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Rpb4p, a Subunit of RNA Polymerase II, Mediates mRNA Export during Stress

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Changes in gene expression represent a major mechanism by which cells respond to stress. We and other investigators have previously shown that the yeast RNA polymerase II subunit Rpb4p is required for transcription under various stress conditions, but not under optimal growth conditions. Here we show that, in addition to its role in transcription, Rpb4p is also required for mRNA export, but only when cells are exposed to stress conditions. The roles of Rpb4p in transcription and in mRNA export can be uncoupled genetically by specific mutations in Rpb4p. Both functions of Rpb4p are required to maintain cell viability during stress. We propose that Rpb4p participates in the cellular responses to stress at the interface of the transcription and the export machineries.

INTRODUCTION

The importance of transcriptional control in the response of cells to external stimuli has been extensively documented (Parvin and Young, 1998; Buratowski, 2000; Young, 2000). Gene expression, however, has also been shown to be controlled at several other levels. One important posttranscriptional event that is used as a regulatory step for gene expression is the export of mRNA from the nucleus to the cytoplasm (reviewed in Cole, 2000; Kuersten *et al.*, 2001; Reed and Hurt, 2002). Relative to our knowledge on transcription, less is known about the contribution of the mRNA export machinery to regulation of gene expression.

The yeast RNA polymerase II (pol II) is composed of 12 subunits, most of which are essential for viability (Young, 1991). Rpb4p, the fourth largest subunit, has unique features. Cells lacking *RPB4* can transcribe and grow comparably to wild-type (WT) cells during optimal growth conditions at moderate temperatures (18–23°C). In contrast, these cells rapidly lose their ability to transcribe most, if not all, genes when exposed to either nonoptimal temperatures (<12°C or >32°C), ethanol (10%), or starvation stresses (Choder and Young, 1993; Rosenheck and Choder, 1998; Maillot *et al.*, 1999; Sheffer *et al.*, 1999; Miyao *et al.*, 2001). Using a promoter-independent transcription assay, we dem-

onstrated that Rpb4p is required for pol II enzymatic activity at temperature extremes but not at intermediate temperatures (Rosenheck and Choder, 1998). Thus, a main role assigned to Rpb4p is to permit transcription when cells experience stress or suboptimal growth conditions. Consistent with this, only a small portion of pol II molecules (~20%) contain Rpb4p during optimal growth, whereas association of Rpb4p with pol II increases fivefold under some stress conditions (to near 100%; Choder and Young, 1993).

Rpb4p is always present in excess over other pol II subunits. Under optimal conditions, only ~2% of Rpb4p molecules are complexed with pol II complexes, and this increases to ~10% under conditions where most pol II complexes contain Rpb4p (Choder and Young, 1993; Rosenheck and Choder, 1998). Moreover, sucrose sedimentation of a whole cell extract indicated that Rpb4p does not sediment as a free protein but seems to be associated with both pol II and, to a much greater extent, smaller complexes (Rosenheck and Choder, 1998). This suggests that Rpb4p performs some function(s) independent of its association with the pol II enzyme. Interestingly, *NSP1*, a nuclear pore complex (NPC) protein, was recently identified as a high-copy suppressor of an *rpb4* mutant, suggesting a connection between the NPC and *RPB4* function (Tan *et al.*, 2000; see DISCUSSION).

The response of eukaryotic cells to heat shock (HS) and to other stress conditions occurs at both transcriptional and posttranscriptional levels (Morano *et al.*, 1998; Cotto and Morimoto, 1999). In response to a relatively moderate HS (37°C), transcription of most yeast genes is inhibited for a

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Table 1. Yeast strains and plasmids used in this study

	Genotype	Source
Strain		
SUB62 (WT)	<i>Mata, lys2-801, leu2-3,2-112, ura3-52, his3-Δ200, trp1-1</i> (<i>am</i>)	D. Botstein
MC11-1	<i>Mata, lys2-801, leu2-3,2-112, ura3-52, his3-Δ200, trp1-1</i> (<i>am</i>) <i>rpb4Δ1::HIS3</i>	This lab
YMF1	<i>Mata, lys2-801, leu2-3,2-112, ura3-52, his3-Δ200, trp1-1</i> (<i>am</i>), GFP- <i>RPB4</i> (GFP is placed immediately upstream and in frame with the <i>RPB4</i> start codon. Both 5' and 3' noncoding regions of <i>RPB4</i> are intact.)	This work
YTN1	<i>Mata, lys2-801, leu2-3,2-112, ura3-52, his3-Δ200, trp1-1</i> (<i>am</i>) <i>rpb4Δ1::HIS3</i> +pMC143	This work
YTN25	<i>Mata, lys2-801, leu2-3,2-112, ura3-52, his3-Δ200, trp1-1</i> (<i>am</i>) <i>rpb4Δ1::HIS3</i> +pGFP- <i>rpb4-2</i>	This work
YTN73	<i>Mata, lys2-801, leu2-3,2-112, ura3-52, his3-Δ200, trp1-1</i> (<i>am</i>) <i>rpb4Δ1::HIS3</i> +pGFP- <i>rpb4-3</i>	This work
YTN75	<i>Mata, lys2-801, leu2-3,2-112, ura3-52, his3-Δ200, trp1-1</i> (<i>am</i>) <i>rpb4Δ1::HIS3</i> +pGFP- <i>rpb4-5</i>	This work
Plasmid		
pMC143	<i>XbaI-BamHI</i> 1960-bp fragment of <i>RPB4p-GFP-RPB4-RPB4t</i> in pRS315 (LEU2 CEN). This fragment was obtained by PCR-amplified GFP- <i>RPB4</i> locus of YMF1. It contains 398 bp of <i>RPB4</i> 5' and 110 bp of the 3' noncoding regions.	

short period of time and there is a modest induction of transcription of HS genes (Saavedra *et al.*, 1996). However, after a short delay, subsequent gene expression is executed efficiently at all levels and cells continue to divide. We have previously reported that severe HS (42°C) or 10% ethanol shock lead to nuclear retention of most poly(A)⁺ RNA. Under these conditions, the transcription of HS genes is strongly induced and HS transcripts are efficiently exported in a process that requires most of the factors essential for normal poly(A)⁺ RNA export (Saavedra *et al.*, 1996, 1997).

A major challenge in current molecular biology is to understand how sequential steps in gene expression are coupled and how coupling is involved in regulation of gene expression in response to external signals. Sophisticated crosstalk between transcription, splicing, polyadenylation, and mRNA export have been described (recently reviewed by Komeili and O'Shea, 2000; Reed and Hurt, 2002). Yra1p and Sub2p are mRNA export factors that couple the transcription machinery to splicing and to mRNA export, by virtue of their interaction with THO complex that functions in transcription elongation (Jimeno *et al.*, 2002; Strasser *et al.*, 2002). Npl3p, a major hnRNP protein in yeast required for mRNA export, is recruited to the mRNA during transcription (Lei *et al.*, 2001). Interestingly, during stress, Npl3p dissociates from the mRNA (or is not recruited to the mRNA; Krebber *et al.*, 1999). This is likely to be one of the mechanisms used to arrest the transport of the bulk mRNA during stress. Nevertheless, the transport of HS mRNAs, which does not require Npl3p, is executed efficiently (Saavedra *et al.*, 1996, 1997). The detailed mechanism of mRNA export during stress is unknown. Neither is it known how coupling between various levels of gene expression is affected by the environment.

Here we show that Rpb4p has a dual role during both moderate and severe HS: a role in transcription and a role in the export of mRNAs to the cytoplasm. We suggest that Rpb4p may couple RNA polymerase II function to mRNA export during stress.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

Yeast strains used are listed in Table 1. Cells were grown in synthetic complete or selective media at 22°C, using standard protocols.

Construction of GFP-*RPB4*, Its Integration at the *RPB4* Locus in the Chromosome, and Its Expression from a Plasmid

A linear fragment encoding GFP-*RPB4* under control of *RPB4* regulatory elements was constructed from three different DNA fragments, each obtained by PCR. One is a 398-base pair fragment spanning positions -398 to -1 upstream of *RPB4* start codon. The second is a GFP fragment that contains the entire GFP coding region, except for the stop codon. The third fragment was placed downstream of the GFP fragment and encodes 10 alanine residues used as a (structurally flexible) linker, followed by the entire *RPB4* coding region and 145 base pairs of its 3'-noncoding region. A *NotI* site was introduced in the region encoding the poly(Ala) linker. The *RPB4p-GFP-RPB4* fragment was introduced into MC11-1 cells by transformation and integrated into the *rpb4* locus by homologous recombination, thus replacing *rpb4Δ1::HIS3* (Woychik and Young, 1989). Transformants were selected on histidine-containing plates that were incubated at 37°C to select for integration of *RPB4*. Integration of the chimeric gene in the correct locus was verified by histidine auxotrophy, PCR-based analyses, and later by a direct sequencing of a DNA fragment that had been PCR amplified using the transformant's genome as the template.

