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Interactions between mRNA Export Commitment, 3′-End Quality Control, and Nuclear Degradation

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Several aspects of eukaryotic mRNA processing are linked to transcription. In *Saccharomyces cerevisiae*, overexpression of the mRNA export factor Sub2p suppresses the growth defect of *hpr1* null cells, yet the protein Hpr1p and the associated THO protein complex are implicated in transcriptional elongation. Indeed, we find that a pool of heat shock *HSP104* transcripts are 3′-end truncated in THO complex mutant as well as *sub2* mutant backgrounds. Surprisingly, however, this defect can be suppressed by deletion of the 3′-5′ exonuclease Rrp6p. This indicates that incomplete RNAs result from nuclear degradation rather than from a failure to efficiently elongate transcription. RNAs that are not degraded are retained at the transcription site in a Rrp6p-dependent manner. Interestingly, the addition of a *RRP6* deletion to *sub2* or to THO complex mutants shows a strong synthetic growth phenotype, suggesting that the failure to retain and/or degrade defective mRNAs is deleterious. mRNAs produced in the 3′-end processing mutants *rna14-3* and *rna15-2*, as well as an RNA harboring a 3′ end generated by a self-cleaving hammerhead ribozyme, are also retained in Rrp6p-dependent transcription site foci. Taken together, our results show that several classes of defective RNPs are subject to a quality control step that impedes release from transcription site foci and suggest that suboptimal messenger ribonucleoprotein assembly leads to RNA degradation by Rrp6p.

The physical separation of mRNA biogenesis and bulk protein synthesis in eukaryotic cells necessitates molecular transport between the nuclear and cytoplasmic compartments. To form a mature translatable mRNA, several processing steps must be completed accurately in the nucleus. In successful formation of mature translatable mRNAs, the 5′ end of the transcript acquires a methylated guanosine cap structure, introns are excised, and the 3′ end is cleaved and polyadenylated (for a review, see reference 31). These pre-mRNA modifications serve to protect the transcript from the cellular degradation systems as well as prepare it for downstream events. Over the past few years, it has been established that pre-mRNA processing is carefully coordinated with transcription, and processing events are thought to occur mostly while the pre-mRNA is nascent, i.e., still emerging from the transcription machinery (29). Nuclear mRNA export is tightly linked to pre-mRNA processing and, consequently, probably also to transcription. In metazoans, although not strictly required for export, pre-mRNA splicing has been found to make loading of export factors onto the mRNA more efficient: the exon junction complex associates with the mRNA as a consequence of splicing, and the exon junction complex-associated proteins Aly/REF and UAP56 are important for productive mRNA export (14, 22, 24, 25, 28, 32). The *Saccharomyces cerevisiae* orthologues of these two proteins (the RNA binding protein Yra1p and the DECD-box RNA helicase Sub2p, respectively) are also involved in mRNA export in this organism (18, 37, 38, 40). Aly/Yra1p is believed to recruit the Mex67p/Mtr2p heterodimer (TAP/p15 in metazoans), which in turn mediates contact to the nuclear pore complex (2, 20, 21, 22, 24, 36, 37, 40, 43). Mex67p/Mtr2p recruitment to the messenger ribonucleoprotein (mRNP) might lead to Sub2p displacement, as in vitro studies have shown that Mex67p/Mtr2p binding and Sub2p binding to Yra1p are mutually exclusive (38).

Only a small fraction of *S. cerevisiae* transcripts contain introns, making pre-mRNA splicing insufficient to link the general mRNA population to nuclear export. Instead, 3′-end formation seems to play a major role in this organism. In temperature-sensitive (*ts*) mutants of the 3′-end processing factors *rna14*, *rna15*, and poly(A) polymerase (*pap1*), mRNA nuclear export is blocked (4, 16). Furthermore, uncapped T7 polymerase-directed transcripts are efficient substrates for nuclear export only if they are cleaved and polyadenylated, whereas unadenylated transcripts terminated due to a T7 terminator sequence are sequestered within the nucleus (11). These results indicate that, in contrast to the 5′-end cap, a proper 3′ end is required for nuclear export.

We recently discovered that a block to nuclear export leads to rapid and dramatic effects on the polyadenylation and subnuclear localization of two heat shock mRNAs, *SSA4* and *HSP104* (19). In a number of export mutants, these transcripts were sequestered at or near their sites of transcription and the mRNAs had poly(A) tails approximately twice as long as those

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found in a wild-type strain. Interestingly, transcripts synthesized without a poly(A) tail in the *pap1-1* mutant were also retained in transcription site foci. This retention required Rrp6p as well as other components of the nuclear exosome, which is a large complex of exonucleolytic enzymes; i.e., in exosome mutants, unadenylated as well as hyperadenylated mRNAs are released from transcription sites and proceed in their metabolism (16). Although the molecular basis for exosome-mediated transcription site retention is not understood, these observations suggest that events closely linked to transcription play important roles in defining an export-competent mRNA. As a consequence, it is highly likely that early mRNA export factors will be found associated with sites of active transcription. Indeed, chromatin immunoprecipitation assays have shown that the RNA binding proteins Yra1p and Npl3p associate with mRNA while it is still in close proximity to chromatin (26). Sub2p/UAP56 is also thought to act at an early step in mRNA metabolism. Yeast *SUB2* interacts genetically with *YRA1*, and the products of the two genes interact physically (38). Furthermore, mutations of *SUB2* lead to a general defect in mRNA export and cause Rrp6p-dependent stalling of *SSA4* and *HSP104* RNAs at or near transcription sites (18, 38). It has recently been shown that both Yra1p and Sub2p are associated with the heterotetrameric THO-protein complex, which is genetically and physically linked to the transcription machinery (7, 8, 39). This observation suggested a role for members of the THO complex in mRNA export, which has indeed previously been determined to exist (39). Although the mechanistic rationale underlying these transport defects at the molecular level is presently unclear, they could be due to inefficient loading of export factor(s) onto the nascent transcript. Alternatively, the lesion could be indirect: for example, through more subtle effects on 3′-end formation.

Inspired by evidence linking *SUB2* genetically and physically to *HPR1* (17, 35), we have analyzed the status of nascent *HSP104* transcripts in *sub2* and *hpr1* mutant strains as well as in strains with deletions of other components of the Hpr1p-containing THO-protein complex (8, 9). We find that a substantial pool of nuclear *HSP104* RNA is 3′-end truncated in these mutants. Moreover, a share of transcripts are rapidly sequestered at or near transcription sites. Deletion of the *RRP6* exonuclease restores a quasinormal level of full-length transcripts and reverses the transcript sequestration phenotype. This indicates that *HSP104* transcripts produced in these mutants are subject to detection and partial degradation by Rrp6p. We have also analyzed transcripts produced in RNA 3′-end cleavage mutants as well as transcripts terminated by a self-cleaving ribozyme. In both cases, transcripts are retained within transcription site foci. All of the results show that several classes of defective RNPs are subject to a quality control step at or near the site of transcription.

MATERIALS AND METHODS

Yeast strains and plasmids. Strains used in this study are listed in Table 1. The *mtr2-26* and *rat7-1* strains were kindly provided by Ed Hurt and Chuck Cole, respectively, and have previously been described (15, 34). In these strains, by using standard procedures, the *RRP6* gene was replaced with the *Escherichia coli* kanamycin resistance (KAN^r) gene to create *mtr2-26*/Δ*rrp6* and *rat7-1*/Δ*rrp6.* All other strains used are derived from W303 or have been backcrossed to W303 multiple times. Double mutants were created by standard genetic crosses. Original strains were kindly provided by H. Klein (Δ*hpr1*), A. Aguilera (Δ*mft1* and Δ*tho2*), and P. Thuriaux (Δ*gcn5*, Δ*ppr2*, and Δ*rbp9*). *rna14-3*, *rna15-2*, and Δ*xrn1* strains were a gift from F. Lacroute and M. Minet.

Plasmids expressing green fluorescent protein (GFP) RNAs terminated by a *cis*-cleaving hammerhead ribozyme element (pRS304-2μm-polII-GFP-RZ) and the glyceraldehyde-3-phosphate dehydrogenase 3 (TDH3) 3′-end processing signal (pRS304-2μm-polII-GFP) were constructed as follows. The 2μm region of Yep24 was PCR amplified and cloned into the *Aat*II site of pRS304 to yield pRS304-2μm. The TDH3 locus, from 670 bp upstream of the start codon to 500 bp downstream of the stop codon, was cloned into pRS304-2μm to yield pRS304-2μm-GPD. *Bam*HI and *Sal*I cloning sites replaced the TDH3 open reading frame (ORF). The GFP ORF was PCR amplified from pJK19-1 (kindly provided by P. A. Silver) and cloned into pRS304-2μm-GPD to yield pRS304-2μm-polII-GFP. pRS304-2μm-polII-GFP-RZ was created similarly, except that the 3′-flanking sequence was replaced with a *cis*-cleaving hammerhead ribozyme element (5′-CCT GTC ACC GGA TGT GTT TTC CGG TCT GAT GAG TCC GTG AGG ACG AAA CAG G).

