The *Saccharomyces cerevisiae* Small GTPase, Gsp1p/Ran, Is Involved in 3′ Processing of 7S-to-5.8S rRNA and in Degradation of the Excised 5′-A0 Fragment of 35S Pre-rRNA, Both of Which Are Carried Out by the Exosome

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ABSTRACT

Dis3p, a subunit of the exosome, interacts directly with Ran. To clarify the relationship between the exosome and the RanGTPase cycle, a series of temperature-sensitive *Saccharomyces cerevisiae dis3* mutants were isolated and their 5.8S rRNA processing was compared with processing in strains with mutations in a *S. cerevisiae* Ran homologue, Gsp1p. In both *dis3* and *gsp1* mutants, 3′ processing of 7S-to-5.8S rRNA was blocked at three identical sites in an allele-specific manner. In contrast, the 5′ end of 5.8S rRNA was terminated normally in *gsp1* and in *dis3*. Inhibition of 5.8S rRNA maturation in *gsp1* was rescued by overexpression of nuclear exosome components Dis3p, Rrp4p, and Mtr4p, but not by a cytoplasmic exosome component, Ski2p. Furthermore, *gsp1* and *dis3* accumulated the 5′-A0 fragment of 35S pre-rRNA, which is also degraded by the exosome, and the level of 27S rRNA was reduced. Neither 5.8S rRNA intermediates nor 5′-A0 fragments were observed in mutants defective in the nucleocytoplasmic transport, indicating that Gsp1p regulates rRNA processing through Dis3p, independent of nucleocytoplasmic transport.

A Ras-like small GTPase, Ran, is regulated by the GTPase-activating protein RanGAP1 and the GDP/GTP exchange protein RCC1 (Azuma and Dasso 2000; Nishimoto 2000). A set of Ran-binding proteins was found to be involved in nucleocytoplasmic transport of macromolecules. Those are proteins of the importin-β family, RanBP1, RanBP2, p10/Ntf2p, and a novel protein, Mog1p (Oki and Nishimoto 1998; reviewed by Melchior and Gerace 1998; Wozniak *et al.* 1998; Gorlich and Kutay 1999; Nakielny and Dreyfuss 1999). Curiously, two Ran-binding proteins, human RanBPM (Nakamura *et al.* 1998) and *Saccharomyces cerevisiae* Dis3p (Noguchi *et al.* 1996), have no clear functional relationship to nucleocytoplasmic transport. Studies of RanBPM first showed that Ran is required for microtubule assembly in Xenopus mitotic extracts (reviewed by Kahana and Cleveland 1999; Nishimoto 1999).

Recombinant *S. cerevisiae* Dis3p binds directly to Gsp1p, the *S. cerevisiae* Ran homologue, and *Schizosaccharomyces pombe* Dis3p is in a complex *in vivo* with *S. pombe* Ran and RCC1 homologues (Noguchi *et al.* 1996). Independently, *S. cerevisiae* Dis3p was identified as Rrp44p, a subunit of the exosome (Mitchell *et al.* 1997), which is composed of 11 subunits (Allmang *et al.* 1999). The exosome carries out not only 3′ processing of 7S-to-5.8S ribosomal RNA (rRNA), but also 3′ → 5′ degradation of *S. cerevisiae* mRNA (Mitchell *et al.* 1996; Jacobs Anderson and Parker 1998). The exosome that is conserved from yeast to human (Allmang *et al.* 1999) is therefore an important RNA processing/degradation machine in eukaryotic cells (van Hoof and Parker 1999). The exosome is localized in both nucleus and cytoplasm (Allmang *et al.* 1999).

Other proteins of the RanGTPase cycle were defined as temperature-sensitive (ts) mutants defective in RNA metabolism. Rna1p, the *S. cerevisiae* homologue of RanGAP1 (Bischoff *et al.* 1995), was identified as *rna1-1*, which is defective in RNA synthesis (Hutchison *et al.* 1969). *rna1-1* also has defects in both tRNA splicing and rRNA processing (Hopper *et al.* 1978). Prp20p, the *S. cerevisiae* RCC1 homologue, was identified as *prp20-1*, which is defective in mRNA splicing (Aebi *et al.* 1990). These phenotypes were thought to be indirect consequences of defects in nucleocytoplasmic transport (Gorlich and Kutay 1999; Nakielny and Dreyfuss

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TABLE 1

Plasmids used in this study

Plasmid	Relevant markers	Descriptions	Source and reference
YCp5072	*DIS3 CEN URA3*	YCp50 with *DIS3* genomic entire gene	Noguchi *et al.* (1996)
pTKSDIS3P	*DIS3*	pBluescript II TKS(+) with *DIS3* genomic fragment	This study
pUCDIS3P	*DIS3*	pUC28 with *DIS3* genomic fragment	This study
p314DIS3P	*DIS3 CEN TRP1*	pRS314 with *DIS3* genomic fragment	This study
p316DIS3P	*DIS3 CEN URA3*	*pRS316* with *DIS3* genomic fragment	This study
p314dis3ts	*dis3ts CEN TRP1*	pRS314 with *dis3* temperature-sensitive mutant	This study
pSKEB2.0	*DIS3-5′*	pBluescript II SK(+) with *DIS3-5′* genomic region	This study
p404DIS3-5′	*DIS3-5′ TRP1*	pRS404 with DIS3-5′ genomic region	This study
p404dis3ts	*dis3ts TRP1*	pRS404 with *dis3* temperature-sensitive mutant	This study
pNS3(pTKS dis3Δ::HIS3)	*dis3Δ::HIS3*	Disruption of *DIS3* ORF by *HIS3*	This study
pTKSURA3α	*URA3*	pBluescript IITKS(+) with *URA3* genomic fragment	This study
pSKHIS3α	*HIS3*	pBluescript IISK(+) with *HIS3* genomic fragment	This study
pTKSdis3Δ::URA3	*dis3Δ::URA3*	Disruption of *DIS3* ORF by *URA3*	This study
p195DIS3P	*2μDIS3 URA3*	YEplac195 with *DIS3* genomic fragment	This study
pGM410	bP_{GAL10}-*MTR4 CEN URA3*	pRS316 with *MTR4* ORF inserted downstream of *GAL10* promoter	Liang *et al.* (1996)
p405mtr4CT	mtr4-1 C-terminal region LEU2	pRS405 with mtr4-1 C-terminal region	This study
p195GSP1	*2μ GSP1 URA3*	YEplac195 with *GSP1* genomic fragment	Oki *et al.* (1998)
pYSKI2	*2μ*$_{ADH}$*SKI2 URA3*	*TRP1* of pYeFlag SKI2 was replaced with *URA3*	Liang *et al.* (1996)

1999), since both small nuclear and nucleolar RNPs are required for RNA metabolism, the maturation of which involves nucleocytoplasmic transport (Vegvar and Dahlberg 1990; Cheng *et al.* 1995; Lafontaine and Tollervey 1995; Maxwell and Fournir 1995; Yu *et al.* 1999). For instance, Xenopus U8 snRNP is required for the endonucleolytic cleavage of 12S pre-rRNA at both 5′ and 3′ ends (Peculis and Steitz 1993). The resulting 12S pre-rRNA is processed to 5.8S rRNAs by exonucleolytic cleavages at both the 5′ and 3′ ends. In yeast, while 5′ → 3′ trimming of pre-rRNA is carried out by Xrn1p and Rat1p (Henry *et al.* 1994), 3′ → 5′ trimming is performed by the exosome, a subunit of which is Dis3p/Rrp44p (Mitchell *et al.* 1997).

To investigate the functional relationship between the exosome and the RanGTPase cycle, we isolated a series of ts mutants of *S. cerevisiae DIS3/RRP44* (henceforth referred to as *DIS3*) by error-prone PCR and compared their rRNA processing with that of *gsp1* alleles that had been isolated by Oki *et al.* (1998). Both *dis3* and *gsp1* mutants showed defects in 3′ processing, but not 5′ processing, of the 5.8S rRNA precursor, as do other exosome mutants. 3′-end processing of 5.8S rRNA was blocked at three identical sites in both *dis3* and *gsp1*, in an allele-specific manner. Furthermore, the 5′-A0 fragment, which is degraded by the exosome (de la Cruz *et al.* 1998), accumulated in both *gsp1* and *dis3*. Taken together with the fact that Dis3p binds directly to Gsp1p (Noguchi *et al.* 1996), it is therefore likely that Gsp1p regulates the exosome through Dis3p.

MATERIALS AND METHODS

Strains and plasmids: *S. cerevisiae* strains and plasmids used in this study are described in Tables 1 and 2. Transformation of *S. cerevisiae* was performed by a modified LiCl method using dimethyl sulfoxide (DMSO; Hill *et al.* 1991). Selection against Ura^+ strains was carried out by culturing on solid synthetic media containing 1 mg/ml 5′-fluoroorotic acid (5′-FOA; Boeke *et al.* 1984).