To express the fusion gene from a plasmid, the gene was inserted in pRS315 as detailed in Table 1.

Immunostaining Assay

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature (addition of formaldehyde, a more conventional fixer, led to a rapid export of Rpb4p-Myc2 before fixation had been completed and could not be used). Spheroplasts were mounted on polylysine-coated slides, permeabilized with cold methanol, blocked with 1% BSA, 0.1% Triton X-100 in PBS, and incubated with primary antibodies in PBS containing 1% BSA for 1 h at room temperature. After extensive washing, cells were reacted with FITC-labeled secondary antibodies in PBS containing 1% BSA, followed by extensive washing. Cell nuclei were stained with DAPI and the slides were examined by fluorescence microscopy.

Random Mutagenesis of the *RPB4* and Identification of Temperature-sensitive Mutants

The *RPB4* coding region was mutagenized using an error-prone PCR technique (Fromant *et al.*, 1995), using pMC143 as the template. oMC129 (5'- CTTCTTGAGTTTGTAACAGCTGCTGGGATTACACA-TGGCATGGATGAAGCGGCCGAGCAGCTGCCGCTGCAGCGGC-3') was used as the forward primer, consisting the 3' end of the GFP

sequence (italicized) upstream of the *NotI* site (underlined), which is present in the poly(A) linker (see plasmid construction above). oMC78 (5'-CGCGGATCCCTTAAAAAGAGCAAGACGAGATTTAA-AATCC-3') was the reverse primer, located 110 base pairs downstream of *RPB4* stop codon. PCR reaction (22 cycles) was carried out as described (Fromant *et al.*, 1995) except that MgCl₂ was added to 4.2 mM and the concentration of the dNTP that was in excess over the others was 1.5 mM. The resulting mutagenized fragment was digested with *Bam*HI, ligated to *Bam*HI digest of pMC143, followed by PCR amplification using standard conditions with the following primers: oMC132 (5'-CTTCTTGAGTTTGAACAGCTGCTGG-GATTACACATGGCATGGATGAG-3') identical to the 5' end of oMC129 and oMC130 (5'-GCTGGCAGCAGGTTTCCC-3'). This oligo recognizes the vector sequence located 290 base pairs downstream of the *Bam*HI and 400 base pairs downstream of the *RPB4* stop codon in pMC143. The mutagenized fragment was integrated into pMC143 by homologous recombination *in vivo* after cotransforming the MC11-1 strain with a *NotI*/*SpeI* digest of pMC143 (*SpeI* is located 20 base pairs downstream of the *RPB4* stop codon; hence the digest lacks the entire *RPB4* sequence). Transformants were allowed to grow at 23°C. After tiny colonies appeared, they were replica plated onto two plates: one plate was incubated at 23°C and the other at 37°C. Colonies that grew at 23°C but not at 37°C were selected, and their plasmid DNA was recovered and introduced again into naïve MC11-1 cells to verify that the ts phenotype was plasmid dependent. The specific mutations in *rpb4* that were responsible for the ts phenotype were determined by sequencing analyses (see Fig. 4).

In Situ Hybridization Assays

In situ hybridization assays to detect poly(A)⁺ RNA or *SSA4* mRNA were performed essentially as described previously (Amberg *et al.*, 1992; Gorsch *et al.*, 1995; Saavedra *et al.*, 1996).

In Vivo Labeling of Proteins and Analysis by Gel Electrophoresis

Cells were grown at 22°C in synthetic complete medium lacking leucine and methionine until midlogarithmic growth phase ($\leq 10^7$ cells/ml), collected and resuspended in 4 ml medium, and then divided into four equal samples in screw-capped 2-ml tubes. One tube was incubated at 22°C and the others at 42°C for the indicated time periods. All tubes were vortexed occasionally. ³⁵S Trans-label (Amersham, Piscataway, N.J.) was then added to a final concentration of 100 μ Ci/ml, 15-min before cell harvest. Labeling was terminated by the addition of cycloheximide to 50 μ g/ml, and cells were immediately collected by centrifugation, resuspended in ice-cold protein extraction buffer A (Choder and Young, 1993), and frozen at -80°C until further use. Proteins were extracted and 30- μ g samples were subjected to electrophoresis in 5–15% gradient polyacrylamide gel (1.5 mm \times 16 cm \times 20 cm). Gels were fluorographed and exposed to x-ray films.

RESULTS

Rpb4p Is a Nuclear Protein under Optimal Growth Conditions but Becomes Primarily Cytoplasmic in Response to Various Stresses

Previously, we observed that the number of Rpb4p molecules in yeast cells is in a vast excess over other pol II subunits. Moreover, sedimentation through sucrose gradients suggested that most Rpb4p molecules are associated with complexes other than pol II complexes (see INTRODUCTION). These results raised the possibility that Rpb4p carries out functions other than those related to pol II activity and led us to examine Rpb4p's subcellular localization.

To this end, we inserted the coding region of green fluorescent protein (GFP) immediately upstream and in frame with the reading frame of *RPB4* (see MATERIALS AND METHODS). In this way, we created a *GFP-RPB4* fusion gene located at the *RPB4* chromosomal locus and containing intact flanking sequences carrying the regulatory elements necessary for *RPB4* expression. Consequently, the fusion protein (GFP-Rpb4p) was present at the same level as Rpb4p in the WT strain (Figure 1A). Cells carrying *GFP-RPB4* in place of *RPB4* (strain YMF1) are phenotypically indistinguishable from WT cells in both their ability to survive conditions of stress and their rate of growth at a range of temperatures (unpublished observations). We conclude that Rpb4p and the fusion protein, GFP-Rpb4p, function similarly if not identically.

Figure 1B shows that, in cells proliferating exponentially at 22°C, GFP-Rpb4p fluorescence was nuclear, because it colocalized with the fluorescence of the DNA stain, DAPI. When cells were exposed to elevated temperatures (ranging from 34 to 42°C), a large portion of GFP-Rpb4p was relocated to the cytoplasm and the fluorescence was detected all over the cell. (Figure 1C, 37°C). Similarly, even at 22°C, starvation or ethanol stress also caused GFP-Rpb4p to relocate and to be detected all over the cell (Figure 1C, Stationary, Ethanol). Thus, relocation of Rpb4p is a characteristic of the stress response and not of high temperature per se. When not fused to Rpb4p, GFP was evenly distributed throughout the cell in both unstressed and stressed cells (our unpublished results), arguing against the possibility that localization of GFP-Rpb4p is governed by the GFP domain.

The kinetics of the relocation of Rpb4p in response to temperature shift was monitored: after a shift to 37°C (moderate temperature stress), GFP-Rpb4p relocation was slow (Figure 1D). When cells were shifted from 22 to 42°C (Figure 1D) or into 15% ethanol (our unpublished results), GFP-Rpb4p accumulated in the cytoplasm much more rapidly; within 2–5 min, ~50% of the cells exhibited cytoplasmic fluorescence, and after 30 min all of the cells exhibited cytoplasmic fluorescence. Significantly, the stress-induced relocation of GFP-Rpb4p was not due to a change in the level of the protein, because its level was similar in unstressed and stressed cells (Figure 1A). Addition of cycloheximide concomitantly with HS did not affect its relocation (Figure 1D), indicating that the GFP-Rpb4p molecules found in the cytoplasm were not newly synthesized and that no newly synthesized factors are required for relocation of Rpb4p. Instead, in response to these stress conditions, most of the nuclear GFP-Rpb4p molecules shifted to the cytoplasm. The data indicate that GFP-Rpb4p is exported from the nucleus in response to various stress conditions. Because Rpb4p is required in the nucleus under these stress conditions, it is quite likely that Rpb4p shuttles during these conditions.

