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Rollenhagen, Christiane; Hodge, Christine A.; and Cole, Charles N., "The Nuclear Pore Complex and the DEAD Box Protein Rat8p/Dbp5p Have Nonessential Features Which Appear To Facilitate mRNA Export following Heat Shock" (2004). *Dartmouth Scholarship*. 1308.
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The Nuclear Pore Complex and the DEAD Box Protein Rat8p/Dbp5p Have Nonessential Features Which Appear To Facilitate mRNA Export following Heat Shock

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Received 9 December 2003/Returned for modification 2 February 2004/Accepted 15 March 2004

Nuclear pore complexes (NPCs) play an essential role in RNA export. Nucleoporins required for mRNA export in *Saccharomyces cerevisiae* are found in the Nup84p and Nup82p subcomplexes of the NPC. The Nup82p subcomplex contains Nup82p, Rat7p/Nup159p, Nsp1p, Gle1p/Rss1p, and Rip1p/Nup42p and is found only on the cytoplasmic face of NPCs. Both Rat7p and Gle1p contain binding sites for Rat8p/Dbp5p, an essential DEAD box protein and putative RNA helicase. Rip1p interacts directly with Gle1p and is the only protein known to be essential for mRNA export after heat shock but not under normal growth conditions. We report that in cells lacking Rip1p, both Gle1p and Rat8p dissociate from NPCs following heat shock at 42°C. Rat8p but not Gle1p was retained at NPCs if *rip1Δ* cells were first shifted to 37°C and then to 42°C, and this was correlated with preserving mRNA export in heat-shocked *rip1Δ* cells. Export following ethanol shock was less dependent on the presence of Rip1p. Exposure to 10% ethanol led to dissociation of Rat8p from NPCs in both wild-type and *rip1Δ* cells. Following this treatment, Rat8p was primarily nuclear in wild-type cells but primarily cytoplasmic in *rip1Δ* cells. We also determined that efficient export of heat shock mRNA after heat shock depends upon a novel 6-amino-acid element within Rat8p. This motif is not required under normal growth conditions or following ethanol shock. These studies suggest that the molecular mechanism responsible for the defect in export of heat shock mRNAs in heat-shocked *rip1Δ* cells is dissociation of Rat8p from NPCs. These studies also suggest that both nuclear pores and Rat8p have features not required for mRNA export in growing cells but which enhance the ability of mRNAs to be exported following heat shock.

Export of mRNAs from their sites of synthesis in the nucleus to sites of translation in the cytoplasm is a complex process and requires several classes of proteins (20, 44). Multiple mRNA-binding proteins are associated with mRNA, yielding an mRNA-ribonucleoprotein complex, mRNP (8). These associations begin during early transcription and require specific interactions between mRNP proteins and the transcriptional machinery (19; reviewed in reference 17). The dependence of mRNA export on these proteins is thought to reflect their role in packaging mRNAs into an exportable configuration. Export also requires that pre-mRNA processing be completed, and this may leave some sort of protein signal or configuration on the mRNP indicating that it is mature and ready for export (reviewed in references 4, 15, and 25).

Export also depends on the nuclear pore complex (NPC), a very large macromolecular complex (60 MDa in yeast and ~120 MDa in higher organisms) assembled using multiple copies of approximately 30 to 35 proteins, called nucleoporins (28, 36). Nucleoporins play both structural and functional roles. Several contain predicted coiled-coil domains thought to be important for maintaining overall pore structure. About one-quarter contain a domain with multiple repeats of a sequence rich in phenylalanine and glycine, called FG repeats, which are thought to serve as docking sites for specific transport factors (20, 25, 27, 40).

Nucleoporins required for mRNA export are found in two subcomplexes of the NPC. The first, the Nup84p subcomplex, contains Nup84p, Nup85p, Nup120p, Sec13p, Seh1p, Nup145p-C, and Nup133p (34). The Nup82p subcomplex contains Rat7p/Nup159p, Nup82p, Nsp1p, Rss1p/Gle1p, and Rip1p/Nup42p and has been a focus of our group's previous studies (1, 2, 6, 9, 12, 13). These two subcomplexes appear to be in close proximity, since fluorescence resonance energy transfer occurs between Gle1p and Nup145p-C (5). Most nucleoporins are present in both the nuclear and cytoplasmic halves of the NPC. Rat7p, Gle1p, Nup82p, and Rip1p are the only nucleoporins found solely on the cytoplasmic side (28). The filaments that extend into the cytoplasm are composed of some of the proteins in the Nup82p and Nup84p subcomplexes (36, 40). In addition to coiled-coil domains which mediate interactions among Nup82p subcomplex components, two of these proteins (Rat7p and Rip1p) contain multiple FG repeats. Cell viability is unaffected by deletion of the repeats from either Rat7p or Rip1p (6, 38, 39).

Cells contain many DEAD box proteins (there are >30 DEAD box and DEXD/H box proteins in *Saccharomyces cerevisiae*), and they are involved in virtually all aspects of mRNA metabolism (reviewed in reference 41). Although the biochemical properties of most DEAD box proteins have not been investigated, those that have been studied biochemically all bind to and hydrolyze ATP. Several have been shown to be able to unwind short regions of double-stranded RNA, and one has been shown to be capable of removing a stably bound protein from RNA (14, 43).

Rat8p/Dbp5p is a DEAD box protein and essential mRNA

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TABLE 1. Yeast strains

Strain	Genotype	Source
FY86	<i>MATα his3Δ200 leu2Δ1 ura3-52</i>	Winston et al. (45)
FY23	<i>MATα trp1Δ63 leu2Δ1 ura3-52</i>	Winston et al. (45)
CHY122	<i>MATα rip1Δ ura3-52 leu2Δ1 trp1Δ63</i>	C. Heath and C. Cole (unpublished)
CRY1	<i>MATα trp1Δ63 leu2Δ1 ura3-52 RAT8-GFP</i>	This study
CRY2	<i>MATα rip1Δ ura3-52 leu2Δ1 trp1Δ63 RAT8-GFP</i>	This study
CSY970	<i>MATα rat7::HIS3 his3Δ200 ura3-52 leu2Δ1 rat7ΔN RAT8-GFP</i>	This study
FSY297	<i>MATα ade2 his3 leu2 trp1 ura3 gle1::HIS3 (pFS1030, LEU2/CEN)</i>	Strahm et al. (37)
FSY298	<i>MATα ade2 his3 leu2 trp1 ura3 gle1::HIS3 rip1::KAN^r (pFS1030, LEU/CEN)</i>	Strahm et al. (37)
CSY550	<i>MATα ura3-52 trp1Δ63 leu2Δ1 rat8-2</i>	Hodge et al. (11)
CSY569	<i>MATα ura3-52 trp1Δ63 leu2Δ1 rat8Δ6</i>	This study
CHY173	<i>MATα rat7::HIS3 his3Δ200 ura3-52 leu2Δ1 rat7ΔN</i>	Hodge et al. (11)
CHY166	<i>MATα rip1Δ leu2Δ1 trp1-63 pCH19</i>	Hammel et al. (10)
CHY167	<i>MATα ura3-52 leu2Δ1 his3Δ200 pCH19</i>	Hammel et al. (10)
CRY3	CSY550 + pCR2 (<i>LEU2</i>)	This study
CRY4	FSY216 + pCR2 (<i>LEU2</i>)	This study
CRY5	CSY569 + pCR2 (<i>LEU2</i>)	This study

export factor which shuttles between the cytoplasm and the nucleus (11). In cells carrying temperature-sensitive (*ts*) alleles of *RAT8*, poly(A)⁺ mRNA accumulates rapidly in the nuclei of all cells following a shift to 37°C (35, 42). The Nup82p subcomplex of the NPC provides docking sites for Rat8p, which binds to sequences within the N-terminal third of Rat7p and the C-terminal region of Gle1p (11, 33, 37). Rapidly occurring defects in mRNA export also occur following a shift to 37°C in cells carrying *ts* mutant alleles of *rat7* and *gle1/rss1* (6, 7, 11). Overexpression of Rat8p suppresses the growth defects of *rat7-1*, *rat7 Δ N*, and *rss1-37* cells (11). In *rat7-1* and *rss1-37* cells, there is a modest decrease in the mRNA export block, and cells are able to grow slowly at the nonpermissive temperature. In contrast, overexpression of Rat8p completely suppresses both the mRNA export and growth defects of *rat7 Δ N* cells (11). Proteins that associate with mRNA in the nucleus are removed from the mRNA at a late stage in mRNA export, allowing them to return to the nucleus for additional rounds of mRNA export. By binding to the Nup82p subcomplex at the cytoplasmic face of the NPC, Rat8p would be positioned to remove mRNP proteins from the mRNA during export.