Culture growth, RNA isolation, and in vivo protein labeling. For experiments involving temperature shifts, yeast cultures were grown in yeast extract-peptone-dextrose medium at 25°C. Temperature shifts were performed by mixing a relevant volume of 25°C culture with an equal volume of 49°C medium, after which incubation was continued at 37°C for the specified times. For experiments involving GFP RNA expression, W303 wild-type cells transformed with pRS304-2μm-polII-GFP-RZ or pRS304-2μm-polII-GFP and Δ*rrp6* cells transformed with pRS304-2μm-polII-GFP-RZ were grown at 30°C in tryptophan dropout medium to an optical density at 600 nm of 0.3. For isolation of total RNA, cells were generally collected by centrifugation, washed once with ice-cold H_2O, and stored at −80°C. Total RNA was extracted using the hot acid phenol method (1).

RNA analysis. For Northern blot analysis of *HSP104* RNA, 20 μg of total RNA was used. In DNA oligonucleotide-RNase H digestion reactions, DNA oligonucleotide DL163 (5′-ACATTTTCATCACGAGATTTACCC, directed against nucleotides [nt] 2583 to 2606 of *HSP104* RNA) and DNA oligonucleotide DL195 (5′-TGAAGGCAGCTAGAATATGTATAGG, directed against nt 94 to 118 of *HSP104* RNA) were used in experiments measuring levels of 3′ and 5′ ends, respectively. To facilitate quantitation of the 3′ ends, an oligo$(dT)_{18}$ oligonucleotide was included for these RNase H reactions. To detect signals from the *HSP104* 5′ and 3′ ends, membranes were probed sequentially with radiolabeled oligonucleotides DL190 (5′-TTGAGCCAACGTCAAAATCGTTAGAG CCCTTTCTGTAAATTGCGTTTGGTCGTTCAT, directed against nt 1 to 57 of *HSP104* RNA) and DL164 (5′-TTATCGTCATCACCTAACGTGTCAGCC CCTATAGTAGCTTCGTGATTTGGTAGAACTTCC, directed against nt 2631 to 2690 of *HSP104* RNA), respectively. Radioactive signals of individual samples were quantitated on a PhosphorImager and normalized to the amount of U4 RNA. The integrity of *HSP104* RNA in mutant strains, at a given time after temperature shift, was calculated as the level of *HSP104* RNA 5′ or 3′ end in the mutant relative to the wild-type strain. RNA was resolved by using 6% denaturing polyacrylamide gel electrophoresis.

RNAs that failed to terminate correctly in the *rna14-3* and *rna14-3*/Δ*rrp6* strains were analyzed using RNase H and oligonucleotides D1 and D2 directed against sequences 221 and 300 nt downstream of the stop codon, respectively.

For Northern blot analysis of GFP RNAs, 2 μg of total RNA was cleaved with RNase H by using oligonucleotide KD290 (5′-GAA CGC TTC CAT CTT CAA TGT TGT) and, where applicable, oligo$(dT)_{18}$. Oligonucleotide KD290 is complementary to the GFP ORF, ~200 nt upstream of the stop codon. Membranes were probed with radiolabeled oligonucleotide KD282 (5′-GCA GCC AGA TCC TTT GTA TAG TTC ATC CAT GCC ATG) and washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 15 min, twice at 25°C and twice at 42°C. Bands were visualized by autoradiography.

FISH analysis. Fixation and preparation of cells for fluorescent in situ hybridization (FISH) analysis were done as previously described (19). Detection of *HSP104* mRNA was done using Cy3-labeled THJ203, THJ204, THJ205, and THJ206 probes (19), poly$(A)^+$ RNA was localized using a Cy3-labeled oligo$(dT)_{70}$ probe, and GFP RNA was localized using CY3-labeled KD209, KD210, KD211, and KD212 probes, as described previously (11).

RESULTS

Genetic interactions between *SUB2* and members of the THO protein complex. It has been recently shown that the severe growth phenotype associated with deletion of *SUB2* is

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

Strain	Genotype	Source or reference
W303	*ura3-1 ade2-1 his3-11,5 trp1-1 leu2-3,112 can1-100*	F. Lacroute
DLY23	W303 but *mat***a** *sub2*::*HIS* pCM188-*SUB2* (*URA3*)	27
DLY33	W303 but *mat***a** *sub2*::*HIS* pCM185-*sub2-201* (*TRP1*)	27
DLY157	W303 but *matα hpr1*::*HIS*	This study[a]
DLY223	W303 but *mat***a** *tho2*::*KAN*^{r}	This study[b]
DLY224	W303 but *mat***a** *mft1*::*KAN*^{r}	This study[b]
DLY200	W303 but *sub2*::*KAN*^{r} *hpr1*::*HIS* pCM188-*SUB2* (*URA3*)	This study
DLY232	W303 but *sub2*::*KAN*^{r} *tho2*::*KAN* pCM188-*SUB2* (URA3)	This study
DLY233	W303 but *sub2*::*KAN*^{r} *mft1*::*KAN* pCM188-*SUB2* (*URA3*)	This study
DLY123	W303 but *matα rrp6*::*KIURA3*	This study
DLY124	W303 but *sub2*::*HIS rrp6*::*KIURA3* pCM185-*sub2-201* (*TRP1*)	18
DLY190	W303 but *rrp6*::*KAN*^{r} *hpr1*::*HIS*	This study
DLY241	W303 but *mft1*::*KAN*^{r} *rrp6*::*KIURA*	This study
DLY207	W303 but *mat***a** *ski2*::*HIS*	This study
DLY208	W303 but *sub2*::*HIS ski2*::*HIS* pCM185-*sub2-201* (*TRP1*)	This study
DLY234	W303 but *hpr1*::*HIS ski2*::*HIS*	This study
DLY199	W303 but *matα xrn1*::*TRP1*	M. Minet
DLY202	W303 but *sub2*::*HIS xrn1*::*TRP1* pCM188-*sub2-201*(*URA*)	This study
DLY210	W303 but *hpr1*::*HIS xrn1*::*TRP1*	This study
DLY142	W303 but *matα rna14-3*	F. Lacroute
DLY128	W303 but *matα rna15-2*	F. Lacroute
DLY171	W303 but *rna14-3 rrp6*::*KAN*^{r}	This study
DLY172	W303 but *rna15-2 rrp6*::*KAN*^{r}	This study
DLY237	W303 but *matα gcn5*::*HIS*	This study[c]
DLY238	W303 but *mat***a** *ppr2*::*URA*	This study[c]
DLY236	W303 but *matα rpb9*::*HIS*	This study[c]
DLY235	W303 but *gcn5*::*HIS rrp6*::*KAN*^{r}	This study
DLY242	W303 but *ppr2*::*URA rrp6*::*KAN*^{r}	This study
DLY243	W303 but *rpb9*::*HIS rrp6*::*KAN*^{r}	This study
rat7-1	*mat***a** *trp1*Δ*63 ura3-52 leu2*Δ*1 rat7-1*	15
rat7-1/Δ*rrp6*	*rat7-1* but *rrp6*::*KAN*^{r}	S. Denome
mtr2-26	*mat***a** *leu2 ura3 trp1 his3 MTR2*::*HIS3* pRS315-*LEU2-mtr2-26*	34
mtr2-26/Δ*rrp6*	*mtr2-26* but *rrp6*::*KAN*^{r}	This study

[a] Original strains provided by H. Klein.
[b] Original strain provided by A. Aguilera.
[c] Original strains provided by P. Thurianx.

partially suppressed by mutations in the *RAD3* gene, a helicase and a component of transcription factor II H (18). *SUB2* overexpression also suppresses the thermosensitive growth defect conferred by deletion of the *HPR1* gene (12). These data inspired a closer investigation of the interaction between *SUB2* and *HPR1*. To this end, we analyzed whether a *SUB2* thermosensitive allele, *sub2-201*, was growth impaired in the absence of the inessential *HPR1* gene. Indeed, the double mutant is inviable (Fig. 1). The *sub2-201* mutation was also synthetically lethal with a *yra1-8* (GFP) allele (kindly provided by F. Stutz), and *yra1-8* (GFP) was synthetically lethal with the Δ*hpr1* mutation (reference 41 and data not shown).

