Messenger RNAs are recruited for nuclear export during transcription

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Following transcription and processing, eukaryotic mRNAs are exported from the nucleus to the cytoplasm for translation. Here we present evidence that mRNAs are targeted for nuclear export cotranscriptionally. Combined mutations in the *Saccharomyces cerevisiae* hnRNP Npl3 and TATA-binding protein (TBP) block mRNA export, implying that cotranscriptional recruitment of Npl3 is required for efficient export of mRNA. Furthermore, Npl3 can be found in a complex with RNA Pol II, indicating that Npl3 associates with the transcription machinery. Finally, Npl3 is recruited to genes in a transcription dependent manner as determined by chromatin immunoprecipitation. Another mRNA export factor, Yra1, also associates with chromatin cotranscriptionally but appears to be recruited at a later step. Taken together, our results suggest that export factors are recruited to the sites of transcription to promote efficient mRNA export.

[*Key Words*: Transcription; mRNA export; chromatin IP; RNA polymerase II; Npl3; Yra1]

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Compartmentalization of the eukaryotic cell necessitates the movement of macromolecules through the nuclear envelope. In particular, mRNAs must be transported from the site of transcription in the nucleus to the cytoplasm for translation to occur. Export of mRNAs requires processing, packaging by RNA-binding proteins, recognition by export factors, and translocation through the nuclear pore complex (NPC) into the cytoplasm.

mRNAs transcribed by RNA Pol II undergo rapid processing in the nucleus. Pre-mRNA processing steps include addition of a 5′ monomethyl cap, splicing of introns, and 3′ cleavage and polyadenylation. 5′ capping occurs on nascent transcripts soon after transcription initiation (Salditt-Georgieff et al. 1980; Jove and Manley 1984; Rasmussen and Lis 1993). Furthermore, capping enzyme is associated with Pol II and can be found associated with promoters of transcribed genes (Cho et al. 1997; McCracken et al. 1997; Komarnitsky et al. 2000; Schroeder et al. 2000). Additionally, the transcription machinery interacts with splicing and 3′ cleavage and polyadenylation factors, suggesting that these events occur either cotranscriptionally or soon after transcription termination (Mortillaro et al. 1996; Yuryev et al. 1996; Dantonel et al. 1997). Coordination of mRNA processing events and transcription is thought to be primarily through Pol II and its carboxy-terminal domain (CTD) in a manner that can be dependent on the state of CTD phosphorylation (for review, see Hirose and Manley 2000).

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In the nucleus, hnRNPs and other RNA-binding proteins package mRNAs into ribonucleoprotein particles (RNPs), and a subset of these proteins remain in the nucleus whereas others accompany the RNP into the cytoplasm, dissociate, and move back into the nucleus for further rounds of export. In the visually tractable *Chironomus tentans*, it is possible to study the very large (35–40 kb) Balbiani ring (BR) RNA throughout its various maturation stages, including transcription, nuclear export, and translation. Previous immunoelectron microscopy studies showed that the hnRNPs hrp36, hrp45, and hrp23 associate with the BR particle during its transcription (Alzhanova-Ericsson et al. 1996; Visa et al. 1996a; Sun et al. 1998). hrp45 and hrp23 dissociate from the BR particle at the nuclear pore whereas hrp36 can accompany the mRNP into the cytoplasm and remain associated during translation. Furthermore, CBP20, which together with CBP80 forms the mRNA cap-binding complex, was also shown to associate with the nascent BR particle (Visa et al. 1996b).

Recent studies have suggested that mechanisms exist to mark mRNAs as fully processed and export competent. In metazoans, splicing can enhance mRNA export (Luo and Reed 1999). The splicing machinery deposits a complex of proteins that mark exon–exon boundaries, and two members of this complex, Aly/REF and Y14, have been suggested to act as markers of spliced and export-competent RNPs (Kataoka et al. 2000; Le Hir et al. 2000; Zhou et al. 2000). Although it remains to be seen whether these proteins directly mediate the export process, both Aly and Y14 shuttle between the nucleus and cytoplasm, and the yeast homolog of Aly, Yra1, is essential for mRNA export (Sträßer and Hurt 2000; Zhou et al. 2000).

Recognition of the RNP for export may be mediated by a number of factors. A strong candidate for a metazoan mRNA export receptor is TAP, which binds and exports the constitutive transport element (CTE)-containing RNA produced by simian type D retroviruses (Gruter et al. 1998; Kang and Cullen 1999). Its yeast homolog, Mex67, along with the small protein Mtr2, is essential for mRNA export (Kadowaki et al. 1994; Santos-Rosa et al. 1998). Both TAP and Mex67 bind mRNA and contact components of the NPC (Katahira et al. 1999; Bachi et al. 2000; Sträßer et al. 2000).

Translocation of RNPs through the NPC undoubtedly requires the action of multiple nucleoporins as various mutations in nucleoporin genes result in blocked mRNA export (for review, see Fabre and Hurt 1997). The actual translocation process may be aided by the action of the ATP-dependent RNA helicase Dbp5/Rat8 and associated protein Gle1, which localize to the cytoplasmic side of the NPC and specifically contact Nup159 (Snay-Hodge et al. 1998; Hodge et al. 1999; Strahm et al. 1999). It has long been postulated and only recently shown that a helicase is able to remodel an RNA-protein complex, and this activity may serve as the mechanism for unwinding and translocating RNPs as well as releasing nonshuttling proteins from the mRNA at the NPC (Jankowsky et al. 2001). In summary, multiple factors are implicated in the translocation step of mRNA export.

Npl3 (also termed Mtr13/Mts1/Nab1/Nop3), a shuttling hnRNP that contains two RNA-recognition motifs (RRMs) and an RS-like domain, is a major mRNA-binding protein in *S. cerevisiae* (Wilson et al. 1994). Mutations in Npl3 cause nuclear accumulation of mRNA, indicating a role for Npl3 in the mRNA export process (Singleton et al. 1995; Lee et al. 1996). It has been postulated that along with other proteins, Npl3 packages pre-mRNA into an export-competent RNP and escorts it through the NPC (Lee et al. 1996; Shen et al. 1998). On arrival in the cytoplasm, Npl3 dissociates from mRNA and is transported back into the nucleus by the importin Mtr10 to allow further rounds of mRNA export (Pemberton et al. 1997; Senger et al. 1998). These characteristics of Npl3 make it an attractive paradigm for the function of hnRNPs with respect to nuclear export of mRNA.

In an effort to study Npl3 and mRNA export, we found that combined mutations in Npl3 and TATA-binding protein (TBP) block mRNA export. Moreover, we show that Npl3 is found in a complex with RNA Pol II and is associated with genes in a transcription dependent manner. Furthermore, the mRNA export factor Yra1 also associates with transcribed chromatin but is recruited at a later step than Npl3. These results suggest that cotranscriptional recruitment of mRNA export factors may be critical for proper mRNA export.