Following heat or ethanol shock, polyadenylated mRNAs accumulate in the nucleus, but transcription of genes encoding heat shock proteins is induced rapidly and heat shock mRNAs are efficiently exported (23, 24, 31). Export of heat shock mRNAs depends on almost all of the proteins required for export of mRNA under nonstress conditions. One mRNP protein required for normal mRNA export, Npl3p, is not required for heat shock export (18, 32). Conversely, export of mRNA after heat shock depends on one protein dispensable for export under nonstress conditions, the nucleoporin Rip1p/Nup42p, a component of the Nup82p subcomplex. A strain lacking Rip1p grows as well as wild type at all temperatures up to 37°C and

has no detectable defects in nucleocytoplasmic transport under any growth conditions (32, 37, 39). We reported previously that a strong defect in export of all mRNA occurs following heat shock in *rip1 Δ* cells (32).

The studies reported here were directed at understanding the molecular basis underlying the requirement for Rip1p for export of mRNAs following heat shock. We report that, in the absence of Rip1p, both Gle1p and Rat8p are lost from NPCs following a shift to 42°C. In addition, we identified a region of Rat8p required for export during heat shock but not under normal growth conditions. This region is absent from all other yeast DEAD box proteins, but it occurs in all known Rat8p orthologs. We also compared export of mRNA in wild-type and *rip1 Δ* cells following ethanol stress. We observed a modest defect in export of heat shock mRNAs in *rip1 Δ* cells following a 5% ethanol shock. Interestingly, neither wild-type nor *rip1 Δ* cells exported heat shock mRNAs after a 10% ethanol shock, and in both, Rat8p was lost from NPCs. The studies reported here underscore the critical role of Rat8p for mRNA export under all conditions and demonstrate that its association with NPCs following heat shock requires Rip1p.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Yeast strains listed in Table 1 were grown at 23°C in yeast extract-peptone-dextrose rich medium or in synthetic complete medium lacking leucine, uracil, or both. Yeast transformation was performed using a standard lithium acetate method (21).

The integration of a green fluorescent protein (GFP) tag at the end of the coding region of the *SSA4* gene was performed by linearizing an integrating plasmid encoding Ssa4p-GFP with *SalI* and transforming it into wild-type cells using the lithium acetate method. Plasmids used in this study are listed in Table 2.

Strains expressing Rat8p-GFP from the genomic *RAT8* locus were obtained by

TABLE 2. Plasmids

Plasmid	Insert	Description	Source
pCR2	Ssa4p-GFP	<i>SSA4-GFP</i> inserted into <i>SacI</i> - <i>SphI</i> -digested Yiplac128 (<i>LEU2</i>)	This study
pCH15	Ssa4p	<i>SSA4</i> inserted into <i>SacI</i> - <i>SphI</i> -digested Yep1ac181 (<i>LEU2</i> 2 μ m)	Heath and Cole (unpublished)
pCH19	Ssa4p-GFP	<i>SSA4-GFP</i> inserted into <i>SacI</i> - <i>SphI</i> -digested Yiplac211 (<i>URA3</i>)	Hammel et al. (10)

inserting the DNA encoding GFP into the *RAT8* locus using homologous recombination so that GFP was fused to the C-terminal end of *RAT8*.

The method used for PCR-based engineering of yeast genome has been described elsewhere (22). The primer we used had the upstream oligonucleotide sequence 5'-TGGGATGAAGTCGAAAAATAGTTAAGAAAGTGTTAAAGGATCGGGATCCCGGGTTAATT and the downstream oligonucleotide sequence 5'-GTGACAAAGGTATCGAAATCACAAGGTATATGCTGTGTTTGTGAATTCGAGCTCGTTTAA.

rat8Δ6 has a deletion of the DNA encoding amino acids 424 to 429 and was made by site-directed mutagenesis. The integration of this allele into cells was accomplished by using the pop-in pop-out method (26). The *rat8Δ6* allele was subcloned into Yiplac211, linearized with *EagI*, and transformed into CSY550 (*rat8-2*). The transformants were selected for growth on plates lacking uracil, then replica plated to plates containing 5-fluoroorotic acid to select for those which had popped out the *URA3* gene and contained either *rat8-2* or the *rat8Δ6* allele. Colonies able to grow at 37°C must have replaced *rat8-2* with *rat8Δ6*. This was confirmed by sequencing.

Localization of GFP fusion proteins in living yeast cells. To determine the location of proteins under stress conditions, strains producing GFP fusion proteins were grown overnight to a maximum optical density at 600 nm (OD_{600}) of 0.5. The cells were shifted to various temperatures as indicated or treated with different ethanol concentrations for 1 h. After incubation for the period stated in the figure legends, 3 μ l of cell solution was pipetted onto prewarmed glass slides and covered with polylysine-coated coverslips. Images were taken within the next 5 min using a Zeiss Axioplan 2 fluorescence microscope equipped with a cooled charge-coupled device camera and 100 \times and 63 \times objective lenses. Differential interference contrast (DIC) microscopy was used to permit visualization of the location of nuclei in the same cells examined for location of GFP fusion proteins. Each experiment was repeated at least twice.

***SS44* in situ assay.** *SS44* in situ hybridization was performed to localize *SS44* mRNA under different temperature and ethanol stress conditions. Yeast strains containing plasmid-based *SS44* on a 2 μ m plasmid were grown overnight to an OD_{600} maximum of 0.5. Cells were temperature shifted or ethanol treated followed by a fixation with formaldehyde. The in situ assay was performed using a standard procedure described previously (31). To detect *SS44* mRNA, we used a digoxigenin-containing probe complementary to the portion of the 3'-untranslated region of *SS44* mRNA not present in other *SS4* mRNAs of *S. cerevisiae*. We used an antidigoxigenin antibody (Roche) to detect the sites at which the digoxigenin-tagged probe hybridized to *SS44* mRNA. Cells were also stained with 4',6'-diamidino-2-phenylindole (DAPI) to locate cell nuclei. Images were obtained using a Zeiss Axioplan 2 fluorescence microscope equipped with a cooled charge-coupled device camera and 100 \times and 63 \times objective lenses. The distribution of *SS44* mRNA and the location of nuclei were visualized in the same cells. Each experiment was repeated at least twice.

Ssa4p-GFP FACS assay. A fluorescence-activated cell sorter (FACS) was used to measure the levels of Ssa4p-GFP produced following various stress or non-stress treatments of yeast cells. Strains containing an integrated *SS44-GFP* allele were grown in selective medium overnight to an OD_{600} maximum of 0.5. The cells were shifted to temperatures as indicated and treated with different ethanol concentrations for 1 h. When *ts* mutants were tested for stress response following ethanol treatment, cells were incubated at 37°C for 0.5 h prior to adding prewarmed ethanol. After incubation, cells were collected by centrifugation at 2,000 rpm and 4°C for 2 min in an Eppendorf microcentrifuge and resuspended in ice-cold phosphate-buffered saline, followed by incubation on ice. The concentrations were approximately 10^6 cells per ml.

For each sample, the GFP signal intensity of 10^5 cells was measured at 4°C using a FACSTAR cell sorter (Becton Dickinson). Graphical plots showing the relative number of cells with various GFP signal intensities were obtained by using Cell Quest software (Becton Dickinson). Each experiment was repeated at least twice.

RESULTS

During mRNA export, important interactions take place between nuclear pores and proteins associated with the translocating mRNP complex. These include interactions between the mRNA export receptor, Mex67p, and FG repeat sequences of nucleoporins. The export factor Rat8p/Dbp5p interacts with the Nup82p subcomplex of the pore. In several strains temperature sensitive for growth (affecting Rat7p, Nup82p, and Gle1p), mutations which reduce or disrupt Rat8p-NPC inter-

actions result in strong defects in mRNA export, underscoring the importance of Rat8p-NPC interactions for mRNA export.

