

Uncoupling of the hnRNP Npl3p from mRNAs during the stress-induced block in mRNA export

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Npl3p, the major mRNA-binding protein of the yeast *Saccharomyces cerevisiae* shuttles between the nucleus and the cytoplasm. A single amino acid change in the carboxyl terminus of Npl3p (E409 → K) renders the mutant protein largely cytoplasmic because of a delay in its import into the nucleus. This import defect can be reversed by increasing the intracellular concentration of Mtr10p, the nuclear import receptor for Npl3p. Conversely, using this mutant, we show that Npl3p and mRNA export out of the nucleus is significantly slowed in cells bearing mutations in *XPO1/CRM1*, which encodes the export receptor for NES-containing proteins and in *RAT7*, which encodes an essential nucleoporin. Interestingly, following induction of stress by heat shock, high salt, or ethanol, conditions under which most mRNA export is blocked, Npl3p is still exported from the nucleus. The stress-induced export of Npl3p is independent of both the activity of Xpo1p and the continued selective export of heat-shock mRNAs that occurs following stress. UV-cross-linking experiments show that Npl3p is bound to mRNA under normal conditions, but is no longer RNA associated in stressed cells. Taken together, we suggest that the uncoupling of Npl3p and possibly other mRNA-binding proteins from mRNAs in the nucleus provides a general switch that regulates mRNA export. By this model, under normal conditions Npl3p is a major component of an export-competent RNP complex. However, under conditions of stress, Npl3p no longer associates with the export complex, rendering it export incompetent and thus nuclear.

[Key Words: hnRNP; Npl3p; mRNA export; *S. cerevisiae*; stress]

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The partition created by the nuclear envelope requires that RNA molecules exit the nucleus in a specific and efficient manner. Most, if not all, RNA molecules exist within cells as ribonucleoprotein (RNP) complexes (Dreyfuss et al. 1993). For mRNAs, their association of various RNA-binding proteins begins as the mRNA precursors are being transcribed (Dreyfuss et al. 1993). The resulting RNP complexes must then move to and pass through the aqueous channels created by the nuclear pore complexes (NPCs). Some proteins appear to dissociate from the RNA before it enters the pore (Pinol-Roma and Dreyfuss 1992), whereas others, such as the cap-binding complex and some heterogeneous nuclear ribonucleoproteins (hnRNPs), remain associated during transit (Daneholt 1997). These proteins are then exchanged for cytoplasmic mRNA-binding proteins and rapidly re-enter the nucleus for new rounds of RNA packaging and transport.

The dynamics of the HIV Rev protein are perhaps the best understood example of the interplay between protein and RNA export. In HIV-infected cells, Rev enters the nucleus by association with the importin β nuclear import receptor (Henderson and Percipalle 1997). Once inside, it binds to RNAs containing the Rev Response Element. The resulting complex moves out of the nucleus and the viral RNA is released in the cytoplasm. Free Rev then re-enters the nucleus for another round of transport. Rev contains a short stretch of leucine-rich amino acids that is both necessary and sufficient for its nuclear export (Fischer et al. 1995). Similar nuclear export signals (NESs) have now been found in a number of proteins known to shuttle between the nucleus and the cytoplasm, such as the protein kinase inhibitor (PKI; Wen et al. 1995; for review, see Lee and Silver 1997; Izaurralde and Adam 1998). This phenomenon of NES-dependent export led to the identification of export receptors (e.g., mammalian exportin and yeast Xpo1p/Crm1p) that bind to NESs and guide their cargo to the cytoplasm (Fornerod et al. 1997; Fukuda et al. 1997; Osareh-Nazari et al. 1997; Stade et al. 1997). The formation of the export complex is supported by the small GTPase, Ran, in its GTP-bound state (Fornerod et al.

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1997; Izaurralde et al. 1997). The induction of Ran's intrinsic GTPase activity by the cytoplasmically localized GAP then causes the dissociation of the cargo from exportin in the cytoplasm (Kutay et al. 1997).

Can the Rev example be generalized to the export of mRNAs? By such a mechanism, one or more of the mRNA-binding proteins that accompany the mRNA out of the nucleus would contain signals to confer interaction with an export receptor. There are a number of mRNA-binding proteins that shuttle between the nucleus and the cytoplasm, including the highly abundant hnRNPA1 (Nakielny and Dreyfuss 1996; Nakielny et al. 1997). However, none have been shown to rely on exportin for its export. In addition, a direct role for exportin in mRNA export in higher eukaryotes remains controversial (Fischer et al. 1995; Fornerod et al. 1997; Pasquinelli et al. 1997). The transported RNP complex, which can consist of transcripts up to 30 kb, covered with proteins and packed into a complex as large as 50 nm, must pass through the 25-nm-active channel formed by the NPC (Ohno et al. 1998). The large size of the transported RNP complex might mandate that it use a number of export proteins such as the recently described Mex67p, Mtr2p, and Gle1p.

The yeast *Saccharomyces cerevisiae* has proven to be particularly useful for studies of mRNA export. In particular, mutations in a number of genes have revealed roles for certain nucleoporins and RNA-binding proteins (for review, see Corbett and Silver 1997). Among the best characterized of the latter class is the abundant mRNA-binding protein Npl3p, also referred to as Nop3p, Mts1p, Nab1p, and Mtr13p (Bossie et al. 1992; Russell and Tollervey 1992; Ellis and Reid 1993; Kadowaki et al. 1994; Wilson et al. 1994). A number of observations have led to the idea that Npl3p is an important mediator of mRNA export. First, loss-of-function temperature-sensitive *NPL3* alleles accumulate poly(A)⁺ RNA in the nucleus at the nonpermissive temperature (Lee et al. 1996). Second, Npl3p shuttles between the nucleus and the cytoplasm and mutations in Npl3p that prevent its nuclear export result in a concurrent block in mRNA export (Lee et al. 1996). Further, Npl3p can be purified with poly(A)⁺ RNA after UV-cross-linking (Russell and Tollervey 1995; Segref et al. 1997). However, it remains an open question as to whether Npl3p is an active carrier of mRNAs or a passive cargo that is required for proper packaging of mRNAs for export. In the latter case, when Npl3p is mutated, mRNA might fail to assemble properly into a complex competent for export.

