A protein that shuttles between the nucleus and the cytoplasm is an important mediator of RNA export

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The connection between RNA and protein export from the nucleus was examined in the budding yeast *Saccharomyces cerevisiae*. *NPL3* encodes an RNA-binding protein that shuttles in and out of the nucleus. Export of poly(A)⁺ RNA has been shown previously to be blocked in *npl3-1* mutants. To understand the role of Npl3p in RNA export, we have developed a novel assay that effectively uncouples nuclear protein export from reimport. With this assay, we show that Npl3p satisfies several of the predicted requirements for a protein carrier for mRNA export. Temperature-sensitive mutations in the RNA recognition motifs of Npl3p result in nuclear accumulation of poly(A)⁺ RNA. One such mutation prevents nuclear export of Npl3p. Moreover, Npl3p export depends on ongoing RNA polymerase II transcription. Export ceases in either the presence of the RNA synthesis inhibitor thiolutin or in a temperature-sensitive RNA polymerase (*rpb1*) mutant. Together, these findings support a model in which Npl3p exits the nucleus in association with poly(A)⁺ RNA, deposits the RNA in the cytoplasm, and is rapidly reimported for another cycle of export.

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RNA export from the nucleus is a facilitated energy-dependent process in which specific factors may mediate the export of certain RNAs (Zasloff 1983; Bataille et al. 1990; Dargemont and Kuhn 1992; Jarmolowski et al. 1994). It is likely that all RNAs exit the nucleus in association with RNA-binding proteins and that the substrate for export is not naked RNA but, instead, an RNP complex (for review, see Mehlin and Daneholt 1993). A recent study has shown that the *Chironomus tentans* pre-mRNA-binding protein Hrp36 is part of a large pre-mRNP complex that accompanies RNA from the site of transcription, through the nuclear pore complex, and into polysomes (Visa et al. 1996).

One of the most abundant classes of nuclear RNA-binding proteins is the heterogeneous nuclear ribonucleoproteins (hnRNP), a family of proteins with proposed roles in multiple steps of mRNA processing and export (for review, see Dreyfuss et al. 1993). hnRNPs have been identified in most eukaryotes, including the budding yeast *Saccharomyces cerevisiae* (Bossie et al. 1992; Anderson et al. 1993; Wilson et al. 1994; Henry et al. 1996). The study of hnRNPs has uncovered a common structural theme: one or more RNA-binding domains and at least one other auxiliary domain proposed to mediate protein interactions. The RNP-type RNA-recognition motif (RRM) present in a number of RNA-binding

proteins is a domain of ~90 amino acids that contains two consensus sequences, RNP-1 and RNP-2 (Dreyfuss et al. 1988; Bandziulis et al. 1989; Kenan et al. 1991; Haynes 1992; Mattaj 1993).

Some nuclear proteins, including hnRNP proteins, exit the nucleus and rapidly re-enter in a process referred to as nucleocytoplasmic shuttling. Although the functional significance of this phenomenon is not yet clear, it has been proposed to be part of the mechanism for the exchange of RNAs between the nucleus and the cytoplasm (Piñol-Roma and Dreyfuss 1992; Piñol-Roma and Dreyfuss 1993; Flach et al. 1994). Specific nuclear export signals (NESs) have now been shown to exist in the cellular proteins hnRNP A1 (Michael et al. 1995) and the protein kinase A inhibitor peptide (PKI; Wen et al. 1995) as well as the viral protein Rev (Fischer et al. 1995), suggesting that protein export is an active, signal-mediated pathway. The existence of specific export signals in proteins that bind RNA (i.e., hnRNP A1 and rev) strengthens the argument that shuttling hnRNPs act as carriers for RNA export.

The *NPL3* gene was originally isolated as a temperature-sensitive mutant defective in nuclear protein localization (Bossie et al. 1992). The same *NPL3* mutants accumulate poly(A)⁺ RNA in the nucleus at the nonpermissive temperature indicating a block in mRNA export (Russell and Tollervey 1995; Singleton et al. 1995; Henry et al. 1996).

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The *NPL3* gene encodes a nuclear protein that shuttles between the nucleus and the cytoplasm in the budding yeast (Bossie et al. 1992; Russell and Tollervy 1992; Flach et al. 1994; Wilson et al. 1994). Npl3p is relatively abundant and can be cross-linked to poly(A)⁺ RNA (Wilson et al. 1994). Moreover, Npl3p has structural features analogous to those found in some hnRNP proteins including two central RRM domains flanked by a carboxy-terminal glycine-rich domain containing a series of RGGY/F repeats. Npl3p also shows some similarity to three members of an evolutionarily conserved family of alternative splicing factors termed SR proteins and is, to date, the only SR-like protein in yeast (Birney et al. 1993). Alignment of the RRM domains of Npl3p with human SF2/ASF, *Drosophila* SRp55, and *Arabidopsis* SR protein reveals homologies far greater than those expected for unrelated RRM domains.

We propose that Npl3p may participate directly in the process of mRNA export by acting as a carrier of mRNA as it is transported to the cytoplasm via the nuclear pore. The requirements for such a carrier, in principle, include the following: It should bind RNA in the nucleus, move out of the nucleus with RNA, and return to the nucleus from the cytoplasm. To demonstrate that Npl3p meets these criteria, we now report a new assay for nuclear export. Previous assays for nucleocytoplasmic shuttling required the formation of heterokaryons and the movement of protein from one nucleus to the other; thus uptake into a second nucleus was required for exit from the first to be assayed. The assay we now present uncouples the process of nuclear export from reimport. Using this assay, we present several lines of evidence to show that Npl3p and poly(A)⁺ RNA export are tightly coupled. First, new mutations in the RRM domains of Npl3p result in defects in poly(A)⁺ RNA export from the nucleus; second, a mutation in the first RRM of Npl3p results in a block in Npl3p export; and third, the export of Npl3p from the nucleus is dependent on RNA polymerase II transcription. We propose a model in which Npl3p must form a complex with poly(A)⁺ RNA for nuclear export to occur.

Results

New NPL3 temperature-sensitive mutants display Npl3p import and RNA export defects

In wild-type cells, Npl3p is located in the nucleus as determined by immunofluorescence, at all growth temperatures tested (Fig. 1A, top row; Bossie et al. 1992). We know, however, that Npl3p is capable of exiting and re-entering the nucleus (Flach et al. 1994). Thus, the observed steady-state nuclear location of Npl3p indicates that it must shuttle rapidly between the nucleus and the cytoplasm. In addition, in wild-type cells, the poly(A)⁺ RNA is distributed diffusely throughout the cytoplasm as determined by *in situ* analysis at all growth temperatures tested (Fig. 1A, top row) and is consistent with rapid RNA export out of the nucleus (Amberg et al. 1992).

