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Ultrastructural localization of rRNA shows defective nuclear export of preribosomes in mutants of the Nup82p complex

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To study the nuclear export of preribosomes, ribosomal RNAs were detected by in situ hybridization using fluorescence and EM, in the yeast *Saccharomyces cerevisiae*. In wild-type cells, semiquantitative analysis shows that the distributions of pre-40S and pre-60S particles in the nucleolus and the nucleoplasm are distinct, indicating uncoordinated transport of the two subunits within the nucleus. In cells defective for the activity of the GTPase Gsp1p/Ran, ribosomal precursors accumulate in the whole nucleus. This phenotype is reproduced with pre-60S particles in cells defective in pre-rRNA processing, whereas pre-40S particles only accumulate in the nucleolus, suggesting a tight control of the exit of the small subunit from the nucleolus. Examination of nucleoporin mutants reveals that preribosome nuclear export requires the Nup82p–Nup159p–Nsp1p complex. In contrast, mutations in the nucleoporins forming the Nup84p complex yield very mild or no nuclear accumulation of preribosome. Interestingly, domains of Nup159p required for mRNP trafficking are not necessary for preribosome export. Furthermore, the RNA helicase Dbp5p and the protein Gle1p, which interact with Nup159p and are involved in mRNP trafficking, are dispensable for ribosomal transport. Thus, the Nup82p–Nup159p–Nsp1p nucleoporin complex is part of the nuclear export pathways of preribosomes and mRNPs, but with distinct functions in these two processes.

Introduction

Molecular exchanges between the nucleus and the cytoplasm in eukaryotic cells are driven by a complex set of mechanisms that direct the trafficking of molecules across the nuclear envelope (Fabre and Hurt, 1997; Mattaj and Englmeier, 1998; Wozniak et al., 1998; Ferrigno and Silver, 1999; Gorlich and Kutay, 1999). In eukaryotic cells, ribosome biogenesis involves a large number of nucleocytoplasmic transport events at various steps. For example, in order to produce ~200,000 ribosomes and thus double the translation machinery over a 100-min cell cycle, ~4,000 ribosomal subunits must be exported every minute from the nucleus in proliferating *Saccharomyces cerevisiae* cells. To assemble these preribosomes, the trafficking of ribosomal proteins should amount to ~1,000 molecules/min/pore (Warner, 1999). This high-rate transfer of preribosomes to the cytoplasm is presumably coordinated with the maturation of the preribosomal RNA (pre-rRNA) and the assembly of the ribosomal proteins. But in contrast to these processes which have been the focus of many studies (Venema and Tollervey, 1999), the mechanisms underlying the nuclear export of preribosomes remain largely unknown.

In *S. cerevisiae*, the small ribosomal subunit (40S) comprises the 18S ribosomal RNA (rRNA) and ~32 proteins, whereas the large subunit (60S) is made of three rRNAs (25S, 5.8S, and 5S) and ~46 proteins. Transcription of ribosomal DNA by RNA polymerase I in the nucleolus yields a large 35S precursor transcript, which immediately undergoes methylation and pseudouridylation guided by snoRNPs (Bachellerie and Cavaille, 1997; Tollervey and Kiss, 1997). This pre-rRNA is converted to the 18S, 5.8S, and 25S mature rRNAs after elimination of two external and two internal spacers by a series of endo- and exonucleolytic cleavages (Venema and Tollervey, 1999). Most steps of this

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maturation process are thought to take place in the nucleus, except conversion of the 20S pre-rRNA to the 18S rRNA, which in *S. cerevisiae* occurs in the cytoplasm (Udem and Warner, 1973; Trapman and Planta, 1976; Vanrobays et al., 2001). A separate gene transcribed by RNA polymerase III encodes the 5S rRNA, which is incorporated into the precursor for the large ribosomal subunit in the nucleus. Early steps of ribosomal biogenesis take place in a large 90S preribosomal particle which divides into 43S and 66S particles, precursors for the 40S and the 60S subunits. The assembly of the ribosomal particles starts in the nucleolus with the binding of some ribosomal proteins to the nascent transcript, and is completed with the late addition of others after export to the cytoplasm (Hadjiolov, 1985).

Transport across the nuclear envelope takes place at the nuclear pore complex (NPC),* a large proteinaceous structure (M = 50–60 MDa in yeast) that forms an aqueous channel (Stoffler et al., 1999). Extensive characterization of the NPC composition in *S. cerevisiae* by various approaches (including genetics, biochemistry, biocomputing, and recently proteomics) has led to the identification of a virtually complete list of ~30 proteins, called nucleoporins (Doye and Hurt, 1997; Fabre and Hurt, 1997; Rout et al., 2000). Physical interactions between some nucleoporins have been established, thus defining a series of subcomplexes including the Nup82p complex (Nup82–Nup159p–Nsp1p) (Grandi et al., 1995a; Hurwitz and Blobel, 1995; Belgareh et al., 1998), the Nup84p complex (Nup84p–Nup85p–Nup120p–Nup145Cp–Seh1p–Sec13p) (Siniossoglou et al., 1996, 2000), the Nic96p complex (Nic96p–Nup49p–Nup57p–Nsp1p) (Grandi et al., 1995b), and the Nup53p complex (Nup53p–Nup59p–Nup170p) (Marelli et al., 1998). Transport across the NPC of most proteins, of tRNAs, and of snRNAs has been shown to require their interaction with a member of the karyopherin family (also called importins/exportins), a group of soluble receptors that recognize nuclear import or export signals and mediate binding to nucleoporins (Mattaj and Englmeier, 1998; Wozniak et al., 1998). Isolation of thermosensitive (ts) mutants accumulating poly(A)$^+$ RNAs in the nucleus has led to the identification of several nucleoporins required for mRNP transport (Doye and Hurt, 1997), including proteins of the Nup84p complex (Heath et al., 1995; Goldstein et al., 1996; Siniossoglou et al., 1996; Fabre and Hurt, 1997), Nup82p (Hurwitz and Blobel, 1995), and Nup159p (Gorsch et al., 1995).

The yeast *S. cerevisiae* has recently been used to develop new assays for identifying proteins involved in the nuclear export of preribosomes. Hurt et al. (1999) have fused the green fluorescent protein (GFP) to Rpl25p, a protein of the large ribosomal subunit, in order to detect the accumulation of pre-60S particles in the nucleus. Mutants affecting the function of the small GTPase Ran/Gsp1p, a key regulator of nucleocytoplasmic transport (Gorlich and Kutay, 1999), as well as mutants of the nucleoporins Nup49p and Nsp1p, displayed nuclear accumulation of Rpl25p-GFP. This assay was used to show that the exportin Xpo1p/Crm1p could act as a nuclear export factor for pre-60S particles through binding to protein Nmd3p, which in turn associates with the ribosomal protein Rpl10p (Ho et al., 2000; Gadal et al., 2001). A similar approach with an Rpl11p-GFP reporter protein was developed by Stage-Zimmermann et al. (2000). Regarding the transport of the pre-40S particles, Moy and Silver (1999) used fluorescence in situ hybridization (FISH) to monitor the fate of the 5′ part of the ITS1, which is cleaved off the 20S pre-rRNA in the cytoplasm and degraded by the exonuclease Xrn1p; in an *xrn1Δ* background, this product accumulates in the cytoplasm unless a second mutation blocks the nuclear export of the small subunit. As for the large subunit, this approach revealed deficient transport of the 20S pre-rRNA upon alteration of Ran function. In addition, mutants of Xpo1p/Crm1p and Nup82p displayed accumulation of the 20S pre-rRNA in the whole nucleus. Mutations affecting other nucleoporins resulted in a strong FISH signal in the nucleolus, a phenotype interpreted as a defect in ribosomal processing rather than in transport. Thus, these first studies in yeast have led to the identification of proteins involved in preribosome trafficking; however, no clear picture has yet emerged regarding the role of the components of the NPC in this process.

Here we present a novel approach using the detection of the ribosomal particles by in situ hybridization and EM, with probes complementary to various regions of the pre-rRNA. This method differs from the previous ones in several aspects: both pre-40S and pre-60S particles can be visualized with the same method; the introduction of a reporter gene or the modification of the genetic background of the cells is not needed; the observation of ultrathin sections gives direct access to the nucleus without interference of the strong signal coming from the cytoplasmic ribosomes; and preribosome distribution within the nucleus can be semiquantitatively analyzed. We first evaluate this ultrastructural approach by examining the phenotypes of mutants known to be impaired in ribosome synthesis or ribosomal nuclear export. Then, in combination with FISH, we show that mutants of the nucleoporins forming the complex Nup82p–Nup159p–Nsp1p, but not the Nup84p complex, are affected in the nuclear export of the ribosomal subunits. Further analysis of deletion mutants of NUP159 demonstrates that the function of Nup159p in mRNP trafficking can be uncoupled from its role in preribosome nuclear export.

Results

Localization of the precursors of the small and large ribosomal subunits in the nucleus of *S. cerevisiae*

The localization of the small and large subunits and of their precursors in wild-type *S. cerevisiae* was first established by in situ hybridization using probes complementary to the 18S and the 25S rRNAs (Fig. 1). As expected, both probes labeled the nucleolus, where rDNA is transcribed, and the cytosol where ribosomes accumulate and carry out translation. However, the labeling level of the nucleoplasm was different with the two probes: this part of the nucleus was poorly labeled with the 18S probe, with only 10–15% of the total nuclear labeling; in contrast, 25–35% of the nuclear 25S rRNA (and its precursors) was detected in this compartment. The rRNAs la-

*Abbreviations used in this paper: FISH, fluorescence in situ hybridization; GFP, green fluorescent protein; NPC, nuclear pore complex; ts, thermosensitive.