Construction of plasmids: The *Eco*RI-*Apa*I fragment of YCp5072 (Noguchi *et al.* 1996) was introduced into the *Eco*RI/*Apa*I site of pBluescript IISK(+), resulting in pTKSDIS3P. The *Sac*I-*Apa*I fragment of pTKSDIS3P was introduced into the *Sac*I/*Apa*I site of pUC28, resulting in pUCDIS3P. The *Nsp*V-*Not*I fragment of pUCDIS3P was introduced into the *Nsp*V/*Not*I site of pRS314, resulting in p314DIS3P. The *Xho*I fragment of p314DIS3P was introduced into the *Xho*I site of pRS316, resulting in p316DIS3P. The *Eco*RI-*Bgl*II fragment of YCp5072 (Noguchi *et al.* 1996) was inserted into the *Eco*RI/*Bam*HI site of pBluescript IISK(+), resulting in pSKEB2.0. The *Eco*RI-*Spe*I fragment of pSKEB2.0 was inserted into the *Eco*RI/*Spe*I site of pRS404, resulting in p404DIS3-5′. The *Pst*I-*Not*I fragment of p314dis3ts was inserted into the *Pst*I/*Not*I site of p404DIS3-5′, resulting in p404dis3ts. The *Pst*I-*Eco*RI fragment of pTKSDIS3P containing the N-terminal noncoding and the coding region of *DIS3* was cut out and exchanged with the *Pst*I-*Eco*RI fragment of pTKSURA3α, resulting in pTKSURA3-3′. Subsequently, the *Bam*HI-*Pst*I fragment of pSKEB 2.0 was inserted into the *Bam*HI/*Pst*I site of pTKSURA3-3′, resulting in pTKSdis3Δ::URA3. Finally, the *Pst*I-*Eco*RI fragment of pTKSdis3Δ::URA3 was exchanged with the *Nsi*I-*Eco*RI fragment of pSKHIS3α, resulting in pNS3 (pTKSdis3Δ::HIS3). The *Sac*I-*Xho*I fragment of pUCDIS3P was inserted into the *Sac*I/*Sal*I site of YEplac195, resulting in p195DIS3P.

The DNA fragment containing the C-terminal open reading frame (ORF) of *mtr4-1* and the 3′ noncoding region of *MTR4*

TABLE 2

Yeast strains used in this study

Strain	Genotype	Source or reference
YPH499	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3*	
37C19	*MAT*α *dis3::HIS3 ade2 his3 leu2 lys2 trp1 ura3 [YCp5072]*	Noguchi *et al.* (1996)
N43	*MAT*α *ade2 his3 leu2 + trp1 ura3* / *MAT***a** *ade2 his3 leu2 lys2 trp1 ura3*	Noguchi *et al.* (1996)
YSN2	*MAT*α *dis3*Δ*::HIS3 ade2 his3 leu2 + trp1 ura3* / *MAT***a** *+ ade2 his3 leu2 lys2 trp1 ura3*	This study
YSN3T-1b	*MAT***a** *dis3*Δ*::HIS3 ade2 his3 leu2 lys2 trp1 ura3 [p316DIS3P]*	This study
dis3-WT	*MAT***a** *dis3-WT::URA3::dis3*Δ*::HIS3 ade2 his3 leu2 lys2 trp1 ura3*	This study
dis3-X[a]	*MAT***a** *dis3-x::TRP1::dis3*Δ*::HIS3 ade2 his3 leu2 lys2 trp1 ura3*	This study
N43-6C-GSP1	*MAT***a** *GSP1::LEU2::gsp*Δ*::HIS3 ade2 his3 leu2 trp1 ura3*	Oki *et al.* (1998)
N43-6C-gsp1[ts]	*MAT***a** *gsp1[ts]::LEU2::gsp*Δ*::HIS3 ade2 his3 leu2 trp1 ura3*	Oki *et al.* (1998)
p79	*MAT***a** *GAL10::prot.A-RRP4 ade2 his3 leu2 ura3*	Mitchell *et al.* (1997)
p108	*MAT***a** *GAL10::RRP4 gal2 gal*Δ*108 his3 leu2 trp1 ura3*	Mitchell *et al.* (1997)
p54	*MAT***a** *rrp4-1 ade2 his3 leu2 ura3*	Mitchell *et al.* (1997)
NN19-5B	*MAT***a** *rnal1-1 ade2 his3 leu2 ura3*	Noguchi *et al.* (1997)
SY1115	*MAT*α *srm1-1 his4 leu2 trp1 ura3*	Clark and Sprague (1989)
prp20/2c	*MAT***a** *prp20-1 ade2 his3 lys2 ura3*	Aebi *et al.* (1990)
T18	*MAT***a** *mtr1-2 ade2 his3 leu2 lys2 ura3*	Kadowaki *et al.* (1993)
MOY1	*MAT***a** Δ*mog1::HIS3 ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52*	Oki *et al.* (1998)
lrc4(ENY9-22)[b]	*MAT***a** *1rc4 ade2 his3 leu2 trp13 ura3*	This study
srp1(NOY612)	*MAT*α *srp1-31 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100*	Yano *et al.* (1994)
Δyrb2(ENY38-6)	*MAT***a** *yrb2-*Δ*1::LEU2 ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52*	This study
prp8-1(SPJ8.31)	*MAT***a** *prp8-1 leu2 trp1 ura3 ade2*	Jamieson *et al.* (1991)
ENY42-2	*MAT***a** *dis3-4::TRP1::dis3*Δ*::HIS3 mrt4-1::LEU2 ade2 his3 leu2 lys2 trp1 ura3*	This study
ENY46-2	*MAT***a** *dis3-6::TRP1::dis3*Δ*::HIS3 mrt4-1::LEU2 ade2 his3 leu2 lys2 trp1 ura3*	This study
ENY47-6	*MAT***a** *dis3-8::TRP1::dis3*Δ*::HIS3 mrt4-1::LEU2 ade2 his3 leu2 lys2 trp1 ura3*	This study
ENY48-1	*MAT***a** *dis3-11::TRP1::dis3*Δ*::HIS3 mrt4-1::LEU2 ade2 his3 leu2 lys2 trp1 ura3*	This study
ENY49-5	*MAT***a** *dis3-12::TRP1::dis3*Δ*::HIS3 mrt4-1::LEU2 ade2 his3 leu2 lys2 trp1 ura3*	This study
ENY50-7	*MAT***a** *dis4-14::TRP1::dis3*Δ*::HIS3 mrt4-1::LEU2 ade2 his3 leu2 lys2 trp1 ura3*	This study

[a] X indicates the number of the *dis3* allele.

[b] lrc4 was isolated as a cold-sensitive mutant synthetically lethal with *prp20*, possessing a single point mutation L283P in the ORF of the *CRM1* gene (K. Ogawa, E. Noguchi, N. Hayashi and T. Nishimoto, unpublished results).

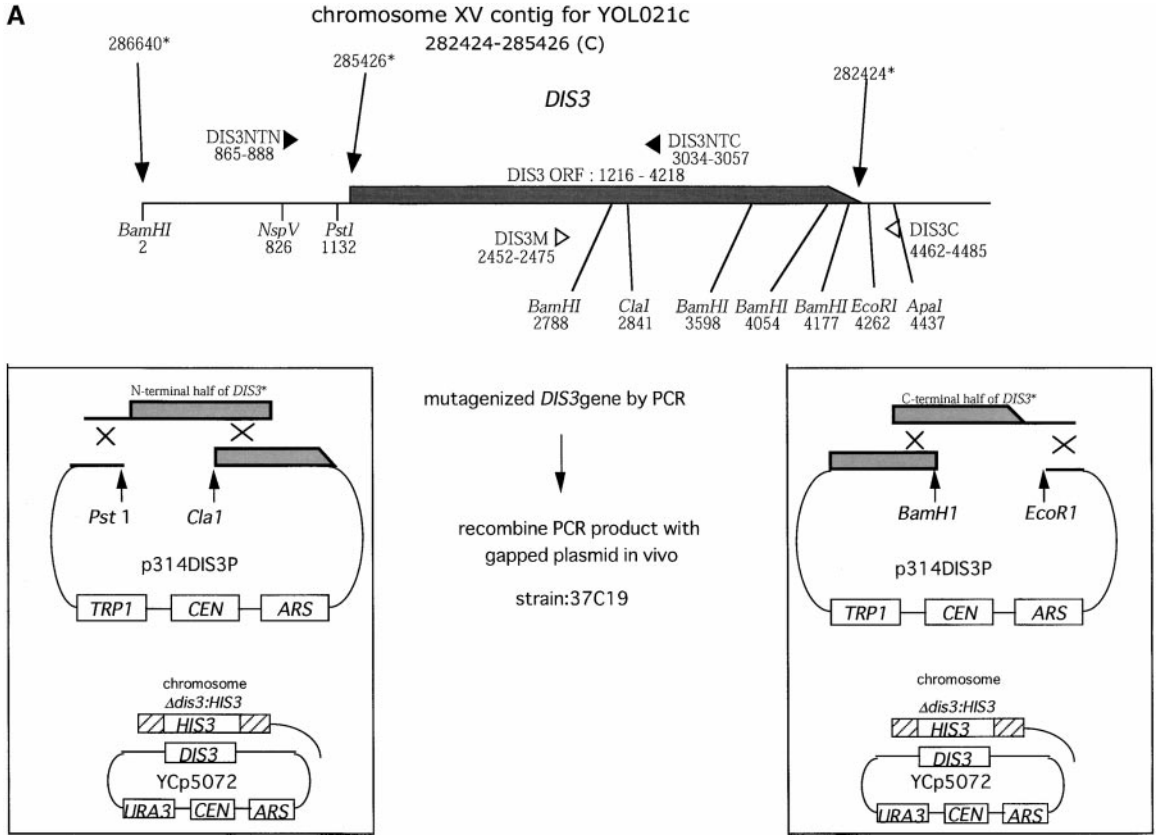
was amplified from the DNA of pRMts (Liang *et al.* 1996), using as the primers MTR4-6 (GAA ACC TGT CGA CCC TAC CTT AC) and MTR4-8 (AAG ATA CTA GTC TGG ATT CTG G), digested with the restriction enzymes *Sal*I and *Spe*I, and then introduced into the *Sal*I/*Spe*I site of pRS405, resulting in p405mtr4CT.