To obtain more information about the functional domains of Npl3p, we generated a panel of new temperature-sensitive *npl3* mutants. These mutants were isolated solely on the basis of their failure to grow on plates at 37°C. Further analysis of the resulting mutations and their phenotypes showed a relationship between mutations in the RRM domains of Npl3p, defects in poly(A)⁺ RNA export from the nucleus, and temperature-sensitive growth. Three phenotypic classes of mutants were identified on the basis of Npl3p protein and poly(A)⁺ RNA localization.

Those in the first class show defects in Npl3p localization at permissive temperatures and accumulation of poly(A)⁺ RNA in the nucleus at the restrictive temperature. One member of this class is the previously described mutant *npl3-1* (Bossie et al. 1992; Kadowaki et al. 1994a; Singleton et al. 1995). This mutant shows severe mislocalization of Npl3p to the cytoplasm at 30°C, a temperature that is semipermissive for growth (Fig. 1B; Bossie et al. 1992). Interestingly, at 37°C, the temperature at which *npl3-1* cells display a poly(A)⁺ RNA export defect and are incapable of growth, Npl3p is localized primarily in the nucleus of the cell (Fig. 1). As shown in Table 1, a similar pattern of Npl3p mislocalization at 30°C and poly(A)⁺ RNA accumulation in the nucleus at 37°C is seen in *npl3-1*, *npl3-38*, and *npl3-48*, each of which contains a mutation within the second RNA recognition motif of Npl3p (RRM2; Fig. 2). The first appearance of the RNA export defect in these cells is apparent 1 hr after a shift to 37°C and is maximal at 3 hr. The mutant *npl3-26* also contains a mutation in RRM2 but displays a much weaker phenotype of Npl3p mislocalization and poly(A)⁺ RNA accumulation in the nucleus, with the latter defect first appearing 3 hr after a shift to 37°C and becoming maximal at 6 hr.

Three of the mutants fall into the second class and show little or no defect in Npl3p localization at any temperature tested but display accumulation of poly(A)⁺ RNA in the nucleus at 30°C as well as at 37°C (Table 1). These mutants include *npl3-3*, *npl3-17*, and *npl3-41*. Each contains at least one mutation in the first RRM of Npl3p (RRM1), and *npl3-17* also contains a mutation in RRM2. Accumulation of poly(A)⁺ RNA in the nucleus of these cells is first observed as early as 30 min after a shift to the nonpermissive temperature (Fig. 1; Table 1).

Although all of the mutants in classes 1 and 2 display some degree of nuclear accumulation of poly(A)⁺ RNA, they also display some cytoplasmic RNA signal. This result suggests that in no case is there a complete block in poly(A)⁺ RNA export. Only one of the mutants analyzed, *npl3-27*, did not display any accumulation of poly(A)⁺ RNA in the nucleus. This mutant did, however, show defects in Npl3p localization, even at the permissive temperature of 25°C. Although *npl3-27* cells fail to grow at 37°C on plates (data not shown), they are capable of logarithmic growth in liquid culture for at least 16 hr after a shift to 37°C (Fig. 1B). This allele of *NPL3* contains one mutation in the carboxy-terminal RGG box domain of Npl3p and is the sole member of the third class (Fig. 1; Table 1). It should also be noted that

Figure 1. Characterization of *npl3* temperature-sensitive mutants. (A) Localization of Npl3p and poly(A)⁺ RNA in *npl3* temperature-sensitive mutants. Strains were grown to 1×10^7 cells/ml in SC medium lacking leucine and shifted to 30°C (middle columns), 37°C (right columns), or incubated at 25°C (left columns) for an additional 4 hr (or 8 hr for wild-type *NPL3* at 37°C). The Npl3p protein was visualized with an FITC anti-rabbit secondary antibody, and the RNA was visualized with the Texas Red sheep anti-digoxigen Fab fragment. (B) Growth curves of *npl3* temperature-sensitive mutants. Overnight cultures of strains containing wild-type *NPL3*, or temperature-sensitive alleles of *npl3*, were diluted into fresh medium and incubated for 8 hr at 25°C (■). Cultures were then shifted (arrow) to either 30°C (●) or 37°C (▲), and growth curves were generated by counting of cells every 4 hr.

Table 1. Summary of *NPL3* mutations and phenotypes

Allele	Mutation ^a	Domain ^b	Npl3p mislocalization (°C)			Nuclear accumulation of poly(A) ⁺ RNA (°C)		
			25	30	37	25	30	37
Class 1								
<i>npl3-1</i>	A254V	RRM2	+	++	+	–	–	++
<i>npl3-26</i>	N223D	RRM2	+	–	–	–	–	+
<i>npl3-38</i>	G241D	RRM2	+	+	–	–	–	++
<i>npl3-48</i>	S230P	RRM2	+	++	–	–	–	++
Class 2								
<i>npl3-3</i>	S193P	RRM1	–	–	–	+	+	++
<i>npl3-17</i>	F160L	RRM1	+	+	–	–	+	++
	I268T	RRM2						
	E121G	N-term	–	–	–	+	+	++
<i>npl3-41</i>	F183I	RRM1						
	S193P	RRM1						
Class 3								
<i>npl3-27</i>	E409K	RGGY	++	++	++	–	–	–

Phenotypes are described as approximate number of cells displaying the defect either Npl3p mislocalization to the cytoplasm or accumulation of RNA in the nucleus. (+) 0%–50%; (++) 50%–100%.

^aAmino acid changes are described using the one-letter amino acid code with the first letter indicating the wild-type amino acid followed by its number and the amino acid to which it is changed in the mutant.

^bThe domain in which the mutation is present.

for all of the mutants tested, the level of the mutant Npl3 protein is unchanged after temperature shift, as determined by Western blotting of total protein from cell extracts (not shown).

A new assay for *Npl3p* nuclear export

To study the relationship between Npl3p and RNA nuclear export, we needed to devise a new assay that effectively uncoupled the two processes from nuclear protein import. A previous assay employed the formation of yeast heterokaryons, where the readout is the re-uptake of a shuttling protein into a second nucleus (Flach et al. 1994). In addition, because the formation of heterokary-

ons requires the mating of two yeast strains, there is inherent technical difficulty in generating heterokaryons in the absence of RNA synthesis or when temperature-sensitive mutants are used. For example, there is ample time for cytoplasmic mixing to occur, which could result in rescue of a recessive mutation. Therefore, we designed a new experimental system to examine specifically the process of protein export in the absence of reimport (Fig. 3A).

