Domain Analysis of the Saccharomyces cerevisiae Heterogeneous Nuclear Ribonucleoprotein, Nab2p

DISSECTING THE REQUIREMENTS FOR Nab2p-FACILITATED POLY(A) RNA EXPORT*

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Kavita A. Marfatia‡, Emily B. Crafton§, Deanna M. Green¶, and Anita H. Corbett**

From the Department of Biochemistry, §Graduate Program in Genetics and Molecular Biology and *Graduate Program in Biochemistry, Cell, and Developmental Biology, Emory University School of Medicine, Atlanta, Georgia 30322

Mature poly(A) RNA transcripts are exported from the nucleus in complex with heterogeneous nuclear ribonucleoproteins (hnRNPs). Nab2p is an essential Saccharomyces cerevisiae hnRNP protein that interacts with poly(A) RNA and shuttles between the nucleus and cytoplasm. Functional Nab2p is required for export of poly(A) RNA from the nucleus. The Nab2 protein consists of the following four domains: a unique N-terminal domain, a glutamine-rich domain, an arginine-glycine (RGG) domain, and a zinc finger domain. We generated Nab2p deletion mutants to analyze the contribution of each domain to the in vivo function of Nab2p. We first tested whether the deletion mutants could replace the essential NAB2 gene. We then examined the impact of these mutations on Nab2p localization, poly(A) RNA localization, and association of Nab2p with poly(A) RNA. Our analyses revealed that the N-terminal domain is required for nuclear export of both poly(A) RNA and Nab2p. We confirm that the RGG domain is important for Nab2p import in vivo. Finally, the zinc finger domain is critical for the interaction between Nab2p and poly(A) RNA in vivo. Our data support a model where Nab2p associates with poly(A) RNA in the nucleus through the zinc finger domain and facilitates the export of the poly(A) RNA through protein interactions mediated by the N-terminal domain.

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¶ To whom correspondence should be addressed: 4117 Rollins Research Center, Emory University, 1510 Clifton Rd., N.E., Atlanta, GA 30322. Tel.: 404-727-4546; Fax: 404-727-3549; E-mail: acorbe2@emory.edu.

** To whom correspondence should be addressed: 4117 Rollins Research Center, Emory University, 1510 Clifton Rd., N.E., Atlanta, GA 30322. Tel.: 404-727-4546; Fax: 404-727-3549; E-mail: acorbe2@emory.edu.
poly(A) RNA-binding protein (12). As with other shuttling hnRNP proteins (13–16), Nab2p localizes to the nucleus at steady state (12) but shuttles between the nucleus and the cytoplasm (17–19). Nab2p is imported into the nucleus by a transport receptor from the karyopherin family, Kap104p (20). Although the mechanism of Nab2p export is unknown, it relies on ongoing synthesis of poly(A) RNA and the arginine methyltransferase, Hmt1p (17). Importantly, in the absence of a functional Nab2 protein, poly(A) RNA accumulates within the nucleus (17, 21). Furthermore, Nab2p binds directly to poly(A) RNA in vitro (12, 21), and cells lacking Nab2p accumulate poly(A) RNA with hyperadenylated poly(A) tails suggesting a role for Nab2p in polyadenylation termination (21). Taken together, these findings support a model where Nab2p plays a role in ensuring proper export of mature mRNA from the nucleus.

In support of this model, the Nab2 protein contains domains similar to those found in other proteins that function in RNA metabolism. Nab2p can be divided into four distinct domains: a unique N-terminal domain, a glutamine-rich domain, an RGG domain encompassing the entire C-terminal half of the protein (see Fig. 1) (12). The RGG domain is found in many proteins involved in RNA metabolism, including the yeast hnRNPs, Npl3p and Hrp1p, and the mammalian hnRNP A1 protein (12, 16, 22, 23). In Nab2p, the RGG domain is the site of methylation by the yeast arginine methyltransferase, Hmt1p (17). This domain also overlaps with the nuclear localization signal/Kap104p-binding site in Nab2p (19, 24). In addition, this domain is a putative RNA binding domain (12). The zinc finger repeats, CXX2CXX2–4CX3H, resemble those found within the largest subunit of RNA polymerases I–III (12). This is a putative site for both protein-RNA interactions (12, 25) and protein-protein interactions (26–29).

For this study, we have generated a series of NAB2 mutants, and analyzed these variant proteins to provide insight into the function of each domain. We find that the N-terminal domain is important for efficient export of both Nab2p and poly(A) RNA from the nucleus. The RGG domain of Nab2p is important, but not absolutely required, for import of Nab2p into the nucleus. Finally, the zinc finger domain is essential for Nab2p function, and our results suggest that the last three zinc finger repeats are critical for the interaction with poly(A) RNA in vivo.