Rat8p and Gle1p are lost from NPCs of *rip1Δ* cells heat shocked at 42°C. Because Rip1p is part of the same NPC subcomplex as Rat7p, we compared the subcellular distribution of a fully functional GFP fusion to Rat8p in *rip1Δ* and wild-type cells under stress and nonstress conditions. The experiments were performed in cells where Rat8p-GFP was integrated into the genome and was the only form of Rat8p present. The absence of Rip1p did not affect Rat8p's ability to associate with the nuclear rim under nonstress (23°C) conditions (Fig. 1A). However, the Rat8p-GFP signal was lost rapidly from NPCs when *rip1Δ* cells were heat shocked by shifting them to 42°C. We also examined the distribution of a Gle1p-GFP fusion (in cells lacking untagged Gle1p); it too dissociated from nuclear pores in *rip1Δ* cells following heat shock (Fig. 1B). Rat8p-GFP exhibited a homogenous cytoplasmic distribution in *rip1Δ* cells shifted to 42°C, whereas Gle1p-GFP appeared in cytoplasmic dots. Loss of Rat8p and Gle1p from NPCs is sufficient to explain the defect in export of all mRNAs in heat-shocked *rip1Δ* cells.

Induction of thermotolerance in *rip1Δ* cells leads to retention of Rat8p but not Gle1p at NPCs. Heat shock results in protein denaturation and aggregation. This can be reduced if cells are exposed transiently to 37°C prior to a shift to 42°C. This modest heat treatment results in the induction of heat shock proteins to levels sufficient to either prevent protein unfolding and aggregation or to reverse it rapidly. We found that Rat8p-GFP was retained at NPCs in *rip1Δ* cells shifted to 42°C if cells were first shifted to 37°C for 1 h (Fig. 1A). Although Gle1p-GFP normally remains at NPCs in *rip1Δ* cells shifted to 37°C, this thermotolerance treatment did not result in retention of Gle1p-GFP at NPCs following a further shift to 42°C (Fig. 1B).

These findings indicate that even with prior exposure to 37°C, NPCs are not likely to be fully functional in *rip1Δ* cells shifted further to 42°C. That this was indeed the case was determined by examining heat shock gene expression under these conditions. *SS44* is one of several genes in *S. cerevisiae* encoding hsp70 species. Although cells produce hsp70 under normal growth conditions, *SS44* is expressed only following stress (3, 31). We examined the induction of *SS44* following various temperature shifts using two assays (Fig. 2). To examine mRNA export directly, we performed in situ hybridization to monitor distribution of *SS44* mRNA. As a functional assay for export of heat shock mRNAs, we used flow cytometry to analyze the synthesis of heat shock protein, in this case Ssa4p-GFP expressed from the *SS44* locus. Neither *SS44* mRNA nor Ssa4p-GFP was detectable in wild-type and *rip1Δ* cells maintained at 23°C (Fig. 2, top line). As reported previously, *SS44* mRNA was exported efficiently and Ssa4p-GFP was produced in wild-type cells heat shocked at 42°C (10, 31). Although there was robust induction of *SS44* mRNA synthesis when *rip1Δ* cells were shifted to 42°C, there was no production of Ssa4p-GFP, and strong nuclear accumulation of *SS44* mRNA was detected in all cells. Note that the level of Ssa4p-GFP is lower in wild-type cells following a shift to 42°C than a shift to 37°C. This suggests that there may be less efficient translation of heat shock mRNA at 42°C than at 37°C. This is discussed below.

In both cell types, *SS44* mRNA was synthesized, exported,

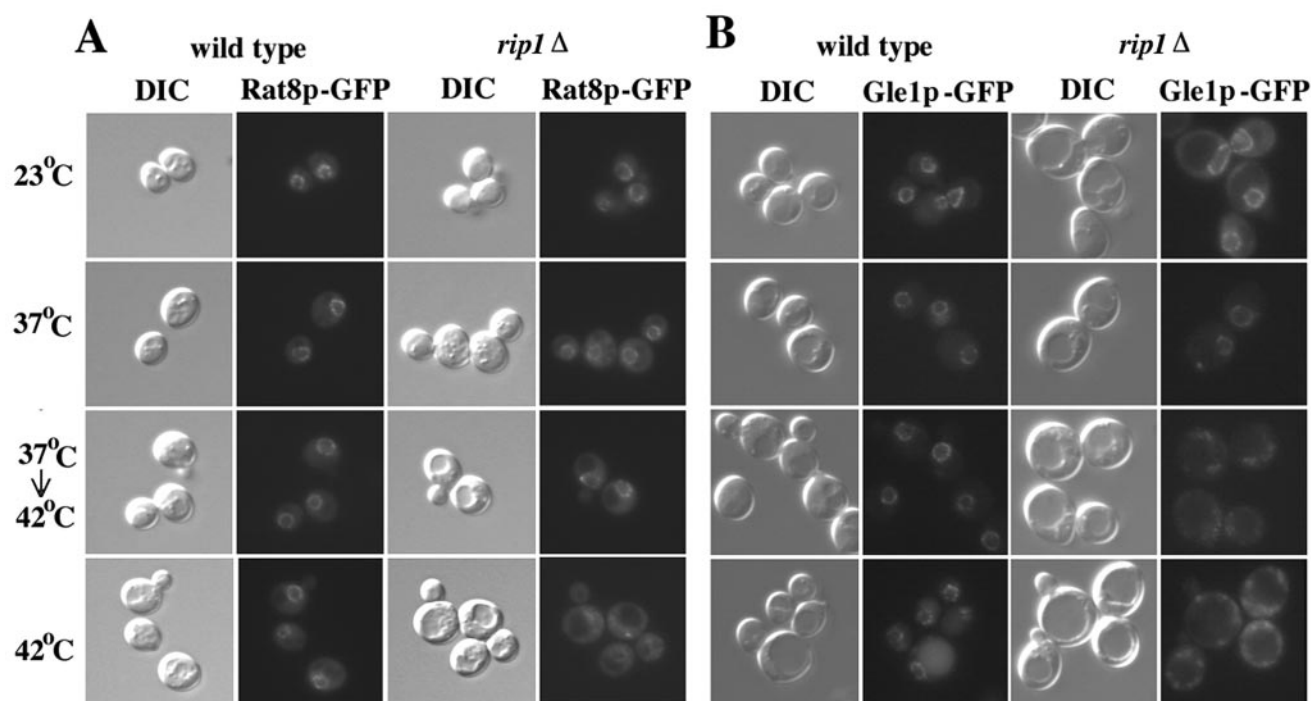


FIG. 1. In *rip1Δ* cells, the loss of Rat8p and Gle1p from the nuclear rim at 42°C can be preserved for Rat8p after induction of thermotolerance. Live cell images of wild-type and *rip1Δ* cells expressing GFP-tagged Rat8p (A) and Gle1p (B) are shown. Cells in early exponential growth were shifted to the indicated temperatures. Where indicated, cells were shifted to 37°C for 1 h and then to 42°C for another hour. DIC images of the same cells visualized for Rat8p-GFP and Gle1p-GFP are also shown.

and translated following a shift to 37°C. When cells were shifted to 42°C following this thermotolerance treatment, the amount of Ssa4p-GFP present increased, though this could have been due to continued translation of mRNA exported prior to the shift to 42°C. However, there was a smaller increase in the Ssa4p-GFP signal in *rip1Δ* cells than in wild-type cells, indicating that the 37°C treatment was not sufficient to maintain a wild-type level of heat shock mRNA export in *rip1Δ* cells. Consistent with this, nuclear accumulation of *SSA4* mRNA was seen in some but not all *rip1Δ* cells shifted from 23 to 37 to 42°C. We conclude that exposure of *rip1Δ* cells to 37°C prior to shifting them to 42°C partially preserves their ability to export mRNA.