Our experimental system is based on the fact that the nucleoporin mutant *nup49-313* is defective in nuclear protein import at the nonpermissive temperature of 36°C but exports RNA under these conditions (Schlenstedt et al. 1993; Doye et al. 1994). When *nup49-313* cells are

Figure 2. Schematic diagram of *npl3* temperature-sensitive mutations. The two RRM of Npl3p are represented. Underlined residues are conserved with members of the SR family of alternative splicing factors. Residues marked with an asterisk are present in a consensus sequence for RRM (Birney et al. 1993). The highly conserved RNP-1 and RNP-2 motifs are indicated. Amino acid substitutions found in the temperature-sensitive mutants generated in this study are indicated with arrows. Also indicated are the previously identified mutants *npl3-1* (Bossie et al. 1992; Kadowaki et al. 1994c; Singleton et al. 1995), *npl3-328* (Bossie et al. 1992; Henry et al. 1996), and *npl3-95* (Loo et al. 1995; Russell and Tol-lervey 1995; Henry et al. 1996).

Figure 3. GFP-Npl3 shuttles in the export assay. (A) Schematic diagram of the export assay. (B) Living cells were photographed after incubation in the export assay. Cells were either incubated at 25°C (panels A, B, E, F, I, J), or at 36°C (panels C, D, G, H, K, L). Cells were photographed either by Nomarski optics (panels A, C, E, G, I, K) or for GFP fluorescence (panels B, D, F, H, J, L). (Panels A–D) Wild-type cells expressing GFP-Npl3; (panels E–H) *nup49-313* cells expressing GFP-Npl3; (panels I–L) *nup49-313* cells expressing NLS-GFP-LacZ. (C) *nup49-313* cells expressing GFP-Npl3 were grown, radiolabeled, lysed, and immunoprecipitates were prepared as described in Materials and methods. Immune complexes were analyzed by SDS-PAGE and autoradiography. Cells were labeled either in medium containing raffinose (lane 1), galactose (lanes 2, 6–8), or after induction in galactose followed by a 2-hr chase in glucose (lanes 3–5). Cells in lanes 1–5 were washed and frozen immediately after labeling; cells in lanes 6–8 were incubated further (chased) in medium containing glucose for another 2 hr at 25°C, then 5 hr at 37° as described for the export assay. In addition, cells were incubated with thiolutin (lanes 4, 7) or cycloheximide (lanes 5, 8) during the 2-hr chase (lanes 4, 5) or during the 7-hr chase (lanes 7, 8). The arrowhead indicates full-length GFP-Npl3, the asterisk indicates a short-lived degradation product of GFP-Npl3.

incubated at 36°C, shuttling proteins should exit the nucleus but should not be reimported, resulting in an accumulation of proteins in the cytoplasm. Therefore, in this assay, the appearance of protein in the cytoplasm is an indicator of protein export.

To visually monitor export, we used a fusion of the green fluorescent protein (GFP) to full-length Npl3p (GFP-Npl3). Expression of this fusion protein was controlled by the inducible *GAL1* promoter. GFP-Npl3 was produced in *nup49-313* cells by growth in galactose-containing medium, after which, the synthesis of the reporter was completely repressed by incubation in medium-containing glucose as a carbon source. If these cells are then incubated at the permissive temperature of 25°C, the GFP-Npl3 remains entirely nuclear (Fig. 3B, panel F), presumably as a result of the re-uptake of GFP-Npl3 into the nucleus from the cytoplasm. When the same cells are incubated at the nonpermissive temperature of 36°C, the GFP-Npl3 signal accumulates in the cytoplasm of the cell (Fig. 3B, panel H) indicating that it is capable of nuclear export. This result is completely dependent on the *nup49-313* mutation, because the

GFP-Npl3 reporter remains nuclear at both temperatures in wild-type cells (Fig. 3B, panels B and D). The same results were obtained with a temperature-sensitive nuclear import mutant in a different nucleoporin, *nsp1* (data not shown).

Typically, after 5 hr at 36°C, 30%–35% of the cells examined showed a strong GFP signal in the cytoplasm (Fig. 4D, large arrowhead) and an additional 30%–35% displayed a weaker but distinctly cytoplasmic GFP signal (Fig. 4D, small arrowhead). In some cells, a bright nuclear GFP-Npl3 signal was also still visible. Many factors could contribute to this remaining nuclear signal. First, the extended time (5 hr) required for the nuclear import defect to occur in *nup49* cells after a shift to the nonpermissive temperature (Schlenstedt et al. 1993; Doye et al. 1994) could prevent the complete accumulation of GFP-Npl3 in the cytoplasm at the time cells are examined. In addition, when GFP-Npl3 is expressed by the *GAL1* promoter, the level of recombinant protein is significantly greater than that of endogenous Npl3p. Therefore, this remaining nuclear signal likely results from excess GFP-Npl3 in the nucleus that may compete

Figure 4. GFP-Npl3-F160L is not exported from the nucleus. *nup49-313* cells expressing GFP-Npl3 (A–D) or GFP-Npl3-F160L (E–H) were processed as described for the export assay and incubated at either 25°C (A,B,E,F) or at 36°C (C,D,G,H). Living cells were either photographed with Nomarski optics (A,E,C,G) or examined for GFP fluorescence (B,D,F,H).

with endogenous Npl3p for saturable components of the export machinery. This remaining nuclear signal is not caused by an export defect in these cells resulting from overexpression of the GFP-Npl3 protein. Although overexpression results in accumulation of poly(A)⁺ RNA in the nucleus immediately after induction (data not shown; L. Gorsch and C. Cole, unpubl.), at the time when cells are examined for export (after repression of GFP-Npl3 synthesis and shift to 36°C), there is no RNA export defect (data not shown).

In contrast to the observed export of GFP-Npl3, a different nuclear protein consisting of the SV40 large T antigen NLS fused to GFP- β -galactosidase (NLS-GFP-LacZ) remains entirely nuclear at both temperatures when examined by use of this assay (Fig. 3B, panels J and L). This result indicates that this NLS-bearing protein can enter the nucleus but is unable to exit once it is there. This result is in agreement with a recently published study (Michael et al. 1995; see Discussion). The continued nuclear localization of NLS-GFP-LacZ also suggests that the appearance of GFP-Npl3 protein in the cytoplasm is not the result of leaky nuclear envelopes.

Because nuclear protein import is blocked in *nup49-313* cells, the accumulation of GFP signal in the cytoplasm must be caused by the export of GFP-Npl3. To confirm this hypothesis further, a pulse-chase experiment was designed to examine the production and stability of GFP-Npl3 during the course of the export assay (Fig. 3C). The appearance of the cytoplasmic signal does not result from synthesis of new GFP-Npl3 because such synthesis is completely blocked in the presence of glucose (Fig. 3C, lane 3). In addition, the fusion protein remains stable throughout the incubation period of the experiment (Fig. 3C, lane 6); therefore, this cytoplasmic accumulation of GFP signal does not result from cleavage of the GFP moiety from the Npl3p.