**Experimental Procedures**

**Strains, Plasmids, and Chemicals—**DNA manipulations were performed according to standard methods (30), and all yeast media were prepared by standard procedures (31). All yeast strains and plasmids used are described in Table I. Chemicals were obtained from Sigma, US Biological, or Fisher unless otherwise noted.

**Construction and Functional Analysis of nab2 Mutant Alleles—**Overlap PCR procedures were performed to generate the NAB2 deletion mutants (32). Proper construction of all mutants was confirmed by sequencing of the resulting clones. The function of each mutant was assessed through a plasmid shuffle technique using 5-fluoro-orotic acid (5-FOA) (33). Nab2 deletion cells (ACY429) maintained by a wild-type URA3-NAB2 plasmid (pAC636) and also containing a LEU2 plasmid carrying the mutant allele of NAB2 to be tested were grown to saturation, counted, and serially diluted in distilled H2O to obtain 10,000, 1000, 100, 10, or 1 cell per 3 μl. These dilutions were spotted onto URA–glucose and 5-FOA plates and incubated at 37, 30, and 18 °C. Cells expressing wild-type NAB2 or vector alone served as controls.

**Immunoblot Analysis—** Cultures were grown to saturation (or as indicated) and then harvested by centrifugation at 3000 rpm for 3 min. Cell pellets were washed twice with distilled H2O and once in PBS/T (PBS, 2.5 mM MgCl2, 5% Triton X-100). Cells were then resuspended in 500 μl of PBS/T supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride and 3 μg/ml each of aprotinin, leupeptin, chymostatin, and pepstatin). One volume of glass beads was added to each sample, and cells were lysed with 60-s pulses in a bead beater (Biospec Products). The lysate was clarified by centrifugation at 13,000 rpm and assayed for total protein concentration using a Bio-Rad protein assay kit. Immunoblot analysis was performed as described previously (34). Nab2 protein was detected with a polyclonal anti-Nab2p antibody (1:500,000) (17); GFP-tagged proteins were detected using a polyclonal anti-GFP antibody (1:5000) (35), and Arf1p was detected using a polyclonal anti-Arf1p antibody (1:1000) (36).

**Microscopy—** All microscopy was carried out using filters from Chroma Technology and an Olympus BX60 epifluorescence microscope.
equipped with a photometrics Quantix digital camera. For direct fluorescence, cells expressing GFP-tagged proteins were grown to 1 × 10^6 cells per ml, and live cells were then examined for the GFP signal through a GFP optimized filter. All images were captured using IP Lab Spectrum software.

**Nuclear Protein Export Assay**—Nuclear export of Nab2p was examined as described previously (37). The wild-type and mutant Nab2-GFP fusion proteins were expressed in *spp4-3D3* (ACY480) yeast (38). Cells were grown to mid-log phase at 25°C in minimal media lacking uracil and supplemented with 2% glucose. To inhibit protein synthesis, cycloheximide (100 μg/ml) was added to each sample for 1 h at 25°C. Cultures were then either maintained at 25°C or shifted to 37°C for 5 h. The GFP signal was examined in live cells by direct fluorescence microscopy as described above.

**Fluorescence in Situ Hybridization (FISH)**—The FISH protocol was adapted from Wong and co-workers (39, 40). Cells expressing wild-type Nab2p (pAC717), ΔN (pAC1152), ΔQQQp (pAC1115), or ΔQQp (pAC957) as their only copy of NAB2 were subsequently permeabilized for 5 min with 0.5% igepal, equilibrated for 2 h at room temperature. Samples were washed and stained with DAPI (1 mg/ml 1:1 mixture of methanol/acetone treatment, blocked with PBS/BSA (1% heat-inactivated fetal calf serum, and 0.3% Triton X-100) for 1 h at room temperature. Fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals) was diluted 1:200 in 1× PBS, 0.5% Tween, and 3% normal donkey serum. Slides were washed several times with dilutions of SSC (2, 1, and 0.5x) before incubation in antibody blocking buffer (0.1 mg/ml BSA, 0.1 mg/ml Tween 20) applied to the wells for overnight incubation at 4°C. The slides were then washed, dried, treated with antifade, and sealed with clear nail polish.

**Indirect Immunofluorescence**—Indirect immunofluorescence was performed according to Wong et al. (39). Cultures were grown to log phase and cells were prepared for immunofluorescence and adhered to slides as described for FISH. Cells were then fixed on slides by methanol/acetone treatment, blocked with PBS/BSA (1× PBS, 0.5% BSA), and then incubated with an anti-GFP antibody (1:1000) before overnight incubation at 4°C. Samples were washed with PBS/BSA, and treated with FITC-conjugated secondary antibody (1:1000) for 1 h at room temperature. The slides were washed, dried, treated with antifade, and sealed with clear nail polish.