The novel NGQADP sequence of Rat8p is required for heat shock mRNA export. The finding that the presence of Rat8p at the nuclear rim following heat shock depends on Rip1p led us to examine the heat shock response of two *RAT8* mutants. DEAD box proteins share eight highly conserved motifs. In addition, the spacing between conserved motifs is very similar among DEAD box proteins, although sequences between conserved motifs vary. Among DEAD box proteins, Rat8p is most closely related to eIF4A (41). However, it differs from eIF4A (TIF1p in *S. cerevisiae*) and from all other DEAD box proteins in having six additional amino acids separating the last (HRI GRTGR) and second-to-last (ARGID) conserved motifs. In Rat8p, the extra six amino acids are NGQADP, and every Rat8p ortholog (including human, mouse, *Xenopus laevis*, *Ara-bidopsis thaliana*, *Drosophila melanogaster*, *Dictyostelium discoideum*, *Schizosaccharomyces pombe*, and *Candida albicans*)

contains a 6-amino-acid insert at this position, with the G and D invariant.

To examine the possible function of this insert, we prepared two mutants, one which encoded a Rat8p lacking these 6 amino acids (*rat8Δ6*) and the other containing point mutations of the G and D (G413A and D416A). Both were indistinguishable from wild type in growth on plates and mRNA export properties at temperatures from 16 to 37°C (data not shown). For comparison, we included *rat8-2* cells in the studies described below. Our investigators reported previously that there is a strong defect in growth and export of polyadenylated mRNA following a shift of *rat8-2* cells to 37°C (11).

The *rat8Δ6* mutant produced a wild-type level of Ssa4p-GFP at 37°C, and nuclear accumulation of *SSA4* mRNA was not detected (Fig. 3B). In contrast, in *rat8-2* cells, *SSA4* mRNA accumulated in nuclei and the level of Ssa4p-GFP produced was very low. Both the *rat8Δ6* and *rat8-2* mutants were defective for heat shock gene expression at 42°C, and *SSA4* mRNA accumulated rapidly in the nuclei of all cells. The level of Ssa4p-GFP produced in heat-shocked *rat8Δ6* cells was low, but clearly above the level seen in the same cells maintained at 23°C (Fig. 3B, compare top and bottom rows) or in either *rip1Δ* cells (Fig. 3B, compare with 2B) or *rat8-2* cells shifted to 42°C (Fig. 3A) (16). We conclude that the conserved 6-amino-acid insert (NGQADP) in Rat8p enhances the ability of Rat8p to function under heat shock conditions.

In contrast to the situation in *rip1Δ* cells, where shifting cells to 37°C for 1 h prior to the shift to 42°C induced thermotolerance and partially preserved *SSA4* mRNA export, thermo-

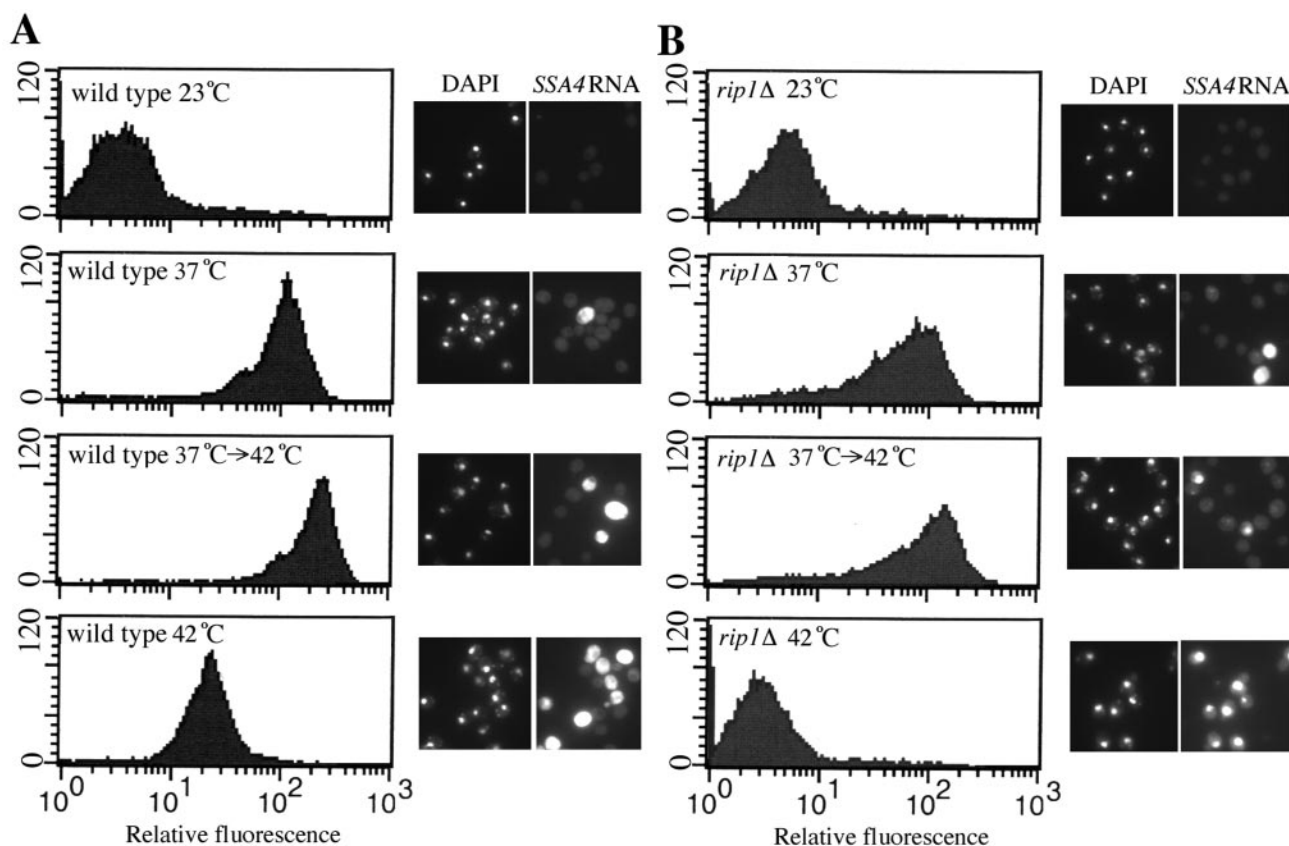


FIG. 2. The *SS44* mRNA export defect in *rip1Δ* cells correlates with the loss of Rat8p-GFP from the nuclear rim. In situ hybridization was performed to monitor the localization of *SS44* mRNA, and flow cytometry was used to analyze the Ssa4p-GFP protein production. (A) Wild-type cells; (B) *rip1Δ* cells. The Ssa4p-GFP protein signal and distribution of *SS44* mRNA were detected in cells shifted to 37 or 42°C for 1 h. Where indicated, cells shifted to 37°C for 1 h were shifted to 42°C for another hour. Cells were also stained with DAPI to permit visualization of nuclei.

tolerance was not induced by similar treatment of *rat8Δ6* cells (Fig. 3B). Most likely, thermotolerance is unable to preserve the enzymatic functions of Rat8Δ6p and Rat8-2p but can preserve the physical interactions between wild-type Rat8p and NPCs lacking a nucleoporin, Rip1p.

The *SS44* mRNA export defect of *rat7ΔN* cells can be suppressed by overexpressing Rat8p. To examine the importance of the binding site for Rat8p on Rat7p, we examined heat shock mRNA export and Ssa4p-GFP synthesis in *rat7ΔN* cells. The *rat7ΔN* allele encodes a Rat7p lacking its N-terminal third (11). A strong defect in mRNA export occurs in *rat7ΔN* cells, and even at 23°C polyadenylated RNA accumulates in nuclei (6). Interestingly, overexpression of Rat8p from a multicopy plasmid completely suppresses both the mRNA export and growth defects of *rat7ΔN* cells (11). As expected, *rat7ΔN* cells were defective for export of heat shock mRNA following the shift to 42°C (Fig. 4B). When we examined the distribution of Rat8p-GFP in *rat7ΔN* cells, it was concentrated at the nuclear rim at 23°C, as in wild-type cells. In contrast, there was substantial nuclear accumulation of Rat8p-GFP in *rat7ΔN* cells shifted to 42°C (Fig. 4C), something not observed in wild-type cells (Fig. 1A). Nuclear accumulation of Rat8p-GFP also occurred when *rat7ΔN* cells were shifted to 37°C (data not shown).

