

## Nucleocytoplasmic Shuttling of the Rpb4p and Rpb7p Subunits of *Saccharomyces cerevisiae* RNA Polymerase II by Two Pathways<sup>∇</sup>

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**Rpb4p and Rpb7p are subunits of the RNA polymerase II of *Saccharomyces cerevisiae* that form a dissociable heterodimeric complex. Whereas the only reported function of Rpb7p is related to transcription, Rpb4p has been found to also act in mRNA export and in the major mRNA decay pathway that operates in the cytoplasm, thus raising the possibility that Rpb4p links between the nuclear and cytoplasmic processes. Here we show that both Rpb4p and Rpb7p shuttle between the nucleus and the cytoplasm. Shuttling kinetics of the two proteins are similar as long as their interaction is possible, suggesting that they shuttle as a heterodimer. Under normal conditions, shuttling of Rpb4p and Rpb7p depends on ongoing transcription. However, during severe stresses of heat shock, ethanol, and starvation, the two proteins shuttle via a transcription-independent pathway. Thus, Rpb4p and Rpb7p shuttle via two pathways, depending on environmental conditions.**

Cellular components function in the context of their location within the cell. Therefore, an important posttranslational control of many cellular proteins determines their precise localization. Coordination between the two main cellular compartments, the nucleus and the cytoplasm, is mediated by the nuclear pore complexes (NPCs) embedded within the nuclear envelope. NPCs are dynamic complexes composed of ~30 proteins called nucleoporins. They facilitate the bidirectional transport of macromolecules across the nuclear envelope (recently reviewed in references 31 and 36). Protein transport is also mediated by cargo-specific machineries that facilitate rapid, directional, and selective transport (40). Transport of various RNA molecules is mediated by transport machineries specific for RNP proteins whose translocation drives the RNA transport (6). The association of several known RNA binding proteins begins during transcription (24). Following mRNP export, or at a late stage during export, some proteins are removed from the mRNP, allowing them to return to the nucleus for an additional round of mRNA export (see references 18 and 25 and references therein). Thus, these proteins shuttle between the two compartments. Whereas the various classes of RNAs are exported by class-specific pathways, recent works have suggested that export of mRNAs is governed by several distinct pathways (12, 13). One distinct pathway is involved in the export of stress-induced mRNAs (29).

Relatively little is known about stress-specific transport pathways. The results of a few studies have suggested that NPC and nucleocytoplasmic transport are modified in response to stress. For example, the DEAD box protein Rat8p/Dbp5p, which plays a role in mRNP remodeling at the cytoplasmic side of the NPC (18), dissociates from the NPC following ethanol (EtOH) stress (25). Export of the bulk of the mRNAs of *Saccharomyces cerevisiae* is blocked during severe stress (e.g.,

upon exposure to 42°C or high concentrations of EtOH), whereas that of stress-induced mRNAs is carried out efficiently in a process that requires most of the factors essential for normal poly(A)<sup>+</sup> RNA export (28, 29). One mRNA protein required for normal export, Npl3p, is not required for export of heat shock (HS) mRNA during HS (14). Conversely, export of HS mRNAs during HS is dependent on Nup42p/Rip1p, a nucleoporin dispensable for export under nonstress conditions (25, 29, 34). Nup42p and Rpb4p are the only proteins known to be essential for mRNA export only during stress (see below).

Rpb4p is a subunit of RNA polymerase II (Pol II), which is composed of 12 subunits (termed Rpb1p to Rpb12p) (43). Crystal structures of the 12 subunits of yeast Pol II have revealed that the 12-subunit enzyme consists of two distinct substructures (2, 3). The first is a 10-subunit core that constitutes the bulk of the Pol II structure and includes the catalytic active site. The major subunits of this core reside in the nucleus under all environmental conditions tested (8). The second part consists of two subunits, Rpb4p and Rpb7p, which form a dissociable heterodimeric complex (recently reviewed in references 5 and 30). Rpb4p and Rpb7p are present in excess over other Pol II subunit quantities (26), suggesting that they also play roles outside the context of the polymerase (5). Indeed, recent studies have demonstrated that Rpb4p can be localized in the cytoplasm and have assigned roles for this Pol II subunit in mRNA export during stress (8) and in mRNA degradation (17). In light of these observations, Rpb4p was proposed to be involved in the linkage between the nuclear and the cytoplasmic machineries that together determine the mRNA level within the cell (17). One prediction of the linkage hypothesis is that Rpb4p shuttles between the nucleus and the cytoplasm. Whether Rpb7p is involved in the posttranscriptional roles that Rpb4p mediates is presently unknown.

Here we show that Rpb7p localization, in similarity to that of Rpb4p, is responsive to the environment. We further show that both Rpb4p and Rpb7p shuttle between the nucleus and the cytoplasm. Interestingly, two distinct shuttling mechanisms transport the two proteins, depending on the environmental conditions. One is dependent on ongoing

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TABLE 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source or reference
yMC286	<i>MATa his3 leu2 ura3 Rpb7-GFP-HIS3X6</i>	This work
yMC310	<i>MATa his3 leu2 ura3 rpb4Δ::HIS3 Rpb7-GFP-HIS3X6</i>	This work
PSy413	<i>MATα his3 leu2 ura3 ade2 ade3 nup49::TRP1 + pUN100 (CEN/LEU2/nup49-313)</i>	15
yMC190	<i>MATa rpb1-1 his3 leu2 ura3 ade3 nup49::TRP1 + pUN100 (CEN/LEU2/nup49-313)</i>	P. Silver
yMS3	PSy413 + pMC190 ( <i>CEN/URA3/GFP-RPB4</i> ) + pMC308 ( <i>CEN HIS3 RPB7-RFP</i> )	This work
yMS4	yMC190 + pMC190 ( <i>CEN URA3 GFP-RPB4</i> ) + pMC308 ( <i>CEN/HIS3/RPB7-RFP</i> )	This work
yMS8	YMC310 + pMC305 ( <i>CEN/URA3/RPB4-RFP</i> )	This work
yMS18	<i>MATa lys801 leu2-3,112 trp1-1 ura3-52 his3Δ200 + pMC190 (CEN URA3 GFP-RPB4) + MC308 (CEN/HIS3/RPB7-RFP)</i>	This work
Fy86	<i>MATα his3Δ200 ura3-52 leu2Δ1</i>	42
yMS19	Fy86 + pMC143 ( <i>CEN/LEU2/GFP-RPB4</i> ) + pMC307 ( <i>CEN URA3 RPB7-RFP</i> )	This work
yMS21	<i>MATα ade2 ade3 his3 leu2 ura3 nsp49::TRP1 rpb4Δ1::HIS3 + pUN100 (CEN LEU2 nup49-313) + p307 (CEN URA3 RPB7-RFP)</i>	This work
yMC140	<i>MATa rpb7Δ::LEU2 ura3-52 his3Δ200 leu2-3,112 lys2Δ201 ade2 pURA3/CEN/RPB7</i>	20
yRL81	<i>MATa rpb7Δ::LEU2 ura3-52 his3Δ200 leu2-3,112 lys2Δ201 ade2 pHIS3/CEN/Rpb7-29<sup>145T,P63T,T64S,F109S,N122S</sup>-RFP p143 (GFP-RPB4/CEN/URA3)</i>	This work
yRL82	<i>MATa rpb7Δ::LEU2 ura3-52 his3Δ200 leu2-3,112 lys2Δ201 ade2 pHIS3/CEN/RPB7-RFP p143 (GFP-RPB4/CEN/URA3)</i>	This work

transcription, linking the functions of Rpb4 and Rbp7 in the nucleus with those in the cytoplasm. The other operates during severe stress conditions and is independent of transcription.

#### MATERIALS AND METHODS

**Yeast strains and growth conditions.** Yeast strains are listed in Table 1. Cells proliferated either in rich medium (2% glucose, 2% peptone, 1% yeast extract [YPD]) or, when selective conditions were required, in standard synthetic media.

Starvation was obtained by shifting the cells to synthetic medium lacking any sugar and lacking amino acids.

**Plasmid construction.** The plasmid carrying *RPB4p*-green fluorescent protein-*RPB4* (*RPB4p-GFP-RPB4*) was described previously (8). Plasmids carrying *RPB7-RFP* or *RPB4-RFP* are derivatives of pRP1084 (*pLSMI-RFP*) (a generous gift of Roy Parker) (32). This plasmid was cut with HindIII and BamHI, and the vector that contained the RFP was ligated either to the *RPB4p-RPB4*-open reading frame (ORF) fragment (*RPB4p-RPB4-ORF*) or to the *RP74p-RPB7-ORF* fragment (both ORF fragments lacking the stop codon were inserted in frame with the RFP ORF). pMC116 is a pCM190 (9) derivative that contains 170 bp of an *RPB7* 5' noncoding region and an *RPB7* open reading frame at the NotI-BamHI site. pMC257 is an *HIS3* derivative of pCM189 (9). All plasmids are centromeric and carry a *URA3*, *LEU2*, or *HIS3* selectable marker.