To examine Rpb4p localization by means other than its fusion to GFP, we tagged *RPB4* at its c-terminus with two copies of the Myc sequence and determined localization of the tagged molecule by an indirect immunostaining technique. Figure 2, *RPB4*-Myc2 panel, demonstrates once again the HS-induced relocation of Rpb4p. Unlike Rpb4-Myc2, Rpb2-HA and Rpb3-HA, two other subunits of RNA polymerase II (Young, 1991), are localized to the nucleus under both HS and moderate temperatures (Figure 2, *RPB2*-HA and *RPB3*-HA panels). Thus, the HS-induced export is not a

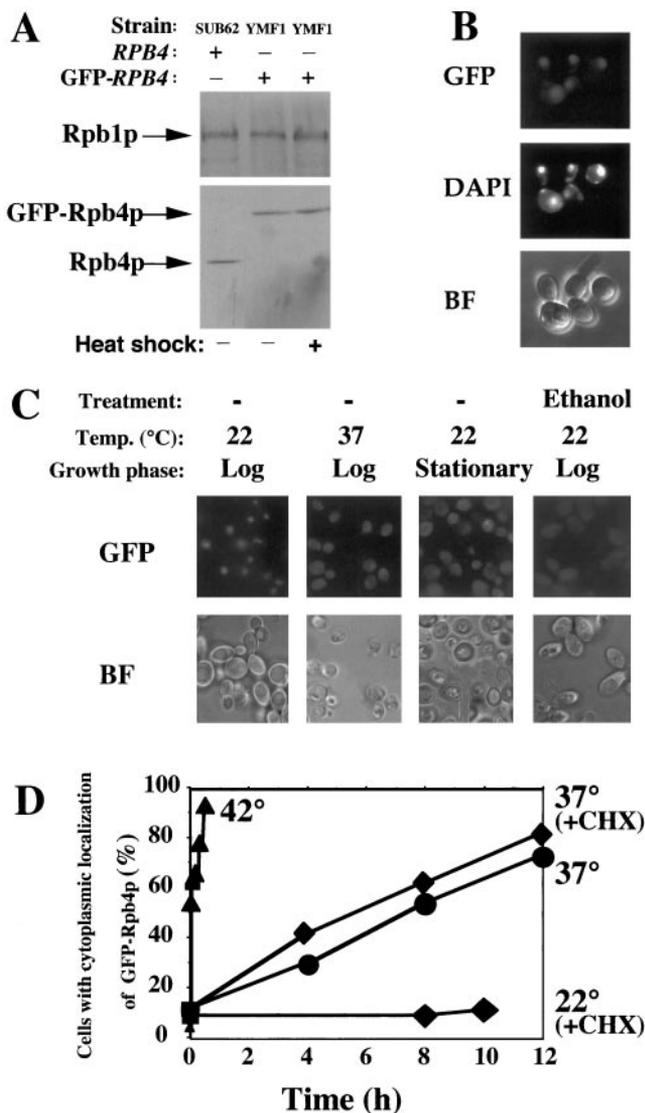


Figure 1. Expression level and localization of GFP-Rpb4p during optimal growth conditions and in response to various stresses. (A) Expression of GFP-*RPB4* fusion gene in YMF1 strain is similar to the expression of *RPB4* in WT cells and is not affected by HS. Proteins were extracted from YMF1 cells that had been growing exponentially at 22°C, or exposed to 42°C for 1 h, as indicated at the bottom. Samples (80 μ g) were analyzed by Western analysis using anti-Rpb4p antibodies (Choder and Young, 1993). To demonstrate equal loading, the same filter was also reacted with anti-Rpb1p antibodies (8WG16 MAb). The GFP-Rpb4p bands also interacted with anti-GFP antibodies (our unpublished results). (B) GFP-Rpb4p localizes to the nucleus during growth under optimal environmental conditions. YMF1 cells were grown in rich medium (YPD) at 22°C and harvested in midlog for inspection using fluorescence microscopy. Top panel: localization of GFP-Rpb4p; middle and bottom panels show DAPI-stained and bright field images (BF), respectively, of the same field shown in the top panel. (C) GFP-Rpb4p is relocated to the cytoplasm in response to various stresses. Cells were grown in rich liquid medium at 22°C till midlog and inspected by fluorescent microscope (designated Log). The remaining of the culture was then divided into three samples. One was allowed to grow at 22°C till cells entered stationary phase (stationary), and the others were

general characteristics of the RNA polymerase II complex or subunits.

Cells lacking *RPB4* are more sensitive than WT cells to some, but not all, types of stress conditions. For example, although *RPB4* is required during temperature, starvation, or ethanol stresses, it is not important for coping with osmotic or oxidative stress under otherwise optimal conditions (Sheffer *et al.*, 1999; Maillat *et al.*, 1999). Interestingly, under conditions where *RPB4* is required, its product is found mainly in the cytoplasm (Figures 1C and 2), whereas under conditions where *RPB4* is dispensable, Rpb4p is nuclear (Figures 1B and 2, and our unpublished results). Note that when cells are under stress, Rpb4p is required for pol II activity; under such conditions some of the Rpb4p molecules must remain in the nucleus. Because there are many more Rpb4p molecules than there are pol II complexes (Rosenheck and Choder, 1998), the nuclear fluorescence would be masked by the strong cytoplasmic one, and fluorescence would be detected throughout the cell.

Analyses of *rpb4* Mutants Indicate a Posttranscriptional Role for Rpb4p during Various Stresses

Cells lacking *RPB4* are unable to grow at elevated temperatures (>32°C) or to synthesize HS mRNAs after severe stress because pol II Δ 4 (pol II lacking Rpb4p) has little or no activity under these conditions (see INTRODUCTION). To determine whether Rpb4p carries a posttranscriptional function and to study further the functions of Rpb4p, *RPB4* was randomly mutagenized. To this end, a centromeric plasmid was constructed carrying GFP-*RPB4* under control of *RPB4* 5' and 3' regulatory regions. The fusion protein expressed from this plasmid was present at approximately the same level as that expressed in the YMF1 strain (Figure 3B). Consistently, this plasmid fully restored the proliferation of the Δ *rpb4* strain either at high temperatures or in the presence of ethanol (Figure 3A). The plasmid also restored pol II transcriptional capacity during HS (42°C; Figure 3C and see also Figure 5A, YTN1 panel). We used error-prone PCR conditions (Fromant *et al.*, 1995) and replaced the WT *RPB4* coding

shifted either to 37°C or to 6% ethanol (at 22°C) for an additional 10 h, as indicated at the top of each panel. Cell samples were then inspected using microscopy as above. (D) Kinetics of stress-induced relocation of GFP-Rpb4p: effect of temperature and cycloheximide. YMF1 cells were grown in rich medium at 22°C until midlog phase. The culture was then split into two samples. Cycloheximide (25 μ g/ml) was added to one sample, and the sample was further divided so that half remained at 22°C (designated 22°C + CHX) and the other half was shifted to 37°C (designated 37°C + CHX). The control culture to which no drug was added was divided into three portions and incubated at 22, 37, or 42°C, as indicated. To determine export kinetics, samples of each culture were examined by microscopy at the indicated times. Each sample was photographed and then ~200 cells were randomly chosen and classified into those exhibiting nuclear localization of GFP-Rpb4p and those whose fluorescence was distributed all over the cell (cytoplasmic localization). The proportion of cells exhibiting cytoplasmic localization was plotted as a function of time. The control sample incubated at 22°C showed no change in the percentage of cells with cytoplasmic localization (~10%) throughout the experiment (our unpublished results).

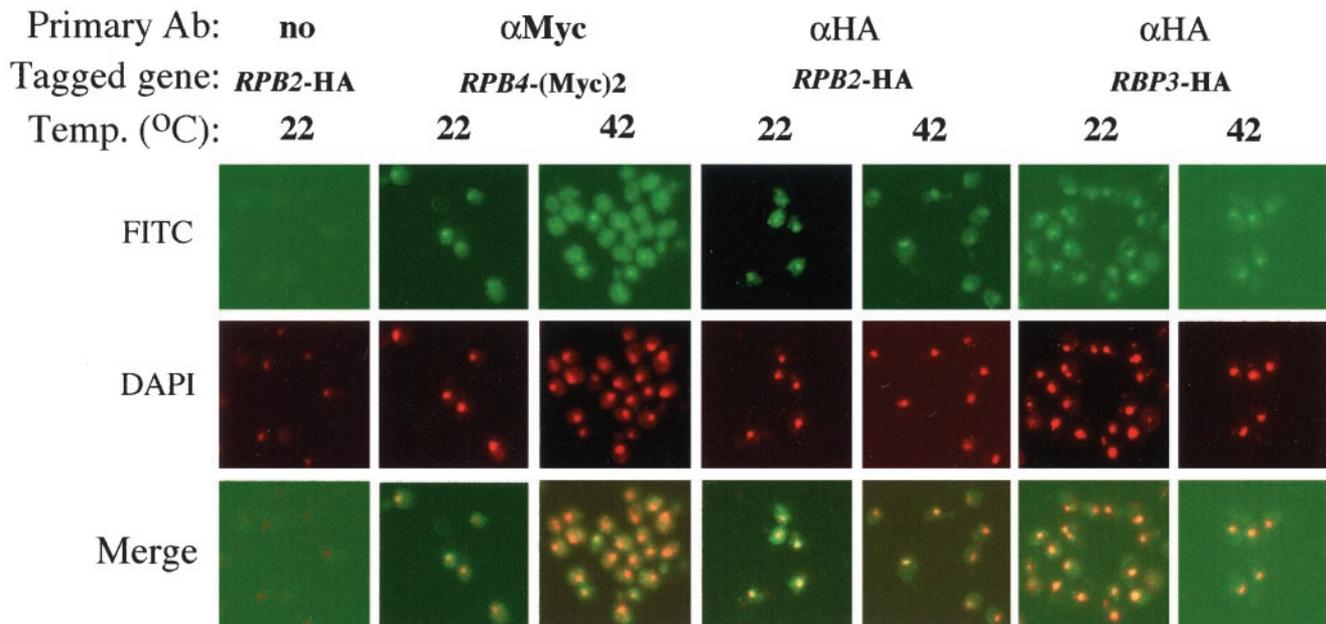


Figure 2. Immunolocalization of Rpb4p-(Myc)2, Rpb2p-HA, and Rpb3p-HA. Indicated strains were immunostained as described in MATERIALS AND METHODS. The bottom panels show the merge of the FITC and DAPI signals. When the two signals colocalize, the resulting color is yellow.