Under various conditions of stress, cells respond by selectively exporting mRNAs encoding heat shock proteins (Saavedra et al. 1996). There is a simultaneous cessation in the export of normal mRNAs (Saavedra et al. 1996; Tani et al. 1996). It remains unknown how this novel form of regulation occurs. Interestingly, in yeast, in which this phenomenon has been studied in the greatest detail, the export of heat shock mRNA does not require the functions of the Ran GTPase or Npl3p, which are required for export of normal mRNAs (Saavedra et al. 1996, 1997). In contrast, hs mRNA export requires the

function of several different export factors, including Rip1p and Gle1p (Saavedra et al. 1997). This result has led to the proposal that there is a special pathway for the export of heat shock RNAs. However, the mechanism by which normal mRNAs fail to be exported under heat shock conditions has not been explored.

Here, we present data that indicate that the reason constitutively exported mRNAs fail to leave the nucleus in stressed cells is because an export incompetent RNP complex, lacking Npl3p, is produced. This hypothesis is based on the observation that Npl3p is exported from the nucleus following onset of stress, whereas mRNA remains nuclear. However, this is not due to exit of Npl3p from the nucleus in association with heat shock mRNAs. In UV-cross-linking experiments, we show that Npl3p is not bound to poly(A)⁺ RNA after the cells were stressed. Taken together, we propose that the uncoupling of Npl3p, and perhaps other hnRNPs, from mRNAs render the RNPs incompetent for export and that it is this uncoupling that provides part of the rapid switch in gene expression observed under stress conditions.

Results

Npl3p has been shown to shuttle rapidly between the nucleus and the cytoplasm (Flach et al. 1994). Its export is thought to be coupled in some manner to the export of poly(A)⁺ RNA. Under steady-state conditions, Npl3p appears to localize exclusively to the nucleus because its rate of reimport into the nucleus following export is quite rapid. We now further analyze the dynamics of Npl3p by taking advantage of a novel mutant allele that allows us to dissect effects on both import and export in more detail.

A single amino acid change in Npl3p slows its nuclear reimport

npl3-27 was originally identified in a screen for plasmid-borne temperature-sensitive alleles of *NPL3* (Lee et al. 1996). Sequencing of the mutated gene revealed a single-point mutation resulting in the change of glutamate 409 to lysine (E409K). A haploid yeast strain was constructed in which genomic *NPL3* was replaced by the *npl3-27* allele. Interestingly, this strain, termed *npl3-27*, was viable at all temperatures; no temperature-sensitive growth defects were observed. This might be because once integrated into the yeast genome, only a single copy of the gene is present. The original mutant was isolated on a multicopy plasmid and may, therefore, have produced a slightly dominant effect on cell growth.

Npl3-27p is localized mainly to the cytoplasm. Yeast cells were analyzed by indirect immunofluorescence microscopy with an anti-Npl3p antibody (Fig. 1A). Wild-type Npl3p is concentrated in the nucleus at all temperatures (Fig. 1A, top). Npl3-27p on the other hand, localizes to the cytoplasm (Fig. 1A, middle). Note that Npl3-27p is not completely excluded from the nucleus but appears to equilibrate between the two compartments at 37°C.

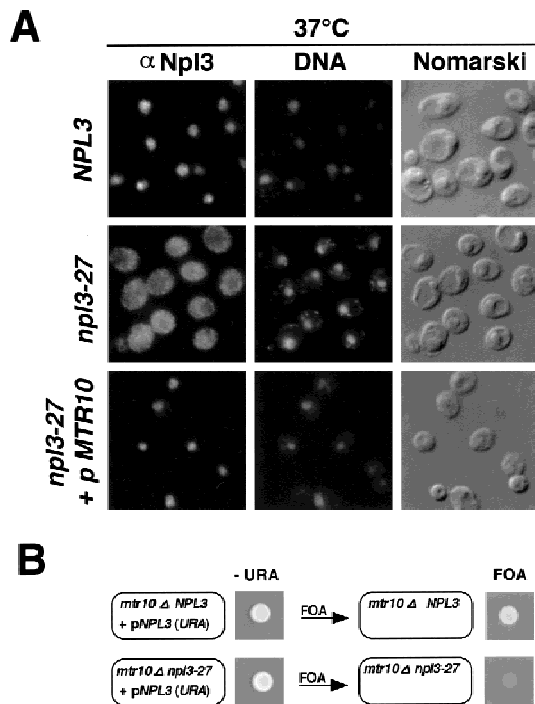


Figure 1. (A) Npl3-27p is slow in nuclear import. Wild-type (*top*), *np13-27* (*middle*), and *np13-27* cells overexpressing *MTR10* on a 2 μ plasmid (*bottom*) were grown to log phase and shifted to 37°C for 30 min. Npl3p localization was analyzed with an α -Npl3p antibody by immunofluorescence, the DNA was stained with DAPI, and the cells were photographed by Nomarski optics. (B) *mtr10*Δ and *np13-27* are synthetically lethal. The *mtr10*Δ *np13-27* double-mutant strain is not able to grow on a plate containing FOA, which allows only those cells to grow that are able to lose the *URA3* plasmid (*bottom, right*). In the presence of wild-type *NPL3* on a *URA3* plasmid, cells are able to grow (*bottom, left*). Growth on FOA-containing medium is not impaired in the mutant *mtr10*Δ that contains genomic wild-type *NPL3* (*top*).

One possible explanation for the behavior of Npl3-27p is that its rate of import is diminished. This is consistent with reports that the carboxyl terminus of Npl3p contains the information for nuclear targeting (Flach et al. 1994; Senger et al. 1998) because the mutation in *np13-27* lies in this carboxy-terminal encoding region. To test whether the import of Npl3-27p was altered, we investigated the effect of overproducing its import receptor, Mtr10p. A multicopy plasmid encoding Mtr10p was introduced into *np13-27* cells and the localization of Npl3-27p was analyzed by immunofluorescence after shifting the cells to 37°C. As shown in Figure 1A (*bottom*), Npl3-27p is completely relocalized to the nucleus when Mtr10p is overproduced. This is consistent with the interpretation that the E409K mutation reduces the efficiency of the Npl3p NLS and that the presence of additional Mtr10p is able to accelerate the slowed import. Together, these results demonstrate that Npl3-27p is still capable of re-entering the nucleus.

Cells deleted for *MTR10* (*mtr10*Δ) are viable at 20°C but mislocalize Npl3p to the cytoplasm (Pemberton et

al. 1997; Senger et al. 1998). However, cells harboring a combination of both mutant alleles, *mtr10*Δ and *np13-27*, are inviable supporting their genetic interaction. This was demonstrated by showing that *mtr10*Δ cells can lose a *NPL3 URA3*-marked plasmid and are thus able to grow on 5-fluoro-orotic acid (FOA)-containing medium (Fig. 1B; *top*), whereas *mtr10*Δ *np13-27* cells cannot (Fig. 1B; *bottom*). These data suggest that *mtr10*Δ cells can live because some Npl3p can still enter the nucleus. However, when the NLS of Npl3p is mutated as in Npl3-27p, cells die because the intranuclear level of the protein is insufficient to support proper growth. Taken together, these data show that import of Npl3p is crucial for the cell. Furthermore, Npl3-27p offers a novel tool for studying the export of Npl3p.