**Creating temperature-sensitive (ts) *rpb7* mutants.** *RPB7* was mutagenized randomly, utilizing a PCR mutagenesis scheme as described previously (8), OMC367 (5'-GCATGCATGTGCTCTGTATG-3'), and OMC368 (5'-GTTACATCGGTACACGCG), with PMC116 as the template. The mutagenized *rpb7*-containing fragment was inserted into NotI-HapI-linearized pMC257 by cotransformation in yMC140. *pRPB7::URA3* present in yMC140 was evicted by selecting cells that lose *URA3* by using a 5-fluoroorotic acid (5-FOA) plate, resulting in cells that carry only the mutagenized *rpb7*. 5-FOA selection was done twice. 5-FOA-resistant strains were allowed to form colonies at 24°C and 37°C. Mutants that were unable to form colonies at 37°C were selected.

**Fluorescent microscopy.** Fluorescent microscopy was done as previously described (8, 17).

**Two-hybrid assay.** The bait *RPB7*-activating domain (AD) or AD-tagged *rpb7* mutants were constructed as described previously (38). Two-hybrid interaction was determined by growth on plates lacking leucine, tryptophane, adenine, and histidine and supplemented with 5 mM 3-amino-1,2,4-triazole and by the β-galactosidase liquid test, as previously reported (38).

#### RESULTS

**Rpb7p is exported to the cytoplasm in response to various stress conditions.** We have previously found that Rpb4 is ex-

ported to the cytoplasm in response to HS, EtOH stress, or starvation conditions (8). Since Rpb4p and Rpb7p are known to function in transcription as a heterodimer (5), we tested whether Rpb7p is also exported to the cytoplasm in response to these stress conditions. To this end, we created a strain that carries the *RPB7-GFP* fusion gene in place of *RPB7* (yMC286). These cells proliferate similarly to wild-type (WT) cells, indicating that the fusion protein that replaces the essential Rpb7p is functionally similar to Rpb7p. As shown in Fig. 1, Rpb7p-GFP was localized to the nucleus during proliferation under optimal conditions, as expected from an RNA Pol II subunit. However, in response to HS, EtOH stress, or starvation, Rpb7p-GFP was relocated and found mainly in the cytoplasm. Addition of the translation inhibitor cycloheximide concomitant with a shift to the stress conditions did not affect Rpb7p-GFP relocation (Fig. 1). These results indicate that the Rpb7p-GFP molecules found in the cytoplasm were not newly synthesized and that no newly synthesized factors are required for relocation of Rpb7p-GFP. Thus, Rpb7p-GFP is exported from the nucleus to the cytoplasm in response to stresses in the form of HS, EtOH, or starvation. Since Rpb7p is required for transcription (5), some portion of it must remain in the nucleus. However, as Rpb7p molecules are present in excess over the Pol II complex quantities (5), the cytoplasmic fluorescence of Rpb7p-GFP masks its nuclear fluorescence. A similar rationale holds for Rpb4p-GFP localization during stress (8).

The cytoplasmic Rpb7p in glucose-starved cells reentered the nucleus when glucose was reintroduced (Fig. 1B). Reentry was insensitive to cycloheximide after reentry, indicating that reentry does not require newly synthesized proteins. Thus, the subcellular localization of Rpb7p is responsive to the environment. In contrast with Rpb4p and Rpb7p, the core Pol II subunits Rpb2p and Rpb3p are localized constitutively in the nucleus during both optimal and stress conditions (8).

**Rpb4p and Rpb7p shuttle between the nucleus and the cytoplasm.** Our observations showing that the cellular localization of Rpb4p and Rpb7p is responsive to the environment

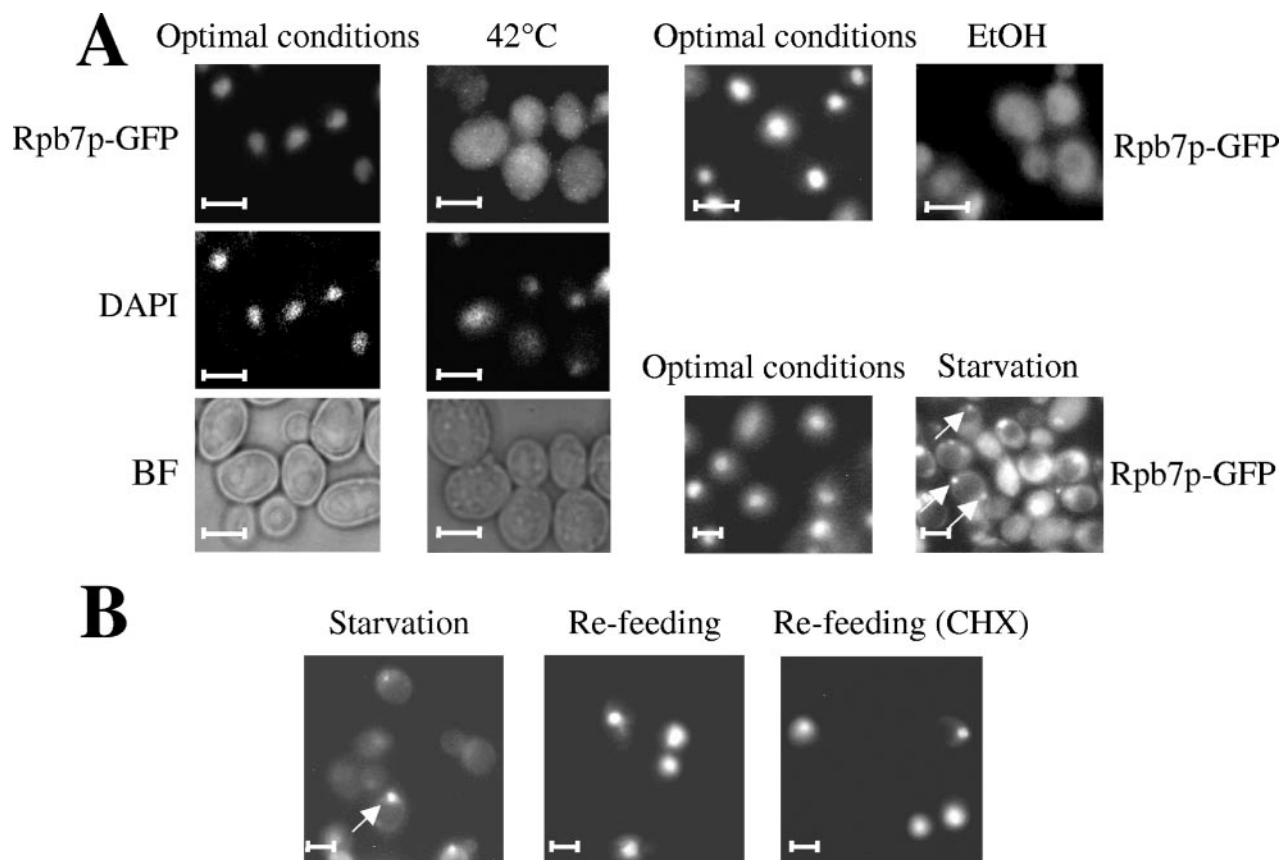


FIG. 1. Rpb7p-GFP localization is responsive to the environment. (A) Rpb7p is exported to the cytoplasm in response to various stress conditions. Cells carrying *RPB7*-GFP in place of *RPB7* (*yMC286*) were allowed to proliferate for at least eight generations under optimal conditions in rich medium (YPD) at either 24°C (HS experiment) or 30°C (other experiments) until the mid-logarithmic phase ( $\leq 5 \times 10^6$  cells/ml). Cells were then treated with 50  $\mu\text{g/ml}$  cycloheximide except for the starvation culture and were challenged with stress as indicated. The nonstressed samples were also treated with 50  $\mu\text{g/ml}$  cycloheximide and were further incubated in parallel with the stress-treated cultures. "Starvation" denotes synthetic medium lacking sugar and amino acids. Rpb7p-GFP localization was examined by fluorescent microscopy 3 h after HS or 12 h after addition of EtOH or after 3 days of starvation. The middle panels of the HS experiment show the position of the nuclei stained by DAPI (4',6'-diamidino-2-phenylindole), and the bottom panels show bright-field (BF) images of the cells; the results shown demonstrate that Rpb7p localization during optimal proliferation is nuclear whereas after HS it is cytoplasmic. (B) Cytoplasmic Rpb7p-GFP in starved cells was reimported to the nucleus after cells were re-fed with glucose. Starved cells were re-fed with 2% glucose and divided into two cultures. One culture (designated CHX) was treated with 50  $\mu\text{g/ml}$  cycloheximide, and the cells were examined microscopically 90 min after addition of glucose. In the absence of the drug, the kinetics of Rpb7p-GFP relocation was similar to that observed in its presence (results not shown). Note that in response to starvation (see panels A and B), Rpb7p-GFP was localized in discrete cytoplasmic foci that are smaller than nuclei. Some are denoted by arrows. These foci probably represent cytoplasmic P bodies that also accommodate Rpb4p (17). The bars represent 5 $\mu\text{m}$ .