Disruption of *DIS3* gene: DNA of the plasmid pNS3 (Δdis3::HIS3) was digested with the restriction enzymes *Sac*I and *Apa*I and introduced into the *S. cerevisiae* diploid strain N43, resulting in the strain YSN2, into which p316DIS3P was introduced. The resulting strain YSN2[p316DIS3P] was sporulated and a haploid segregant YSN2T-1α was isolated and mated with the YPH499. The resulting diploid was sporulated and a haploid strain YSN3T-1b was isolated.

Mutagenesis of *DIS3* gene: The N-terminal and C-terminal parts of the *S. cerevisiae* Dis3p ORF carried on p314DIS3P were separately amplified by the error-prone PCR (Beckman *et al.* 1985; Leung *et al.* 1989) as described by Oki *et al.* (1998) using two sets of primers: the N-terminal set, ATT CAG TAG CAC ATG GCG GAA AAG and AGA ACA CAG GTC GGT ACC TAG AAG [amino acids (aa) 1–614], and the C-terminal set, GAT CCA CAA AGC AGT AGT ACA CAG and TGA AAG CGC GCA AGT GGT TTA GTG (aa 413–1001). Site-directed mutagenesis of *DIS3* was performed using p314DIS3P by the site-directed mutagenesis system Mutan-K (TaKaRa, Kyoto, Japan).

Extraction of total RNA: Cultures of 10 ml sampled at the indicated time points were centrifuged at 10,000 rpm for 5 min. Cell pellets were washed with ice-cold ddH_2O containing diethyl pyrocarbonate (DEPC; 0.1%) and suspended in 0.2 ml of lysis buffer (0.5 M NaCl, 0.2 M Tris-HCl, pH 7.6, 0.01 M EDTA, 1% SDS). After addition of glass beads and 0.2 ml of chloroform-saturated phenol, cells were vortexed for 3 min and then received 0.3 ml of lysis buffer and 0.3 ml of chloroform-saturated phenol. After further vortexing for 3 min, the mixture was centrifuged at 10,000 rpm for 5 min, and the supernatant received 0.3 ml of chloroform-saturated phenol and was vortexed and then centrifuged at 10,000 rpm for 5 min. Sodium acetate was added to 0.3 M, followed by 1 ml of 100% ethanol, and the mixture was kept at −20° overnight and then centrifuged at 10,000 rpm for 15 min. The precipitate was washed twice with 100% ethanol and dried. Afterward, the precipitates were suspended in TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) containing 0.1% DEPC at the final RNA concentration (2.0 mg/ml) and stored at −80°. All procedures were carried out at 4° except where otherwise indicated.

Northern hybridization: In the 5.8S rRNA processing analysis, 4 μl of total RNA for each lane was electrophoresed at 250 V, 16 mA for 12 hr in TBE buffer containing 8.3 M urea, 5.0% Long Ranger (FMC, Rockland, ME), 0.05% ammonium persulfate (APS), and 0.07% TEMED (*N,N,N′,N′*-tetrameth-



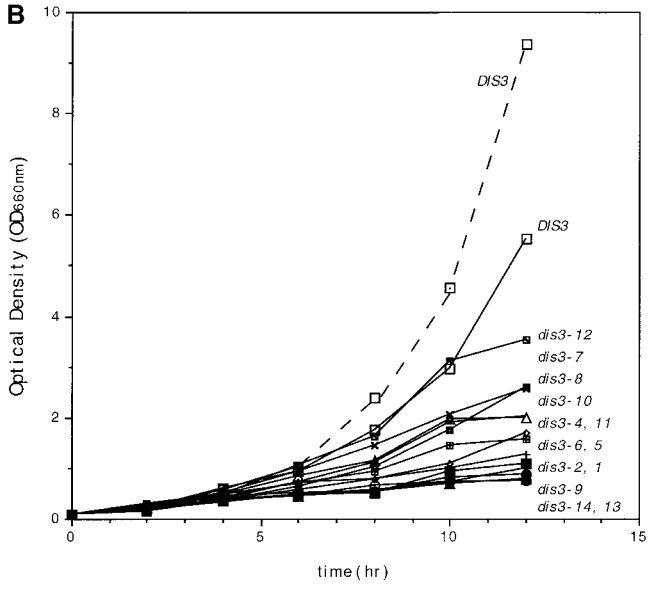


FIGURE 1.—Construction and growth of ts *dis3* strains. (A) Localized mutagenesis of the *DIS3* gene. The *DIS3* gene was amplified by mutagenic PCR using two sets of primers, DIS3NTN/DIS3NTC and DIS3M/DIS3C. The resulting PCR products were cotransformed into the strain 37C19 with the plasmid p314DIS3P treated with restriction enzymes as shown. Transformants (Ura^+, Trp^+) were selected for ts growth as described in MATERIALS AND METHODS. *, mutagenized *DIS3* gene. The number with an asterisk (*) is the nucleotide number on *S. cerevisiae* chromosome XV, and the number without an asterisk is the arbitrary number of the nucleotide on the cloned *DIS3* DNA fragment. (B) Growth curve of *DIS3* and *dis3* strains. Overnight cultures of *DIS3* and ts *dis3* strains were diluted into YPD medium and grown to $OD_{660\ nm} = 0.1$. Cultures (5 ml) were centrifuged, suspended in the same volume of prewarmed (37°) YPD medium, and then incubated at 37° (solid line). Another 5-ml culture was incubated at 26° (dotted line). At the indicated times (hr), the $OD_{660\ nm}$ was measured. For *dis3* strains, only the growth curve at 37° is shown.

ylethylenediamine) and electrotransferred onto Hybond-N (Amersham Pharmacia Biotech) in 60 mM sodium acetate buffer at 120 mA for 15 hr as described (MITCHELL *et al.* 1996).

In the mRNA analysis, 2 μl of total RNA was electrophoresed at 50 V, 20 mA for 8 hr in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer containing 1% agarose, 1× MOPS (20 mM MOPS, 10 mM $CH_3COONa \cdot 3H_2O$, and 0.5 M EDTA), and formamide (16%) and transferred onto Hybond (Amersham Pharmacia Biotech, Arlington Heights, IL) in 20× SSC.

The prepared RNA filters were prehybridized twice with 100 μg/ml of single-strand salmon sperm DNA at 50° for 2 hr in buffer containing 0.5% SDS, 50% formamide, 6× SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), and 5× Denhardt's solution and were then incubated with ^{32}P-labeled oligodeoxyribonucleotides for 8 hr. After hybridization, filters were washed in the following manner: once in 2× SSC plus 0.1% SDS for 10 min at room temperature, once in 1× SSC plus 0.1% SDS for 10 min at 42°, and twice in 1× SSC plus 0.1% SDS for 10 min at 42°. Finally, filters were dried and analyzed by Fuji Bioimage analyzer.

The oligodeoxyribonucleotide probes 1, 2, 3, 4, 5, 6, and 7 that were used for 5.8S rRNA analysis were as follows: 1, CGA ACG ACA AGC CTA CTCG; 2, CAT GGC TTA ATC TTT

TABLE 3

***dis3* alleles, mutated amino acids, and the phenotypes of 5.8S rRNA processing and pre-mRNA splicing**

dis3 allelic No.[a]	Amino acid change[b]	5.8S rRNA intermediate[c]	pre-mRNA[d]
dis3-1	G465T, H488R	M	Not visible
dis3-2	L48H,D140N, V158E, M238T, Y259H, S291C, N361S, P457A	M	Not visible
dis3-4	L102V, K143R, E154D, I174K, N212K, F255S, N312D, D351E, E482K, I539T	L	Not visible
dis3-5	V3A, D230G, S251P, T254K, Y259S, L292S	L	Not visible
dis3-6	G562D, D632E, P698H, V714E, D744G, F746I, F952I, V962E	None	2 hr
dis3-7	I862F, V934L, V982A	M	Not visible
dis3-8	I570N	L, S	8 hr
dis3-9	C502R, A521T, D536E, A589T, E728G	S	Not visible
dis3-10	A588T, T592S, E655D, Q656L, E669D, E715V, L934S	L	Not visible
dis3-11	V577I, N712S, F746S, V794F, D837V, D873N, Y901F, L997I	M, S	2 hr
dis3-12	V566G	S	2 hr
dis3-13[e]	P463L	M	Not visible
dis3-14	E565K	S	2 hr
mtr17-1[f]	Not determined	None	4 hr

[a] The third *dis3* allele was discarded, due to unsatisfactory temperature sensitivity.

[b] The changes of amino acid residues indicated by underlining were introduced into the *DIS3* gene by site-directed mutagenesis.

[c] The lengths of 5.8S rRNA intermediates, long (L), middle (M), and short (S), are indicated. "None" indicates that 5.8S rRNA intermediates were not observed.

[d] The initial time of pre-mRNA appearance upon incubation at 37° is shown. After shifting up to 37°, total RNAs were extracted at 2, 4, and 8 hr. "Not visible" indicates that no pre-mRNA appeared within 8 hr of incubation at 37°.

[e] This mutant was from Dr. S. J. Elledge.

[f] Isolated as a mutant that accumulates nuclear poly(A)$^+$ RNA, by A. Tartakoff.

GAG AC; 3, CCA GTT ACG AAA ATT CTTG; 4, TTT CGC TGC GTT CTT CATC; 5, TGA GAA GGA AAT GAC GCT; 6, GGC CAG CAA TTT CAA GTTA; and 7, GAA CAT TGT TCG CCT AGA, as described by de la Cruz *et al.* (1998). The 1.2 kb of *CRY1* genomic DNA (from 175,773 to 176,958 on *S. cerevisiae* chromosome III) containing an intron was amplified by PCR, using as the 5′ primer GGA AAG CTT ACA AGT TCT GGT ATA TTCTAT and as the 3′ primer AAC TCA TAA GCT TCT ACC TCT TCT ACC, to be used as a probe for mRNA analysis.