Factors affecting export of Npl3p

In principle, mutations that slow the rate of Npl3p export will result in accumulation of Npl3-27p in the nucleus instead of the cytoplasm. To test this idea, double mutants were constructed containing the *np13-27* allele and either the NES-receptor mutant *xpo1-1* or the nucleoporin mutant, *rat7-1*. Following a shift of the single and double mutants to 37°C for 30 min, cells were fixed and the localization of Npl3-27p was determined by immunofluorescence. The distribution of poly(A)⁺ RNA in the same cells was visualized by in situ hybridization with an oligo[d(T)₅₀] probe. Under these conditions, Npl3-27p and mRNA were both cytoplasmic in the *np13-27* single mutant, indicating no defect in export (Fig. 2, *left*). However, Npl3-27p and poly(A)⁺ RNA both accumulated in the nuclei of *xpo1-1* and *rat7-1* cells following the shift to the nonpermissive temperature (Fig. 2, *center and right*).

To investigate whether the export of other proteins is altered at the nonpermissive temperature in *xpo1-1* and *rat7-1* cells, the localization of Srp1p/Kap65p, the NLS

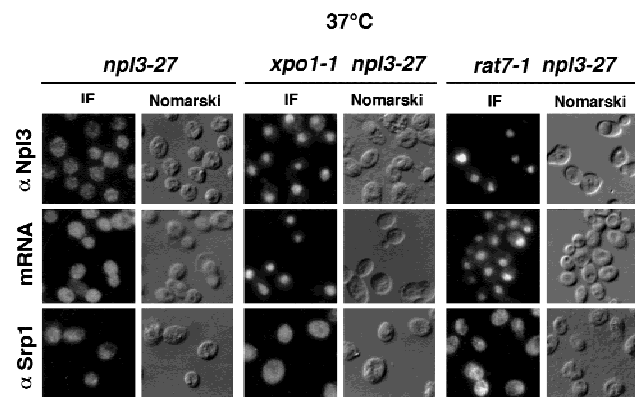


Figure 2. *xpo1-1* and *rat7-1* exhibit Npl3-27p localization and mRNA export defects. *np13-27* (*left*), *xpo1-1 np13-27* (*middle*), and *rat7-1 np13-27* (*right*) strains were grown to log phase then shifted to 37°C for 30 min before they were prepared for immunofluorescence (IF) with α -Npl3p (*top*), oligo[d(T)₅₀] probe (*middle*), or α -Srp1p (*bottom*).

receptor was also analyzed by immunofluorescence (Fig. 2, bottom). Srp1p shuttles between the cytoplasm and the nucleus. Its export from the nucleus depends on the importin- β homolog Cse1p; in *cse1-1* cells, Srp1p accumulates inside the nucleus (Hood et al. 1998; Kunzler and Hurt 1998; Solsbacher et al. 1998). However, in *npl3-27*, *npl3-27 xpo1-1*, and *npl3-27 rat7-1* cells, Srp1p was partly cytoplasmic as it is in wild-type cells, demonstrating that export can occur through the NPCs at the restrictive temperature. Together these data demonstrate that the mRNA-export factors Xpo1p and Rat7p are important for Npl3p export as well.

Nuclear export of Npl3-27p during conditions of heat shock

Cells undergoing various forms of stress such as heat shock retain a substantial amount of their mRNA in the nucleus. Heat shock mRNAs, however, exit rapidly to allow the cell to selectively cope with the stress situation (Saavedra et al. 1996). Given this rapid switch in mRNA export, it was of interest to analyze the behavior of Npl3p under conditions in which mRNA export is perturbed.

Surprisingly, Npl3-27p was still exported rapidly under heat shock conditions. *npl3-27* cells were shifted for 15 min to 42°C and analyzed for the intracellular location of Npl3-27p and poly(A)⁺ RNA, as well as for the *SSA4* mRNA, which encodes Hsp70p. Following heat shock, an accumulation of poly(A)⁺ RNA in the nucleus was observed (Fig. 3A) as has been described previously (Saavedra et al. 1996). Similarly, under conditions of heat shock, the *SSA4* mRNA moved to the cytoplasm as has been reported previously (Saavedra et al. 1996; Fig. 3A). However, Npl3-27p remained in the cytoplasm in the heat-shocked cells, indicating that it was still being ex-

ported from the nucleus (Fig. 3A; top). Even under conditions in which Npl3-27p is normally trapped inside the nucleus, such as in the *xpo1-1* mutant, Npl3-27p instead exited the nucleus after a 15 min shift to 42°C. The poly(A)⁺ RNA, on the other hand, accumulated in the nucleus following heat shock of the *xpo1-1* cells. Interestingly, although bulk mRNA export is blocked in *xpo1-1* cells, heat shock mRNA (*SSA4*) export is not impaired (Fig. 3A, bottom). The same results were obtained following heat shock treatments of 5 to 30 min and in the presence of the protein synthesis inhibitor cycloheximide (data not shown). Moreover, Npl3-27p remains intact under all conditions as determined by immunoblot analysis (Fig. 3B). These data show that under heat shock conditions, Npl3-27p can dissociate from the mRNA and exit the nucleus. As is the case for cells grown at 37°C, overexpression of Mtr10p at 42°C drives Npl3-27p into the nucleus (Fig. 3C). Thus, Npl3-27p is still capable of nuclear entry even under heat shock conditions.

Wild-type Npl3p also accumulates in the cytoplasm to some extent under conditions of stress. Wild-type cells were shifted to 42°C for 15 and 30 min and 1, 2, and 3 hr. The intracellular localization of Npl3p was determined by immunofluorescence with anti-Npl3p antibodies (Fig. 3D). At 15 and 30 min, some Npl3p is observed in the cytoplasm. However, the effect is not as complete as that observed for Npl3-27p, presumably because the reimport of Npl3p is faster than that of Npl3-27p. As cells adapt to the heat shock, after ~1 hr, cytoplasmic Npl3p disappears.