We showed previously that overexpression of Rat8p from a

multicopy plasmid completely suppressed both the growth and mRNA export defects of *rat7ΔN* cells at 37°C (11). Therefore, we analyzed the effect of overexpressing Rat8p on heat shock gene expression at 42°C. In *rat7ΔN* cells, we saw both increased production of Ssa4p-GFP and suppression of nuclear accumulation of *SS44* mRNA.

Effects of ethanol shock on export of *SS44* mRNA and location of Rat8pGFP. Exposure of cells to ethanol also induces a stress response (23, 24, 31). Our group reported previously that 10% ethanol leads to nuclear accumulation of polyadenylated mRNAs and induction of heat shock gene expression. To investigate the importance of Rip1p and Rat8p for mRNA export following ethanol treatment, we exposed both wild-type and *rip1Δ* cells to 5 and 10% ethanol at room temperature for 1 h.

The location of Gle1p-GFP was unchanged in response to either 5% (data not shown) or 10% ethanol in both wild-type and *rip1Δ* cells (Fig. 5B). In both types of cells, Rat8p-GFP remained at the nuclear rim following 5% ethanol treatment. However, in response to 10% ethanol, Rat8p became mislocalized in both wild-type and *rip1Δ* cells. Interestingly, Rat8p was detected in different locations in the two strains. Rat8p-GFP accumulated in nuclei of wild-type cells but primarily in the cytoplasm of similarly treated *rip1Δ* cells (Fig. 5A).

To determine how mislocalization of Rat8p-GFP affected

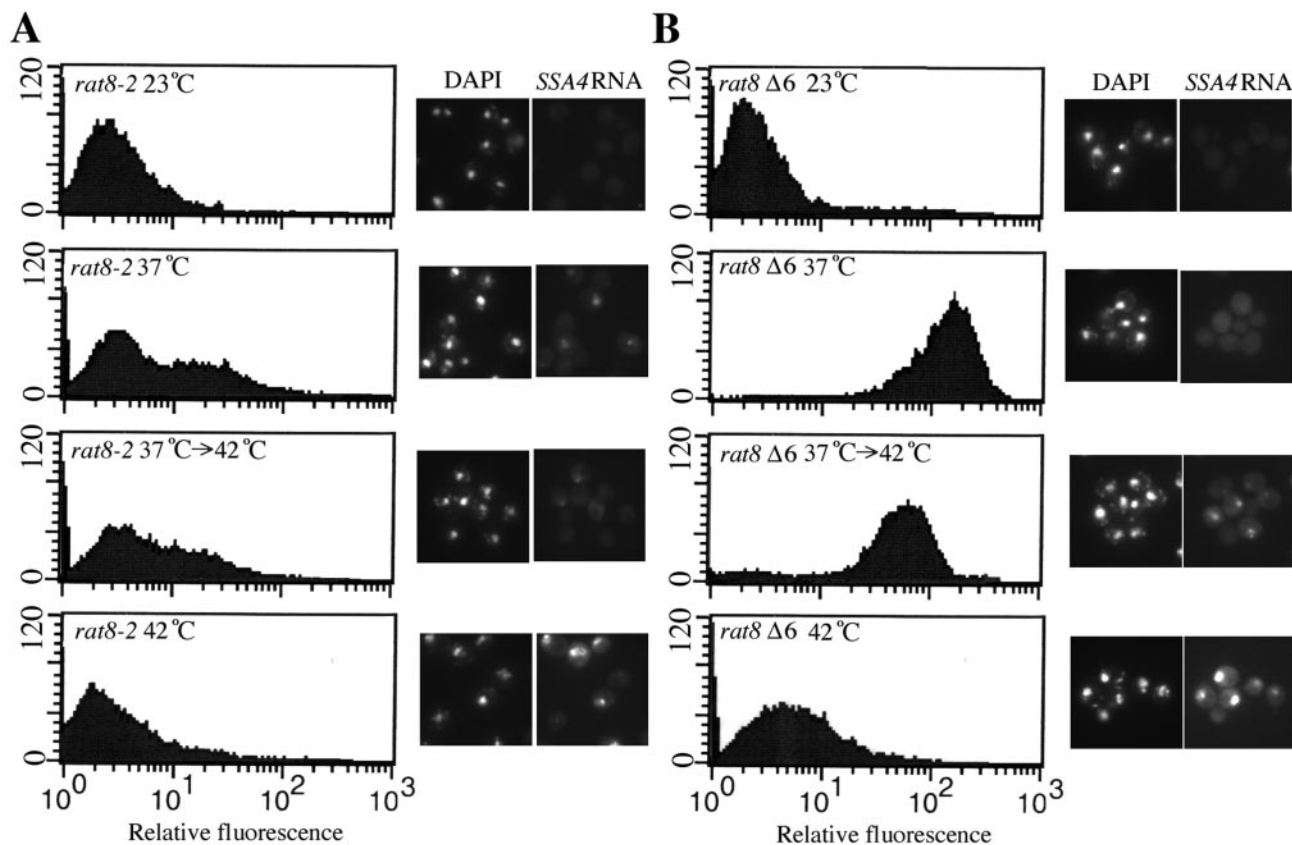


FIG. 3. *RAT8* mutations lead to a defect in export of *SSA4* mRNA at 42°C. In situ hybridization and flow cytometry were used to examine the distribution of *SSA4* mRNA and production of Ssa4p-GFP in *rat8-2* (A) and *rat8Δ6* (B) cells incubated as indicated.

mRNA export following ethanol stress, we analyzed both the location of *SSA4* mRNA and the production of Ssa4p-GFP. Following 5% ethanol treatment, *SSA4* mRNA was induced and exported to the cytoplasm in wild-type cells, whereas this mRNA accumulated in the nuclei of about 20 to 30% of similarly treated *rip1Δ* cells. Consistent with this, the FACS profile of *rip1Δ* cells indicated a lower level of expression of Ssa4p-GFP compared to wild-type cells. Note also that less Ssa4p-GFP was produced in wild-type cells exposed to 5% ethanol than when these cells were heat shocked at 42°C (Fig. 6, middle row, compare with Fig. 2).

Surprisingly, after 10% ethanol treatment, *SSA4* mRNA was detected in nuclei of both wild-type and *rip1Δ* cells (Fig. 6), and no Ssa4p-GFP was produced. This finding strengthens the connection between mislocalization of Rat8p-GFP and the inability to export heat shock mRNAs, in this case even in wild-type cells treated with 10% ethanol. The observations that wild-type and *rip1Δ* cells behave similarly when treated with 10% ethanol and that *rip1Δ* cells show only a modest defect in *SSA4* mRNA export following treatment with 5% ethanol indicate that Rip1p is less important for export following ethanol treatment than following heat shock.

We found that response to ethanol shock was quite sensitive to the concentration of ethanol used, and we therefore exposed cells to a range of ethanol concentrations (0 to 10% in 1% steps). Although the concentration where synthesis of Ssa4p-

GFP was greatest varied somewhat in both cell types from experiment to experiment, we noted interesting differences between *rip1Δ* and wild-type cells. The concentration where Ssa4p-GFP was maximal in any one experiment was always slightly lower for *rip1Δ* cells than for wild-type cells (3 to 6% in different experiments). Although high concentrations of ethanol (>9%) resulted in no Ssa4p-GFP production in either *rip1Δ* or wild-type cells, *rip1Δ* cells were more sensitive to increasing ethanol concentration and produced little or no Ssa4p-GFP as the concentration was increased above that where maximum induction occurred (data not shown). In contrast, the level of Ssa4p-GFP produced in wild-type cells dropped more gradually as the ethanol concentration was raised above the level where Ssa4p-GFP production was maximal. We conclude that the absence of Rip1p increases the sensitivity of cells to ethanol.