A mutation in the RRM impairs Npl3p nuclear export

Several of the mutants generated in this study encode amino acid changes in the RRM of Npl3p (Fig. 2). In addition, a subset of these mutants shows some cytoplasmic localization of Npl3p at permissive temperatures; however, this subset shows only nuclear localization of the protein at the nonpermissive temperature (Table 1). One possible explanation for this aberrant

Npl3p localization is that at the permissive temperature, the import of Npl3p in these mutants is slowed, resulting in its appearance in the cytoplasm, but at the nonpermissive temperature, Npl3p export from the nucleus is blocked, resulting in nuclear accumulation. We therefore hypothesized that *npl3* mutants with substitutions in the RRM may be defective in Npl3p nuclear protein export. To test this idea, we examined the behavior of an Npl3p mutant containing a substitution in RRM1 and found it to be defective in export from the nucleus.

The reporter construct used for this analysis was a fusion of GFP with Npl3p in which phenylalanine 160 of Npl3p is changed to leucine (GFP-Npl3-F160L). This substitution was originally identified as one of the mutations in *npl3-17* and is located in RRM1 (Fig. 2). When produced in wild-type cells under the regimen used for the shuttling assay, the GFP-Npl3-F160L fusion protein remains localized entirely to the nucleus (data not shown). The localization of this fusion protein was then examined in the *nup49-313* protein export assay. As seen in Figure 4, although the GFP-Npl3 accumulates in the cytoplasm of *nup49-313* cells at 36°C (Fig. 4D), the GFP-Npl3-F160L mutant remains in the nucleus at this temperature (Fig. 4H). These results show that a mutation in RRM1 of Npl3p can result in defective export of Npl3p from the nucleus.

RNA synthesis and nuclear protein export

If Npl3p is participating in RNA export, we might expect the export of Npl3p to be coupled to the presence of mRNA in the nucleus. To explore this possibility, we examined the effect of RNA synthesis on GFP-Npl3 nuclear export.

The antifungal agent thiolutin is an inhibitor of all three RNA polymerases in yeast (Tipper 1973). When the export assay is performed in the presence of thiolutin, GFP-Npl3 does not accumulate in the cytoplasm but, instead, remains in the nucleus of the *nup49-313* cells (Fig. 5J). This block in GFP-Npl3 export is not a result of a lack of new protein synthesis because GFP-Npl3 accumulates in the cytoplasm to the same extent when the export assay is performed in the presence (Fig. 5L) or absence (Fig. 5H) of the protein synthesis inhibitor cycloheximide. In the nuclei of cells treated with cycloheximide the GFP-Npl3 signal appears to fill a larger

Figure 5. GFP-Npl3 export is dependent on new RNA synthesis. Wild-type (A–F), or *nup49-313* (G–L) cells expressing GFP-Npl3 were processed as described for the export assay. Cells were incubated at 36°C in the presence of no addition (control, A,B,G,H) or in the presence of thiolutin (C,D,I,J) or cycloheximide (E,F,K,L). Living cells were either photographed with Nomarski optics (A,C,E,G,I,K) or examined for GFP fluorescence (B,D,F,H,J,L).

area than it does in the absence of the drug (Fig. 5F,L). Because incubation of *nup49-313* cells with cycloheximide results in a more rapid cessation in cellular division than does shift to 36°C, the relative amount of GFP signal per nucleus in cycloheximide-treated cells is greater than that in untreated cells. Therefore, this apparent increase in size does not result from larger nuclei but, instead, from a much brighter GFP-Npl3 signal in these cells.

The inhibition of only poly(A)⁺ RNA synthesis in yeast can also be accomplished by use of a strain that contains a temperature-sensitive allele of RNA polymerase II, *rpb1-1* (Nonet et al. 1987). When the export assay is performed with a strain containing both the *rpb1-1* and *nup49-313* mutations, there is no accumulation of GFP-Npl3 in the cytoplasm (Fig. 6H). These results indicate that the synthesis of poly(A)⁺ RNA by RNA polymerase II is required for the export of Npl3p from the nucleus. Together, the two experimental results indicate that new RNA synthesis, and in particular, RNA polymerase II transcription, is absolutely required for the export of Npl3p from the nucleus.

Npl3p colocalizes with nucleolar antigens in the absence of RNA synthesis

During the course of these studies, we observed that in the absence of RNA synthesis, the intranuclear distribution of the GFP-Npl3 signal was different from its normal wild-type distribution (Fig. 6, cf. B and F with D and H; Fig. 5, cf. D and J with B). In the absence of RNA

synthesis, the Npl3p signal appears condensed into one or two small points within the nucleus. In many yeast mutants blocked in mRNA export, and in the absence of RNA polymerase I (Pol I) transcription, the nucleolus appears fragmented as assayed by immunofluorescence with antibodies to nucleolar antigens (Oakes et al. 1993; Kadowaki et al. 1994a,b,c). Therefore, we wondered whether the intranuclear localization of Npl3p, when export is blocked, coincided with the redistribution of nucleolar proteins.

Cells producing GFP-Npl3 were treated with thiolutin and prepared for immunofluorescence microscopy (Fig. 7A). To detect nucleolar antigens, we used the monoclonal antibody 9C4, which reacts with a 32-kD nucleolar protein (Bossie et al. 1992; Loeb et al. 1995). In wild-type cells expressing GFP-Npl3, the GFP signal is distributed throughout the nucleoplasm (panel C), and the nucleolus stains as a crescent-shaped body to one side of the nucleus (panel D). In cells treated with thiolutin, however, the GFP-Npl3 signal is found in a small structure within the nucleus (panel G) that colocalizes with similar structures stained by the 9C4 antibody (panel H). To ensure that the appearance of these structures does not result from the presence of recombinant GFP-Npl3 in the nucleus, we examined endogenous Npl3p and nucleolar staining in *rpb1-1* cells (Fig. 7B). In *rpb1-1* cells at 25°C, endogenous Npl3p is located throughout the nucleoplasm, and the nucleolus has its characteristic crescent shape (panels C and D, respectively). In *rpb1-1* cells incubated at 36°C for as little as 30 min, however, the endogenous Npl3p is again found in a condensed spot in

Figure 6. GFP-Npl3 export is dependent on RNA Pol II transcription. *rpb1-1* cells (A–D) or *nup49-313 rpb1-1* double mutants (E–H) expressing GFP-Npl3 were processed as described for the export assay. Cells were incubated at either 25°C (A,B,E,F) or 36°C (C,D,G,H). Living cells were either photographed with Nomarski optics (A,C,E,G) or examined for GFP fluorescence (B,D,F,H).

