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Following Temperature Stress, Export of Heat Shock mRNA Occurs Efficiently in Cells with Mutations in Genes Normally Important for mRNA Export▽

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Heat shock leads to accumulation of polyadenylated RNA in nuclei of *Saccharomyces cerevisiae* cells, transcriptional induction of heat shock genes, and efficient export of polyadenylated heat shock mRNAs. These studies were conducted to examine the requirements for export of mRNA following heat shock. We used in situ hybridization to detect *SSA4* mRNA (encoding Hsp70) and flow cytometry to measure the amount of Ssa4p-green fluorescent protein (GFP) produced following heat shock. Npl3p and Yra1p are mRNA-binding proteins recruited to nascent mRNAs and are essential for proper mRNA biogenesis and export. Heat shock mRNA was exported efficiently in temperature-sensitive *npl3*, *yra1*, and *npl3 yra1* mutant strains. Nevertheless, Yra1p was recruited to heat shock mRNA, as were Nab2p and Npl3p. Interestingly, Yra1p was not recruited to heat shock mRNA in *yra1-1* cells, suggesting that Npl3p is required for recruitment of Yra1p. The THO complex, which functions in transcription elongation and in recruitment of Yra1p, was not required for heat shock mRNA export, although normal mRNA export is impaired in growing cells lacking THO complex proteins. Taken together, these studies indicate that export following heat shock depends upon fewer factors than does mRNA export in growing cells. Furthermore, even though some mRNA-binding proteins are dispensable for efficient export of heat shock mRNA, those that are present in nuclei of heat shocked cells were recruited to heat shock mRNA.

An emerging theme in our understanding of gene expression is the integration and coordination of the many nuclear events of mRNA biogenesis. mRNA export depends upon both accurate completion of pre-mRNA processing and proper packaging of mRNAs into ribonucleoprotein complexes (mRNPs). The THO complex (Tho2p, Hrp1p, Mft1p, Thp2p) is believed to play a major role in the formation of export-competent mRNPs in *Saccharomyces cerevisiae* by recruiting key proteins to the mRNA before export (37, 45; for a review, see reference 29). An example of this coordination is the recruitment of Sub2p and Yra1p to the THO complex, forming the TREX complex, which is required for efficient elongation and for subsequent recruitment of the mRNA export receptor Mex67p to the elongating mRNA. The overall pathway for gene expression and almost all of the proteins required for packaging and export of mRNA are very highly conserved among eukaryotes, suggesting that mRNA export occurs by the same mechanisms in all eukaryotic cells.

Multiple mechanisms are present to ensure that mRNPs with incompletely or incorrectly processed mRNAs are retained in the nucleus (for reviews, see references 1, 7, and 33). The nuclear exosome, a complex of 3′-to-5′ exoribonucleases, acts to retain defective mRNPs at or near their sites of transcription (14, 19; for a review, see reference 39). Surveillance also involves the Mlp proteins (Mlp1p and Mlp2p), which are associated with the nuclear basket of the nuclear pore complex (NPC) and are thought to participate in quality control over mRNA export by interacting with mRNP proteins, including Nab2p (8, 11).

Heat shock and other stresses cause a radical shift in the pattern of gene expression. At the level of transcription, many genes, including those encoding heat shock proteins, are induced and transcribed at a high rate, while the expression of many others ceases (9). At the level of mRNA processing, splicing is blocked following heat shock (5, 42, 43). Because heat shock mRNAs generally lack introns, they are unaffected by inactivation of splicing. After heat shock, polyadenylated mRNAs accumulate in nuclei of both budding and fission yeast, whereas heat shock mRNAs are exported efficiently (24, 27, 38). In the cytoplasm, the translation of many mRNAs is interrupted following heat shock, and this facilitates efficient synthesis of large amounts of heat shock proteins.

The mechanistic basis for differential mRNA export following heat shock in yeast is not known. Export after stress requires the same nucleoporins as export under normal growth conditions (3, 4, 15, 22, 26). The mRNA export receptor Mex67p (34) and the export factor Dbp5p (15, 18, 26, 32) are also required for heat shock mRNA export.

It is not known how distinct the pathways for general mRNA export in growing cells and export of heat shock mRNAs following heat shock are. Krebber et al. showed that Npl3p, an hnRNP protein that associates with mRNAs during transcription, dissociates from mRNAs following heat shock (23). We reported previously that heat shock mRNA export was not affected when cells carrying the temperature-sensitive (TS) *npl3-1* allele were shifted to 42°C (28). Because Npl3p is nor-

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TABLE 1. Yeast strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
Strains		
FY86 (WT)	*mat*α *his3200 leu21 ura3-52*	40
FY23 (WT)	*mat***a** *trp163 leu21 ura3-52*	40
CSY1037	*mat*α *Yra1*::*KAN ura3-52 leu2Δ1 his3Δ200 trp1Δ63* pRS316 (*URA CEN YRA1*)	This study
FSY1136	*mat***a** *yra12*:: *KAN yra1*::*HIS ade2 his3 leu2 trp1 ura3* pFS1876 (*URA CEN YRA1*)	44
PSY777	*mat*α *ura3-1 his3-11 ade2-1 npl3-1*	P. Silver
CRY12	*mat*α *YRA1*::*KAN ura3-52 leu2Δ1 his3Δ200 trp1Δ63* pFS2142	This study
CRY13	*mat*α *YRA1*::*KAN ura3-52 leu2Δ1 his3Δ200 trp1Δ63* pFS2145	This study
CRY14	*mat*α *YRA1*::*KAN ura3-52 leu2Δ1 his3Δ200 trp1Δ63* pFS2156	This study
CRY16	*mat*α *YRA1*::*KAN ura3-52 leu2Δ1 his3Δ200 trp1Δ63* pFS2168	This study
CRY26	*mat*α *YRA1*::*KAN ura3-52 leu2Δ1 his3Δ200 trp1Δ63* pL20GSTYRA1-C1	This study
CRY27	*mat*α *YRA1*::*KAN ura3-52 leu2Δ1 his3Δ200 trp1Δ63* pL20GSTYRA1-N1	This study
CRY28	*mat*α *YRA1*::*KAN ura3-52 leu2Δ1 his3Δ200 trp1Δ63* pL20GSTYRA1-ΔRRM	This study
CRY26	*mat*α *YRA1*::*KAN ura3-52 leu2Δ1 his3Δ200 trp1Δ63* pL20GSTYRA1-1	This study
*tho2*Δ	*mat***a** *his3Δ1 leu2Δ0 lys2D0 ura3Δ0 THO2*::*kanMX4*	EUROSCARF
*thp2*Δ	*mat***a** *his3Δ1 leu2Δ0 lys2D0 ura3Δ0 THP2*::*kanMX4*	EUROSCARF
sub2-85	*mat***a** *ura3 his3 leu2 trp1 sub2*::*kanMX4* pTrp-*CEN sub2-85*	35
Plasmids		
pFS2142	*YRA1*-HA *CEN TRP1*	44
pFS2145	*YRA1*-HA *1-167 CEN TRP1*	44
pFS2156	*YRA1*-HA 77-227 *CEN TRP1*	44
pFS2168	*YRA1*-HA ΔRRM *CEN TRP1*	44
pL20GSTYRA1-C1	*YRA1* ΔC1 184-209 *CEN TRP1*	35
pL20GSTYRA1-N1	*YRA1* ΔN1 1-17 *CEN TRP1*	35
pL20GSTYRA1-RRM	*YRA1* ΔRRM *CEN TRP1*	35
pL20GSTYRA1-1	*YRA1-1 CEN TRP1*	35
pCR2	*SSA4-GFP*; integrating; *LEU2*	26
pCH15	*SSA4 CEN LEU2*	26
pCH19	*SSA4-GFP*; integrating; *URA3*	12
pCH16	*SSA4 CEN URA3*	6