Because of the correlation between mislocalization of Rat8p and a defect in heat shock gene expression, even in wild-type cells exposed to 10% ethanol, we analyzed the response of two *rat8* mutants to ethanol stress (Fig. 7A). We first shifted *rat8-2* cells to 37°C for 30 min and then treated cells for an additional 60 min with 10%, 5%, or no ethanol. The data from Fig. 3A (now shown in the top row of Fig. 7A) showed that a very low level of Ssa4p-GFP was produced in *rat8-2* cells (at 37°C) which received no ethanol. Treatment with 5% ethanol reduced this level of expression, and 10% ethanol prevented

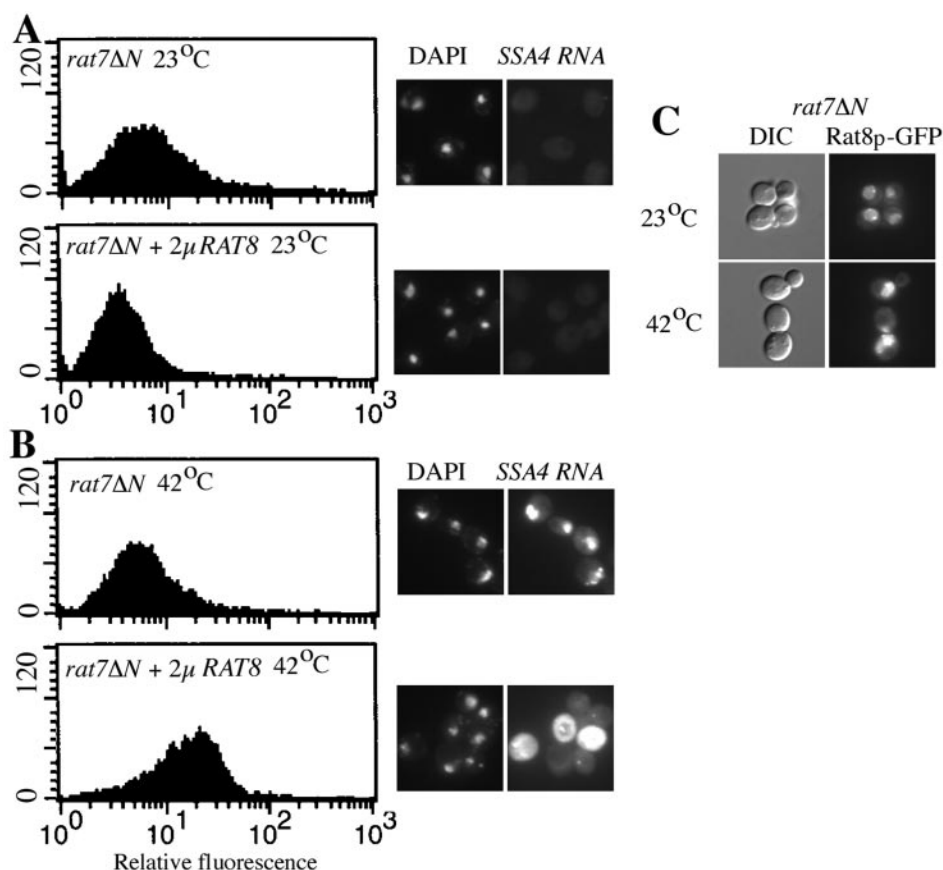


FIG. 4. The *SSA4* mRNA export defect at 42°C in *rat7ΔN* cells can be suppressed by expression of Rat8p from a high-copy plasmid. (A and B) In situ hybridization and flow cytometry were used to examine the distribution of *SSA4* mRNA and production of Ssa4p-GFP. (A) *rat7ΔN* cells and *rat7ΔN* cells overexpressing Rat8p and in exponential growth phase at 23°C are shown. (B) *rat7ΔN* cells and *rat7ΔN* cells overexpressing Rat8p shifted to 42°C are shown. Cells examined by in situ hybridization were also stained with DAPI to permit visualization of nuclei. (C) Live cell images of the location of Rat8p-GFP, expressed from the *RAT8* chromosomal locus, in *rat7ΔN* cells incubated at 23°C or shifted to 42°C. DIC images of the same cells visualized for Rat8p-GFP are also shown.

Ssa4p-GFP expression. We conclude that Rat8p function and proper localization is important for export following ethanol treatment.

The response of *rat8Δ6* maintained at 23°C to ethanol treatment was very similar to that of wild-type cells (Fig. 7B). *SSA4* mRNA was induced and exported following 5% ethanol treatment, and Ssa4p-GFP was produced. Following 10% ethanol treatment, *SSA4* mRNA export was blocked and there was little or no Ssa4p-GFP production in *rat8Δ6* cells. This indicates that the NGQADP sequence in Rat8p is important for expression of heat shock genes after heat shock but not after ethanol shock. We conclude that these 6 amino acids render Rat8p more heat stable but play no role in permitting Rat8p to function in response to ethanol stress.

DISCUSSION

Yeast NPCs contain multiple copies of approximately 30 nucleoporins, most of which are not essential. Strains lacking any one of approximately one-third of yeast nucleoporins are able to grow at 23°C but are defective for growth at elevated temperatures. For another one-third, strains lacking any one nucleoporin are able to grow at both 23°C and also at elevated

temperatures. The final one-third of nucleoporins are essential, but in many cases the deletion of substantial portions of these nucleoporins results in temperature-sensitive growth (for reviews, see references 29, 30, and 40; see also entries on specific nucleoporins in the Yeast Proteome Database at the Incyte Bioknowledge Library maintained by Incyte, Inc., Palo Alto, Calif.). However, there is substantial synthetic lethality among nucleoporin mutations, and in most cases cells are inviable at 23°C if they lack any two nucleoporins (30). Based on nuclear accumulation of polyadenylated mRNA, nucleoporins required for mRNA export include some which are essential and several which are required only at elevated temperatures.

Why is Rip1p required for mRNA export following heat shock? Among the nucleoporins not required for growth at any temperature, only Rip1p is defective in export of heat shock mRNA following heat shock (32). The studies presented here were directed at understanding why Rip1p becomes important for mRNA export after heat shock. There are at least two possible explanations for this. Rip1p could provide a binding site for mRNA export factors which function uniquely during mRNA export after stress; alternatively, Rip1p could contrib-

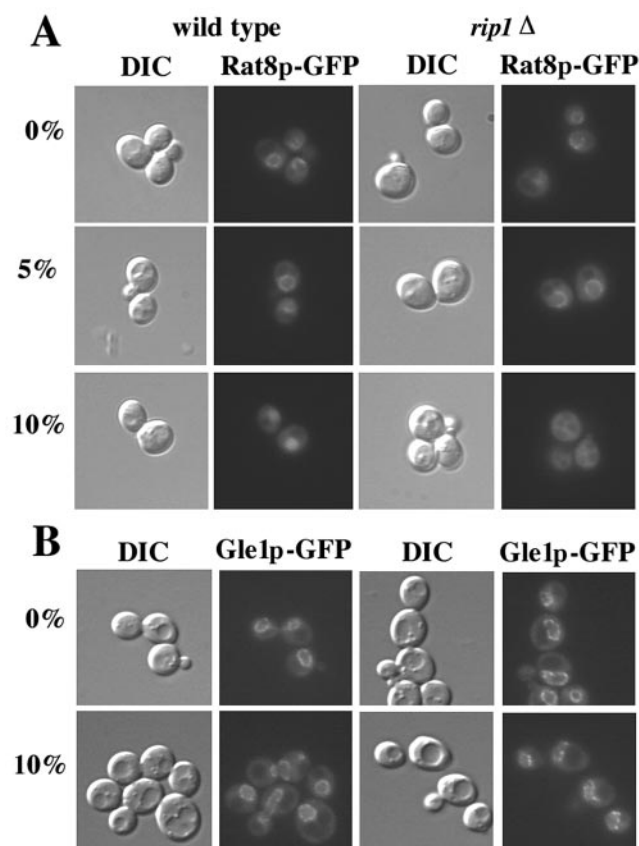


FIG. 5. Rat8p-GFP but not Gle1p-GFP is mislocalized in cells exposed to 10% ethanol. Live cell images of the location of Rat8p-GFP (A) and Gle1p-GFP (B) in wild-type and *rip1*Δ cells expressing GFP-tagged Rat8p and Gle1p are shown. Where indicated, cells were incubated with 5 or 10% ethanol for 1 h. Live cell DIC images of the same cells visualized for Rat8p-GFP and Gle1p-GFP are also shown.

ute to the overall integrity or thermal stability of the NPC. The data presented here favor the second model and suggest that Rip1p stabilizes the sites on NPCs where Rat8p binds. Since Rip1p is dispensable for export of heat shock mRNAs following a 5% ethanol shock, Rip1p is not a general stress response factor.