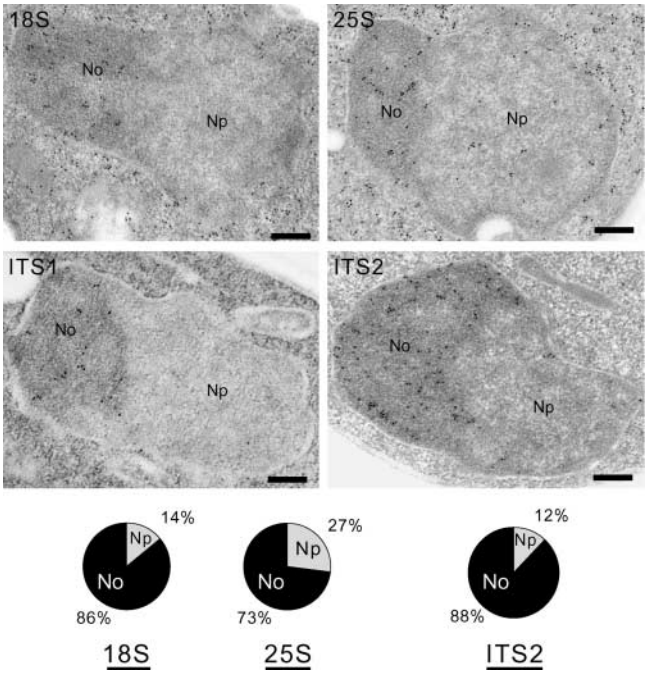


Figure 1. **Ultrastructural localization of preribosomes by in situ hybridization in the nucleus of *S. cerevisiae*.** In situ hybridization was performed on sections of wild-type cells with probes complementary to the 18S rRNA, the 25S rRNA, the ITS1, or the ITS2. The majority of the nuclear labeling is found over the nucleolus (No), which is more electron-dense than the nucleoplasm (Np). However, a significant fraction of pre-60S particles is detected in the nucleoplasm. The distribution of the labeling in the nucleoplasm and the nucleolus was assessed by counting the gold particles on a series of pictures of nuclei taken randomly. Bars, 200 nm.

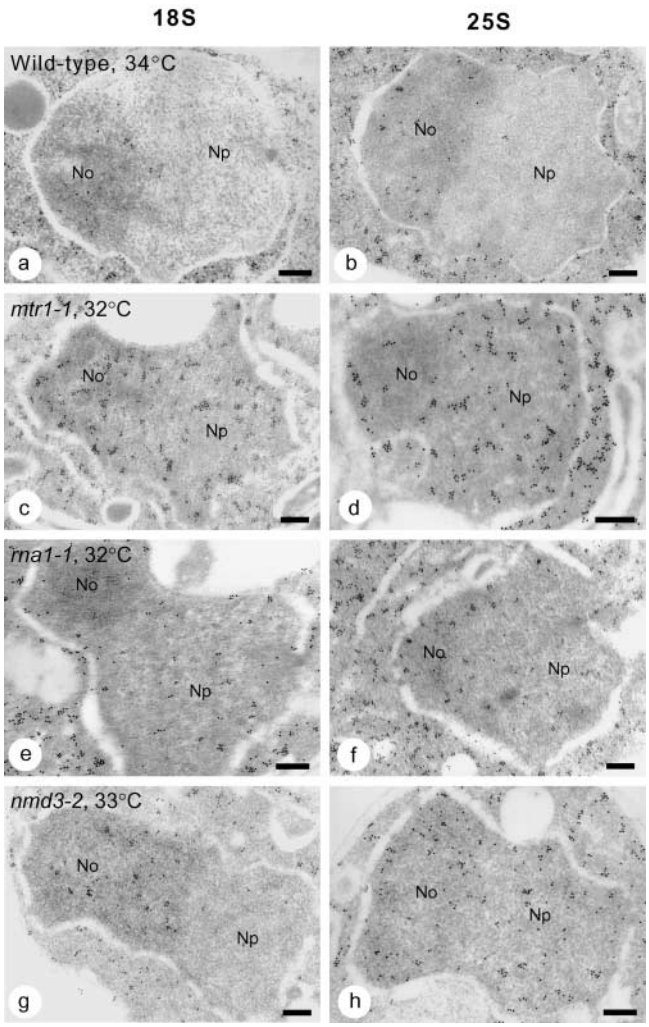


Figure 2. **Localization of preribosomes in strains defective in ribosomal nuclear export.** The *mtr1-1* and *rna1-1* strains, grown for 4 h at 32°C, strongly accumulate pre-40S (18S probe) and pre-60S (25S probe) particles in the whole nucleus (c–f), whereas the *nmd3-2* mutant (shifted to 33°C for 3 h) is specifically affected in the export of the large subunit (g and h). Wild-type cells cultured at 34°C for 4 h are shown in pictures a and b. No, nucleolus; Np, nucleoplasm. Bars, 200 nm.

beled with the 25S probe in the nucleoplasm mostly corresponded to mature 25S RNA and not to its precursors, as a probe complementary to the ITS2 was primarily detected over the nucleolus (10% in the nucleoplasm). In accordance with the labeling obtained with the 18S probe, a probe directed to the 5′ part of the ITS1 (D-A2 segment), which is present in the 20S pre-rRNA, decorated the nucleolus almost exclusively. This difference in the distribution of the pre-40S and pre-60S particles in the nucleolus and the nucleoplasm suggests that the two subunits exit the nucleus along distinct intranuclear pathways or with different kinetics. This is in accordance with previous biochemical data showing that the small subunit is exported to the cytoplasm faster than the large one, and is more rapidly incorporated into polysomes (Udem and Warner, 1973; Trapman and Planta, 1976).

Distribution of ribosomal RNA upon a defect in nucleocytoplasmic transport

One result expected from a defect in the nuclear exit of preribosomes is the rapid buildup of (pre-) rRNA level in the nucleus, as compared with the wild-type situation. In parallel, the amount of rRNA in the cytoplasm should decrease, but this may occur slowly due to the long half-life of the ribosomal subunits, and the fact that nuclear export may not be completely stopped in the mutant context. For this reason, we mostly concentrated on the nuclear retention of (pre-) rRNA. In addition to a quantitative change in the amount of rRNA in the nucleus, we also considered a possible shift in the distribution of preribosomes from the nucleolus to the nucleoplasm. Data from Moy and Silver (1999) suggest that a mutation affecting an early step of the pre-40S particle synthesis pathway would result in the accumulation of pre-18S rRNA in the nucleolus, whereas disruption of a late step (i.e., passage through the NPC) would lead to the accumulation of pre-18S rRNA in the whole nucleus.

The activity of the GTPase Gsp1p/Ran was previously shown to be necessary for the nuclear export of both subunits (Hurt et al., 1999; Moy and Silver, 1999), as for many transport substrates. Thus, we examined ts alleles of MTR1 (*mtr1-1*) and RNA1 (*rna1-1*), two genes whose products act as the GTP exchange factor and the GTPase activating protein, respectively, of Ran (Fig. 2). In wild-type cells, raising the temperature above 30°C (up to 37°C) for 3–4 h only led to a slight decrease of the amount of preribosomes in the nucleus, and did not change their nuclear distribution (Fig. 2, a and b).

In contrast, the *mtr1-1* and *rna1-1* strains, grown for 4 h at 32°C, strongly accumulated (pre-) rRNA in the whole nucleus, as seen with both the 18S and the 25S probes (Fig. 2, c–f). The increase of the labeling was particularly conspicuous in the nucleoplasm in comparison with wild-type cells. The subunits did not accumulate at the vicinity of the NPCs, but instead were distributed throughout the entire volume of the nucleus. To look more specifically at ribosomal trafficking, we tested a ts mutant of Nmd3p (*nmd3-2*), a protein shown to act as an adapter between the pre-60S particle and the exportin Crm1p (Ho et al., 2000; Gadal et al., 2001). After 3 h at 33°C, a strong labeling was observed with pre-60S particles both in the nucleolus and in the nucleoplasm, whereas the pre-40S particles were mostly observed in the nucleolus (Fig. 2, g and h). This observation is consistent with a transport defect specific for the large subunit. Thus, mutations known to affect preribosome export result in the buildup of the amount of (pre-) rRNA in the entire nucleus, particularly in the nucleoplasm, as compared with wild-type cells. These observations are reminiscent of the data obtained with these mutants using functional assays based on fluorescence microscopy.