mally required for mRNA export, its dissociation from mRNA following heat shock could be part of the mechanism underlying selective mRNA export. Whether another protein performs the functions of Npl3p for heat shock mRNAs is not known. The studies described here were conducted to examine the effects of mutations affecting other mRNA-binding proteins and additional mRNA export factors on heat shock mRNA export. We also analyzed recruitment of mRNA-binding proteins to *SSA4* mRNA (encoding Hsp70).

Heat shock mRNA export was not affected by mutation of several other mRNA-binding proteins, including Yra1p. Interestingly, double mutants carrying the *npl3-1* allele and several *yra1* mutant alleles were still capable of heat shock mRNA export. Yra1p was loaded onto heat shock mRNAs in wild-type (WT) cells but not in *npl3-1* cells, suggesting that Npl3p participates in the recruitment of Yra1p to mRNA. Export of *SSA4* mRNA following heat shock does not require the THO complex. Because heat shock mRNA export occurs efficiently in strains that contain mutations affecting proteins normally important for efficient mRNA export, export after heat shock may have a reduced requirement for accurate formation of mRNPs.

MATERIALS AND METHODS

Yeast strains, genetic methods, growth conditions, and antibodies. Yeast strains were grown in yeast extract-peptone-dextrose (YPD)-rich medium or in synthetic complete medium lacking leucine, tryptophan, or both. Yeast transformation was performed using a standard lithium acetate method (25). The yeast strains and plasmids used in these studies are listed in Table 1.

The *YRA1* shuffle strain (CSY1037) was obtained by replacement of one copy of the *YRA1* gene in a wild-type diploid with a kanamycin resistance gene. The strain was transformed with a *URA3 CEN* plasmid containing *YRA1*. The desired strain was isolated following sporulation and selection for haploids that are kanamycin resistant and able to grow on media lacking uracil.

The *YRA1* mutant strains were obtained by transforming plasmids encoding mutants of *YRA1* both with and without HA epitope tags into the *YRA1* shuffle strain. The former were obtained from Francoise Stutz (44) and the latter from Ed Hurt (35). After transformation, the cells were plated on synthetic complete plates lacking tryptophan. After 2 days, a single colony was streaked onto a 5-fluoro-orotic acid (5-FOA) plate and incubated an additional 2 days. Colonies from those plates lack the wild-type copy of *YRA1* and contain a mutant form of *YRA1* as the only copy.

yra1 npl3-1 double mutants were made by crossing the *YRA1* shuffle strain

CSY1037 with the *npl3-1* strain PSY777. Colonies were replica plated, and those that did not grow at 37°C and on 5-FOA plates were detected as the double-mutant strains containing both the *yra1* disruption and the *npl3-1* allele. *yra1* mutant plasmids were transformed into the double-mutant strain. Selection for cells able to grow on 5-FOA plates permitted the isolation of strains lacking the wild-type copy of *YRA1*.

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.

Monoclonal antibodies to Npl3p and Nab2p were obtained from M. Swanson, University of Florida, Gainesville, FL.

Growth assay. Cells were grown overnight in 5 ml of YPD. Cell suspensions were diluted to an optical density at 600 nm (OD_{600}) of 0.05, and 1:5 serial dilutions were spotted onto YPD plates. The plates were incubated for 4 days at both 30°C and 34°C.

SSA4 in situ hybridization assay. In situ hybridization was performed to localize *SSA4* mRNA, as described previously (2). Yeast strains containing plasmid-based *SSA4* on a 2μ high-copy plasmid were grown overnight to a maximum OD_{600} of 0.5. Images were obtained using a Zeiss Axioplan 2 fluorescence microscope equipped with a cooled charge-coupled-device camera and 100× and 63× objective lenses. The distribution of *SSA4* mRNA and the locations 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 temperature stress. Yeast strains containing an integrated *SSA4-GFP* allele were grown in selective media overnight to a maximum OD_{600} of 0.5. Cultures were shifted to 42°C for 30 min, collected by centrifugation at 2,000 rpm at 4°C for 2 min, and resuspended in ice-cold phosphate-buffered saline, followed by incubation on ice. Cell 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 numbers of cells with various GFP signal intensities were obtained by using Cell Quest software (Becton Dickinson). Each experiment was repeated at least twice.

***SSA4* mRNP IP experiments.** The experiments were performed based on a previously published protocol (17). Wild-type and mutant strains encoding hemagglutinin (HA)-tagged proteins were grown in 50 ml of YPD overnight at room temperature. The OD_{600}s of the cultures did not exceed 0.5. Cells were shifted to 42°C for 30 min, collected by centrifugation, and washed once with Tris-buffered saline. Cell pellets were resuspended in 1 ml of RNA-immunoprecipitation (IP) buffer (25 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.2% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride) supplemented with a protease inhibitor mixture (Roche), 5 mM dithiothreitol, and 10 units/ml RNasin (Invitrogen). The cells were lysed by vortexing them with glass beads in the cold room for 10 min. The lysate obtained after centrifugation (15 min, 13,000 rpm) was used for immunoprecipitations. Thirty microliters of immunoglobulin G beads (EZview Red Protein G affinity gel; Sigma Chemical Co.) was used for each immunoprecipitation and was washed in RNA-IP buffer. Antibodies were added to the beads on ice. Thirty microliters of the bound antibody-bead fraction was added to 400 μl of lysate, RNA-IP buffer was added to a 1.5-ml total volume, and the samples were rotated overnight at 4°C. The beads were then washed five times with RNA-IP buffer. The RNAs in the immunoprecipitates and the total lysate samples were precipitated by adding a 1/10 volume of 3 M sodium acetate and 1 ml of 100% ethanol. The pellet was washed with 70% ethanol, dried, and resuspended in 40 μl of RNase-free water. One microliter of DNase (RNase free; Roche) was added to each sample and incubated at 37°C for 10 min. DNase was deactivated by incubation for 1 h at 65°C. A 12.5-μl volume of each sample and 2.5 μl from the total lysate sample were used for a 20-μl reverse transcription reaction mixture (Invitrogen). From this reaction mixture, 4 μl of each sample was used for a 50-μl PCR mixture. The same *SSA4* primers used to produce the *SSA4* in situ hybridization probe were used. Ten microliters of each PCR was loaded onto a 1% agarose gel. Photographs were taken following electrophoresis. Each experiment was repeated at least twice.