The Sub2p, Yra1p, and Hpr1p proteins have recently been purified, together with THO protein complex members (9, 17, 39). The core THO complex consists of the Tho2p, Hpr1p, Mft1p, and Thp2p proteins, and deletion of any of these four factors leads to accumulation of 3′-end-truncated transcripts (9). To assess whether the genetic interactions linking *SUB2*, *YRA1*, and *HPR1* extend to other members of the THO complex, we attemped the construction of *sub2-201*/Δ*tho2* and *sub2-201*/Δ*mft1* double mutants. As shown in Fig. 1, neither double mutant was viable. This result is consistent with that of a recent report from the Hurt laboratory of work performed with the *sub2-85* mutant allele (39).

***HSP104* transcripts are sequestered in Rrp6p-dependent transcription site foci in strains from which individual THO components are deleted.** Thermosensitive mutants of Sub2p show nuclear accumulation of mRNA at the restrictive temperature (18, 38). Because of the genetic and physical association between *SUB2* and *HPR1*, we analyzed the fate of transcripts produced in an *hpr1* null environment. The Δ*hpr1* mutant was shifted to 37°C, and *HSP104* RNA localization was assayed by FISH analysis utilizing sequence-specific probes. Under these experimental conditions, heat shock gene expression is transiently induced and *HSP104* expression follows a pseudo-pulse-chase profile. At the 15-min time point, a single nuclear dot appeared in the majority of the cells (Fig. 2A, second row), which was a result identical to that previously observed with a number of *ts* mutant strains that inhibit mRNA export (16, 18, 19). The dot reflects retention of the transcript at or near its site of transcription (reference 19 and data not shown). However, this nuclear sequestration phenotype was more transient than previously observed for other mRNA export mutants; i.e., the *HSP104* dot disappeared after a 60-min incubation at 37°C (Fig. 2A, third row). Since *HSP104* RNA levels, as measured by Northern blotting, do not decrease over time (Fig. 3A), this result indicates an incomplete block to transcription site release, leading to the disappearance of the

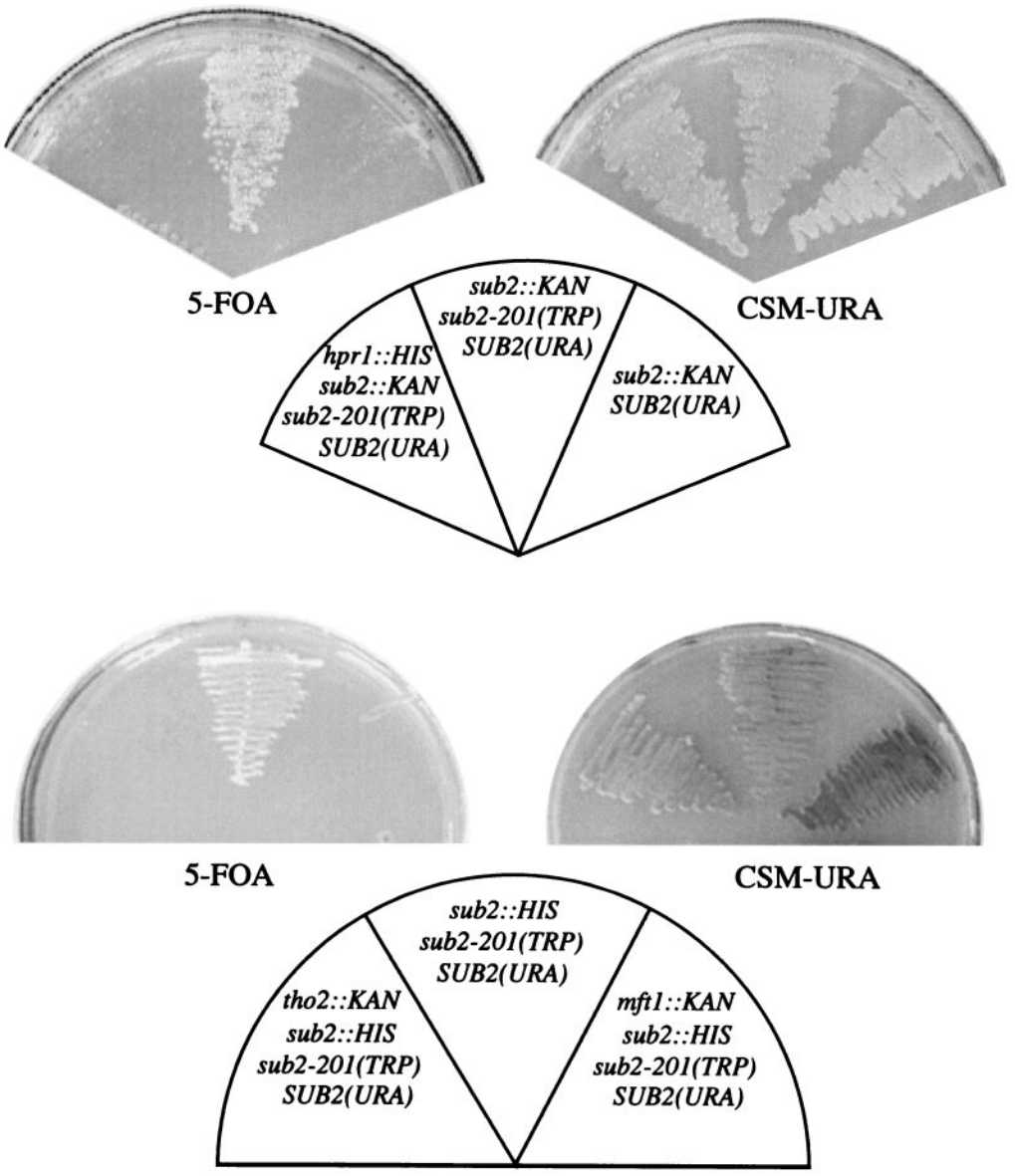
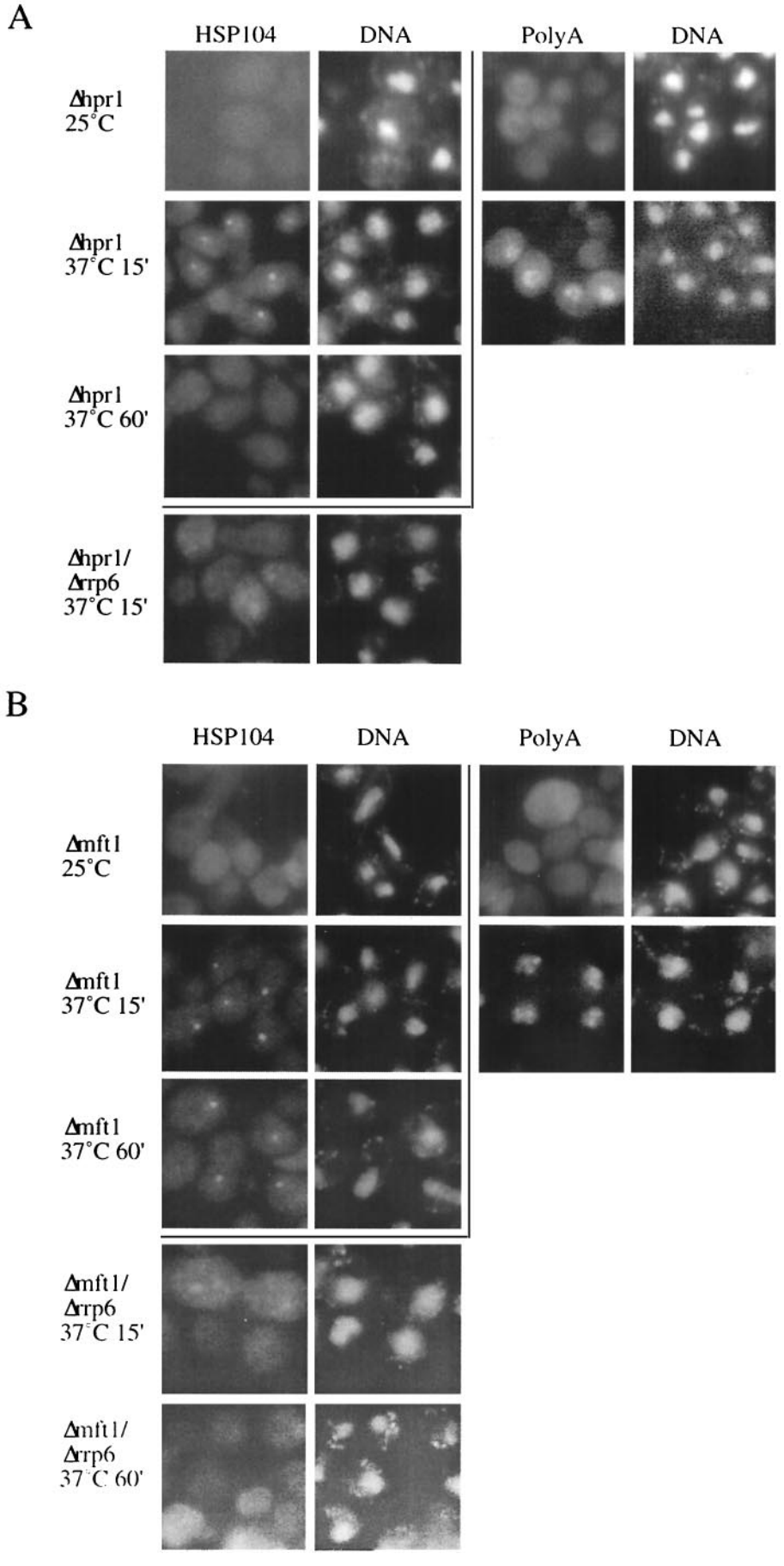