Effect of ribosomal RNA processing defects on the nuclear transport of preribosomes

Although accumulation of preribosomes in the whole nucleus is expected from an alteration of the export machinery per se (namely the NPC, karyopherins, and Ran), such a phenotype could also result from the release from the nucleolus of aborted preribosomes incompetent for nuclear export (due to a defect in pre-rRNA processing or in the assembly of a subunit), or from the failure of a maturation step that normally occurs in the nucleoplasm. For example, we have shown previously that preribosomes strongly accumulate in the nucleus of cells bearing a mutated 5S rRNA (Dechampesme et al., 1999). To gain more insight into the fate of preribosomes upon a defect in pre-rRNA processing, we looked at strains bearing conditional mutations of NOP1 and GAR1, two genes encoding nucleolar proteins found in C + D and H/ACA box snoRNPs, respectively. Depletion of Nop1p in a *GAL::NOP1* strain leads to a general defect in pre-rRNA processing and methylation (Tollervey et al., 1991). The ts *gar1-1* mutant is mostly affected in the synthesis of 18S rRNA, and pre-rRNA pseudouridylation is abolished (Bousquet-Antonelli et al., 1997). Observation of these mutants under nonpermissive conditions revealed different accumulation patterns for the small and large subunits (Fig. 3, a–f). The nucleolar labeling was higher with both probes (18S and 25S) under nonpermissive conditions, probably due to accumulation of ill-processed pre-rRNAs. In addition, the amount pre-60S particles dramatically increased in the nucleoplasm in both *gar1-1* and *GAL::NOP1* mutants. The ITS2 probe revealed a large amount of pre-

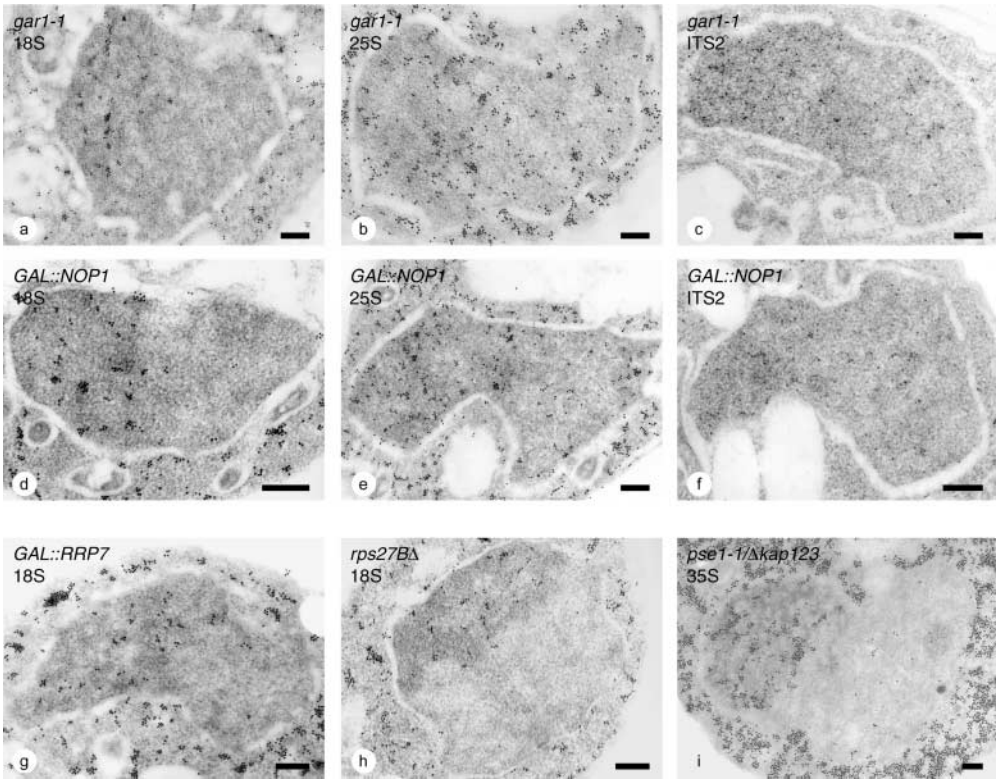


Figure 3. **Strains defective in pre-rRNA maturation or ribosomal protein trafficking accumulate pre-60S, but not pre-40S particles in the nucleoplasm under nonpermissive conditions.** EM in situ hybridization was performed with the 18S, 25S, and ITS2 probes on the pre-rRNA processing mutants *gar1-1*^{ts} (transferred for 3 h to 37°C) (a–c) and *GAL::NOP1* (grown for 20 h with glucose) (d–f). Pre-60S particles are detected in high amount in the whole nucleus, whereas pre-40S particles accumulate in the nucleolus. Nucleolar, but not nucleoplasmic, accumulation of pre-40S particles is also observed in *GAL::RRP7* cells cultured with glucose for 16 h (g) and in *rps27BΔ* cells (h). In the nucleus of *pse1-1*^{ts}*/Δkap123* cells shifted to 37°C for 2 h, pre-rRNAs are found in the nucleolus, when detected with a probe against the entire ribosomal transcription unit (35S probe) (i). Bars, 200 nm.

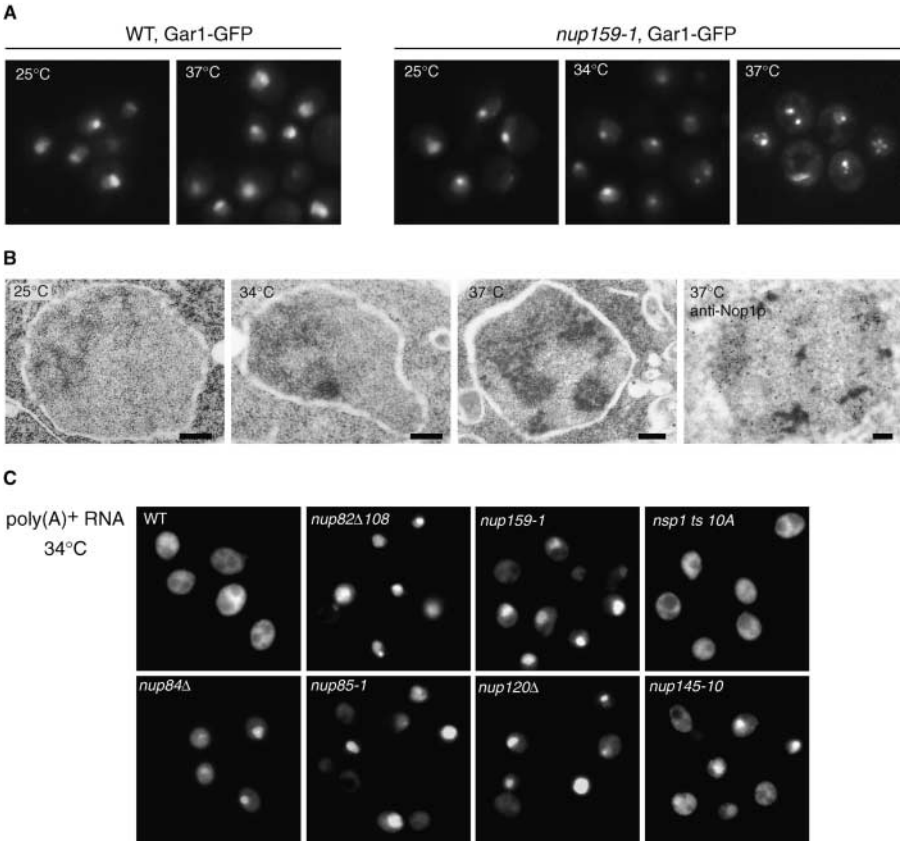


Figure 4. **Setting appropriate conditions to study the trafficking of preribosomes in nucleoporin ts mutants.** (A) Visualization of the nucleolus in live wild-type and *nup159-1* cells at various temperatures with a Gar1-GFP reporter protein. The nucleolus is fragmented in *nup159-1* cells at 37, but not at 34°C. (B) Ultrastructural morphology of the nucleus in *nup159-1* cells at various temperatures; after 2 h at 37°C, several nucleolar fragments containing the protein Nop1p are detected. The nucleolus remains intact at 34°C. (C) FISH with an oligo-dT probe in nucleoporin mutants cultured for 3 h at 34°C. Poly(A)$^+$ RNA nuclear accumulation is seen in all mutant strains except *nsp1 ts10A*. Bars (EM), 200 nm.

25S/pre-5.8S rRNA in the nucleoplasm of these cells, indicating the release of immature pre-60S particles from the nucleolus (compare with wild-type cells in Fig. 1). Although these cells were shown to accumulate aberrant precursors (23S) of the 18S rRNA, the loss of Gar1p or Nop1p function did not result in the accumulation of 18S precursors in the nucleoplasm. Because Nop1p or Gar1p act in early steps of pre-rRNA processing, we studied a strain conditionally expressing RRP7 (*GAL::RRP7*), an essential gene whose product is required downstream of Nop1p and Gar1p in the synthesis of the small subunit (Baudin-Baillieu et al., 1997). Upon depletion of Rrp7p, no nucleoplasmic accumulation of pre-18S rRNA was observed (Fig. 3 g); a similar result was obtained with a mutant deleted of the ribosomal protein gene RPS27B (Fig. 3 h), which can act as a multicopy suppressor of RRP7 deletion, and whose absence severely impairs growth and 18S rRNA production (Baudin-Baillieu et al., 1997). Last, in a double mutant of Kap121p/Pse1p and Kap123p (*pse1-1Δkap123*), two karyopherins involved in import of ribosomal proteins (Rout et al., 1997), (pre-) rRNAs accumulated in the nucleolus but not in the nucleoplasm, as checked with a probe to the entire ribosomal transcription unit (Fig. 3 i).

Thus, a strong defect in pre-rRNA processing is not sufficient to induce accumulation of pre-40S particles in the nucleoplasm, in contrast to a defect in Ran activity or in some nucleoporin mutants (see below). This suggests that the exit of these preribosomes from the nucleolus is tightly controlled and gives a criterion by which to distinguish a defect in ribosomal synthesis from a defect in the nuclear export of the 43S particles. In contrast, both kinds of defects result in a high level of 60S particles in the entire nucleus. For this reason, we mainly concentrated on the nuclear export of the small subunits in the subsequent experiments.