Figure 7. Npl3p colocalizes with nucleolar antigens in the absence of RNA synthesis. (A) Wild-type cells containing the GFP-Npl3 expression plasmid pPS811 were grown and induced as described for the export assay. After induction, GFP-Npl3 synthesis was repressed by growth in YPD for 2 hr in the presence (panels E–H) or absence (panels A–D) of thiolutin. Cells were then fixed and processed as described for immunofluorescence. Cells were photographed by use of Nomarski optics (panels A,E) or DNA was stained with DAPI (panels B,F). Npl3p was visualized by the GFP signal (panels C,G), and nucleolar antigens were visualized by use of the 9C4 monoclonal antibody (panels D,H). (B) *rpb1-1* cells were grown to 1×10^7 cells/ml in YPD and incubated at either 25°C (panels A–D) or shifted to 36°C for 30 min (panels E–H). Cells were fixed and processed as described for immunofluorescence. Cells were photographed by use of Nomarski optics (panels A,E) or DNA was stained with DAPI (panels B,F). Npl3p was visualized with anti-Npl3 antisera (panels C,G), and nucleolar antigens were visualized with the 9C4 monoclonal antibody (panels D,H).

the nucleus (panel G) that colocalizes with nucleolar proteins (panel H).

Discussion

Our examination of the yeast RNA-binding protein Npl3p has led to the proposal that the processes of Npl3p and poly(A)⁺ RNA export from the nucleus are tightly coupled. We isolated mutations in the two RRM of Npl3p that result in defects in export of poly(A)⁺ RNA from the nucleus. To establish a link between the processes of protein and poly(A)⁺ RNA, we developed a new system to study the export of Npl3p from the nucleus. Using this assay, we have shown that a mutation in one

of the RNA-binding domains of Npl3p results in a block in Npl3p export from the nucleus. Furthermore, the export of Npl3p from the nucleus is dependent on the RNA synthesis by RNA polymerase II. We propose a model in which Npl3p acts as a carrier for poly(A)⁺ RNA export from the nucleus.

A new assay for nuclear protein export

We have developed an assay system to examine the export of proteins from the yeast nucleus. This assay is based on the properties of yeast temperature-sensitive nucleoporin mutants that are defective in nuclear protein import at the nonpermissive temperature. In *nup49* and *nsp1* mutant cells, export is scored as accumulation of the reporter protein in the cytoplasm at the nonpermissive temperature. A previous test for nuclear protein shuttling relied on the production of heterokaryons (Flach et al. 1994). Our system has advantages over the use of heterokaryons; in particular, it allows us to uncouple nuclear protein export from reimport.

The yeast heterokaryon experiments were based on the fact that yeast karyogamy mutants are defective in nuclear fusion after mating (Flach et al. 1994). When a heterokaryon is formed, the two cells exchange cytoplasms, resulting in a rescue of any defects not shared by both cells, thereby preventing the use of yeast mutants to examine the process of export. Because the assay presented here does not rely on the mating of yeast cells, it can be performed in the presence of drugs and mutations that otherwise block cellular processes required for mating.

The search for a specific signal for protein export from the nucleus has led to differing conclusions about the requirements for this process. The retention of nuclear proteins by interaction with nuclear structures or chromatin is likely to counteract the passive diffusion of proteins out of the nucleus (Schmidt-Zachermann et al. 1993). Nuclear export signals (NESs), however, can drive the rapid and active export of proteins from the nucleus (Fischer et al. 1995; Michael et al. 1995; Wen et al. 1995). Here we report that an artificial nuclear protein containing a classic NLS does not exit the nucleus. This result, along with similar recently published data (Michael et al. 1995), is in conflict with the previous suggestion that a classic NLS can promote the efflux of protein from the nucleus (Guiochon-Mantel et al. 1994). We believe, however, that our export assay is a more faithful representation of the *in vivo* situation where proteins are targeted into the nucleus by their NLS and exit only if they contain export signals or interact with another normally exported protein.

Npl3p mutants affect transport of RNA out of the nucleus

One temperature-sensitive allele, *npl3-1*, has been shown previously to accumulate mRNA abnormally in the nucleus at the nonpermissive temperature (Singleton et al. 1995; Henry et al. 1996). This result suggests that

Npl3p might somehow be involved in export of mRNA from the nucleus. Moreover, Npl3p is a major poly(A)⁺ RNA-binding protein that shuttles between the nucleus and the cytoplasm. We now present several lines of evidence to support the hypothesis that Npl3p and poly(A)⁺ RNA export are tightly coupled processes and that Npl3p is a mediator of RNA export.

First, mutations in the RRM of Npl3p result in defective poly(A)⁺ RNA export. Six of the seven temperature-sensitive mutants generated in this study contain one or more mutations within the two RRMs of Npl3p. All of these mutants display nuclear localization of Npl3p, and accumulation of poly(A)⁺ RNA in the nucleus at the nonpermissive temperature indicating a defect in RNA export. At face value, these results are difficult to interpret because the immunofluorescence localization reflects only the steady-state distribution of Npl3p. It is only when we employ the export assay, which measures the dynamics of Npl3p, that we can get a clearer picture of what is happening. Then we find that one of the mutations in a highly conserved amino acid in the RRM results in defective export of Npl3p from the nucleus. Moreover, by two different approaches we show that the export of Npl3p is dependent on the synthesis of new RNA. One approach utilizes the drug thiolutin to block synthesis of all RNA, whereas the other uses the *rpb1-1* temperature-sensitive mutant to specifically block RNA polymerase II (Pol II) transcription. Under both of these circumstances, Npl3p does not exit the nucleus. Together, these results indicate that Npl3p and poly(A)⁺ RNA each require the presence of the other for proper export from the nucleus.

A model incorporating these results is presented in Figure 8. We propose that Npl3p (and other RNA-binding proteins) forms a complex with poly(A)⁺ RNA in the nucleus, and at some point this complex becomes competent for export. Once in the cytoplasm, the complex would dissociate and Npl3p would be reimported for an-

other round of RNA binding and export. Our results suggest that mutations in the RRMs of Npl3 block RNA binding or prevent binding of RNA in the proper conformation, resulting in a complex that is not competent for export. In this case, both Npl3p and poly(A)⁺ RNA remain in the nucleus. Our results also suggest that if RNA synthesis is blocked, the RNA–Npl3p complex would never form, resulting in an inability of Npl3p to exit the nucleus.

