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The Transcription Elongation Factor Spt5 Influences Transcription by RNA Polymerase I Positively and Negatively*[S]

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Spt5p is a universally conserved transcription factor that plays multiple roles in eukaryotic transcription elongation. Spt5p forms a heterodimer with Spt4p and collaborates with other transcription factors to pause or promote RNA polymerase II transcription elongation. We have shown previously that Spt4p and Spt5p also influence synthesis of ribosomal RNA by RNA polymerase (Pol) I; however, previous studies only characterized defects in Pol I transcription induced by deletion of *SPT4*. Here we describe two new, partially active mutations in *SPT5* and use these mutant strains to characterize the effect of Spt5p on Pol I transcription. Genetic interactions between *spt5* and *rpa49*Δ mutations together with measurements of ribosomal RNA synthesis rates, rDNA copy number, and Pol I occupancy of the rDNA demonstrate that Spt5p plays both positive and negative roles in transcription by Pol I. Electron microscopic analysis of mutant and WT strains confirms these observations and supports the model that Spt4/5 may contribute to pausing of RNA polymerase I early during transcription elongation but promotes transcription elongation downstream of the pause(s). These findings bolster the model that Spt5p and related homologues serve diverse critical roles in the control of transcription.

Ribosome synthesis involves all three eukaryotic RNA polymerases and consumes the majority of cellular resources during periods of rapid growth and proliferation (1). Synthesis of ribosomal RNA (rRNA)[3] by RNA polymerase (Pol) I is the first step in this complex biosynthetic pathway. As such, transcription of rRNA is a critical point for regulation of this process (for review see Ref. 2). Thus, detailed characterization of the cellular mechanisms that control and optimize ribosome synthesis is essential to more fully understand or control cell proliferation.

Synthesis of rRNA is regulated at multiple steps. Transcription by Pol I is controlled at the initiation step (3–7), by alteration of the fraction of actively transcribed rDNA repeats (8, 9), or at the elongation step (10, 11). The transcription initiation step is the best characterized target for regulation. The activity of transcription initiation factors Rrn3 (TIF-1A in mouse) (3, 5, 6) and SL1 (12, 13) is modified in response to demands for protein synthesis. Several studies have validated these factors as targets for the control of Pol I transcription initiation; however, recent data demonstrate that other steps in transcription by Pol I are also targets of regulation (10, 11).

In eukaryotic cells, approximately half of the ribosomal DNA repeats is transcriptionally active, whereas the other half is epigenetically silent (14). It has been shown in yeast and in mammalian cells that this ratio of active to inactive repeats can change in response to growth conditions (8, 9). However, this mechanism of regulation is not essential for proper control of rRNA output (3, 15–17). Indeed, recent findings suggest that the role of the inactive rDNA copies may be in genome stability rather than for transcriptional control of ribosome synthesis (18).

Until recently, transcription elongation by Pol I was not heavily investigated. However, studies in mammalian and yeast models have now shown that multiple factors influence the elongation phase of transcription by Pol I and that proper control of this step in transcription is critical both for the regulation of rRNA synthesis and the efficiency of rRNA processing (10, 19–22). Thus, there is a need to understand the mechanism of transcription elongation by Pol I and the factors that affect it.

Spt4p and Spt5p form a heterodimer (for simplicity referred to as Spt4/5 here) that influences transcription elongation (23, 24). In fact, Spt5p is the only known transcription factor conserved throughout all kingdoms of life (25). *SPT4* and *SPT5* were originally identified for their ability to suppress defects in transcription induced by retrotransposition of Ty1 elements in yeast (26). It was later shown in both yeast and mammalian cells that the Spt4/5 complex (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity inducing factor in human) acts primarily as a transcription elongation factor (23, 27).

A series of biochemical and genetic studies have discovered multiple distinct roles for Spt4/5 in RNA polymerase II transcription. It was originally shown in *Drosophila* that Spt4/5 (together with the negative elongation factor) is required to induce a promoter-proximal pause on the *hsp70* gene (28). Covalent modification of Spt5p by P-TEFb (Bur1p/Bur2p and Ctk1p complex in yeast) promotes pause site clearance (29). These data together with the observation that mRNA capping enzymes functionally interact with Spt4/5 and the negative

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[3] The abbreviations used are: rRNA, ribosomal RNA; Pol, polymerase.