RESULTS

Dependence of heat shock mRNA export on export factors and mRNA-binding proteins. We employed two assays to analyze heat shock gene expression: in situ hybridization to determine how various mutations affected the subcellular distribution of *SSA4* mRNA (encoding Hsp70) and flow cytometry to analyze production of Ssa4p. In all cases, Ssa4p-GFP was expressed from the genomic *SSA4* locus. Flow cytometry has a large dynamic range and can readily distinguish between a complete and a nearly complete defect in export of heat shock mRNA. In contrast, the in situ assay can readily distinguish between normal export of *SSA4* mRNA and limited nuclear accumulation under conditions where some *SSA4* mRNA is exported, but this approach is considerably less able to distinguish between a complete and nearly complete block in *SSA4* mRNA export.

Using these assays, we compared mutant strains (the *npl3-1*, *mex67-5*, and *rip1*Δ strains) whose heat shock response we had analyzed previously but not by flow cytometry (Fig. 1). At 23°C in all strains, there was virtually no *SSA4* mRNA detected by in situ hybridization and the very low Ssa4p-GFP signal seen using flow cytometry represents the background and is equal to the signal that would be obtained if GFP were not fused to heat shock mRNA. Heat shock in wild-type cells led to robust expression of Ssa4p-GFP; *SSA4* mRNA could be detected throughout the cell, and a high level of Ssa4p-GFP was produced (Fig. 1A). *rip1*Δ cells served as a negative control since there is no export of any mRNA after heat shock at 42°C (28). *SSA4* mRNA accumulated in nuclei, and there was no increase in the Ssa4p-GFP signal (Fig. 1B). In *npl3-1* cells (Fig. 1C), there was a strong induction of *SSA4* mRNA production. A small fraction of *npl3-1* cells showed limited nuclear accumulation of *SSA4* mRNA. Although Ssa4p-GFP was produced, a broad range of fluorescence intensities was seen and less Ssa4p-GFP was present than in wild-type cells. A very low level of *SSA4* mRNA could be detected in the cytoplasm of *mex67-5* cells shifted to 42°C, but all cells accumulated *SSA4* mRNA in their nuclei and there was no production of Ssa4p-GFP (Fig. 1D). These results serve as a basis for evaluating the requirements for mRNA export following heat shock in other mutant yeast strains.

The FACS assay reflects both the amount of heat shock mRNA exported and the efficiency of its translation for production of Ssa4p-GFP. Because the translatability and stability of *SSA4* mRNA can be affected by various mutations, the amount of Ssa4p-GFP can be lower than that in wild-type cells even when the same amount of *SSA4* mRNA is exported.

***yra1* mutant strains are able to export heat shock mRNA following heat shock.** Yra1p plays an important role in mRNA biogenesis. It is recruited to the mRNA during mRNA synthesis by the THO complex (21, 37) and then recruits Mex67p to the mRNP (36). Poly(A)$^+$ mRNA accumulates in nuclei when Yra1p is depleted or when *yra1-1* cells are shifted to 37°C. The central region of Yra1p is essential and contains RNA recognition motifs (RRMs). Deletions that remove the N or C terminus or the central RRMs are all viable, though some grow less well than the wild type at 37°C and show nuclear accumulation of poly(A)$^+$ RNA at elevated temperatures (35, 44). Both the N- and the C-terminal domains of Yra1p can bind to Mex67p, and a mutant lacking both termini is inviable.

We examined export of heat shock mRNA at 42°C in several *yra1* mutant strains (35). No defect in export of *SSA4* mRNA at 42°C was seen in *yra1* mutants lacking either the N terminus (*yra1*ΔN, lacking aa 1 to 17), the C terminus (*yra1*ΔC, lacking aa 184 to 209), or the RRM domain (*yra1*ΔRRM, lacking aa 76 to 183). Surprisingly, no defect in *SSA4* mRNA export was

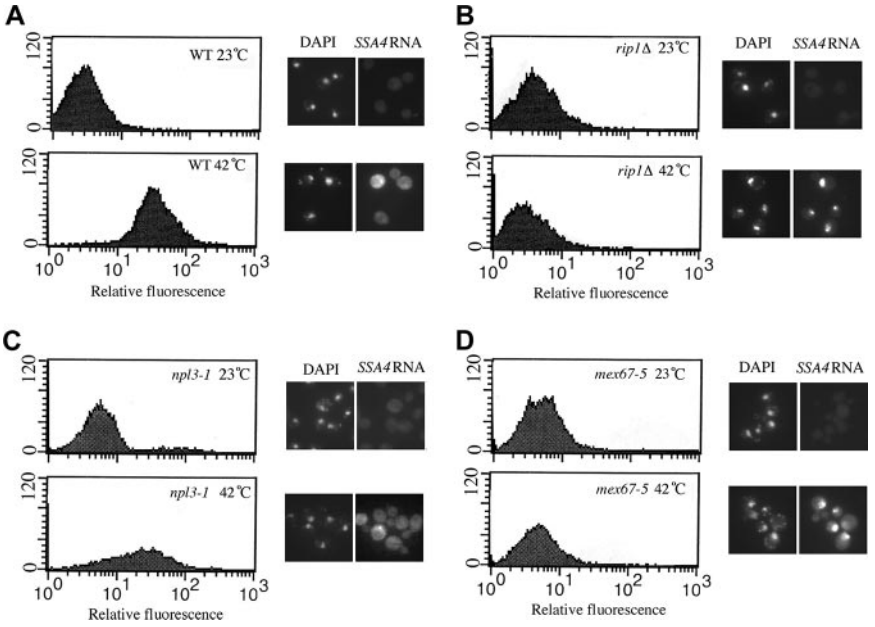

FIG. 1. The temperature-sensitive transport factor mutant *mex67-5* and the nucleoporin *rip1*Δ have a defect in *SSA4* gene expression. In situ hybridization was performed to monitor the localization of *SSA4* mRNA, and flow cytometry was used to analyze Ssa4p-GFP protein production. (A) Wild type; (B) *rip1*Δ; (C) *npl3-1*; (D) *mex67-5*. Cells were shifted to 42°C for 1 h prior to analyses. A DAPI (4′,6′-diamidino-2-phenylindole) stain was used to permit visualization of nuclei. For FACS, 10,000 cells were analyzed.

seen with the temperature-sensitive *yra1-1* allele, even though this mutant is quite defective in export of polyadenylated mRNA at 37°C. The FACS analyses showed that all four mutant strains produced approximately the same amount of Ssa4p-GFP (Fig. 2) as the wild type (Fig. 1). Yra2p is a nonessential homolog of Yra1p produced at a much lower level than Yra1p. Overexpression of Yra2p permits growth of cells lacking Yra1p. We constructed double-mutant strains by deleting *YRA2* from each of the *yra1* mutants under study. Deletion of *YRA2* had no effect on the export of *SSA4* mRNA following heat shock (data not shown). We conclude that neither Yra1p nor Yra2p is required for stress mRNA export.

