The Yeast mRNA-binding Protein Npl3p Interacts with the Cap-binding Complex*

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A number of RNA-binding proteins are associated with mRNAs in both the nucleus and the cytoplasm. One of these, Npl3p, is a heterogeneous nuclear ribonucleoprotein-like protein with some similarity to SR proteins and is essential for growth in the yeast S. cerevisiae. Temperature-sensitive alleles have defects in the export of mRNA out of the nucleus (1). In this report, we define a genetic relationship between NPL3 and the nonessential genes encoding the subunits of the cap-binding complex (CBP80 and CBP20). Deletion of CBP80 or CBP20 in combination with certain temperature-sensitive npl3 mutant alleles fail to grow and thus display a synthetic lethal relationship. Further evidence of an interaction between Npl3p and the cap-binding complex was revealed by co-immunoprecipitation experiments; Cbp80p and Cbp20p specifically co-precipitate with Npl3p. However, the interaction of Npl3p with Cbp80p depends on both the presence of Cbp20p and RNA. In addition, we show that Cbp80p is capable of shuttling between the nucleus and the cytoplasm in a manner dependent on the ongoing synthesis of RNA. Taken together, these data support a model whereby mRNAs are co-transcriptionally packaged by proteins including Npl3p and cap-binding complex for export out of the nucleus.

While in the nucleus, mRNA precursors, referred to as pre-mRNAs or heterogeneous nuclear RNAs undergo a series of processing events before entering the cytoplasm. These maturation events include co-transcriptional capping at the 5'-end, splicing, and cleavage and polyadenylation at the 3'-end. The proper execution of these steps affects the export of mRNA (reviewed in Refs. 2–4). Thus, the process of mRNA export commences long before the RNA actually reaches the nuclear membrane.

Pre-mRNAs and snRNAs1 are first modified co-translationally at the 5'-end by a monomethyl cap after reaching a length of 20–30 nucleotides (5, 6). This modification consists of a guanosine residue methylated at the N-7 position joined to a guanosine residue methylated at the N-2 position and is essential for growth in the yeast S. cerevisiae. The cap structure appears to be important for efficient splicing and export of RNAs (reviewed in Ref. 2).

Evidence has suggested that the effects of the cap structure on RNA metabolism are protein-mediated (7). A cap binding activity was purified from HeLa cell nuclear extracts and was found to consist of two proteins termed cap-binding protein 80 (Cbp80p) and cap-binding protein 20 (Cbp20p) (8–10). Neither Cbp80p nor Cbp20p has been found to bind capped RNA alone, suggesting that a complex of the two proteins is required for binding of a cap (10). This complex is referred to as the cap-binding complex (CBC). Homologues of both CBC proteins have been identified in all eukaryotes examined thus far (e.g. see Refs. 10–13).

Although the 5' cap structure is not required for successful export, it has been shown to enhance the rate of mRNA export from the nucleus (7, 14). Studies of the Balbiani ring pre-mRNA particle in the insect Chironomus tentans show that Cbp20p binds the pre-mRNA nascent transcript and remains bound to the pre-mRNA throughout splicing and as the mRNA is translocating through the nuclear pore complex (13). Because the 5'-end is in the lead as the mRNA exits the nucleus, these data raised the possibility that the CBC mediates the effect of the cap on mRNA export.

In the budding yeast, Saccharomyces cerevisiae, CBP80 has been isolated through both genetic and biochemical approaches (15, 16). CBP80 is not an essential gene (15). However, deletion of the gene results in a severe growth defect. Yeast CBP20 was originally identified as MUD13, a gene that when mutated causes synthetic lethality in combination with a mutant form of U1 snRNA (12). Like CBP80, CBP20 is not an essential gene in yeast. A strain carrying null alleles of both CBP80 and CBP20 is also viable (17).