**Poly(A) RNA-Protein Cross-linking**—The protocol for UV cross-linking was adapted from Krebber et al. (41). Cultures were grown to mid-log phase and shifted to 18°C until cultures reached early saturation. Cell pellets were washed once and resuspended in PBS. Proteins and RNA were cross-linked by UV (λ = 365 nm) irradiating the cells four times for 2.5 min each time using a Stratalinker (Stratagene). Cells were washed, treated with PBS, and resuspended in lysis buffer (10 mM Tris, pH 7.5, 50 mM LiCl, 1% SDS, 1 mM EDTA, and protease and RNase inhibitors) with glass beads. Samples were vortexed for 20 min at 4°C to lyse the cells and clarified by centrifugation at 13,000 rpm for 15 min. Lysates were incubated with LiCl, oligo(dT) binding buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 0.5% SDS, 0.5 mM LiCl), 0.2 g of oligo(dT), and 2 μg of New England Biolabs RNeasy mini kit (phenol/mercaptoethanol) for 30 min at room temperature. The complexes bound to the oligo(dT)_2 cellulose were washed, resuspended in oligo(dT) binding buffer, and poured into a polypropylene column (Bio-Rad). Complexes were eluted from the oligo(dT)_2-cellulose with elution buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 0.05% SDS). The eluate was heated at 65°C for 10 min, supplemented with 10 μl of 5 M LiCl, and loaded onto the cellulose column. The column was washed with oligo(dT) binding buffer, and complexes were eluted with elution buffer. The eluate was subsequently concentrated by extraction with butanol. RNA and protein complexes were precipitated with 2 μl LiCl and 100% ethanol overnight at −80°C and resuspended in RSB (10 mM Tris, pH 7.4, 100 mM NaCl, 2.5 mM MgCl2, 1 mM CaCl2, and protease inhibitors). Units of RNA in each sample were measured at A260. Samples were treated with RNase and micrococcal nuclease at 30°C for 30 min. For each sample, 35 units of RNA in gel loading dye was resolved on a 0.8% PAGE gel and immunoblotted with anti-Nab2p antibody (1:5000) (17), anti-GFP antibody (1:5000) (35), or anti-Arf1p antibody (1:10,000) (12).

The amount of protein bound to poly(A) RNA was quantified by fluoromaging using Labworks software from UVP Bioimaging Systems. The amount of protein in the bound and lysate lanes of each immunoblot was quantified using non-linear equations. The background density was subtracted from each lane resulting in a value corresponding to density of protein in each lane. The amount of bound protein was calculated by setting each lysate value to 1. The fold difference in the amount of bound protein for the mutant proteins as compared with wild-type Nab2p was calculated by setting the amount of bound wild-type Nab2p to 1 for each matched experiment. Experiments were performed and analyzed independently three to four times. Standard deviations were calculated for each set of experiments.

**RESULTS**

**Characterization of Nab2p Deletion Mutants**—To probe the function of each domain of Nab2p, we generated Nab2p mutants that precisely deleted each of the major predicted domains (Fig. 1). Because NAB2 is essential (12), a plasmid shuffle technique was used to assess the function of each of the deletion mutants (Fig. 2A). The positive control for growth is the wild-type Nab2p, and the negative control is the vector alone. Cells expressing a deletion of the N-terminal domain (AN) of Nab2p as their only form of Nab2p grow slowly at 30°C and 37°C and are not viable at 18°C. In contrast, deletion of the glutamine-rich domain (ΔQQQP) causes no apparent growth defect at any temperature examined. Cells expressing a deletion of the RGG domain (ΔRGG) of Nab2p grow slowly at 18°C and 37°C but show no growth defect at 30°C. Deletion of the entire zinc finger domain (ΔCCHC) yields a non-functional protein that cannot replace the essential Nab2 protein (Fig. 2A). Identical results were obtained when each of the Nab2 deletion mutant proteins was C-terminally tagged with GFP and analyzed in this assay (data not shown).

To confirm that each of the mutant proteins was expressed, quantitative immunoblot analysis of the GFP-tagged wild-type and mutant Nab2 proteins was performed. Because cells expressing the zinc finger domain deletion mutant (ΔCCHC) as their only copy of NAB2 are viable, we analyzed the expression of each of the mutant proteins in a wild-type background where the endogenous NAB2 gene is intact. As shown in Fig. 2B, all the mutant proteins were expressed at their predicted sizes at levels comparable with that of wild-type Nab2p-GFP. Similar results were obtained for the untagged mutant proteins when they were detected with an anti-Nab2p antibody (data not shown). Furthermore, we analyzed the expression of the ΔN-GFP mutant protein, which confers strong cold sensitivity, at both 30 and 18°C. The GFP-tagged N-terminal deletion mutant was expressed at the same levels as wild-type Nab2p-GFP at both temperatures tested (data not shown).