TABLE 1
Strains used in this study

Strain	Description	Reference
NOY396	*MATα ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100*	11
NOY886	Same as NOY396, except *rpa135Δ::LEU2 fob1Δ::HIS3* pNOY117 [*CEN, RPA135, TRP1*] rDNA copy number = ~40	16
NOY1051	Same as NOY886, except rDNA copy number = ~140	16
NOY1064	Same as NOY396, except *MATa fob1Δ::HIS3* rDNA copy number = ~190	51
NOY1071	Same as NOY1064, except rDNA copy number = ~25	51
NOY2167	*MATa ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100 spt4Δ::HIS3mx6*	20
DAS50	*MATa ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100 rpa49Δ::LEU2*	11
DAS208	Same as NOY396, except *rrp6Δ::HIS3*	This study
DAS540	Same as NOY396, except *spt5(C292R)*	This study
DAS541	Same as NOY396, except *spt5(S324P, E427K)*	This study
DAS570	Same as NOY396, except *spt5(C292R)-(HA)$_3$-his$_7$::URA3*	This study
DAS573	*MAT? ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100 rpa49Δ::LEU2 spt5(C292R)*	This study
DAS574	*MAT? ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100 rpa49Δ::LEU2 spt5(S324P, E427K)*	This study
DAS576	Same as DAS50, except *MATα*	This study
DAS577	*MAT?* haploid segregant of DAS578 *rpa49Δ::LEU2 SPT5*	This study
DAS578	*MATa/α* diploid resulting from cross of DAS50 × DAS540	This study
DAS579	*MATa/α* diploid resulting from cross of DAS50 × DAS541	This study
DAS581	*MATa/α* diploid resulting from cross of DAS576 × NOY2167	This study
DAS604	*MAT? ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100 spt5(C292R)-(HA)$_3$-his$_7$::URA3 rrp6Δ::HIS3*	This study

elongation factor led to the model that this Spt4/5-mediated pause-and-release serves as a quality control checkpoint in mRNA synthesis (30, 31). After clearance of the pause, Spt4/5 remains associated with the transcription elongation complex where it has been shown to enhance Pol II transcription elongation by increasing the processivity and/or elongation rate of the complex as well as by recruiting other transcription elongation factors (23, 27, 32–35). The roles for Spt4/5 in Pol II transcription elongation are robust.

We have shown previously that Spt4/5 can associate with Pol I in addition to Pol II and that deletion of *SPT4* leads to a small net increase in the synthesis rate of rRNA per transcribing Pol I enzyme in the *spt4*Δ strain relative to WT (20). Thus, we proposed that Spt4p (and by connection Spt5p) acted to inhibit Pol I transcription elongation rate. This was the first evidence that Spt4/5 could inhibit Pol I transcription in any eukaryotic cell. An additional finding was that deletion of *SPT4* resulted in slowed rRNA processing. Here we have extended our study of the role(s) for Spt4/5 in Pol I transcription by characterizing newly isolated, partially active mutants of *SPT5*. Genetic studies support the previous model that Spt4/5 can inhibit Pol I transcription *in vivo*. However, rRNA synthesis rate, Pol I occupancy, and electron microscopic analyses suggest that wild-type Spt5p also plays one or more positive roles in Pol I transcription. These findings are consistent with working models for the role of Spt5p in Pol II transcription and further demonstrate that Spt4/5 is a critical regulator of gene expression in eukaryotic cells.

EXPERIMENTAL PROCEDURES

Strains and Media—Strains used in this study are described in Table 1. The cells were grown in YEPD unless indicated otherwise (*e.g.* SD−Met; see Fig. 3*A*; for recipes see Ref. 11). Because *spt5* strains are sensitive to high temperature and *rpa49*Δ strains are cold-sensitive, the cells were grown at 27 °C with aeration for all of the experiments unless otherwise noted in the figure legends. Partially active mutations of *SPT5* were generated using error-prone PCR to produce a pool of plasmid-encoded mutations and identified by screening for complementation of an *spt5*Δ::*HIS3mx6* mutation at 23 °C. The mutants that exhibited slower than WT growth were selected for further characterization. All of the mutants characterized exhibited high temperature sensitivity at 37 °C (data not shown). Confirmed mutations were integrated into the *SPT5* locus using standard methods and reconfirmed by sequencing the entire gene. Protein stability/abundance was confirmed by Western blot, by comparison with WT Spt5p (data not shown). Diploid strains were sporulated in liquid sporulation medium (10 g/liter potassium acetate and 5 mg/liter zinc chloride) plus required nutrients for 5 days with mild aeration at room temperature. Tetrads were dissected using a Zeiss Axioskop 40 tetrad micromanipulator.