From the time that they leave the transcription complex, pre-mRNAs are also associated with proteins in complexes referred to as heterogeneous nuclear ribonucleoprotein particles (hnRNPs) (for a review, see Ref. 18). The hnRNPs are among the most abundant proteins found in the nucleus (19) and are proposed to function in nearly every maturation step of mRNA including splicing, polyadenylation, and export (for reviews, see Refs. 20 and 21). One of the most studied hnRNPs in mammalian cells is hnRNPA1, which belongs to the class of hnRNPs that shuttles between the nucleus and cytoplasm (22). Along with other hnRNPs in its class, hnRNPA1 contains two RNA recognition motifs (RRMs) and a glycine-rich region at the carboxyl terminus (23). Because hnRNPA1 is bound to poly(A)+ RNA in both the nucleus and the cytoplasm and is able to shuttle, it has been proposed to play a role in mRNA export (for a review, see Ref. 20). Shuttling proteins like hnRNPA1 could be escorting the RNA to the cytoplasm, where other RNA-binding proteins take over.

Several hnRNPs have been identified in S. cerevisiae, allowing for a genetic approach to the study of their functions.

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1 The abbreviations used are: snRNA, small nuclear RNA; CBC, cap-binding complex; hnRNP, heterogeneous nuclear ribonucleoprotein; RRM, RNA recognition motif; 5'-FOA, 5-fluoroorotic acid; GFP, green fluorescent protein; ts, temperature-sensitive.

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One of the most studied hnRNPs is Npl3p (24, 25). Cells bearing mutant npl3 alleles accumulate poly(A)⁺ RNA in their nuclei at the nonpermissive temperature, consistent with a block in mRNA export (1, 25–27). Npl3p shares structural features found in some mammalian hnRNPs such as hnRNP A1. These include two RRMs and a glycine-rich domain (RGG box) in the form of 15 RGG repeats (23). Npl3p is methylated at arginine residues in this glycine-rich domain by the major arginine methyltransferase, Hmt1p/Rmt1p, in yeast cells (28–30). In addition, Npl3p contains a domain characterized by a series of serine-arginine repeats that overlaps with the RGG box (1, 31). Npl3p has been shown to cross-link to poly(A)⁺ RNA in vivo (1). Our results suggest that these RNA-binding proteins may work together to promote the export of mRNAs.

**Materials and Methods**

**Plasmids and Strains**—Many of the plasmids carrying temperature-sensitive alleles of NPL3 were published earlier (1). The remaining mutant alleles come from the same screen for temperature-sensitive alleles. Briefly, a polymerase chain reaction mutagenesis and plasmid shuffling protocol was used, using the strain MYH132 and the plasmid PSY865 (24, 34). This construct was digested with SmaI–Myc was inserted into a total volume of 500 µl. Lysates were then clarified by centrifugation for 10 min at 14,000 × g at 4 °C. Protein concentrations of the lysates were determined using a protein assay kit (Bio-Rad). 1.5 mg of total protein of each lysate was incubated responding antibodies as follows: anti-GFP antibody, 1:5000; anti-Myc conjugated secondary antibodies (Jackson Immunoresearch Laboratories). For immunoblotting, gels were transferred to nitrocellulose membranes (Protran; Schleicher & Schuell). The membranes were then washed three times for 3 min in water and then developed using soaking the gel in destain.

**Silver Staining**—For silver staining, the gel was fixed for 15 min in destain (30% methanol, 10% acetic acid) and then washed in water overnight. The gel was soaked for 15 min in 30% methanol, 10% acetic acid and then washed in water overnight. The gel was soaked for 15 min in 30% methanol, 10% acetic acid and then washed in water overnight. The gel was soaked for 15 min in 30% methanol, 10% acetic acid and then washed in water overnight. The gel was soaked for 15 min in 30% methanol, 10% acetic acid and then washed in water overnight. The gel was soaked for 15 min in 30% methanol, 10% acetic acid and then washed in water overnight.
RESULTS

Cbp80p Is Able to Exit the Nucleus—Once bound to mRNAs, the cap-binding complex has been proposed to move out of the nucleus together with the RNA, as has been demonstrated for Npl3p (1). In yeast, Cbp80p localizes predominantly to the nucleus at steady state (16). To determine if Cbp80p is able to exit the nucleus, we utilized the in vivo nuclear export assay (1). This assay takes advantage of a temperature-sensitive mutant allele of the nucleoporin NUP49 (38). At the nonpermissive temperature of 37 °C, nup49–313 cells are defective in the nuclear import of proteins. However, no defect in export of proteins or RNAs has been observed in these cells. Therefore, if a protein that localizes in the nucleus at steady state is able to exit the nucleus, it will accumulate in the cytoplasm after the temperature shift in the absence of new protein synthesis.