Yra1p lacking the RNA-binding domain (RRM) is unable to bind to heat shock RNA. We determined whether the proteins encoded by *yra1* mutants were bound to *SSA4* mRNA. Strains producing HA epitope-tagged forms of Yra1p as the only form of Yra1p present (44) were heat shocked at 42°C for 30 min. Yra1p-HA and associated RNA were immunoprecipitated, and reverse transcription (RT)-PCR was used to determine whether *SSA4* mRNA had been coprecipitated (Fig. 2E). For controls, immunoglobulin G Sepharose beads were incubated with the HA antibody, either without any lysate (Fig. 2E, lane 1) or with a lysate from a wild-type strain producing untagged Yra1p. The *SSA4* PCR product was barely detectable in the untagged control, indicating a very low level of nonspecific binding of *SSA4* mRNA to the beads or antibody. In Fig. 2E, the even-numbered lanes show *SSA4* in lysates and the odd-numbered lanes reflect *SSA4* RNA pulled down in association with HA-tagged Yra1p. A prominent *SSA4* band was observed in cells producing wild-type Yra1p-HA, indicating that Yra1p was bound to *SSA4* mRNA at 42°C (Fig. 2E, lane 5). *SSA4* mRNA was also pulled down with Yra1pΔC (Fig. 2E, lane 7) and Yra1pΔN (Fig. 2E, lane 9). Yra1p lacking the RRM did not interact with *SSA4* mRNA in this assay (Fig. 2E, lane 11). Although Yra1p is not required for export of heat shock mRNA, the data indicate that wild-type Yra1p and some mutant Yra1ps were recruited to heat shock mRNA.

***npl3-1 yra1* double mutants are capable of heat shock response.** We mated *npl3-1* to *yra1* mutants and attempted to isolate *npl3 yra1* double mutants. *yra1-1* and *npl3-1* are synthetically lethal (data not shown), but we were able to isolate double mutants containing *yra1*ΔC, *yra1*ΔN, or *yra1*ΔRRM. Growth assays (Fig. 3A) indicate that the single *yra1* mutants grew in nearly the same fashion at 30°C and 34°C, while the double mutants were temperature sensitive and grew very poorly or not at all at 34°C.

We next examined the abilities of the double mutants to produce *SSA4* mRNA and Ssa4p-GFP following heat shock. In all cases, *SSA4* mRNA was induced and exported after heat shock (Fig. 3B to D). In the *npl3-1 yra1*ΔC strain (Fig. 3C), most cells showed nuclear foci of *SSA4* mRNA along with a cytoplasmic *SSA4* mRNA signal with an intensity similar to that seen with the other mutants, but this is often seen even with wild-type cells. The levels of Ssa4p-GFP produced were similar to those seen with *yra1* single mutants (Fig. 2).

***npl3-1* cells are defective in recruiting Yra1p to *SSA4* mRNA.** The *npl3-1* mutant does not have an *SSA4* mRNA export defect. We wondered whether the mutant protein could be recruited to *SSA4* mRNA and whether recruitment of other mRNA-binding proteins would be affected by the *npl3-1* mutation. We performed *SSA4* mRNA pulldown experiments with *npl3-1 yra1* double-mutant strains (encoding HA-tagged Yra1p), as described above for the single *yra1* mutants. Interestingly, wild-type Yra1p was not bound to *SSA4* mRNA after heat shock in *npl3-1* mutant cells

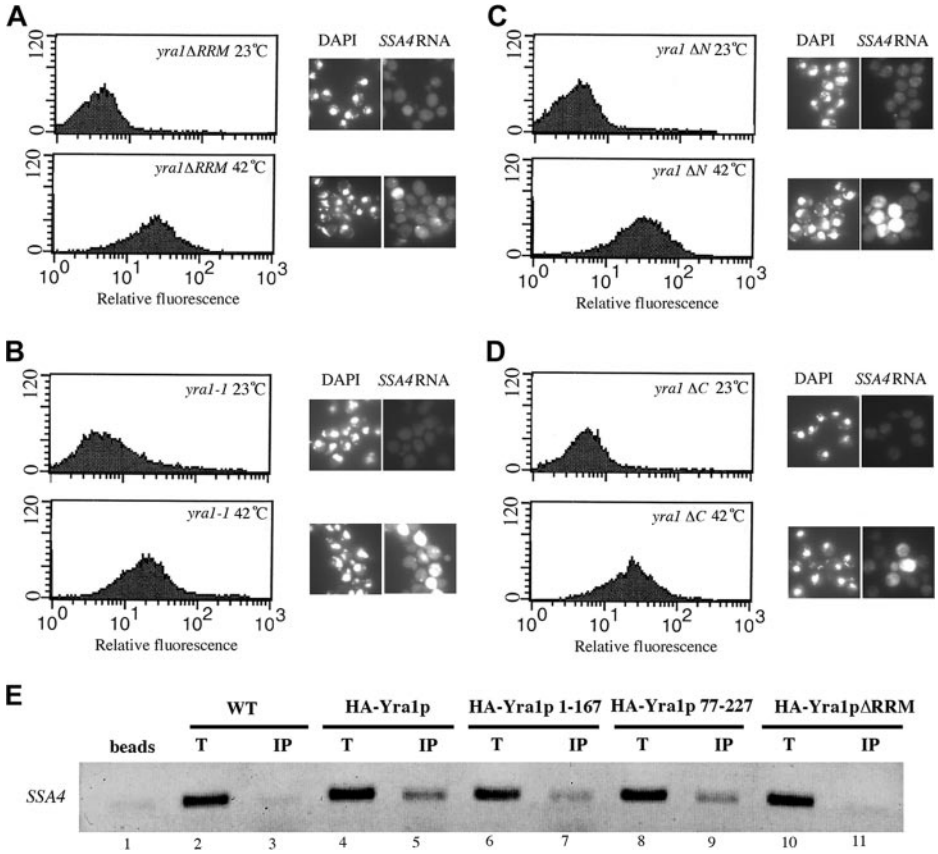

FIG. 2. *yra1* mutants are not defective for heat shock gene expression. In situ hybridization and flow cytometry were used to examine the distribution of *SSA4* mRNA and production of Ssa4p-GFP in *yra1*ΔRRM (lacking aa 76 to 183) (A), *yra1-1* (B), *yra1*ΔN (lacking aa 1 to 17) (C), and *yra1*ΔC (lacking aa 184 to 209) (D) cells that were heat shocked for 1 h. Nuclei were DAPI (4′,6′-diamidino-2-phenylindole) stained, and 10,000 cells were analyzed by flow cytometry. (E) Yra1p is able to bind SSA4 mRNA but is not required for export of *SSA4* mRNA. Shown are *SSA4* gene PCR products from reverse-transcribed RNA that was coimmunoprecipitated (lanes IP) using an anti-HA antibody and HA-tagged *yra1* mutants. These constructs have deletions slightly different from those used for panels A to D. As controls, immunoprecipitation of WT cells that do not express an HA-tagged protein (lane 3), analysis of material bound to beads in the absence of antibody (lane 1), and RT-PCRs using as a template total whole-cell extracts prior to immunoprecipitation (lanes T) are shown. The amount of material in the T lanes represents 1/10 that in the IP lanes.