raised the possibility that these proteins are capable of shuttling between the nucleus and the cytoplasm. To examine this possibility, we performed a standard nuclear export assay (15). This assay takes advantage of its nucleoporin mutant *nup49-313*. At the restrictive temperature, import into the nucleus is blocked whereas export out of the nucleus is unaffected. Thus, if the fluorescent proteins normally shuttle between the nucleus and the cytoplasm, they should accumulate in the cytoplasm of the *nup49-313* cells when these cells are shifted to 37°C, the nonpermissive temperature. Several shuttling factors have been identified using this assay (see, e.g., references 15 and 25). This assay was also useful in determining that several mRNA transport proteins, e.g., Rna14p, Rna15p, and Pcf11p, do not shuttle (11). *RPB7*-red fluorescent protein (*RPB7*-RFP) and GFP-*RPB4* were coexpressed in the mutant cells carrying *nup49-313*. As shown in Fig. 2A, both proteins were found

mainly in the nucleus upon incubation of *nup49-313* cells at 24°C; however, they accumulated in the cytoplasm when the cells were shifted to the nonpermissive temperature (Fig. 2A and control results in 2D). Since cycloheximide was added before the increase in temperature, we concluded that the appearance of tagged Rpb4p and Rpb7p in the cytoplasm upon increasing the temperature was not due to accumulation of newly synthesized protein. In contrast with the rapid relocation of Rpb4p and Rpb7p in *nup49-313* cells (Fig. 2A), incubation of WT cells at 37°C resulted in much slower relocation kinetics, so that by 5 h both Rpb4p and Rpb7p were still mainly in the nucleus (Fig. 2B). Immunoblotting of cell lysates made before and after the temperature increase confirmed that the Rpb4p and Rpb7p levels as well as integrity were not affected by incubating cells at 37°C (4, 8). Therefore, the cytoplasmic signal was due to presence of the intact fusion proteins. This

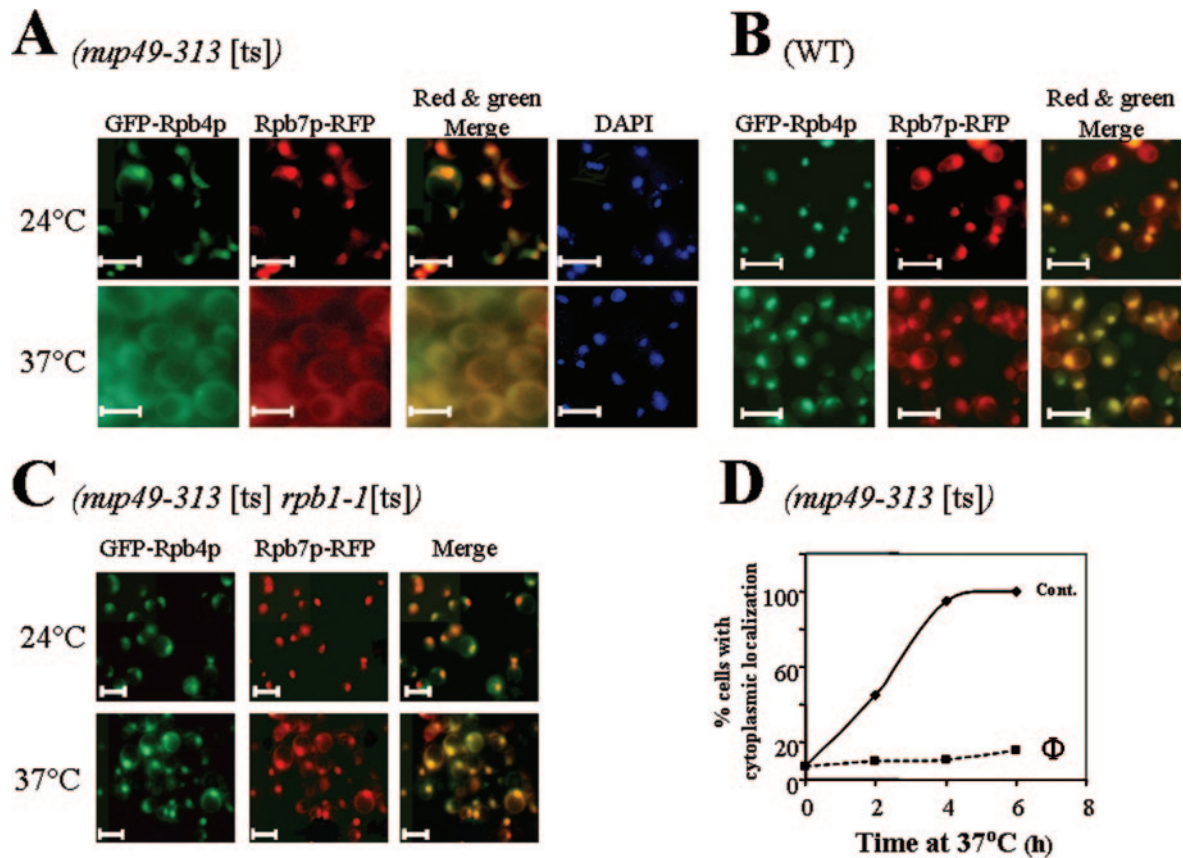


FIG. 2. GFP-Rpb4p and Rpb7p-RFP shuttle by a transcription-dependent mechanism. (A) Shuttling of both proteins was demonstrated using *nup49-313*(*ts*) mutant cells that are defective in protein import at elevated temperatures. Cells expressing both GFP-Rpb4p and Rpb7p-RFP (yMS3) were allowed to proliferate for at least eight generations under optimal conditions in selective media at 24°C. Both fluorescent proteins were then localized in the nucleus (results not shown). Cycloheximide (50  $\mu$ g/ml) was then added, and the culture was divided into two samples. One was incubated for an additional 5 h at 24°C and the other for 5 h at 37°C. Images were then taken using green (GFP-Rpb4p), red (Rpb7p-RFP), or blue (to detect nuclei stained by DAPI) filters, as indicated. (B) Images of WT cells expressing Rpb4p-RFP and Rpb7p-GFP (yMS8) at 24°C or 5 h after incubation at 37°C in the presence of 50  $\mu$ g/ml cycloheximide. (C) Export is blocked when transcription is arrested. Images of *nup49-313*(*ts*) *rpb1-1*(*ts*) cells expressing both GFP-Rpb4p and Rpb7p-RFP (yMS4) at 24°C or 5 h after incubation at 37°C are shown. Cycloheximide was added as described for panel A. (D) Export kinetics of Rpb7p-RFP after a shift from 24°C to 37°C in the absence (Cont.) or presence ( $\Phi$ ) of the transcription inhibitor 1,10-phenanthroline (100  $\mu$ g/ml). Cycloheximide was added as described for panel A. To monitor export kinetics, samples of each culture were examined microscopically at the indicated time points and photographs of random fields were taken using both the green and red channels. More than 200 cells were classified into those exhibiting nuclear or whole-cell (i.e., cytoplasmic) localization of the respective fluorescent protein. The proportion of cells exhibiting cytoplasmic localization was plotted as a function of time. The merged images in panels A and C (created using Adobe PhotoShop) demonstrate that the distributions of the two fluorescent proteins were similar. The bars represent 5 $\mu$ .

localization pattern is indicative of shuttling activity and is characteristic of other shuttling proteins (see, e.g., references 11 and 15).

**Rpb4p and Rpb7p shuttling is dependent on active transcription.** One of the features that characterizes some shuttling factors involved in mRNA export is their dependency on transcription (1). Since they are Pol II subunits that also shuttle, it was important to determine whether their shuttling capacity was dependent upon ongoing transcription. To address this possibility, we performed the nuclear export assay using a strain carrying both *nup49-313* and *rpb1-1* encoding a *ts* mutant form of the Pol II subunit Rpb1p (22). Upon increase of the temperature of cells to 37°C, the products of both *ts* alleles became inactive, resulting in both import and transcription defects. In contrast to their capacity to exit the nucleus of *nup49-313* cells upon heat inactivation of Nup49-313p, both

GFP-Rpb4p and Rpb7p-RFP were unable to leave the nucleus of cells carrying both the *nup49-313* and *rpb1-1* alleles (Fig. 2C). These results indicate that efficient export of these proteins requires Rpb1p activity. To examine the dependency of GFP-Rpb4p and Rpb7p-RFP transport on transcription by other means, we used the drug 1,10-phenanthroline, which blocks transcription (see references 10 and 17 and references therein). Treating *nup49-313* cells that carry WT *RPB1* with this drug before the temperature shift upward blocked the export of both the tagged Rpb4p (data not shown) and the tagged Rpb7p (Fig. 2D). Taken together, the results lead us to conclude that ongoing transcription is necessary for export of Rpb4 and Rpb7.