Ribosomal trafficking defect in nucleoporin mutants

To get further insight into the function of the NPC in the nuclear export of preribosomes, we next examined ts mutants of nucleoporins. We focused on two sets of nucleoporins which have been shown to be involved in mRNA trafficking and to physically associate in two distinct complexes: (a) Nup82p, Nup159p, and Nsp1p (Belgareh et al., 1998); and (b) Nup84p, Nup85p, Nup120p, and Nup145-Cp- which form the so-called Nup84p complex with Sec13p and Seh1p (Siniossoglou et al., 1996, 2000). All these proteins are required for the nuclear export of mRNAs, with the exception of Nsp1p (Doye and Hurt, 1997).

Upon transfer to 37°C, we observed that all the mutants displayed a fragmented nucleolus, as already reported for some of them (Aitchison et al., 1995; Iovine et al., 1995; Del Priore et al., 1996; Goldstein et al., 1996; Dockendorff et al., 1997); an example is given with *nup159-1* in Fig. 4. Nucleolar fragmentation could be monitored by fluorescence microscopy in live cells transformed with a Gar1–GFP fusion (Fig. 4 A), starting as soon as 5 min after the shift to 37°C. Under these conditions, the nucleolar protein Nop1p was localized throughout the nucleus around dense bodies that did not contain rRNA (Fig. 4 B), and the in situ hybridization signal in the nucleus was low, probably due in part to alteration of rRNA synthesis upon heat shock

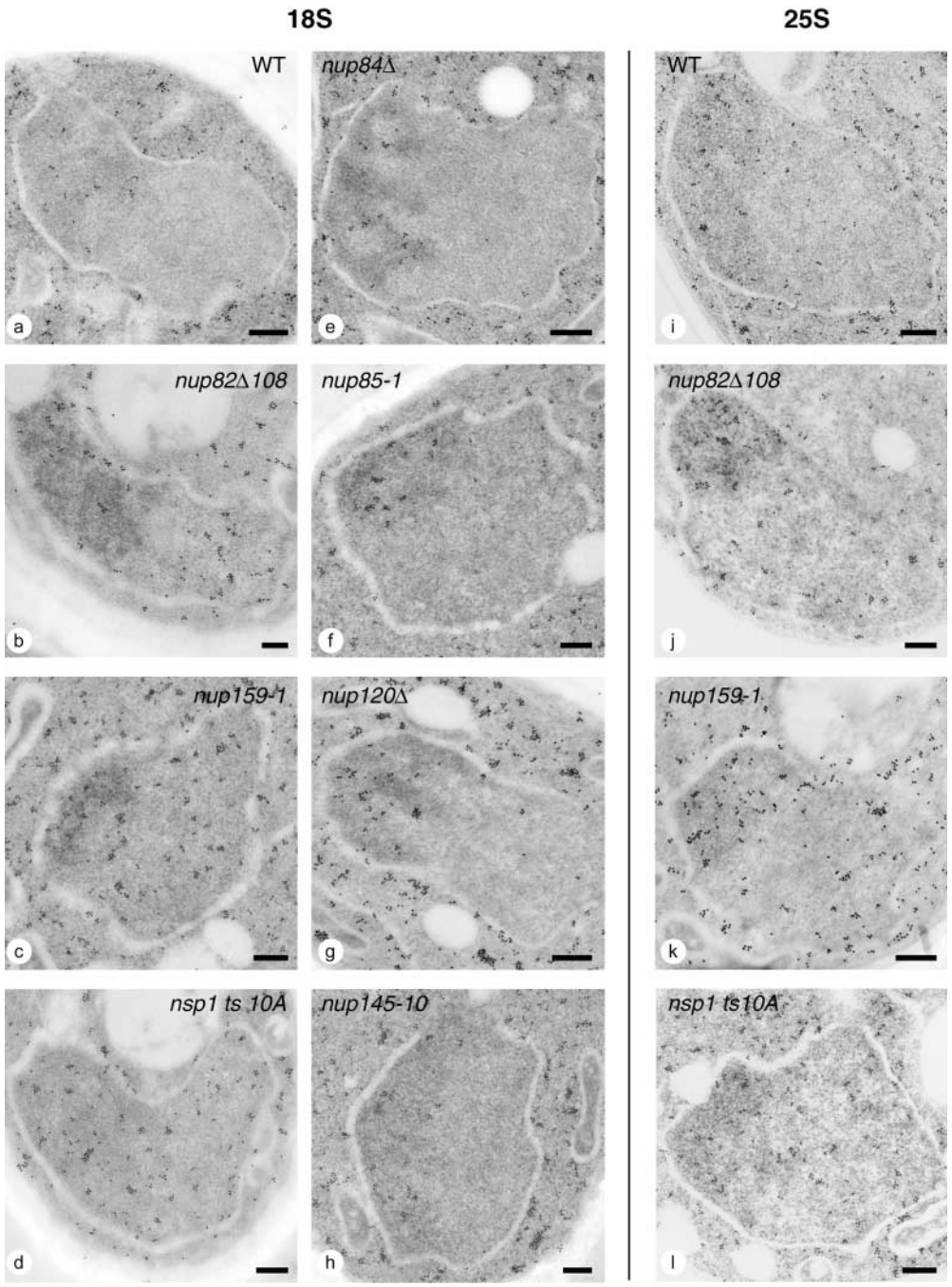


Figure 5. **Nuclear export of preribosomes is altered in mutants of the Nup82p complex, but not in mutants of the Nup84p complex.** Exponentially growing cells were shifted to 34–35°C for 3 h and processed for EM. In situ hybridization with the 18S probe reveals the accumulation of pre-40S particles in the whole nucleus of mutants *nup82Δ108*, *nup159-1*, and *nsp1 ts10A* (b–d), as compared with wild-type cells (a). The same labeling pattern is also observed with the 25S probe in these mutants (i–l). In contrast, labeling with the 18S probe is limited to the nucleolus in mutants *nup84Δ*, *nup85-1*, *nup120Δ*, and *nup145-10* (e–h). Bars, 200 nm.

(Warner and Udem, 1972; Veinot-Drebot et al., 1989). Therefore, to observe the effect of the ts mutations on preribosome export under conditions that preserve the nuclear organization, we first determined the threshold temperature above which the nucleolar structure is altered, using Gar1–GFP as a marker. We found that in most mutants, the nucleolus was not fragmented when the temperature was raised up to 34–35°C, as shown for *nup159-1* cells in Fig. 4, A and B. Nevertheless, this temperature was sufficient to induce accumulation of poly(A)$^+$ RNAs in the nucleus, as checked by FISH with an oligo-dT probe (Fig. 4 C).

Thus, exponentially growing wild-type and mutant cells were transferred to 34–35°C for 4 h and prepared for EM. In situ hybridization was performed with the 18S and 25S probes (Fig. 5), and the distribution of the 18S probe in the nucleus was analyzed semiquantitatively (Fig. 6). This temperature switch had no effect on the amount of preribosomes in the nucleus of wild-type cells (Fig. 5 a). Under these con-

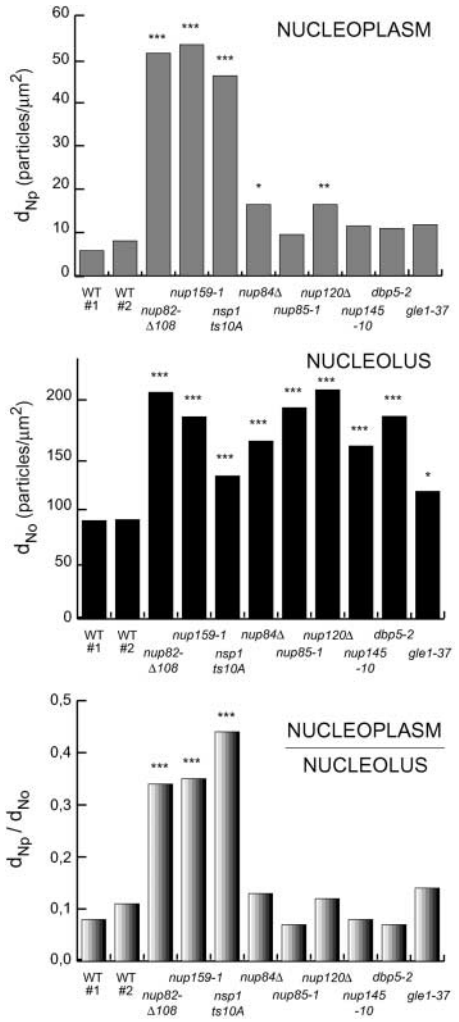


Figure 6. **Semiquantitative analysis of the labeling distribution in the nucleolus and the nucleoplasm after in situ hybridization with the 18S probe.** Pictures of cells grown at 34°C and prepared as in Fig. 5 were taken randomly; on each picture, the labeling density of the nucleolus (d_{No}) and of the nucleoplasm (d_{Np}) was evaluated as described in Materials and methods, and the ratio d_{No}/d_{Np} was calculated. The graph shows the mean values for each strain. A one to one comparison of the mean value of the wild-type strain (FY86) with the others was performed (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). Two independent in situ hybridization experiments were necessary to examine all the strains. The results obtained for the wild-type cells in these two experiments are shown (WT #1 and WT #2).

ditions, mutants *nup84Δ*, *nup85-1*, *nup120Δ*, and *nup145-10*, in which the corresponding protein is either absent (*nup84Δ, nup120Δ*) or extensively truncated (*nup85-1*, *nup145-10*), showed a 1.5–2× increase in the labeling of the nucleolus with the 18S probe as compared with the wild-type cells, and either no change (*nup85-1*, *nup145-10*) or a modest 2× increase (*nup84Δ*, *nup120Δ*) in the labeling of the nucleoplasm (Figs. 5, e–h, and 6). In contrast, in the strains *nup82Δ108*, *nup159-1*, and *nsp1 ts10A* cells, the nucleoplasm was 5–6× more labeled than in the wild-type cells, whereas the labeling of the nucleolus was multiplied by 1.5–2 (Figs. 5, b–d, and 6). As shown in Fig. 6, the labeling of the nucleoplasm significantly increased relative to the labeling of the nucleolus in these three mutants, whereas in the mutants of the nucleoporins forming the Nup84p complex, this ratio was equivalent to that found in the wild-type cells. The *nup82Δ108*, *nup159-1*, and *nsp1 ts10A* cells, but not the other strains (unpublished data), also displayed a high labeling of the nucleoplasm with the 25S probe, showing that the export defect was not restricted to the small subunit (Fig. 5, i–l). Similar to the situation in the *rna1-1* and *mtr1-1* mutants, the preribosomes did not accumulate at the NPC. Accumulation of pre-40S and pre-60S particles in the nucleoplasm could also be observed at the permissive temperature (25°C) in these three mutant strains (unpublished data). Thus, these results indicate that the Nsp1p–Nup159p–Nup82p complex, but not the Nup84p complex, is required for efficient nuclear export of the ribosomal subunits.