Metabolic Labeling of rRNA—The cells were grown in SD−Met medium to an A_{600} = ~0.3. Five ml of culture was then pulse-labeled with 25 μCi/ml [methyl-^{3}H]methionine (PerkinElmer Life Sciences) for 5 min and then treated with excess cold methionine (500 μg/ml) for an additional 5 min. RNA was then extracted and analyzed as described previously (11).

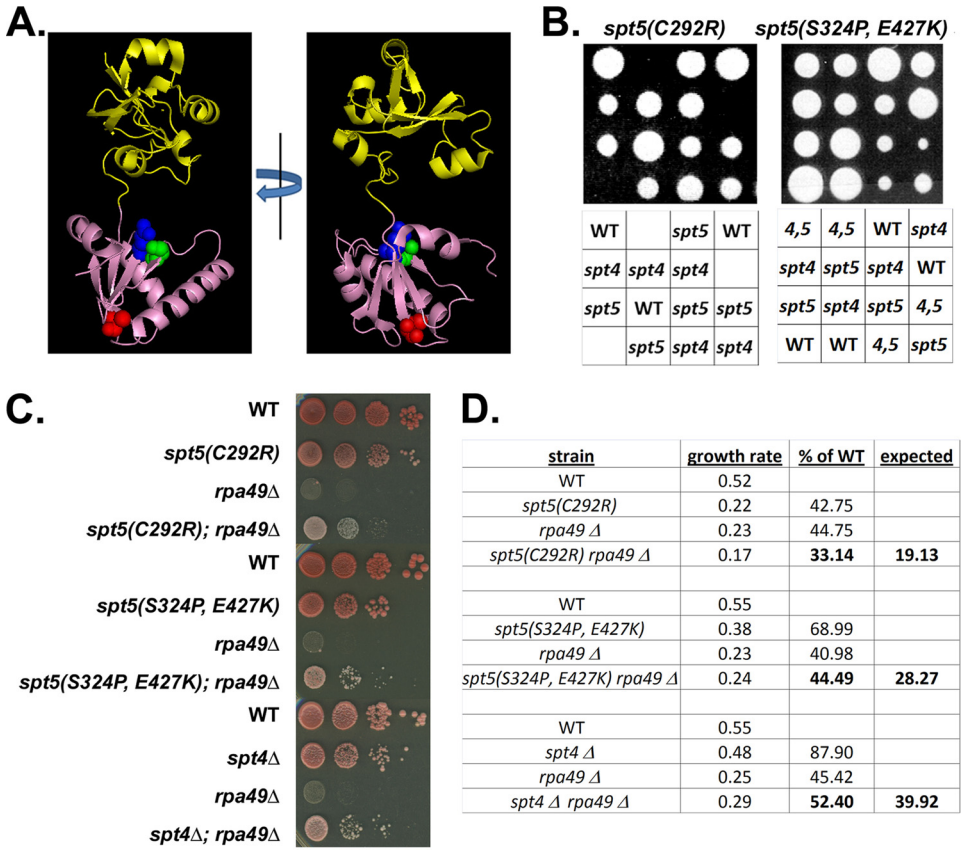
Southern Blot Hybridization—Preparation of DNA for contour-clamped homogeneous field electrophoresis, electrophoresis conditions, capillary transfer, Southern blot hybridization, and quantification were performed as described previously (22).

Chromatin Immunoprecipitation—ChIP was performed exactly as described previously using a rabbit polyclonal antibody against the A190 subunit of Pol I (22). Sequences of primers used for real time PCR are provided in the supplemental text.