Npl3-27p does not exit the nucleus with heat shock mRNAs

The observation that both Npl3-27p and heat shock mRNA exit the nucleus, whereas bulk mRNA remains

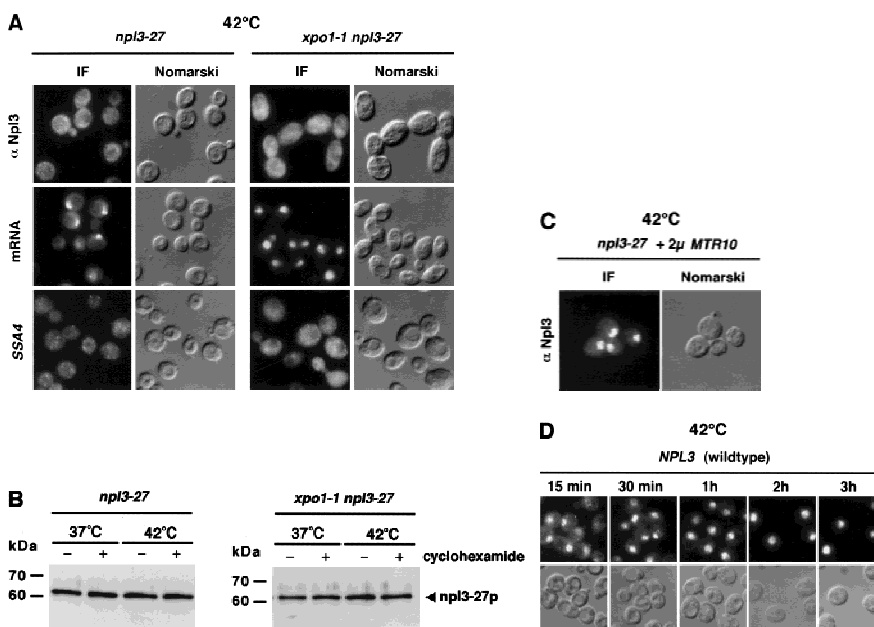


Figure 3. (A) At 42°C, Npl3-27p, but not mRNA exits the nucleus. Both strains, *npl3-27* (left) and *xpo1-1 npl3-27* (right), were grown to log phase, then shifted to 42°C for 15 min, before they were prepared for IF with α -Npl3p (top), oligo[d(T₅₀)] probe (middle), and a *SSA4* probe (bottom). (B) Yeast lysates of the same strains were prepared after they were grown to log phase and either incubated at 37°C for 3 hr (lanes 1,2) or to 42°C for 15 min (lanes 3,4) without [- (lanes 1,3)] or with cycloheximide addition [+ (lanes 2,4)]. The lysates were separated on a 12% SDS-polyacrylamide gel and analyzed on a Western blot with antibodies against Npl3p. (C) *npl3-27* cells overexpressing *MTR10* on a 2 μ plasmid were grown to log phase and shifted to 42°C for 15 min. (D) Wild-type strains were grown to log phase before they were shifted to 42°C for different times. The localization of Npl3p was monitored by immunofluorescence.

nuclear after heat shock, indicates that the export of Npl3p and mRNA have become uncoupled. There are at least three possible explanations for this apparent separation of Npl3p from most of the mRNA. By one mechanism, Npl3p might exit the nucleus alone and unbound to mRNA. By a second mechanism, Npl3p could move out of the nucleus with a specific pool of mRNA that is not detectable in the *in situ* hybridization experiments. A third possibility is that Npl3p exits the nucleus together with heat shock mRNA under conditions of stress.

It has been reported previously that the nuclear pore-associated proteins Rat7p and Rip1p are essential for the nuclear export of heat shock mRNAs (Saavedra et al. 1997). To test whether or not Npl3-27p is exported together with *SSA4* heat shock mRNA, the localization of Npl3-27p was investigated in *rat7-1* and *rip1Δ* mutants. *rat7-1 npl3-27* and *rip1Δ npl3-27* double mutants were constructed and shifted for 15 min to 42°C to induce the heat shock stress response. The cells were then analyzed by immunofluorescence with anti-Npl3p antibodies and by *in situ* hybridization with a probe specific for *SSA4* mRNA. As expected, a strong accumulation of the *SSA4* mRNA in the nucleus was observed in both strains (Fig. 4, bottom). However, Npl3-27p was largely localized in the cytoplasm (Fig. 4, top). These results indicate that under heat shock conditions, Npl3p exits the nucleus without heat shock mRNAs.

Uncoupling of Npl3p from mRNA export is a general response to different stress conditions

As we have shown that Npl3p exits the nucleus under heat shock conditions independently of poly(A)⁺ RNA, the question arose as to whether this represented a general stress response of the cell. To determine this, the double mutant strains *xpo1-1 npl3-27* and *rat7-1 npl3-27* were shifted to 37°C for 30 min, conditions that trap

Npl3-27p and mRNA inside the nucleus (Fig. 2). The cells were then exposed to either high salt (0.4 M NaCl) for 10 min or 10% ethanol for 30 min. As shown in Figure 5, although poly(A)⁺ RNA accumulated inside the nucleus, Npl3-27p was separated from the bulk mRNA and transported into the cytoplasm. These data suggest a rapid dissociation of Npl3p from mRNA as a response to stress.

Cross-linking of Npl3p to poly(A)⁺ RNA

From the data shown above, we suggest a model in which Npl3p is released from poly(A)⁺ RNA prior to stress. To further test this proposal, we performed UV cross-linking experiments, which have shown previously that Npl3p can be photocross-linked to poly(A)⁺ RNA (Russell and Tollervey 1995; Segref et al. 1997). A wild-type strain was grown to log phase and then separated into three portions. One was not UV-irradiated, one was UV-irradiated, and the third portion was exposed to high salt (0.4 M NaCl) for 10 min before UV-irradiation. Approximately the same amounts of poly(A)⁺ RNA were isolated by oligo(dT)-cellulose chromatography (as determined by OD₂₆₀) and tested for the presence of Npl3p by Western blot analysis, loading 10 OD₂₆₀ units per lane. Npl3p was UV-cross-linked to poly(A)⁺ RNA under normal growth conditions (Fig. 6, top, eluate; -salt shock +UV). However, Npl3p was completely absent from purified poly(A)⁺ RNA when isolated under the identical conditions from cells that had been salt shocked before the UV-irradiation (Fig. 6, eluate; +salt shock +UV). The same experiment was performed on cells incubated at 42°C for 15 min (heat shock conditions). Following the heat treatment, cells were transferred to ice and subjected to UV irradiation just as with the salt-shocked cells. As predicted and as in the case of salt-shocked cells, very little Npl3p was found associated with the poly(A)⁺ RNA under these conditions (Fig. 6, bottom, cf. eluate -heat shock +UV with +heat shock +UV). It could be that this small amount of Npl3p reassociates with the mRNA during the incubation on ice.