region with the mutagenized one, making sure that it was in-frame with (nonmutagenized) GFP (see MATERIALS AND METHODS). Transformants were first grown on selective plates at 22°C where Rpb4p function is dispensable. Colonies were then replica-plated and screened at a nonpermissive temperature (37°C). Plasmids were isolated from the temperature-sensitive (ts) transformants and then reintroduced into naive cells of the same $\Delta rpb4$ strain to ensure that the ts phenotype was due to mutations in the plasmid-encoded *RPB4* and not to mutations elsewhere in the genome. Finally, expression of the fusion proteins was determined by Western analysis. Mutations in *RPB4* that were responsible for the ts phenotype were determined by DNA sequence analysis. The sequences of these alleles are shown in Figure 4. The alleles that were chosen for further studies and the strains that express these alleles are listed in Table 1.

Thus far, 13 mutants have been sequenced and further analyzed. Two classes of ts *rpb4* mutants were obtained, referred herein as class I and class II: class I mutants (9 of 13 analyzed) are defective in supporting pol II transcription at high temperatures; as shown in Figure 4, all these mutant forms carry at least one mutation in the C-terminal region (position 129–212). Five of them carry mutations exclusively in the c-terminus. Class II mutants, 4 of 13 analyzed, can support transcription at high temperatures like the WT counterpart, yet (like all the mutants identify in this screen) they cannot confer upon the cells the ability to cope with the stress. Mutations in three of these mutant forms fall within the N-terminal region (position 33–80; see Figure 4). GFP-Rpb4-40p carries two cysteines at positions 143 and 149. We suspect that these two closely located cysteines adversely affect the native conformation of Rpb4p. Western analyses of the mutant fusion proteins raised the possibility that part of

the reasons why some of these mutants are defective lays in their abnormal turnover (our unpublished results). Nevertheless, abnormal turnover characterizes both classes. We therefore surmise that abnormal turnover is not the only parameter that affects the abnormal function of the various mutant forms.

One example for the different defect of the two classes of mutants is shown in Figure 5A in which the level of the global poly(A)⁺ RNA was assessed by its hybridization to radiolabeled poly(dT). Although exposure of cells carrying WT GFP-*RPB4* to 37°C had little effect on the global mRNA level, when cells expressing the GFP-*rpb4-5* mutant were shifted from 22 to 37°C, their global mRNA level gradually declined (Figure 5A; cf. YTN1 with YTN75). This decline is a characteristic of cells lacking *RPB4* (Choder and Young, 1993; Sheffer *et al.*, 1999; our unpublished results), indicating that GFP-Rpb4-5p cannot support transcription. YTN75 was therefore classified as class I mutant and the protein it carries, GFP-Rpb4-5p, as class I mutant form. The transcriptional defect of class I mutants during HS is sufficient to explain why cells carrying these mutants are temperature sensitive and cannot survive stress. In contrast with YTN75 strain, YTN25 and YTN73 strains could transcribe their genome at 37°C at a level similar to their WT counterpart (Figure 5A, compare YTN25 and YTN73 panels with YTN1 panel). YTN25 and YTN73 were therefore classified as class II mutants and the proteins they carry, GFP-Rpb4-2p and GFP-Rpb4-3p, as class II mutant forms.

During abrupt and severe HS (e.g., a shift from 22 to 42°C) yeast cells express preferentially HS genes. We therefore examined the capacity of our mutants to function also under severe HS. Results shown in Figure 5B demonstrate that although the strain expressing the WT GFP-*RPB4* responded