Ribosomal RNA processing in mutants *nup159-1, nsp1 ts10A,* and *nup82Δ108*

We checked the status of pre-rRNA processing in mutants *nup159-1*, *nup82Δ108*, and *nsp1 ts10A* at the permissive and semipermissive temperatures by Northern blot analysis with probes complementary to the internal transcribed spacers. As shown in Fig. 7 A, no rRNA precursor strongly accumulated in these mutants, although a slight increase in 35S pre-rRNA could be observed. As in wild-type cells, 20S pre-rRNA was the most abundant species detected with a probe hybridizing to the D-A2 fragment, which reveals all the precursors of the 18S rRNA. Pre-rRNA overall signal in *nup159-1* and *nup82Δ108* was dimmer at the semipermissive temperature, consistent with a lower rDNA transcription rate. Despite the general decrease in rRNA signal, the amount of 20S pre-rRNA in these cells at the semirestrictive temperature remained as high as at the permissive temperature, indicating a relative accumulation of this species. We thus checked by in situ hybridization where the 20S pre-rRNA accumulated in these cells, using a probe complementary to the ITS1 upstream of the A2 cleavage point (Fig. 7 B for *nup159-1*). As with the 18S probe, labeling was mostly restricted to the nucleolus in wild-type cells, whereas it spread to the nucleoplasm in the mutants. Thus, the small subunits accumulating in the nucleoplasm contain 20S pre-rRNA, in accordance with early results showing that cleavage at point D, which yields 18S rRNA, takes place in the cytoplasm in yeast (Udem and Warner, 1973; Trapman and Planta, 1976). Unlike in pre-rRNA processing mutants (Fig. 3), the probe to the ITS2 mostly labeled the nucleolus in *nup159-1* cells (Fig. 7 B), strongly suggesting that the rRNA detected with the 25S probe in the nucleoplasm corresponds to mature 25S rRNA, in agreement with the Northern blot analysis showing no accumulation of 27S pre-rRNA in this mutant.

To confirm the results obtained by EM with the ITS1 probe, we performed FISH with an oligonucleotidic probe also directed to the 5′ part of the ITS1. As shown in Fig. 7 C, wild-type cells were mostly labeled by this probe in the nucleolus (no colocalization with DNA). Additionally, a dim signal was detected in the cytoplasm. The pattern observed in the strains mutated in nucleoporins of the Nup84p complex was similar, except that the nucleolar labeling appeared to be stronger. In contrast, in the *nup82Δ108*, *nup159-1*, and *nsp1 ts10A* mutants, some cells displayed a

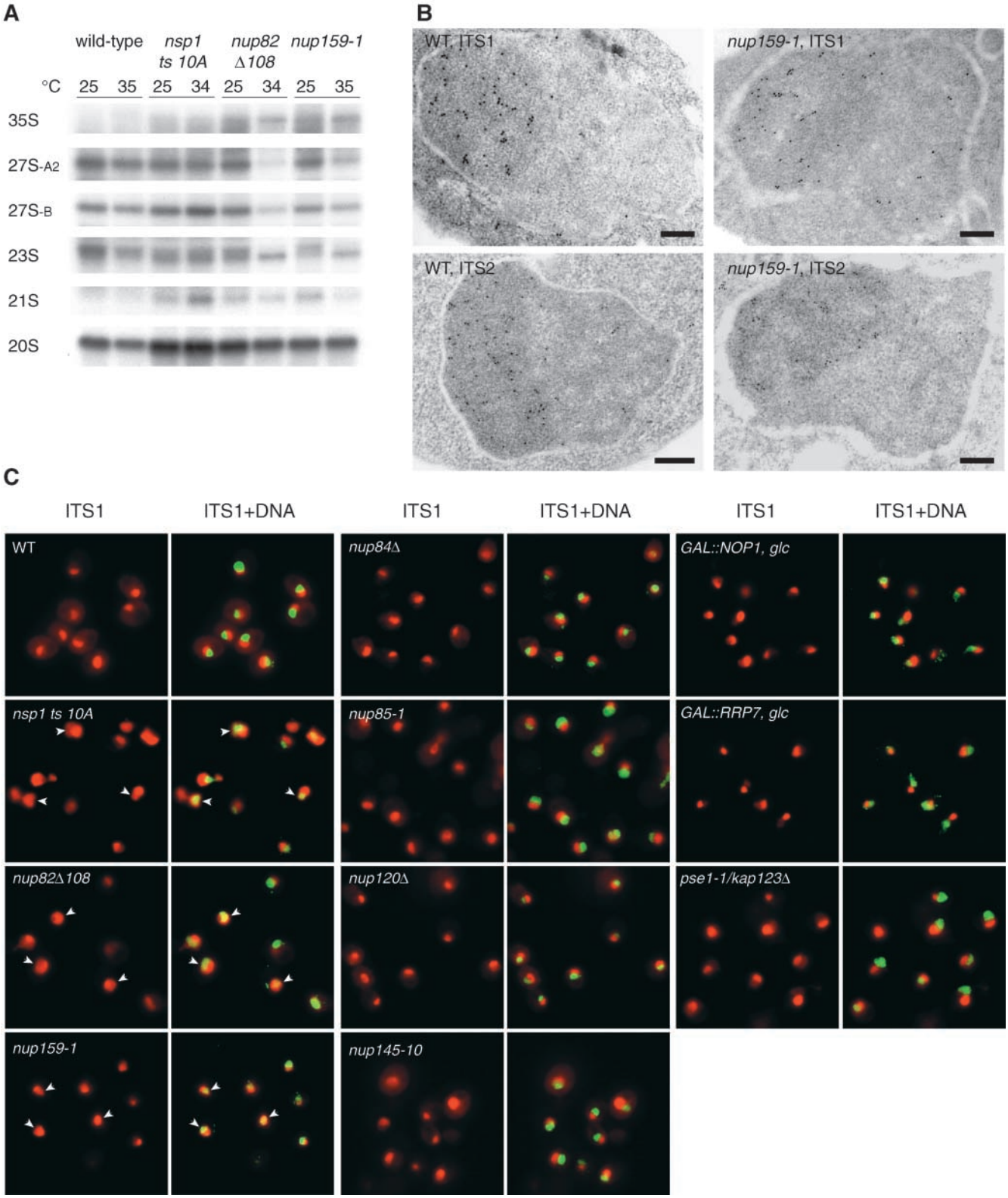


Figure 7. **Pre-rRNA processing in mutants *nup82Δ108, nup159-1,* and *nsp1 ts10A*.** (A) Northern blot analysis of pre-rRNA processing. No gross defect in pre-rRNA processing is detected, although a slight increase in 35S pre-rRNA is visible in the mutant strains as compared with the wild-type cells. Pre-rRNA overall signal in *nup159-1* and *nup82Δ108* is dimmer at semipermissive temperature, except the amount of 20S pre-rRNA which remains constant, indicating a relative accumulation of this species. (B) EM in situ hybridization with probes complementary to the D-A2 fragment of the ITS1 and to the ITS2. The ITS1 probe labels the nucleoplasm in *nup159-1* cells grown at 34-35°C for 3 h, but not in a wild-type strain. No accumulation of pre-5.8S/25S rRNA is detected with the ITS2 probe in the nucleoplasm of this mutant. (C) FISH with a probe to the D-A2 domain of the ITS1 (red), and DNA staining with DAPI (green). Mutations in the Nup82p, but not in the Nup84p complex, lead to the accumulation of pre-18S rRNA in the whole nucleus (nuclei designated by arrows) and a very low cytoplasmic signal. In cells defective in pre-rRNA processing (*GAL::NOP1* and *GAL::RRP7* mutants grown for 20 h on glucose) and in nuclear import of ribosomal proteins (*pse1-1/Δkap123* transferred for 3 h to 34°C), the ITS1 is localized to the nucleolus. Bars (EM), 200 nm.

strong labeling of the whole nucleus, whereas the cytoplasm was not fluorescent, consistent with the nuclear retention of pre-40S particles. As a comparison, no signal was either detected in the cytoplasm of the *GAL::NOP1* and *GAL::RRP7* strains grown on glucose, but the ITS1 remained confined to the nucleolus, suggesting very early sequestration of the pre-18S rRNAs in this domain. Similarly, the *pse1-1/Δkap123* cells displayed strongly labeled hypertrophied nucleoli; in this strain, a dim signal could still be detected in the cytoplasm.