**In response to severe stresses, export of Rpb4p and Rpb7p is independent of transcription.** It was previously found that, in response to severe HS (42°C) but not more moderate HS

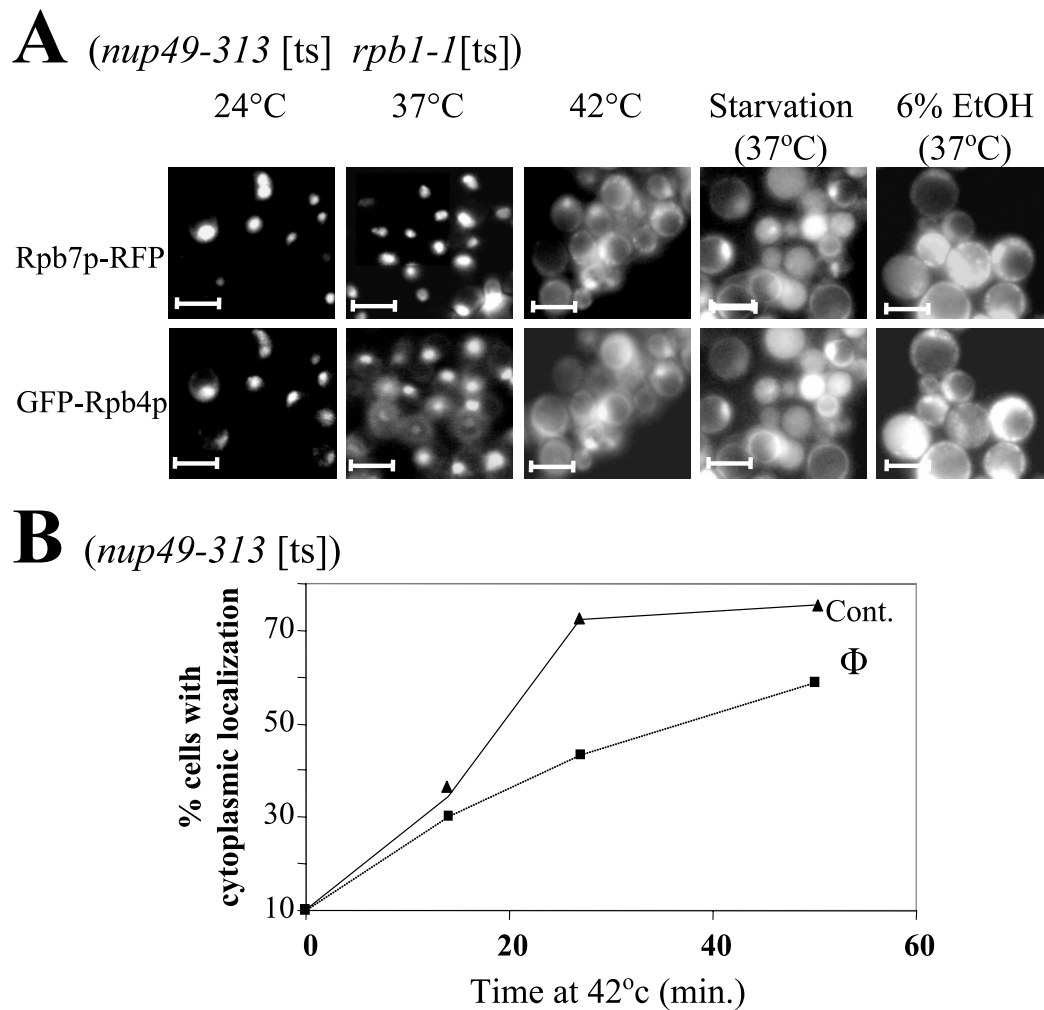


FIG. 3. Export of GFP-Rpb4p and Rpb7p-RFP during severe stresses is independent of transcription. (A) Images of *nup49-313*(ts) *rpb1-1*(ts) cells expressing both GFP-Rpb4p and Rpb7p-RFP (yMS4) in the mid-logarithmic phase at 24°C (left panels) or after 2 h under the indicated stress conditions in the presence of 50  $\mu$ g/ml cycloheximide. The nonstressed sample was equally treated with cycloheximide and further incubated in parallel with the stress-treated cultures. The starved and EtOH-treated cultures were incubated at 37°C to inactivate transcription. The bars represent 5 $\mu$ . (B) Export kinetics, determined as described for Fig. 2D, of Rpb7p-RFP after a shift from 24°C to 42°C in the absence (Cont.) or presence ( $\Phi$ ) of the transcription inhibitor 1,10-phenanthroline (100  $\mu$ g/ml).

(e.g., 37°C), the export of most mRNAs is blocked and only HS mRNAs are exported (14, 28, 29, 34). Thus, different transport pathways are employed depending on the environment. We therefore compared the capacities of Rpb4p and Rpb7p to be exported under moderate or severe stress conditions. During moderate HS (37°C), GFP-Rpb4p and Rpb7p-RFP were retained in the nuclei in double-mutant *nup49-313 rpb1-1* cells (Fig. 2C and 3A). In contrast, after these cells were shifted to severe HS (42°C), Rpb4p and Rpb7p were exported to the cytoplasm even under conditions that inactivate Rpb1-1p, i.e., in the absence of transcription (Fig. 3A). We next tested export of Rpb4p and Rpb7p during other severe stresses. Briefly, cells carrying both the *nup49-313* and *rpb1-1* alleles were shifted to 37°C to inactivate transcription and immediately thereafter challenged with either 6% EtOH or with starvation medium (see Materials and Methods). As in the response to severe HS, both Rpb4p and Rpb7p were also

exported in response to EtOH and starvation, while transcription was blocked (Fig. 3A).

We next examined whether transcription-independent export of Rpb4p and Rpb7p can be observed by blocking the transcription of cells carrying WT Pol II by use of the drug 1,10-phenanthroline. Consistent with the results shown in Fig. 3A, Fig. 3B shows that the drug did not block export at 42°C, although it had a relatively minor inhibitory effect (compare Fig. 2D with 3B). These results indicate that during the tested severe stress conditions, export of Rpb4p and Rpb7p is largely independent of transcription (see Discussion).

**Rpb4p and Rpb7p shuttle between the nucleus and the cytoplasm also during severe stresses.** During severe stresses, when Rpb4p and Rpb7p are exported by a transcription-independent pathway and are found mainly in the cytoplasm (Fig. 1 and reference 8), a portion of these proteins must be located in the nucleus to enable transcription (5). This raises

