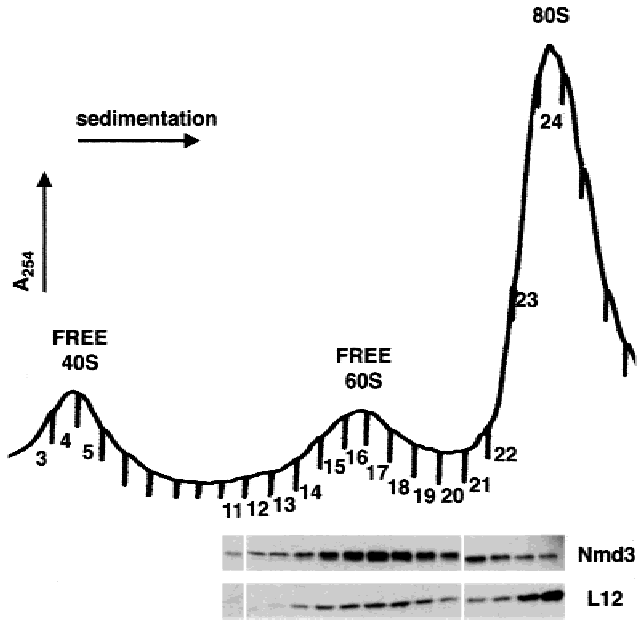

FIGURE 9. Stoichiometry of Nmd3p to free 60S subunits. **A**: Ten A_{260} units of an extract from wild-type cells were fractionated on a 15–35% sucrose gradient as described in Materials and Methods. Fractions were collected and proteins were precipitated by the addition of TCA and separated by SDS-PAGE on 12% acrylamide gels. Proteins were transferred to nitrocellulose membrane and western blots were carried out using affinity-purified anti-Nmd3p and anti-L12 antibodies.

DISCUSSION

We have recently shown that Nmd3p shuttles and provides the nuclear export signal for the large ribosomal subunit (Ho et al., 2000). Nmd3p thereby acts as an adapter protein between the 60S subunit and the export receptor protein Crm1p/Xpo1p to carry the Nmd3p-60S complex to the nuclear pore complex for export. Although we initially reported that Nmd3p was cytoplasmic (Ho & Johnson, 1999), we now know that its ability to shuttle in and out of the nucleus is essential for 60S export. Thus at steady state it is primarily cytoplasmic, but it must transiently be in the nucleus to recruit 60S subunits for export. Here we have shown that Nmd3p binds directly to 60S subunits, that this interaction is stable and that it can be used to coimmunoprecipitate free 60S subunits. This coimmunoprecipitation of free 60S subunits provides a novel and powerful technique for characterizing free 60S subunits. To our knowledge, no other nonribosomal protein has been described that forms a stable complex with the free 60S subunit.

Using this coimmunoprecipitation technique, we have shown that nascent 60S subunits are in the Nmd3p-bound pool. Indeed, labeled 60S subunits were present in the Nmd3p-bound pool within 5 min of labeling, suggesting that Nmd3p binds to nascent subunits very early in their biogenesis. This is consistent with our finding that Nmd3p loads onto nascent subunits before they exit the nucleus. More surprising was the finding that mature 60S subunits were bound by Nmd3p. This conclusion was supported by three lines of evidence: (1) pulse-chase analysis showed that labeled 60S subunits persisted in the Nmd3p-bound fraction long after the addition of an excess of unlabeled chase. This was most easily explained by the continued exchange of labeled subunits as they were diluted into the pool of actively recycling subunits. (2) 60S subunits remained

associated with Nmd3p under conditions in which ribosome biogenesis was inhibited. (3) A truncated Nmd3p protein (Nmd3Δ120) lacking its essential NLS and NES bound 60S subunits in vivo. Although we have not proven that Nmd3Δ120 does not enter the nucleus, deletion only of the NES leads to nuclear accumulation of the truncated protein. Further deletion of the NLS in addition to the NES (Nmd3Δ120) leads to a cytoplasmic distribution. If this protein entered the nucleus, we would expect accumulation of the truncated protein because of its lack of an NES. Thus, the cytoplasmic localization of Nmd3Δ120 implies that the protein is restricted to the cytoplasm and that subunits bound by Nmd3Δ120 have already exited the nucleus.

What role does Nmd3p serve on cytoplasmic free 60S subunits?