To monitor the localization of Cbp80p, GFP was fused to the C terminus of the Cbp80p coding region. The resulting fusion protein was placed under the control of the regulatable GAL1 promoter. This construct is functional because it rescues the synthetic lethality between hmt1Δ and cbp80–1 (data not shown). Expression of Cbp80p-GFP was induced by growth in galactose-containing medium. A fusion protein of the correct size, approximately 120 kDa, is seen by Western blot with anti-GFP antibody, with no free GFP detected (Fig. 1A). When cells expressing Cbp80p-GFP were viewed by fluorescence microscopy, all of the Cbp80p-GFP was in the nucleus (Fig. 1B).

In the nuclear export assay, expression of Cbp80p-GFP was induced for 2 h. Following repression of new protein synthesis by growth in glucose-containing medium, the cells were either left at the permissive temperature of 25 °C or shifted to the nonpermissive temperature of 37 °C for 5 h. As expected, Cbp80p-GFP localizes to the nucleus in nup49–313 cells that were grown at the permissive temperature (Fig. 2, A and B). However, when the nup49–313 cells were shifted to the nonpermissive temperature, Cbp80p-GFP is seen accumulating in the cytoplasm (Fig. 2, C and D), indicating that Cbp80p-GFP is able to exit the nucleus. Immunoblotting of cell lysates made after the temperature shift confirm that there is no degradation of Cbp80p-GFP (data not shown). Therefore, the cytoplasmic signal is due to the intact Cbp80p-GFP.

To test if the ability of Cbp80p-GFP to exit the nucleus is dependent on RNA polymerase II transcription, we performed the nuclear export assay in a strain carrying both the nup49–313 and rpb1–1 mutant alleles (1). At the nonpermissive temperature, Cbp80p-GFP is no longer able to exit the nucleus, suggesting that active RNA polymerase II transcription is required (Fig. 2, G and H).

CBC Interacts with NPL3 Genetically—We have previously reported that a strain containing a null allele of CBP80 (cbp80Δ strain) is synthetically lethal with a strain containing a null allele of HMT1 (35). In addition, a strain containing a null allele of HMT1 is synthetically lethal with the temperaturesensitive (ts) allele of NPL3, npl3–1 (28). To see if CBP80 also interacted genetically with NPL3, we crossed the cbp80Δ strain to the npl3–1 strain. Tetrad analysis revealed that progeny containing both npl3–1 and cbp80Δ were unable to germinate, indicating a synthetic lethal relationship.

To further test synthetic lethality of the null allele of CBP80 with other ts mutants of NPL3, a strain was constructed that contained null alleles of both CBP80 and NPL3 as well as a plasmid carrying a wild type copy of NPL3 and the URA3 gene. This strain was transformed with a series of plasmids that carry a ts allele of NPL3 and the LEU2 gene (1). Transformants were then streaked on plates lacking leucine and containing 5-FOA, a drug that selects against the presence of the URA3 gene. Therefore, if the null allele of CBP80 and the temperature-sensitive allele of interest are synthetically lethal, the transformants will not be able to grow on a leu– 5-FOA plate due to the lack of a wild type copy of NPL3. For instance, when the double mutant strain is transformed with a LEU2 plasmid carrying the npl3–1 allele, it is unable to survive on a leu– 5-FOA plate (Fig. 3A). Similarly, the double mutant strain transformed with the LEU2 plasmid is unable to survive on a leu– 5-FOA plate, whereas the strain is able to survive when it carries a LEU2 plasmid expressing wild type Npl3p. The latter strain gives rise to heterogeneous colonies. Analysis of this strain confirmed that both the large and small sized colonies contain both the null alleles of NPL3 and CBP80 (data not shown). The results from the leu– 5-FOA plate tests are summarized in Table II.

To further characterize the genetic interaction between NPL3 and the CBC, we decided to test whether the npl3 alleles that were also synthetic lethal with cbp80Δ were also synthetic lethal with a deletion of CBP20. Therefore, we also constructed strains containing the null allele of CBP20 with the NPL3 null allele as well as the URA3 plasmid carrying NPL3. These strains were also transformed with plasmids carrying various ts alleles of NPL3. Results of the genetic analyses are summarized in Table II.