FIG. 1. The *sub2-201* mutation is synthetically lethal with deletion of any one of the *HPR1*, *THO2*, and *MFT1* genes of the THO complex. Δ*sub2*, Δ*sub2*/Δ*hpr1*, Δ*sub2*/Δ*tho2*, and Δ*sub2*/Δ*mft1* cells, expressing Sub2-201p from a TRP1-marked plasmid and Sub2p from a URA3-marked plasmid, were grown at 24°C on medium containing 5-FOA (to rid the cells of Sub2p) or on URA dropout plates (CSM-URA), as indicated.

HSP104 RNA in situ signal after the transcriptional shutoff. The mRNA nuclear export phenotype of the Δ*hpr1* strain was also evident when poly(A)$^+$ RNA localization was assayed using an oligo(dT) in situ probe (Fig. 2A, right panel).

Retention of aberrant transcripts in transcription site foci requires components of the nuclear exosome (16). The 3′-5′ exonuclease Rrp6p likely defines the nuclear exosome, as it localizes to the nucleus and does not purify with the cytoplasmic exosome (6, 30). Deletion of *RRP6* from the Δ*hpr1* strain resulted in almost the full disappearance of the *HSP104* RNA nuclear dot at the 15-min time point. Instead, a diffuse *HSP104* signal could be detected, which in some cells showed a mild nuclear accumulation (Fig. 2A, lower row). Disappearance of the nuclear dot is not due to lack of *HSP104* RNA, since transcript levels were actually increased in the Δ*hpr1*/Δ*rrp6* double-mutant strain compared to the Δ*hpr1* single-mutant strain (Fig. 3A). Furthermore, deletion of cytoplasmic degradation components (i.e., *XRN1* or *SKI2*) did not result in dot relief in the Δ*hpr1* context (data not shown). Finally, Hsp104p protein synthesis was diminished in the Δ*hpr1* strain after 15 min compared to a wild-type strain and normal levels were restored in the Δ*hpr1*/Δ*rrp6* double-mutant strain (data not shown). We conclude that Rrp6p, and possibly the nuclear exosome, are involved in *HSP104* RNA transcription site retention in the Δ*hpr1* strain.

We next wanted to evaluate the involvement of other components of the THO complex in nuclear mRNA export and their relationships with *RRP6*. To this end, we analyzed the


A
HSP104
DNA
PolyA
DNA
Δhpr1
25°C
Δhpr1
37°C 15'
Δhpr1
37°C 60'
Δhpr1/
Δrrp6
37°C 15'
B
HSP104
DNA
PolyA
DNA
Δmft1
25°C
Δmft1
37°C 15'
Δmft1
37°C 60'
Δmft1/
Δrrp6
37°C 15'
Δmft1/
Δrrp6
37°C 60'

status of *HSP104* transcripts in strains from which the inessential THO complex components *MFT1* and *THO2* (kindly provided by A. Aguilera) were deleted. As in Δ*hpr1* cells, in Δ*mft1 HSP104* RNA localized to a nuclear dot after a 15-min incubation at 37°C (Fig. 2B, second row). The signal intensity decreased slightly over time, although not as much as in the Δ*hpr1* strain (compare Fig. 2A and B). Under similar conditions, nuclear accumulation of poly(A)$^+$ RNA was also detectable in this strain (Fig. 2B, right panel). Similar results were obtained for the Δ*tho2* strain (data not shown).

The contribution of Rrp6p to transcription site retention was evaluated by constructing a Δ*mft1*/Δ*rrp6* strain (a Δ*tho2*/Δ*rrp6* strain was not viable; see below). *HSP104* RNA localization in Δ*mft1*/Δ*rrp6* resembled that in Δ*hpr1*/Δ*rrp6* (Fig. 2B, fourth and fifth rows). Northern blot analysis confirmed the presence of *HSP104* RNA in the Δ*mft1*/Δ*rrp6* strain (data not shown).

What is the nature of the transcription site-sequestered *HSP104* transcripts in these strains? *HSP104* RNA was detected with FISH probes directed towards the 3′ end of the ORF (Fig. 3B). When we utilized a single probe (THJ203) that hybridizes to a *HSP104* region immediately upstream of the stop codon, nuclear dots could also be detected (data not shown). We conclude that *HSP104* transcripts with sequences at or very close to the stop codon are retained in transcription site foci.

Rrp6p-mediated degradation of *HSP104* transcripts in the Δ*hpr1*, Δ*mft1*, and *sub2-201* mutant strains. Using our transcriptional pseudo-pulse-chase heat shock assay, we analyzed *HSP104* transcripts biochemically by Northern blotting. In wild-type cells, *HSP104* transcription is transiently induced at 37°C and a polyadenylated species first appears that is exported from the nucleus, deadenylated, and degraded in the cytoplasm (a representative gel is shown in Fig. 3A). In contrast, *HSP104* RNA expressed in *hpr1* null cells did not seem to proceed in its metabolism but accumulated with slightly longer poly(A) tails (Fig. 3A; compare lanes 1 to 4 with lanes 5 to 8). The overall amount of *HSP104* RNA was increased in the Δ*hpr1*/Δ*rrp6* double mutant compared to the Δ*hpr1* single mutant (Fig. 3A; compare lanes 15 and 16 with lanes 17 and 18; see below for quantitation). Removal of *HSP104* RNA poly(A) tails by oligo(dT) and RNase H revealed that the clear majority of transcripts had been polyadenylated.

Members of the THO complex have previously been implicated in transcription elongation (10). To determine Hpr1p, Mft1p, and Sub2p contributions to *HSP104* RNA quality, we analyzed the status of *HSP104* RNA 5′ ends and 3′ ends in W303, Δ*hpr1*, Δ*mft1*, and *sub2-201* cells. Total RNA, harvested after a temperature shift, was subjected to oligonucleotide-directed RNase H cleavage and subsequent Northern analysis. Use of specific cleaving oligonucleotides and hybridization probes allowed independent assessment of 5′- and 3′-end abundance (Fig. 3B). In the Δ*hpr1* and *sub2-201* background, the amount of 3′-end fragments derived from the *HSP104* RNA was significantly reduced at the 5- and 30-min time points (to approximately 20% of wild-type levels) (Fig. 3C [see right panels for quantitation]). On the other hand, the 5′ ends were much less affected, which indicates the existence of 3′-end-truncated *HSP104* transcripts in these two mutant strains. The presence of incomplete transcripts was even more pronounced in the Δ*mft1* strain, with the 3′ ends almost undetectable at the 5- and 15-min time points (Fig. 3C, lower part).

Given the proposed role for the THO protein complex, incomplete RNAs could be due to defective transcriptional elongation. However, deletion of the *RRP6* gene from the Δ*hpr1*, *sub2-201*, and Δ*mft1* backgrounds resulted in the almost complete restoration of *HSP104* 3′-end levels relative to *HSP104* 5′-end levels (Fig. 3C). This was not due to a general Rrp6p-mediated increase in mRNA abundance, since the levels of *HSP104* 3′ ends (and 5′ ends) were unaffected in *rrp6* null cells compared to those of a wild-type strain (data not shown).

mRNA degradation in the cytoplasm occurs by two principal mechanisms: a major cap-dependent 5′-3′ pathway and a minor cap-independent 3′-5′ pathway (6). The exonuclease Xrn1p and the helicase Ski2p are nonessential, cytoplasmic factors involved in the 5′-3′ and 3′-5′ degradation pathways, respectively. Deletion of *XRN1* (and to a minor extent, *SKI2*) in a wild-type strain resulted in stabilization of *HSP104* RNA at the 30-min time point, as would be expected when cytoplasmic degradation of mRNA is inhibited (data not shown). However, deletion of *XRN1* or *SKI2* in a *sub2-201* or Δ*hpr1* background did not result in general *HSP104* RNA stabilization or restoration of a proper 5′-end:3′-end ratio, in contrast to what was observed in the Δ*hpr1*/Δ*rrp6* and *sub2-201*/Δ*rrp6* double-mutant strains (data not shown). This strongly indicates that significant proportions of RNAs produced in Δ*hpr1*, *sub2-201*, and Δ*mft1* environments are specifically degraded in the nucleus and suggests that Rrp6, and possibly the nuclear exosome, plays a major role in this process.