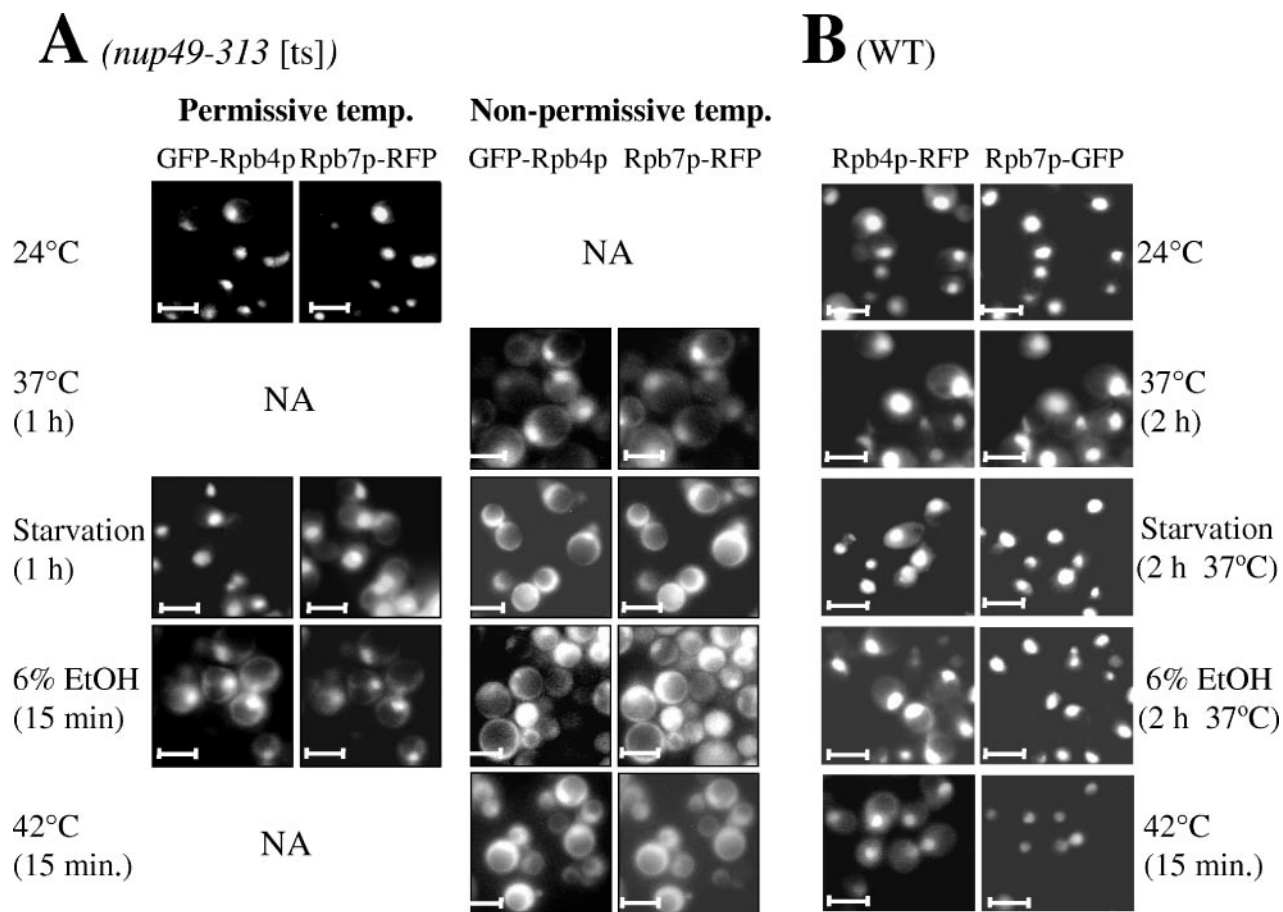


FIG. 4. Rpb4p and Rpb7p shuttle during severe stress conditions. (A) Relocation in *nup49-313* cells is faster at the nonpermissive temperature. *nup49-313*(ts) cells expressing both GFP-Rpb4p and Rpb7p-RFP (yMS3) were allowed to proliferate for at least eight generations under optimal conditions in selective media at 24°C. Cells were then challenged with the indicated stress at 24°C (Permissive temp.) (left panels) or at 37°C, or 42°C when indicated (Non-permissive temp.) (right panels). Cycloheximide (50  $\mu$ g/ml) was added to all samples as described for Fig. 2A. (B) Little relocation occurs in WT cells. Images of WT cells expressing both GFP-Rpb4p and Rpb7p-RFP (yMS8) similarly challenged with the indicated conditions in the presence of cycloheximide are shown. NA, not applicable. The bars represent 5 $\mu$ .

the question whether the stress-induced relocation reflects a one-way transport. Alternatively, these proteins also shuttle back and forth during severe stresses, but because their export is faster than their import, they gradually accumulate in the cytoplasm. In the latter case, accumulation of Rpb4p and Rpb7p in the cytoplasm under severe stress conditions is expected to be accelerated when their import is blocked due to Nup49-313p inactivation. To address the issue of shuttling during stress, we shifted *nup49-313* cells producing either GFP-Rpb4p or Rpb7p-RFP to 37°C to inactivate protein import (see the results for nonpermissive temperature in Fig. 4A) and concomitantly challenged them with starvation or 6% EtOH or by further increasing the temperature to 42°C (severe HS). After a relatively short incubation period under these stress conditions, complete relocation was observed at the nonpermissive temperature, while relatively little relocation was observed at 24°C—a temperature that permits import (Fig. 4A). Although some relocation occurred at 37°C without additional stress, it was incomplete, as nuclear fluorescence was clearly detected (Fig. 4A). Moreover, little relocation was observed in WT

cells exposed to even longer periods of the same stress conditions (Fig. 4B). These results indicate that, although Rpb4p (8) and Rpb7p (unpublished observation) are relocated very slowly in response to a temperature of 37°C, this temperature is not responsible per se for the rapid relocation in the mutant cells. Rather, the rapid relocation was due to inactivation of Nup49-313p. Taking these results together, we conclude that Rpb4p and Rpb7p shuttle under the examined stress conditions, as they do under more-hospitable conditions (see Fig. 2). We propose that under severe stress conditions Rpb4p and Rpb7p export is faster than their import and, hence, that they gradually accumulate in the cytoplasm.

**Rpb4p and Rpb7p are exported at similar kinetics in response to stresses of HS, starvation, and EtOH.** Rpb4p plays a role both in transcription within the nucleus and in the major mRNA decay pathway in the cytoplasm (17). An unresolved issue is whether Rpb4p, which functions in transcription together with Rpb7p, also functions in mRNA decay in the context of the Rpb4-Rpb7 heterodimer. If the two proteins function in the cytoplasm as a heterodimer, it is likely that they

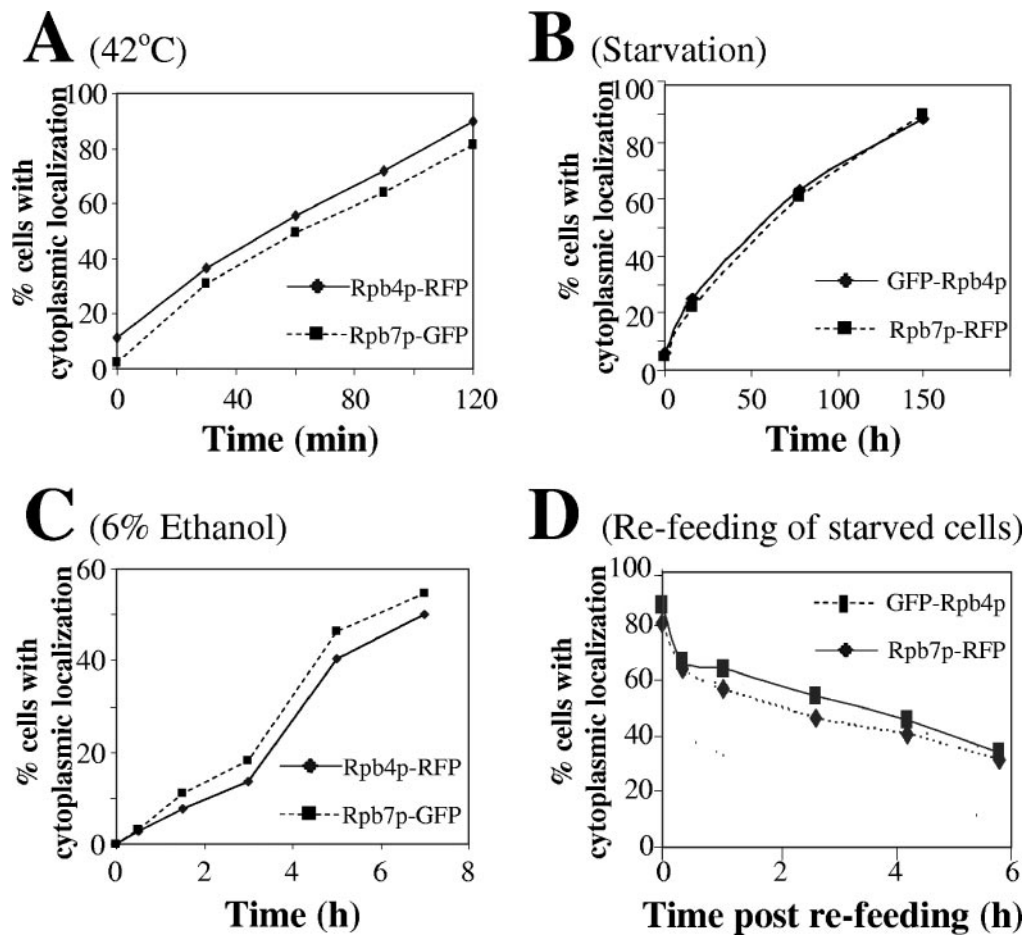


FIG. 5. In response to various stresses, Rpb4p and Rpb7p are exported to the cytoplasm at similar kinetics. (A to C) yMS4 (A), yMS18 (B), and yMS8 (C) cells expressing the indicated fluorescence-tagged proteins were allowed to proliferate for at least eight generations under optimal conditions in selective media at either 24°C (A) or 30°C (B, C, and D) until the mid-logarithmic phase ( $5 \times 10^6$  cells/ml). Cultures were then challenged under the indicated stress condition as described for Fig. 1. Export kinetics experiments were performed as described for Fig. 2D. (D) Import kinetics of GFP-Rpb4p and Rpb7p-RFP are comparable. yRL82 cells were starved until both fluorescent proteins were localized in the cytoplasm in 80% or 90% of the cells. Cells were then collected by centrifugation and resuspended in synthetic complete medium (containing glucose and amino acids) supplemented with 50  $\mu$ g/ml cycloheximide (similar results were obtained when cycloheximide was not added). Import kinetics results were determined as described for panels A to C.

shuttle together. If this is the case, some predictions can be made, the first being that the export kinetics of the two proteins are similar. We also predict that disruption of Rpb4p and Rpb7p interaction, either by deleting the nonessential *RPB4* or by mutating the essential *RPB7*, would have an effect on the kinetics.