Although the mutations in NPL3 lie throughout the gene (Fig. 3B), the majority are found in the two RRMs. Several of the temperature-sensitive alleles result in a change in more than one amino acid. Therefore, in these alleles, it is not known which mutation causes the temperature sensitivity. For all mutants except npl3–27, the mutated Npl3p is located in the nucleus (1, 33),2 and all mutants have some degree of defect in mRNA export (1). While no clear relationship between the

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2 E. C. Shen, T. Stage-Zimmerman, P. Chui, and P. A. Silver, unpublished observations.
location of the mutation in NPL3 and synthetic lethality with the null allele of CBP80, or CBP20 arose, several observations can be made. First, a mutation in a residue of NPL3 that is conserved in both RRM s and SR proteins is either dead in combination with both null alleles (e.g. npl3–38), or alive in combination with all null alleles (e.g. npl3–48). Second, there are no npl3 alleles with mutations in the RGG box that result in synthetic lethality in combination with either of the null alleles. Finally, if the mutation in Npl3p is in an unconserved residue that is next to other unconserved residues, as is the case for npl3–15 and npl3–26, the temperature-sensitive allele is not synthetically lethal with any of the null alleles.

FIG. 3. A, the null allele of CBP80 and npl3–1 are synthetically lethal. The cbp80Δ npl3Δ strain carrying NPL3 on a URA3 plasmid was transformed with the LEU2 vector alone or with the vector carrying wild type NPL3 or the npl3–1 allele. The transformants were then streaked on a leu− 5-FOA plate and assessed for ability to grow. B, schematic diagram of npl3 temperature-sensitive mutations. The protein sequence of NPL3 consists of an N terminus that contains repeats of the amino acid sequence APQE, two RRMs, and a C-terminal RGG box. The ts mutations above the diagram of Npl3p show no synthetic lethality with cbp80Δ and cbp20Δ. The ts mutations below the diagram of Npl3p show synthetic lethality with both null alleles.

Table II
Genetic interactions between NPL3, CBP80, and CBP20

| Allele  | Mutation | Domain   | Growth on leu− 5-FOA |
|---------|----------|----------|----------------------|
| npl3–1  | A254V    | RRM2     | cbp80Δ               |
| npl3–3  | S193P    | RRM1     | cbp80Δ               |
| npl3–15 | F237L    | RRM2     | cbp80Δ               |
| npl3–16 | C211R    | RRM2     | cbp80Δ               |
| npl3–17 | F160L    | RRM1     | cbp80Δ               |
| npl3–21 | I268T    | RRM2     | cbp80Δ               |
| npl3–26 | N223D    | RRM2     | cbp20Δ               |
| npl3–27 | E409K    | RGG      | cbp20Δ               |
| npl3–31 | I268F    | RGG      | cbp20Δ               |
| npl3–32 | V237C    | RGG      | cbp20Δ               |
| npl3–37 | Q91P     | N-terminal| cbp20Δ               |
| npl3–38 | P131L    | RRM1     | cbp20Δ               |
| npl3–41 | W213R    | RRM2     | cbp20Δ               |
| npl3–43 | P64T     | N-terminal| cbp20Δ               |
| npl3–44 | V191A    | RRM1     | cbp20Δ               |
| npl3–45 | L216P    | RRM2     | cbp20Δ               |
| npl3–46 | G241D    | RRM2     | cbp20Δ               |
| npl3–47 | E121G    | RRM1     | cbp20Δ               |
| npl3–48 | F183I    | RRM1     | cbp20Δ               |
| npl3–49 | S193P    | RRM1     | cbp20Δ               |
| npl3–52 | Y83H     | N-terminal| cbp20Δ               |
| npl3–52 | V232D    | RRM2     | cbp20Δ               |
containing a null allele of NPL3 and a plasmid expressing untagged Npl3p (Fig. 4A, lane 1). Cbp80p was not observed in this sample. Therefore, Cbp80p interacts with Npl3p-Myc in vivo.