Results

A screen for Npl3 export factors identifies SPT15, *which encodes TATA-binding protein*

The export of Npl3 is intimately tied to that of mRNA; therefore, we postulated that studying Npl3 export would yield information about general mRNA export. To identify factors important for Npl3 and mRNA nuclear export, a mutant *npl3-27* was employed. Npl3-27 contains a single point mutation (E409K), and cells bearing this mutation are viable at all temperatures (Lee et al. 1996). However, the cellular distribution of Npl3-27 is altered with respect to wild-type Npl3. At steady state, wild-type Npl3 localizes predominantly to the nucleus (Fig. 1A, panels a–c) whereas the Npl3-27 mutant localizes to both the nucleus and cytoplasm at 37°C (Fig. 1A, panels d–f) as detected by indirect immunofluorescence using polyclonal α-Npl3 antibodies (Bossie et al. 1992; Krebber et al. 1999). Npl3-27 appears to have a slowed rate of import because overexpression of the Npl3 import receptor gene, *MTR10*, results in restoration of Npl3-27 nuclear localization (Krebber et al. 1999). The E409K mutation maps to the region that is important for Npl3 nuclear localization, further supporting this explanation (Flach et al. 1994). It has been shown previously that mutations that cause mRNA export defects also cause nuclear accumulation of Npl3-27, indicating that Npl3-27 export is subject to the same requirements as mRNA export (Krebber et al. 1999). As seen by in situ hybridization using an oligo $(dT)_{50}$ probe, wild-type (Fig. 1B, panels a–c) and *npl3-27* cells (Fig. 1B, panels d–f) display the same distribution of mRNA throughout the nucleus and cytoplasm (Amberg et al. 1992; Krebber et al. 1999). Therefore, Npl3-27 is functional for mRNA export despite its altered cellular localization. Furthermore, as has been shown with similar mutant forms of Npl3, Npl3-27 is capable of binding mRNA in vivo at 25°C and 37°C as determined by UV-crosslinking (data not shown; Gilbert et al. 2001). The cellular level of Npl3-27 is equivalent to that of wild type at both 25°C and 37°C (Krebber et al. 1999).

Taking advantage of the altered localization but wild-type activity of Npl3-27, we performed a genetic screen to identify genes required for Npl3-27 export. A temperature-sensitive (*ts*$^-$) library from the *npl3-27* strain was created by EMS mutagenesis and was screened for nuclear accumulation of Npl3-27. An intergenic mutation (F183I) in an RRM domain of *NPL3* was obtained that also causes an mRNA export defect. Moreover, a mutation in the mRNA export factor *MTR2* was also found (data not shown). Identification of these two mutations verified that this screen is capable of identifying genes that affect Npl3 and mRNA export. This screen also identified a mutation in *SPT15*, which encodes the transcription initiation factor TBP. Sequencing of this mutant revealed that the mutation is identical to a previously reported *ts*$^-$ *spt15* mutant termed *spt15-ts1* (Cormack and Struhl 1992). This mutant displays a nuclear accumulation of Npl3-27 at the nonpermissive temperature of 37°C indicating a block of Npl3-27 export (Fig. 1A, panels g–i). Levels of Npl3-27 are not affected (data not shown).

NPL3 *and* SPT15 *genetically interact to promote mRNA export*

Unexpectedly, in addition to nuclear accumulation of Npl3-27, *spt15-ts1 npl3-27* cells display an mRNA ex-

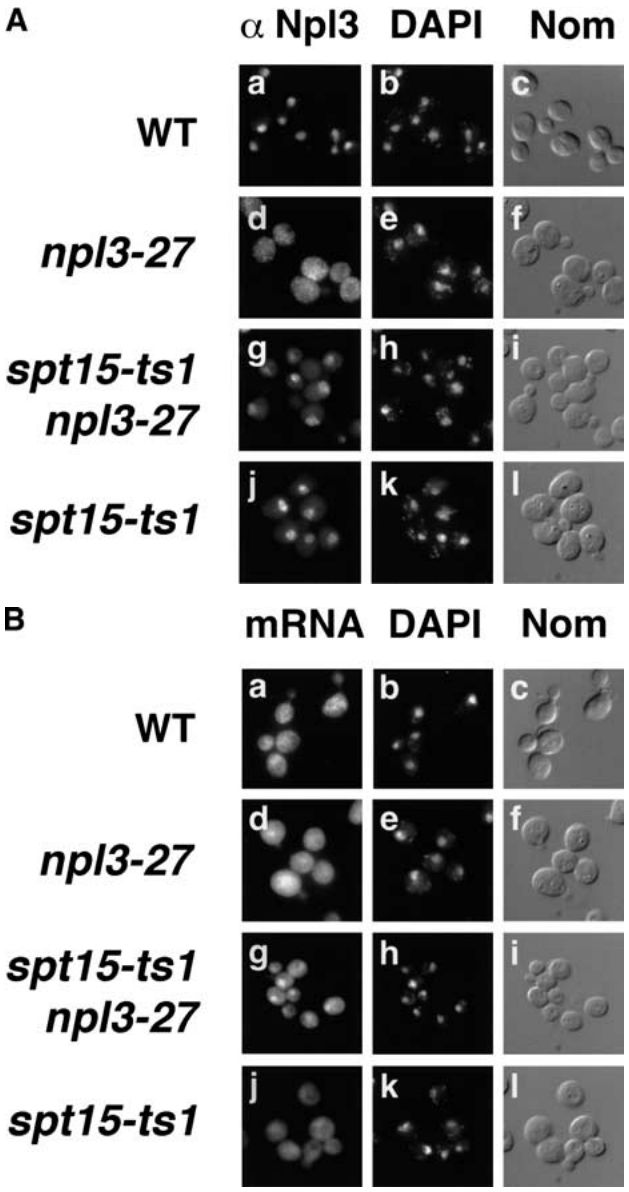

Figure 1. *spt15-ts1 npl3-27* cells are slowed for Npl3-27 export and display a synthetic mRNA export defect. (*A*) Localization of Npl3 or Npl3-27 in wild type (PSY580, *a–c*), *npl3-27* (PSY1031, *d–f*), *spt15-ts1 npl3-27* (PSY1698, *g–i*) and *spt15-ts1* cells (PSY1702, *j–l*). Cells were shifted to 37°C for 1 h. Indirect immunofluorescence with polyclonal antibodies to Npl3 (*left*), DAPI (*center*), and Nomarski images (*right*) are shown. (*B*) Localization of poly(A)$^+$ RNA in wild type (*a–c*), *npl3-27* (*d–f*), *spt15-ts1 npl3-27* (*g–i*), and *spt15-ts1* cells (*j–l*). Cells were shifted to 37°C for 1 h. in situ hybridization with an oligo $(dT)_{50}$ probe (*left*), DAPI (*center*), and Nomarski images (*right*) are shown.

port defect. Poly(A)$^+$ RNA accumulates in the nucleus of *spt15-ts1 npl3-27* cells shifted to 37°C for 1 h (Fig. 1B, panels g–i). Neither the single *spt15-ts1* mutation (Fig. 1B, panels j–l) nor the *npl3-27* mutation (Fig.1B, panels d–f) in isolation cause nuclear accumulation of mRNA. Consistent with a defect in transcription at 37°C, *spt15-ts1* cells display a lower intensity poly(A)$^+$ RNA signal distributed throughout the cells relative to wild type (Fig. 1B, panels j–l). Double mutant *spt15-ts1 npl3-27* cells (Fig. 1B, panels g–i) display a nuclear RNA signal that is of considerably higher intensity than that of *spt15-ts1* cells. One explanation is that blocked export and subsequent concentration of mRNA in the nucleus causes a brighter signal. Another possibility is that *npl3-27* decreases the rate of mRNA degradation in the nucleus, resulting in an increase of the overall poly(A)$^+$ RNA signal and an apparent accumulation in the nucleus.