(Fig. 4A, lane 7) but was bound to *SSA4* mRNA in the wild-type (*NPL3*) strain (Fig. 4A, lane 5). This is additional evidence that Yra1p is not required for export of *SSA4* heat shock mRNA following heat shock, because the *npl3-1* strain is capable of heat shock mRNA export. Moreover, it suggests that Npl3p may be involved in recruitment of Yra1p to *SSA4* mRNA, although this could be indirect. In contrast, in *npl3-1* cells under normal growth conditions (23°C, permissive for *npl3-1* cells), Yra1p was bound to the mRNA of a normal housekeeping gene, *ADH1* (Fig. 4A). The levels of *ADH1* and other non-stress response mRNAs were too low following heat shock to determine what mRNA-binding proteins were bound to the mRNAs (data not shown). None of the mutant *yra1* proteins was recruited to heat shock mRNA in *npl3-1* cells (data not shown).

Because Yra1p was not detected on *SSA4* mRNA after heat shock in *npl3-1* cells, we wondered whether Npl3-1p or Nab2p was bound to *SSA4* mRNA. The data in Fig. 4B indicate that Npl3p (Fig. 4B, lane 5) and mutant Npl3-1p (Fig. 4B, lane 9) were each bound to *SSA4* mRNA. This finding was verified in a pulldown experiment using GFP-tagged Npl3p (data not shown). We also observed that Nab2p was bound to *SSA4* mRNA in both the wild type (Fig. 4B, lane 7) and the *npl3-1* mutant strain (Fig. 4B, lane 11). Taken together, the data indicate that Yra1p, Npl3p, and Nab2p become part of the *SSA4* mRNP after stress in wild-type cells. In addition, Nab2p but not Yra1p can be recruited to *SSA4* mRNP in *npl3-1* cells, even though Npl3-1p itself associates with *SSA4* mRNA.

The THO complex is not required for export of heat shock mRNA. The THO complex of four proteins (Tho2p, Thp2p, Hpr1p, and Mft1p) is thought to function in transcription elongation and in recruitment of Yra1p and Sub2p to nascent mRNPs. Each of the genes encoding THO complex proteins is not essential, but cells lacking any of the four proteins are ts for growth and mRNA export (37). The phenotypes of mutants lacking two THO complex subunits are very similar to those of mutants lacking a single subunit (A. Aguilera, personal communication), suggesting that in the absence of any one subunit, the complex does not form (16). Since the complex is not essential under some conditions, alternate pathways for recruiting Yra1p and Sub2p are thought to exist (20). We analyzed export of *SSA4* mRNA in the single-mutant strains each

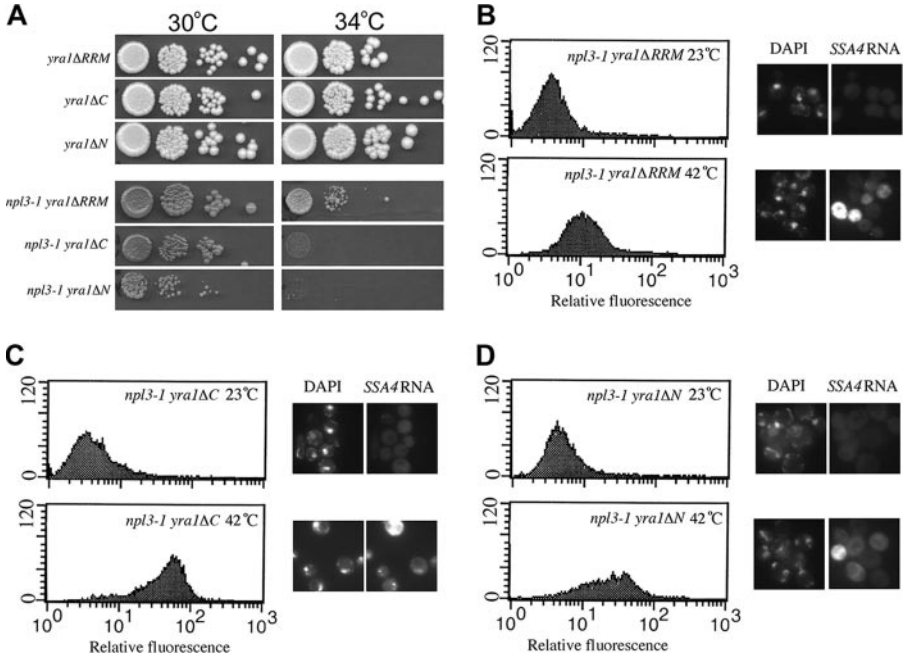

FIG. 3. *npl3-1* and *npl3-1 yra1* double mutants do not exhibit an *SSA4* heat shock message export defect and are capable of Ssa4p-GFP stress protein production. (A) Growth behavior of *npl3 yra1* double mutants. Strains were grown on selective media and diluted to an OD_{600} of 0.05, and 1:5 serial dilutions were spotted onto plates, which were incubated for 4 days at the temperatures indicated. (B to D) In *situ* hybridization and flow cytometry were employed to investigate the distribution of *SSA4* mRNA and production of Ssa4p-GFP in *npl3-1 yra1*ΔRRM (B), *npl3-1 yra1*ΔC (C), and *npl3-1yra1*ΔN (D) cells that were heat shocked for 1 h. Nuclei were DAPI (4′,6′-diamidino-2-phenylindole) stained, and 10,000 cells were measured by flow cytometry.

lacking one THO complex component. The data for *thp2*Δ and *tho2*Δ are shown in Fig. 5A and B, but the same phenotypes were seen for each of the mutant THO mutant strains (data not shown). Each of the THO mutants produced approximately wild-type levels of Ssa4p-GFP (compare Fig. 5 with Fig. 1). As is the case with wild-type cells, poly(A)$^+$ mRNA accumulated in nuclei of THO complex mutants following heat shock (31, 37; also data not shown).