Pulse-chase labeling experiments: *dis3*, *gsp1*, and, as a control, wild-type *Dis3* strains were grown to OD_{660} = 0.2 in 100 ml of YPD medium and harvested by centrifugation. Cells were resuspended in SD medium lacking uracil at a density of 20 OD_{660}/ml in a total volume of 1 ml. The culture was preincubated for 15 min at 37° with shaking, 0.8 mCi of prewarmed [5.6-^{3}H]uracil (TRK408; Amersham Pharmacia Biotech) was added to the culture, and it was incubated for 20 min (Sachs and Davis 1990; Tollervey *et al.* 1993). Following centrifugation, cells were suspended in 100 ml of prewarmed SD medium containing 0.24 mg/ml of uracil (final concentration) and incubated at 37°. Every 10 min, 20 ml of culture was sampled and total RNA was extracted as described above. Total RNAs of 20,000 cpm were resolved on 5.75% polyacrylamide gel containing 7.0 m urea (TaKaRa Long Ranger single pack 373; 34 cm), using 12-cm-long, 1-mm-thick glass, at 10 mA (constant current) at 4° for 400 min. After electrophoresis, gels were fixed with 10% methanol, 10% CH_3COOH, and then RNA was transferred to Hybond (Amersham Pharmacia Biotech). The membranes were sprayed with ENLIGHTNING (New England Nuclear Life Science Products, Boston) and were exposed to X-ray films for 96–120 hr at −80° (de la Cruz *et al.* 1998).

RESULTS

Construction of temperature-sensitive *S. cerevisiae dis3* strains: To introduce mutations into the *S. cerevisiae DIS3* gene, the ORF of Dis3p was divided into an N-terminal half (aa 1–614) and a C-terminal half (aa 413–1001). Subsequently, each region was separately amplified by error-prone PCR. The amplified DNA fragments were introduced into the strain 37C19 (Δ*dis3::HIS3* [YCp5072]; Table 1), along with the plasmid p314DIS3P (*DIS3*, *CEN*, *TRP1*), from which a part of the *DIS3* gene had been removed using either *Pst*I and *Cla*I (N-terminal half) or *Bam*HI and *Eco*RI (C-terminal half), as shown in Figure 1A. Transfected cells were plated onto synthetic medium lacking tryptophan and uracil at 26°. Of 200,000 Trp$^+$, Ura$^+$ colonies, 2000 grew on synthetic medium containing 1 mg/ml of 5′-FOA. Colonies were replated onto YPD plates and incubated either at 26°, the permissive temperature, or at 37°, the nonpermissive temperature. A total of 12 ts *dis3* alleles were obtained (Table 3). Some *dis3* alleles possessed multiple amino acid changes. In these cases, the amino acid changes of conserved residues (under-

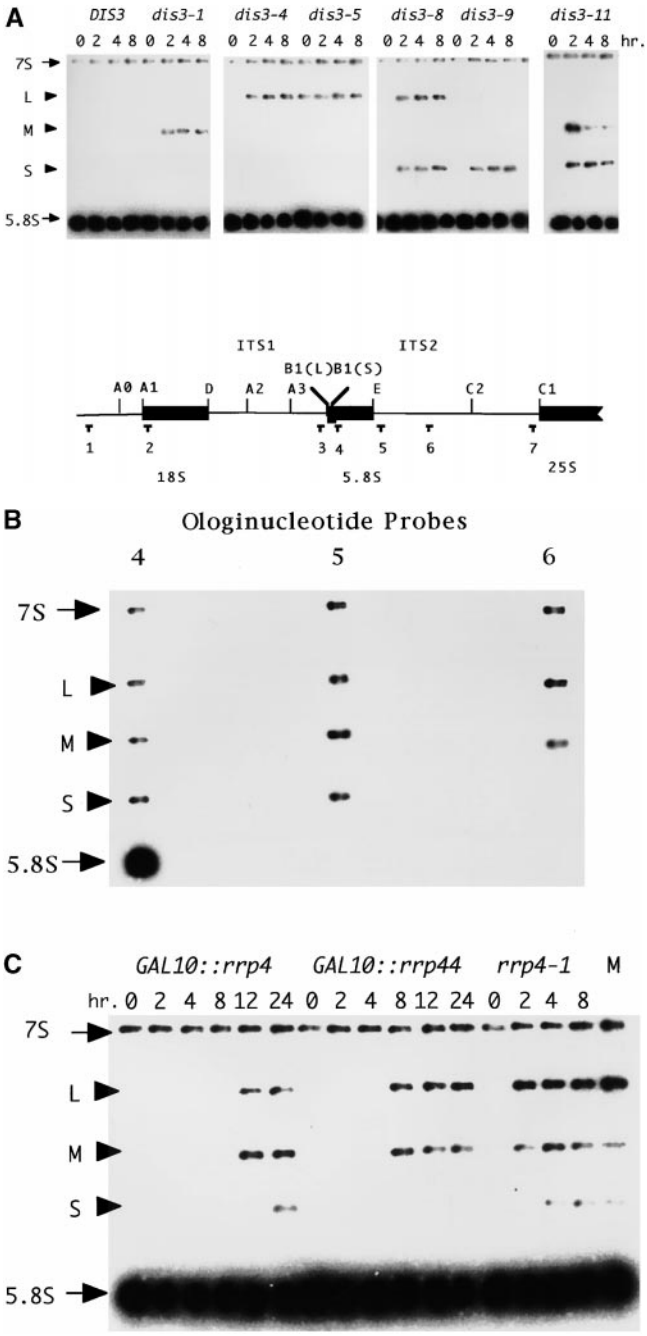


FIGURE 2.—*dis3* strains have defects in 3′ end processing of 5.8S rRNA, similar to other exosome mutants. (A) Ladders of intermediate 5.8S rRNA fragments observed in *dis3*. Cultures (50 ml) of *dis3* strains and, as a control, the wild-type strain *DIS3* were grown at 26° to OD_{660} = 0.8, centrifuged, and resuspended in the same volume of prewarmed (37°) YPD medium. After incubation at 37° for 0, 2, 4, and 8 hr as indicated, 10 ml was centrifuged and the extracted total RNA was subjected to Northern blot analysis with oligonucleotide 4 (bottom). Mutants showing a representative pattern are shown. Arrows indicate the position of 7S pre-RNA and 5.8S rRNA, and arrowheads indicate the long (L), middle (M), and short (S) intermediates. The bottom indicates the position of the nucleotide probes in the 35S rRNA. (B) 3′ end processing was blocked in *dis3* mutants. Total RNAs (10 μl) extracted from *dis3-1*, *-4*, and *-9* strains were mixed, and then 5 μl of the mixture was electrophoresed, transferred to filters, and hybridized with oligonucleotide 4, 5, or 6 as indicated. Arrows indicate the position of 7S pre-RNA and 5.8S rRNA, and arrowheads indicate the long (L), middle (M), and short (S) intermediates. (C) Cultures (100 ml) of strains *Gal10::RRP4* and *GAL10::RRP44* were grown at 26° to OD_{660} = 0.8 in synthetic medium without uracil containing 2% galactose, centrifuged, and resuspended in the same volume of a synthetic medium containing raffinose. After incubation for 0, 2, 4, 8, 12, and 24 hr, 10 ml was centrifuged. For comparison, 50 ml of the strain *rrp4-1*, grown at 26° to OD_{660} = 0.8, was centrifuged and resuspended in the same volume of prewarmed (37°) YPD medium. After incubation at 37° for 0, 2, 4, and 8 hr, 10-ml samples were collected by centrifugation. Total RNA was analyzed by Northern hybridization with oligonucleotide 4 (A, bottom). Lane M contains the mixture of total RNA of *dis3-1*, *-4*, and *-9* strains. Arrows indicate the position of 7S pre-RNA and 5.8S rRNA, and arrowheads indicate the long (L), middle (M), and short (S) intermediates.

lined in Table 3) were introduced into the wild-type *DIS3* gene by site-directed mutagenesis, to identify single amino acid changes responsible for the ts phenotype. Finally, 4 ts *dis3* alleles had single amino acid changes (Table 3). To characterize in a uniform genetic background, all of the ts *dis3* genes listed in Table 3 and, as a control, the wild-type *DIS3* were inserted into the haploid strain YSN3T-1b (Δdis3::HIS3[p316DIS3P]) by homologous recombination, and colonies (Trp^+, Ura^+) were incubated in synthetic medium containing 1 mg/ml of 5′-FOA. All of the resulting *dis3* strains ceased to grow after incubation at 37° for 12 hr in liquid medium (representative results are shown in Figure 1B). Southern hybridization analysis revealed that the *dis3* alleles were correctly integrated into the chromosome (data not shown) and all of the *dis3* mutant strains could be complemented by the wild-type *DIS3* gene.

3′ end processing of 5.8S rRNA is blocked at three distinct sites: A series of the *dis3* strains and, as a control, the wild-type *DIS3* strain (dis3-WT) were cultured at 26° to OD_{660} = 0.8 and then incubated at 37°. After incubation for 2, 4, and 8 hr, total RNA was extracted, resolved by polyacrylamide gel electrophoresis, and hybridized with oligonucleotide 4, which is complementary to the mature 5.8S rRNA (Figure 2A, bottom). In the *DIS3* strain, two bands corresponding to 7S and 5.8S rRNAs were detected. Additionally, one or two fragments intermediate in length between 7S and 5.8S rRNA appeared in 12 out of 13 *dis3* strains upon incubation at 37° (Table 3; representative results are shown in Figure 2A).