As a first approach, we determined the export kinetics of the two fluorescent proteins in response to stresses of HS, EtOH, and starvation and found them to be identical (Fig. 5A, B, and C). To rule out the possibility that the fluorescent tag influenced the export kinetics, we exchanged the tags between the two proteins and found that the tags had no specific effect. To illustrate this observation, we show in Fig. 5A and C the results obtained using Rpb4p-RFP and Rpb7p-GFP and in Fig. 5B the results obtained using GFP-Rpb4p and Rpb7p-RFP. In both cases similar export kinetics results were observed. Moreover, the import kinetics results obtained with GFP-Rpb4p and Rpb7p-RFP in response to shifting starved cells to rich medium were also identical (Fig. 5D). Careful microscopic exam-

ination of these proteins under various environmental conditions indicated that their localization patterns were always similar (see, for example, the merge panels in Fig. 2A and C; other results not shown). Only in cases in which the heterodimer could not be formed did we observe differing results (see Discussion).

Note that stress-induced relocation of Rpb4p and Rpb7p in the WT cells (shown in Fig. 5) was slower than it was in *nup49-313(ts)* cells at 37°C (shown in Fig. 3A). For example, after 2 h at 37°C in starvation medium, Rpb4p and Rpb7p were localized mainly in the cytoplasm in all mutant cells (see results for starvation at 37°C in Fig. 3A). In contrast, relocation of these proteins after starving WT cells for 2 h was marginal, as only a small fraction of these cells exhibited complete relocation (Fig. 5B). These differences are in accord with our results presented in Fig. 4 that show that Rpb4p and Rpb7p shuttle under stress conditions such that by blocking import (as was the case in the experiment whose results are shown in Fig. 3A) their relocation is accelerated (see also legend to Fig. 8).

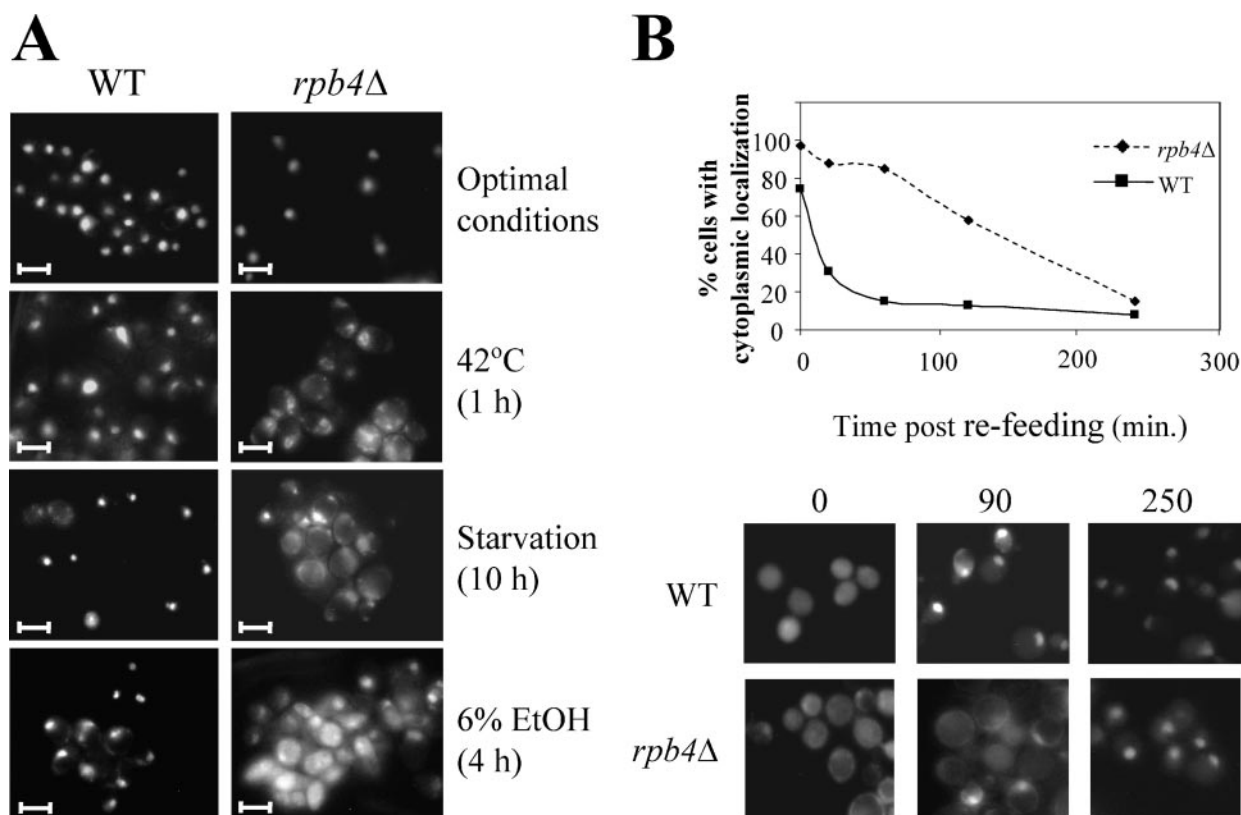


FIG. 6. Transport of Rpb7p-GFP during severe stresses is affected by *RPB4*. (A) Stress-induced relocation of Rpb7p-GFP is accelerated in the absence of *RPB4*. Images of WT cells carrying *RPB7*-GFP in place of *RPB4* (yMC286) and isogenic *rpb4Δ*-derivative cells (yMC310) that were challenged with various stresses for relatively short periods of time are shown. The kinds of stresses and their durations are indicated on the right side of each panel. Starvation conditions were simulated by use of synthetic medium lacking sugar and amino acids. Note that after a brief period of stress, relatively little export was detected in the WT cells (left panels) whereas export in the mutant was more advanced (right panels). (B) Efficient import of Rpb7p-GFP is dependent on Rpb4p. The strains described for panel A were incubated in starvation medium for 72 h to permit relocation of Rpb7p-GFP in 77% of WT cells and 100% of the mutant cells. Cells were then collected by centrifugation and resuspended in synthetic complete medium (containing glucose and amino acids) supplemented with 50  $\mu$ g/ml cycloheximide. Import kinetics results were determined as described for Fig. 2. Note that in response to starvation or HS, Rpb7p-GFP can be localized in discrete cytoplasmic foci that are smaller than nuclei. We suspect that these foci represent cytoplasmic P bodies (17).

**Rpb4p is required for efficient import of Rpb7p from the cytoplasm to the nucleus.** To further examine whether Rpb4p and Rpb7p shuttle independently or as a heterodimer, we determined whether disrupting the heterodimer affected their export or import kinetics. We first deleted *RPB4* and found that the stress-induced relocation of Rpb7p-GFP was substantially faster in the absence of Rpb4p than it was in its presence. This can be demonstrated by the cytoplasmic localization of Rpb7p-GFP in the *rpb4Δ* cells after a relatively short exposure to stress while Rpb7p-GFP localization in the WT cells was still mainly nuclear (Fig. 6A). The rapid relocation may be due to accelerated export or, since Rpb7p-GFP shuttles (Fig. 3C), may be due to slow import (or to both). We therefore determined whether import of Rpb7p-GFP was affected by Rpb4p by refeeding starved cells whose Rpb7p-GFP was localized in the cytoplasm with fresh medium that contained all the necessary nutrients. As shown in Fig. 6B, Rpb7p-GFP was imported to the nucleus more slowly in the absence of Rpb4p than in its presence, suggesting that the rapid relocation of Rpb7p-GFP when *rpb4Δ* cells were exposed to stress was due at least in part to slow import.