Because Cbp80p and Cbp20p form a tight complex, the immunoprecipitations were repeated to check the presence of Cbp20p. As above, cell lysates containing Npl3p-Myc (Fig. 4B, left panel, lanes 2–4 and 6) or a control plasmid (Fig. 4B, left panel, lanes 1 and 5) were subjected to immunoprecipitation with anti-Myc antibody. The resulting immunoprecipitates were probed with anti-Myc antibody to confirm the presence of Cbp80p and Cbp20p in the samples. As controls, lysates from npl3Δ and cbp20Δ cells carrying a plasmid expressing untagged Npl3p were also used (both panels, lanes 1, 5, 6, and 10). The asterisk denotes the location of the antibody light chain, while the carat denotes the location of the antibody heavy chain.

Expression of Cbp20p is highly decreased in the strain containing the null allele of CBP80 (Fig. 4B, right panel, lanes 3 and 17) such that we cannot determine if Cbp80p is required for the interaction between Cbp20p and Npl3p-Myc. In contrast, Cbp80p is present in cell lysate from cbp20Δ cells (Fig. 4B, right panel, lanes 4 and 5). However, it is not detected in the sample from the immunoprecipitation using this lysate (Fig. 4B, right panel, lane 9), indicating that Cbp20p is required for the interaction between Cbp80p and Npl3p.

The Interaction between CBC and Npl3p Is Dependent on RNA—To test if the interaction between the cap-binding complex and Npl3p is RNA-dependent, we treated the lysate with RNase at room temperature before performing the immunoprecipitation. The amount of Npl3p-Myc immunoprecipitated remained the same in comparison with lysate that received no RNase treatment (Fig. 5A, compare lanes 2 and 3). However, the amount of Cbp80p and Cbp20p co-precipitated with Npl3p-Myc dramatically diminished when the lysates were treated with RNase (Fig. 5B, compare lanes 1 and 2). To rule out the possibility that Cbp80p and Cbp20p were degraded during RNase treatment, lysates were immunoblotted for Myc was dramatically diminished when the lysates were treated with RNase (Fig. 5B, compare lanes 1 and 2). To rule out the possibility that Cbp80p and Cbp20p were degraded during RNase treatment, lysates were immunoblotted for

FIG. 4. Interaction between the CBC and Npl3p-Myc. A, anti-Myc beads were used to immunoprecipitate complexes containing Npl3p-Myc from yeast lysates from wild type, npl3Δ, and cbp80Δ cells carrying a plasmid expressing Npl3p-Myc (lanes 3 and 4). Samples were separated on a 12% gel by SDS-polyacrylamide gel electrophoresis and analyzed by silver staining. As a control, lysate from npl3Δ cells carrying a plasmid expressing untagged Npl3p was also used (lanes 1 and 2). B, anti-Myc beads were used to precipitate complexes from lysates of npl3Δ, cbp80Δ, and cbp20Δ cells carrying a plasmid expressing Npl3p-Myc (both panels, lanes 7–9). These samples, along with the corresponding lysates (both panels, lanes 2–4), were separated on a 12% gel by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose. The left panel shows a blot probed with anti-Myc antibodies. The right panel shows a blot with identical samples. The upper portion was probed with anti-Cbp80p antibodies, and the bottom half was probed with anti-Cbp20p antibodies. As controls, lysates from npl3Δ and cbp20Δ cells carrying a plasmid expressing untagged Npl3p were also used (both panels, lanes 1, 5, 6, and 10). The asterisk denotes the location of the antibody heavy chain, while the carat denotes the location of the antibody light chain.

FIG. 5. Dependence of the CBC and Npl3p-Myc interaction on RNA. A, immunoprecipitations with anti-Myc beads were performed from lysates of npl3Δ cells carrying a plasmid expressing Npl3p-Myc that were either untreated (lane 2) or treated with RNase (lane 3). Untreated lysate (lane 1) was separated along with samples of the immunoprecipitations on a 12% gel, transferred to nitrocellulose membrane, and blotted with anti-Myc antibodies. B, a blot of the identical immunoprecipitation samples in A was cut in half, and the upper portion was probed with anti-Cbp80p antibodies while the bottom half was probed with anti-Cbp20p antibodies. C, a blot of equal amounts of untreated and RNase-treated lysates from npl3Δ (lanes 1 and 2) expressing Npl3p-Myc was cut in half as in B. Once again, the upper half was probed with anti-Cbp80p antibodies, and the bottom half was probed with anti-Cbp20p antibodies.
Cbp80p and Cbp20p. Both proteins were present at the same level before and after treatment (Fig. 5c, lanes 1 and 2). In conclusion, the lower levels of Cbp80p and Cbp20p observed in immunoprecipitations using RNase-treated lysates appear to be due to a weakened ability of these proteins to bind Npl3p-Myc, suggesting that the interactions are mediated at least in part by RNA. All attempts to reconstitute a direct interaction between Npl3p and CBC with recombinant proteins have thus far been negative, further suggesting a need for RNA to support the interaction.

