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A Yeast RNA-Binding Protein Shuttles between the Nucleus and the Cytoplasm

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RNA-binding proteins have been suggested to move in association with RNA as it leaves the nucleus. The *NPL3* gene of the yeast *Saccharomyces cerevisiae* encodes a nuclear protein with consensus RNA-binding motifs and similarity to heterogeneous nuclear ribonucleoproteins and members of the S/R protein family. We show that although Npl3 is located in the nucleus, it can shuttle between nuclei in yeast heterokaryons. In contrast, other nucleus-targeted proteins do not leave the nucleus under similar conditions. Mutants missing the RNA-binding motifs or the N terminus are still capable of shuttling in and out of the nucleus. Npl3 mutants missing the C terminus fail to localize to the nucleus. Overproduction of Npl3 in wild-type cells slows cell growth. This toxicity depends on the presence of a series of unique repeats in the N terminus and localization to the nucleus. We suggest that the properties of Npl3 are consistent with it being involved in export of RNAs from the nucleus.

Proteins and RNAs are continuously moving in and out of the nucleus via the nuclear pore. Proteins destined for the nucleus often contain short stretches of amino acids, termed nuclear localization sequences (NLSs), that can direct them to the nucleus (15, 17, 34). Other proteins may enter the nucleus by associating with an NLS-bearing protein (38). Once at the pore, the protein to be imported binds and is transported in a reaction that requires ATP (26, 28) and proteins of the nuclear pore complex (12, 13, 24, 32). Much less is known about exit of macromolecules from the nucleus. All classes of RNAs are thought to be exported to the cytoplasm as RNA-protein complexes via the nuclear pores (10). Most likely this is also an active process that may involve specific targeting of macromolecules. Experiments with microinjected RNAs indicate that exit of RNA from the nucleus is an energy-dependent, facilitated, and saturable process (7, 37).

Some nuclear proteins, in particular those that bind RNA, repeatedly shuttle in and out of the nucleus (9). The functional significance of this export-reimport is still unclear. However, one proposal is that nuclear shuttling proteins may be directly involved in nucleocytoplasmic exchange of macromolecules. By one model, certain RNA-binding proteins would be part of the export substrate that moves with the RNA from the nucleus via the pore. Following release of their RNA "cargo" by exchange with cytoplasmic RNA-binding proteins, the proteins that accompanied RNA out would rapidly reenter the nucleus. Implicit in this model is the idea that some proteins would contain information that not only targets them to the nucleus but also allows for their specific exit. Proteins such as the pre-mRNA-binding protein heterogeneous ribonucleoprotein (hnRNP) A1 have been proposed to play such a role (27).

Two different mechanisms have been proposed to explain how nuclear proteins shuttle: (i) some proteins contain signals for export, or (ii) export is a default process and nonshuttling proteins are retained via intranuclear interactions. In support of the second model, it has been shown that none of the domains of the shuttling nucleolin protein (3) are essential for its nuclear export and that cytoplasmic pyruvate kinase fused to an NLS is targeted to the nucleus but shuttles in and out (33). Taken together, these results suggest that specific export signals do not exist. However, retention of proteins such as nucleoplasmin core pentamers (8) after introduction into the nucleus argues against export being a default process (18). These conflicting results underscore the need for additional experiments.

The yeast *NPL3* gene encodes a protein of 414 amino acids that contains RNA recognition motifs (RRMs) and a series of RGGF/Y repeats at the C terminus (4). On the basis of the presence of these conserved motifs, Npl3 is similar to a large family of proteins implicated in RNA processing and movement, including hnRNPs and nucleolar RNA-binding proteins (2). To date, Npl3 is the only yeast protein with similarity to the S/R family of RNA-binding proteins previously identified in mammals and flies (2).

Npl3 was originally isolated because temperature-sensitive mutants were defective for nuclear localization of some proteins. The same mutants also accumulate poly(A)$^+$ RNA in the nucleus, consistent with a concomitant block in mRNA export (14). Npl3 is located in the nucleus, as judged from immunofluorescence with anti-Npl3 antibodies (4). Npl3 was also identified as Nop3 because it contained amino acid repeats with the sequence RGGF/Y. It was suggested that Npl3/Nop3 was also located, at least in part, in the nucleolus, where it played a role in processing of rRNA (30). Recently, Npl3 has been shown to be cross-linked in vivo with poly(A)$^+$ RNA (1).

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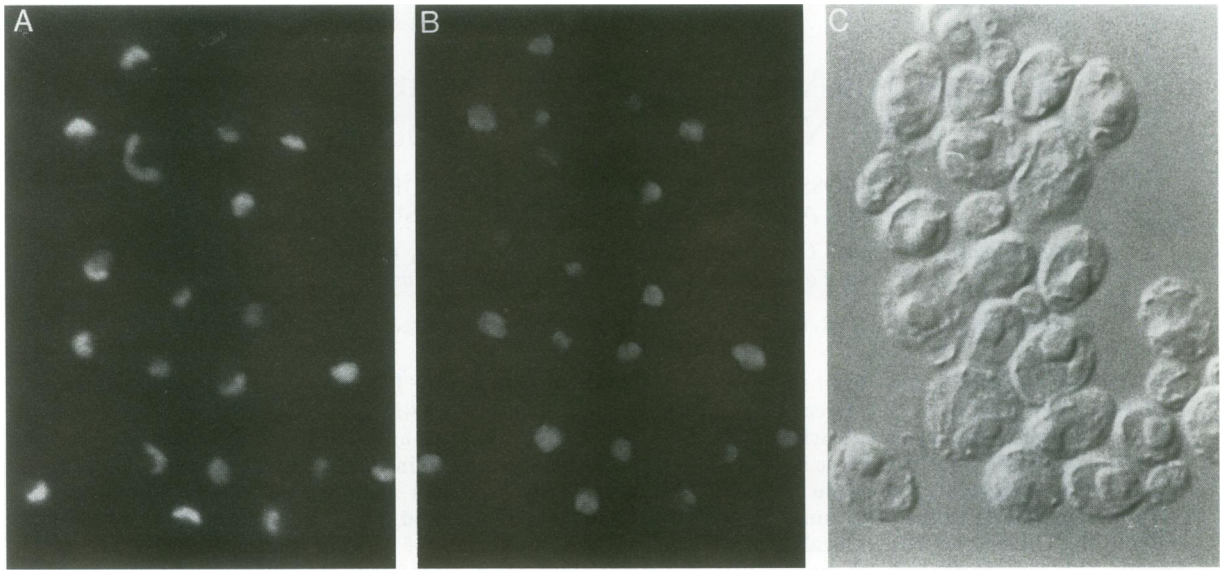