The association of Nmd3p with mature free 60S subunits suggests that Nmd3p has a role in the recycling of free 60S subunits in the cytoplasm. The stoichiometry of Nmd3p to free 60S subunits is 1:6 to 1:9, showing that Nmd3p binds only a fraction of free 60S subunits. Several explanations could account for this stoichiometry. First, it is possible that there is heterogeneity to the free 60S subunit pool and that Nmd3p binds a particular class of subunits. These could be subunits that are localized to specific compartments or cellular structures or that are translating a specific subset of mRNAs. Alternatively, the recycling of 60S subunits may involve distinct steps, one of which requires Nmd3p binding. In this case, Nmd3p could bind all 60S subunits but only at a particular step during recycling. Intriguingly, Rpl10p is substoichiometric on free 60S ribosomal subunits. Only about 10% of the free subunits contain Rpl10p (Eisinger et al., 1997). Because this number is similar to the ratio of free 60S subunits that contain Nmd3p, it is possible that these two proteins define a specific species of the recycling 60S subunit.

Both nascent and mature 60S subunits are bound by Nmd3p. Because there is no reason a priori to believe that these two classes of subunits are released from Nmd3p at different times, it is reasonable to assume that nascent subunits are bound by Nmd3p until they enter the translation cycle. In addition, Nmd3p may be serving a common function on nascent subunits after export from the nucleus and on recycling mature subunits. Nmd3p could serve a protective function on the free 60S, perhaps masking or stabilizing a sensitive site on the free subunit. However, a temperature-sensitive *nmd3* mutant that is unable to produce stable 60S subunits at nonpermissive temperature displays normal stability of subunits that were made before shifting to restrictive conditions (Ho & Johnson, 1999). Thus, Nmd3p does not appear to play a significant role in the stability of cytoplasmic 60S subunits. Nmd3p could help to localize 60S subunits in the cytoplasm or to maintain a pool of 60S subunits in the vicinity of polysomes. Lastly, Nmd3p could serve a more active role in translation, possibly recruiting the 60S subunit to the 40S subunit. Although temperature-sensitive mutants of *NMD3* do not reveal translation initiation defects, the genetic interaction of *NMD3* with *RPL10*, encoding a large subunit protein required for efficient subunit joining (Dick et al., 1997), may indicate such a role in subunit joining. Dominant *NMD3* mutations (Karl et al., 1999) or high-copy *NMD3* (Zuk et al., 1999) suppress temperature-sensitive mutations in *RPL10*. Such suppression could be the result of increased subunit joining by Nmd3p. Indeed, preliminary results using purified Nmd3p and 40S and 60S subunits suggest that Nmd3p enhances the formation of 80S complexes in vitro in a subunit joining assay (G. Kallstrom and A.W. Johnson, unpubl.). Furthermore, the presence of low levels of Nmd3p in the polysomal portion of the gradient could reflect a transient involvement in 60S recruitment during translation initiation. Translation initiation factors associated with the 40S subunit also show a small fraction cosedimenting with polysomes. Although Nmd3p could alternatively function during subunit dissociation at the end of the translation cycle, the genetic suppression of mutations in Rpl10p, required for subunit joining, more strongly suggests a role for Nmd3p in joining.

Does the genetic interaction of *NMD3* and *RPL10* reflect nuclear functions?

Considering the role of Nmd3p in 60S subunit export (Ho et al., 2000), the genetic interaction between *NMD3* and *RPL10* (Karl et al., 1999; Zuk et al., 1999) could be due to effects on nuclear transport of 60S subunits. Although Rpl10p exchanges on the 60S subunit in the cytoplasm (Dick et al., 1997; Nguyen et al., 1998), it is possible that Rpl10p is initially loaded in the nucleus and that it is required on the nascent 60S subunit for efficient export. QM protein, the human homolog of Rpl10, is reported to be restricted to the cytoplasm (Nguyen et al., 1998). However, a transient presence of QM protein in the nucleus, perhaps in the nucleoplasm, could not be ruled out. Nmd3p, which also appears restricted to the cytoplasm at steady state, does, in fact, bind to nascent 60S subunits in the nucleus (Ho et al., 2000). It should also be noted that Rpl10p has a putative NLS and that the nucleoplasmic protein Rsa1p is required for efficient Rpl10p loading onto the 60S subunit (Kressler et al., 1999a), supporting the notion that Rpl10p may enter the nucleus. If Rpl10p does initially load onto the nascent 60S subunit in the nucleus and is required for efficient export, suppression by *NMD3* may be due to increasing the rate of transport. We are currently carrying out experiments to distinguish between these models for Nmd3p function.