The results of the FISH experiments are in agreement with the pictures obtained by EM in situ hybridization with the 18S and ITS1 probes. Overall, these data show that the nucleoplasmic accumulation of rRNA associated with the mutations *nup82Δ108*, *nup159-1*, and *nsp1 ts10A* does not correlate with a major alteration of pre-rRNA processing in the nucleus, and is likely to reflect a defect in the nuclear export process.

The function of Nup159p in the nuclear export of ribosomal particles and mRNPs is different

As mentioned above, mutation of *NUP159* as well as *NUP82* impairs mRNP nuclear export, which might result from the loss of function of one of the proteins interacting with Nup159p, in particular the DEAD-box RNA helicase Dbp5p/Rat8p (Hodge et al., 1999) and the mRNA transport factor Mex67p (Strasser et al., 2000). Dbp5p, which was isolated in a genetic screen for mRNA transport mutants, is bound to the NPC through the NH_2-terminal domain of Nup159p. Deletion of this domain in Nup159p results in the loss of Dbp5p from the NPC, confers thermosensitivity, and severely affects mRNA export, even at 25°C (Del Priore et al., 1997; Hodge et al., 1999). Dbp5p in turn forms a complex with the nucleoporins Gle1p/Rss1p and Nup42p/Rip1p (Hodge et al., 1999; Strahm et al., 1999), which are also involved in mRNA export (Murphy and Wente, 1996; Saavedra et al., 1997).

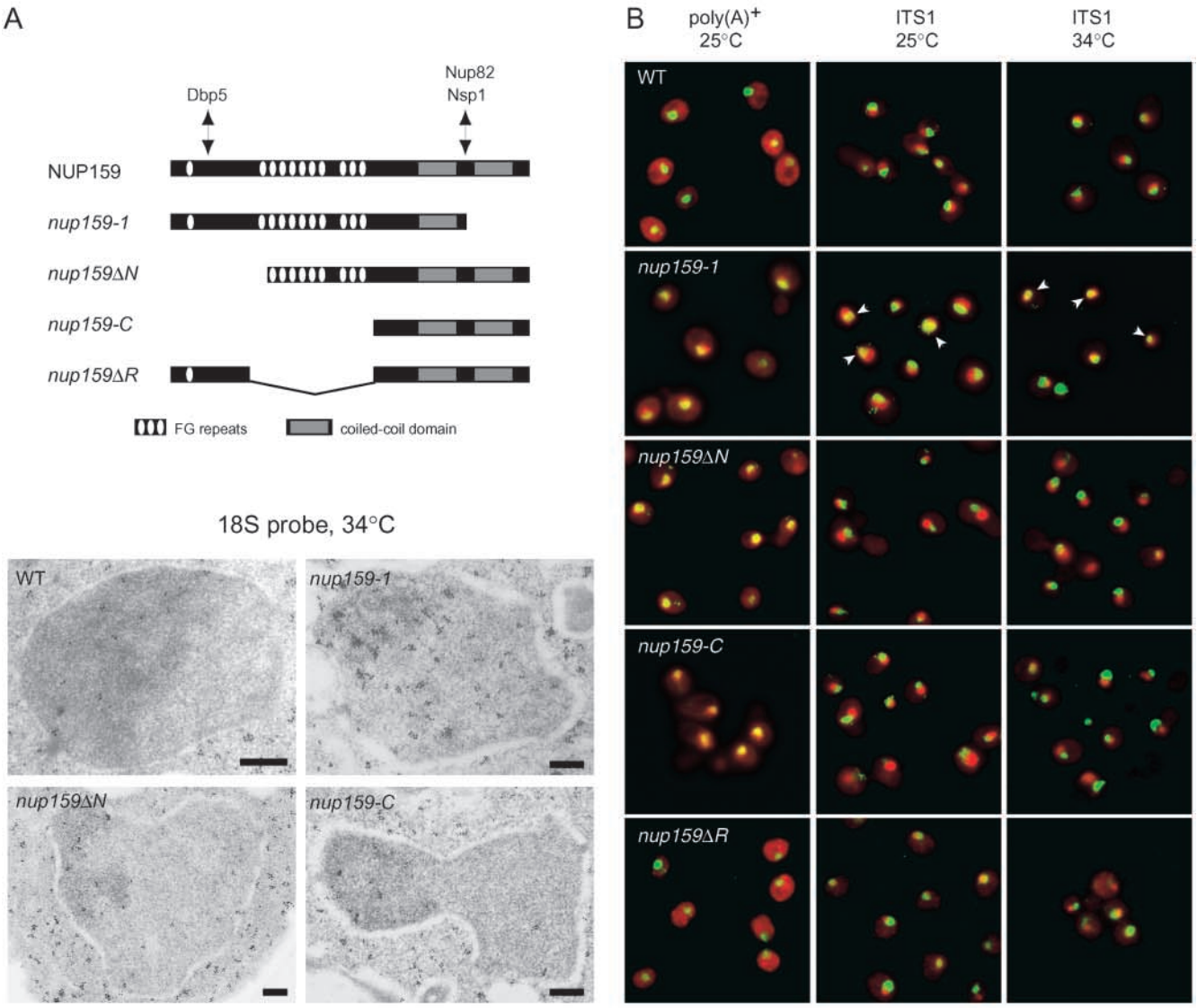
First, we established whether the domains of Nup159p involved in the nuclear export of the ribosomal subunits were the same as for mRNPs by looking at mutants expressing Nup159p deleted of its NH_2-terminal domain (*nup159ΔN*), its central region (*nup159ΔR*), or both (*nup159-C*) (Fig. 8). As described previously, the mRNA transport defect in mutants *nup159ΔN* and *nup159-C* was more pronounced than in *nup159-1* cells, with 100% of the cells showing nuclear accumulation of poly(A)$^+$ RNAs at the permissive temperature (Fig. 8 B). However, under the same conditions (25°C; unpublished data) or at 34°C, these strains displayed no accumulation of the small ribosomal subunits in the nucleus, as visualized by EM in situ hybridization with the 18S probe (Fig. 8 A), and as confirmed by FISH with the 5′-ITS1 probe on cells grown (Fig. 8 B). Deletion of the central domain (mutant *nup159ΔR*) had no effect. Thus, expression of the sole COOH-terminal domain of Nup159p was sufficient to ensure the proper trafficking of the pre-40S particles.

We then examined the conditional mutants *rat8-2* (*dbp5^{ts}*), *GAL::GLE1*, and *gle 1-37^{ts}*. At 34°C, the *rat8-2* strain displayed a higher labeling in the nucleolus with the 18S probe than wild-type cells (2× increase; Fig. 6), but no accumulation was seen in the nucleoplasm (Fig. 8 C). Similarly, FISH with the 5′-ITS1 showed no nuclear retention of pre-40S particles, whereas poly(A)$^+$ RNAs were primarily detected in the nucleus at this temperature (Fig. 8 D). Depletion of Gle1p after a 12-h growth on glucose resulted in a mild nucleoplasmic accumulation of rRNA (Fig. 8 C), although not as dramatic as in mutants *nsp1 ts10A*, *nup159-1*, or *nup82Δ108*. Cells bearing the GLE1 ts mutant allele *gle1-37* grown at 34°C displayed no clear increase of the EM nuclear labeling with the 18S probe (Fig. 6, semiquantitative analysis), and FISH revealed a strong nuclear accumulation of poly(A)$^+$ RNA, but no obvious defect in 20S rRNA export (Fig. 8 D). Deletion of the nonessential NUP42 gene had no effect either on ribosomal trafficking (unpublished data). GLE1 was also isolated as a weak multicopy suppressor of the ts phenotype of mutant *nup159-1* (Del Priore et al., 1996). It restores slow growth at 37°C and partially corrects the mRNA transport defect in *nup159-1* cells. Strikingly, there was no pre-40S nuclear accumulation in *nup159-1* cells overexpressing Gle1p (Fig. 8, C and D), indicating that GLE1 is an efficient multicopy suppressor of the rRNA trafficking defect observed in this mutant at 25 and 34°C. In contrast, a high level of Gle1p did not correct the ribosomal transport defect in mutant *nsp1 ts10A* (Fig. 8 C).

Thus, the transport defect observed for the ribosomal particles upon alteration of the Nup82p–Nup159p–Nsp1p complex is not due to the loss of Dbp5p from the NPC, as described for mRNPs. In addition, GLE1 corrects the preribosome and mRNP transport defects to different extents in *nup159-1* cells. These observations indicate that Nup159p, although involved both in mRNP and preribosome nuclear export, does not fulfill the same function in these two pathways.