We next examined poly(A) RNA localization in NAB2 mutant cells to determine which domains of Nab2p are critical for poly(A) RNA export. In wild-type cells, poly(A) RNA localizes throughout the cell at both 30 and 18°C (Fig. 2C). In contrast, AN mutant cells show nuclear accumulation of bulk poly(A) RNA at both 30°C, where they grow very slowly, and 18°C, where they do not grow at all (Fig. 2C). Cells that express the ΔQQQP mutant have no apparent defect in poly(A) RNA export (Fig. 2C). Cells expressing ΔRGG accumulate poly(A) RNA in the nucleus at 18°C in ~50% of the population but export poly(A) RNA efficiently at 30°C. Localization of poly(A) RNA could not be analyzed in cells expressing the ΔCCHC mutant as the only copy of NAB2 because these cells are inviable (see Fig. 2C).
Taken together, these results demonstrate that the N-terminal domain and RGG domain of Nab2p are both required for efficient export of poly(A) RNA from the nucleus.

To examine the steady state localization of each of the mutant Nab2 proteins, GFP-tagged mutant and wild-type proteins were expressed from the NAB2 promoter on a low copy plasmid in wild-type cells (Fig. 3A). Similar to wild-type Nab2p-GFP, the ΔN-GFP and ΔQQQP-GFP mutant proteins were localized in the nucleus at steady state. The zinc finger deletion mutant (ΔCCCH-GFP) protein was localized in the nucleus with a low level also present in the cytoplasm. We determined that the nuclear localization of ΔCCCH-GFP is dependent on the Nab2p import receptor, Kap104p (20) (data not shown), suggesting that, despite the large deletion, this protein is sufficiently folded to be targeted to the nucleus by the same mechanism as wild-type Nab2p. In contrast to the other mutant proteins, the ΔRGG-GFP mutant protein is localized throughout the cell (Fig. 3A). We also observed this mislocalization with an untagged ΔRGG protein detected with an anti-Nab2p antibody (data not shown). These results are consistent with previous work (19, 20, 24) demonstrating that the RGG domain mediates the interaction between Nab2p and its import receptor, Kap104p. Identical localization patterns were observed when cells were fixed, and GFP-tagged Nab2 proteins were visualized using an anti-GFP antibody (Fig. 3B).

The ΔRGG-GFP mutant protein, which lacks the Kap104p-binding site (19, 24), is localized throughout the cell at steady state (Fig. 3A). Based solely on this observation, it is difficult to determine whether this mutant protein never enters the nucleus or, alternatively, whether the mutant protein still shuttles but enters the nucleus at a slower rate than wild-type Nab2p so that the steady state localization is now cytoplasmic. To distinguish between these two possibilities, we localized the ΔRGG-GFP protein in cells containing a temperature-sensitive allele (rat7-1) of the nucleoporin, Rat7p/Nup159p (42). rat7-1 cells are defective in macromolecular export at the non-permis-
that any cytoplasmic accumulation of protein observed is because of protein export rather than new protein synthesis, cycloheximide is added to the cultures prior to the shift to the non-permissive temperature.

In this assay, wild-type Nab2p-GFP can be observed in the cytoplasm (Fig. 3D) as has been demonstrated previously (17, 18). Because the NUP49 nuclear export assay relies on monitoring the appearance of the protein of interest in the cytoplasm, a prerequisite for this assay is that the protein of interest must be localized strictly to the nucleus under steady state conditions. For this reason, only the ΔN and ΔQQQP mutant proteins, which are both strictly nuclear at steady state, could be analyzed using this assay. As shown in Fig. 3D, the ΔN-GFP mutant protein remains within the nucleus indicating that export of this mutant protein is blocked. In contrast, the ΔQQQP-GFP mutant protein is detected in the cytoplasm demonstrating that deletion of this domain does not impact Nab2p export from the nucleus (Fig. 3D). Thus, analysis of the Nab2p deletion mutants reveals that the N-terminal domain of Nab2p is required for export of both poly(A) RNA and Nab2p from the nucleus.

Analysis of the N-terminal Domain of Nab2p—Our experiments show that the Nab2p N-terminal deletion mutant (ΔN) is not efficiently exported from the nucleus (see Fig. 3D). One possible explanation for this defect is that this N-terminal domain may contain a signal for Nab2p export. To test this possibility, we generated a nuclear localized reporter protein containing an SV40 bipartite nuclear localization signal (NLS) fused to two GFP molecules (NLS-GFP)2 (45) and a modified NLS-GFP protein that also incorporates the N-terminal domain (amino acids 1–97) of Nab2p (NLS-Nab2p-GFP). Unlike the strictly nuclear NLS-GFP protein, the NLS-Nab2p-GFP shows some cytoplasmic signal (Fig. 4, A and C). The NLS-NES-GFP reporter protein, which contains the classical leucine-rich NES (41), is exported from the nucleus more efficiently than the NLS-N(Nab2p)-GFP reporter protein (Fig. 4E).