To determine whether the observed intermediates are 3′ end-extended forms of 5.8S rRNA, total RNAs of *dis3-1*, *-4*, and *-9* strains, which contain the middle, long, and short intermediate fragments, respectively, were mixed and hybridized with the oligonucleotides 3, 4, 5, or 6. The positions of these oligonucleotides in pre-rRNA are shown in the bottom of Figure 2A. As expected, probe 4 hybridized with all three intermediate fragments, in addition to both 7S and 5.8S rRNA (Figure

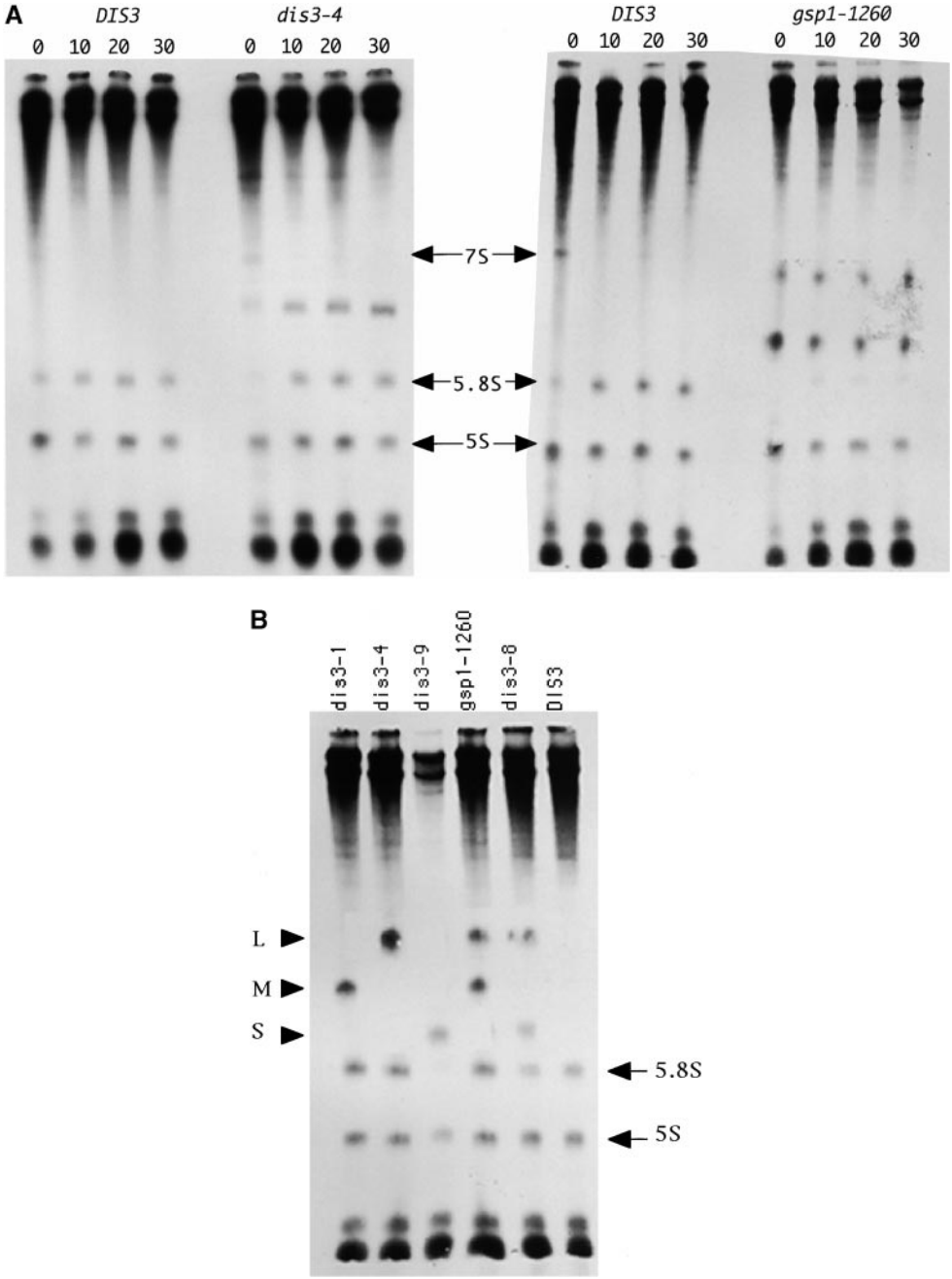


FIGURE 3.—In both *dis3* and *gsp1* mutants, 3′ end processing of 5.8S rRNA was blocked at three distinct sites. Cultures of dis3-WT(*DIS3*), *dis3-1*, *dis3-4*, *dis3-8*, *dis3-9*, and *gsp1-1260* were grown in YPD medium, transferred to SD medium lacking uracil, labeled with [5.6-^{3}H]uracil for 20 min, and then chased with an excess of cold uracil for 60 min. Immediately after labeling (0), and then after chasing for 10, 20, 30, and 60 min, total RNA was extracted, separated on 5.75% polyacrylamide-7.0 M urea gels, transferred to nylon membrane, and visualized by fluorography. Approximately 20,000 cpm was loaded in each lane. (A) Representative results of time-course experiments. (B) Total RNAs extracted from indicated strains after a 60-min chase were analyzed for comparison. The positions of the 7S, 5.8S, and 5S rRNA are indicated by arrows. Arrowheads indicate the long (L), middle (M), and short (S) intermediates.

2B, probe 4). Probe 5, which is partially complementary to both the 3′ end of 5.8S rRNA and the 5′ end of the ITS2 spacer, hybridized with all three intermediate fragments and with 7S rRNA, but not with the mature 5.8S rRNA, as previously reported (MITCHELL *et al.* 1996) (Figure 2B, probe 5). On the other hand, probe 6, which is complementary to a site in the ITS2 ~50 nucleotides farther 3′ from the mature 5.8S rRNA, hybridized with 7S rRNA and the two long (L) and middle (M) intermediate fragments (Figure 2B), but not with the short (S) intermediate fragment (Figure 2B, probe 6). No 5.8S rRNA fragments hybridized with probe 3 (data not shown), which is complementary to a site in ITS1 proximal to the 5′ end of 5.8S rRNA. These observations prove that the fragments of 5.8S rRNA intermediate in length between 7S and 5.8S rRNAs are 3′ extended forms of 5.8S rRNA.

To learn whether loss of another exosomal component also inhibits 5.8S rRNA maturation in a similar pattern, the strains *GAL10::rrp4*, *rrp4-1*, and *GAL10::rrp44* were precultured at 26° in synthetic medium containing 2% galactose or YPD medium and then incubated under nonpermissive conditions. At the indicated times, total RNAs were extracted, coelectrophoresed with the mixture of total *dis3* RNAs, and hybridized with oligonucleotide 4. As reported (MITCHELL *et al.* 1996, 1997), ladders of 5.8S rRNA fragments appeared in *GAL10::rrp4* and *GAL10::rrp44*, both after 8–24 hr of in-

TABLE 4

5.8S rRNA processing and pre-mRNA splicing in *gsp1*

gsp1 allelic no.	5.8S rRNA intermediate[a]	pre-mRNA[b]
gsp1-1757	None	4 hr
gsp1-1268	L, M	4 hr
gsp1-479	M, S	4 hr
gsp1-16	None	Not visible
gsp1-322	M, S	Not visible
gsp1-882	None	8 hr
gsp1-1547	L, M, S	8 hr
gsp1-1907	L, M, S	Not visible
gsp1-245	M, S	Not visible
gsp1-1178	L	Not visible
gsp1-1568	L	Not visible
gsp1-1598	S	Not visible
gsp1-1651	L	Not visible
gsp1-1819	L	8 hr
gsp1-1894	M, S	Not visible
gsp1-1060	None	4 hr
gsp1-640	S	Not visible
gsp1-1486	M, S	Not visible
gsp1-1518	L	8 hr
gsp1-1582	M, S	Not visible
gsp1-1778	L	Not visible
gsp1-1968	L	Not visible
gsp1-1260	L, M	Not visible
gsp1-1763	L, M	Not visible
gsp1-1817	None	4 hr

[a] The lengths of 5.8S rRNA intermediates, long (L), middle (M), short (S), are indicated. "None" indicates that 5.8S rRNA intermediates were not observed.

[b] The initial time of pre-mRNA appearance upon incubation at 37° is shown. After shifting up to 37°, total RNAs were extracted at 2, 4, and 8 hr. "Not visible" indicates that no pre-mRNA appeared within 8 hr of incubation at 37°.