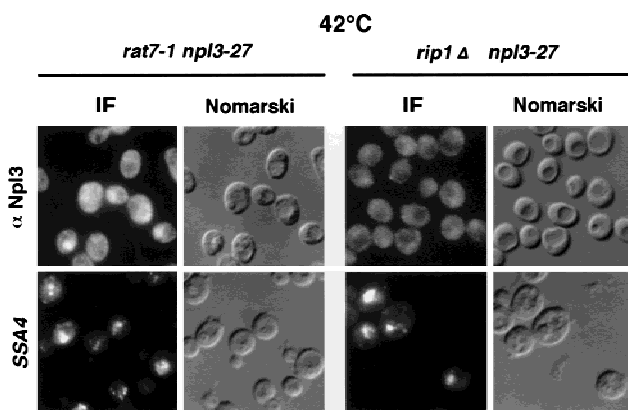


Figure 4. Npl3p does not exit the nucleus coupled to heat-shock mRNA. Both double mutants *rat7-1 npl3-27* (left columns) and *rip1Δ npl3-27* (right columns) were grown to log phase before they were shifted to 42°C for 15 min. Then they were analyzed by immunofluorescence with α -Npl3p (top) and by *in situ* hybridization with a *SSA4* probe (bottom).

Discussion

In all cells, mRNAs are bound by a number of hnRNPs (Nakielny and Dreyfuss 1996; Nakielny et al. 1997). The precise role(s) of the numerous hnRNP proteins in mRNA processing and transport remains one of the outstanding questions in RNA biology. In the yeast *S. cerevisiae*, *NPL3* encodes an essential and abundant mRNA-binding protein that shuttles between the nucleus and the cytoplasm (Bossie et al. 1992; Flach et al. 1994; Wilson et al. 1994). Moreover, loss-of-function mutations in *NPL3* result in a block in export of mRNA out of the nucleus (Kadowaki et al. 1994; Lee et al. 1996). Thus, Npl3p has been implicated in the proper export of mRNAs out of the nucleus.

We now present data defining further the role of Npl3p in mRNA export. By using a novel mutant form, Npl3-

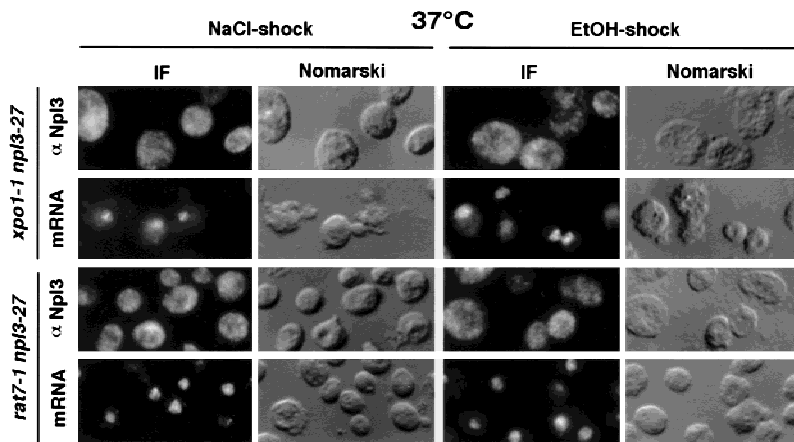


Figure 5. Salt- and ethanol shocks result in the export of Npl3-27p. Both double-mutant strains *xpo1-1 npl3-27* (top two rows) and *rat7-1 npl3-27* (bottom two rows) were grown to log phase and shifted to 37°C for 30 min before they were stressed with 0.4 M NaCl for 10 min (left) or with 10% ethanol for 30 min (right). Cells were then prepared for immunofluorescence (IF) with α -Npl3p (first and third row) and a dT probe (second and fourth row).

27p, that is slowed in nuclear reuptake, we are able to monitor the requirements for its nuclear export. We show that under conditions of stress, Npl3p is not associated with mRNA and is thus capable of exiting the nucleus while the mRNA remains nuclear. We propose a model in which the uncoupling of Npl3p from mRNAs results in the failure of the majority of mRNA to exit the nucleus under conditions of stress. This model is supported by UV-cross-linking experiments in which the Npl3p binding to poly(A)⁺ RNA is greatly reduced under two different stress conditions. We propose that this uncoupling, in turn, allows for the rapid export of heat shock mRNAs in an Npl3p-independent manner. This switch in mRNA export is thus necessary for the rapid onset of the ability of cells to cope with stress conditions.

A novel mutant to study hnRNP nuclear protein export

The experiments presented here were carried out with a novel form of Npl3p that contains a single amino acid change in the carboxyl terminus. Cells bearing the *npl3-27* allele are still viable at all temperatures, but the amount of Npl3-27p in the cytoplasm under steady-state conditions is greatly increased as compared with wild-type Npl3p. Previous studies have indicated that the carboxyl terminus of Npl3p is important for its nuclear import (Flach et al. 1994; Senger et al. 1998). Removal of amino acids 397–414 results in the failure of Npl3p to enter the nucleus (Senger et al. 1998). However, the amino acid sequence important for Npl3p nuclear targeting bears no similarity to the classical NLS.

MTR10 encodes one of several nuclear import receptors and, as such, promotes the nuclear import of Npl3p and other potential RNA-binding proteins (Pemberton et al. 1997; Senger et al. 1998; E.P. Lei and P.A. Silver, unpubl.). In fact, *MTR10* was originally identified as a mutant defective in mRNA export (Kadowaki et al. 1994). Cells missing *MTR10* accumulate Npl3p in the cytoplasm and show a simultaneous accumulation of poly(A)⁺ RNA in the nucleus. In contrast, *npl3-27* cells, which also have cytoplasmic Npl3p, do not have a pronounced mRNA export defect. Possible explanations for this apparent paradox are as follows: (1) there still might be sufficient Npl3p moving in and out of the nucleus to sustain mRNA export in *npl3-27*; and/or (2) additional mRNA-binding proteins important for mRNA export may not be imported into the nucleus in *mtr10* mutants.

Npl3p has been shown to interact directly with Mtr10p and amino acids 397–414 are important for this interaction, further confirming their function as an NLS (Senger et al. 1998). To reflect the decreased rate of Npl3-27p import because of a mutation in its NLS, we show that overexpression of Mtr10p can drive Npl3-27p into the nucleus. Taken together, these data indicate that Npl3-27p is still capable of being exported from the nucleus but is slowed in its reimport, thus resulting in a net accumulation of protein in the cytoplasm but not a block in mRNA export.