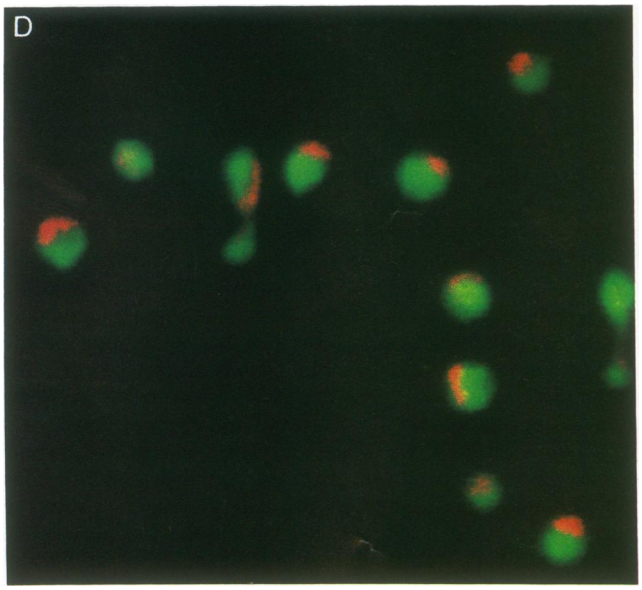

FIG. 1. Npl3 is located in the nucleus. Diploid yeast cells were prepared for immunofluorescence and stained with anti-Nop1 monoclonal antibodies followed by Texas red anti-mouse antibody (A) or affinity-purified anti-Npl3 antibodies followed by FITC anti-rabbit antibodies (B). The same cells were viewed by Nomarski optics (C) and by confocal laser microscopy (D) (red indicates anti-Nop1 staining, and green indicates anti-Npl3 staining).

Because of its similarity to other RNA-binding proteins known to shuttle in and out of the nucleus and its potential role in nucleocytoplasmic transport, we show that Npl3 can move rapidly in and out of the nuclei of yeast heterokaryons. We propose that Npl3 is a multidomain protein that plays a role in transport of RNA from the nucleus.

MATERIALS AND METHODS

Plasmids. PCRs were performed by using *Pfu* DNA polymerase (Stratagene) as instructed by the manufacturer. Fragments corresponding to the four regions of Npl3 (see Fig. 7) were amplified with appropriate restriction sites incorporated into the amplified DNA and cloned into pBS to obtain the various deletion derivatives. The inserts were then subcloned under the *GAL1* promoter in pPS311 (*LEU2 CEN*). N-terminal deletions were obtained by PCR amplification of the C-terminal part of *NPL3* as a *Bam*HI fragment. The PCR product was cloned into pBS. The 5′ primer provided an ATG; following sequencing, the *NPL3* derivatives were placed in pPS311 under control of the *GAL1* promoter. Additional N-terminal deletions were obtained by exonuclease III-mung bean digestion of *NPL3*. A cassette providing an ATG and a 5′ overhang (*Sph*I) was placed upstream of *NPL3* in pBS. The resulting vector was digested by *Sph*I and *Cla*I and then by exonuclease III-mung bean for different periods of time. The DNA was self-ligated, and individual clones were sequenced. An N-terminal deletion of 190 amino acids was obtained. The resulting *Bam*HI-*Cla*I fragment was subcloned under control of the *GAL1* promoter in pPS310 (*URA3 CEN*) and pPS311 (*LEU2 CEN*). *NPL3-myc* was generated by inserting an oligonucleotide encoding the c-*myc* epitope (PEQKLISEEDLN) into the unique *Pml*I site of *NPL3*.

Strains. Yeast strains used in these studies are W303 (*MAT***a** *ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100*) and MS739 (*MAT*α *leu2-3,112 ura3-52 ade2-101 kar1-1* [from M. Rose, Princeton University]).

Protein localization. W303 was transformed with the p*GAL-NPL3* derivatives. The resulting transformants were grown overnight in 5 ml of leucine dropout medium with 2% glucose, diluted, and grown overnight in 10 ml of leucine dropout medium with 2% raffinose. When the cell density reached 10^7 cells per ml, galactose was added (2%, final concentration). The cells were incubated for 1.5 to 2 h at 30°C. Fixation and immunofluorescence were done as previously described (4). For double-labeling experiments, affinity-purified rabbit anti-Npl3 (1:500) and mouse monoclonal anti-Nop1 (1:10) followed by fluorescein isothiocyanate (FITC)-labeled anti-rabbit (1:

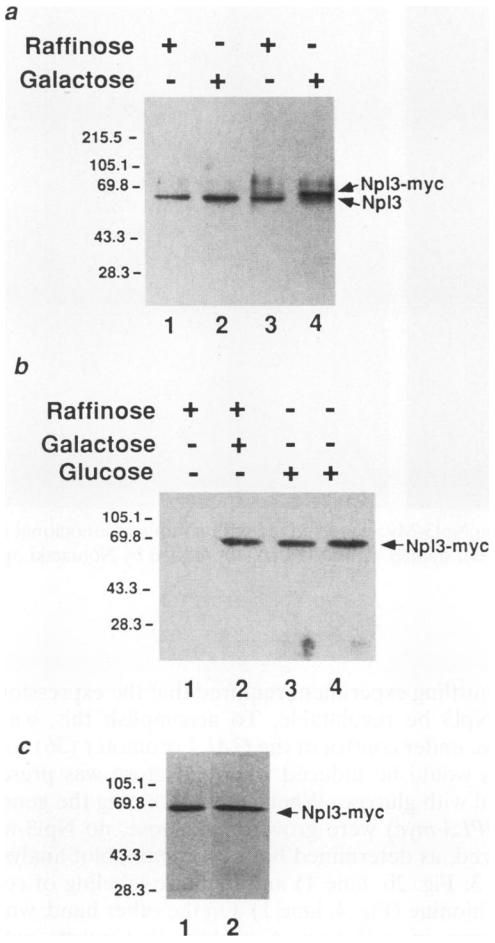

FIG. 2. Npl3-Myc is stable. (a) Cell extracts prepared from yeast strain W303 (lanes 1 and 2) or W303 bearing plasmid pGAL-Npl3-myc (lanes 3 and 4) grown in raffinose (lanes 1 and 3) or in galactose (lanes 2 and 4) were analyzed by immunoblotting with anti-Npl3 antibody. (Sometimes an unidentified protein that appears to cross-react with anti-Npl3 was visible). (b) Cell extracts prepared from yeast strain W303 bearing plasmid pGAL-Npl3-myc grown in raffinose (lane 1), after the addition of 2% galactose for 1.5 h (lane 2) and after transfer to 5% glucose for 2.5 h (lane 3) and 5 h (lane 4) were analyzed by immunoblotting with the mouse monoclonal anti-Myc antibody 9.E10. (c) Cell extracts prepared from yeast strain W303 carrying plasmid pGAL-Npl3-myc after growth for 1.5 h in 2% galactose (lane 1) and after shifting to 4% glucose for 2 h and mating with *kar1-1* cells for 2 h (lane 2) were analyzed by immunoblotting with mouse monoclonal anti-Myc antibody 9.E10. Sizes are indicated in kilodaltons.

1,000) and Texas red-labeled anti-mouse (1:1,000) antibodies were used to simultaneously visualize both proteins.

Protein shuttling. W303 was transformed with pGAL-Npl3, the various deletion derivatives, pGAL-SUC2, or pGAL-H2B-GFP (20, 31). The resulting transformants were grown overnight in 5 ml of leucine dropout medium with 2% glucose, diluted, and grown overnight in 10 ml of leucine dropout medium with 2% raffinose. When the cell density reached 5×10^6 cells per ml, galactose was added (2%, final concentration). The cells were incubated for 90 min at 30°C, washed and resuspended in the same volume of YEPD, and incubated for 2 h more at 30°C. For matings, 1 ml of culture was mixed with 10^7 *kar1-1* cells and concentrated on a 0.45-μm-pore-size filter. The filter was placed on a YEPD plate and incubated at 30°C for 3 h, at which time heterokaryon formation was optimal (approximately 1 of 20 cells was a heterokaryon). The yield of heterokaryons at times earlier than 3 h was too low for routine observation. The cells were recovered by vortexing the filter in 2 ml of YEPD. Fixation and immunofluorescence were performed as previously described (4). Histone H2B-green fluorescent protein (GFP) was observed directly in living cells or following fixation and spheroplasting (3), using a Zeiss Axioskop. Approximately 30 to 50 heterokaryons were observed in each experiment.

Radiolabeling and immunoprecipitation. Labeling of cells and immunoprecipitations of protein were performed essentially as described previously (29), with the following modifications. Following an overnight growth in raffinose, cells were subjected to a variety of conditions to induce or repress expression of Npl3-Myc protein from the *GAL1* promoter. Induction for 1.5 h in 2% galactose was followed by repression in 2% glucose for 2 or 4 h. Alternatively, cells were induced in 2% galactose for 1.5 h, repressed in 2% glucose for 2 h, and then mated for 2 h as done in the shuttling experiments. Cells were pulse-labeled by the addition of 150 μCi of TRANS-LABEL (ICN) for 10 min. Labeling was terminated by the addition of azide (10 mM, final concentration) or a mix of unlabeled cysteine and methionine (0.04%, final concentration). Cells were disrupted by vortexing with glass beads in lysis buffer (20 mM mannitol, 100 mM NaCl, 25 mM $NaPO_4$ [pH 7.5], 2 mM EDTA, 10% glycerol, 1.0% Triton X-100). Npl3-Myc was immunoprecipitated by the addition of 10 μl of anti-Myc antibody (9E10; from the Department of Molecular Biology, Princeton University) followed by the addition of protein A-Sepharose beads (Pharmacia). Proteins were separated on a 10% polyacrylamide gel, fixed and enhanced by using AMPLIFY (Amersham), and exposed to X-ray film.

RESULTS

***NPL3* encodes a protein located in the nucleoplasm.** Npl3 is located in the nucleus, as determined by indirect immunofluorescence and confocal microscopy. We have raised antibodies specific to the unique regions contained in the N-terminal half of Npl3 (4). When these antibodies were used for immunofluorescence on wild-type yeast cells, only nuclear staining was observed (Fig. 1B). Simultaneous staining with antibodies against the nucleolar protein, Nop1 (16), yielded a crescent-like staining pattern and suggested that Npl3 was not completely coincident with the yeast nucleolus (Fig. 1A). Further analysis of cells costained with anti-Npl3 and anti-Nop1 antibodies by confocal microscopy confirmed this observation (Fig. 1D). The red signal represents Nop1, the green signal represents Npl3, and any regions of overlap are represented by yellow. There is only a small amount of overlap between the two signals at the nucleolar border. A portion of Npl3 had previously been suggested to be located in the nucleolus and, for this reason, was termed Nop3 (nucleolar protein [30]).

Npl3 shuttles in and out of the yeast nucleus. Because of its similarity to other hnRNPs known to shuttle in and out of the nucleus, we designed the following experiment to test whether this was also true for Npl3 in yeast. We wished to test whether Npl3 could move out of one nucleus and into another (via the cytoplasm) in a yeast heterokaryon. The basic design of the experiment was to construct heterokaryons between yeast cells bearing an epitope-tagged version of Npl3 and cells bearing the *kar1-1* mutation (6), which prevents nuclei from fusing