To verify that *spt15-ts1 npl3-27* cells exhibit a true decrease in mRNA export and not a defect in mRNA stability, we harvested total RNA from wild-type, *spt15-ts1*, *spt15-ts1 npl3-27*, and *npl3-27* cells and determined the amount of steady state poly(A)$^+$ RNA in strains grown at 25°C or shifted to 37°C for 1 h. The amount of poly(A)$^+$ RNA was determined by hybridization to a radiolabeled poly dT probe. Levels of 18S rRNA were verified to be constant among samples (data not shown). Single *spt15-ts1* mutant cells (Fig. 2A, lanes 5,6) and double *spt15-ts1 npl3-27* cells (Fig. 2A, lanes 3,4) showed an identical decrease in poly(A)$^+$ RNA after the shift to 37°C. The poly(A)$^+$ RNA level in single *npl3-27* mutant cells (Fig. 2A, lanes 7,8) matched that of wild type (Fig 2A, lanes 1,2), indicating that *npl3-27* has no effect on total mRNA levels. To rule out the effects of poly(A) tail length, Northern analysis was performed on the *ACT1* transcript, and these results mirrored those of the poly(A)$^+$ RNA slot blot. Wild-type *ACT1* levels (Fig. 2B, lanes 1,2) matched those of *npl3-27* cells (Fig. 2B, lanes 7,8), and *spt15-ts1 npl3-27 ACT1* levels (Fig. 2B, lanes

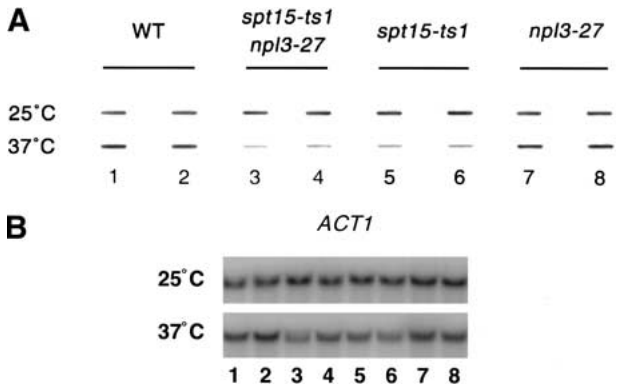

Figure 2. *npl3-27* has no effect on total mRNA levels. (*A*) Slot blot hybridization of total poly(A)$^+$ RNA of wild type (lanes *1–2*, PSY580 and 603), *spt15-ts1 npl3-27* (lanes *3–4*, PSY1698 and 1699), *spt15-ts1* (lanes *5–6*, PSY1702 and 1703), and *npl3-27* cells (lanes *7–8*, PSY1031 and 1032). Cells were grown to log phase at 25°C, cultures were split, one half was shifted to 37°C for 1 h, and total RNA was isolated. Two μg of total RNA was probed with a ^{32}P-labeled poly dT probe. (*B*) *ACT1* Northern of wild type (lanes *1–2*), *spt15-ts1 npl3-27* (lanes *3–4*), *spt15-ts1* (lanes *5–6*), and *npl3-27* cells (lanes *7–8*). Cells were grown to log phase at 25°C, cultures were split, one half was shifted to 37°C for 1 h, and total RNA was isolated. Fifteen μg of total RNA separated by agarose gel electrophoresis was probed with a ^{32}P-labeled *ACT1* probe.

3,4) matched those of *spt15-ts1* cells (Fig. 2B, lanes 5,6) after a shift to 37°C for 1 h. Differences in reduction of transcription levels seen in the *spt15-ts1* and *spt15-ts1 npl3-27* cells between the two assays are likely attributable to the higher stability of the *ACT1* transcript relative to total mRNA. Therefore, nuclear mRNA accumulation in *spt15-ts1 npl3-27* cells results from decreased mRNA export and not altered mRNA stabilization.

These results indicate that *NPL3* and *SPT15* genetically interact, ensuring proper mRNA export. Furthermore, *npl3-27* exerts a dominant effect on *spt15-ts1* cells and not wild-type cells in that expression of exogenous *npl3-27* over endogenous wild-type *NPL3* causes an mRNA export defect in *spt15-ts1* cells. We tested other *ts*⁻ alleles of *spt15* (*spt15-328* and *spt15-341*; Arndt et al. 1995) in combination with *npl3-27* and found that these alleles also displayed nuclear accumulation of Npl3-27 and mRNA. To determine the specificity of this genetic interaction, we examined strains mutated for RNA Pol II that decrease transcription (*rpb1-1*, *rpb1Δ101*, *rpb1Δ103*), but these strains do not display Npl3-27 or mRNA export defects (data not shown). Therefore, there is a specific genetic interaction between *SPT15* and *NPL3*, which suggests interplay between the mRNA transcription and nuclear export machinery.

Npl3 and RNA Pol II exist in a complex

The genetic interaction between *NPL3* and *SPT15* suggested that Npl3 and TBP or some component of the transcription machinery may interact physically. Therefore, we performed α-Npl3 immunoprecipitation experiments to isolate endogenous Npl3 and bound proteins from yeast cell extracts. We were unable to detect TBP in a complex with Npl3 (data not shown). However, we found that a small amount of RNA Pol II coimmunoprecipitates with α-Npl3 antibodies (Fig. 3, lane 2). Furthermore, we found that this association is not RNA-dependent as Pol II is still found associated with Npl3 after incubation with RNase A (Fig. 3, cf. lanes 2 and 3). These results indicate that Npl3 is associated with the transcription machinery and may be recruited to transcribing genes by RNA Pol II.

Npl3 is associated with the chromatin of genes in a transcription-dependent manner

To determine whether Npl3 is localized in the vicinity of transcribed genes, we tested whether Npl3 is recruited to mRNAs cotranscriptionally by the method of chromatin immunoprecipitation (Orlando et al. 1997). We chose to analyze the promoter and coding region of the constitutively and highly transcribed *PMA1* gene as well as a nontranscribed region devoid of open reading frames (ORFs) on a different chromosome. Chromatin of an average size of 200 bp was prepared from wild-type cells, and as a positive control, TBP was immunoprecipitated using polyclonal α-TBP antibodies. Similarly, Npl3 was immunoprecipitated using polyclonal α-Npl3 antibodies. The amount of DNA associated with each protein was determined by performing quantitative PCR using primer sets spanning the indicated regions (Fig. 4A). For illustrative purposes, PCR products from a single dilution of input and a single dilution of each immunoprecipitate that are known to be in the linear range of PCR are shown. In accordance with previous studies, α-TBP antibodies preferentially immunoprecipitate promoter DNA (Fig. 4B, center panel, lane 1) in comparison to DNA spanning the coding sequence (Fig. 4B, center panel, lanes 2,3) and intergenic region (Fig. 4B, center panel, lane 4; Komarnitsky et al. 2000; Kuras and Struhl 1999).

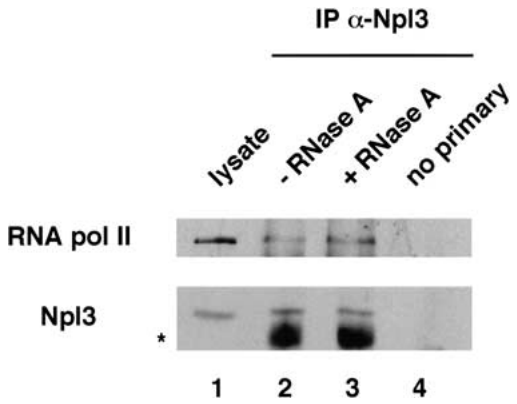

Figure 3. Npl3 and RNA Pol II form a complex that is not dependent on RNA. Npl3 from cell lysates is immunoprecipitated with α-Npl3 antibodies (*bottom*). Heavy chain of the primary antibody is denoted with an asterisk. RNA Pol II is coimmunoprecipitated with Npl3 (*top*). Total cell lysate is loaded (lane *1*). After binding and washing, samples were treated with 50 μg/mL RNase A in wash buffer (lane *3*) or wash buffer alone (lane *2*) and incubated at RT for 20 min. Samples were washed once and raised in sample loading buffer, run on a 7% SDS-PAGE gel and Western blotted with α-Npl3 antibodies or 8WG16 against Pol II CTD. No primary samples were mock IPs lacking primary antibody (lane *4*). Blots shown are from two different gels from the same experiment. Approximately 20% of Npl3 and 0.02% of Pol II were immunoprecipitated from the lysate.