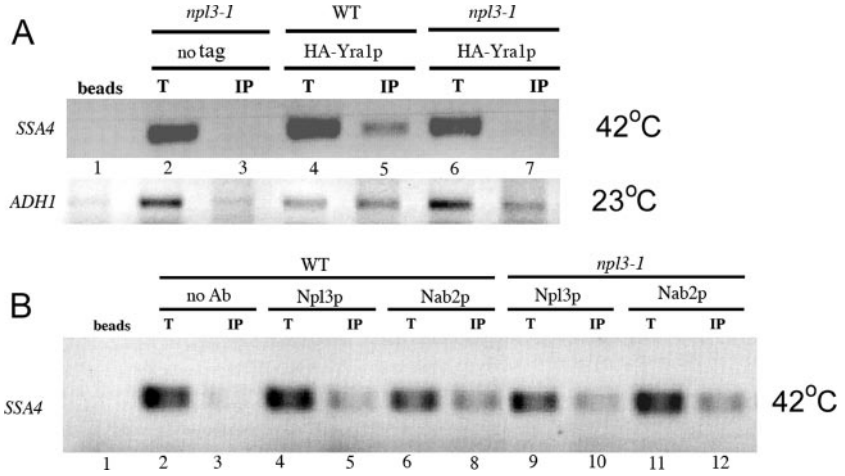

FIG. 4. (A) Yra1p does not bind *SSA4* mRNA in *npl3-1* cells at 42°C. Shown are *SSA4* PCR products from reverse-transcribed RNA that was coimmunoprecipitated (lanes IP) by using an anti-HA antibody and lysates prepared from cells expressing HA-tagged Yra1p. As controls, immunoprecipitation of wild-type cells expressing Yra1p-HA (lane 5), *npl3-1* cells not expressing HA-Yra1p (lane 3), analysis of beads only (lane 1), and RT-PCR of whole-cell extracts as a template (lanes T) without immunoprecipitation (lane 4) are shown. Also shown is a similar analysis of *ADH1* mRNA in cells grown at 23°C. (B) *SSA4* mRNA interacts with Npl3p and Nab2p in WT and *npl3* cells at 42°C. Shown are *SSA4* gene PCR products from reverse transcribed RNA that was coimmunoprecipitated (IP) from lysates of WT and *npl3-1* cells using antibodies against Npl3p and Nab2p. As controls, immunoprecipitation of WT cells using only secondary antibody, analysis of beads only, and RT-PCR of whole-cell extracts as a template (lanes T) are shown. The amount of material in the T lanes represents 1/10 that in the IP lanes.

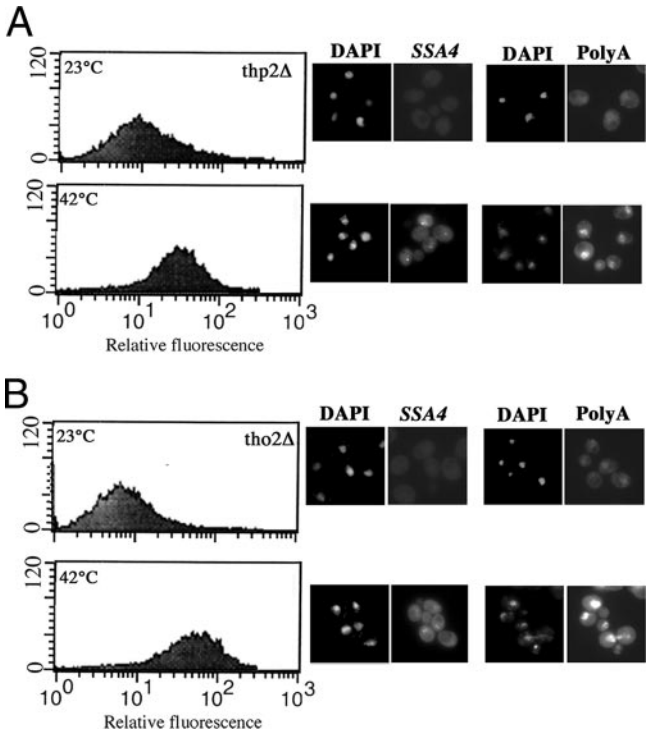

FIG. 5. The THO complex is not required for export of *SSA4* mRNA and production of Ssa4p-GFP. In situ hybridization and flow cytometry were employed to examine the distribution of *SSA4* mRNA and the levels of Ssa4p-GFP in *thp2*Δ (A) and *tho2*Δ (B) cells. Cells were shifted to 42°C for 1 h prior to analyses. A DAPI (4′,6′-diamidino-2-phenylindole) stain was used to permit visualization of nuclei. For FACS, 10,000 cells were analyzed.

DISCUSSION

The only protein identified previously as important for normal mRNA export and dispensable for export of heat shock mRNAs is the mRNP protein Npl3p, which dissociates from polyadenylated RNA following heat shock, and much of it moves to the cytoplasm (23). mRNA export could be regulated following stress if the loss of Npl3p from most mRNAs prevented their export. Heat shock mRNA might associate specifically with one or more mRNA-binding proteins that are not associated with other mRNAs following heat shock, thereby permitting heat shock mRNAs to achieve an exportable configuration. The data presented here show that Npl3p was bound to *SSA4* mRNA following heat shock (Fig. 4B) and might contribute to its export.

Defining the requirements for export of heat shock mRNA. Yra1p is thought to recruit Mex67p to mRNA (36, 37, 44) and, unlike Npl3p, is believed to be removed from mRNPs prior to export. To examine whether Yra1p was required for heat shock mRNA export, we used both a ts point mutant, *yra1-1*, and three viable mutants whose deletions remove sequences from the N-terminal, C-terminal, or central RRM regions of the protein (35). None was defective for export of *SSA4* mRNA, and the amount of Ssa4-GFP produced in the *yra1* mutant strains was nearly the same as that produced in wild-type cells (compare Fig. 2A to D with Fig. 1A). Even though Yra1p does not appear to be required for heat shock mRNA export, wild-type Yra1p as well as Yra1p-ΔC and Yra1p-ΔN became associated with heat shock mRNA (Fig. 2E). We also found that Nab2p and mutant Npl3-1p associated with *SSA4* mRNA in wild-type cells (Fig. 4B). Together, these findings indicate that while Npl3p and Yra1p may not be required for heat shock mRNA export, both wild-type and mutant Yra1p and Npl3p can associate with *SSA4* mRNA if they are present.

npl3-1 yra1 double-mutant strains were also capable of exporting heat shock message and producing Ssa4p-GFP (Fig. 3). Interestingly, recruitment of Yra1p to heat shock mRNA did not occur in *npl3-1* cells (Fig. 4). One possibility is that other mRNA-binding proteins perform the function of Yra1p in cells where it is not recruited to heat shock mRNA. Hrb1p and Gbp2p are two nonessential mRNA-binding proteins. Each could be recruited to heat shock mRNA, and heat shock mRNA export was unaffected in Δ*hrb1* Δ*gbp2* double-mutant cells (unpublished results).

None of the THO complex components of TREX are required for heat shock mRNA export (Fig. 5 and data not shown), but Sub2p, which is recruited by THO, is essential for mRNA export, both in growing cells and after heat shock (data not shown). Because THO complex components are not essential for growth or heat shock gene expression, it is likely that Sub2p has another way to associate with the nascent heat shock mRNP. The finding that overexpression of Sub2p suppresses the mRNA export defect of *hpr1*Δ is consistent with this (20).

Pathways for mRNA export. Whether multiple distinct pathways for mRNA export exist is not known. At one extreme, export of all mRNAs might utilize all of the same factors. At the other, there could be two or more classes of mRNA, each using a different set of mRNA export and packaging factors. Whether NPCs that are specialized for export of a subset of mRNAs exist is also not known. Even though all NPCs contain the same nucleoporins, NPCs juxtaposed to the nucleolus are not associated with Mlp1p (8) and these NPCs are thought to be used primarily for export of ribosomal subunits. There is no evidence that any NPCs are specific for export of a subset of mRNAs.