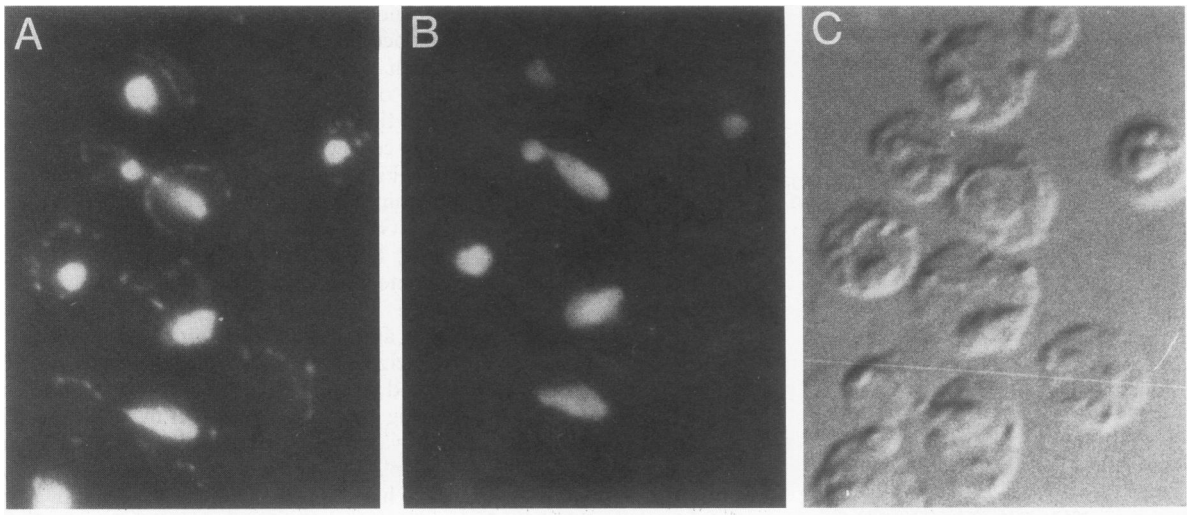

FIG. 3. Npl3-Myc is located in the nucleus. Yeast cells bearing a plasmid encoding Npl3-Myc were stained with a mouse monoclonal antibody (9.E10) against the c-Myc epitope followed by Texas red-labeled anti-mouse antibody (B), stained with DAPI (A), or viewed by Nomarski optics (C).

after mating of haploid yeast cells. Use of karyogamy mutants such as *kar1* allows for the rapid production of large numbers of heterokaryons under physiological conditions.

To tag Npl3, we constructed a gene fusion that encodes Npl3 with the 9.E10 c-*myc* epitope (11) inserted at amino acid 407. This protein (Npl3-Myc) is recognized by the monoclonal antibody 9.E10 raised against the c-*myc*-derived epitope (Fig. 2b, lanes 2 to 4) and is functional in that it rescues the growth defect of a strain lacking *NPL3* as well as the two temperature-sensitive alleles *npl3-1* and *npl3-328* (4) when introduced into these strains on a plasmid (data not shown). Npl3-Myc is located in the nucleus, as judged by immunofluorescence (Fig. 3B) and coincidence with the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 3A).

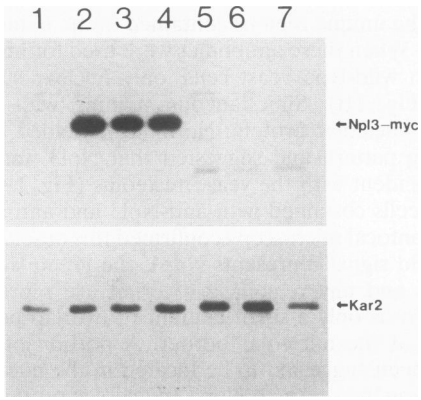

FIG. 4. Npl3-Myc synthesis is repressed in glucose. Cells bearing pGAL-Npl3-Myc grown in raffinose (lane 1), in galactose for 1.5 h (lane 2), in galactose for 1.5 h followed by glucose for 2 h (lane 5) or 4 h (lane 6), and after 4 h of mating with *kar1-1* cells (lane 7) were pulse-labeled with [^{35}S]methionine, and lysates were immunoprecipitated with anti-Myc antibodies and anti-Kar2 antibodies as a control. (The faint non-Npl3-Myc-size bands represent proteins that bind nonspecifically to the protein A-Sepharose beads.) Lanes 3 and 4 show immunoprecipitates from cells grown in galactose (1.5 h), pulse-labeled, and chased for 2 and 4 h with unlabeled methionine.

The shuttling experiment required that the expression of the tagged Npl3 be regulatable. To accomplish this, we placed Npl3-Myc under control of the *GAL1* promoter (36) so that its synthesis would be induced when galactose was present and repressed with glucose. When cells containing the gene fusion (*GAL-NPL3-myc*) were grown in raffinose, no Npl3-myc was synthesized, as determined both by immunoblot analysis (Fig. 2a, lane 3; Fig. 2b, lane 1) and by pulse-labeling of cells with [^{35}S]methionine (Fig. 4, lane 1). On the other hand, when cells were grown in galactose, a protein that reacts with both anti-Npl3 (Fig. 2a, lane 4) and anti-Myc (Fig. 2b, lane 2) antibodies was made. As expected, Npl3-Myc is slightly larger than Npl3 (Fig. 2a, lane 4). Following induction of Npl3-Myc, cells were shifted to glucose to repress further synthesis. No new protein was synthesized, as shown by pulse-labeling after 2 and 4 h in glucose (Fig. 4, lanes 5 and 6). The Npl3-Myc that was made during induction in galactose remained stable for 5 to 6 h, as shown both by immunoblot analysis (Fig. 2b, lanes 3 and 4) and by pulse-chase analysis (Fig. 4, lanes 3 and 4).

To test for shuttling, nuclei of wild-type cells were preloaded with Npl3-Myc by induction in galactose followed by glucose repression. Heterokaryons were prepared by mating to *kar1-1* cells lacking Npl3-Myc. The resulting heterokaryons were stained with anti-Myc monoclonal antibody to determine the distribution of Npl3-Myc. In all heterokaryons that stained positive for Npl3-Myc, we found that Npl3-Myc was present in both nuclei (Fig. 5A, E, and I), indicating that it moved through the cytoplasm from one nucleus to the other. In a mixed field of heterokaryons and unmated cells, the intensity of the Npl3-Myc signal in each heterokaryon nucleus was less than in unmated cells. This finding suggests that the Npl3-Myc has been redistributed between the two nuclei of the heterokaryons. There was no breakdown of Npl3-Myc throughout the course of the experiment, as indicated by immunoblotting of extracts prepared before and after mating (Fig. 2c, lanes 1 and 2). In addition, pulse-labeling experiments showed that no new synthesis of Npl3-Myc occurred during the course of the mating (Fig. 4, lane 7).