Because the NLS-N(Nab2p)-GFP protein is detected in the cytoplasm of wild-type cells, the N-terminal domain of Nab2p could either act as a nuclear export signal or an inhibitor of import of the NLS-GFP reporter protein. To distinguish between these possibilities, we took advantage of the rat7-1 temperature-sensitive mutant (42). If the N-terminal domain can act as an export signal, we would predict that NLS-N(Nab2p)-GFP protein export would be blocked, and the protein would accumulate within the nucleus of rat7-1 cells at the non-permissive temperature. However, if the N-terminal domain inhibits import of the NLS-GFP protein, we would expect no change in localization for the NLS-N(Nab2p)-GFP protein in rat7-1 cells as compared with wild-type cells at 37 °C. In rat7-1 cells at the permissive temperature (25 °C), the NLS-N(Nab2p)-GFP protein is localized to the nucleus with some cytoplasmic signal (Fig. 4K). In contrast, the NLS-N(Nab2p)-GFP protein is detected only within the nucleus at the non-permissive temperature (37 °C) (Fig. 4M). As a control, the NLS-GFP protein is strictly nuclear in rat7-1 cells at both 25 and 37 °C, and the NLS-NES-GFP reporter protein accumulates in the nucleus in rat7-1 cells at 37 °C (Fig. 4, I and Q). Together, these results suggest that the N-terminal domain contains within it a nuclear export signal that could facilitate Nab2p export from the nucleus. Our analysis of the ΔN mutant suggests a model where Nab2p associates with poly(A) RNA in the nucleus (12), and the N-terminal domain of Nab2p facilitates export of both poly(A) RNA and Nab2p.

The finding that the ΔN Nab2 mutant protein cannot exit the

Fig. 3. Localization of the Nab2p deletion mutants. A, wild-type (pAC753) or each mutant Nab2-GFP protein was expressed in wild-type cells (ACY192), and their localization was examined by direct fluorescence microscopy. Corresponding DIC images are shown. B, wild-type (pAC753) or each mutant Nab2-GFP protein was expressed in wild-type cells (ACY192). The cells were fixed and prepared for indirect immunofluorescence, and Nab2p-GFP proteins were detected with an anti-GFP antibody (35). The nuclei are indicated by the DAPI-stained chromatin. Corresponding DIC images are shown. C, wild-type (ACY192) or rat7-1 (ACY194) cells expressing ΔRGG-GFP (pAC980) were grown to log phase at 25 °C and shifted to 37 °C for 15 min, and GFP-tagged proteins were viewed by direct fluorescence microscopy. Corresponding DIC images are shown. D, the nuclear export assay was performed using nup49-313 cells (ACY480) as described under "Experimental Procedures." Cells expressing Nab2p-GFP (pAC719), ΔN-GFP (pAC1050), or ΔQQQP-GFP (pAC1051) were treated with cycloheximide and shifted to 37 °C. The localization of GFP-tagged proteins was analyzed by direct fluorescence microscopy. Corresponding DIC images are shown.

2 M. T. Harreman, G. Truscott, and A. H. Corbett, unpublished data.

3 M. Hodel and A. Hodel, unpublished data.
of the GFP-tagged proteins was examined by direct fluorescence microscopy. Corresponding DIC images are shown (Fig. 2). Perhaps, the ΔN mutant protein still binds poly(A) RNA within the nucleus but then cannot exit the nucleus and consequently accumulates in the nucleus bound to poly(A) RNA. This explanation is only valid if the ΔN Nab2p mutant binds to poly(A) RNA. To determine whether the ΔN Nab2p protein binds to poly(A) RNA, we performed an in vivo RNA/protein cross-linking experiment. Cells expressing either wild-type or ΔN Nab2p were UV cross-linked, and poly(A) RNA complexes were isolated. Nab2p present in the lysate (L), bound (B), or unbound (U) fraction was detected by immunoblot analysis. For each experiment, an equal amount of cross-linked RNA from each sample was analyzed (Fig. 5A), and the amount of Nab2p protein bound was quantified (Fig. 5B) by fluorimaging of the immunoblots as described under “Experimental Procedures.” Wild-type Nab2p is detected in complex with poly(A) RNA in this experiment as indicated by the band in the bound fraction (Fig. 5A, middle row, lanes 2 and 5, and 5B). The ΔN mutant protein also binds to poly(A) RNA at both 18 °C and 30 °C (Fig. 5A, top row, lanes 2 and 5, and 5B). In fact, more ΔN Nab2p is detected in complex with poly(A) RNA than wild-type Nab2p, which could be a result of increased levels of ΔN Nab2p in the nucleus because ΔN does not appear to exit the nucleus (see Fig. 3D). The cytoplasmic GTPase, Arf1p, was used as a control for nonspecific binding. The anti-Arf1p immunoblot from the wild-type cross-linking experiment is shown in Fig. 5A (bottom row). In addition, we did not observe any nonspecific Nab2p binding to the oligo(dT) column when UV cross-linking was not performed prior to isolation of poly(A) RNA (data not shown).