**WT Rpb7p is required for efficient import of Rpb4p from the cytoplasm to the nucleus.** To determine whether the export and import of Rpb4p are dependent upon its capacity to interact with Rpb7p, we randomly mutagenized the essential *RPB7* and selected for ts mutants, a phenotype characteristic of *rpb4Δ* cells (see Materials and Methods). As was reported previously, single-point mutations in Rpb7p in highly conserved residues are incapable of compromising Rpb7p function (44). Consistently, all our ts mutants carried more than one mutation; for example, Rpb7-29p carried five point mutations (see Table 1). We then selected mutants that were defective in their capacity to form two-hybrid interactions with Rpb4p. The following experiments were performed with three mutants that exhibited defective interaction with Rpb4p and gave very similar results. Only one mutant, Rpb7-29p, which binds Rpb4p poorly even at the permissive temperature (Fig. 7A), is described hereafter. Rpb7-29p was tagged with RFP, and its relocation in response to stress was examined. Consistent with its defective interaction with Rpb4p, Rpb7-29p-RFP was relocated faster than WT Rpb7p-RFP during starvation (Fig. 7B) or during HS (results not shown), much like its accelerated relocation in cells



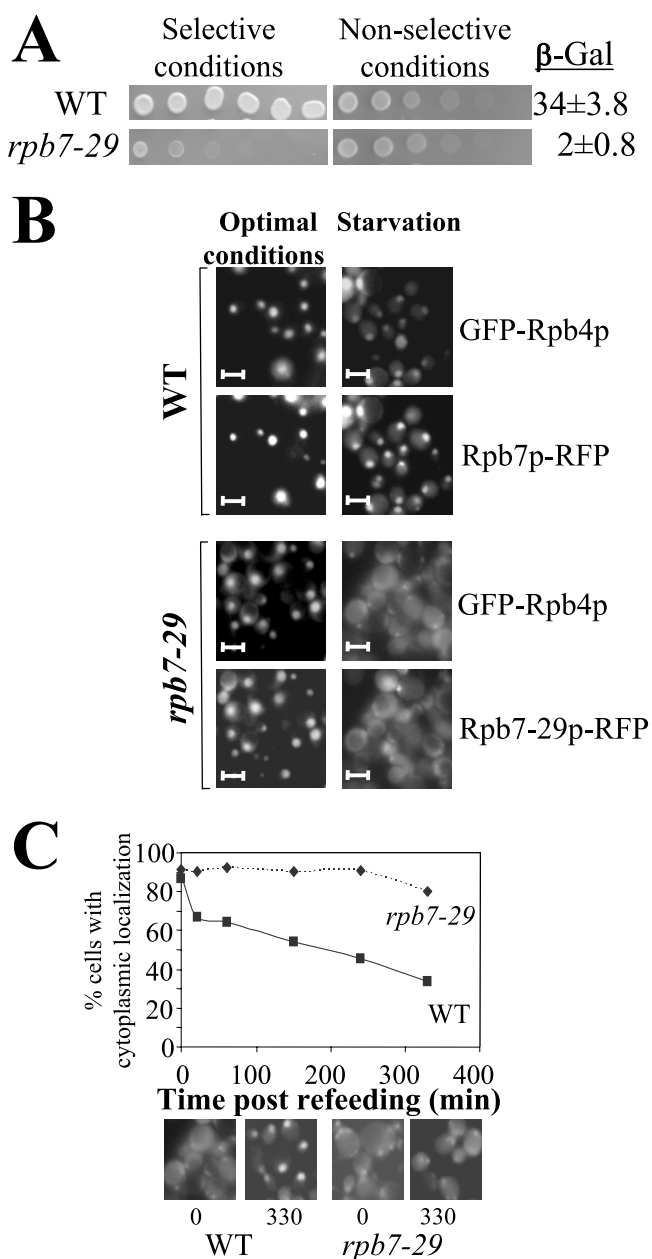


FIG. 7. Transport of GFP-Rpb4p during starvation is affected by *RPB7*. (A) Two-hybrid interaction between Rpb7p-DBD and Rpb4p-AD (upper row) and between Rpb7-29p-DBD and Rpb4p-AD (lower row). Cells were spotted in threefold serial dilutions starting with  $10^6$  cells/spot on an indicator plate (Selective conditions) as described in Materials and Methods. Growth on this plate indicates interaction (38). To demonstrate spotting of equal amounts of cells, cells were spotted on a nonselective plate that allowed growth of cells that carry both the bait and prey plasmids independently of the two two-hybrid interactions (using medium lacking only leucine and tryptophane). Beta-galactosidase ( $\beta$ -Gal) values, quantitative means used to determine interactions (38), are indicated at the right. They reflect the average values determined for three independent experiments. Background values obtained with cells expressing only the bait plasmid were subtracted. (B) Starvation-induced relocation of GFP-Rpb4p and Rpb7p-RFP is faster in *rpb7-29* cells than in WT cells. Images of WT cells carrying *RPB7*-RFP in place of *RPB7* and GFP-*RPB4* (yRL82) or cells carrying a *rpb7-29*-RFP derivative thereof (yRL81) that were challenged with starvation (using a synthetic medium lacking sugar and amino acids) at 37°C for a relatively short period of time (45 min) are

lacking *RPB4* (see results for Rpb7p-RFP in Fig. 6A). We then examined stress-induced relocation of WT GFP-Rpb4p in *rpb7-29* cells and found it to be faster than it was in WT cells (Fig. 7B). Again, this rapid relocation might be due either to accelerated export or, since Rpb4p shuttles (Fig. 2 and 4), to slow import. To determine whether GFP-Rpb4p import was affected by the mutations in Rpb7p, we re-fed starved cells whose GFP-Rpb4p was localized in the cytoplasm with fresh medium that contained all the necessary nutrients. Import of WT GFP-Rpb4p was much slower in *rpb7-29* mutant cells than it was in the parental *RPB7* cells (Fig. 7C). In contrast, the import kinetics results obtained with WT GFP-Rpb4p and WT Rpb7p-RFP in WT cells, in which the heterodimer can be formed, were identical (Fig. 5D).

The results shown in Fig. 6 and 7 indicate that the import kinetics of both proteins are severely impaired when the heterodimer integrity is compromised either by deleting *RPB4* or by mutating *RPB7*. Thus, disruption of the heterodimer should affect GFP-Rpb4p or Rpb7p-GFP localization much as inactivation of Nup49-313p does, because in both cases import is compromised. Indeed, in both cases the net relocation of the tagged Rpb4p and Rpb7p from the nucleus to the cytoplasm in response to stress was faster than it was in WT cells. Thus, these results and those shown in Fig. 3C indicate that both Rpb4p and Rpb7p shuttle under the examined stress conditions. Moreover, the results shown in Fig. 5, 6, and 7 suggest that both proteins normally shuttle as a heterodimer.

## DISCUSSION

Rpb4p is unusual among Pol II subunits in that it couples between transcription and mRNA transport (8). Moreover, Rpb4p is the only factor of the basal transcription apparatus that is known to also control mRNA decay in the cytoplasm, thus regulating mRNAs at both their synthesis and decay levels (17). Therefore, unlike some other export factors that are reimported immediately following mRNA export (18, 25, 33), Rpb4p remains associated with the mRNA in the cytoplasm until its degradation in the P bodies (17). It was not known whether Rpb4p is degraded after its role in the cytoplasmic P bodies is completed or is imported back to the nucleus for recycling.

Here we show that Rpb4p shuttles between the nucleus and cytoplasm by a cycloheximide-insensitive pathway. Thus, a

shown. The same field was photographed using either the green (for detection of GFP-Rpb4p) or red (for detection of Rpb7p-RFP) channel, as indicated. Note that after a short period of starvation, relatively little export was detected in the WT cells (upper right panels) whereas export of both fluorescent proteins in the mutant was more advanced (lower right panels). (C) Import of GFP-Rpb4p is impaired in *rpb7-29* mutant cells. WT cells (yRL82) or *rpb7-29* mutant cells (yRL81) expressing GFP-*RPB4* were starved until GFP-Rpb4p was localized in the cytoplasm in ~90% of the cells. Cells were then collected by centrifugation and resuspended in synthetic complete medium (containing glucose and amino acids) supplemented with 50  $\mu$ g/ml cycloheximide (similar results were obtained when cycloheximide was not added). Import kinetics results were determined as described for Fig. 5D. Examples of photographs of cells taken at the indicated time points after refeeding are shown below the graph.