To control for the possibility that the nuclei transiently fuse or are leaky, we simultaneously tested the behavior of a

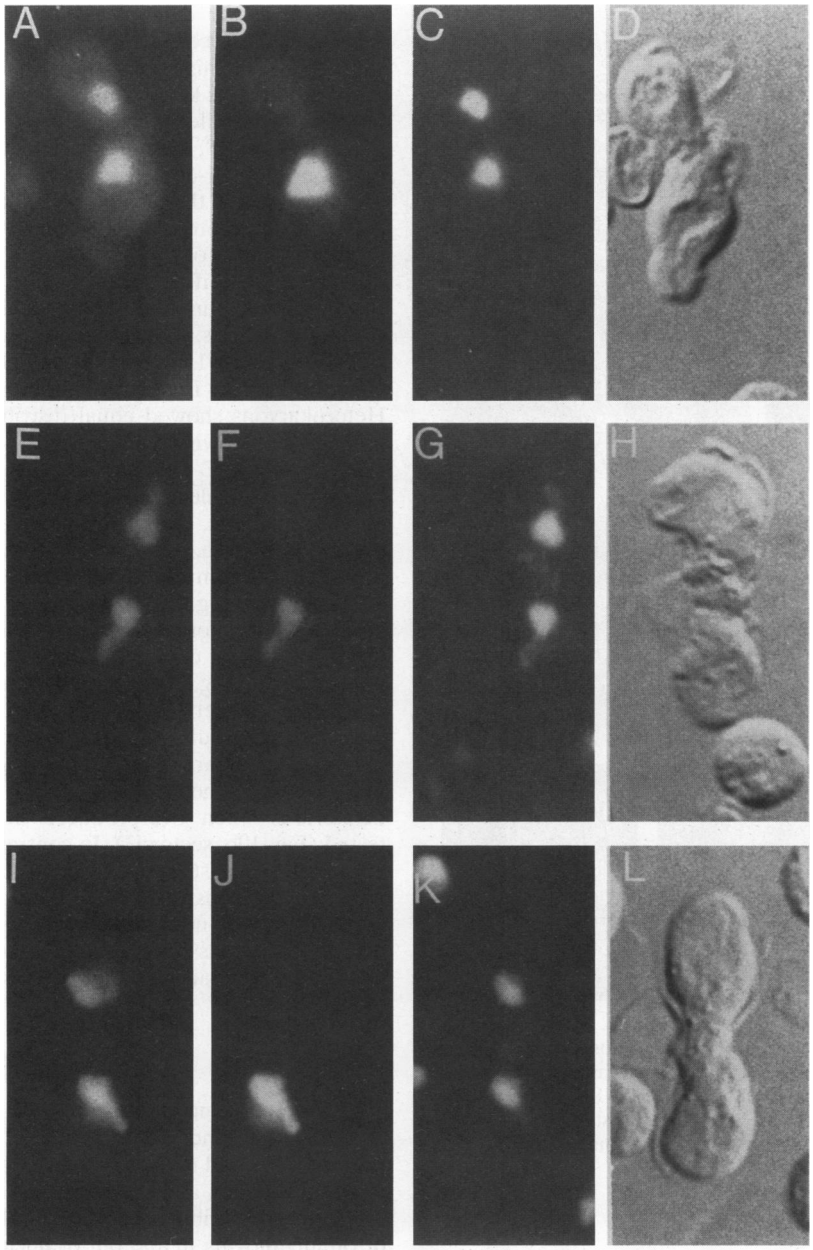

FIG. 5. Protein shuttling in yeast heterokaryons. Heterokaryons were stained with anti-Myc monoclonal antibody followed by FITC-labeled anti-mouse antibody to visualize Npl3-Myc (A, E, and I), with anti-invertase antibody followed by Texas red-labeled anti-rabbit antibody to visualize SV40-invertase (B, F, and J), and with DAPI (C, G, and K) and viewed by Nomarski optics (D, H, and L).

nucleus-targeted protein consisting of the simian virus 40 (SV40) large-T-antigen NLS fused to invertase (SV40-invertase). When expressed from the *GAL1* promoter, SV40-invertase is located in the nucleus (25). Following repression of further synthesis and mating with *kar1-1* cells, all SV40-invertase remained associated with only one nucleus (Fig. 5B, F, and J), indicating that it did not shuttle. Shuttling was not a property of a special subset of heterokaryons, since in all cases in which both proteins were present, we saw distribution of Npl3-Myc in both nuclei and retention of SV40-invertase in only a single nucleus.

We also examined the ability to shuttle of a fusion protein containing histone H2B and the naturally fluorescent GFP (5, 20, 32). After 3 h of induction in galactose, all visible fluorescence was concentrated in the nucleus (Fig. 6A and B) and remained there during the 2.5 h of repression in glucose (Fig. 6C and D). When H2B-GFP cells were mated to *kar1-1* cells, all of the H2B-GFP appeared in only one of the two nuclei in every heterokaryon observed (over 40 heterokaryons were scored for a given experiment), indicating that it did not shuttle (Fig. 6E to J).

Effects of mutations in *NPL3* on protein localization, shuttling, and cell growth. Attempts were made to determine if certain regions of Npl3 were important for shuttling between the nucleus and the cytoplasm, as well as for other *NPL3*-related phenotypes. Npl3 can be thought of as having four potential functional domains (Fig. 7). The first, defined by amino acids 1 to 125 (termed region I), is not similar to any