Analysis of the ΔN mutant supports a model where Nab2p binds poly(A) RNA in the nucleus and the N-terminal domain mediates a critical interaction that facilitates export of poly(A) RNA-mRNP complex to the cytoplasm. This model predicts that if the wild-type and ΔN Nab2p proteins are co-expressed, the ΔN Nab2p protein should compete with wild-type Nab2p for binding to poly(A) RNA. Consequently, cells that overexpress ΔN Nab2p, even in the presence of wild-type Nab2p, should accumulate poly(A) RNA in the nucleus. To test this prediction, we examined poly(A) RNA localization and cell growth in wild-type cells that overexpress ΔN Nab2p. For these experiments, ΔN NAB2 was placed under the control of a galactose-inducible promoter. As shown in Fig. 6A, wild-type cells that overexpress ΔN Nab2p accumulate poly(A) RNA in the nucleus (Fig. 6A, panel J) whereas cells that overexpress wild-type Nab2p export poly(A) RNA to the cytoplasm (Fig. 6A, panel D). Because overexpression of ΔN in wild-type cells blocks the essential process of poly(A) RNA export, we predict that overexpression of ΔN should also inhibit cell growth. Thus, growth of wild-type cells either expressing (Gal plate) or not expressing (Glu plate) ΔN Nab2p was examined. Cells that overexpress ΔN Nab2p grow more slowly than cells that express either wild-type Nab2p or an empty vector (Fig. 6B, Gal plate). A dominant negative mutant of yeast Ran (gsp1r79E (46)) was included as a control (Fig. 6B, Gal plate). Immunoblot analysis demonstrates that the galactose-inducible Nab2 and ΔN proteins are expressed at similar levels upon induction with galactose (data not shown). These results are consistent with the predictions of our model.

Analysis of the Nab2p Zinc Finger Domain—Deletion of the entire C-terminal zinc finger domain yields a non-functional Nab2 protein (see Fig. 2A). Closer analysis of the zinc finger domain reveals that the seven CCCH repeats cluster into two
groups, a set of four repeats and a set of three repeats. To
dissect further the function of the zinc finger domain, we
generated mutants that deleted the first four repeats (ΔC4), the
last three repeats (ΔC3), or the last 47 amino acids of Nab2p (ΔCT),
which lie outside of the zinc finger domain (see Fig. 1). The C-terminal mutants were expressed in Δnab2 cells that
were maintained by a plasmid-borne copy of NAB2
(mutant proteins are non-functional. In contrast, deletion of the
last three zinc finger repeats (ΔC3) greatly diminishes, but does not completely elim-
nate, the interaction between Nab2p and poly(A) RNA (Fig.
6A), whereas deletion of the last three
zinc finger repeats results in a non-functional
these mutant proteins interact with poly(A) RNA. Because
deletion of the zinc finger deletion variants (ΔC3, ΔC4, and ΔCT) were strictly
nuclear, we could determine the impact of these deletions on
Nab2p shuttling using the NUP49-based nuclear export assay.
As shown in Fig. 7D, the ΔC4 and ΔCT proteins are exported to the
cytoplasm of the nab2 mutant cells at 37 °C in a
manner similar to wild-type Nab2p. However, deletion of the
last three zinc finger repeats (ΔC3) blocks nuclear export of
Nab2p. These results indicate that the last three zinc finger
repeats are more important for Nab2p export than the first four
repeats.