Specific genetic interactions between the THO complex and components of the nuclear exosome. Rrp6p modulation of *HSP104* localization and 3′-end abundance suggests a functional relationship between the THO protein complex and the nuclear exosome. Indeed, Δ*hpr1*/Δ*rrp6* and Δ*mft1*/Δ*rrp6* double-mutant strains are severely growth impaired at 24 and 30°C and inviable at all temperatures above 30°C (Table 2). Furthermore, a Δ*tho2*/Δ*rrp6* strain is inviable at all temperatures tested (data not shown). We have previously reported an interaction between the *sub2-201* mutation and deletion of *RRP6* (18), and this observation extends to mutant *YRA1* alleles, which are growth impaired or lethal in combination with exosome component mutations (41). Thus, *SUB2*, *YRA1*, *HPR1*, *MFT1*, and *THO2* all show a genetic interaction with *RRP6.* Growth impairment of the *sub2-201*/Δ*rrp6* and Δ*hpr1*/Δ*rrp6* mutants could be linked to general aspects of mRNA degradation. However, deletion of *SKI2* or *XRN1* does not affect growth of the *sub2-201* mutant at any temperature. Similarly, Δ*hpr1*/Δ*xrn1* and Δ*hpr1*/Δ*ski2* double-mutant strains show

FIG. 2. Deletion of *HPR1* or *MFT1* leads to Rrp6p-dependent transcription site focus accumulation of *HSP104* RNA. (A) *HSP104* and poly(A)$^+$ RNA FISH analysis of Δ*hpr1* cultures grown at 25°C or temperature shifted to 37°C for 15 or 60 min and *HSP104* RNA FISH analysis of Δ*hpr1*/Δ*rrp6* cultures temperature shifted to 37°C for 15 min, as indicated. (B) RNA FISH analysis of Δ*mft1* and Δ*mft1*/Δ*rrp6* cultures, as described for panel A. For the Δ*hpr1*/Δ*rrp6* strain, *HSP104* RNA FISH analysis was also performed on a culture grown for 60 min at 37°C. DNA was stained with DAPI (4′,6′-diamidino-2-phenylindole).

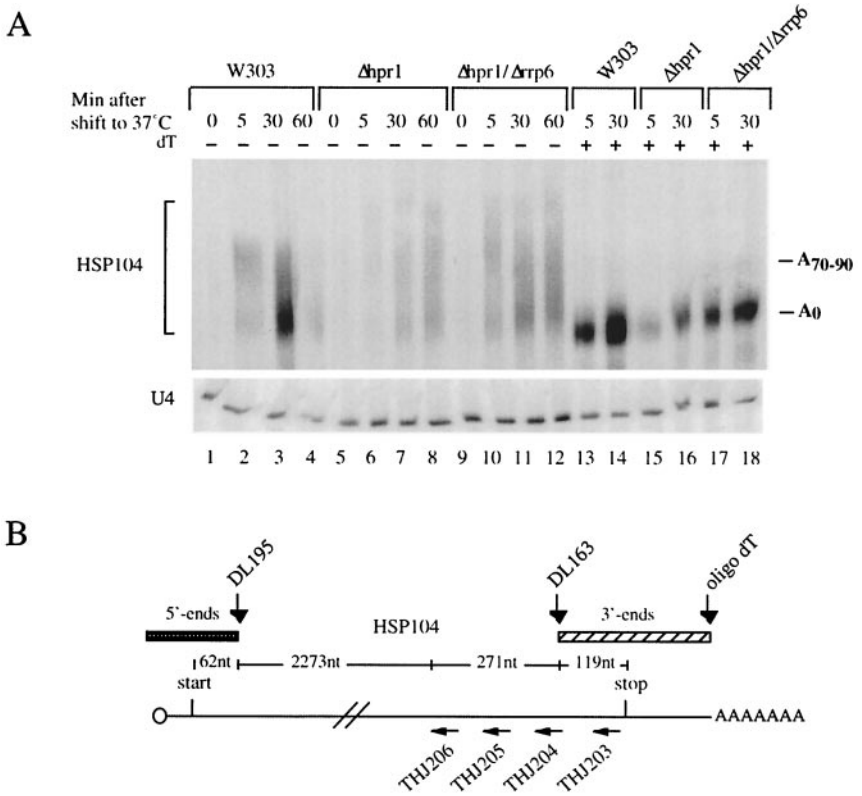


FIG. 3. Rrp6p-dependent generation of 3′-end-truncated transcripts in Δ*hpr1*, *sub2-201*, and Δ*mft1* mutant backgrounds. (A) Northern analysis of *HSP104* RNA (or U4 snRNA as a control) isolated from W303, Δ*hpr1*, and Δ*hpr1*/Δ*rrp6* cultures harvested at the indicated time points after a 37°C temperature shift. Prior to gel electrophoresis, all samples were treated with RNase H and the DL163 oligonucleotide complementary to nt 2583 to 2606 of the *HSP104* gene. The row above the upper gel (dT) indicates whether the RNA sample was also treated with oligo(dT) to remove the poly(A) tail. *HSP104* RNA was visualized using a radiolabeled DNA oligonucleotide (DL164) complementary to nt 2631 to 2690 of the *HSP104* gene. (B) Schematic representation of the *HSP104* RNA, indicating positions cleaved by RNase H and the DNA oligonucleotides DL195, DL163, and oligo(dT). Positions recognized by the *HSP104* RNA FISH probes (THJ203 to THJ206) are also shown. (C) Top row, Northern analysis of *HSP104* RNA isolated from W303, Δ*hpr1*, and Δ*hpr1*/Δ*rrp6*; middle row, Northern analysis of *HSP104* RNA isolated from W303, *sub2-201*, and *sub2-201*/Δ*rrp6*; bottom row, Northern analysis of *HSP104* RNA isolated from W303, Δ*mft1*, and Δ*mft1*/Δ*rrp6*. All cultures were harvested at the indicated time points after a 37°C temperature shift. To measure 5′-end levels, oligonucleotide DL195 complementary to nt 94 to 118 of the *HSP104* gene was used in the RNase H cleavage reaction and a radiolabeled DNA oligonucleotide spanning the first 57 nt of *HSP104* was used for hybridization. *HSP104* 3′-end abundance was measured as described for panel A. All samples were treated with oligo(dT) in the RNase H reaction to facilitate quantitation analysis. To quantitate relative *HSP104* 5′- and 3′-end levels, signals were normalized to the amount of U4 RNA and expressed in comparison to the percentage of RNA produced in a wild-type strain (set to a value of 100) at the same time point.

growth rates identical to that of a Δ*hpr1* single mutant (Table 2). Thus, the link between *HPR1* and *SUB2* on the one hand and the RNA degradation machinery on the other appears to be restricted to the nucleus.

Sub2p and Yra1p have been shown to be involved in mRNA export (18, 37, 38). Therefore, we sought to determine whether the synthetic phenotype of nuclear exosome mutations with lesions in *SUB2*, *HPR1*, *MFT1*, *THO2*, and *YRA1* would extend to other nuclear mRNA export factors. Growth of strains harboring two known mRNA export factor mutations combined with an *RRP6* deletion was tested at various temperatures. None of these double mutants (*rat7-1*/Δ*rrp6* or *mtr2-26*/Δ*rrp6*) grew slower than their single-mutant counterparts (Table 2). This genetic interaction pattern might reflect the fact that Yra1p, Sub2p, and the THO protein complex function at early transcription-associated steps in mRNA export, whereas the roles of Rat7p and Mtr2p are linked to later steps associated with nuclear pore complexes.

We also tested for a genetic interaction between *RRP6* and bona fide transcription elongation factors. As shown in Table 2, growth of cells from strains with a deletion of the *PPR2* gene (transcription elongation factor TFIIS), *RPB9* (RNA polII component), or *GCN5* (SAGA- and ADA-complex-associated histone acetyltransferase) was unaffected by deletion of the *RRP6* gene. Together, the genetic results strongly suggest that the interaction of THO complex components and the *SUB2* helicase with *RRP6* is related to a function that is specific for this set of proteins.