EM Analysis of Miller Chromatin Spreads—EM analysis was performed essentially as described previously (16), except the cells were grown at 27 °C. Chromatin spreads from multiple cell cultures of both strains were examined. For quantitative analysis, multiple entire EM grids were scanned. All of the rRNA genes visualized were photographed, and all of the genes that could be unambiguously followed from 5′ to 3′ end were analyzed for number of polymerases/gene and for the relative polymerase density at the 5′ and 3′ ends as described in Fig. 5.

RESULTS

Characterization of Partially Active Mutations in SPT5—*SPT4* is not essential for yeast viability, whereas *SPT5* is. Thus,



D.

strain	growth rate	% of WT	expected
WT	0.52		
spt5(C292R)	0.22	42.75	
rpa49 Δ	0.23	44.75	
spt5(C292R) rpa49 Δ	0.17	**33.14**	**19.13**
WT	0.55		
spt5(S324P, E427K)	0.38	68.99	
rpa49 Δ	0.23	40.98	
spt5(S324P, E427K) rpa49 Δ	0.24	**44.49**	**28.27**
WT	0.55		
spt4 Δ	0.48	87.90	
rpa49 Δ	0.25	45.42	
spt4 Δ rpa49 Δ	0.29	**52.40**	**39.92**

FIGURE 1. **Isolated mutations in *SPT5* map to different faces of the protein and suppress cold sensitivity of *rpa49*Δ strains.** *A*, ribbon diagram adapted from the published structure of Spt4p-Spt5 (NusG-like domain) fusion protein (Protein Data Bank code 2EXU) (36). Spt4p is colored *yellow*, and the NusG-like domain of Spt5p is *pink*. Residues in Spt5p implicated in binding Spt4p, Glu-338 and Ser-324, are shown in spacefill and colored *blue* and *green*, respectively. Cys-292 is colored *red* (also in spacefill) and is positioned on the opposite face of the domain. *B*, segregants resulting from dissection of tetrads of *spt4 spt5* heterozygous diploids (constructed by mating NOY2167 to DAS540&541) are shown with the relevant genotypes of individual haploid strains indicated. The *spt4 spt5* double mutants are indicated by *4,5*. The plates were incubated 5 days at 27 °C. No viable *spt4*Δ *spt5*(*C292R*) double mutants were recovered. *C*, 10-fold dilutions of individual haploid segregants resulting from sporulation of DAS578, DAS579, and DAS581 were spotted onto YEPD plates and grown at 23 °C for 5 days before imaging. *D*, haploid segregants shown in *C* were grown in YEPD liquid culture at 27 °C with aeration and growth rates (doublings per hour) were calculated. The "expected" growth rate is the product of the growth rates of the parental haploid mutants as a percentage of the WT growth rate.

to characterize potential additional roles for Spt5p in Pol I transcription, we isolated partially active mutants of *SPT5*. Using standard genetic approaches and PCR-mediated mutagenesis of *SPT5*, we identified several independent mutations in *SPT5* that resulted in slower than WT growth rates (Fig. 1 and data not shown). The cells used for the screen were grown at 23 °C; however, all of the mutants were scored for growth at higher temperatures. Most candidates exhibited poor growth at 30 °C and no growth at 37 °C. Two of these mutations were integrated into the chromosomal *SPT5* locus and sequenced.

One of the mutants had a substitution of a serine at position 324 for a proline (Fig. 1*A*, *green spacefill*). This residue lies within the conserved NusG-like domain of Spt5p and was shown previously to be required for binding Spt4p (36). It was shown previously that a mutation of serine 324 to phenylalanine was sufficient to induce temperature-sensitive growth, consistent with its role in interaction with Spt4p (36). Thus, it is likely that the proline at this position also resulted in poor growth (despite the presence of an additional point mutation in the 3′ end of the gene; Fig. 1). The other mutant carried a single point mutation leading to an arginine substitution for cysteine at position 292 (*spt5*(*C292R*); Fig. 1*A* shown in *red*). This residue also lies within the NusG-like domain of Spt5p but was not previously implicated in binding Spt4p. Indeed, Cys-292 is positioned on the opposite face of the domain from the residues involved in association with Spt4p. Furthermore, the growth rate of the *spt5*(*C292R*) strain was ~2.5-fold slower than WT at 27 °C; much slower than either the *spt4*Δ strain or the *spt5*(*S324P*) mutant strain (Fig. 1*D*). Thus, we predicted that the *spt5*(*C292R*) mutation yielded a protein impaired for functions that do not require Spt4p.