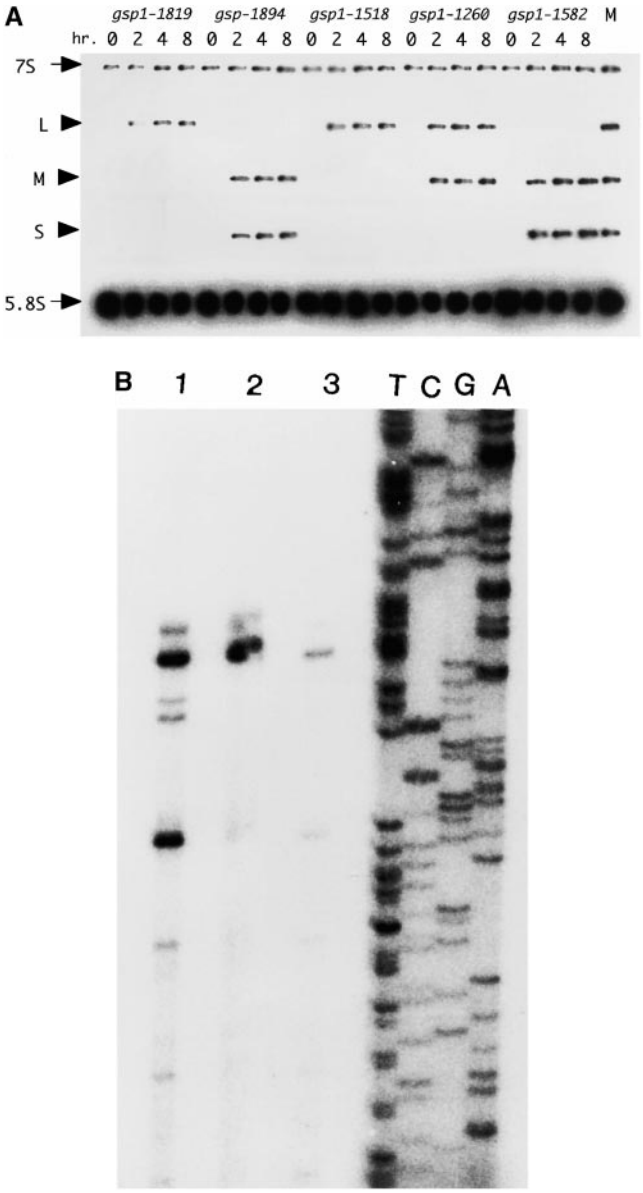


FIGURE 4.—*gsp1* strains have a defect in 3′ end, but not 5′ end, processing of 5.8S rRNA, similar to *dis3*. (A) *gsp1* mutations blocked 3′ processing of 5.8S rRNA at three sites. Cultures (50 ml) of *gsp1* strains indicated (*gsp1-1819*, *gsp1-1894*, *gsp1-1518*, *gsp1-1260*, and *gsp1-1582*) were prepared as for *dis3* and samples of total RNA extracted after incubation at 37° for 0, 2, 4, and 8 hr were analyzed by Northern hybridization with oligonucleotide 4 (Figure 2A, bottom). Lane M contains the mixture of total RNAs of *dis3-1*, *-4*, and *-9* strains. Arrows indicate the position of 7S pre-RNA and 5.8S rRNA, and arrowheads indicate the long (L), middle (M), and short (S) intermediates. (B) Primer extension analysis of the 5′ end of 5.8S rRNA. Total RNA was extracted from *gsp1-1907* (lane 1), *dis3-1* (lane 2), and *DIS3* (lane 3) after incubation at 37° for 8 hr. Primer extension was performed using oligonucleotide 5 (Figure 2A, bottom). A sequencing reaction on the rDNA repeat using the same primer is also shown.

cubation in the absence of galactose, and also in *rrp4-1* after 2 hr of incubation at 37° (Figure 2C). Strikingly, the lengths of 5.8S rRNA fragments observed were identical to those found in *dis3* mutants (Figure 2C, compare with lane M), indicating that the 3′ end processing of 5.8S rRNA from 7S rRNA was inhibited at the same three sites by loss of exosome function.

Northern analysis cannot show whether 5.8S rRNA maturation was terminated at these three distinct sites, as opposed to being paused for an extended period. In the latter case, intermediate fragments will ultimately be processed to mature 5.8S rRNA. To address this issue, several *dis3* mutants and, as a control, wild-type *DIS3* strains were labeled *in vivo* with [5.6-^{3}H]uracil for 20 min at 37° and then chased with an excess of cold uracil for 60 min. Every 10 min, total RNA was extracted and low molecular weight RNA species were analyzed as described (DE LA CRUZ *et al.* 1998). We chose *dis3* strains *dis3-1*, *dis3-4*, *dis3-9*, and *dis3-8*, which accumulate one or two intermediate fragments at 37° (Table 3). In *DIS3* cells, mature 5.8S rRNA appeared immediately after 20-min pulse labeling. On the other hand, in the *dis3* mutants, fragments intermediate in length between 7S and 5.8S rRNA appeared before labeling of 5.8S rRNA

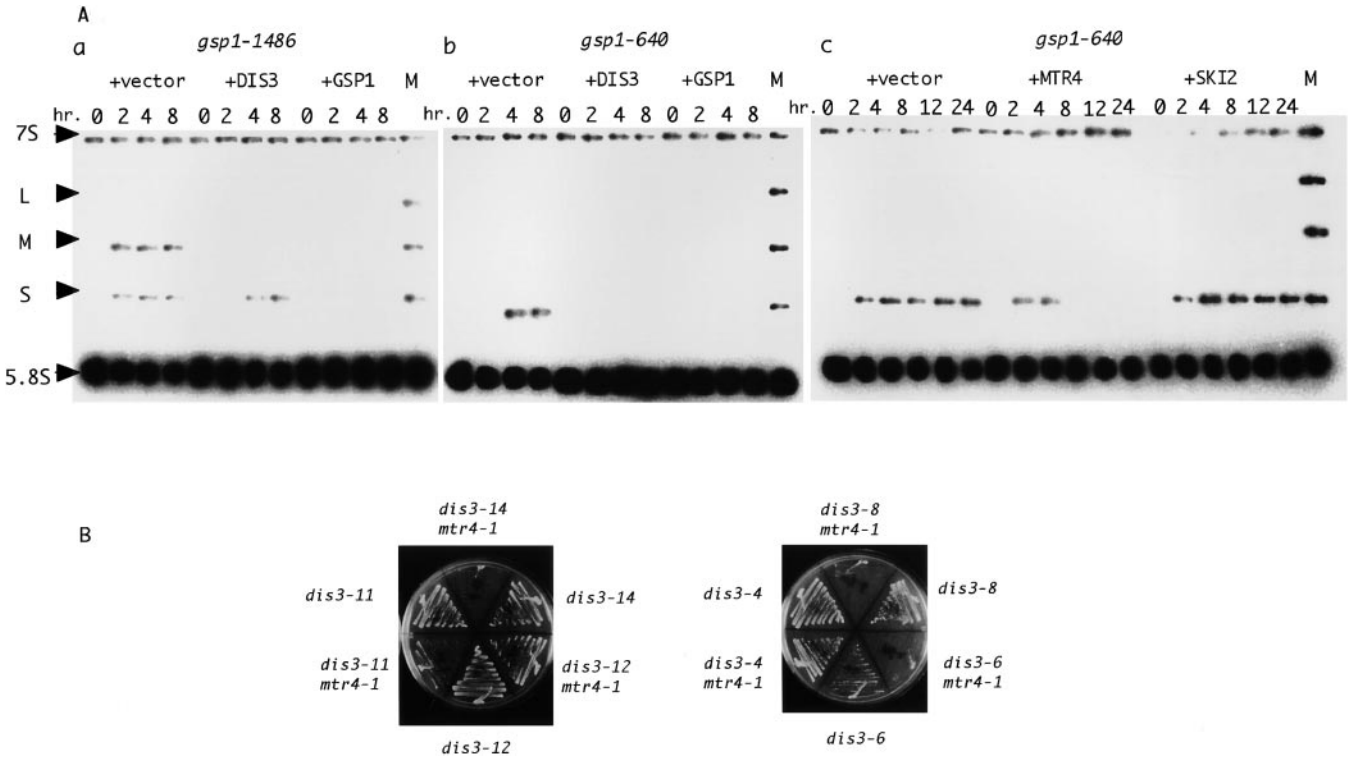


FIGURE 5.—Overexpression of Dis3p and Mtr4p, but not Ski2p, rescues 3′ end processing defects of 5.8S rRNA maturation. (A) Cultures of *gsp1-1486* (a) and *gsp1-640* (b) containing p195DIS3P, p195GSP1, or YEplac195 vector alone, as indicated, were incubated at 36° for 0, 2, 4, and 8 hr. Cultures of *gsp1-640* (c) containing pGM410 (*GAL10*::MTR4), pYSKI2 (*ADH*::SKI2), or YEplac195 vector alone, as indicated, which had been grown to OD_{660} = 0.8 at 26°, were precultured in synthetic medium containing 2% galactose for 1 hr at 26° and then cultured in the same medium at 37° for 0, 2, 4, 8, 12, and 24 hr. Total RNAs extracted at the indicated times were analyzed by Northern hybridization with oligonucleotide 4 (Figure 2A, bottom). Lane M contains the mixture of total RNA of *dis3-1*, *-4*, and *-9* strains. Arrowheads indicate 7S pre- and 5.8S rRNAs, and the long (L), middle (M), and short (S) intermediates. (B) *dis3* and *mtr4* are synthetic lethal. The strains ENY42-2 (*mtr4-1 dis3-4*), ENY46-2 (*mtr4-1 dis3-6*), ENY47-6 (*mtr4-1 dis3-8*), ENY48-1 (*mtr4-1 dis3-11*), ENY49-5 (*mtr4-1 dis3-12*), ENY50-7 (*mtr4-1 dis3-14*), and, as controls, *dis3* strains, all carrying the plasmid p195DIS3, were streaked on 5′-FOA-containing plates to counterselect p195DIS3 and were incubated at 26° for 3 days.

was seen. Representative results are shown in Figure 3A. Even after a 60-min chase, the amount of the intermediate species did not decrease (Figure 3B). To compare the number and length of labeled intermediates, total RNA extracted after a 60-min chase was analyzed (Figure 3B). Strikingly, labeled intermediates showed three distinct lengths. For instance, *dis3-1* accumulated a single M-intermediate while *dis3-8* accumulated two intermediates (L and S; Figure 3B, lanes 1 and 6), consistent with the Northern analysis (Figure 2A). These results indicate that 5.8S rRNA maturation is blocked at three distinct sites in an allele-specific manner.