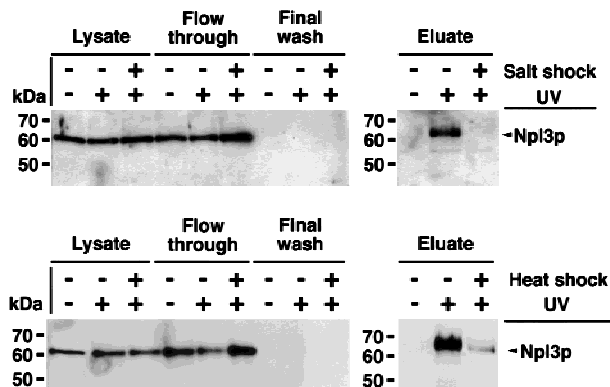


Figure 6. UV cross-linking of Npl3p to poly(A)⁺ RNA. (Top) Cells were either treated with or without high salt (+ or – Salt shock) and exposed to UV for cross-linking (+ or –UV-irradiation). The lysates were taken before application to the oligo(dT)-cellulose-column. (Flow through) The material that did not bind to the column. The final wash is derived from the last wash before elution (see Materials and Methods). The eluate contains the cross-linked poly(A)⁺ RNA-associated proteins. (Bottom) Cells were either shifted to 42°C for 15 min and exposed to UV (+heat shock +UV) or UV cross-linked without a heat shock (– heat shock +UV). The immunoblot was carried out with a polyclonal antibody against Npl3p.

Why can cells still survive normally with so much Npl3p in the cytoplasm? One interpretation is that at any given time there is still enough Npl3p in the nucleus to package mRNAs and escort them into the cytoplasm. Similarly, in *mtr10Δ* cells, Npl3p is localized mainly to the cytoplasm, but the cells are still viable (Pemberton et al. 1997; Senger et al. 1998). It is possible that there is a second importer for Npl3p that has reduced affinity for its cargo, thus resulting in reduced nuclear uptake. However, when *MTR10* is deleted from *npl3-27* cells, they can no longer survive, suggesting that any backup import system is not sufficient when the NLS is mutated.

The mechanism by which most hnRNPs are exported from the nucleus to the cytoplasm remains an open question. Our data suggest that in yeast there may be an interplay between the export of Npl3p and the function of the NES nuclear export receptor encoded by *XPO1*. However, our failure to demonstrate a direct interaction between Npl3p and Xpo1p (data not shown) leaves open the possibility that these effects may be indirect.

In higher eukaryotes, the role of the Xpo1p homolog exportin in mRNA export remains ambiguous. On one hand, treatment with the exportin inhibitor leptomycin B results in a block in NES-dependent export but not in mRNA export in *Xenopus* oocytes (Fischer et al. 1995; Fornerod et al. 1997). Moreover, overexpression of the Nup214 carboxyl terminus in COS cells causes a coincident relocalization of exportin to the nucleoplasm and a block in NES-dependent export, but no block in mRNA export (Bogerd et al. 1998). In contrast, others report inhibition of mRNA export when certain NESs are injected at high concentration into the *Xenopus* oocyte nucleus (Pasquinelli et al. 1997).

Npl3p is uncoupled from mRNA when cells are exposed to stress

Eukaryotic cells respond to stress in a number of ways, including the retention of mRNA. To make the response to stress as efficient as possible, mRNAs encoding heat shock proteins are rapidly transcribed and exported from the nucleus to the cytoplasm for translation (Saavedra et al. 1996). At the same time, there is a shutdown in the export of bulk mRNAs, presumably to allow for the selective and rapid translation of the heat shock mRNAs (Saavedra et al. 1996; Tani et al. 1996). One outstanding question is how the cell executes this rapid switch in RNA export.

We now present the novel finding that the major yeast hnRNP protein, Npl3p, is no longer mRNA associated under conditions of stress that result in a general block in poly(A)⁺ RNA nuclear export. The first indication for the dissociation of Npl3p from mRNA came from our observations of the behavior of Npl3-27p in *xpo1-1* and *rat7-1* mutants, in which at the restrictive temperature both Npl3-27p and mRNA remain in the nucleus. However, when salt or ethanol is then added to these shifted mutants, Npl3-27p is exported, whereas the bulk of the mRNA remains nuclear. These findings regarding the behavior of Npl3p can be interpreted in at least three

different ways: Under conditions of stress, Npl3p could (1) leave the nucleus together with heat shock mRNAs, (2) fail to re-enter the nucleus, and/or (3) dissociate from the majority of the mRNA and redistribute independently.

Whether Npl3p leaves the nucleus together with heat shock mRNAs was tested by use of mutants that are defective in heat shock mRNA export, *rat7-1* and *rip1Δ*. At the nonpermissive temperature, heat shock mRNA export was blocked, whereas Npl3-27p export was not affected, indicating movement of Npl3p independent of heat shock mRNAs. This finding is further supported by the fact that heat shock mRNA export is not blocked in *npl3* mutants in which poly(A)⁺ RNA export is severely slowed (Saavedra et al. 1997).

Blockage of Npl3p nuclear import following stress is contraindicated by the mostly nuclear localization of wild-type Npl3p at all times. Could the accumulation of mRNA during stress be due to a reduced rate of Npl3p import? If cytoplasmic Npl3p is sufficient to block mRNA export, we would also expect to see trapped mRNA in the *npl3-27* strain, in which much of the protein is cytoplasmic. The cytoplasmic localization of mRNA in *npl3-27* strains at 25°C and 37°C argues that the cytoplasmic appearance of Npl3p is not sufficient for the accumulation of mRNA during stress.

Failure of Npl3p to maintain its mRNA association following stress is perhaps the simplest explanation. In this way, any protein no longer bound to mRNA would appear where it is localized at steady state, that is, wild-type protein mostly in the nucleus and much of Npl3-27p in the cytoplasm. Support for this view comes from the results of our UV cross-linking experiments. Although wild-type Npl3p was detected bound to mRNA in unstressed cells, association was greatly reduced in heat-shocked and salt-stressed cells. Thus, stress may lead to the dissociation of Npl3p and mRNA.

Whereas mRNA is most likely always protein bound, the composition of the RNA-protein complex is probably dynamic. For example, proteins important for holding the mRNA in the correct conformation for splicing and transport might come on and off in a temporal manner. Once bound to the mRNA, proteins such as Npl3p might further promote its interaction with the export machinery. From our findings of the dynamic behavior of Npl3p during stress conditions, we suggest a model in which Npl3p (and perhaps other proteins) dissociates from the RNP, leaving an export-incompetent complex behind (Fig. 7). The biological consequences of this dissociation and the resulting block in mRNA export could then be to allow the cell to cope with stress, for example by reducing the general traffic at the nuclear pore. The transport machinery and nuclear pores would then be free for the necessary rapid export of heat shock mRNAs.