Rat7p and Gle1p are the two nucleoporins to which Rat8p is known to bind directly. Rip1p interacts directly with Gle1p (37), and both Rat8p and Gle1p are lost from NPCs following heat shock at 42°C (Fig. 1). Since Rat7p remains at NPCs in heat-shocked *rip1*Δ cells (data not shown), the interaction between Rat8p and Rat7p is insufficient to retain Rat8p at NPCs, at least when cells are shifted from 23 to 42°C. However, this interaction appears to be sufficient if *rip1*Δ cells are first incubated at 37°C prior to the shift to 42°C (Fig. 1). No other mutations or treatments have been described which result in dissociation of Gle1p from NPCs.

The binding site for Rat8p on NPCs. The data presented here and other data presented earlier suggest that a complex binding site for Rat8p is formed through the interactions among Rat7p, Nup82p, Nsp1p, Gle1p, and Rip1p at the cytoplasmic fibrils of the NPC. Each of these nucleoporins appears to interact with multiple other nucleoporins, and some have

interactions with nucleoporins not listed above. Within the complex, Rat8p can bind directly to Rat7p's N-terminal third and to Gle1p's C-terminal half (11, 37), but precise binding sites have not been mapped. Our studies of this NPC subcomplex indicate that these nucleoporins perform both structural and functional roles. The N terminus of Rat7p and its repeat regions appear to play functional roles in nuclear transport by serving as docking sites for transport factors, but they probably contribute little to the structural stability and integrity of the pore. A common feature of *rat7*ΔN, *rat7-1*, and *nup82*Δ108 cells is the loss of the Rat7 binding site for Rat8p, constitutively in *rat7*ΔN cells and following a temperature shift to 37°C in *rat7-1* and *nup82*Δ108 cells (which causes dissociation of Rat7p from NPCs) (2, 11). The growth and mRNA export defects of *rat7*ΔN cells can be completely suppressed by overexpression of Rat8p. The experiments reported here indicate that overexpression of Rat8p can also completely suppress the defect in export of heat shock mRNAs in *rat7*ΔN cells shifted to 42°C. In contrast, overexpression of Rat8p in *rat7-1* and *nup82*Δ108 cells permits only slow growth at 37°C, and these cells still show strong accumulation of polyadenylated mRNA in nuclei (2, 11). The most likely explanation for the ability of Rat8p overexpression to suppress completely the growth and export defects of *rat7*ΔN cells is that the absence of the N terminus of Rat7p affects only its interaction with Rat8p but not overall NPC structure or stability. In contrast, we suggest that loss of Rat7p in *nup82*Δ108 or *rat7-1* cells also disrupts NPC structure and explains why Rat8p overexpression is only able to suppress the growth defects of *rat7-1* and *nup82*Δ108 cells to a very limited extent.

We also found that we could not suppress the heat shock export defect of *rip1*Δ cells by overexpressing Rat8p. This suggests that the loss of Gle1p from NPCs in heat-shocked *rip1*Δ cells leads to both disruption of overall NPC structure and loss of one of Rat8p's NPC binding sites. Exposure to 37°C appears to have stabilized NPC structure, since these cells still bound Rat8p and exported heat shock mRNA following a subsequent shift to 42°C.

Thermotolerance and export of mRNA after heat shock. Some cellular processes and structures can be protected from the adverse effects of heat shock by brief exposure to a sub-heat shock temperature. We found that the ability of NPCs lacking Rip1p to maintain export of heat shock mRNA at 42°C could be partially preserved by incubating cells at 37°C before shifting them to 42°C. This was correlated with retaining Rat8p-GFP at NPCs. Interestingly, this thermotolerance treatment did not result in retention of Gle1p-GFP at NPCs. We conclude that a critical function performed by Rip1p during heat shock is to help retain Rat8p at NPCs.

The data also indicate that protein synthesis is adversely affected by heat shock at 42°C and can be protected if cells are first shifted to 37°C and then to 42°C. The data in Fig. 2A show that there was greater production of Ssa4p-GFP if cells were first shifted to 37°C and then to 42°C than in cells shifted directly to 42°C. This can also be seen in the data in Fig. 3B. Although overexpression of Rat8p completely suppressed the defect in heat shock mRNA export in *rat7*ΔN cells (Fig. 4B), the level of Ssa4p-GFP produced was considerably lower than is seen in wild-type cells shifted to 37°C and was similar to the level seen in wild-type cells shifted directly to 42°C.

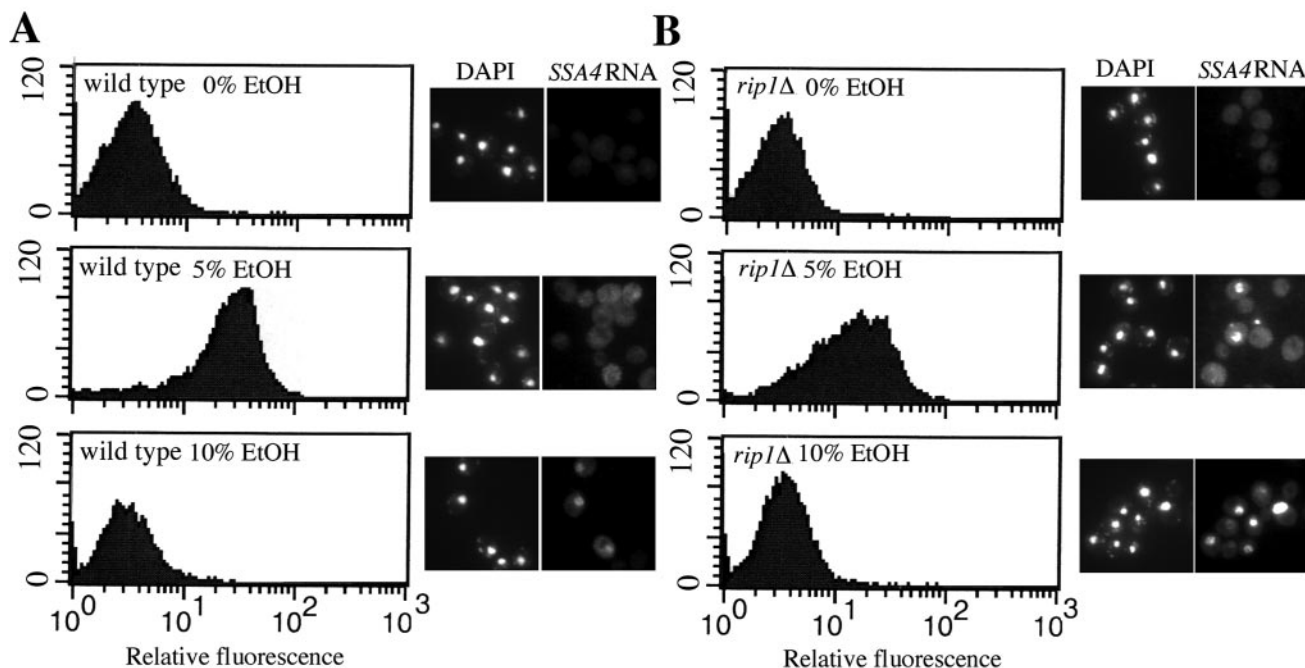


FIG. 6. Rip1p is not required for export of *SSA4* mRNA following ethanol shock. In situ hybridization and flow cytometry were used to analyze *SSA4* RNA export and Ssa4p-GFP protein production in wild-type (A) and *rip1Δ* (B) cells exposed to 5 or 10% ethanol. Cells were also stained with DAPI to permit visualization of nuclei.

We also studied how export of heat shock mRNA was affected by mutations of *RAT8*. The data in Fig. 3A indicate that *rat8-2* cells were defective for export of heat shock mRNA at both 37 and 42°C.