Hieronymus and Silver showed that the set of mRNAs that could be coimmunoprecipitated with Mex67p overlapped only partially with the set for Yra1p (13), and some mRNAs were not coprecipitated with either protein. One interpretation of these results is that other proteins are present and provide to some mRNAs the functions of Mex67p and Yra1p. However, it is not known whether an mRNA with a single molecule of Mex67p or Yra1p can be coprecipitated efficiently, so it is possible that all species of mRNA utilize Mex67p and Yra1p. Furthermore, some mRNAs may be sufficiently inabundant in nuclei that very few of these mRNAs would be coprecipitated even if associated with Mex67p or Yra1p. Interestingly, Mex67p is not required for mRNA export in *Schizosaccharomyces pombe* (41). In contrast, *S. pombe* requires Rae1p whereas its orthologue in *S. cerevisiae*, Gle2p, is not required for mRNA export.

The DEAD box protein Dbp5p is essential, and mutations affecting Dbp5p result in very rapid onsets of accumulation of poly(A)$^+$ mRNA in all nuclei at nonpermissive temperatures (32) and after heat shock (28). Although Dbp5 is also essential in mammalian cells (30), it does not appear to be required for mRNA export in *Drosophila* (10). Because so many of the

proteins important for mRNA export are highly conserved, it is likely that mRNA export occurs by identical mechanisms in all nucleated cells. We think it most likely that some organisms contain two or more proteins able to perform the same function during mRNA export while others contain a single gene product.

We have conducted multiple screens to identify proteins important for heat shock mRNA export and have not identified any transport factors in yeast that are required only for export after stress. In the screen performed using the strain that produces Ssa4p-GFP under control of the *SSA4* promoter, we mutagenized cells, sorted using flow cytometry, collected the darkest 1% of cells, and then identified those strains in this set that were temperature sensitive for growth (12). We also screened our original collection of temperature-sensitive strains for those that accumulated *SSA4* mRNA in their nuclei following heat shock (12). In this screen, we studied further only those strains that were temperature sensitive, because this allowed us to identify the mutant genes by complementation. We attempted to identify novel nonessential genes important for heat shock mRNA export by performing transposon-mediated mutagenesis in the Ssa4p-GFP strain and again sorting cells to isolate the darkest 1%. Using a transposon as a tool to identify mutated genes, we studied dark strains that were not temperature sensitive for growth, and the only gene found to be inactivated by the transposon was *SSA4-GFP* (C. A. Heath and C. N. Cole, unpublished results). This suggests that there are very few or no nonessential genes whose inactivation would result in a defect specifically in heat shock mRNA export.

In summary, the data presented here indicate that under stress conditions there are more relaxed requirements for packaging mRNA for export, in that Yra1p and Npl3p are not required. This could lead to decreased accuracy in formation of export-competent heat shock mRNPs. However, the levels of Ssa4pGFP are approximately the same in wild-type cells and in many mutants that affect mRNA-binding proteins. Possibly, heat shock mRNAs are more readily packaged into an exportable configuration than are some normal cellular mRNAs. This could result from their having less potential for formation of secondary structure or from other features of heat shock mRNA biogenesis. There is at present no evidence for the existence of heat shock-specific mRNA export factors or for the existence of more than one mRNA export pathway.

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REFERENCES

1. **Casolari, J. M., and P. A. Silver.** 2004. Guardian at the gate: preventing unspliced pre-mRNA export. Trends Cell Biol. **14:**222–225.
2. **Cole, C. N., C. V. Heath, C. A. Hodge, C. M. Hammell, and D. C. Amberg.** 2002. Analysis of RNA export. Methods Enzymol. **351:**568–587.
3. **Del Priore, V., C. Heath, C. Snay, A. MacMillan, L. Gorsch, S. Dagher, and C. Cole.** 1997. A structure/function analysis of Rat7p/Nup159p, an essential nucleoporin of Saccharomyces cerevisiae. J. Cell Sci. **110:**2987–2999.
4. **Del Priore, V., C. A. Snay, A. Bahr, and C. N. Cole.** 1996. The product of the Saccharomyces cerevisiae RSS1 gene, identified as a high-copy suppressor of the rat7-1 temperature-sensitive allele of the RAT7/NUP159 nucleoporin, is required for efficient mRNA export. Mol. Biol. Cell **7:**1601–1621.
5. **Denegri, M., I. Chiodi, M. Corioni, F. Cobianchi, S. Riva, and G. Biamonti.** 2001. Stress-induced nuclear bodies are sites of accumulation of pre-mRNA processing factors. Mol. Biol. Cell **12:**3502–3514.
6. **Dunn, E. F., C. M. Hammell, C. A. Hodge, and C. N. Cole.** 2005. Yeast poly(A)-binding protein, Pab1, and PAN, a poly(A) nuclease complex recruited by Pab1, connect mRNA biogenesis to export. Genes Dev. **19:**90–103.
7. **Fasken, M. B., and A. H. Corbett.** 2005. Process or perish: quality control in mRNA biogenesis. Nat. Struct. Mol. Biol. **12:**482–488.
8. **Galy, V., O. Gadal, M. Fromont-Racine, A. Romano, A. Jacquier, and U. Nehrbass.** 2004. Nuclear retention of unspliced mRNAs in yeast is mediated by perinuclear Mlp1. Cell **116:**63–73.
9. **Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown.** 2000. Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell **11:**4241–4257.
10. **Gatfield, D., H. Le Hir, C. Schmitt, I. C. Braun, T. Kocher, M. Wilm, and E. Izaurralde.** 2001. The DExH/D box protein HEL/UAP56 is essential for mRNA nuclear export in Drosophila. Curr. Biol. **11:**1716–1721.
11. **Green, D. M., C. P. Johnson, H. Hagan, and A. H. Corbett.** 2003. The C-terminal domain of myosin-like protein 1 (Mlp1p) is a docking site for heterogeneous nuclear ribonucleoproteins that are required for mRNA export. Proc. Natl. Acad. Sci. USA **100:**1010–1015.
12. **Hammell, C. M., S. Gross, D. Zenklusen, C. V. Heath, F. Stutz, C. Moore, and C. N. Cole.** 2002. Coupling of termination, 3′ processing, and mRNA export. Mol. Cell. Biol. **22:**6441–6457.
13. **Hieronymus, H., and P. A. Silver.** 2003. Genome-wide analysis of RNA-protein interactions illustrates specificity of the mRNA export machinery. Nat. Genet. **33:**155–161.
14. **Hilleren, P., T. McCarthy, M. Rosbash, R. Parker, and T. H. Jensen.** 2001. Quality control of mRNA 3′-end processing is linked to the nuclear exosome. Nature **413:**538–542.
15. **Hodge, C. A., H. V. Colot, P. Stafford, and C. N. Cole.** 1999. Rat8p/Dbp5p is a shuttling transport factor that interacts with Rat7p/Nup159p and Gle1p and suppresses the mRNA export defect of xpo1-1 cells. EMBO J. **18:**5778–5788.
16. **Huertas, P., M. Garcia-Rubio, R. E. Wellinger, R. Luna, and A. Aguilera.** 2006. An hpr1 point mutation that impairs transcription and mRNP biogenesis with increasing recombination. Mol. Cell. Biol. **26:**7451–7465.
17. **Hurt, E., M. J. Luo, S. Rother, R. Reed, and K. Strasser.** 2004. Cotranscriptional recruitment of the serine-arginine-rich (SR)-like proteins Gbp2 and Hrb1 to nascent mRNA via the TREX complex. Proc. Natl. Acad. Sci. USA **101:**1858–1862.
18. **Hurt, E., K. Strasser, A. Segref, S. Bailer, N. Schlaich, C. Presutti, D. Tollervey, and R. Jansen.** 2000. Mex67p mediates nuclear export of a variety of RNA polymerase II transcripts. J. Biol. Chem. **275:**8361–8368.
19. **Jensen, T. H., K. Patricio, T. McCarthy, and M. Rosbash.** 2001. A block to mRNA nuclear export in S. cerevisiae leads to hyperadenylation of transcripts that accumulate at the site of transcription. Mol. Cell **7:**887–898.
20. **Jimeno, S., R. Luna, M. Garcia-Rubio, and A. Aguilera.** 2006. Tho1, a novel hnRNP, and Sub2 provide alternative pathways for mRNP biogenesis in yeast THO mutants. Mol. Cell. Biol. **26:**4387–4398.
21. **Jimeno, S., A. G. Rondon, R. Luna, and A. Aguilera.** 2002. The yeast THO complex and mRNA export factors link RNA metabolism with transcription and genome instability. EMBO J. **21:**3526–3535.
22. **Kendirgi, F., D. J. Rexer, A. R. Alcazar-Roman, H. M. Onishko, and S. R. Wente.** 2005. Interaction between the shuttling mRNA export factor Gle1 and the nucleoporin hCG1: a conserved mechanism in the export of Hsp70 mRNA. Mol. Biol. Cell **16:**4304–4315.
23. **Krebber, H., T. Taura, M. S. Lee, and P. A. Silver.** 1999. Uncoupling of the hnRNP Npl3p from mRNAs during the stress-induced block in mRNA export. Genes Dev. **13:**1994–2004.
24. **Lei, E. P., H. Krebber, and P. A. Silver.** 2001. Messenger RNAs are recruited for nuclear export during transcription. Genes Dev. **15:**1771–1782.
25. **Moerschell, R. P., S. Tsunasawa, and F. Sherman.** 1988. Transformation of yeast with synthetic oligonucleotides. Proc. Natl. Acad. Sci. USA **85:**524–528.
26. **Rollenhagen, C., C. A. Hodge, and C. N. Cole.** 2004. The nuclear pore complex and the DEAD box protein Rat8p/Dbp5p have nonessential features which appear to facilitate mRNA export following heat shock. Mol. Cell. Biol. **24:**4869–4879.
27. **Saavedra, C., K. S. Tung, D. C. Amberg, A. K. Hopper, and C. N. Cole.** 1996. Regulation of mRNA export in response to stress in Saccharomyces cerevisiae. Genes Dev. **10:**1608–1620.
28. **Saavedra, C. A., C. M. Hammell, C. V. Heath, and C. N. Cole.** 1997. Yeast heat shock mRNAs are exported through a distinct pathway defined by Rip1p. Genes Dev. **11:**2845–2856.
29. **Saguez, C., J. R. Olesen, and T. H. Jensen.** 2005. Formation of export-competent mRNP: escaping nuclear destruction. Curr. Opin. Cell Biol. **17:**287–293.
30. **Schmitt, C., C. von Kobbe, A. Bachi, N. Pante, J. P. Rodrigues, C. Boscheron, G. Rigaut, M. Wilm, B. Seraphin, M. Carmo-Fonseca, and E. Izaurralde.** 1999. Dbp5, a DEAD-box protein required for mRNA export, is recruited to