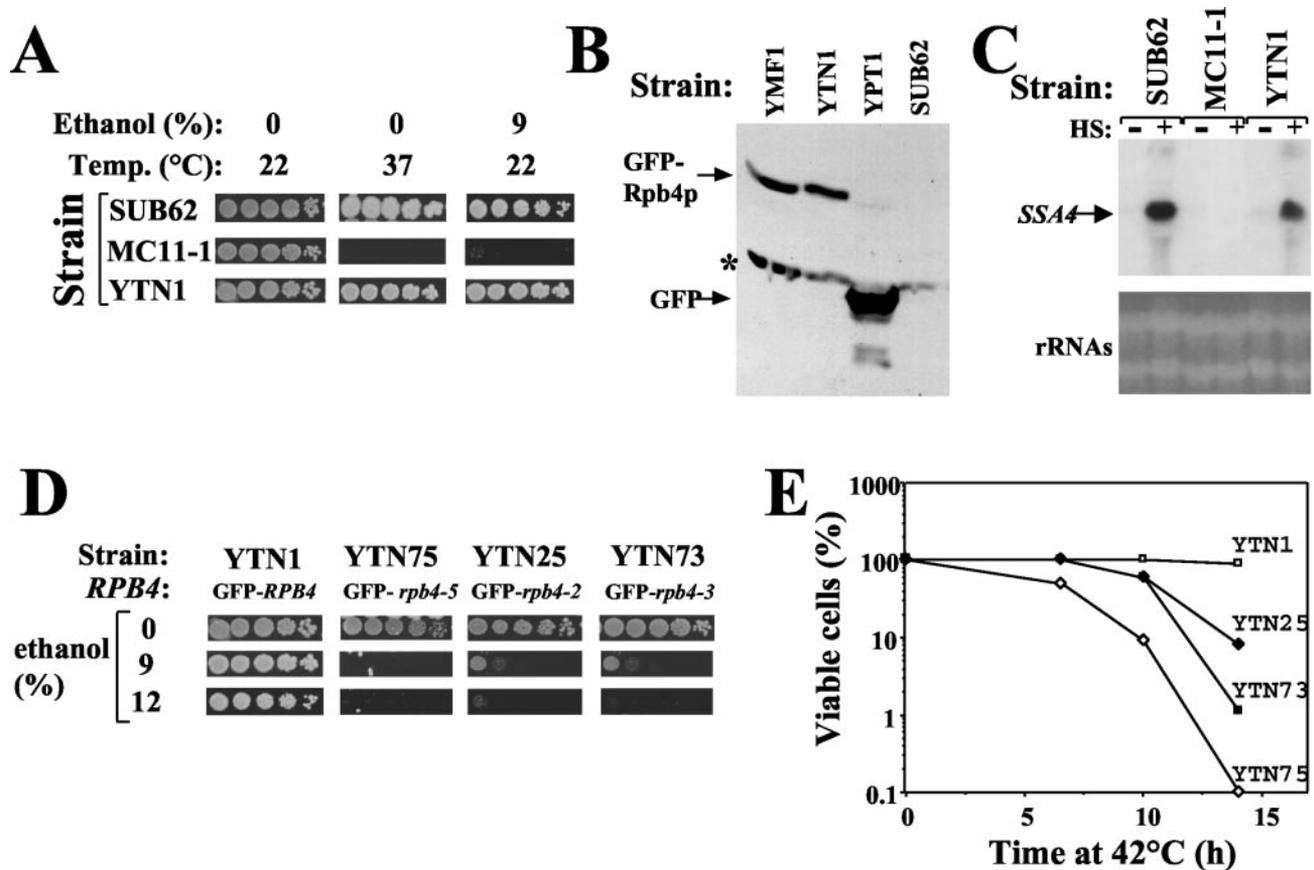


Figure 3. Growth and viability of cells carrying GFP-*RPB4* or various mutant derivatives thereof under stress. (A–C) A plasmid expressing GFP-*RPB4* (pMC143) restores the functions of Rpb4p. (A) Expression of pMC143 (see Table 1) recovers growth defects of $\Delta rpb4$ cells. WT Cells (SUB62) and $\Delta rpb4$ cells (MC11-1) were cultured in a synthetic complete medium, whereas $\Delta rpb4$ cells carrying pMC143 (YTN1) were cultured in a selective medium. Cells were allowed to grow at 22°C till midlogarithmic phase. Equivalent amounts of cells (diluted in fivefold steps) were spotted onto YPD plates supplemented with the indicated amounts of ethanol and were allowed to grow at the indicated temperatures for either 4 d (37°C) or 7 d (22°C). (B) Expression of GFP-*RPB4* fusion gene in YTN1 strain is similar to the expression of GFP-*RPB4* in YMF1 cells. Proteins were extracted from the indicated cells that had been grown logarithmically at 22°C till midlogarithmic growth phase. PT1 is isogenic to SUB62 strain and carrying a high-copy plasmid expressing GFP under *ACT1* promoter. Samples (80 μ g) were analyzed by Western analysis using anti-GFP antibodies. A band that was cross-reacted with the antibodies, marked by asterisk, demonstrates equal loading. (C) Expression of pMC143 recovers the transcriptional defect of $\Delta rpb4$ cells (MC11-1). Cells were grown in a selective medium at 22°C till midlogarithmic phase. The cultures were then split. One sample was left at 22°C, and the other was shifted rapidly to 42°C and incubated for 30 min at this temperature. Cell samples were collected and RNA was extracted as described previously (Choder, 1991; Sheffer *et al.*, 1999). RNA samples (5 μ g) were analyzed by Northern blot hybridization (Choder, 1991; Sheffer *et al.*, 1999). The filter was hybridized with *SSA4* DNA probe. rRNAs were detected by 0.5 μ g/ml EtBr staining and shown at the bottom panel. (D and E) Both class I and class II mutants are sensitive to ethanol and temperature stresses. (D) Growth on YPD containing ethanol. Cells were grown in selective medium at 22°C till midlogarithmic phase. Equivalent amounts of cells (diluted in fivefold steps) were spotted onto YPD plates supplemented with the indicated amounts of ethanol. Plates were allowed to grow at 22°C for 7 d. (E) Cell viability as a function of time at 42°C. Experiment was done as described previously (Choder and Young, 1993).

to the HS by a rapid and robust transcription of HS genes *SSA3* and *SSA4*, transcripts of these genes could not be detected in class I mutants (cf. YTN75 panel with YTN1 panel). Note that within 45 min of the severe HS, transcription of non-HS genes (e.g., *ACT1* and *NUP157* shown in Figure 5B) was compromised and transcript levels gradually declined, both in the WT and in the mutants, consistent with previous results (Saavedra *et al.*, 1997). Unlike class I mutants, transcription of *SSA3* and *SSA4* during the HS was as efficient in class II mutants as it was in WT cells (Figure 5B, cf. YTN25 and YTN73 panels with YTN1 panel). It is worth

noting that Northern blot hybridization analyses indicated that the sizes of HS mRNAs produced in class II mutant cells were identical to those transcribed in WT cells under both optimal and suboptimal conditions cells (Figure 5B and our unpublished results). Significantly, in spite of their capacity to synthesize full-size transcripts efficiently, class II mutants were still temperature sensitive.

The *rpb4* mutant were selected based on their inability to form colonies at 37°C. Yet, further study indicated that both class I and class II mutants were also sensitive to the presence of ethanol (see Figure 3D). Moreover, consistent with

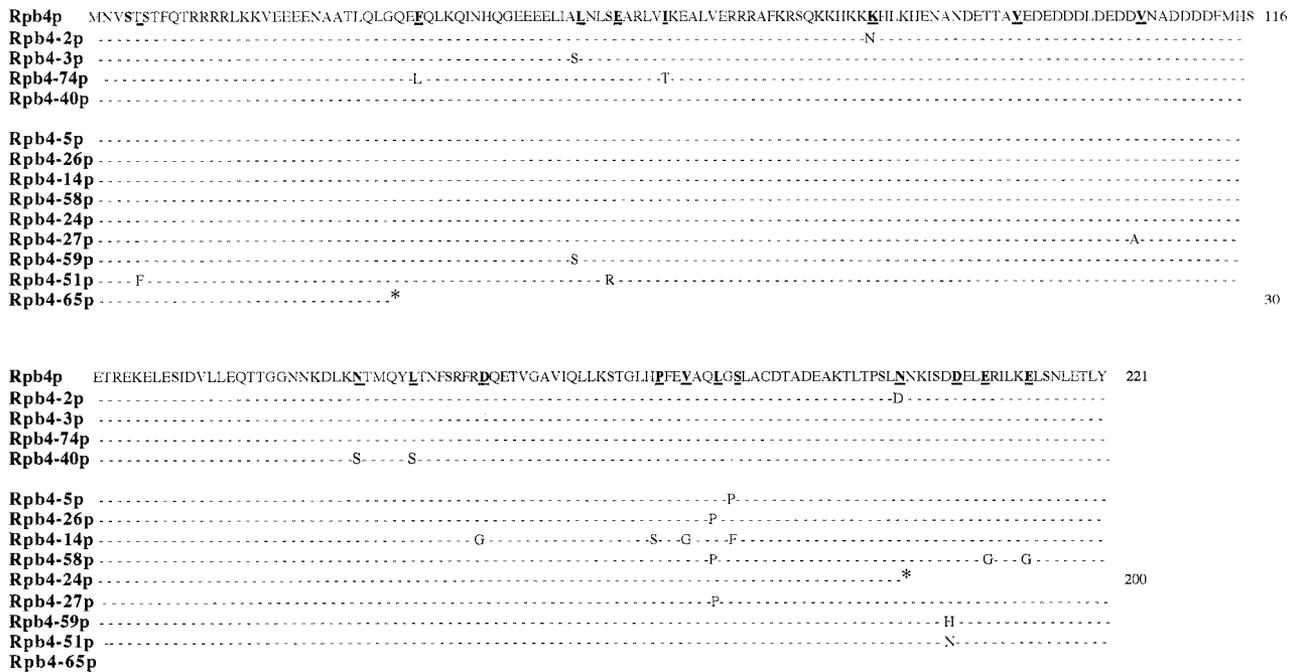


Figure 4. Sequence alignment of Rpb4p and its mutant forms. The predicted amino acid sequence of WT Rpb4p and the Rpb4p moiety of the GFP-*rpb4* mutants that conferred the ability to cope with stress upon *rpb4*Δ cells is presented here. Residues that underwent mutations are underlined. Star represents a stop codon. The four mutant forms that we classified as class II are depicted at the top. The rest are class I mutants.

their inability to proliferate at 37°C, both class I and class II mutants died much more rapidly than WT at 42°C (Figure 3E). In addition, some of the mutants (e.g., YTN25) died much more rapidly than the WT strain during starvation (our unpublished results).

The identification of *rpb4* mutants that transcribe normally but are defective in their ability to cope with various stresses (class II) suggests that Rpb4p plays an additional posttranscriptional role under these stress conditions. The nature of this posttranscriptional function is discussed in the following section.

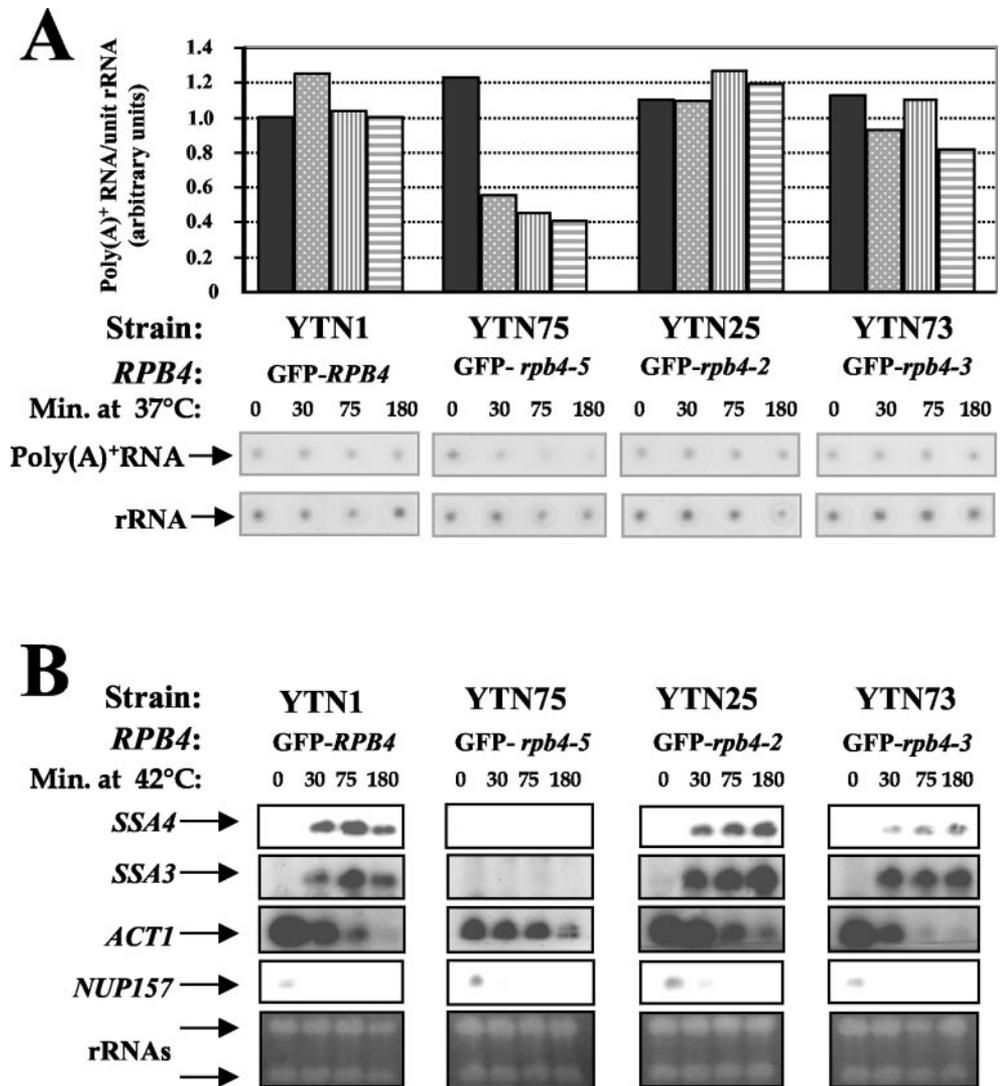
***Rpb4p* Is Required for the Export of poly(A)⁺ RNA at 37°C and for Export of Heat Shock mRNAs during Severe Temperature Stress (42°C)**