Npl3 immunoprecipitates modest levels of the *PMA1* promoter region (Fig. 4B, right panel, lane 1) and high levels of ORF DNA (Fig. 4B, right panel, lanes 2,3) in comparison with the intergenic region (Fig. 4B, right panel, lane 4). For each primer set, percentages of input DNA present in the α-Npl3 immunoprecipitate were graphed with quantitation error for a single experiment (Fig. 4C). Values were normalized by dividing the percentage obtained for each primer set by the percentage of intergenic region (Fig. 4C, bottom). Approximately 1.5-fold more promoter DNA (Fig. 4C, bar 1) and 12- to 17-fold more coding sequence (Fig. 4C, bars 2,3) immunoprecipitate with α-Npl3 antibodies compared to the intergenic region (Fig. 4C, bar 4), indicating that Npl3 is associated with chromatin of a transcribing gene. Similar results were obtained using a monoclonal antibody to Npl3 (1E4) and α-myc antibodies to a myc epitope-tagged version of Npl3. Background signal (<0.047%) was ob-

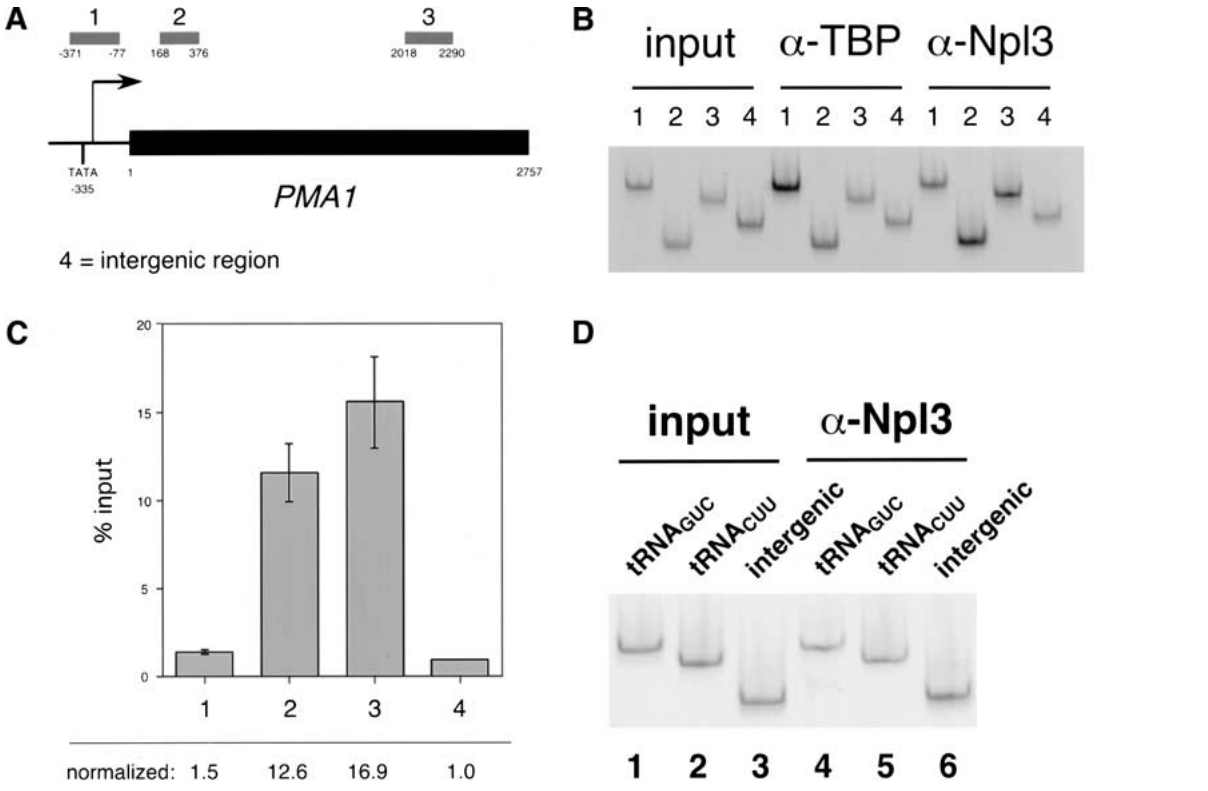

Figure 4. Npl3 crosslinks to the promoter and coding sequence of the constitutively expressed Pol II transcribed *PMA1* gene. (*A*) Diagram of *PMA1*. Primer set 1 spans the promoter including the TATA box. Primer set 2 spans the 5′ region of the coding sequence. Primer set 3 spans the 3′ region of the coding sequence. (ATG = +1). Primer set 4 spans a nontranscribed intergenic region. (*B*) Npl3 immunoprecipitates promoter and coding sequence of *PMA1*. Quantitative PCR of input (*left*), α-TBP immunoprecipitate (*center*) and α-Npl3 immunoprecipitate (*right*) using primers sets 1–4 spanning regions as indicated in *A*. PCR products were separated on an 8% TBE polyacrylamide gel. Single dilutions of each template are shown and are in the linear range of PCR (data not shown). (*C*) Quantitation of α-Npl3 immunoprecipitated material. Raw values are expressed in graphical form as percentage of input. Error bars >0.1 are shown for a single experiment. Normalized values were obtained by dividing the percentage obtained for each primer set by the percentage obtained of primer 4 (*bottom*). (*D*) Npl3 does not associate with Pol III transcribed genes. Quantitative PCR of input (lanes *1–3*) and Npl3 immunoprecipitate (lanes *4–6*) of DNA spanning the $tRNA_{GUC}$ gene (lanes *1,4*), $tRNA_{CUU}$ gene (lanes *2,5*), and intergenic region (lanes *3,6*).

tained with α-myc antibodies in a strain in which Npl3 was not tagged with myc (data not shown). To examine the specificity of Npl3 chromatin association with Pol II transcribed genes, we analyzed two Pol III transcribed tRNA genes (Fig. 4D). In comparison to input (Fig. 4D, lanes 1–3), Npl3 does not associate with regions containing the genes encoding $tRNA_{GUC}$ (Fig. 4D, lane 4) and $tRNA_{CUU}$ (Fig. 4D, lane 5) or the intergenic region (Fig. 4D, lane 6). These results show that Npl3 associates specifically with Pol II transcribed genes.

To determine if the association of Npl3 with genes is dependent on transcription, we analyzed the highly transcribed galactose inducible *GAL10* gene using primer sets spanning the promoter and coding sequence (Fig. 5A). Chromatin was prepared from cells grown in noninducing glucose-containing media as well as cells grown in inducing galactose-containing media. In cells grown in glucose, *GAL10* expression is repressed, and α-TBP antibodies immunoprecipitate background levels of DNA (Fig. 5B, center panel, lanes 1–5). With galactose induction, α-TBP antibodies immunoprecipitate a significant level of upstream activating sequence (UAS) DNA (Fig. 5B, center panel, lane 1) and DNA directly downstream of the TATA-box and 5′ coding sequence (Fig. 5B, center panel, lane 2) but not DNA well into the coding region of *GAL10* (Fig. 5B, center panel, lanes 3,4) or the intergenic region (Fig. 5B, center panel, lane 5).