the cytoplasmic fibrils of nuclear pore complex via a conserved interaction with CAN/Nup159p. EMBO J. **18:**4332–4347.

31. **Schneiter, R., C. E. Guerra, M. Lampl, G. Gogg, S. D. Kohlwein, and H. L. Klein.** 1999. The *Saccharomyces cerevisiae* hyperrecombination mutant hpr1Delta is synthetically lethal with two conditional alleles of the acetyl coenzyme A carboxylase gene and causes a defect in nuclear export of polyadenylated RNA. Mol. Cell. Biol. **19:**3415–3422.
32. **Snay-Hodge, C. A., H. V. Colot, A. L. Goldstein, and C. N. Cole.** 1998. Dbp5p/Rat8p is a yeast nuclear pore-associated DEAD-box protein essential for RNA export. EMBO J. **17:**2663–2676.
33. **Sommer, P., and U. Nehrbass.** 2005. Quality control of messenger ribonucleoprotein particles in the nucleus and at the pore. Curr. Opin. Cell Biol. **17:**294–301.
34. **Strasser, K., J. Bassler, and E. Hurt.** 2000. Binding of the Mex67p/Mtr2p heterodimer to FXFG, GLFG, and FG repeat nucleoporins is essential for nuclear mRNA export. J. Cell Biol. **150:**695–706.
35. **Strasser, K., and E. Hurt.** 2001. Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. Nature **413:**648–652.
36. **Strasser, K., and E. Hurt.** 2000. Yra1p, a conserved nuclear RNA-binding protein, interacts directly with Mex67p and is required for mRNA export. EMBO J. **19:**410–420.
37. **Strasser, K., S. Masuda, P. Mason, J. Pfannstiel, M. Oppizzi, S. Rodriguez-Navarro, A. G. Rondon, A. Aguilera, K. Struhl, R. Reed, and E. Hurt.** 2002. TREX is a conserved complex coupling transcription with messenger RNA export. Nature **417:**304–308.
38. **Tani, T., R. J. Derby, Y. Hiraoka, and D. L. Spector.** 1995. Nucleolar accumulation of poly (A)+ RNA in heat-shocked yeast cells: implication of nucleolar involvement in mRNA transport. Mol. Biol. Cell **6:**1515–1534.
39. **Vinciguerra, P., and F. Stutz.** 2004. mRNA export: an assembly line from genes to nuclear pores. Curr. Opin. Cell Biol. **16:**285–292.
40. **Winston, F., C. Dollard, and S. L. Ricupero-Hovasse.** 1995. Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. Yeast **11:**53–55.
41. **Yoon, J. H., D. C. Love, A. Guhathakurta, J. A. Hanover, and R. Dhar.** 2000. Mex67p of *Schizosaccharomyces pombe* interacts with Rae1p in mediating mRNA export. Mol. Cell. Biol. **20:**8767–8782.
42. **Yost, H. J., and S. Lindquist.** 1991. Heat shock proteins affect RNA processing during the heat shock response of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**1062–1068.
43. **Yost, H. J., and S. Lindquist.** 1986. RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. Cell **45:**185–193.
44. **Zenklusen, D., P. Vinciguerra, Y. Strahm, and F. Stutz.** 2001. The yeast hnRNP-Like proteins Yra1p and Yra2p participate in mRNA export through interaction with Mex67p. Mol. Cell. Biol. **21:**4219–4232.
45. **Zenklusen, D., P. Vinciguerra, J. C. Wyss, and F. Stutz.** 2002. Stable mRNP formation and export require cotranscriptional recruitment of the mRNA export factors Yra1p and Sub2p by Hpr1p. Mol. Cell. Biol. **22:**8241–8253.