MATERIALS AND METHODS

Strains and plasmids

The following yeast strains were used in this work: BJ5464 (*MATα ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1*), CH1305 (*MATa ade2 ade3 leu2 lys2-801 ura3-52*), AJY272, (*MATa ade2 ade3 leu2 lys2-801 ura3-52 NMD3-13c-myc::KanMX6*), W303, (*MATa leu2-3,112 his3-11 trp1-1 ura3-1 ade2-1 can1-100 ssd1-1*), and 312XX (*MATa leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100 ssd1-1 sly1-1*). Rich (YPD) or synthetic complete dropout medium were prepared as described (Rose et al., 1990).

Epitope tagging Nmd3p

The oligonucleotides AJO230 5′-AGAAGATGGAGTCGAGA ACACACCCGTTGAATCTCAGCAGCGGATCCCCGGGTTA ATTAA and AJO231 5′-ACTGAAATGCTAGTGTTTGTTAAG AGTATATACTACTCTCGAATTCGAGCTCGTTTAAAC were used to amplify a 13Myc-kanMX6 cassette from pFA6a-13Myc-kanMX6 (Longtine et al., 1998) by PCR. The resulting product was transformed into yeast strain CH1305 and transformants were selected on G418-containing plates giving strain AJY272. Correct integration was confirmed by PCR of the genomic *NMD3* locus. The 13c-myc-tagged *NMD3* locus was rescued to a centromeric plasmid by transforming into AJY272 plasmid pAJ123 (Ho & Johnson, 1999) in which the 3′ end of *NMD3* had been removed by digestion with *Bgl*II and *Hin*dIII giving plasmid pAJ401. C-myc-tagged NMD3Δ120, and c-myc-tagged full-length *NMD3* on centromeric vectors (pAJ536 and pAJ538, respectively) are described elsewhere (Ho et al., 2000).

The 2 μm plasmid pAJ235, expressing GST-Nmd3p under control of the *GAL10* promoter, was made by ligating the *NMD3*-containing *Xho*I-*Hin*dIII fragment from pAJ118 (Ho & Johnson, 1999) into the *Sal*I and *Hin*dIII sites of pEG(KT).

In vitro Nmd3p-60S binding

GST-Nmd3p and free GST were expressed in wild-type yeast (CH1305) and purified from total-cell extracts by glutathione sepharose column chromatography as directed by the manufacturer (Pharmacia). 60S subunits were purified from strain CH1305 as described (Raué et al., 1991). Protein binding reactions were carried out as follows. GST-Nmd3p (2 μg) or free GST (2 μg) were incubated with 0.2 A_{260} U of purified 60S subunits in 50 μL of joining buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 8 mM $MgCl_2$, 6 mM β-mercaptoethanol) for 30 min at 30 °C. Reactions were brought to 0.5 mL by the addition of joining buffer and then 20 μL of BSA-coated glutathione sepharose 4B beads were added. Samples were incubated with rocking for 1 h. Beads were pelleted by brief centrifugation and washed five times in joining buffer. Bound proteins were then eluted by the addition of buffer containing 50 mM glutathione.

Anti-Nmd3p antibody

GST-Nmd3p was expressed at high levels and purified from the protease-deficient yeast strain BJ5464 containing plasmid pAJ235. A total of 190 μg of purified GST-Nmd3p was used to inoculate one rabbit three times at 2-week intervals. Serum was collected after an additional 2 weeks and was affinity purified using free Nmd3p cleaved from GST with thrombin and immobilized on nitrocellulose membrane. The affinity-purified antibody was highly specific for Nmd3p and did not cross-react on western blots with other yeast proteins (data not shown).

Immunoprecipitation

Cultures of 250 mL were grown to 1×10^7 cells/mL in YPD or dropout medium as required. Cells were collected, washed once in IP buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 40 mM NaCl, 0.1% NP_{40}, 5 mM β-mercaptoethanol, 1 mM PMSF, and leupeptin and pepstatin each at 0.5 μg/mL), and resuspended in 1 mL of the same buffer. Extracts were prepared by vortexing with glass beads. After clarification by centrifugation two times for 10 min at 13,000 × *g*, 50 μL of BSA-coated protein A agarose beads were added and the samples were incubated for 30 min with rocking at 4 °C. The beads were removed and 2 μL of anti-c-myc antibody was added. After rocking for 2 h, 20 μL of BSA-coated protein A beads were added. After an additional 30 min of incubation with rocking, the supernatant was removed from the beads. The beads were washed three times with IP buffer and bound proteins were eluted in Laemmli sample buffer.