Discussion

Early cell fractionation experiments by Udem and Warner (1973) showed that nuclear export of the pre-40S particles in *S. cerevisiae* takes place within 5–10 min after synthesis of the 35S pre-rRNA, whereas the pre-60S particles are transferred to the cytoplasm after 15–20 min. Incorporation of the neosynthesized 60S subunits into polysomes happens with the same delay. Comparable results were found in *Saccharomyces carlsbergensis* (Trapman and Planta, 1976), strongly suggesting that the intranuclear pathways of the two subunits derived from a given 90S precursor are not temporally coordinated in yeast. Indeed, we see here at the ultrastructural level a clear difference in the distributions of the precursors for the pre-40S and pre-60S particles within the nucleus of wild-type cells: the 18S probe mainly labeled the nucleolus (10–15% only in the nucleoplasm), whereas a substantial part (25–35%) of the signal obtained with the 25S probe was found within the nucleoplasm. This observation provides ultrastructural evidence that the two subunits travel through these nuclear compartments along distinct pathways and/or with different kinetics. The occurrence of pre-60S particles in the nucleoplasm is consistent with some late assembly steps taking place in this part of the nucleus. Accordingly, Rsa1p, a protein involved in the biogenesis of the 60S subunits, has recently been shown to be exclusively nucleoplasmic (Kressler et al., 1999). In contrast, the 43S particles may be entirely assembled in the nucleolus and rapidly exported to the cytoplasm, although the low but repro-


A
Dbp5
Nup82
Nsp1
NUP159
nup159-1
nup159ΔN
nup159-C
nup159ΔR
FG repeats
coiled-coil domain
18S probe, 34°C
WT
nup159-1
nup159ΔN
nup159-C
B
poly(A)+ 25°C
ITS1 25°C
ITS1 34°C
WT
nup159-1
nup159ΔN
nup159-C
nup159ΔR
C
18S probe
dbp5-2, 34°C
GAL::GLE1, glc
nup159-1 / GLE1, 34°C
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gle1-37
nup159-1 / GLE1

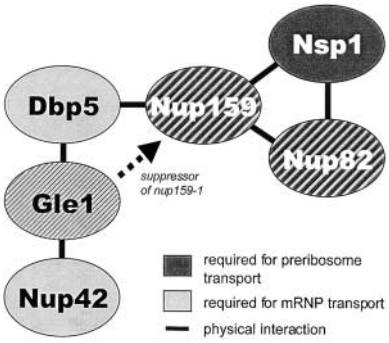

Figure 9. **Schematic overview of the involvement of the Nup82p complex in nuclear export of preribosomes and mRNPs.** The proteins required for mRNP transport are in light gray, whereas involvement in ribosomal export is indicated in dark gray. Nup159p and Nup82p are acting in both processes, whereas Nsp1p is not required for mRNP trafficking. Gle1p overexpression corrects the ribosomal export defect in mutant nup159-1, but direct involvement of Gle1p in this transport remains to be shown.

ducible labeling of the nucleoplasm with the 18S probe shows that some small subunits, if not all, transit through this part of the nucleus.

This difference in the nuclear distributions of the pre-40S and pre-60S particles was exacerbated in mutants affected in pre-rRNA processing: the 25S and ITS2 probes strongly labeled the nucleoplasm, whereas the 18S probe was only detected over the nucleolus. Nuclear accumulation of pre-60S subunits was also observed in mutants affected in 5S rRNA production (Dechampesme et al., 1999). Recently, Stage-Zimmermann et al. (2000) reported nuclear retention of pre-60S particles in the ribosomal assembly mutants *dob1-1*, *nsr1Δ*, *nop1-3*, and *fal1-1*. Therefore, ill-matured pre-60S particles appear to be released from the nucleolus and accumulate in the nucleoplasm in a variety of situations that are not directly related to a defect in the nuclear export machinery. In contrast, the nucleolar exit of aberrant processing products of the 18S pathway seems tightly controlled. These intermediates may be rapidly degraded by the exosome complex (Allmang et al., 2000) before exiting the nucleolus, and the signal detected in the nucleolus with the 18S probe in these mutants corresponds in part to accumulation of 32S and 35S pre-rRNAs. These results show that the small subunits do not necessarily accumulate in the nucleoplasm upon a defect in rRNA processing. A similar conclusion was reached by Moy and Silver (1999) when they examined by FISH the localization of the 20S pre-rRNA in ts mutants affected in 18S production, including *fal1-1*, *nop1-3*, and *nsr1Δ*. Nucleoplasmic accumulation of pre-40S particles in the whole nucleus may be more confidently interpreted as a defect in nuclear export than in the case of the pre-60S.

Examination of different nucleoporin mutants revealed a clear correlation between a function in preribosome export and known structural data on nucleoporin subcomplexes. The three nucleoporins whose mutation was found to trigger a strong nuclear accumulation of ribosomal particles, namely Nup82p, Nup159p, and Nsp1p, were previously shown to be physically associated within the NPC (Belgareh et al., 1998). In contrast, mutants of the four nucleoporins of the Nup84p complex (Siniossoglou et al., 1996, 2000) that we looked at (Nup84p, Nup85p, Nup120p, and Nup145Cp) displayed very mild or no nuclear retention of preribosomes. All of these nucleoporins, Nsp1p excepted, were found to be involved in mRNP export (Doye and Hurt, 1997), whereas only a subset of them are shown here to be necessary for trafficking of the ribosomal subunits. Using a functional assay based on the use of a GFP fused ribosomal protein (Rpl25p–GFP), Hurt et al. (1999) also found no requirement for Nup84p and Nup85p in the trafficking of the large ribosomal subunit. Similarly, detection of 20S pre-rRNA by FISH did not show overall nuclear accumulation of the pre-40S particle in mutants of the nucleoporins composing the Nup84p complex. Our data extend these observations by showing, with a single assay, that both subunits are correctly exported from the nucleus in mutants of the Nup84p complex. Interestingly, *nup84Δ*, *nup85-1*, *nup120Δ*, and *nup145-10* share with mutant *nup159-1*, and to a lesser extent *nup82Δ108*, the property of displaying NPC aggregation (Doye and Hurt, 1997). In addition, all these nucleoporin mutants have fragmented nucleoli at 37°C. Our results show that there is no direct correlation between these phenotypes and a defect in preribosome trafficking.

Nsp1p, Nup82p, and Nup159p are associated through interactions of coiled-coil domains contained in their COOH-termini (Belgareh et al., 1998). These domains in Nup159p and Nsp1p are necessary and sufficient for cell viability (Nehrbass et al., 1990; Del Priore et al., 1997). The *nup159-1*, *nup82Δ108*, and *nsp1 ts10A* mutations all affect coiled-coil domains, and result at nonpermissive temperature in the disruption of the complex; thus, our data suggest that the integrity of the whole complex is required for preribosome export. Two other ts alleles of NSP1, *nsp1-ala6* and *nsp1-5*, were also reported by Hurt et al. (1999) to accumulate L25-GFP in the nucleus. In contrast, Moy and Silver (1999) detected overall nuclear accumulation of the 20S pre-rRNA in the *nup82Δ108* mutant at 37°C, but not in the *nup159-1* and *nsp1 ts10A* mutants. However, this discrepancy may be related to the temperature chosen to trigger the ts phenotype, as we observed that shifting these mutants to 37°C leads to the disruption of the nucleolus and a drop in the rRNA in situ hybridization signal.

The transport defect seen here for preribosomes upon alteration of the Nup82p complex could be explained as the result of a pleiotropic effect on nucleocytoplasmic transport, but there is no general defect of protein nuclear import and export in *nup159-1* and *nup82Δ108* cells, as shown with re-

Figure 8. **Function of Nup159p in the trafficking of the pre-40S particles: structural requirements and interacting proteins.** (A and C) EM in situ hybridization with the 18S probe. (B and D) FISH with the poly-dT probe or the ITS1 (D-A2) probe (red), and DNA staining with DAPI (green). Arrows indicate nuclei displaying overall accumulation of the ITS1. In A and B, the cells were grown at 25°C or shifted to 34°C for 3 h as indicated. In C and D, all the cells were cultured at 34°C for 3 h, except the GAL::GLE1 strain, which was grown for 12 h on glucose. The domains in Nup159p interacting with Dbp5p, Nsp1p, and Nup82p are indicated on the schematic representation of the wild-type and mutated forms of Nup159p in A. Bars (EM), 200 nm.

Table I. **Yeast strains**

Strain	Genotype	References
FY86	*MATα ura3-52 leu2Δ1 his3Δ200*	(Winston et al., 1995)
LGY101	*MATα ura3-52 leu2Δ1 his3Δ200 rat7-1/nup159-1*	(Gorsch et al., 1995)
VDPY107	*MATα ura3-52 leu2Δ1 his3Δ200 nup159-1 + pVΔP7 (RSS1/GLE1 LEU2 2μ)*	(Del Priore et al., 1996)
VDPY114	*MATα ura3-52 leu2Δ1 his3Δ200 trp1D63 + pVΔP14 (GAL1::gle1 LEU2 2μ)*	(Del Priore et al., 1996)
VDPY121	*MATα ura3-52 leu2Δ1 his3Δ200 nup159::HIS3 + pVΔP16 (nup159ΔN LEU2 CEN)*	(Del Priore et al., 1997)
VDPY122	*MATα ura3-52 leu2Δ1 his3Δ200 nup159::HIS3 + pVΔP17 (nup159-C LEU2 CEN)*	(Del Priore et al., 1997)
AMY101	*MATα ura3-52 leu2Δ1 his3Δ200 nup159::HIS3 + pAM1 (nup159ΔR LEU2 CEN)*	(Del Priore et al., 1997)
LGY108	*MATα ura3-52 leu2Δ1 his3Δ200 nup159::HIS3 + pLG4 (NUP159 URA3 CEN)*	(Del Priore et al., 1997)
nup82Δ108	*MATα ura3-52 leu2,3-112 his3Δ200 trp1-1 nup82::HIS3 + p(nup82Δ108 LEU2 2μ)*	(Hurwitz and Blobel, 1995)
YV179	*MATα ura3-52 leu2 his3 ade2 trp1 nup84::KanR*	(unpublished data)
nup85-1	*MAT? ura3-52 leu2Δ1 his3Δ200 rat9-1/nup85-1*	(Goldstein et al., 1996)
CHY104	*MATα ura3-52 leu2Δ1 his3Δ200 rat2/nup120::HIS3*	(Heath et al., 1995)
TDY105	*MATa ura3-52 leu2Δ1 trp1Δ63 nup145-10*	(Dockendorff et al., 1997)
nsp1 ts10A	*MAT? ade2-1 can1-100 leu2-3 lys1-1 ura3-52 nsp1::URA3 nsp1 ts10A/URA3*	(Nehrbass et al., 1990)
SPY23	*MAT? ura3-52 leu2Δ1 his3Δ200 rss1-37*	(Hodge et al., 1999)
CHY119	*MATα ura3-52 leu2Δ1 his3Δ200 rip1/nup42::HIS3*	(Saavedra et al., 1997)
CSY550	*MATa ura3-52 leu2Δ1 trp1Δ63 rat8-2*	(Snay-Hodge et al., 1998)
BMA59-1B	*MATa ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 can1-100 GAL::RRP7/URA3*	(Baudin-Baillieu et al., 1997)
BMA67-1B	*MATa ura3-1 ade2-1 leu2-3,112 his3Δ200 trp1-1 can1-100 rpS27B::HIS3*	(Baudin-Baillieu et al., 1997)
pse1-1/Δkap123	*MATa ura3-52 leu2Δ1 his3Δ200 pse1-1 kap123::HIS3*	(Seedorf and Silver, 1997)
EE1b-6	*MATa ura3-52 his4 his7 ade1 tyr1 Gal-rna1-1 rnh1::URA3*	(Hopper et al., 1990)
nmd3-2	*MATa ura3 his3 leu2 lys2 nmd3::kanMX4 + p(LEU2 nmd3-2 ARS/CEN)*	(Gadal et al., 2001)
YO126	*MATa ade2 ade3 his4-260 tyr7-1 lys2 ura3 leu2 trp1 gar1::LEU2 + pJPG224 (gar1-1)*	(Bousquet-Antonelli et al., 1997)