Materials and methods

Plasmid constructs

All plasmid constructs used in this study are listed in Table 1. To integrate *npl3-27* into the genome, a 4.5-kb *ClaI* fragment

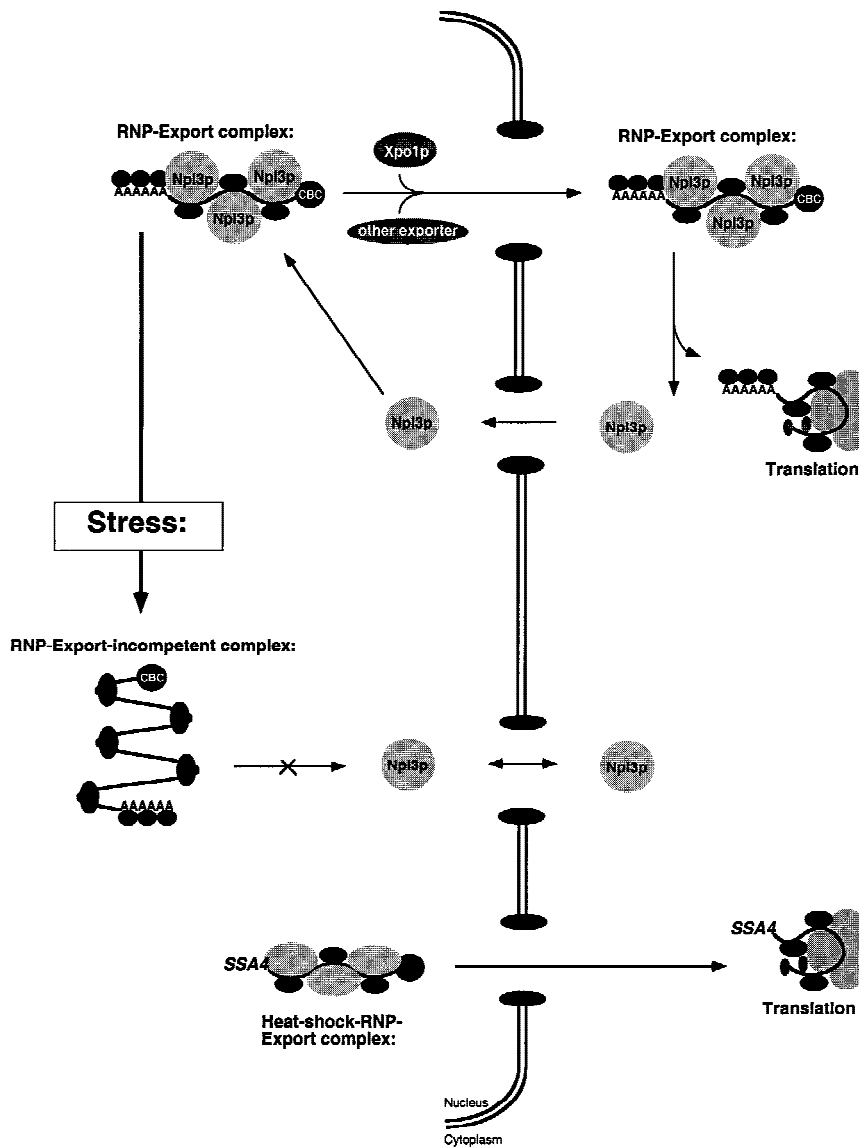


Figure 7. Model for RNP export under normal conditions and during stress. According to this model, mRNAs are packaged into export complexes by proteins such as Npl3p, which may interact with the as yet to be defined mRNA export machinery. Under conditions of stress, Npl3p dissociates from the RNP leaving it incompetent for export.

from pPS873 containing *npl3-27* was ligated into *Cla*I-linearized integration vector pPS750 (Sikorski and Hieter 1989).

Yeast strains

Growth and maintenance of yeast strains, as well as all genetic manipulations were performed as described (Rose and Broach

1990). The strains used in this study are listed in Table 2. To integrate *npl3-27* into the genome, the diploid strain PSY1121 was transformed with the integration vector pPS878 linearized within *npl3-27* with *SexA*I. After selection on plates lacking uracil, single colonies were streaked to FOA to loop out one copy of *NPL3*. The diploid strain was sporulated, genomic DNA was prepared from the viable spores, and a part of the *NPL3* gene

Table 1. Plasmids used in this study

Plasmid	Features	Source
pPS750	CEN <i>URA3</i> yeast integration vector	Sikorski and Hieter (1989)
pPS873	4.2-kb <i>Cla</i> I fragment containing <i>npl3-27</i> in YCp50 <i>LEU2</i>	this study
pPS878	4.2-kb <i>Cla</i> I fragment from pPS873 into pPS750 <i>URA3</i>	this study
pPS1164	3.5-kb <i>Sca</i> I fragment of <i>NPL3</i> in YEp352 <i>URA3</i> (pMH7)	Lee et al. (1996)
pPS1370	chromosomal sequence containing <i>MTR10</i> in YEp24 <i>URA3</i>	A.M. Tartakoff (Case Western Reserve University School of Medicine, Cleveland, OH)
pPS1743	chromosomal sequence containing <i>SSA4</i> in 2 μ <i>LEU2</i>	C. Cole (Dartmouth Medical School, Hanover, NH)
pPS1745	chromosomal sequence containing <i>SSA4</i> in 2 μ <i>URA3</i>	C. Cole