Most WT lab yeast strains can grow at temperatures as high as 38°C. Nevertheless, when yeast cells are shifted from moderate temperatures (18–23°C) to 37°C, a modest stress response occurs: expression of HS genes is transiently induced and cells transiently decrease transcription of most other genes. Later, cells adjust to the high temperature, and the expression of non-HS genes becomes efficient again, allowing cells to continue to divide, albeit at a slower rate (Lindquist, 1986; Nonet *et al.*, 1987; Choder and Young, 1993). In light of the observations, discussed in the previous section, that at ≥37°C Rpb4p performs a posttranscriptional function and is also exported to the cytoplasm, we tested the possibility that Rpb4p plays a role in mRNA export when cells are exposed to elevated temperatures. To test this pos-

sibility we used the YTN25 strain expressing GFP-*Rpb4p-2*, a class II mutant allele. Although this strain can transcribe efficiently at 37 and at 42°C (see Figure 5), it is unable to grow at 37°C and rapidly dies at 42°C (Figure 3E). To determine what kind of posttranscriptional defect characterizes YTN25, we determined the location of poly(A)⁺ RNA. Figure 6, panel MC11-1, shows that 1 h after the shift of Δ*rpb4* cells from 22°C to 37°C, the level of the poly(A)⁺ RNA declined, reflecting the transcriptional defect of these cells (see INTRODUCTION). In contrast, 1 h after the shift of WT to 37°C, cells contained a high level of poly(A)⁺ RNA detected mainly in the cytoplasm (Figure 6, YTN1). This indicates that at 37°C, mRNAs were synthesized and exported efficiently to the cytoplasm in wild-type cells (see also Lee *et al.*, 1996; Krebber *et al.*, 1999). In contrast, after the shift of YTN25–37°C, cells produced poly(A)⁺ RNA, but it accumulated in nuclei (Figure 6, YTN25, 37°C). Note that not all cells accumulated mRNAs in their nuclei. This suggests that, at 37°C, the penetrance of the mutation was not complete or that the function of Rpb4p is merely to enhance export efficiency (see DISCUSSION). In contrast with nuclear accumulation at 37°C, poly(A)⁺ RNA was normally distributed in the cytoplasm of these cells grown at 22°C (Figure 6, MC11-1 and YTN25, 22°C). These results indicate that, under conditions where *RPB4* is dispensable (22°C), both transcription and mRNA export are carried out efficiently and independently of Rpb4p. However, at 37°C, Rpb4p is required for the efficient execution of both processes.

When yeast cells are exposed to 42°C, a strong stress response occurs and transcription of HS genes is strongly

Figure 5. Unlike class I (YTN75), class II mutants exhibit transcriptional capacity comparably to wild type. Temperature-sensitive mutants were selected as described in MATERIALS AND METHODS. (A) Levels of global mRNA. Cells were grown in selective medium at 22°C till mid-log and then shifted rapidly to 37°C. Cell samples were collected either before HS (time 0) or at the indicated time points after temperature shift. After all samples had been collected, RNA was extracted. RNA samples (2 μ g) from the indicated strains were dot-blotted onto nitrocellulose filter, in two sets of triplicates. One set was hybridized with 32 P-poly(dT), to detect the global mRNA, and the other set with 32 P-rDNA to detect rRNA. Only one dot of each set is shown. The intensities of the radioactive dots was quantified by phosphoimager. The average of the mRNA triplicate was divided by the average of the rRNA triplicate. The ratio obtained at time 0 of YTN1 was arbitrarily defined as 1, whereas the other columns are plotted relative to this ratio. Variation was <12%. This procedure was described previously (Choder, 1991). (B) Levels of specific mRNAs. Samples (5 μ g) of RNA from the indicated strains were analyzed by Northern blot hybridization (Choder, 1991; Sheffer *et al.*, 1999). The filter was sequentially hybridized with the indicated DNA probes. rRNA was detected by EtBr staining.



induced. HS transcripts are then selectively exported, while export of the bulk poly(A)⁺ RNA is blocked (Saavedra *et al.*, 1996, 1997). A similar pattern was observed after stressing cells with 10% ethanol (Saavedra *et al.*, 1996). The role of Rpb4p in poly(A)⁺ RNA at 37°C led us to determine if Rpb4p is required also for HS mRNA export at 42°C. To this end, we used in situ hybridization to examine the localization of *SSA4* mRNA, encoding one of the yeast Hsp70 proteins. As expected, very little *SSA4* mRNA was visible when these cells were grown at 22°C (Figure 7A, see columns designated 0, and Figure 7B, 22°C panels). After a shift to 42°C, *SSA4* mRNA was not produced in cells lacking *RPB4* (Figure 7, A and B, strain MC11-1). In cells expressing WT Rpb4p, GFP-Rpb4p, or class II mutant Rpb4p, *SSA4* mRNA was produced and readily detected. However, cellular distribution of the *SSA4* transcript was dependent on the nature of Rpb4p. In cells carrying WT-Rpb4p or WT GFP-Rpb4p, *SSA4* mRNA was exported and detected in the cytoplasm as early as 15 min after temperature shift (Figure 7A, strains SUB62 and YTN1, and Figure 7B, SUB62 panel).

In contrast, strong nuclear accumulation of *SSA4* mRNA was detected within 15 min after shifting isogenic cells expressing GFP-Rpb4-2p or GFP-Rpb4-3p (class II mutants) to 42°C (Figure 7A, strains YTN25 and YTN73, 15 min; Figure 7B YTN25 panel). Thus, class II mutants are defective not only in export of the bulk poly(A)⁺ RNA at 37°C (Figure 6), but also in the export of *SSA4* mRNA at 42°C. The two class II mutants differed in their penetrance. Although cells expressing GFP-*rpb4-2* were almost completely defective in *SSA4* mRNA export, those expressing GFP-*rpb4-3* supported *SSA4* mRNA export but at a reduced rate. Consequently, cytoplasmic *SSA4* mRNA could be detected in cells expressing GFP-*rpb4-3*, but only after 60 min at 42°C (Figure 7A, cf. strains YTN25 and YTN73, 60-min), reflecting a partial export block.