Mislocalization of Rat8p following ethanol stress. Although Rip1p is important for maintaining Rat8p and Gle1p at NPCs after heat shock, we were surprised to find that Rat8p was mislocalized even in wild-type cells following exposure to 10% ethanol. Furthermore, it became primarily nuclear in wild-type cells but mainly cytoplasmic in *rip1Δ* cells exposed to 10% ethanol. We cannot tell whether it is also present at the nuclear rim in wild-type cells, because the nuclear signal is too strong to permit clear visualization of the nuclear rim. The different distribution of Rat8p in wild-type versus *rip1Δ* cells treated with 10% ethanol suggests that nucleocytoplasmic transport of Rat8p is affected by the deletion of *RIP1*. Since Rat8p is a shuttling protein, the data suggest that exposure to 10% ethanol results in reduced export in wild-type cells, reduced import in *rip1Δ* cells, or both. Additional studies will be required to determine the basis for this difference, but other studies in our lab suggest a role for the Nup82p subcomplex (containing Rip1p) in the shuttling and distribution of Rat8p (C. A. Hodge and C. N. Cole, unpublished results).

Interestingly, we found that *rip1Δ* cells were more sensitive to ethanol treatment than were wild-type cells. In addition, the production of Ssa4p-GFP fell off much more rapidly in *rip1Δ* cells than in wild-type cells as the ethanol concentration was increased further. This differential sensitivity is reflected in the data shown in Fig. 5, where we observed nuclear accumulation of *SSA4* mRNA in approximately 30 to 40% of *rip1Δ* cells exposed to 5% ethanol. In the same experiment, there was no

nuclear accumulation of *SSA4* mRNA in wild-type cells. The presence of Rip1p confers on cells the ability to maintain export of stress mRNAs at higher concentrations of ethanol than in its absence. Depending on the growth condition, we were able to observe normal export of *SSA4* mRNA in wild-type cells and its nuclear accumulation in *rip1Δ* cells at some ethanol concentrations and accumulation in both wild-type and *rip1Δ* cells at higher concentrations.

Role of the novel 6-amino-acid motif absent in Rat8pΔ6.

The only defect we have been able to identify for *rat8Δ6* cells is in the heat shock response at 42°C. Since export of heat shock mRNAs induced by exposure to 5% ethanol was not affected by the deletion, the 6 amino acids appear to contribute to maintenance of Rat8p function following heat shock. Although expression of Rat8pΔ6 from a multicopy plasmid suppressed the heat shock mRNA defect, expression from a centromeric plasmid was insufficient for suppression. Although the amounts of Rat8pΔ6 and wild-type Rat8p expressed from the genomic locus were approximately equal in cells incubated at 23 or 37°C, the level of Rat8pΔ6 was approximately one-third that of wild-type Rat8p in cells shifted to 42°C, whereas wild-type Rat8p levels were unchanged (C. Rollenhagen and C. N. Cole, unpublished results). We think it likely that there is more than a quantitative defect in Rat8pΔ6, because cells are able to grow in glucose when Rat8p is expressed from the *GAL1* promoter and Rat8p levels under these conditions are quite low (less than one-fifth as much as in cells grown on glucose) (Hodge and Cole, unpublished). Most likely, Rat8pΔ6 is less functional than wild-type Rat8p but retains sufficient activity that multicopy overexpression provides sufficient Rat8p activity for a strong heat shock response. The phenotype of the

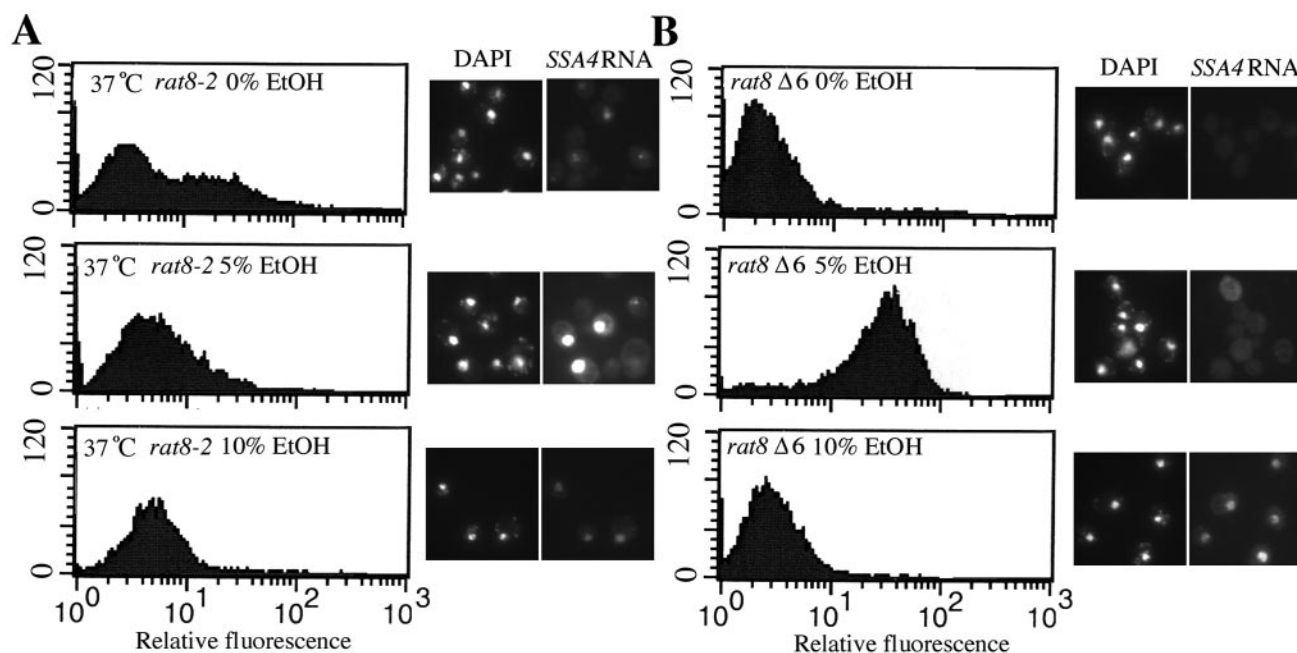


FIG. 7. *RAT8* mutants have a distinct response to ethanol shock. In situ hybridization and a flow cytometry assay were performed on *rat8-2* (A) and *rat8Δ6* (B) cells following exposure to 5 or 10% ethanol. The temperature-sensitive mutant *rat8-2* was shifted to 37°C for 0.5 h followed by 5 or 10% ethanol treatment for 1 h at 37°C. *rat8Δ6* cells were treated with 5 or 10% ethanol for 1 h at room temperature. Cells were also stained with DAPI to permit visualization of nuclei.

rat8Δ6 mutant was completely recessive, as heat shock mRNAs were exported normally in cells carrying both the *rat8Δ6* and wild-type alleles.

Both growth and heat shock mRNA export were normal in *rat8Δ6* cells at temperatures up to 37°C, but these cells were unable to export heat shock mRNAs at 42°C (Fig. 3B and data not shown). In addition, incubation of *rat8Δ6* cells at 37°C did not preserve mRNA export when these cells were subsequently shifted to 42°C. This contrasts with similar treatment of wild-type cells, which retain the ability to export mRNAs normally at 42°C if first incubated at 37°C (data not shown). This inability to preserve the function of a temperature-sensitive Rat8p by thermotolerance treatment contrasts with the ability to preserve wild-type Rat8p's interaction with NPCs in *rip1Δ* cells under the same conditions.

Rat8p is the only DEAD box protein with this insertion, and it is present in Rat8p orthologs found in unicellular organisms, higher plants, and animals. Induction of gene expression at the level of transcription is a major component of how cells respond to stress, so maintaining mRNA export under stress conditions is critical for survival. We speculate that the NGQADP sequence found in Rat8p and all known orthologs evolved to permit cells to maintain mRNA export under conditions of elevated temperature.

ACKNOWLEDGMENTS

We thank Francoise Stutz for providing strains. We thank the Cole laboratory for helpful discussions and Catherine Heath for critically reading the manuscript.

This research was supported by Public Health Service grant GM33998 to C.N.C. from the National Institute of General Medical Sciences, National Institutes of Health.

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