Table 2. *Strains used in this study*

Strain	Genotype	Source
PSY580	<i>MATα ura3 leu2 trp1</i>	Winston et al. (1995)
PSY635	<i>MATα rat7-1 ura3 leu2 his3</i>	C. Cole
PSY814	<i>MATα npl3::HIS3 ura3 leu2 his3 lys1 trp1 ade2 ade8 can1 + YCp50 NPL3 URA3</i>	Henry and Silver (1996)
PSY1031	<i>MATα npl3-27 ura3 leu2 trp1 lys1 his3 ade2 ade8 can1</i>	this study
PSY1032	<i>MATα npl3-27 ura3 leu2 lys1 his3 ade2 ade8 can1</i>	this study
PSY1033	<i>MATα npl3-27 ura3 leu2 lys1 his3 ade2 ade8 can1</i>	this study
PSY1034	<i>MATα npl3-27 ura3 leu2 trp1 lys1 his3 ade2 ade8 can1</i>	this study
PSY1105	<i>MATα xpo1::LEU2 ura3 leu2 trp1 his3 ade2 can1 + pKW457 xpo1-1 HIS3</i>	Stade et al. (1997)
PSY1121	<i>MATα npl3::HIS3/NPL3 ura3/ura3 leu2/leu2 trp1/TRP1 lys1/lys1 his3/his3 ade2/ade2 ade8/ade8 can1/can1</i>	Shen et al. (1998)
PSY1169	<i>MATα rip1::HIS3 ura3 leu2 his3</i>	C. Cole
PSY1637	<i>MATα npl3-27 rat7-1 ura3 leu2 his3 ade⁻</i>	this study
PSY1638	<i>MATα npl3-27 xpo1::LEU2 ura3 lys1 his3 ade2 + pKW457 xpo1-1 HIS3</i>	this study
PSY1639	<i>MATα npl3-27 rip1::HIS3 ura3 leu2 his3</i>	this study
PSY1670	<i>MATα mtr10::HIS3 ura3 leu2 lys1 his3 ade⁻ + pMTR10 URA3 CEN</i>	this study
PSY1671	<i>MATα mtr10::HIS3 npl3-27 ura3 leu2 trp1 lys1 his3 ade⁻ + pMTR10 URA3 CEN</i>	this study

was amplified with the following primers: forward, 5'-GA-CAATCCTCCCAATCAGAAGATCAAAA-3'; reverse, 5'-TTATTCTATCTACTGGAGTGAAGTCG-3', under standard PCR conditions. The resulting 705-bp fragment was analyzed for the loss of a *PmlI* site because the mutation (E409K) is based on a single nucleotide exchange (GAA to AAA) within this restriction site. In each tetrad, two spores had lost the *PmlI* site, indicating retention of the *npl3-27* allele, whereas two spores retained the *PmlI* site, indicating retention of the wild-type *NPL3* allele. In this way, the haploid strains PSY1031, 1032, 1033, and 1034 containing *npl3-27* were obtained. Crosses of these strains with PSY653, 1105, 1169, sporulation of the resulting diploid stains, and subsequent tetrad analysis resulted in the double-mutant strains PSY1637, PSY1638, and PSY1639.

Indirect immunofluorescence

Cells were grown to 1×10^7 to 5×10^7 cells/ml at 25°C. Cells (5 ml) were then shifted to 37°C or 42°C in a shaking water bath for different time points, before 350 μ l of 37% formaldehyde was added for 30 min to fix the cells. Cells were washed once in 0.1 M K_2HPO_4 - KH_2PO_4 at pH 6.5 and once in P solution (0.1 M K_2HPO_4 - KH_2PO_4 at pH 6.5, 1.2 M Sorbitol), spheroplasted with Zymolase (100T, ICN) at a concentration of 500 μ g/ml, placed on slides coated with 0.3% polylysine, and permeabilized by addition of 0.5% Triton X-100 for 5 min. Antibodies against Npl3p were used at a 1:1000 dilution, those against Srp1p at a 1:2000 dilution, and a fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody was used at a 1:1000 dilution (Lee et al. 1996). Nuclei were stained with 10 μ g/ml DAPI as described in Lee et al. (1996).

In situ poly(A)⁺ RNA hybridization

Localization of poly(A)⁺ RNA by in situ hybridization was performed essentially as described (Amberg et al. 1992), with some modifications. Cells were fixed, washed, spheroplasted, and permeabilized as described for immunofluorescence. After incubation with a digoxigenin-labeled oligo[d(T₅₀)] probe, cells were then processed for immunofluorescence as described in Lee et al. (1996), beginning with the antibody-blocking buffer step. A sheep anti-digoxigenin Fab-FITC antibody (Boehringer) was used at a 1:200 dilution.

In situ SSA4 RNA hybridization

Localization of *SSA4* by in situ hybridization was performed as described for localization of poly(A)⁺ RNA, with the PCR-labeled probe described below at a final concentration of 1 ng/ μ l. To generate the *SSA4* probe, PCR with the following primers (forward, 5'-ATGTCAAAGCTGTTGGTATTGATTTAGG-3'; reverse, 5'-GGCCCTTTCAGCGCGCGTCC-3') resulted in a 800-bp fragment, incorporating digoxigenin-11-dUTP (Boehringer Mannheim) into the product when used at 40 μ M concentration. A signal was only obtained when *SSA4* was overexpressed (pPS1743 or pPS1745). No signal was observed in stressed cells not carrying the plasmid (data not shown).

Stress conditions

Heat shock conditions used in this study were as follows: Cells were grown to 1×10^7 to 5×10^7 cells/ml and then incubated in a shaking water bath for 5, 10, 15, and 30 min at 42°C, before fixation. Both salt and ethanol shock were performed at the same cell density. However, cells were shifted to 37°C for 30 min before 0.4 M NaCl or 10% ethanol was added to a final concentration. Salt-shocked cells were incubated for 5, 10, and 15 min at 37°C before fixation. Ethanol-shocked cells were further incubated for 30 and 60 min at 37°C, before fixation. Cultures (10 ml) were grown under the same conditions to 2×10^7 cells/ml. Cyclohexamide was added before the temperature shift at a final concentration of 10 μ g/ml. Cells were then lysed in 50 μ l of SDS-PAGE sample buffer. Five microliters of these extracts were separated on a 12% SDS-polyacrylamide gel before Western blotting. α -Npl3p was used at a 1:1000 dilution.

UV cross-linking of Npl3p to poly(A)⁺ RNA

Isolation of UV-cross-linked poly(A)⁺ RNA-RNP complexes and analysis of cross-linked Npl3p by SDS-PAGE and Western blotting were done essentially as described (Anderson et al. 1993). A total of 1.5 liters of the wild-type strain PSY580 was grown to OD₆₀₀ of 1.0. Cells were harvested, washed twice in PBS, and split in three equal portions. The first portion was not UV-irradiated, the second portion was UV-irradiated for 2×2.5 min on ice in the Stratlinker (Stratagene). The third portion was treated with 0.4 M NaCl for 10 min before it was UV-irradiated and treated as portion two. For the heat shock, cells were incu-

bated at 42°C for 15 min. All samples were washed in PBS and lysed in lysis buffer. The poly(A)⁺ RNA was purified and the proteins analysed as described previously (Anderson et al. 1993; Russell and Tollervey 1995). All eluate samples were loaded normalized to the purified RNA and 10 OD₂₆₀ units were loaded per lane.

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