To characterize further the zinc finger deletion mutants, we
used in vivo cross-linking experiments to examine whether these
mutant proteins interact with poly(A) RNA. Because
deletion of the zinc finger repeats results in a non-functional
protein (see Fig. 7A), we performed these experiments in wild-
type cells expressing GFP-tagged alleles of Nab2p, ΔCCCH,
ΔC3, and ΔC4. Wild-type Nab2p-GFP interacts with poly(A) RNA in our experiments (Fig. 8A, lane 2). Analysis of the zinc
finger domain shows that deletion of the entire zinc finger
domain (ΔCCCH) abolishes all Nab2p-poly(A) RNA binding
(Fig. 8A, lane 5, and 8B), whereas deletion of the last three
repeats (ΔC3) greatly diminishes, but does not completely elimin-
ate, the interaction between Nab2p and poly(A) RNA (Fig,
Fig. 8. In vivo cross-linking of the zinc finger deletion mutants to poly(A) RNA. Wild-type cells (ACY192) expressing Nab2p-GFP (pAC719), ΔCCCH-GFP (pAC1048), ΔC3-GFP (pAC1153), or ΔC4-GFP (pAC1154) were grown to saturation, diluted, and incubated at 30 °C. Cells were collected by centrifugation and cross-linked by UV irradiation. Cell lysates were incubated with oligo(dT)-cellulose and washed, and complexes bound to the cellulose were eluted and examined by SDS-PAGE and immunoblotting. L indicates lysate, B indicates bound/eluted fraction, and U indicates unbound fraction. A typical experiment is shown in A, and quantification of the data from three independent experiments is shown in B. A. poly(A) RNA-protein complexes were examined from cross-linked cells expressing the wild-type Nab2p-GFP (top row, lanes 1–3) or ΔCCCH-GFP (top row, lanes 4–6), ΔC3-GFP (bottom row, lanes 7–9), or ΔC4-GFP (bottom row, lanes 10–12) mutants. Immunoblots were probed with an anti-GFP antibody (35). B, the amount of bound mutant Nab2p proteins was quantified and compared with the corresponding band for bound wild-type Nab2p, which was set to 1.0. The error bars indicate the standard deviations in the data.

8A, lane 8, and 8B). Deletion of the first four repeats (ΔC4) modestly diminishes the Nab2p/poly(A) RNA interaction (Fig. 8A, lane 11, and Fig. 8B).

DISCUSSION

This study characterizes the domains of the essential S. cerevisiae hnRNPs, Nab2p. We generated a series of deletion mutants of Nab2 and tested whether each mutant could replace the essential Nab2 protein in vivo. To characterize the impact of the mutations on Nab2 function, we examined the effect of these deletions on Nab2 localization, poly(A) RNA export, and interaction between Nab2p and poly(A) RNA. Our conclusions are summarized in Fig. 9A. From our analysis, we have characterized the in vivo roles for three functionally important domains of Nab2p: the N-terminal, RGG, and zinc finger domains.

Cells expressing the N-terminal deletion mutant of Nab2p (ΔN) as the only copy of NAB2 accumulate both poly(A) RNA and Nab2p within the nucleus. Because our experiments also demonstrate that deletion of the N-terminal domain does not decrease the interaction between poly(A) RNA and Nab2p, we propose that the ΔN protein binds poly(A) RNA and sequesters it within the nucleus. Consistent with this idea, we find that overexpression of the non-shuttling ΔN protein in wild-type cells causes accumulation of poly(A) RNA in the nucleus. In addition, the N-terminal domain can act as a weak nuclear export signal in the context of a heterologous protein, SV40 NLS-GFP-GFP. Taken together, these data could suggest that the N-terminal domain of Nab2p mediates a critical interaction required for export of both Nab2p and poly(A) RNA. However, because the ΔC3 mutant protein, which contains an intact N-terminal domain but has decreased interaction with poly(A) RNA, is also not exported from the nucleus, we conclude that the export signal within the N-terminal domain is necessary but not sufficient for export of the full-length Nab2 protein.

We show here that the RGG domain is important, but not absolutely required, for the essential function of Nab2p. Cells expressing the RGG deletion mutant grow well at 30 °C, but slowly at 18 °C, where a defect in poly(A) RNA export is observed. We have no evidence that the RGG domain plays a direct role in mediating poly(A) RNA export. Instead, we believe that the primary role of the RGG domain is to target Nab2p to the nucleus as suggested previously (19, 20, 24). Although we predict that the poly(A) RNA export defect observed in ΔRGG cells at 18 °C results from a decreased nuclear pool of Nab2p, we cannot rule out the possibility that the RGG domain plays direct roles both in nuclear targeting of Nab2p and in Nab2p-facilitated poly(A) RNA export.

It is necessary to reconcile the results of this study with previous work that demonstrates that, as for other essential arginine-methylated yeast hnRNP proteins (16), the arginine methyltransferase, Hmt1p, is required for export of full-length Nab2p from the nucleus (17). The inference from this finding is that arginine methylation of Nab2p is essential for its export from the nucleus. Although several studies (16, 17) have demonstrated that the HMT1 gene is essential for export of arginine-methylated hnRNP proteins, none of these studies have demonstrated that it is methylation of the actual hnRNP being studied that is required for its export. Thus, it could be methylation of any number of described methylation targets or as yet undiscovered targets that are actually required for hnRNP export. In the case of Nab2p, a number of observations must be reconciled with our model for export. The RGG domain of Nab2p is methylated in an Hmt1p-dependent manner, and Hmt1p is required for Nab2p export (17), but the RGG domain is not essential for Nab2p export as the ΔRGG protein is apparently exported from the nucleus. One model (Fig. 9B) consistent with these observations is that Nab2p interacts with a protein in the nucleus via the RGG domain, and this interaction is regulated by arginine methylation. The simplest idea is that this interaction is disrupted by methylation. Once this interaction is disrupted, Nab2p can exit the nucleus. For the Nab2 protein that lacks the RGG domain, the interaction with the nuclear protein is absent, and now there is no dependence on arginine methylation for export. Obviously, further work will be required to understand how methylation influences hnRNP function.