***gsp1* has a defect in 3′ end but not 5′ end processing of 5.8S rRNA, similar to *dis3*:** The maturation of 5.8S rRNA was examined in 25 *gsp1* strains, to learn whether there is any functional relationship between the Ran-GTPase and the exosome. Total RNAs extracted from *gsp1* strains after incubation for 2, 4, and 8 hr at 37° were analyzed by Northern hybridization with oligonucleotide 4. In 20 out of 25 *gsp1* strains, intermediate 5.8S rRNA fragments appeared (Table 4). Representative results are shown in Figure 4A. Remarkably, three intermediates seen in *gsp1* mutants have the same lengths as those of *dis3* mutants (Figure 4A, compare with lane M), suggesting that 3′ end processing of 5.8S rRNA is inhibited in a similar manner in both *gsp1* and *dis3* mutants. This is also the case after chasing of [5.6-^{3}H]uracil-labeled RNA with an excess of cold uracil. *gsp1-1260* accumulated both L- and M-length intermediates (Figure 3, A and B), as observed by Northern analysis (Table 4). Thus, 5.8S rRNA maturation is blocked at three distinct sites in *gsp1* mutants at 37°, as in *dis3* mutants.

To determine the 5′ end of 5.8S rRNA, primer extension analysis of total RNAs extracted from *gsp1-1907* was carried out using as a primer the oligonucleotide 5, which hybridizes to all intermediate 5.8S rRNA fragments and to 7S pre-rRNA, but not to mature 5.8S rRNA (Figure 2B, probe 5). *dis3-1* and *gsp1-1907* accumulate one and three intermediates, respectively, at 37° (Figure 2A and Table 4). In both mutants, the 5′ end of 5.8S rRNA terminates near nucleotides 2855(A) and 2864(A),

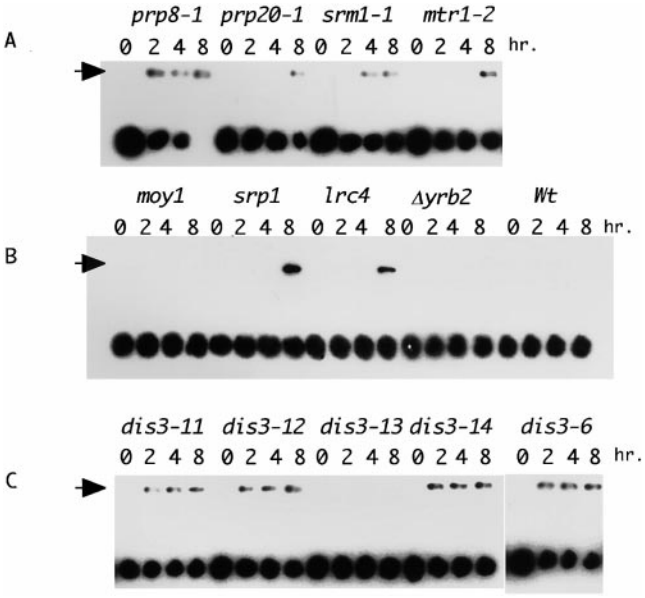


FIGURE 6.—mRNA splicing is affected by *dis3* and nucleocytoplasmic transport mutants. Total RNA was prepared as described above from cultures of strains (A) *prp8-1* (SPJ8.31), *prp20-1* (prp20/2c), *srm1-1* (SY1115), and *mtr1-2* (T18); (B) *moy1*, *srp1* (NOY612), *lrc4* (ENY9-22), Δ*yrb2* (ENY38-6), and *wt* (wild type); and (C) *dis3-11*, *dis3-12*, *dis3-13*, *dis3-14*, and *dis3-6* after incubation at 37° for 0, 2, 4, and 8 hr as indicated and was then analyzed by using a *CRY1* probe. Arrows indicate the position of unspliced *CRY1* mRNA.

corresponding to the B1(L) and B1(S), respectively, as reported (HENRY *et al.* 1994; Figure 4B). Therefore, the 5′ end of 5.8S rRNA was terminated correctly in both *gsp1* and *dis3*, even when the 3′ end processing was blocked. Consistent with a previous report (HENRY *et al.* 1994), there was much less 5.8S(L) than 5.8S(S) in both *dis3* and *gsp1*.

These results indicate that loss of RanGTPase activity inhibits 3′ end processing, but not 5′ end processing, of 5.8S rRNA maturation. It is notable that the number of intermediate fragments depends on the *gsp1* allele, as for *dis3* mutants.

Overexpression of Dis3p and Mtr4p enhances 3′ end processing of 5.8S rRNA: To further examine the relationship between the RanGTPase and the exosome, a high copy *DIS3* plasmid, p195DIS3P, was introduced into the *gsp1* strains and 3′ processing of 5.8S rRNA was examined in the resulting transformants. Representative results are shown in Figure 5A. Upon overexpression of Dis3p, the ladders of 5.8S rRNA intermediates were shifted toward the shorter species (Figure 5A, a), while all 5.8S rRNA intermediates were removed by overexpression of Gsp1p. In the case of *gsp1-640*, all 5.8S rRNA intermediates disappeared upon overexpression of Dis3p (Figure 5A, b). The same change occurred upon overexpression of Rrp4p, although Dis3p and Rrp4p did not rescue the ts growth phenotype of *gsp1* (data not shown).

A putative ATP-dependent RNA helicase, Mtr4p/Dob1p, functions as a cofactor for the exosome (DE LA CRUZ *et al.* 1998). When the *MTR4* gene was overexpressed in *gsp1-640*, growth was still temperature sensitive, but a 5.8S rRNA intermediate disappeared (Figure 5A, c). Moreover, when the *mtr4-1* mutation (LIANG *et al.* 1996) was introduced into *dis3*, strains carrying some alleles of *dis3* did not grow even at 26° (Figure 5B). Thus, there is an allele-specific synthetic lethality between *mtr4-1* and *dis3*. Taken together with the previous report that a strain (*dob1-1 rrp4-1*) grows poorly (DE LA CRUZ *et al.* 1998), these results indicate a functional interaction between Dob1p/Mtr4p and the exosome. Indeed, Mtr4p was reported to be required for the nuclear exosome, which carries out 5.8S rRNA maturation, but not for the cytoplasmic exosome, which performs 3′-to-5′ mRNA degradation (VAN HOOF *et al.* 2000). Accordingly, overexpression of Ski2p, another putative ATP-dependent RNA helicase that is required for mRNA degradation (JACOBS ANDERSON and PARKER 1998), did not eliminate the 5.8S rRNA intermediates (Figure 5A, c).

Nucleocytoplasmic transport mutants show no defect in exosomal activity: Several snoRNPs and ribosomal proteins are involved in rRNA processing (WOOLFORD 1991; LAFONTAINE and TOLLERVEY 1995; MAXWELL and FOURNIR 1995; BURGE *et al.* 1999). Those proteins and RNAs are imported into the nucleolus for ribosome genesis. Therefore, inhibition of Ran-dependent nucleocytoplasmic transport could cause defects of 5.8S rRNA maturation. To address this possibility, 5.8S rRNA maturation was investigated in strains defective in the nucleocytoplasmic transport. We chose four mutants defective in a general nuclear import or export pathway: Δ*mog1*, in which both classic and nonclassic nuclear localization signal-dependent nuclear-protein imports are defective (OKI and NISHIMOTO 1998); *srp1*, which is a ts mutant of the importin-α homologue (YANO *et al.* 1994); *lrc4*, an allele of *crm1* that is required for leucine-rich nuclear export signal-dependent nuclear export (STADE *et al.* 1997); and Δ*yrb2*, which is defective in the Ran-dependent nuclear export (NOGUCHI *et al.* 1999).

After incubation at 37° for 0–8 hr, total RNAs were analyzed by Northern hybridization using oligonucleotide 4. Even after incubation for 8 hr at 37°, no intermediates were seen (data not shown). To confirm that the nucleocytoplasmic transport defects were induced under our experimental conditions, the presence of pre-mRNA was examined in the same RNA extracts by using a *CRY1* probe. As controls for pre-mRNA splicing, we used *prp8-1* (JAMIESON *et al.* 1991) and three alleles of *prp20* (SEKI *et al.* 1996). After incubation at 37° for 8 hr, pre-mRNA, which appeared after 2 hr of incubation at 37° in *prp8-1*, was seen in NOY612 (*srp1*) and ENY9-22 (*lrc4*) strains, as for *prp20* alleles (Figure 6, A and B).