α-Npl3 antibodies immunoprecipitate background levels of DNA from cells grown in glucose. Under transcription-inducing conditions, α-Npl3 antibodies immunoprecipitate a significant amount of *GAL10* UAS DNA (Fig. 5B, right panel, lane 1) and a much greater amount of DNA corresponding to coding sequence (Fig. 5B, right panel, lanes 2–4). For each primer set, percentages of input DNA present in the α-Npl3 immunoprecipitate after galactose induction (Fig. 5C, gray bars) and noninducing conditions (Fig. 5C, white bars), were graphed with quantitation error for a single experiment (Fig. 5C). Values were normalized by dividing the percentage obtained for each primer set by the percentage of intergenic region. The ratio of these normalized values of galactose to glucose are indicated (Fig. 5C, bottom). Approximately a

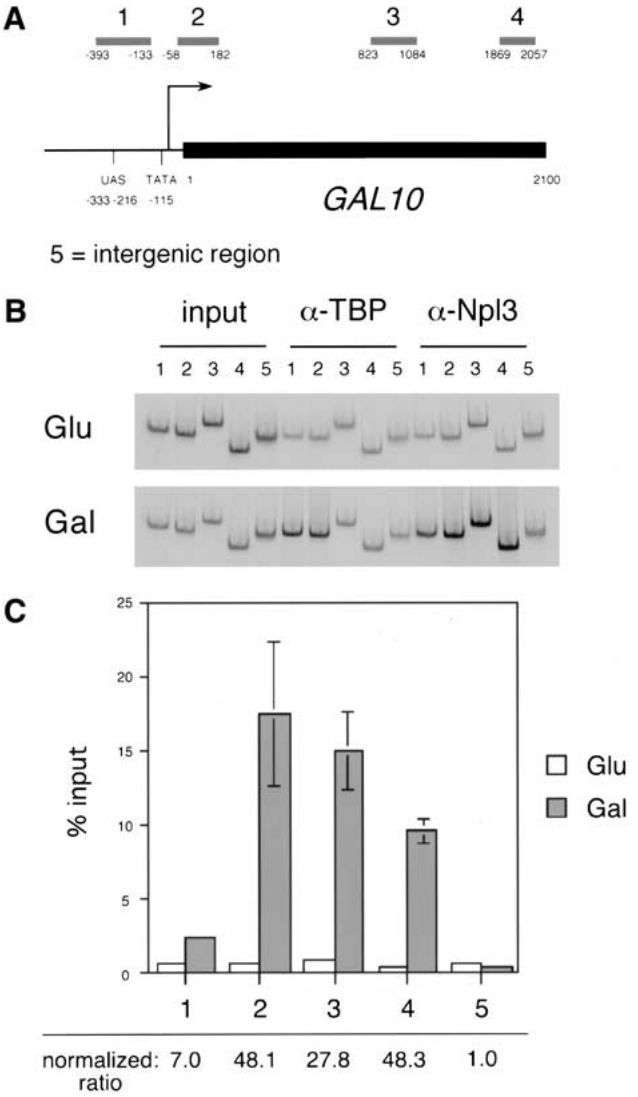

Figure 5. Npl3 crosslinks to the promoter and coding sequence of *GAL10* in a transcription dependent manner. (*A*) Diagram of *GAL10*. Primer set 1 spans the upstream activating sequence. Primer set 2 spans the promoter and 5′ coding sequence. Primer set 3 spans the middle of the coding sequence. Primer set 4 spans the 3′ coding sequence. Primer set 5 spans a nontranscribed intergenic region. (*B*) Npl3 immunoprecipitates promoter and coding sequence of *GAL10* under inducing conditions. Quantitative PCR of input (*left*), α-TBP immunoprecipitate (*center*), and α-Npl3 immunoprecipitate (*right*) using primers sets 1–5 spanning regions as indicated in *A*. Cells were grown in glucose (*top*) or galactose (*bottom*). (*C*) Quantitation of α-Npl3 immunoprecipitated material. Raw values are expressed for cells grown in glucose (white) and galactose (gray) in graphical form as a percentage of input. Error bars >0.3 are shown for one experiment. Normalized ratio values were obtained by dividing galactose values by glucose values for each primer set and normalizing to primer set 5.

7-fold increase of UAS DNA (Fig. 5C, bar 1) and 30- to 50-fold increase of ORF DNA (Fig. 5C, bars 2–4) was immunoprecipitated with α-Npl3 antibodies after galactose induction compared to noninducing conditions, indicating that Npl3 is associated with *GAL10* in a transcription dependent manner.

Yra1 associates with chromatin at a later step of transcription

To determine whether cotranscriptional recruitment of mRNA export factors is a general phenomenon, we examined whether Yra1 associates with genes by chromatin immunoprecipitation. Chromatin was prepared from cells containing a myc-epitope tagged version of Yra1 (K. Straesser and E.C. Hurt, unpubl.), and Yra1-myc was immunoprecipitated with α-myc antibodies. Quantitative PCR was performed for regions of *PMA1* as described in Figure 5A. In comparison to the intergenic reference primer (Fig. 6A, bar 4), Yra1-myc does not associate with *PMA1* promoter DNA (Fig. 6A, bar 1) but does so modestly with *PMA1* 5′ coding sequence (Fig. 6A, bar 2) and

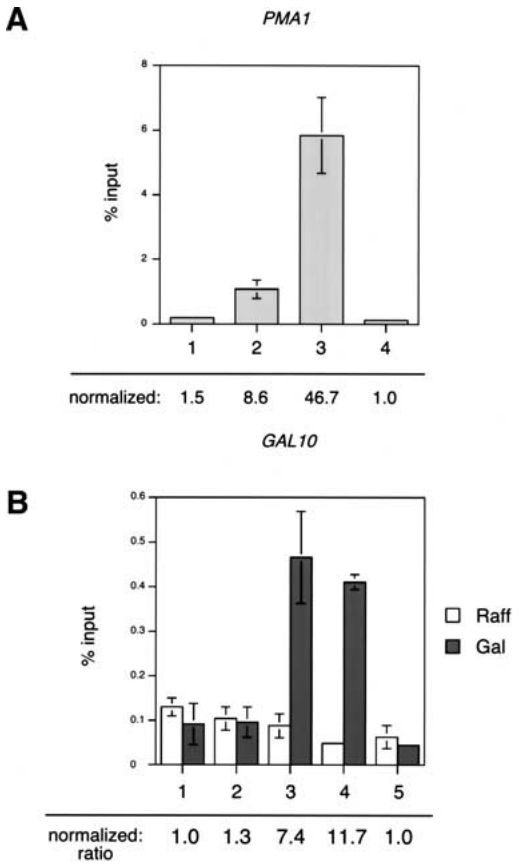

Figure 6. Yra1 is associated with the 3′ end of genes in a transcription dependent manner. (*A*) Quantitation of Yra1-myc association with the *PMA1* promoter (bar *1*), 5′ coding sequence (bar *2*), and 3′ coding sequence (bar *3*) and intergenic region (bar *4*). Values normalized to intergenic region are shown (*bottom*). (*B*) Quantitation of Yra1-myc association with the *GAL10* UAS (bars *1*), 5′ coding sequence (bars *2*), middle coding sequence (bars *3*), 3′ coding sequence (bars *4*), and intergenic region (bars *5*) in cells grown in raffinose (white) and galactose (gray). Normalized ratio values are shown (*bottom*).

strongly with 3′ coding sequence (Fig. 6A, bar 3). Values were normalized to the intergenic region, indicating an eightfold increase of 5′ coding sequence and a 40-fold increase of 3′ coding sequence (Fig. 6A, bottom). Therefore, Yra1 associates with the coding sequence of a transcribing gene. Furthermore, Yra1 association is biased toward the 3′ of *PMA1* in contrast to Npl3, which associates strongly at the 5′ end of *PMA1* (Fig. 4).