Read-through transcripts are retained and partially degraded in an Rrp6p-dependent manner. It has been suggested that nonadenylated, 3′-OH ends trigger the degradation activity of Rrp6p (5). Data shown in the previous sections show that *HSP104* RNA produced in *sub2*- or THO complex-defective mutants are retained and degraded in the nucleus. It is not entirely clear whether these transcripts are adenylated (see Discussion). With polyadenylation and free 3′-OH ends in

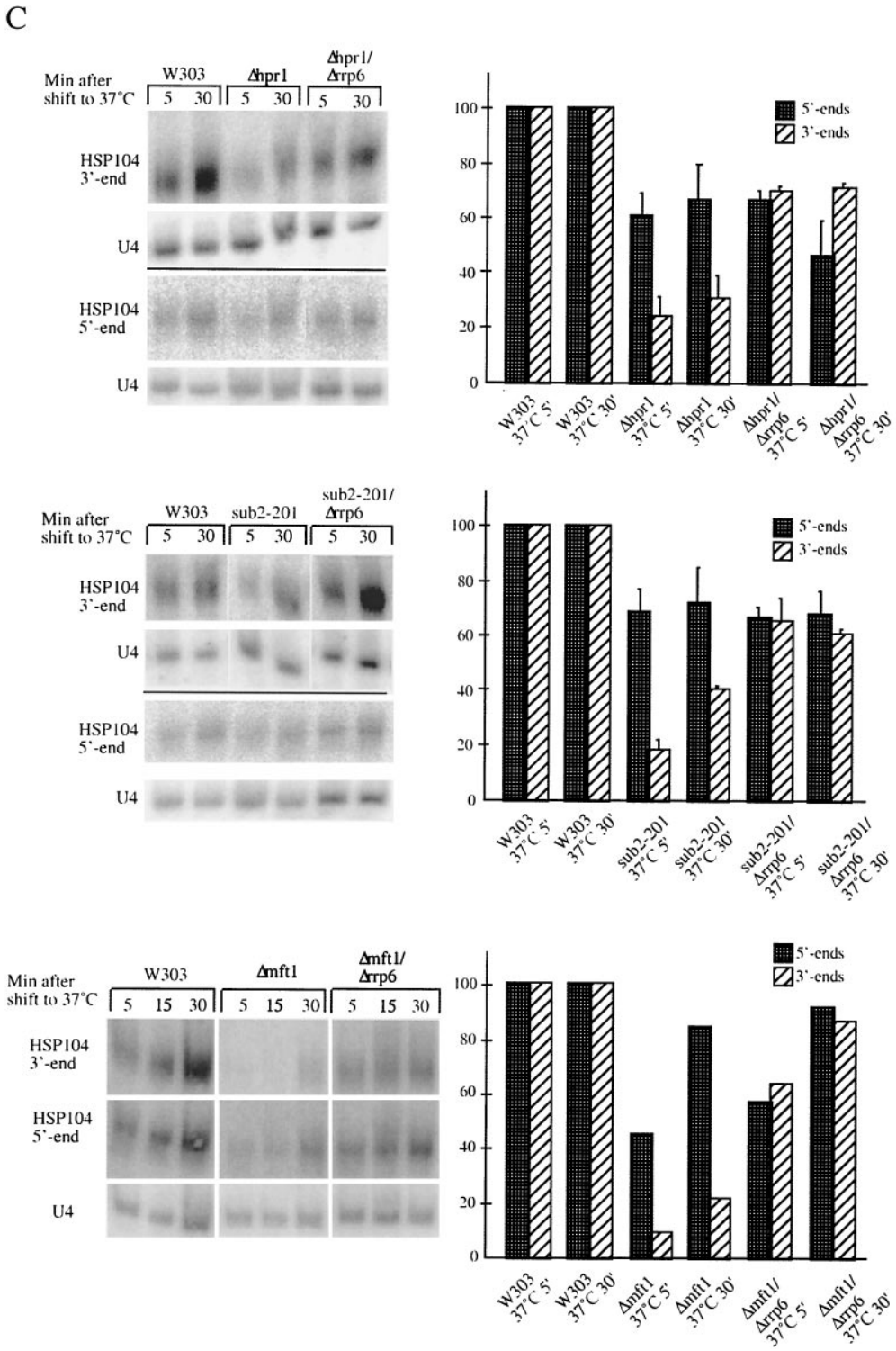


FIG. 3—*Continued.*

mind, we turned to the 3′-end cleavage and transcription termination mutants *rna14-3* and *rna15-2* (31). As shown in Fig. 4A, *HSP104* transcripts accumulate in an intranuclear dot at the nonpermissive temperature in these strains. Deletion of *RRP6* significantly reduces this signal in both mutant backgrounds, despite a general increase in *HSP104* RNA levels (Fig. 4B to E). A fraction of the *HSP104* transcripts produced in the *rna14-3* and *rna15-2* strains appear much longer and heterogeneous than in a wild-type strain, which results in a diffuse Northern blot signal (Fig. 4B [also Fig. 4C; compare

TABLE 2. Temperature sensitivity of strains

Strain	Growth rate at a temp (°C) of:			
	24	30	34	37
W303	+++	+++	+++	+++
Δ*rrp6*	+++	+++	+++	+
sub2-201	+++	+++	+++	−
Δ*xrn1*	++	++	++	++
Δ*ski2*	+++	+++	+++	+++
sub2-201/Δ *rrp6*	+++	+	−	−
sub2-201/Δ *xrn1*	+++	+++	+++	−
sub2-201/Δ *ski2*	+++	+++	+++	−
Δ*hpr1*	+++	+++	+++	+
Δ*hpr1*/Δ*rrp6*	++	+	−	−
Δ*hpr1*/Δ*xrn1*	+++	+++	+++	+
Δ*hpr1*/Δ*ski2*	+++	+++	+++	+
Δ*mft1*	+++	+++	+++	+
Δ*mft1*/Δ*rrp6*	++	+	−	−
Δ*tho2*	++	++	+	−
Δ*tho2*/Δ*rrp6*	−	−	−	−
rna14-3	+++	++	+	−
rna14-3/Δ*rrp6*	+++	++	++	−
rna15-2	+++	++	++	−
rna15-2/Δ*rrp6*	+++	++	++	−
mtr2-26	+++	++	−	−
mtr2-26/Δ*rrp6*	+++	++	−	−
rat7-1	+++	+++	+++	−
rat7-1/Δ*rrp6*	+++	+++	+++	−
Δ*rpb9*	++	+++	+++	++
Δ*rpb9*/Δ*rrp6*	++	+++	+++	+
Δ*gcn5*	+++	+++	+++	+++
Δ*gcn5*/Δ*rrp6*	+++	+++	+++	+
Δ*ppr2*	+++	+++	+++	++
Δ*ppr2*/Δ*rrp6*	+++	+++	+++	+

lanes 1 and 4] and data not shown). These longer transcripts derive from defective 3′-end cleavage and/or transcriptional termination in these strains, as read-through *HSP104* transcripts can be cleaved by RNase H and DNA oligonucleotides D1 and D2, positioned downstream of the canonical poly(A) addition site (Fig. 4C and data not shown). *HSP104* transcripts polyadenylated at the correct site could also be detected (Fig. 4C to D). Both read-through and correctly polyadenylated transcripts appear to be substrates for Rrp6p, since the amount of both species increases approximately threefold at the 15-min time point in the *rna14-3*/Δ*rrp6* double mutant compared to the *rna14-3* single mutant (Fig. 4D and E).

Interestingly, the *rna14-3* growth phenotype was slightly suppressed by deletion of the *RRP6* gene (Table 2). This mirrors data in the literature on the partial suppression of the *pap1-1* phenotype by an *RRP6* deletion (5).