The ΔRGG protein can still enter the nucleus arguing for the existence of a Kap104p-independent mechanism for import of Nab2p. This idea of an alternate import pathway is supported by the following observations. Nab2p is essential for cell viability (12) which demonstrates that it is absolutely essential for some cellular process. As suggested previously for other hnRNPs, such as Hrp1p (13), it is likely that the essential function of Nab2p is within the nucleus (17, 21), and therefore import of Nab2p into the nucleus should be essential for cell viability. However, the known Nab2p import receptor, Kap104p, is not essential (47, 48) suggesting that in the absence of Kap104p there must be another mechanism to target Nab2p into the nucleus. This is consistent with our finding that the RGG domain is not absolutely required for Nab2p import. Thus, it is likely that an alternate import receptor or adaptor protein can recognize another domain within Nab2p and mediate its import. There is precedent for the existence of multiple import mechanisms for essential nuclear proteins because a number of different karyopherins have been identified that can
mediate the import of other essential nuclear proteins such as the TATA-binding protein (49, 50) and histones (51, 52). Further studies will be required to characterize the alternate import pathway for Nab2p.

Our study highlights the importance of the zinc finger domain for Nab2p function because deletions within this domain result in a non-functional protein. Although the deletions could impact the overall folding of the Nab2 protein, it is important to note that all of the C-terminal deletion mutants are imported into the nucleus in a Kap104p-dependent manner suggesting that they are sufficiently folded to be correctly recognized by the major Nab2p import receptor. We propose that the C-terminal domain, consisting of seven zinc finger repeats clustered into two groups, is essential for the interaction between Nab2p and poly(A) RNA in vivo. Support for this idea comes from previous in vitro data showing that deletion of the zinc finger domain specifically eliminates Nab2p binding to poly(A) homopolymers (12) and our in vitro cross-linking data where deletion of the entire zinc finger domain completely abolishes the interaction between Nab2p and poly(A) RNA. Significantly, deletion of the last three zinc finger repeats (∆C3) decreases the interaction between Nab2p and poly(A) RNA to ~10% of wild-type levels. This finding suggests that the last three zinc finger repeats comprise the primary binding site for poly(A) RNA in vivo. Interestingly, Hectors et al. (21) have recently isolated a cold-sensitive allele of Nab2, nab2-21, which contains a deletion corresponding to most of the C3 domain. They found that cells expressing this mutant accumulate poly(A) RNA within the nucleus (21). This mutant phenotype could be consistent with our finding that the last three zinc finger repeats of Nab2p are required for binding poly(A) RNA in vivo.

The data from these analyses suggest a model where efficient export of poly(A) RNA from the nucleus requires both the N-terminal domain of Nab2p and Nab2p association with poly(A) RNA via the zinc finger domain. In this model, Nab2p is imported into the nucleus primarily by Kap104p (20), which binds to the RGG domain of Nab2p (19, 24). Once in the nucleus, Nab2p dissociates from Kap104p and binds to poly(A) RNA; a previous study has shown that Nab2p release from Kap104p requires both binding to poly(A) RNA and the GTP-bound form of the small GTPase, Ran (19). We predict that the zinc finger domain of Nab2p mediates binding to poly(A) RNA, which could cause a conformational change in Nab2p to facilitate dissociation from Kap104p. Nab2p then associates with the maturing mRNA transcript within the nucleus, and as recent evidence suggests, Nab2p may play a role in polyadenylation termination of the pre-mRNA (21). It is likely that Nab2p bound to poly(A) RNA associates with other hnRNP proteins to form an mRNP complex that is exported from the nucleus. We propose that the N-terminal domain of Nab2p mediates an interaction that facilitates the efficient export of the mRNP complex. Finally, Nab2p is released from the mRNP complex in the cytoplasm and is recycled back into the nucleus by Kap104p.

From this study, we conclude that Nab2p requires both binding to poly(A) RNA via the zinc finger domain and interactions with the N-terminal domain to facilitate proper export of poly(A) RNA from the nucleus. Ultimately, in order to understand the detailed steps required for poly(A) RNA processing and export, it will be necessary to define the protein complexes that mediate each step in the process. Furthermore, it will be important to understand how the individual proteins within these complexes interact with one another and with the poly(A) RNA substrate. The nab2 mutants that we have generated will be useful in characterizing the relationships between Nab2p and other poly(A) RNA-binding proteins and in identifying the critical interactions required for export of the mRNP complex from the nucleus.

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