To examine further the transport defect of cells expressing a class II mutant (GFP-*rpb4-2*) and to determine whether the mutations in the expressed protein affect the export of other classes of HS mRNAs, we pulse-labeled cells with [35 S]methionine+cysteine for 10-min periods before or at

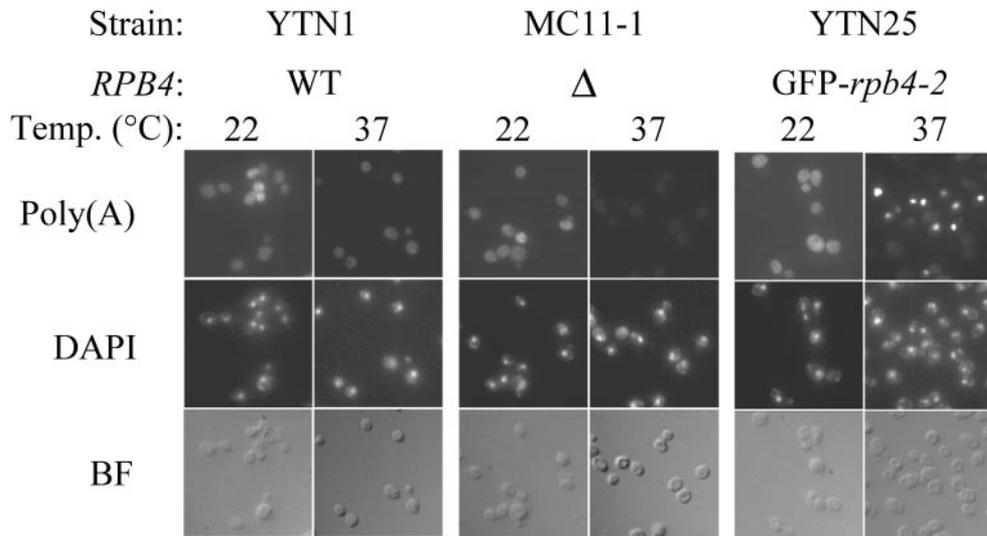


Figure 6. During incubation at 37°C, class II mutant cells synthesize poly(A)⁺ RNA that accumulates in the nucleus. The indicated strains were grown at 22°C till midlog. Cultures were then split into two samples. One was maintained at 22°C, and the other was shifted to 37°C for 1 h. Cells were then harvested and poly(A)⁺ RNA was detected by in situ hybridization using an oligo-dT50 probe (see MATERIALS AND METHODS). Preliminary test were made to make sure that the GFP fluorescence was not detected after cell fixation and permeabilization. See, for example, the 22°C panels in this figure. Nuclei were detected by DAPI staining.

different times after HS. Results are shown in Figure 8A. In WT cells, the expected set of HS proteins was synthesized and readily detected within 15 min of HS (lane 2). As observed previously (Saavedra *et al.*, 1997), within 30 min after temperature shift (lane 3) and more clearly after 45 min (lane 4), the HS proteins were the predominant proteins synthesized. In contrast, in cells expressing GFP-*rpb4-2*, the synthesis of all HS proteins was severely compromised (Figure 8A, lanes 10–12). Because *SSA4* mRNA accumulates in the nuclei of cells carrying GFP-*rpb4-2*, the defect in synthesis of *Ssa4p* is most likely a reflection of the block in export of *SSA4* mRNA. Most likely, the defect in the synthesis of the other HS proteins is also due to a defect in the export of their

mRNAs. Figure 8A, YTN75 (lanes 9–12) shows that no HS proteins were detected in class I mutant cells that do not transcribe HS genes at 42°C (see Figure 5B, YTN75). Thus, the inability of class I mutant cells to synthesize proteins during HS is similar to that of Δ *rpb4*, as analyzed by two-dimensional gel electrophoresis (Maillet *et al.*, 1999). This defect is more severe than that observed in the class II mutant (which is not defective in transcription), as the latter did exhibit limited synthesis of HS proteins (Figure 8A, cf. lanes 10–12 with 6–8). This suggests that leaky export of HS mRNAs did occur in YTN25 cells (unless some translation was carried out in the nucleus). Nevertheless, the leaky export was insufficient to protect the cells from the damag-

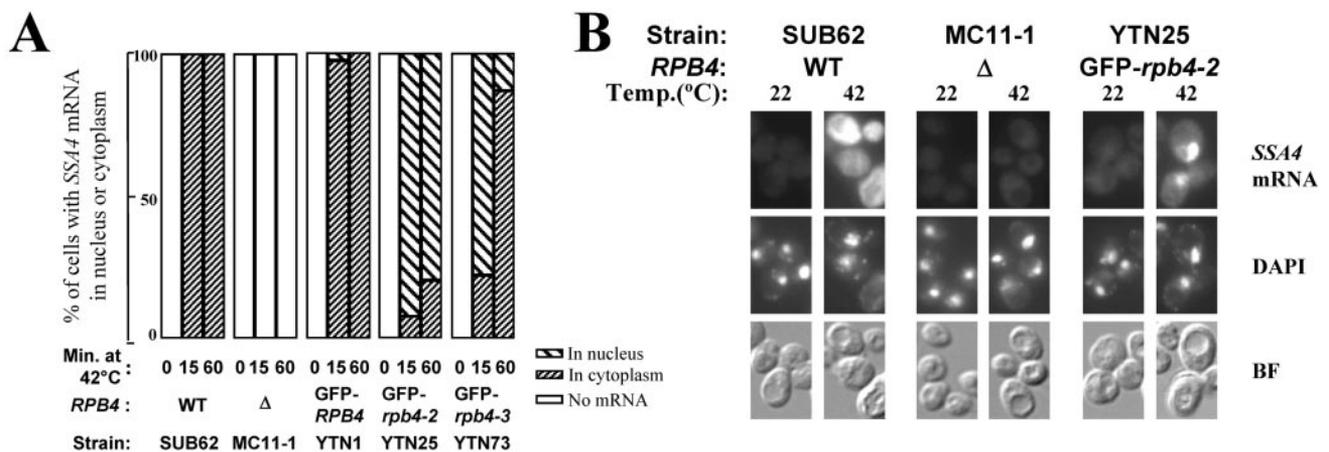


Figure 7. During incubation at 42°C, class II mutant cells synthesize *SSA4* mRNA that accumulates in the nucleus. (A) Quantitative analysis of *SSA4* mRNA expression and localization. Indicated strains were grown in a selective medium at 22°C until midlog. Cultures were then divided into three samples. One was maintained at 22°C, and the others were shifted to 42°C for the indicated time. Cells were then harvested and the level of *SSA4* mRNA and its localization was detected by in situ hybridization technique as described in MATERIALS AND METHODS. After taking pictures of a fluorescent microscope, cells were classified into those where *SSA4* mRNA was detected in the cytoplasm, in the nucleus, or not detected at all. The code for the different expression and localization classes is depicted at the bottom right side of panel A. (B) Images of selected samples of cells that were visualized either before (22°C panels) or 15 min after a temperature shiftup (42°C panels), as described in Figure 1.

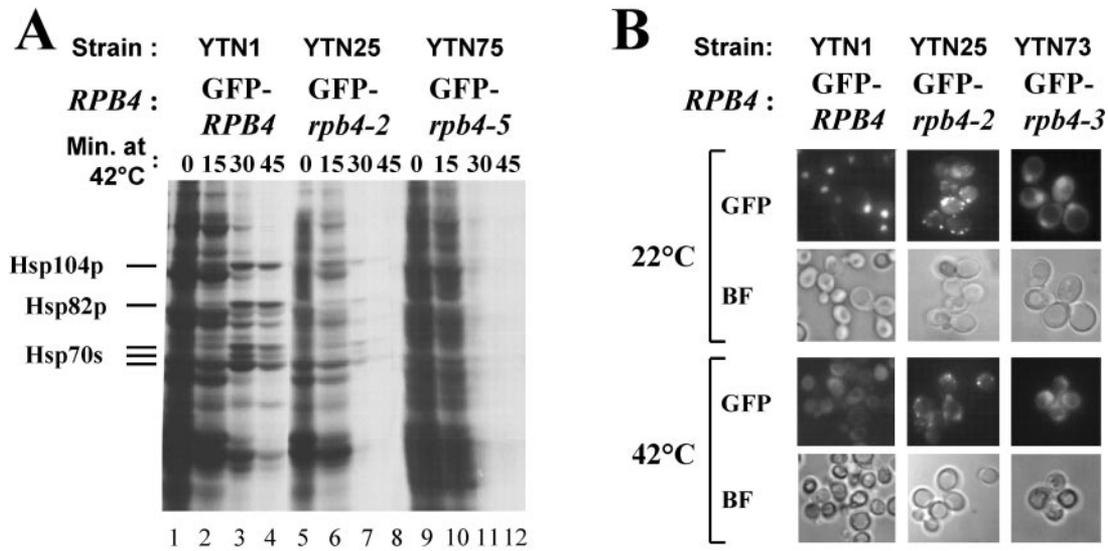


Figure 8. Effect of class I and class II mutations in GFP-RPB4 on the pattern of protein synthesis and localization of GFP-Rpb4p. (A) Pattern of protein synthesis. Strains, indicated at the top, were grown in a selective medium at 22°C till midlog and then incubated at 42°C for the indicated time periods, as detailed in MATERIALS AND METHODS. Cells were radiolabeled for 15 min before cell harvest as described in MATERIALS AND METHODS. Proteins were extracted and equal amounts were analyzed by SDS-PAGE (see MATERIALS AND METHODS), followed by fluorography and autoradiography. (C) Localization of class II GFP-Rpb4p mutants. Strains, indicated at the top, were grown in a selective medium at 22°C till midlog. The culture was then divided into two samples; one was further incubated at 22°C and the other was shifted to 42°C. One hour after the temperature shift, cell samples were inspected using microscopy as in Figure 1.

ing effects of the severe stress, resulting in their death (Figure 3E).