One of the mutants generated in this study, *npl3-27*, does not display any defect in poly(A)⁺ RNA export, and the steady-state distribution of the mutant Npl3p in these cells is predominantly cytoplasmic. How can these results be reconciled with a model in which Npl3p is required for poly(A)⁺ RNA export? One possibility is that although the *npl3-27* mutant protein appears mostly cytoplasmic, enough of this protein is able to enter the nucleus to support poly(A)⁺ RNA export, as a result of either a reduced rate of nuclear import or a defect in retention of the mutant Npl3p within the nucleus. By the latter scenario, premature export of the mutant Npl3p–RNA complex could result in the temperature-sensitive growth defect in *npl3-27* cells. These possibilities are supported by two separate observations: First, the mutant Npl3p does not appear to be excluded from the nucleus in *npl3-27* cells, and second, *npl3-27* mutant protein accumulates in the nucleus in a strain that is also defective in poly(A)⁺ RNA export (data not shown). Alternatively, the *npl3-27* defect may result in a bypass in the requirement for Npl3p in poly(A)⁺ RNA export. In this event, the *npl3-27* protein must play some essential role independent of poly(A)⁺ RNA export, which results in the temperature-sensitive growth defect.

Although the majority of the mutants analyzed display accumulation of poly(A)⁺ RNA in the nucleus, the presence of a cytoplasmic RNA signal indicates that in no case is there a complete block in export. This may be explained in part by the fact that Npl3p is an extremely stable protein. When the synthesis of Npl3p is repressed in a strain where the *GAL1* promoter controls its expression, the protein is still present after 18 hr of growth in a glucose-containing medium (Russell and Tollervey 1992), and poly(A)⁺ RNA export still appears normal at this time (M. Henry and P. Silver, unpubl.). In addition, although the expression of GFP–Npl3 controlled by the *GAL1* promoter has ceased completely after 2 hr of growth in a glucose-containing medium, the protein persists for at least 5 hr more in *nup49-313* cells (Fig. 3C). If Npl3p is present in a stable hnRNP complex, this could explain its long half-life in the absence of expression. A stable hnRNP complex could partially protect the heat labile mutants used in this study and result in the leaky RNA export phenotype that we have observed.

What targets the Npl3p–RNA complex for export from the nucleus? Npl3p does not contain amino acid sequences similar to the NESs identified previously in Rev, PKI, and hnRNP A1 (Fischer et al. 1995; Michael et al. 1995; Wen et al. 1995). The heterogeneity of export signals identified to date, however, indicates the possibility

Figure 8. Model for Npl3p transport. Npl3p forms a complex with poly(A)⁺ RNA in the nucleus. This complex is then exported to the cytoplasm via the nuclear pore complex. Once in the cytoplasm, the complex dissociates and Npl3p is reimported for another round of RNA-binding and export.

that additional types of export sequences exist. A mutation in the RRM of Npl3p blocks its efficient export from the nucleus, suggesting that sequences within this domain have a role in targeting Npl3p for export. It is also possible that correct association of the RNA–Npl3p complex results in the appearance of an export signal. It is not known whether RNA is required for the export of Rev or hnRNP A1, although the NESs identified in these proteins lie outside of their RNA-binding domains. A final possibility is that the interaction between Npl3p and poly(A)⁺ RNA results in association of this complex with an unidentified NES-containing protein capable of targeting the Npl3p–RNA complex for export.

Similarities and differences with hnRNP A1 protein

Like Npl3p, hnRNP A1 protein is an RNA-binding protein that shuttles between the nucleus and the cytoplasm in mammalian cells (Piñol-Roma and Dreyfuss 1992, 1993). The hnRNP A1 protein has also been proposed to act as a carrier for poly(A)⁺ RNA export from the nucleus (Piñol-Roma and Dreyfuss 1991, 1992). hnRNP A1 contains two tandemly arranged RRMs and a carboxy-terminal glycine-rich domain (for review, see Dreyfuss et al. 1993). Also, like many other RNA-binding proteins, hnRNP A1 contains an RGG box domain that confers full RNA-binding capacity (Biamonti and Riva 1994). A 39-amino-acid nuclear import signal has been identified in the glycine-rich carboxyl terminus of human hnRNP A1 (Siomi and Dreyfuss 1995; Weighardt et al. 1995). This signal, termed M9, also functions as an NES (Michael et al. 1995).

There are noteworthy parallels between hnRNP A1 and Npl3p. They share a significant degree of structural similarity. Neither contains prototypical NLSs, and Npl3p does not contain a sequence similar to the NLS defined by hnRNP A1. Like hnRNP A1, however, sequences required for proper nuclear targeting of Npl3p lie within its glycine-rich carboxyl terminus. Removal of the carboxy-terminal RGG box domain from Npl3p results in cytoplasmic localization of the protein (Flach et al. 1994). Also, in the *npl3*–27 mutant, a substitution in the extreme carboxyl terminus of Npl3p results in cytoplasmic accumulation.

Our findings also indicate that the dynamics of Npl3p and hnRNP A1 localization differ significantly in their dependence on the new synthesis of RNA. We show that the nuclear export of Npl3p requires the synthesis of Pol II transcripts, which is what one might expect for an RNA carrier protein. This is not the case for human hnRNP A1. This protein instead requires ongoing Pol II transcription for its proper reimport into the nucleus (Piñol-Roma and Dreyfuss 1991). Our experiments cannot address whether Pol II transcription is required for Npl3p import because Npl3p does not exit the nucleus in its absence. Therefore, our results illustrate both similarities and differences between Npl3p and hnRNP A1 and provide the first example of a shuttling protein whose export is dependent on the new synthesis of RNA.

Mutations in conserved amino acids of Npl3p affect RNA export

When the two RRMs of Npl3p are subjected to alignment with 68 other RRMs from proteins thought to be involved in RNA binding and processing, each of the mutants displaying defects in poly(A)⁺ RNA export contains substitutions mapping to residues at or near sites of high conservation. In particular, phenylalanine 160 in Npl3p lies in position 35 of the RRM consensus sequence. This is one of the three solvent-exposed aromatic positions in the RRM consensus that has been implicated in RNA-binding (Birney et al. 1993). In position 35, phenylalanine is present 60% of the time (of 179 aligned sequences), although this position can tolerate substitutions in some RRM-containing proteins. Therefore, it is possible that mutation of phenylalanine 160 to leucine disrupts the proper interaction of Npl3p with RNA, and this disruption is responsible for its inability to exit the nucleus. One prospect is that this mutant protein no longer binds RNA, although it is also possible that RNA binds the mutant with a different conformation than it does with the wild-type protein. If the nature of this RNA–Npl3p complex is important for proper export from the nucleus, then either possibility could result in the observed block in export seen for these two species. It is also noteworthy that there appears to be a bias toward recovering temperature-sensitive mutations that correspond to changes in the RRMs—six of seven mutants that we studied fall into this category.