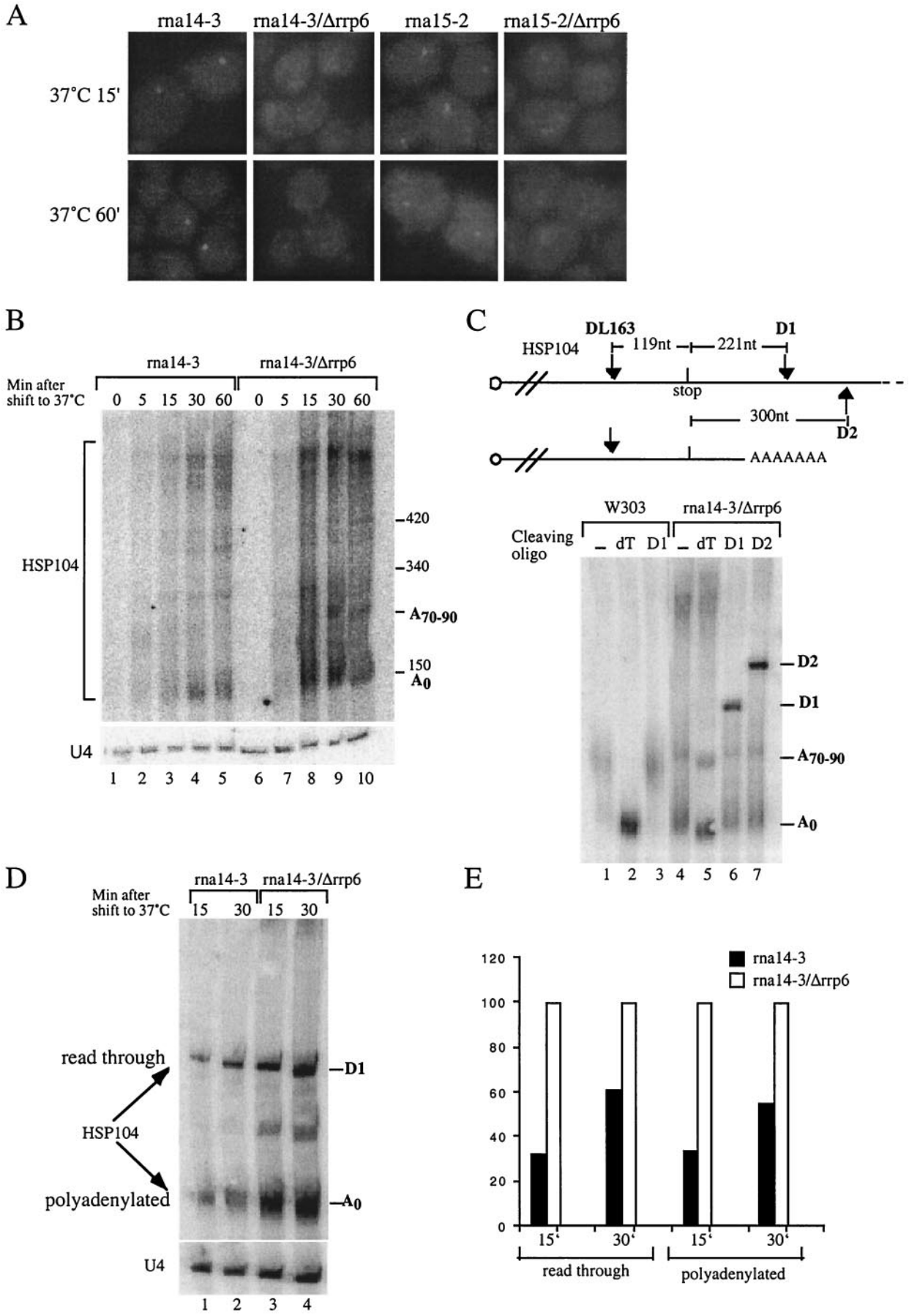
Formation of free 3′ ends in a wild-type strain results in transcription site retention. To generate transcripts with free 3′ ends without *ts* mutants and the consequent temperature change, we constructed a plasmid expressing a GFP RNA with a 3′ end formed by a self-cleaving hammerhead ribozyme (33). The DNA was transformed into wild-type or Δ*rrp6* cells, and the GFP RNA localization at 30°C was analyzed by using RNA FISH with probes specific for the GFP sequence. In wild-type cells, GFP RNA accumulated in nuclear granules, reminiscent of the signal obtained when transcription site-restricted transcripts are expressed from high-copy-number 2μm reporter plasmids (11, 19). The sequestration was dependent on the presence of Rrp6p, as the nuclear signal was significantly reduced in the Δ*rrp6* strain (Fig. 5A, middle row). Furthermore, mRNA accumulation in nuclear granules was dependent on the ribozyme-produced 3′ ends, as substitution of the hammerhead ribozyme sequence with a conventional yeast polyadenylation signal resulted in a GFP RNA signal dispersed throughout the cell (Fig. 5A, lower row). Northern analysis confirmed that the polyadenylation signal-containing construct produced RNAs with poly(A) tails of the expected length, whereas the ribozyme construct led to the synthesis of unadenylated transcripts (Fig. 5B). We conclude that Rrp6p is linked to the nuclear retention of transcripts with unadenylated 3′ ends, regardless of their origin.

DISCUSSION

The DECD-box helicase Sub2p has been implied in splicing, polyadenylation, and nuclear export (13, 14, 18, 23, 27, 28, 38, 42). A connection between Sub2p function and transcription was suggested by the genetic and functional interactions of *SUB2* with *RAD3* and *HPR1*, whose protein products associate with the transcription machinery (8, 12, 18). Hpr1p belongs to the heterotetrameric THO complex, which was first described on the basis of genetic and physical interactions and was assigned a role in transcription elongation and mitotic recombination (9). More recently, Sub2p and Yra1p have been found to be associated with the THO complex in a large-scale yeast protein complex analysis (17), which suggested that this complex functions in nuclear mRNA export. Support for this notion was recently presented by Strasser et al. (39) while the manuscript for the present report was in preparation.

Data presented in this paper strengthen the functional interactions between export factors and members of the THO complex. Not only is the *sub2-201* mutation synthetically lethal with deletion of the *HPR1* gene, but a similar relationship also extends to the two other THO complex genes tested, *THO2* and *MFT1*. Moreover, Sub2p interacts directly with Hpr1p and

FIG. 4. HSP104 transcripts harboring extended 3′ untranslated regions accumulate in Rrp6p-dependent transcription site foci. (A) *HSP104* mRNA FISH analysis of *rna14-3*, *rna14-3*/Δ*rrp6*, *rna15-2*, and *rna15-2*/Δ*rrp6* cultures temperature shifted to 37°C for 15 or 60 min. DNAs were stained with DAPI. (B) Northern analysis of *HSP104* mRNA (or U4 snRNA as a control) isolated from *rna14-3* and *rna14-3*/Δ*rrp6* cultures harvested at the indicated time points after a 37°C temperature shift. Prior to gel electrophoresis, samples were treated with the DL163 oligonucleotide complementary to nt 2583 to 2606 of the *HSP104* gene and RNase H. Approximate size markers are indicated. (C) Northern analysis of *HSP104* RNA isolated from W303 and *rna14-3*/Δ*rrp6* cultures 15 min after a 37°C temperature shift. Prior to gel electrophoresis, samples were treated with RNase H and the indicated oligonucleotide [none (−), oligo(dT), D1, or D2] complementary to the region of the *HSP104* gene (as schematized above the gel) for read-through as well as polyadenylated transcripts. Migration of bands corresponding to oligo(dT), D1, and D2 cleavage is shown. (D) Northern analysis of read-through as well as polyadenylated *HSP104* RNA content in *rna14-3* and *rna14-3*/Δ*rrp6* strains at 15- and 30-min time points after a 37°C temperature shift. To facilitate concomitant detection of read-through and polyadenylated *HSP104* RNA, samples were treated with RNase H and a mix of oligonucleotide D1 and oligo(dT) prior to gel electrophoresis. (E) Quantitation of the result described for panel D. Signals were normalized to the amount of U4 RNA and expressed in comparison to the percentage of RNA produced in the *rna14-3*/Δ*rrp6* strain (set to a value of 100) at the same time point.


A
rna14-3
rna14-3/Δrrp6
rna15-2
rna15-2/Δrrp6
37°C 15'
37°C 60'
B
rna14-3
rna14-3/Δrrp6
Min after shift to 37°C
0 5 15 30 60 0 5 15 30 60
HSP104
420
340
A70-90
150
A0
U4
1 2 3 4 5 6 7 8 9 10
C
DL163
D1
HSP104
119nt
221nt
stop
300nt
D2
AAAAAAA
W303
rna14-3/Δrrp6
Cleaving oligo
– dT D1 – dT D1 D2
D2
D1
A70-90
A0
1 2 3 4 5 6 7
D
rna14-3
rna14-3/Δrrp6
Min after shift to 37°C
15 30 15 30
read through
HSP104
polyadenylated
D1
A0
U4
1 2 3 4
E
rna14-3
rna14-3/Δrrp6
120
100
80
60
40
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read through
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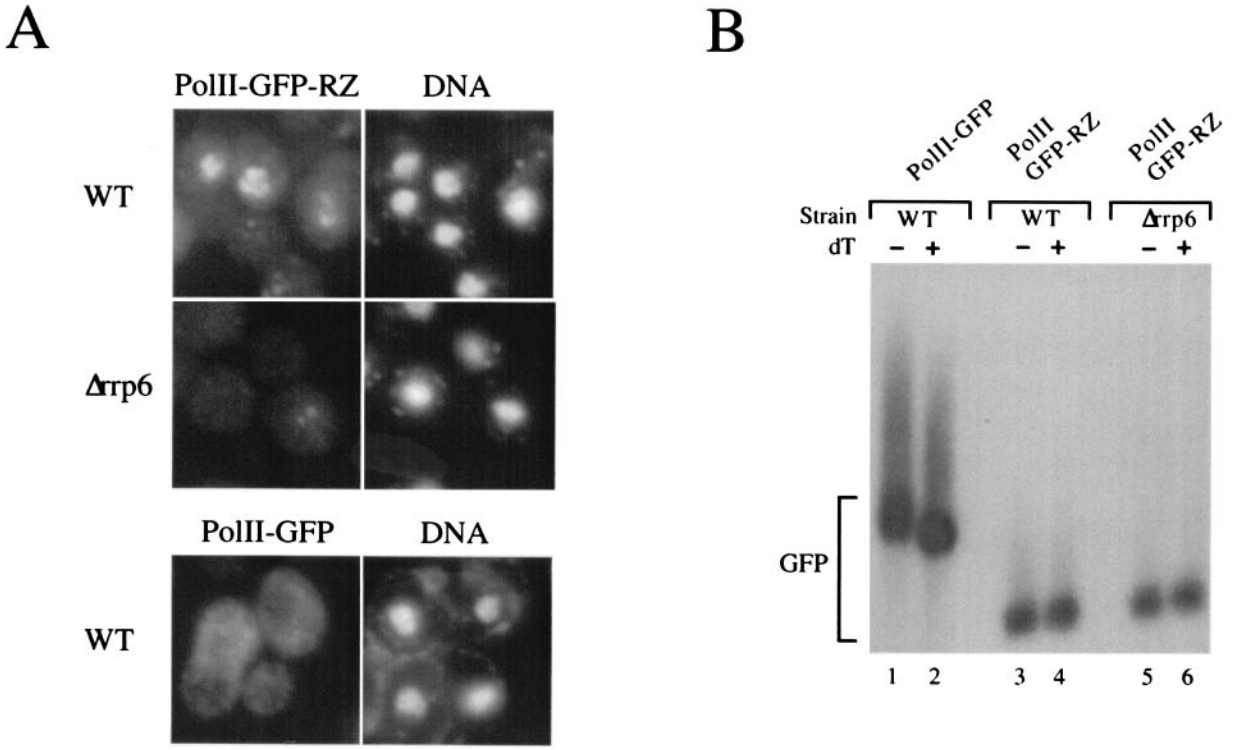