Figure 8B shows the localization of class II mutants. GFP-Rpb4-2p was constitutively mislocalized to the cytoplasm and its localization was not affected by the environmental conditions (Figure 8B, YTN25 panels). Note, however, that at least during stress, some GFP-Rpb4-2p molecules should be present in the nucleus to support transcription. Localization of GFP-Rpb4-3 in optimally growing cells was partially defective as it was partly cytoplasmic (Figure 8B, cf. YTN73 panel with that of YTN1); during HS, localization of some GFP-Rpb4-3p molecules was still nuclear. These results demonstrate that the localization of class II mutant proteins is abnormal. Their mislocalization may contribute to their defect in supporting mRNA export or may be the result of their compromised transport capability.

Taken together, our results indicate that Rpb4p plays an important role in mRNA export both under conditions of moderate temperature stress, under which cells continue to grow (37°C), and during severe HS (42°C). Export of both bulk poly(A)⁺ RNA and HS mRNAs is compromised in these mutants at temperatures where WT Rpb4p becomes essential for transcription. However, during optimal growth, Rpb4p is required neither for transcription nor for mRNA export, and $\Delta rpb4$ cells grow like WT (Choder and Young, 1993, and compare MC11-1 with SUB62 in Figure 3A, 22°C).

DISCUSSION

In this study, we show that Rpb4p, a pol II subunit, is required for the export of poly(A)⁺ RNA at 37°C. During severe HS (42°C), Rpb4p is also essential for the selective

export of HS mRNAs. Thus, at 37°C, Rpb4p plays a key role in gene expression, both at the level of transcription and at the level of mRNA export. During severe HS, Rpb4p plays a key role in the expression of HS genes, also at both levels. Significantly, the two functions of Rpb4p can be uncoupled genetically by specific mutations in *RPB4*. We have identified mutants that support transcription during HS, but are defective in Rpb4p's export role and are unable to survive various stress conditions (class II mutants). Transcription in these mutants is comparable to that in WT both at 37 and at 42°C. The existence of these mutants indicates that the transcriptional function of Rpb4p is not sufficient to complement the lack of *RPB4*, because the export function, which is defective in class II mutants, is also essential. Although it is not inconceivable that Rpb4p performs different roles in export of the bulk mRNA at 37°C and that of HS mRNAs at 42°C, it seems more likely that Rpb4p performs the same roles in transcription and mRNA export under all conditions where it is required.

What might Rpb4p be doing in transcription and in mRNA export? Does Rpb4p perform two different functions, one in transcription and one in export, or do phenotypic defects in both processes reflect a single operational defect in Rpb4p? The possibility that the interaction of Rpb4p with pol II, which helps to maintain pol II integrity during stress (Choder and Young, 1993; Jensen *et al.*, 1998; Rosenheck and Choder, 1998), is responsible for both Rpb4p functions is appealing because of its simplicity. Indeed, recent studies indicated that pol II plays an important role in various posttranscriptional processes including capping, 3' processing, and mRNA maturation (Hammell, Heath, and Cole, unpublished results; for recent reviews see Cramer *et al.*,

2001; Maniatis and Reed, 2002; Zhao *et al.*, 1999). Maintaining pol II integrity is therefore important for both transcription and pre-mRNA processing. RNA processing, in turn, appears to be coupled to export of the mature mRNA. However, if the sole role of Rpb4p is to maintain pol II integrity and/or to help recruiting other factors to pol II complex, why would most Rpb4p molecules be found in the cytoplasm under conditions (stress) when their function is important in the nucleus? More significantly, class II mutants are fully functional in transcription, suggesting that pol II integrity is not impaired in these mutants; yet these mutants are defective in mRNA export. This suggests that maintaining pol II integrity when Rpb4p is mutant is not sufficient to ensure mRNA export. We propose that Rpb4p has a dual role during stress; a role in transcription and a role in mRNA export.

Previously, Woychik and her coworkers have selected *NSP1* as a high-copy suppressor of the $\Delta rpb4$ ts phenotype at 34°C (a very mild temperature stress that can readily be tolerated by WT strains but not by $\Delta rpb4$ strains; Tan *et al.*, 2000). The identification of *NSP1*, which encodes a component of the nuclear pore complex, is intriguing. Nsp1p could not suppress the *in vitro* transcriptional defects of $\Delta rpb4$ cell extracts (Tan *et al.*, 2000), consistent with the possibility that this suppressor affects a posttranscriptional function of Rpb4p. Although it was proposed that suppression by Nsp1p was indirect (Tan *et al.*, 2000), the results presented here suggest that overexpression of *NSP1* suppresses the export defect that characterizes $\Delta rpb4$ mutant cells in a direct manner. The ability of *NSP1* to suppress the ts defect of $\Delta rpb4$ under moderate temperature stress suggests that Rpb4p plays a role in enhancing the efficiency of the export machinery; it is not essential for export if export is enhanced by other means (e.g., by Nsp1p overproduction). Indeed, Figure 6 shows that, even at 37°C, a severe defect in export was observed in some, but not all, YTN25 cells.

Our attempts to demonstrate a physical interaction of Rpb4p with mRNA either directly or via the export complex have thus far not been successful. This suggests that either the interaction of Rpb4p with the export complex is transient or unstable when extracted from the cell or that the essential role of Rpb4p in export is indirect. Interestingly, however, Rpb4p complexed with Rpb7p is capable of binding RNA *in vitro* (Orlicky *et al.*, 2001), which leads us to favor the possibility proposed by Orlicky *et al.* (2001) that the heterodimer interacts (transiently?) with the emerging transcript.

Our study was based, to a substantial degree, on our earlier studies of mRNA distribution after stress, using *in situ* hybridization (Saavedra *et al.*, 1996, 1997). Subsequently, another study suggested that HS mRNA was not exported selectively after heat shock (42°C) but instead competed for export with bulk poly(A)⁺ RNA (Vainberg *et al.*, 2000). Unlike our previous results (Saavedra *et al.*, 1996, 1997), no nuclear accumulation of poly(A)⁺ mRNA was detected by Vainberg *et al.* We tested the WT and $\Delta rip1$ strains (derivatives of W-303-1a) used by Vainberg *et al.* and found that, in our laboratory, they behaved similarly to our WT strains (FY23 and FY86, derivatives of S288C; Winston *et al.*, 1995). We therefore suspect that the different conclusions from the two studies reflect differences in how the *in situ* hybridization assays were performed. Consistent with our previous results (Saavedra *et al.*, 1996, 1997), selective mRNA export

during stress has also been observed in both *Schizosaccharomyces pombe* (Tani *et al.*, 1996) and mammalian cells (Imed-Eddine *et al.*, 2000). In addition, Brodsky and Silver (2000) used a novel RNA-binding GFP fusion protein assay to localize individual species of mRNA and reported efficient export of *SSA4* mRNA and nuclear accumulation of *PGK1* and *ASH1* mRNAs in stressed cells.

Many studies over the past several years indicate that there is an intricate coupling between transcription, mRNA processing, and mRNA export. Recent studies are beginning to define the mechanisms of coordination. The recruitment of nuclear export factors onto nascent transcripts during transcription involves a variety of factors, including Npl3p (Lei *et al.*, 2001), Yra1p, Sub2p, and the THO complex (containing 4 subunits; Jimeno *et al.*, 2002; Strasser *et al.*, 2002 and references therein). Moreover, genetic analyses revealed the possible involvement of TBP (Lei *et al.*, 2001) and TAF (*ptr6*⁺ of *S. pombe*; Shibuya *et al.*, 1999) whose specific roles in export are not clear. Recently, eight proteins whose roles in export are not known were reported to be tightly associated with THO/Yra1p/Sub2p complex (Strasser *et al.*, 2002). Taken together these data indicate that much remains to be uncovered about nuclear mRNA metabolism and export. In particular, how these components are involved in the regulation of both transcription and export in response to environmental signals is still unknown. This work assigns a transport role to the pol II subunit Rpb4p. Because both the transcriptional and export roles of Rpb4p are important only during stress, we propose that Rpb4p participates in the cellular responses to stress at the interface of the transcription and export machineries. The recruitment of Rpb4p to the transcription apparatus in response to starvation stress has been demonstrated previously (Choder and Young, 1993). It remains to be determined whether or not Rpb4p is recruited to the transport complex, or to auxiliary complexes involved in transport, also only during stress.

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