During the course of this study, we observed that in the absence of Pol II transcription, both GFP–Npl3 and endogenous Npl3p appear to accumulate in a discrete location in the nucleus that colocalizes with a nucleolar antigen. This is similar to the mininucleolar bodies in which Npl3p is localized in the absence of Pol I transcription (Russell and Tollervey 1992; Oakes et al. 1993). Some have suggested that RNA may transit through the nucleolus on its way out of the nucleus (Kadowaki et al. 1994b, 1995; Tani et al. 1995). By this scenario, Npl3p would be trapped at this intermediate stage in the absence of poly(A)⁺ RNA. Alternatively, in the absence of poly(A)⁺ RNAs, Npl3p could associate with RNAs in the nucleolus, or the nucleolus may act as a repository for proteins involved in RNA processing and trafficking.

Conclusions

In sum, we have demonstrated a link between the nuclear export of poly(A)⁺ RNA and that of a shuttling RNA-binding protein. These results support a model in which Npl3p functions as a carrier for poly(A)⁺ RNA out of the nucleus. The study of the process of molecular export from the nucleus has provided a greater understanding of the processes of protein and RNA export. Our results provide a connection between these two rapidly growing fields of study.

Materials and methods

Plasmid constructs

The plasmid constructs used in this study are listed in Table 2. Unless otherwise stated, all PCR amplifications were performed with *Pfu* DNA polymerase (Stratagene). A 1.5-kb insert containing the entire *NPL3* gene was generated by PCR amplification, digested with *Bam*HI, and ligated into *Bam*HI-linearized pBlue-script (Stratagene) to create pPS425. A fusion of GFP to the amino terminus of Npl3p (pPS811) was created by ligation of the 1.5-kb *Bam*HI fragment from pPS425 into *Bam*HI-linearized pCGF-1A (Kahana and Silver 1996). To create pPS817, a *Bam*HI-*Sph*I fragment from pPS815 (a gift from Jim Haseloff, Medical Research Council, Cambridge, UK), containing the SV40 T-antigen NLS fused to a GFP- β -galactosidase fusion protein, was ligated into *Bam*HI/*Sph*I-linearized pPS293, a high-copy, YEp352-based vector containing the *GAL1/10* promoter (Yocum et al. 1984) and a polylinker sequence. The plasmid pPS877 was created by ligation of a 4.5-kb *Cl*aI fragment from the original plasmid pPS870 containing *npl3-17* into *Cl*aI-linearized pRS306 (Sikorski and Hieter 1989). pPS879 was created by ligation of a 278-bp *B*glII fragment from pPS877 into *B*glII-linearized pPS811. The plasmid pMHY11 was constructed in two steps as follows. First, the complete *NPL3* gene as a 3.5-kb *S*caI fragment was inserted into the *S*maI site of YEp352 to yield pMHY7. The complete *NPL3* gene was then excised from pMHY7 as a *S*acI fragment and inserted into pPS709 (2 μ *ADE3 URA3*) to yield pMHY11.

Yeast strains

Growth and maintenance of yeast strains, as well as all genetic manipulations were performed as described previously (Rose et al. 1990). The strains used in this study are listed in Table 3. MHY132 contains a disruption of *NPL3* in a genetic background in which this gene is essential (Russell and Tollervy 1992;

Table 3. Yeast strains

Strain	Genotype	Source
MHY132	<i>mata npl3::HIS3 ura3-1 lys2 leu2 ade2 ade3</i> plus pMHY11	this study
nup49-313	<i>mata nup49::TRP1 ura3 leu2 his3 ade2 ade3</i> plus pUN100 <i>nup49^{ts}-LEU2</i>	Doye et al. (1994)
FY23	<i>mata ura3-52 leu2Δ1 trp1Δ63</i>	F. Winston
Y262	<i>mata rpb1-1 ura3-52 his4-539</i>	Nonet et al. (1987)
CH1305	<i>mata ura3 leu2 ade2 ade3 lys2 can1</i>	Kranz and Holm (1990)
PSY704	<i>mata rpb1-1 ura3-52 leu2Δ1 his4-539</i>	this study
PSY814	<i>mata npl3::HIS3 ura3-52 leu2-3 his3 trp1-1 ade2-1 lys1-1 can1-100</i> plus YCp50-NPL3-3	Henry et al. (1996)
PSY844	<i>mata rpb1-1 nup49::TRP1 ura3 leu2 —ade3his</i> plus pUN100 <i>nup49^{ts}-LEU2</i>	this study

Henry et al. 1996) that is rescued by a plasmid carrying a wild-type copy of *NPL3* (pMHY11). This strain was created by the mating of PSY814 with CH1305 (Kranz and Holm 1990). Diploids were grown on 5-fluoro-orotic acid (FOA), transformed with pMHY11, and sporulated. PSY704 was created by the crossing of Y262 (Nonet et al. 1987) with the wild-type strain FY23 (a gift from Fred Winston, Harvard Medical School, Boston, MA). A double mutant containing both *nup49-313* and *rpb1-1* was generated by the crossing of PSY704 with *nup49-313* (Doye et al. 1994).

Generation of temperature-sensitive mutants

A panel of new temperature-sensitive *npl3* mutants was generated by use of a PCR mutagenesis and plasmid shuffling proto-

Table 2. Plasmid constructs

Plasmid	Features	Source
pPS293	yeast high-copy galactose-inducible expression vector <i>URA3</i>	G. Schlenstedt and P. Silver (unpubl. plasmid)
pPS425	PCR-amplified 1.5-kb <i>Bam</i> HI fragment of <i>NPL3</i> in pBluescript	M. Bossie and P. Silver (unpubl. plasmid)
pRS306	yeast integration vector <i>URA3</i>	Sikorski and Hieter (1989)
pCGF-1A	yeast high-copy galactose-inducible expression vector for fusion to the carboxyl terminus for GFP	Kahana and Silver (1996)
YEp352	high-copy yeast shuttle vector <i>URA3</i>	Hill et al. (1993)
pMHY3	3.8-kb chromosomal sequence containing <i>NPL3</i> in YCp50- <i>LEU2</i>	Henry et al. (1996)
pMHY3- <i>npl3-1</i>	3.8-kb chromosomal sequence containing <i>npl3-1</i> in YCp50- <i>LEU2</i>	Henry et al. (1996)
pMHY7	3.5-kb <i>S</i> caI fragment of <i>NPL3</i> in YEp352	this study
pMHY11	3.5-kb <i>S</i> acI fragment containing <i>NPL3</i> in pPS709	this study
pPS709	High-copy yeast vector <i>ADE3 URA3</i>	D. Koepp and P. Silver (unpubl. plasmid)
pPS811	1.5-kb <i>Bam</i> HI fragment from pPS425 containing <i>NPL3</i> in pCGF-1A	this study
pPS815	SV40 NLS fused to GFP- β -galactosidase fusion in pVT103-U, a high-copy constitutive yeast expression vector <i>URA3</i>	Jim Haselhoff (unpubl. plasmid)
pPS817	4.2-kb <i>Bam</i> HI- <i>Sph</i> I fragment from pPS815 containing SV40-GFP- β -galactosidase in pPS293	this study
pPS870	<i>npl3-17</i> allele gap repaired into pMHY3	this study
pPS877	4.5-kb <i>Cl</i> aI fragment from pPS870 in pRS306	this study
pPS879	278-bp <i>B</i> glII fragment from pPS877 in pPS811	this study