To assess transcriptional dependence of Yra1 association with genes, we examined the *GAL10* inducible gene (Fig. 5A) in chromatin prepared from cells grown in noninducing raffinose-containing media or inducing galactose-containing media. Under noninducing conditions (Fig. 6B, white bars), Yra1-myc did not immunoprecipitate a significant amount of *GAL10* sequence (Fig. 6B, bars 1–4) compared to the intergenic reference primer (Fig. 6B, bar 5). However, under inducing conditions (Fig. 6B, gray bars), Yra1-myc preferentially associated with the middle and 3′ regions of *GAL10* coding sequence (Fig. 6B, bars 3,4) and not UAS or 5′ sequences (Fig. 6B, bars 1,2). Normalized ratios were obtained by dividing percent input values obtained in galactose by values obtained in raffinose and show that there is a 10-fold induction of association with *GAL10* middle and 3′ coding sequence (Fig. 6B, bottom). These results indicate that Yra1 associates with genes in a transcription-dependent manner. Furthermore, Yra1 binds preferentially to the 3′ ends of both *PMA1* and *GAL10* in contrast to Npl3, which binds strongly to the 5′ ends and throughout the coding sequence of both genes.

Discussion

hnRNP proteins are proposed to play essential roles in pre mRNA processing and nuclear export. The highly conserved process of mRNA export is perhaps best understood in the yeast *S. cerevisiae* where many factors have been defined, one of which is the major mRNA-binding protein Npl3. In an effort to understand how Npl3 is involved in mRNA export, we designed a genetic screen to identify mutations that block Npl3 export, and we successfully identified known mRNA export factors. As a result of this screen, we also found that mutation of TBP could affect both Npl3 and mRNA export. Next, we showed that Npl3 exists in a complex with RNA Pol II and that Npl3 association with genes is transcription dependent. Finally, we have shown that another mRNA export factor, Yra1, is also cotranscriptionally recruited at a later step of transcription. Taken together, these results suggest that the process of mRNA nuclear export begins at the level of transcription.

SPT15 *and* NPL3 *interact to promote mRNA export*

The mRNA that is produced in *spt15-ts1 npl3-27* double mutant cells is not efficiently exported from the nucleus. One possibility is that reduced transcription levels give rise to this defect. In *spt15-ts1* cells, TBP is reduced in its ability to bind promoter DNA at the nonpermissive temperature, thereby compromising its role in transcription initiation and recruitment of the preinitiation complex to the promoter (Cormack and Struhl 1992). However, the reduced level of transcription cannot be the sole cause of the mRNA export defect because transcription levels are equally reduced in the *spt15-ts1* single mutant and *spt15-ts1 npl3-27* double mutant, and *npl3-27* alone does not affect mRNA levels. Furthermore, when *npl3-27* is combined with mutations in Pol II that reduce transcription levels, mRNA export is not affected. In addition, we found that Npl3-27 and mRNA export is also affected in two other *spt15 ts*$^-$ mutants. Therefore, there is a specific genetic interaction between *NPL3* and *SPT15* to promote mRNA export that may not be entirely dependent on transcription levels.

Another possibility is that in *spt15-ts1 npl3-27* cells, decreased activity of TBP combined with the smaller population of Npl3-27 in the nucleus causes an accumulation of export incompetent mRNAs. This hypothesis assumes that *npl3-27* is a loss-of-function mutation, and thus we would expect that expression of wild-type *NPL3* would rescue the mRNA export defect of *spt15-ts1 npl3-27* cells; however, we have found that this is not the case (E. Lei and P. Silver, unpubl.). Moreover, we have found that *npl3-27* exerts a dominant effect over wild-type *NPL3* in *spt15-ts1* cells to cause an mRNA export defect. Therefore, *npl3-27* does not appear to be a simple loss-of-function mutation, suggesting that an alteration of Npl3-27 function with respect to wild type is important for the synthetic mRNA export defect when combined with *spt15* mutations.

Although Npl3-27 clearly displays altered localization compared to wild type, the precise nature of the Npl3-27 mutant is unclear. The point mutation E309K lies in a region that contains the NLS. It has been proposed that Npl3 binds to mRNA in the nucleus, remains associated throughout export, and releases from the RNA during reimport (Pemberton et al. 1997). Although the assay is not quantitative, our UV-crosslinking studies consistently reveal that a larger population of Npl3-27 than wild-type Npl3 may exist bound to mRNA. Therefore, cytoplasmic accumulation of Npl3-27 may be tied to an inability to release RNA as efficiently as wild type. Changes in the behavior of Npl3-27 with respect to RNA binding could affect many aspects of mRNA export such as loading of Npl3-27 onto the nascent transcript, packaging of the RNP, and interaction of the RNP with transport factors. We were unable to test the behavior of Npl3-27 by chromatin immunoprecipitation because of the inability to immunoprecipitate the mutant protein (data not shown). Further studies of Npl3-27 function will shed light on the intriguing genetic interaction between *NPL3* and *SPT15*.

Recruitment of Npl3 to transcribing genes

Our results show that Npl3 is recruited to genes in a transcription-dependent manner. Promoter DNA found in the chromatin immunoprecipitations, albeit at lower levels than the coding region, suggest that the earliest step of Npl3 recruitment is at the site of transcription

initiation. Although the method of chromatin immunoprecipitation has limited resolution, we were able to see differences between the footprints of Npl3 and Yra1, which associates strongly with the 3′ ends of coding sequences, suggesting that Npl3 promoter association is significant. We predicted that there might be a physical interaction between Npl3 and some component of the transcription machinery. Although attempts to coimmunoprecipitate Npl3 with TBP and other transcription factors yielded negative results, we were able to detect an interaction of Npl3 with RNA Pol II, indicating that Npl3 physically interacts with the transcription machinery.

Pol II undergoes cycles of phosphorylation and dephosphorylation at its CTD, and this cycle coincides with Pol II activity (Hirose and Manley 2000). Pol II is hypophosphorylated while engaging in initiation and hyperphosphorylated during elongation. Furthermore, multiple elongation and mRNA processing factors are recruited by the Pol II CTD in a manner that is dependent on the state of CTD phosphorylation. We detected Pol II using an antibody that recognizes the unphosphorylated CTD (8WG16) and not with lower affinity antibodies directed to two different phosphorylation sites in the CTD (H14 and H5). Although elongating Pol II may be partially dephosphorylated in these experiments, these results are consistent with the possibility that Npl3 is recruited by unphosphorylated Pol II in the preinitiation complex and transferred to the nascent RNA at the start of elongation (Fig. 7). Alternately, Npl3 may be recruited to elongating polymerase soon after transcription initiation and then transferred to RNA. Moreover, Npl3 may be further recruited by cooperative self-association or by the RNA itself.

To address whether RNA may be necessary for Npl3 recruitment, we performed chromatin immunoprecipitation after extensive RNase A treatment and obtained similar results as described previously, indicating that Npl3 is not associated with chromatin entirely through RNA contacts (E. Lei and P. Silver, unpubl.). However, it should be noted that RNase A treatment cannot be performed before crosslinking; therefore, it is impossible to rule out that Npl3 association relies on contacts that are mediated by large RNA/protein complexes. Therefore, it remains a possibility that the growing nascent transcript further recruits Npl3. Future studies will determine how RNA, Pol II CTD phosphorylation, and other transcription factors affect Npl3 recruitment.