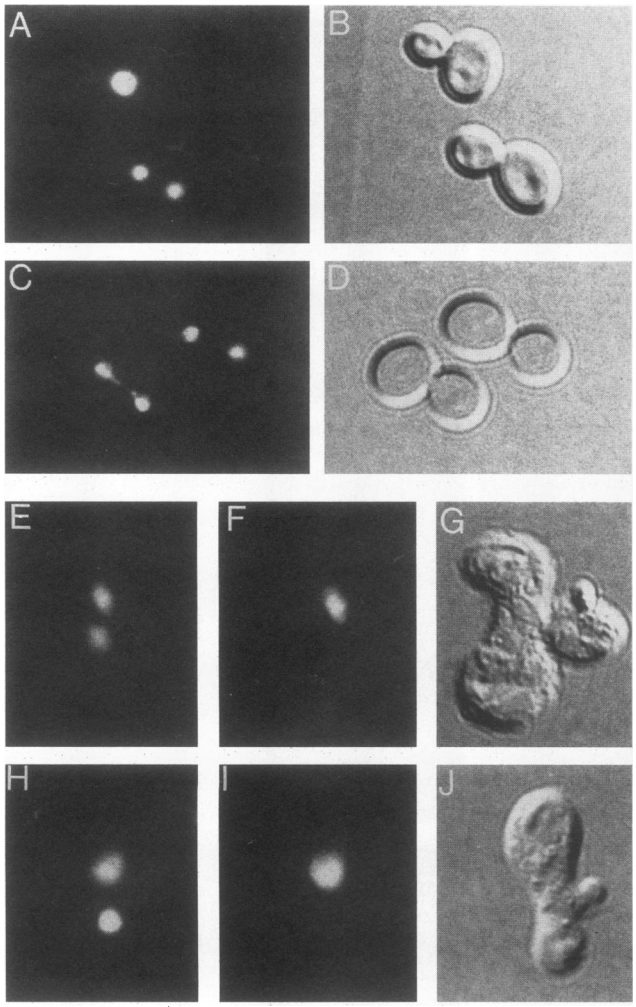

FIG. 6. Chimeric H2B-GFP does not shuttle. Cells bearing a plasmid encoding H2B-GFP were grown in galactose for 3 h (A and B), repressed for 2.5 h in glucose (C and D), and mated with *kar1-1* cells to form heterokaryons (E to J). Panels A, C, F, and I represent the GFP signal, panels E and H are heterokaryons stained with DAPI, and panels B, D, G, and J are cells viewed by Nomarski optics.

known proteins in the current database but does contain four copies of the repeated amino acid sequence APQE. The second, defined by amino acids 126 to 196 (termed region II), was previously identified as containing the canonical RRM. The third, contained within amino acids 197 to 276 (termed region III), contains a sequence corresponding to an atypical RRM and with a high degree of similarity to ASF and the SR protein family (2). Finally, the C terminus (amino acids 277 to 414 [region IV]) contains 17 repeats of the sequence RGGF/Y.

To test the importance of the various regions for protein shuttling and other functions, a number of constructs were made by deleting different domains of Npl3 (see Materials and Methods) and then assayed to determine their effects on Npl3 functions. Figure 7 shows the domains of *NPL3* remaining after the deletions were made. These were placed under control of the regulatable *GAL1* promoter so that their synthesis could be controlled by the addition of galactose and tagged with the c-*myc* epitope so that their ability to shuttle could be tested in the same manner as was demonstrated for Npl3. All of the deleted versions of Npl3 are produced in wild-type yeast cells (Fig. 8). As previously observed (4), wild-type Npl3 migrates with a higher molecular weight than predicted (Fig. 8, lanes 1 and 6). All of the deletions migrated faster than the wild-type protein, consistent with their reduced size (Fig. 7; Fig. 8, lanes 3 to 5 and 7 to 8). The amino-terminal deletion (Npl3Δ1-66) migrated closer to the predicted size of 38 kDa. Perhaps the aberrant migration of Npl3 is due to the proline-rich region containing the APQE repeats in the amino-terminal portion of the protein.

To determine if there was a portion of Npl3 necessary for shuttling, we examined the intracellular localization of the various deletions. Deletions removing the APQE repeats (region I), RRM1 (region II), or RRM2 (region III) had no effect on nuclear import or shuttling (Fig. 9B and C; Fig. 7). Heterokaryons showed equal distribution of each protein in both nuclei following 2 h of mating (data not shown). However, removal of the C-terminal domains of the protein abolished (or reduced) nuclear localization of the mutant protein (Fig. 8D). As a result, we were unable to test the ability of this protein to shuttle, since the shuttling assay requires that the protein be first imported into the nucleus.

While analyzing the localization of the various deletions, we noticed that prolonged overproduction of wild-type Npl3 was toxic to the yeast cell at 30°C and, to some extent, even more toxic at 36°C (Fig. 10b, sectors B and G). Overproduction of most of the deletions also resulted in toxicity. This finding confirms the results of our experiments showing that these mutant proteins are indeed made (Fig. 8). However, a deletion that removed the first 66 amino acids of Npl3 was not deleterious when overproduced at all three temperatures tested (Fig. 10b, sector H). In contrast, removing only the first 29 amino acids still left Npl3 toxic (Fig. 10b, sector I). In addition, the construct that was not localized to the nucleus (Npl3-I+II) was not toxic (Fig. 10b, sector D). In conclusion, overproduction-associated toxicity required that the protein be nuclear and contain the APQE repeats.

DISCUSSION

The protein encoded by the *NPL3* gene has similarity to proteins that bind to RNA. Here we show that the Npl3 protein is located mainly in the nucleoplasm with little, if any, in the nucleolus, as determined by costaining with nucleolar Nop1, the yeast fibrillarin homolog. However, Npl3 is capable of rapidly moving in and out of nuclei in yeast heterokaryons. The C terminus, which contains repeats of RGGF/Y, is necessary for proper localization to the nucleus. Overexpression of regions containing the N-terminal repeats of APQE is toxic when in the nucleus.

We have developed a novel method for assaying the shuttling of proteins from one nucleus to another in *Saccharomyces cerevisiae*. Previous experiments created heterokaryons by fusing chemically treated mammalian cells. In addition, these shuttling experiments required prolonged treatment of cells with protein synthesis inhibitors. In contrast, by using the *kar* mutants, we can rapidly create large numbers of heterokaryons under physiological conditions. Also, by using the strongly regulated *GAL1* promoter, we can avoid prolonged treatment with metabolic inhibitors. Similarly, recent use of *kar* mutants has been reported for assaying membrane fusion in vivo (19).