FIG. 5. Transcripts whose 3′ end is generated by a self-cleaving hammerhead ribozyme accumulate in Rrp6p-dependent transcription site foci. (A) GFP RNA FISH analysis of wild-type (top row) and Δ*rrp6* (middle row) strains transformed with a 2μm high-copy-number plasmid construct expressing GFP RNA terminated by a self-cleaving hammerhead ribozyme (polII-GFP-RZ) and wild-type cells transformed with a 2μm plasmid expressing GFP RNA terminated by a conventional 3′-end processing signal (polII-GFP) (bottom row). Δ*rrp6* cells were cotransformed with either a plasmid expressing the Rrp6p protein (top and bottom rows) or empty vector (middle row). Log-phase cultures growing at 30°C were fixed and subjected to RNA FISH utilizing probes specific for the GFP ORF. DNA was stained with DAPI. dT, oligo(dT). (B) Northern analysis of GFP RNA from the cultures described above. WT, wild type.

Yra1p, which indicates that the observed genetic interaction between *SUB2*, *YRA1*, and the THO complex is due to direct protein-protein interactions (39, 41).

Deletion of all tested members of the THO complex leads to sequestration of newly synthesized *HSP104* transcripts in transcription site foci. This mRNA retention is Rrp6p dependent, which is a finding identical to the previously reported finding for inactivation of *SUB2* (18). The exact nature of sequestered *HSP104* transcripts in *sub2* and THO complex mutants remains undetermined, as we cannot ascertain whether 3′-end-truncated transcripts are retained in transcription site foci or not. However, we note that *HSP104* RNA is detectable with an in situ probe complementary to a sequence immediately upstream of the stop codon. Thus, complete, or nearly complete, transcripts are retained. Moreover, polyadenylated RNAs accumulate in the nucleus in THO complex mutants (39; this study). It is therefore likely that the *HSP104* RNAs, which can be detected in a nuclear dot, belong to the class of transcripts that either have not yet been degraded (see below) or escaped degradation and await further processing steps (e.g., formation of export-competent mRNP).

Read-through *HSP104* RNAs produced in *rna14-3* and *rna15-2* mutants are also retained in Rrp6p-dependent transcription site foci. These transcripts harbor extended 3′ untranslated regions, which are presumably unadenylated. Furthermore, transcripts generated by a self-cleaving hammerhead ribozyme are also subject to transcription site retention by Rrp6p. All of our results show that detection by Rrp6p, and possibly by the nuclear exosome, controls the release of several different incompletely and/or improperly processed mRNAs. Although it is presently unclear how recognition by Rrp6p is achieved, our results with the self-cleaving ribozyme imply that recognition of aberrant mRNA can occur independently of a polyadenylation signal and thus without binding of all factors normally involved in 3′-end formation.

THO complex mutants were originally described as defective in transcription of the bacterial *LacZ* gene, as well as of long and/or GC-rich yeast ORFs (9, 10). In the present study, we observed 3′-end-truncated *HSP104* transcripts in THO complex and *sub2-201* mutants. However, deletion of *RRP6* leads to almost complete restoration of full-length *HSP104* transcripts. Consistent with our results, *LacZ* transcript levels are also restored in a *yra1* mutant upon deletion of *RRP6* (41). Although an inhibitory role of Rrp6p on transcriptional elongation cannot be excluded, the specific restoration of *HSP104* 3′ ends (as opposed to an overall stabilization of *HSP104* RNA) supports the hypothesis that incomplete *HSP104* transcripts derive from partial degradation by Rrp6p. It is unclear why the exosome does not degrade *HSP104* RNA to completion, but this might be due to inhibitory features of the substrate mRNP.

What is the substrate for Rrp6p? Oligo(dT)/RNase H diges-

tion showed that the majority of restored *HSP104* RNAs in double mutants are polyadenylated. Thus, either RNAs degraded in the presence of Rrp6p were originally polyadenylated, or RNAs produced in a THO-mutant environment can undergo normal polyadenylation only in the absence of competitive Rrp6p degradation. Consistent with these possibilities, we found that polyadenylated as well as read-through transcripts are stabilized when *RRP6* is deleted in an *rna14-3* mutant background (Fig. 4). This suggests that a nuclear degradation process might outcompete a defective mRNP formation step, in the THO and *sub2* as well as the *rna14-3* mutant backgrounds. Alternatively, polyadenylation took place in these mutants before degradation. Inefficiently spliced RNAs are also substrates of the nuclear exosome, and nuclear degradation has been shown to compete with inefficient splicing (3). Although these observations suggest that nuclear RNA degradation is a general and important way of controlling aberrant (and maybe even normal) transcript abundance, it is not known how this is controlled.

A distinctive feature of *SUB2*, *YRA1*, and THO complex genes is their strong genetic interaction with the nuclear exosome component *RRP6*. This appears to be specific, as deletion of cytoplasmic mRNA degradation factors *XRN1* and *SKI2* does not affect growth of Δ*hpr1* or *sub2-201* strains. Furthermore, growth of a *RRP6* deletion strain is unaffected when combined with lesions in three other classes of genes involved in mRNA metabolism: (i) conventional mRNA export factors *MTR2* and *RAT7*, (ii) 3′-end formation components *RNA14*, *RNA15*, and *PAP1*, and (iii) bona fide transcriptional elongation factors *GCN5*, *PPR2*, and *RPB9*. The latter result suggests that a transcriptional elongation failure per se is insufficient to explain the genetic interaction of the THO complex with the nuclear exosome. The synthetic growth defects between mutants of *yra1*, *sub2*, or the THO complex with mutations of the nuclear exosome might be due to transcription site release or to reduced degradation. However, mRNA release is not toxic in the context of tested polyadenylation, transcription termination, or mRNA export mutants. This suggests that the released mRNP in THO complex, *sub2*, and *yra1* double mutants has a different composition and/or might improperly sequester an important subset of factors.

The hypothesis of involvement of the THO complex in transcription is based on the assessment of transcript abundance in mutants and on run-on experiments. In light of the results presented in this report, we suggest that transcript abundance in THO complex and *sub2* mutants is at least partially dependent on the extent of nuclear degradation. This, in turn, is probably triggered by defects in late events in transcription, 3′-end processing, and/or mRNP formation. Chromatin immunoprecipitation experiments indeed suggest a role of the THO complex in mRNP formation and specifically in the recruitment of Sub2p and Yra1p to nascent RNA (39, 41). Interestingly, *LacZ* sequences affect transcript abundance in mutants more markedly when located at the 3′ end of chimeric ORFs (10), which is consistent with the progressive cotranscriptional constitution of a functional mRNP. This hypothesis does not exclude functional feedback of mRNP formation on transcription elongation efficiency or pausing, possibly implicating factors recruited by the THO complex in one or more aspects of these processes. In this view, the primary effect of the THO complex is on nascent mRNP formation.

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