Another mRNA export factor, Yra1, is cotranscriptionally recruited

The mRNA export factors Aly/REF in metazoans and Yra1 in yeast have been proposed to mark fully processed mRNAs for nuclear export (Luo and Reed 1999; Sträßer and Hurt 2000). Although Aly/REF has been shown to preferentially bind to spliced and not unspliced RNAs, there is evidence that Aly/REF mediates the nuclear export mRNAs regardless of whether they once contained an intron (Luo and Reed 1999; Rodrigues et al. 2001). Our chromatin immunoprecipitation experiments, which provide a snapshot of the in vivo association of Yra1 with *PMA1* and *GAL10*, two genes that do not contain introns, are in agreement with Yra1 being a general export factor. Furthermore, Yra1 has been shown to interact directly with the mRNA export factor Mex67, which localizes primarily to nuclear pores, suggesting that Yra1 can function to bridge the formation of the RNP with the actual translocation machinery (Sträßer and Hurt 2000). If this is indeed the case, our results imply that full

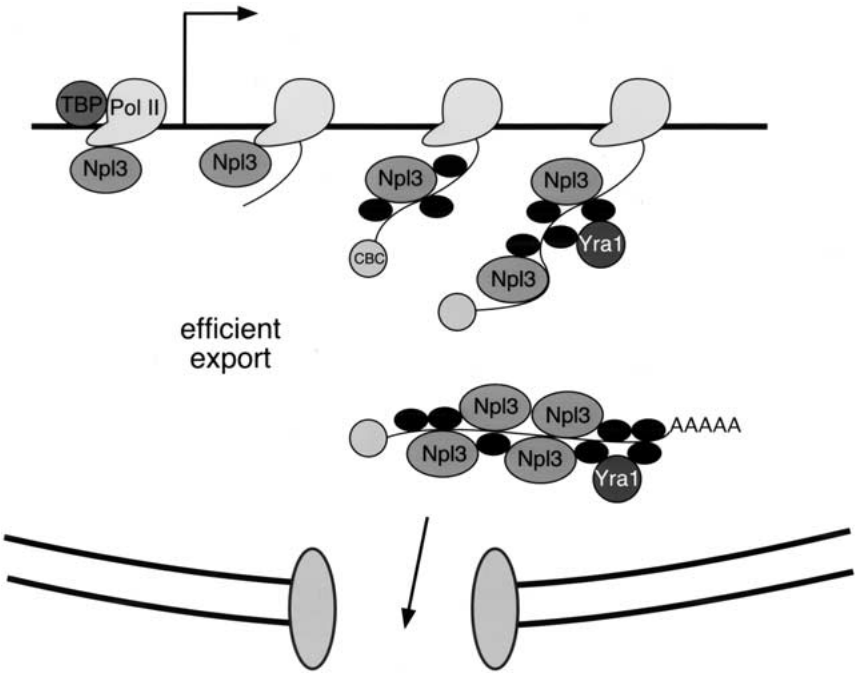

Figure 7. Model for cotranscriptional recruitment of mRNA export factors. Npl3 is recruited to the transcription machinery perhaps by Pol II in the preinitiation complex and/or elongating Pol II and then transferred to the nascent transcript. Further recruitment of Npl3 continues as elongation proceeds. CBC is also recruited to the nascent transcript possibly dependent on Npl3. Yra1 associates with the nascent RNA at a late step of transcription. Other RNA-binding proteins are indicated (black). Cotranscriptional recruitment of these factors promotes efficient export of the RNP.

maturation of the RNP can occur cotranscriptionally. Mounting evidence for cotranscriptional recruitment of multiple factors involved in pre-mRNA processing supports this hypothesis.

Transcription coordinates assembly of the export-competent RNP

NPL3 lies at the center of an increasingly complex network of genes. One such gene is *HRP1*, which encodes another abundant hnRNP (Henry et al. 1996). Hrp1 is involved in 3′ processing and nonsense mediated decay and recently has been shown to be associated with transcribed genes similar to the pattern exhibited by Npl3 (Kessler et al. 1997; Gonzalez et al. 2000; Komarnitsky et al. 2000). Like Npl3, Hrp1 shuttles between the nucleus and cytoplasm dependent on ongoing transcription (Shen et al. 1998). Furthermore, *NPL3* genetically and physically interacts with the CBC gene *CBP80* in that combinations of mutant alleles of *npl3* and *cbp80* display synthetic lethal interactions, and Npl3 is strongly associated with the CBC proteins Cbp80 and Cbp20 in an RNA-dependent manner (Shen et al. 2000). We have found that *CBP80* is not required for Npl3 recruitment to transcribing genes (E. Lei and P. Silver, unpubl.). Although CBC has not been shown to bind to RNA cotranscriptionally in yeast, this result raises the possibility that Npl3 promotes CBC recruitment to nascent RNAs.

In combination with previously published reports, our data show that mRNA export and processing factors are cotranscriptionally recruited to perform their function in maturation of the RNA for nuclear export. This mechanism may explain early observations in which mRNA export in *Xenopus* was stimulated by injection of promoter DNA containing an intact consensus TATA-box (de la Pena and Zasloff 1987). Additionally, identification of *ptr6*$^+$, which encodes a putative TBP-associated factor (TAF), in a screen for mRNA export genes in *S. pombe* suggests that additional transcription factors may be involved in this process (Shibuya et al. 1999). In sum, we have shown that chromatin immunoprecipitation is a powerful technique that can be used to order the steps of recruitment of proteins to RNA, an outstanding question in the field of mRNA processing and export. Our results suggest that Npl3 recruitment is an early event, possibly occurring at transcription initiation and that Yra1 recruitment is a later event, perhaps signaling the complete maturation of the RNP for export (Fig. 7). Future studies will further understanding of RNP assembly.

Materials and methods

Yeast strains and genetic manipulations

Standard yeast methods and media were utilized (Guthrie and Fink 1991). Strains used in this study: FY23/PSY580 MAT**a** *ura3-52 trp1*Δ*63 leu2*Δ*1*; PSY603 MAT**a** *his3 ade2 can1 leu2 lys1 ura3 ade8*; PSY 1031 MATα *npl3-27 ura3-52 leu2-3,112 his3 lys1-1 trp1-1 ade2-1 ade8 can1-100*; PSY 1032 MAT**a** *npl3-27 ura3-52 leu2-3,112 his3 lys1-1 ade2-1 ade8 can1-100*; PSY 1698 MATα *npl3-27 spt15-ts1 ura3-52 leu2-3,112 his3 lys1-1 ade2-1 ade8 can1-100*; PSY 1699 MAT**a** *npl3-27 spt15-ts1 ade2-1 can1-100 his3 leu2-3,112 lys1-1 ura3-52 ade8*; PSY 1702 MATα *spt15-ts1 ade2-1 ura3-52 leu2-3,112 his3 lys1-1 trp1-1 ade8 can1-100*; PSY 1703 MATα *spt15-ts1 ade2-1 can1-100 his3 leu2-3,112 lys1-1 ura3-52 ade8*; YRA1-MYC is identical to YRA1 shuffle MAT**a** *ade2 his3 leu2 trp1 ura3 yra1::HIS3* (Sträßer and Hurt 2000) but contains the plasmid pNOPMYCA1L-YRA1, which was constructed by cloning the entire ORF of *YRA1* from pNOPPATA1L-YRA1 into the *Pst*I site of pNOPMYCA1L (K. Straesser and E. Hurt, unpubl.). Strains were provided generously by F. Winston, Harvard Medical School (*spt15-328*, *spt15-341*), R. Young, Whitehead Institute for Biomedical Research (*rpb1-1*, CTD truncations), and E. Hurt, Biochemie Zentrum Heidelberg (YRA1-MYC).

Indirect immunofluorescence and in situ poly(A)$^+$ RNA hybridization

These procedures were performed as described previously (Krebber et al. 1999).