Other nuclear proteins that have been shown to shuttle between the nucleus and the cytoplasm include hnRNP A1 (27), nucleolin (3), Hsc70 (21), and Nopp140 (22). For some of these proteins, it has been suggested that their ability to cycle

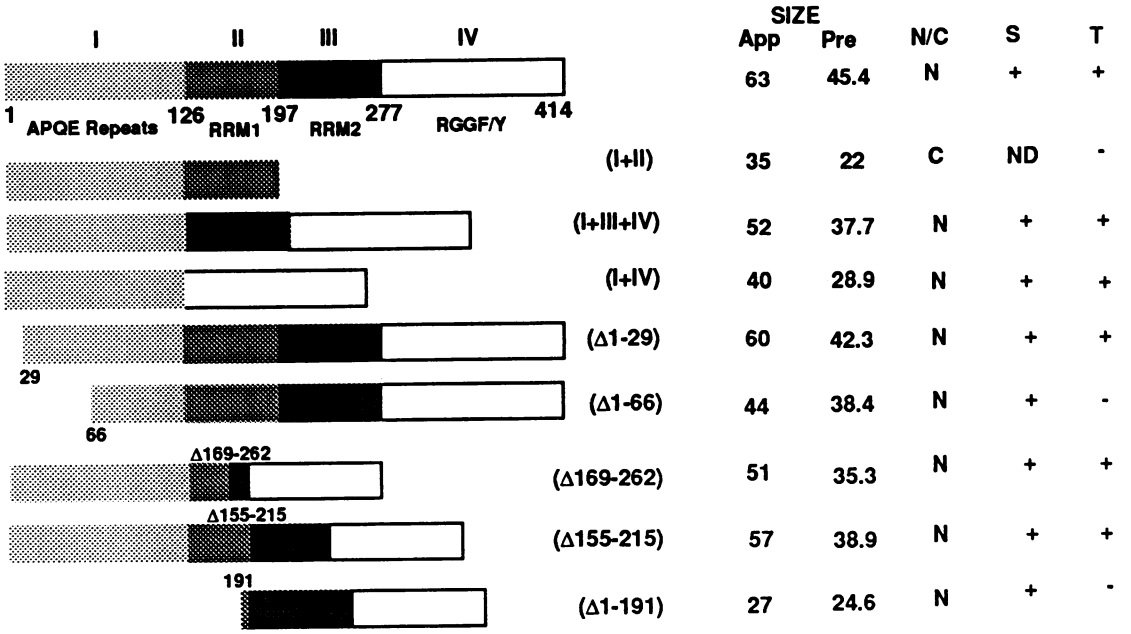

FIG. 7. Deletion mutants of Npl3. Various deletion mutants were generated as described in Materials and Methods. The four different regions of Npl3 are indicated by roman numerals I to IV. Region I contains the four APQE repeats; region II contains similarity to RNA recognition motifs (RRM1); region III contains a second region similar to RNA-binding proteins (RRM2), in particular the family of alternate splicing factors and S/R proteins; region IV contains 17 repeats of the sequence RGGY/F. The apparent (App) sizes (indicated in kilodaltons) were determined by electrophoresis on a sodium dodecyl sulfate–10% polyacrylamide gel. Pre, predicted N, nuclear; C, cytoplasmic; S, shuttling; T, toxic; ND, not determined.

in and of the nucleus is key to their function. For example, Nopp140 binds to NLSs and is proposed to move along tracks inside the nucleus (23). Perhaps it is acting as a protein and/or RNA carrier. Conversely, at least some mRNAs appear to be exported from the nucleus in association with proteins (35). It is likely that all RNAs leave the nucleus in association with proteins. Npl3 is the first yeast protein shown to shuttle between the nucleus and cytoplasm. Shuttling of proteins like hnRNP A1 and Npl3 may play an important role in transport of RNA. Consistent with this idea is that mutations in *NPL3* and overexpression of Npl3 result in accumulation of mRNA in the nucleus (14).

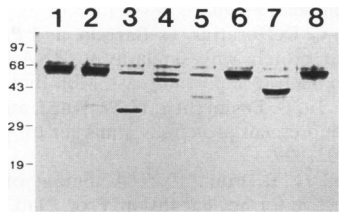

FIG. 8. Npl3 mutant proteins are made. Lysates were prepared from wild-type cells producing from the *GAL1* promoter Myc-tagged Npl3 (lanes 1 and 6), Npl3-G241D (lane 2) Npl3-I+II (roman numerals designate regions as defined in the legend to Fig. 7) (lane 3), Npl3-I+III+IV (lane 4), Npl3-I+IV (lane 5), Npl3 Npl3Δ1-66 (lane 7), and Npl3Δ1-29 (lane 8), electrophoresed on a sodium dodecyl sulfate–10% polyacrylamide gel, and analyzed by immunoblotting with anti-Npl3 antibodies. Mutant proteins missing Npl3 amino acids 169 to 262, 155 to 215, and 1 to 191 were similarly analyzed (data not shown), and the results are summarized in Fig. 7. Note that all samples also contain the wild-type Npl3. Npl3-I+IV may be less abundant or react less well with the anti-Npl3 antibodies. There is an unidentified 70-kDa cross-reacting protein. Sizes are indicated in kilodaltons.

It has been proposed that proteins may have export as well as import signals in their primary sequences (8). For Npl3, one idea is that it might leave the nucleus in association with RNA. Therefore, its ability to bind RNA might define an export signal. We tested this idea by removing the region of Npl3 that is similar to the canonical RNA-binding domains and found that these mutant proteins still shuttled. We cannot say at this point if there is an alteration in the rate of their nucleocytoplasmic movement. However, we also found that the C terminus can mediate binding to ribohomopolymers. Perhaps this region is important for shuttling. We could not test this idea directly because proteins missing the C terminus were no longer localized to the nucleus. This could be because they do not efficiently enter the nucleus.

In contrast to Npl3, chimeric proteins containing either an NLS fused to normally cytoplasmic invertase or histone H2B fused to GFP remain in the nucleoplasm and do not shuttle. Several explanations are possible. It could be that, in fact, proteins like Npl3 do contain specific information for export and reimport and SV40-invertase lacks this information. Alternatively, once inside the nucleus, SV40-invertase might aggregate or bind to something inside the nucleus, thus being retained (33). This explanation is perhaps more plausible for H2B-GFP, in which case one might expect the protein to associate with DNA inside the nucleus. We do not think that molecular size influences our results, since Npl3 and invertase are comparable in size.