porter proteins carrying an NLS or an NES (Belgareh et al., 1998; Hurwitz et al., 1998), and tRNA export is not affected in the *nup159-1* mutant either (Sarkar and Hopper, 1998). Because of the diversity of the karyopherins and of the nuclear import routes, one cannot formally exclude that the mutations in the nucleoporins of the Nup82p complex primarily affect a particular pathway required for the nuclear import of factors involved in preribosome maturation and transport. Here we show that the phenotype observed in *nup159-1*, *nup82*Δ108, and *nsp1 ts10A* cells is not reproduced in the double mutant *pse1-1Δkap123*, and, concerning the small subunit, in cells mutated for some pre-rRNA processing factors.

The roles of the Nup82p complex in the nuclear export of preribosomes and mRNPs are compared in Fig. 9. The deletion of Nup159p NH_2-terminal domain results in the loss of Dbp5p from the NPC, which has been proposed to be the reason why poly(A)$^+$ RNA accumulate in the nucleus of this mutant (Hodge et al., 1999). Here we show that the release of Dbp5p may not account for the negative effect of *nup159-1* mutation on preribosome trafficking, as this process is neither affected by the deletion of Nup159p NH_2-terminal domain, nor by the loss of *DBP5* function in the ts mutant *rat8-2*. Interestingly, our data show that GLE1 is an efficient high-copy suppressor of the defect in preribosome transport in *nup159-1* cells, whereas it poorly corrects nuclear retention of mRNPs in this mutant (Del Priore et al., 1996). Although an explanation of the suppressive effect of *GLE1* remains elusive, this observation supports the idea that Nup159p plays different roles in the transport of the two types of RNPs. Because Nsp1p is lost from the Nup82p complex in mutant *nup159-1* (Belgareh et al., 1998), one possibility would be that an excess of Gle1p directly or indirectly allows Nsp1p to stay on the cytoplasmic side of the NPC. From our observations, we propose that Nup159p has a dual role in RNP export: (a) the NH_2-terminal region mediates a function which is critical for nuclear export of mRNPs, but not of preribosomes (presumably anchoring Dbp5p and other proteins to the NPC); and (b) the COOH-terminal region, containing the coiled-coil domains, is necessary and sufficient for preribosome export, at least by ensuring the stability of the Nup82p complex. Further insight into this question may be gained from the analysis of *GLE1* suppressive effect in the *nup159-1* mutant and Nsp1p function in ribosomal trafficking.

Materials and methods

Yeast strains

Yeast cells were culture in standard YP supplemented with 2% glucose (YPD) or 2% galactose (YPG), and in YNB supplemented with amino acids and bases as required. The strains studied in this article are listed in Table I.

Probes for ultrastructural in situ hybridization

To make probes complementary to the 18S and the 25S rRNAs, fragments 859–2371 (XbaI-StuI) and 3378–6471 (KpnI-NheI) from *S. cerevisiae* ribosomal transcription unit were inserted into vector pGEM4. These fragments span at least 80% of the 18S rRNA and of the 25S rRNA, respectively. Labeling with biotin was performed by nick translation on the entire plasmids in the presence of dUTP-16-biotin (Enzo). The 35S probe used in Fig. 3 was prepared according to the same protocol with a plasmid bearing the full transcription unit.

To obtain a probe complementary to the 5′ part of the ITS1, the D-A2 rDNA fragment from *S. cerevisiae* was amplified by PCR and subcloned into pGEM4. The resulting plasmid was linearized at a restriction site close to site D, and used for multiple cycles of DNA synthesis in a thermocycler using Goldstar DNA polymerase (Roche), primer 5′-CTC CAC AGT GTG TTG TAT TG, which encompasses site A2, and a mix of dNTPs containing dUTP-biotin (Roche). The single strand DNA fragment thus obtained was purified from free dUTP-biotin by salt-precipitation and used as a probe for in situ hybridization on ultrathin sections.

An antisense probe to the ITS2 was generated by in vitro transcription of the corresponding DNA fragment subcloned into pGEM4. Synthesis of the antisense RNA was performed with the Sp6 polymerase in the presence of UTP-digoxigenin.

Ultrastructural in situ hybridization

Embedding of yeast cells for EM was performed as described (Léger-Silvestre et al., 1997, 1999). In brief, cells were cultured at permissive (25°C) or semirestrictive temperature as indicated (OD_{600nm} final = 0.2–0.6), and fixed with 4% paraformaldehyde in the culture medium for 45 min. The cell wall was permeabilized by oxydizing polysaccharides with 1% sodium metaperiodate for 1 h, followed by a 1-h treatment with 500 mM NH_4Cl to reduce aldehydes. Dehydration was performed with ethanol, and the cells were embedded in LR White resin (London Resin Company) according to the manufacturer. Ultrathin sections were performed with an Ultracut E ultra-microtome (Reichert-Jung) and recovered on nickel grids. The ultrastructural observations were performed at the EM facility of the Institut d'Exploration Fonctionnelle des Génomes (IFR 109) of the Centre National de la Recherche Scientifique (Toulouse, France).

For in situ hybridization, the grids were incubated with 1 ng/μl of labeled probe in 50% formamide/10% dextran sulfate/2× SSC for 5 min at 75°C, and then for 3 h at 37°C. The grids were washed three times in 50% formamide/2× SSC for 5 min at 65°C, three times in 0.1× SSC for 5 min at 45 °C, and once in 4× SSC for 5 min at room temperature. The grids were first incubated in 4× SSC/5% BSA for 30 min at 37°C, and then in 4× SSC containing anti-biotin antibodies conjugated to 10- nm gold particles (British Biocell International) for 1 h at 37°C. After washing three times for 5 min in 4× SSC, the grids were plunged repeatedly in water to remove salts. Finally, the sections were contrasted by staining with uranyl acetate and lead citrate, and observed in a Jeol 1200-EX electron microscope at 80 kV.

Semiquantitative analysis of the labeling on micrographs

To evaluate the distribution of rRNAs in the nuclei of mutant cells after EM in situ hybridization (Fig. 6), 20–30 sections of nuclei displaying both the nucleolus and the nucleoplasm were randomly photographed per strain. On each picture, the labeling density of the nucleolus and of the nucleoplasm was calculated as the ratio of the number of gold particles by the surface. For statistical analysis, a one to one comparison of the mean value of FY86 cells (wild-type) with the others was performed, based on the test of normal means in the two populations case (with or without equality of variance); there was no significant violation of the assumption underlying this test.

FISH

FISH with Cy3 labeled oligonucleotides was performed as described (Bertrand et al., 1998). The probe complementary to the D-A2 fragment (5′-ITS1) had the following sequence: TT*CGAACATT*CAAAGAAAGAAC-GAT*AAGGTTTGCCACT*CTCTAAAGACACGT*T, where the asterisks represent amino-modified nucleotides coupled with Cy3 (Amersham Pharmacia Biotech). The 50-mer poly-dT probe for poly(A)$^+$ detection was also labeled with five Cy3 molecules. Images were captured with a CoolSnap cooled CCD camera (Photometrics) mounted on a DMRB microscope (Leica). Composites were prepared using Adobe Photoshop.

Northern blot analysis

Extraction of total RNAs from yeast was performed as described by Tollervey and Mattaj (1987). Pre-rRNAs were detected on Northern blots as reported elsewhere (Vanrobays et al., 2001) using the following probes: probe 20S.3 (5′-TTA AGC GCA GGC CCG GCT GG-3′) complementary to the D-A2 fragment of ITS1; probe 23S.1 (5′-GAT TGC TCG AAT GCC CAA AG-3′) hybridizing within the A3-B1 segment of ITS1; and probe rRNA2.1 (5′-GGC CAG CAA TTT CAA GTT A-3′) complementary to the C2-E segment of ITS2.

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