Using the same probe, we then sought to detect pre-

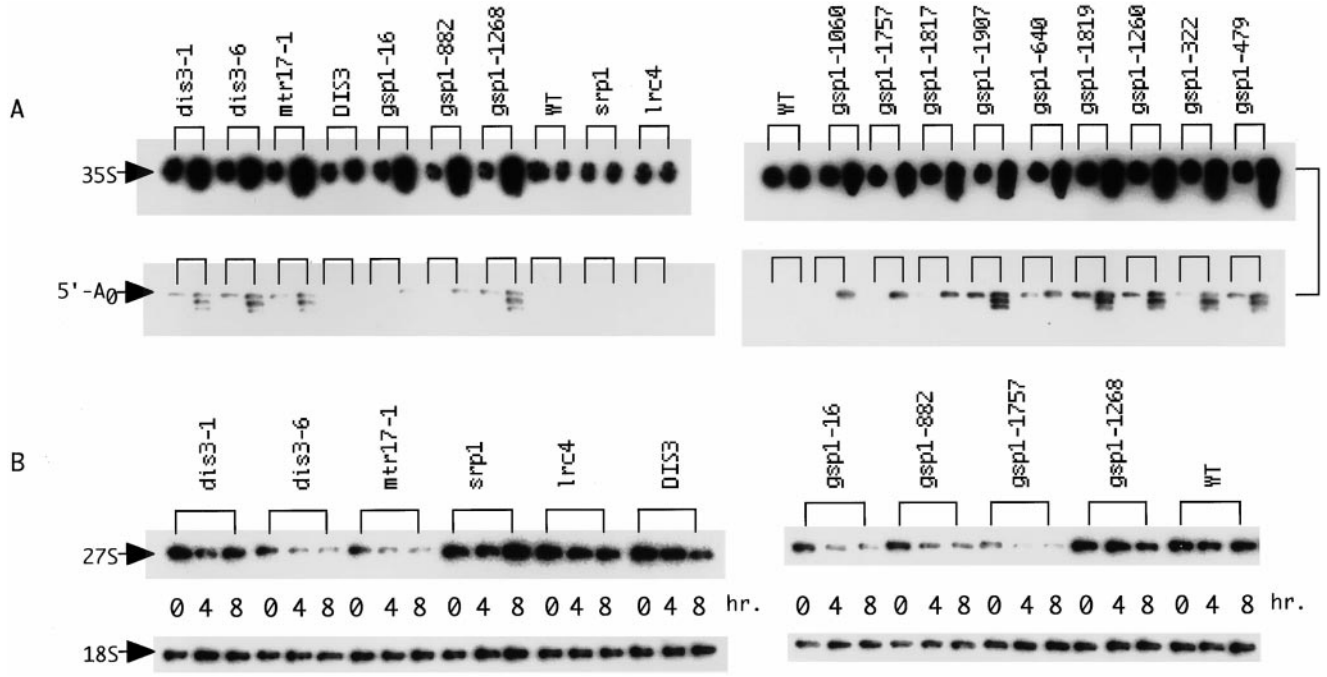


FIGURE 7.—Effects of *dis3* and *gsp1* mutations on steady-state levels of products of rRNA processing. Cultures (50 ml) were grown at 26° to OD_{660} = 0.8, centrifuged, and resuspended in the same volume of prewarmed (37°) YPD medium. After incubation at 37° for 0, 2, 4, and 8 hr, 10 ml of cultures was centrifuged, and total RNA was extracted. Equal amounts of total RNA were resolved on 8.3 M urea-5.0% Long Ranger (A) or 1.5% agarose-formaldehyde gels (B) and transferred to a nylon membrane for Northern hybridization. (A) Hybridization with oligonucleotide probe 1 (Figure 2A, bottom). Each lane contains total RNA extracted from indicated strains. Zero- (left) and 8-hr (right) samples were analyzed. Positions of 35S rRNA and 5′-A0 fragments are shown by arrows. (B) Hybridization with oligonucleotide probe 7 (27S) and 2 (18S). Each contains total RNA extracted from indicated strains. Zero-, 4-, and 8-hr incubation samples were analyzed. Positions of 27S and 18S are shown by arrows.

mRNA in total RNA extracts from *gsp1* and *dis3* strains, to learn whether mRNA splicing occurred in our experimental conditions. No pre-mRNA appeared in more than half of *dis3* and *gsp1* mutant strains that accumulate intermediate 5.8S rRNA fragments upon incubation at 37° (Tables 3 and 4). Only four *dis3* alleles showed inhibition of mRNA splicing, equivalent to *prp8-1* (Figure 6C).

Early steps of rRNA processing are defective in *gsp1*, as in *dis3*: The absence of 5.8S rRNA intermediates does not imply that the exosome functions normally. Using oligonucleotide 1, the position of which is shown at the bottom of Figure 2A, we have therefore evaluated accumulation of the 5′-A0 fragment, which is generated by processing of 35S-to-33S rRNA and is degraded by the exosome (DE LA CRUZ *et al.* 1998). As shown in Figure 7A, the 5′-A0 fragment accumulated in most of *gsp1* and *dis3* mutant strains examined. It is notable that the 5′-A0 fragment accumulated even in those *dis3* and *gsp1* mutants that show no 5.8S rRNA intermediates (Figure 7A, *dis3-6*, *mtr17-1*, and *gsp1-882*, *-1060*, *-1757*, and *-1817*). Thus, all *gsp1* mutants examined are defective in exosomal function. In contrast, the 5′-A0 fragment was not detected in nucleocytoplasmic transport mutants even after 8 hr of incubation at 37° (Figure 7A, *srp1* and *lrc4*).

The steady-state level of 35S pre-rRNA increased in both *dis3* and *gsp1* mutants that accumulated the 5′-A0 fragment (Figure 7A, compare 35S and 5′-A0). This finding may indicate that the pre-rRNA cleavage at the A0 and A1 sites was affected in these mutants, which is consistent with the report that depletion of each of the individual exosome components inhibits the early pre-rRNA cleavage at sites A0, A1, A2, and A3 (ALLMANG *et al.* 2000). In this context, we examined the steady-state level of 27S and 18S rRNA using nucleotide probes 2 and 7. As reported, some *dis3* strains showed a strong reduction of 27S rRNA (Figure 7B, *dis3-6* and *mtr17-1*). Similarly, the level of 27S rRNA was reduced in some alleles of *gsp1* (Figure 7B, right side). In contrast, nucleocytoplasmic transport mutants did not show any change in the level of 27S (Figure 7B, *srp1* and *lrc4*). We did not detect a significant reduction in the level of 18S rRNA.

DISCUSSION

We mutagenized the *DIS3* gene with error-prone PCR. The lethality of the PCR product was >90%. Such a high lethality may be important for efficient isolation of ts mutants. All 12 new *dis3* alleles contain distinct amino acid change(s). Using these mutants, the func-

tional interaction between Dis3p and Gsp1p was investigated.

Almost all *dis3* mutants show a ladder(s) of 3′ extended fragments intermediate in length between 7S and 5.8S rRNAs at 37°, the nonpermissive temperature. This is consistent with the previous report that Dis3p is a subunit of the exosome (Mitchell *et al.* 1997). The lengths of 5.8S rRNA intermediates were grouped into three size classes. Although 5.8S rRNA intermediates were previously reported to appear upon loss of exosome function (Mitchell *et al.* 1996, 1997; de la Cruz *et al.* 1998; Allmang *et al.* 1999), there is no report indicating that 3′ processing can be blocked at three distinct points. According to the model of van Nues *et al.* (1995), the arrest site proximal to the 3′ end of 5.8S rRNA seems to be at helical domain II in the ITS2 region of the 35S pre-rRNA unit, since the shortest intermediate fragment hybridizes with nucleotide 5, but not with nucleotide 6. On the other hand, the 3′ end of the longest intermediate fragment could be near the 3′ end of helical domain IV in the ITS2 region. Pulse-chase experiments revealed that 5.8S rRNA maturation was blocked at these three sites by loss of exosome function.

Since Ran/Gsp1p is required for nucleocytoplasmic transport, 5.8S rRNA maturation could be indirectly inhibited in *gsp1* due to defects in nucleocytoplasmic transport. But it is unlikely for the following reasons. First, the majority of *gsp1* mutants showing intermediate 5.8S rRNA fragments did not accumulate a pre-mRNA, which does accumulate in nucleocytoplasmic transport mutants. Therefore, any nucleocytoplasmic transport is active enough to carry out pre-mRNA splicing in most of *gsp1* strains. Second, the 5′ end of 5.8S rRNA intermediates was correctly terminated in *gsp1*, as in *dis3*. A large number of *trans*-acting factors are required for rRNA processing, which have been characterized as nucleases, ribonucleoprotein particles, putative RNA helicases, and ribosome assembly factors (Allmang *et al.* 2000). The fact that the 5′ end of 5.8S rRNA is correctly terminated therefore indicates that at least some rRNA processing factors function correctly in *gsp1* strains. Finally, in both *dis3* and *gsp1*, 5.8S rRNA maturation was blocked at the same three distinct sites. Such an allele-specific phenotype is consistent with there being a direct interaction between Gsp1p and the exosome. In fact, *S. cerevisiae* Dis3p does bind directly to Gsp1p (Noguchi *et al.* 1996). Dis3p binds to either GTP-Gsp1p or GDP-Gsp1p and enhances RCC1-stimulated nucleotide exchange. Recently, Ran was reported to induce spindle assembly by releasing importin-α from the TPX2 complex (Gruss *et al.* 2001) or importin-β from the NuMA complex (Nachury *et al.* 2001). Ran was already known to stimulate both assembly and disassembly of protein complexes during nucleocytoplasmic transport of macromolecules (Mattaj and Englmeier 1998; Gorlich and Kutay 1999). In this context, Ran/Gsp1p may regulate assembly/disassembly of the exosome, which comprises 11 components (Allmang *et al.* 1999; van Hoof and Parker 1999). In this case, overexpression of Dis3p may rescue the exosome when the interaction between Dis3p and mutated Gsp1p is weak, and overexpression of Mtr4p may increase exosomal activity, which is weakened by binding to mutated Gsp1p. It is notable that Ski2p, which is an RNA helicase like Mtr4p, did not rescue a defect of 5.8S rRNA processing. Since Ski2p is required for 3′-to-5′ mRNA degradation (Jacobs Anderson and Parker 1998), this finding indicates that a defect in Gsp1p specifically inhibits 5.8S rRNA maturation, which is carried out by the nuclear exosome. Consistently, early steps of rRNA processing, which are carried out by the nuclear exosome (van Nues *et al.* 1995), are also affected in *gsp1* as in *dis3*. Recently, Ski7p, which belongs to the GTPase family, was reported to be required for 3′-to-5′ mRNA degradation (van Hoof *et al.* 2000). In this context, Ran/Gsp1p may substitute for Ski7p in 5.8S rRNA processing.

It is notable that *dis3* strains, which quickly accumulate pre-mRNA, contain amino acid changes in the region from residue 562 to 566 [G562D (*dis3-6*), E565K (*dis3-14*), and V566G (*dis3-12*)]. V566 is conserved from yeast to humans (Shiomi *et al.* 1998). These residues may be required for a function of Dis3p in pre-mRNA splicing, although it is not known how the exosome may function in mRNA splicing. Alternatively, Dis3p could function in pre-mRNA splicing by virtue of its being part of distinct complexes other than the exosome.

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