In sum, since Npl3 shuttles between the nucleus and the cytoplasm and shares homology with RNA-binding proteins, we suggest that Npl3 accompanies RNAs out of the nucleus. A

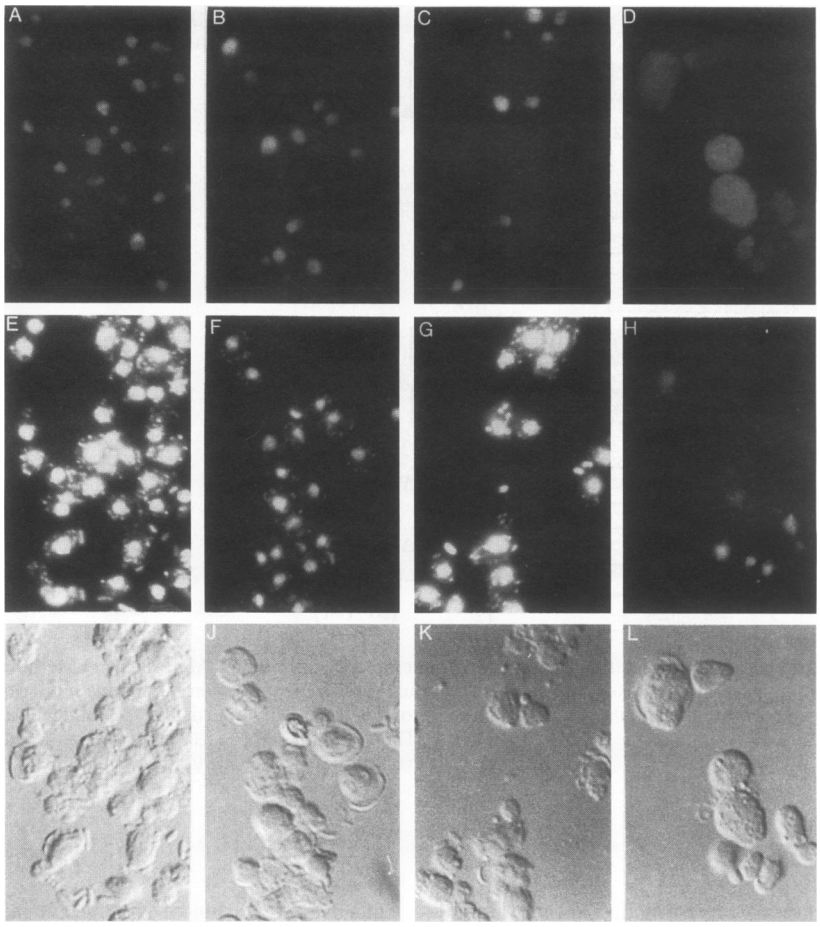

FIG. 9. Localization of mutant proteins. Cells expressing Npl3-Myc (A, E, and I), Npl3-I+III+IV (roman numerals designate regions as defined in the legend to Fig. 7) (B, F, and J), Npl3-I+IV (C, G, and K), and Npl3-I+II (D, H, and L) were analyzed by immunofluorescence with anti-Npl3 antibodies (A to D), stained with DAPI (E to H), or viewed by Nomarski (I to L). Staining of the same cells with anti-Myc antibodies yielded the same results (data not shown).

signal for export could, for example, be exposed when Npl3 binds RNA in the nucleus. Once RNA reaches the cytoplasm, Npl3 then would dissociate and return to the nucleus. Whether Npl3 plays an active or passive role in RNA and/or protein import requires further investigation.

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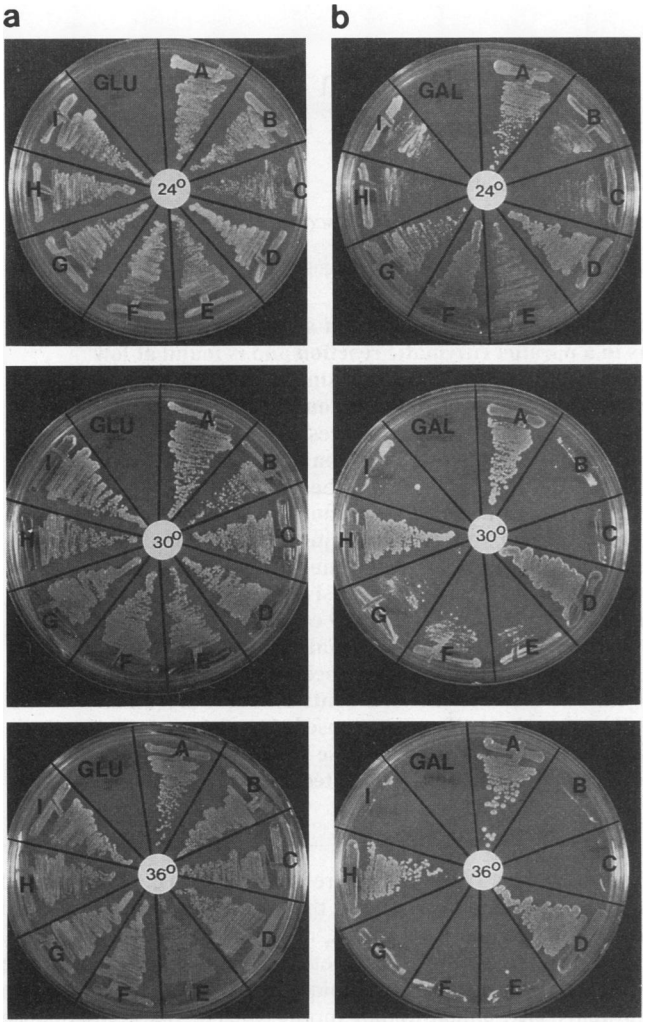

FIG. 10. Expression of Npl3 and some mutant derivatives is toxic. Plasmid-bearing cells were streaked on Ura dropout plates containing glucose (a) or galactose (b) and incubated at the indicated temperatures. The sectors contain cells harboring plasmids producing no protein (A), Npl3 (B), Npl3-G241D (C), Npl3-I+II (roman numerals designate regions as defined in the legend to Fig. 7) (D), Npl3-I+III+IV (E), Npl3-I+IV (F), Npl3 (G), Npl3Δ1-66 (H), and Npl3Δ1-29 (I).

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