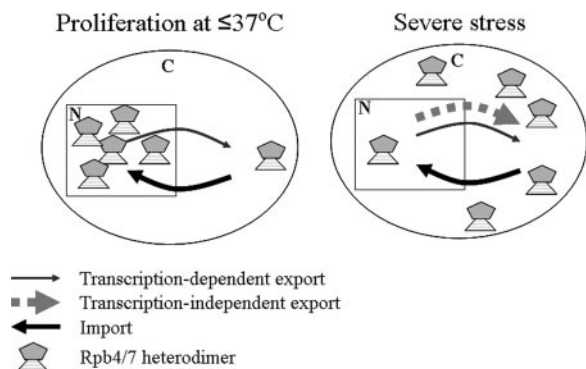


FIG. 8. Model of the proposed shuttling pathways of the Rpb4-Rbp7 heterodimer. Rpb4p and Rpb7p shuttle as a heterodimer. Under conditions that support proliferation, export of the Rpb4-Rbp7 heterodimer is dependent on transcription. Import of the heterodimer (thick arrow) is faster than its export (thin arrow), resulting in mainly nuclear localization. In response to some severe stress conditions, a relatively robust transcription-independent mechanism is induced (thick dashed arrow) in addition to a relatively weak transcription-dependent mechanism (thin arrow). Export is then faster than import, and the general direction of the net flow is from the nucleus to the cytoplasm. Because the Rpb4-Rbp7 heterodimer must be located in the nucleus to enable transcription during stress (5), this flux of the Rpb4-Rbp7 heterodimer from the nucleus to the cytoplasm must be leveled off at some point during prolonged incubation under these stress conditions and balanced by the retrograde flux. While export of Rpb4p or Rpb7p does not seem to be inhibited by disruption of the heterodimer integrity, the import pathway is very sensitive to this integrity. Thus, import of Rpb4p or Rpb7p is severely compromised when the heterodimer is disrupted either by deleting *RPB4* or by mutating *RPB7*. Import is also blocked by inactivating Nup49p. In all these cases the net flow of these molecules from the nucleus to the cytoplasm is faster than normal. N denotes the nucleus; C denotes the cytoplasm.

likely scenario is that Rpb4p is imported back to the nucleus to start a new cycle of transcription, mRNA transport, and degradation only after its role in mRNA decay is completed. Rpb4p may represent a new kind of shuttling protein that shuttles between the nucleus and P bodies. It is possible that this shuttling contributes to the proposed role of Rpb4p in communicating between transcription in the nucleus and mRNA decay in the cytoplasm. In addition, we found that Rpb7p, the close partner of Rpb4p, also shuttles between the two compartments. Moreover, Rpb7p's cellular location is responsive to the environment in a cycloheximide-insensitive manner, most likely due to environmentally regulated changes in the balance between its import and export rates (see Fig. 8).

Although Rpb4p and Rpb7p function as a heterodimer in transcription, it was not known whether they also function as a heterodimer outside this context. In fact, whereas Rpb4p was known to carry out functions outside the Pol II context, there were no indications that this is also the case for Rpb7p. Moreover, because *RPB4* and *RPB7* are found as two separate genes in archeal organisms and in all eukaryotes whose genomes have been sequenced (5), it is likely that the two proteins would physically separate at some point. Nevertheless, in spite of our efforts to find such a separation in WT cells, our current results do not support this possibility. Instead, it seems that Rpb4p and Rpb7p shuttle as a heterodimer under the studied conditions. First, Rpb4p import was compromised when Rpb7p was defective (Fig. 7). Likewise, in the absence of

Rpb4p, Rpb7p import was compromised (Fig. 6). Second, in response to severe stresses, the two proteins were exported to the cytoplasm with similar kinetics results. Importantly, export kinetics was not influenced significantly by the nature of the tag (see text discussing Fig. 5). Only in mutant cells whose Rpb4-Rbp7 heterodimer cannot be formed normally did we find different relocation kinetics or different localization patterns. For example, in response to challenging *rpb7-29* cells (yRL82) with starvation for 72 h, GFP-Rpb4p exhibited whole-cell localization in 90% of the cells, whereas Rpb7-29p-RFP exhibited similarly distributed localization in only 50% of the cells while in the other 50% of the cells it was mainly in the nucleus. This is consistent with our observation that Rpb4p import is more sensitive than Rpb7p import to disruption of the heterodimer integrity (compare the very slow import of Rpb4p shown in Fig. 7C with the faster import kinetics of Rpb7p shown in Fig. 6B). The mechanistic basis for these differences remains to be determined. Third, both Rpb4p and Rpb7p are constituents of two other complexes, i.e., cytoplasmic P bodies (17; R. Lotan and M. Choder, unpublished observation) and Npl3p-containing RNAs (17). Taken together, all these observations suggest that the role of Rpb4p within the cytoplasm is carried out in the context of the Rpb4-Rbp7 heterodimer. Although our current results do not support separate functions, we still believe that under yet-unexplored conditions the two proteins would function independently of each other.

Whether or not Rpb4p and Rpb7p have nuclear localization and nuclear export sequences remains to be determined. In this regard, it should be mentioned that studies that have dissected the transport signals responsible for localization of multiprotein complexes have uncovered numerous examples of a single subunit providing the transport signal for the entire complex (16, 19, 23, 35, 41). Therefore, it is possible that the Rpb4-Rbp7 heterodimer does not carry functional nuclear localization and nuclear export sequences of its own but instead shuttles as a component of larger complexes.

Recently it was shown that the Rpb4-Rbp7 heterodimer interacts *in vitro* with the transcript as soon as it emerges from Pol II (39). This interaction is mediated by at least one of the two RNA-binding domains of Rpb7p (21, 37). Thus, the Rpb4-Rbp7 heterodimer is probably the first protein that assembles with the transcript and is probably involved in recruiting other RNA-interacting proteins (39). We provided an *in vivo* demonstration that the Rpb4-Rbp7 heterodimer interacts with the Npl3p-containing mRNP (17). These observations and our observation that the two partners seem to export as a heterodimer in a transcription-dependent manner provoked us to propose that the Rpb4-Rbp7 heterodimer is exported together with the transcript that it helps synthesize, as is the case for other shuttling factors, e.g., Npl3p (15). Consistently, Rpb4p is essential for exporting the transcript (8).

Rpb4p's role in the export of poly(A)<sup>+</sup> mRNA is detected under conditions of mild temperature stress (37°C), as is consistent with its capacity to shuttle under these conditions. However, at a moderate temperature of 22°C, Rpb4p is not essential for mRNA export (8). Since Rpb4p's role in mRNA decay is evident under optimal conditions at 24°C (17), we suspect that Rpb4p is also involved in mRNA export at 22°C but that under these conditions it does not play a critical role. Consistent with this possibility is the observation that nucleocytoplasmic

mic transport is relatively resilient to perturbations when cells are incubated under optimal conditions but that it is very sensitive to the environmental temperature. Specifically, yeast strains lacking any one of approximately a third of their nucleoporins are able to grow at 22°C but die at elevated temperatures. Regarding another third of the essential nucleoporins, deletion of substantial portions thereof results in temperature-sensitive proliferation (27, 36).

Unexpectedly, we found that while Rpb4p and Rpb7p shuttle under all environmental conditions tested, including severe stress (Fig. 4, 6, and 7), in response to severe stress in the form of HS (42°C), EtOH ( $\geq 6\%$ ), or starvation, these proteins are exported by a transcription-independent mechanism. We suspect that during severe stress, the Rpb4-Rbp7 heterodimer is also exported by the transcription-dependent pathway because (i) export at 42°C is partially inhibited by the transcription inhibitor 1,10-phenanthroline (Fig. 3B) and (ii) Rpb4p is required for the export of HS mRNAs at 42°C, thus suggesting that the Rpb4-Rbp7 heterodimer is exported together with these mRNAs (8). We therefore propose that the transcription-dependent pathway is constitutive. The simplest scenario explaining all our current and previous observations suggests the existence of two shuttling mechanisms, which are likely to employ common factors, as schematized in Fig. 8. While Rpb4p and Rpb7p always shuttle, the balance between these two pathways determines the localization patterns of these proteins. This balance is responsive to the environment. It is likely that this balance affects transcription and mRNA decay. There has been another report of two pathways that control transport of an RNA binding protein, Pab1p. More specifically, Pab1p can exit the nucleus by transcription-dependent and transcription-independent pathways (7). However, it is not known how the two pathways of Pab1p export respond to the environment. Our results show that the two export pathways can regulate the transport of a given factor in an environmentally responsive manner.

Why have cells evolved a transcription-independent export pathway during stress? A normal response to stress involves not only transcriptional induction of stress-induced genes but also repression of many other genes whose expression is unfavorable under these conditions (see reference 17 and references therein). We surmise that one mechanism for rapid repression of the stress-repressed genes is exportation of their transcription factors from the nucleus. The Rpb4-Rbp7 heterodimer is a transcription factor that is required for the transcription of stress-induced and stress-repressed genes. Because Rpb4-Rbp7p's level in the nucleus exceeds that of other Pol II subunits (26), we speculate that it would be beneficial to export a surplus of but not all of the molecules of the Rpb4-Rbp7 heterodimer. As a result, a nuclear environment with limiting levels of Rpb4-Rbp7 heterodimer-containing Pol II will be created. This will permit promoter competition on Rpb4-Rbp7 heterodimer-containing Pol II, resulting in transcriptional induction of stress-induced genes and repression of others. An alternative explanation for the accelerated export of the Rpb4-Rbp7 heterodimer during severe stresses is that surplus of the Rpb4-Rbp7 heterodimer is harmful because it titrates some of the transcription factors necessary for the normal response to the stress. It remains to be determined whether eviction of

other transcription factors from the nucleus is a normal response to severe stress.

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