For analysis of immunoprecipitated RNA, 150-mL cultures were grown at 30 °C to a density of 1×10^7 cells/mL in PO_4-depleted medium (Warner, 1991). The cells were collected, resuspended in 4 mL of the same medium, prewarmed to 30 °C. After 10 min, 200 μCi of $^{32}PO_4$ were added and cells were incubated for 90 min. Extracts were prepared and a sample was removed for preparation of total RNA. Immunoprecipitation was carried out as described above. The immunoprecipitated material was resuspended in 0.5 M LiCl before RNA was extracted with phenol/chloroform and precipitated with ethanol. 25S and 18S rRNAs were separated on 1% formaldehyde agarose gels. The gels were then fixed with 10% trichloroacetic acid (TCA), dried, and autoradiographed. 5.8S and 5S rRNAs were separated on 6% polyacrylamide-urea gels that were then fixed with 30% methanol and 10% acetic acid, dried, and autoradiographed.

[^{35}S] methionine pulse-chase

A 240-mL culture of strain AJY272 was grown in SCMet− medium to a density of 1×10^7 cells/mL. The cells were collected and resuspended in 9 mL of the same medium. A 1.5-mL sample was removed and 2.3 mCi of [^{35}S]methionine (EXPRE^{35}S^{35}S protein labeling mix, Dupont) were added. After 5 min of labeling, a 1.5-mL sample was removed and unlabeled methionine was added to a final concentration of 200 μg/mL. Additional samples were taken after 10, 20, 40, and 90 min. Extracts were prepared and immunoprecipitated as described above. Immunoprecipitated proteins were separated by SDS-PAGE on a 8–20% polyacrylamide gel, transferred to nitrocellulose, and Nmd3p and ribosomal protein L12 were visualized by western blotting. [^{35}S]-labeled proteins were visualized by autoradiography.

sly1-1 work

Cultures of 200 mL of 312XX (*sly1-1*) or W303 (WT) expressing c-mycNmd3p from a centromeric plasmid (pAJ401) were grown at 26 °C to a density of 1×10^7 cells/mL. The cultures were split in two and one half of each culture was shifted to 37 °C. After 2 h, cells were collected, extracts were prepared, and c-mycNmd3p was immunoprecipitated as described above. Immunoprecipitated proteins were visualized by Coomassie blue staining after separation by SDS-PAGE through a 8–20% acrylamide gel. rRNA transcription was monitored in wild-type and *sly1-1* strains as follows. For RNA analysis, cells were grown at 26 °C in phosphate-depleted YPD (Warner, 1991) to 1.3×10^7 cells/mL. Cultures were split into two and one sample of each culture was shifted to 37 °C. After 1 h, cells were concentrated 17-fold in the same medium and labeled for 30 min with $^{32}PO_4$ at a final concentration of 2 μCi/μL. RNA was prepared and analyzed as described above with the exception that electrophoresis used a 1% agarose gel in TAE buffer. For polysome analysis, cultures were grown at 26 °C in YPD to 1×10^7 cells/mL. Cultures were then divided into two and one sample was shifted to 37 °C. After incubation for another 2 h, cycloheximide was added and extracts were prepared and analyzed by sedimentation through 7–47% sucrose gradients as described previously (Ho & Johnson, 1999). Sucrose-gradient fractions were precipitated with TCA, separated by SDS-PAGE and c-mycNmd3p and L3 were visualized by western blotting.

Thiolutin treatment

A culture of AJY272 in YPD was grown to 1×10^7 cells/mL, at which point thiolutin (Pfizer) was added to 20 μg/mL. Samples were taken after 20 or 40 min of incubation in thiolutin. Extracts were prepared and immunoprecipitated as described above. After SDS-PAGE separation of the immunoprecipitated proteins, c-mycNmd3p and ribosomal protein L12 were visualized by western blotting using anti-c-myc and anti-L12 antibodies, respectively. For RNA analysis, cells were grown in phosphate-depleted rich medium (Warner, 1991), concentrated 10-fold in the same medium, and thiolutin was added to 20 μg/mL. After 10 min, $^{32}PO_4$ was added to a final concentration of 150 μCi/mL. Cells were harvested after 50 min and total RNA was prepared (Ho & Johnson, 1999). RNA was analyzed by electrophoresis through a 1% agarose gel in TAE buffer. After staining with ethidium bromide for visualizing total RNA, the RNA was transferred to nylon membrane and autoradiographed.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant GM53655 to A. Johnson. We thank J. Warner for providing strains 312XX and W303, C. Chan for plasmid pEG(KT), J. Ballesta for anti-L12 antibody, and J. Brown for comments on the manuscript. We thank Dr. E. Weiss, Pfizer, for the generous gift of thiolutin.

Received July 12, 2000; returned for revision August 17, 2000; revised manuscript received August 23, 2000

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