Generation of npl3-27 ts$^-$ *library*

The integrated *npl3-27* strains of both mating types, PSY1031 and 1032, were used to generate *ts*$^-$ mutants using a modified protocol as described (Lawrence 1991). The strains were grown to a density of 6×10^7 cells/mL in YPD and were collected by centrifugation and resuspended in 5 mL 0.1 M sodium phosphate at pH 7.0. Cells were mutagenized with 130 µL ethyl methanesulfonate (EMS) to a killing rate of 50%. Cells were incubated for 30 min at 30°C on a roller drum. The mutagenesis was stopped by the addition of 1 mL of 10% (w/v) sodium thiosulfate, and the cells were collected by centrifugation, washed in 5 mL H_2O and resuspended in 1 mL H_2O. Dilutions were plated on YPD plates to obtain ~300 cells per plate. After 5 d of growth at 25°C, colonies were replica plated to YPD and incubated at 37°C for 5 d. Four hundred and fifteen colonies that did not grow at 37°C were selected for the *npl3-27 ts*$^-$ library.

Isolation of npl3-27 *export mutants*

The collection of 415 *npl3-27 ts*$^-$ mutants was analyzed for localization of Npl3-27 by indirect immunofluorescence after a shift to 37°C for 30 min for mutants ts300–415 and 4 h for mutants ts1–400. Mutants that displayed >20% of cells with nuclear accumulation of Npl3-27 were backcrossed to a parental *npl3-27* strain to determine if the *ts*$^-$ mutation was recessive. The resulting diploids were sporulated and analyzed for the presence of a single *ts*$^-$ mutation. *ts*$^-$ spores were compared against Ts$^+$ spores using indirect immunofluorescence to verify that accumulation of Npl3-27 was linked to the *ts*$^-$ mutation. Each strain was backcrossed to the parent *npl3-27* strain two more times to remove undesired mutations resulting from the mutagenesis procedure. Mutations were cloned by complementation of the *ts*$^-$ phenotype by transformation with a CEN *URA3* library (Rose et al. 1987).

Northern analysis

Determination of poly(A)$^+$ RNA levels Fifty mL of cells were grown to log phase (1×10^7 cells/mL) at 25°C and half the culture was shifted to 37°C for 1 h. Total RNA was isolated by the hot acid phenol method, and 2 µg of total RNA was hybridized to a nitrocellulose membrane by slot blot hybridization and

probed as described previously (Thompson and Young 1995). The radiolabeled poly dT probe was produced as described previously (Kuldell and Buratowski 1997). Signal was detected and quantitated by PhosphorImager (Molecular Dynamics).

Northern blotting Fifteen µg of total RNA were separated by agarose gel electrophoresis and transferred to a Hybond N^+ membrane (Amersham Pharmacia) by vacuum transfer in 20× SSC. A ^{32}P-labeled 300-bp specific probe for *ACT1* was labeled by random priming and hybridized to the membrane in hybridization buffer (50 mM PIPES at pH 7, 100 mM NaCl, 50 mM sodium phosphate at pH 6.9, 1 mM EDTA, 5% SDS, 60 µg/mL sheared salmon sperm DNA) overnight at 65°C and washed three times in 0.5× SSC + 5% SDS at 65°C for 15 min. Signal was quantitated by PhosphorImager.

Chromatin immunoprecipitation

Chromatin IPs were performed essentially as described (Dudley et al. 1999; Kuras and Struhl 1999). Briefly, 400 mL of cells were grown to early log phase (~1 × 10^7 cells/mL) at 30°C in YPD or YP containing 2% raffinose. For galactose induction, galactose was added to raffinose cultures to a final concentration of 2% and cultures were induced for 20 min. Formaldehyde was added to a final concentration of 1% and cells were incubated at room temperature (RT) for 25 min. Glycine was added to a final concentration of 360 mM. Cells were washed twice in 1× TBS and lysed with glass beads in breaking buffer (0.1 M Tris pH at 8.0, 20% glycerol, 1 mM PMSF) using two 30 sec pulses at speed 6.5 m/sec in a vortexer. Cross-linked chromatin was collected by centrifugation at 14,000 rpm for 1 min, and pellets were washed twice in FA buffer (50 mM HEPES-KOH at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% DOC) and resuspended in 1.5 mL FA buffer. Cells were sonicated until DNA was of 200 bp average size. Soluble chromatin was separated from insoluble material by centrifugation at 14,000 rpm for 10 min and adjusted to 8 mL with FA buffer. Chromatin was stored in 800 µL aliquots at –80°C.

Immunoprecipitations were performed as described (Kuras and Struhl 1999). For α-TBP, 8 µL of antibody was coupled to protein A-sepharose beads. For α-Npl3, 25 µL of antibody was coupled to fixed *Staphylococcus aureus* bacteria. For α-myc, 1 µL of 9E11 antibody was coupled to fixed *S. aureus*. Input and immunoprecipitated DNA was subjected to quantitative PCR as described (Komarnitsky et al. 2000) except 0.15 mCi/mL [α-^{33}P]dATP was used. PCR products were separated on an 8% TBE polyacrylamide gel and quantitated by PhosphorImager. A standard curve of six twofold serial dilutions of input in the linear range of PCR was obtained. Four twofold serial dilutions of immunoprecipitate were plotted against the standard curve to determine the percentage of input DNA in each immunoprecipitate. Experiments were performed at least twice, and each experiment yielded similar results.

The sequence of *PMA1* primers are the same as described (Komarnitsky et al. 2000). The sequence of *GAL10* primers used are *GAL10*-1: GAGCCCCATTATCTTAGCC and TTACTGC CAATTTTTCCTCT; *GAL10*-2: TTAAACTTCTTTGCGTC CATCC and TGCTTGGTCAAGACCTCTAACC; *GAL10*-3: TGTCGTGAGTGGAACTTGGGTT and GCATATCTTCAG CGGAAAATCTGGC; *GAL10*-4: TGTCAAGGCTTTTCATC CCGATTC and TTGGACCCGTAAGTTTCACCGT; Intergenic region: GAAAAAGTGGGATTCTGCCTGTGG and GT TTGCCACAGCGACAGAAGTATAACC.; $tRNA_{GUC}$: STR2963 CACCACAAATGGAAAAGCGACTTTC and STR2964 CCT GTTATTTCCAAAGAACTGGGTTC; $tRNA_{CUU}$ STR2967 GCACTAGTTGATTCTTGTTCCAACAG and STR2968 CC GTTTTTCCCCAGAGCACTTTTA (L. Kuras and K. Struhl, unpubl.).

Immunoprecipitation

Immunoprecipitations were performed essentially as described (Hartzog et al. 1998). Cells (50 mL) were grown in YPD at 30°C to mid log phase (2.5 × 10^7 cells/mL) and lysed with glass beads by vortexing at speed 6.5 for 30 sec in lysis buffer (25 mM $NaPO_4$ at pH 6.8, 0.1 M KOAc, 2 mM MgOAc, 10% glycerol, 1 mM PMSF, 3 ng/mL pepstatin, 3 ng/mL leupeptin, 3 ng/mL aprotinin, 3 ng/mL chymostatin, 0.2 mM Na_3VO_4, 5 mM β-glycerophosphate, 1 mM NaF). Total lysate (1 mg) was added to IP/wash buffer (lysis buffer with 0.5 M NH_4OAc, 0.1% Tween 20) to a final volume of 900 µL. Lysates were precleared with 3 µL α-Npl3 preimmune serum and 100 µL *S. aureus* in IP/wash buffer for 1 h. Fourteen µL α-Npl3 was added to lysates and incubated overnight. One hundred µL *S. aureus* was added and incubated for 1 h, and immune complexes were washed five times in 1 mL IP/wash buffer and raised in 20 µL IP/wash buffer. Samples containing RNase A were at a concentration of 50 µg/mL. Samples were incubated for 20 min at RT then washed once with 1 mL of IP/wash buffer and resuspended in sample loading buffer. Samples were run on a 7% SDS-PAGE gel, transferred to nitrocellulose in 10 mM CAPS (pH 11), 1% MeOH and Western blotted with 8WG16 (1:500) and α-Npl3 (1:10,000).

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