**DISCUSSION**

In this report, we have demonstrated a genetic and biochemical interaction between one of the major yeast mRNA-binding proteins, Npl3p, and the proteins of the cap-binding complex. Mutations in **NPL3** show allele-specific synthetic lethal relationships with both **CBP80** and **CBP20**. Moreover, a complex containing Npl3p, Cbp80p, and Cbp20p can be isolated from yeast and is dependent on the presence of RNA.

Deletion of either component of the cap-binding complex, **CBP80** or **CBP20**, is dead when combined with certain temperature-sensitive **npl3** mutations. Moreover, **CBP80** and **CBP20** show the same allele-specific synthetic lethality with the **npl3** ts mutants, supporting the idea that Cbp80p and Cbp20p work as a complex with relation to Npl3p. Taken together, these genetic data support the idea that the CBC works with Npl3p to ensure proper RNA metabolism.

The genetic interaction between the genes that encode the CBC and **NPL3** is further supported by our finding that a complex containing both the CBC and Npl3p can be isolated from yeast. This complex requires Cbp20p because binding of Cbp80p to Npl3p is not detected in a strain lacking **CBP20**. It is possible that Cbp80p interacts with Npl3p only after Cbp20p binds the capped RNA. However, it appears that neither Cbp80p nor Cbp20p is able to bind capped RNA alone (10). Perhaps the interaction between the CBC and Npl3p can take place only after the CBC has bound the capped RNA. We could not determine if Cbp80p is necessary for the binding of Cbp20p to Npl3p, because expression of Cbp20p is barely detectable in a strain lacking **CBP80** (17). We have found that the interaction between the CBC and Npl3p is sensitive to RNase treatment, supporting the idea that capped RNA may also need to be present for the interaction. A direct interaction between the CBC and Npl3p has not yet been found.

In addition to binding the cap structure of mRNAs, the cap-binding complex is also known to bind the cap structure of snRNAs. Interestingly, Npl3p has also been associated with snRNAs in that it has been found as a potential component of the yeast U1 snRNP (42). In the same study, it was shown that the association of Npl3p with the U1 snRNP is weak, salt-sensitive, and only moderately specific. Similarly, Mattaj and colleagues (43) have found that Npl3p and Cbp80p can be co-immunoprecipitated with the U1 snRNP protein Luc7. These interactions with Luc7 have also been shown to be weak and easily dissociated. However, the formal possibility still exists that interaction between the CBC and Npl3p exists in the context of snRNAs and not mRNAs.

We found that, like Npl3p and other mRNA-binding proteins, Cbp80p shuttles between the nucleus and the cytoplasm. This is consistent with the observation that Cbp20p is bound to the Balbiani ring RNP as it traverses across the nuclear pore (13). We also report that, like Npl3p, export of Cbp80p depends on ongoing mRNA synthesis. This result suggests that Cbp80p may need to be bound to mRNA in order to be exported. It has been previously shown that Cbp20p binds to nascent transcripts before they are released from the chromosome (13). Because of the interdependence shown between **CBP20** and **CBP80**, Cbp80p is also most likely bound at this time. Capping itself has been shown to occur co-transcriptionally (5). This occurs through direct interactions between the capping machinery and the phosphorylated C-terminal domain of RNA polymerase II (44, 45). Perhaps Cbp80p export depends on RNA polymerase II because binding of the CBC to capped RNA is also occurring co-transcriptionally.

Taken together, we propose that these data lend further support to a model where mRNAs are packaged co-transcriptionally by proteins such as Npl3p and Cbp80/20p. It is possible that the binding of the CBC to the cap structure of mRNAs as they are being synthesized promotes the binding of other mRNA-binding proteins such as Npl3p (Fig. 6). These and other proteins then leave the nucleus together with the mRNA and are released in the cytoplasm, where they rapidly return to the nucleus for another round of packaging and export.

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