col. Briefly, a 2492-bp PCR product was generated that contains the entire *NPL3* open reading frame by use of *TaqI* polymerase (Promega) according to the manufacturers' instructions and was purified with a Qiaquick column (Qiagen). The plasmid pMHY3 (Henry et al. 1996) was linearized with *NcoI* and *PmlI*, and a repair ligation (Orr-Weaver and Szostak 1983) was achieved by cotransformation of the strain MHY132 with the PCR product and linear pMHY3. Colonies were selected on synthetic complete medium (SC) lacking leucine, followed by replica plating to 25°C and 37°C on SC lacking leucine and containing FOA and 7.5 mg/liter of the vital dye erythrocyanin B. Of 49 strains, 48 remained temperature sensitive after rescue of the plasmid, retransformation into MHY132, and selection on SC lacking leucine and containing FOA. Of these, seven were chosen, and the *NPL3* ORF was sequenced entirely by use of a Stratagene sequencing kit. To generate growth curves, strains were inoculated into 10 ml of SC lacking leucine and grown overnight at 25°C. Cells were counted with a hemacytometer, and cultures diluted to 2×10^6 cells/ml in fresh medium. Cell counts were generated every 4 hr thereafter. After 8 hr at 25°C, each culture was split and shifted to either 30°C or 37°C.

Indirect immunofluorescence

Indirect immunofluorescence microscopy was performed essentially as described (Bossie et al. 1992), with the following modifications. Cells were spheroplasted with Zymolyase (100T, ICN) at a concentration of 300 µg/ml, placed on slides coated with 0.3% polylysine, and permeabilized by addition of 0.5% NP-40 in solution P (0.1 M K_2HPO_4 – KH_2PO_4 at pH 6.5, 1.2 M sorbitol) for 5 min. Cells were washed once with solution P, blocked for 1 hr with antibody blocking buffer (0.1 M Tris-HCl at pH 9.0, 0.15 M NaCl, 5% heat-inactivated fetal calf serum, 0.3% Triton TX-100), followed by overnight incubation with the primary antibody in antibody blocking buffer. Cells were washed extensively after primary and secondary antibody incubations with buffer 1 (0.1 M Tris-HCl at pH 9.0, 0.15 M NaCl) and buffer 2 (0.1 M Tris-HCl at pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$). Affinity-purified rabbit anti-NPL3 (Bossie et al. 1992) was used at a 1:1000 dilution, mouse monoclonal 9C4 (Flach et al. 1994; Loeb et al. 1995) was used at a 1:100 dilution, and fluorescein isothiocyanate (FITC) or Texas Red-conjugated secondary antibodies (Jackson) were used at a 1:1000 dilution. Nuclei were stained with 10 µg/ml of 4' 6-diamido-2-phenylindole dihydrochloride (DAPI) for 5 min in buffer 2, followed by two washes in buffer 2.

In situ RNA hybridization

Localization of poly(A)⁺ RNA by in situ hybridization was performed essentially as described (Amberg et al. 1992), with some modifications. Cells were fixed, washed, spheroplasted, and permeabilized as described for immunofluorescence. After incubation with a digoxigenin-labeled oligo[d(T₅₀)] probe, cells were then processed for immunofluorescence as described above, beginning with the antibody blocking buffer step. The Texas Red sheep anti-digoxigenin Fab fragment (Boehringer Mannheim) was used at a 1:100 dilution, and rabbit anti-NPL3 at a 1:1000 dilution overnight at 25°C. An FITC-conjugated anti-rabbit secondary antibody (Jackson) was used at a 1:1000 dilution.

Nuclear export assay

nup49–313 (Doye et al. 1994) cells containing vectors for expression of GFP-fusion proteins were grown at 25°C to mid-log phase in SC medium lacking uracil and containing 2% glucose

as a carbon source. Cultures were diluted 1:200 into SC medium lacking uracil and containing 2% raffinose as a carbon source and grown at 25°C to a density of 0.5×10^7 to 1×10^7 cells/ml. GFP fusion proteins were induced by the addition of galactose to a final concentration of 2%. After 2 hr at 25°C, cells were collected by centrifugation at 2000g for 5 min and resuspended in an equal volume of YPD. This step was repeated, and cells were incubated for 2 hr at 25°C in YPD. Cultures (10 ml) were then either shifted to 36°C or remained at 25°C for an additional 5 hr. Cells were then pelleted by centrifugation at 12,000g for 1 min and resuspended in SC lacking uracil. Cells were then examined for GFP fluorescence signal in the FITC channel of an Axioscop fluorescence microscope (Zeiss). Thiolutin was used at a final concentration of 15 µg/ml, and cycloheximide at a concentration of 10 µg/ml. When used, these drugs were added after the second YPD wash.

Radiolabeling and immunoprecipitation

nup49–313 cells containing the pPS811 expression plasmid were grown as described for the export assay except that the growth medium lacked methionine. For labeling, cell cultures were pelleted by centrifugation at 2000g for 5 min, and all but 500 µl of medium was aspirated. Cells were resuspended in the remaining medium and 150 µCi (15 µl) of EXPRE^{35S} Protein Labeling Mix (>1000 Ci/mmol, NEN) was added, and labeling was allowed to proceed for 15 min at room temperature. Cells were pelleted by centrifugation at 12,000g for 1 min and washed twice with ice-cold water. Cell pellets were frozen at –20°C. For samples that were chased after labeling, cells were washed twice in YPD, resuspended in the original volume of YPD, and carried through the rest of the export assay. For immunoprecipitation, frozen cell pellets were resuspended in 50 µl of lysis buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 5 mM EDTA 1% Triton X-100) supplemented with protease inhibitors (0.5 mM PMSF and 3 µg/ml each of pepstatin, leupeptin, aprotinin, and chymostatin). Glass beads (5 mg, 0.5 mm) were added, and the cells were lysed by repeated vortexing at 4°C (3× for 30 sec each). Lysates were clarified by centrifugation for 10 min at 12,000g at 4°C. Lysates were incubated with 5 µl of rabbit anti-Npl3p antiserum for 2 hr at 4°C with rocking. Npl3p-antibody complexes were precipitated by addition of 100 µl of a 50% slurry of protein A-Sepharose (Pharmacia) and incubation for 1 hr with rocking at 4°C. Beads were washed three times with lysis buffer, and immunoprecipitated proteins were visualized by separation on a 10% SDS-polyacrylamide gel followed by autoradiography.

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