To confirm that the *spt5*(*C292R*) strain had defects in addition to those induced by deletion of *SPT4*, we mated the individual *spt5* strains to a strain carrying a deletion of *SPT4*. When we sporulated the resulting diploids and scored the cells for growth at 23 °C, we found that the *spt5*(*C292R*) mutation was lethal in combination with *spt4*Δ, whereas the *spt5*(*S324P*) *spt4*Δ double mutant was viable (Fig. 1*B*). These data suggest, as predicted from structural and phenotypic data (36), that the S324P mutant form of Spt5p is defective in its ability to bind Spt4p, whereas the *spt5*(*C292R*) mutation affects Spt4p-independent functions of Spt5p.

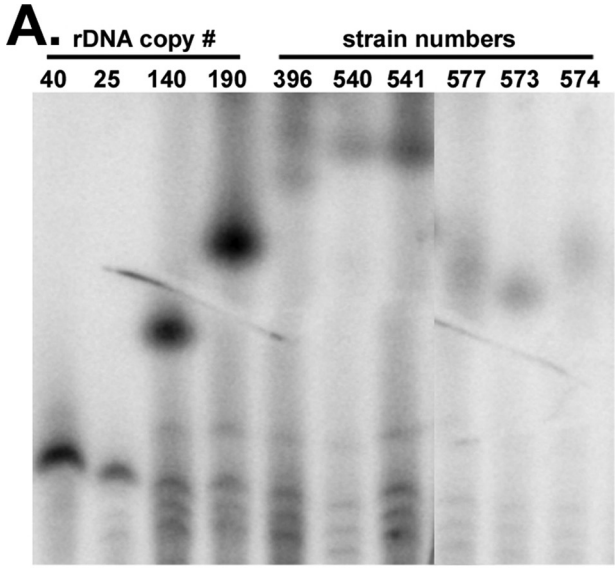
Previous data demonstrated that deletion of *SPT4* leads to a small increase in the net transcription elongation rate of Pol I (20). Thus, Spt4 can inhibit Pol I transcription. To test whether wild-type Spt5p also influences transcription by Pol I nega-

tively, we mated each of the *spt5* strains as well as an *spt4*Δ strain to a strain carrying a deletion of *RPA49*. The *RPA49* gene encodes the A49 subunit of Pol I. The A49 subunit serves as an intrinsic transcription elongation factor for Pol I (37), but it is not essential for survival. If a candidate mutation interacts genetically with the *rpa49*Δ mutation, this interaction supports the conclusion that the candidate factor participates (directly or indirectly) in transcription elongation by Pol I. *rpa49*Δ strains are cold-sensitive, exhibiting almost undetectable growth at 23 °C (Fig. 1*C*). We grew haploid segregants representing all four genetic possibilities resulting from sporulation of *spt rpa49*Δ heterozygous diploids at 23 °C to test for phenotypic suppression of the *rpa49*Δ mutation. In each case, we observed robust growth of the *spt rpa49*Δ double mutant strain, whereas the *rpa49*Δ haploid segregants were not viable (Fig. 1*C*). Thus, we conclude that mutations in *SPT4* or *SPT5* at least partially suppress the cold sensitivity of the *rpa49*Δ strain. These data support the previously proposed model that Spt4/5 plays a negative role in transcription elongation by Pol I.

To quantify the suppression of the *rpa49*Δ growth defect, we measured the exponential growth rate of individual haploid segregants. The cells were grown in YEPD at 27 °C, because *rpa49*Δ mutants are cold-sensitive and *spt* mutants are highly temperature-sensitive. Consistent with the spot test, the observed growth of the *spt rpa49*Δ double mutants was better than expected based on the growth rates of parental single mutant strains (Fig. 1*D*). We note, however, that suppression of the *rpa49*Δ growth defect was not complete at this temperature, most likely because of the opposing temperature sensitivities of the candidate mutations. These data, together with previous results using *spt4*Δ mutants (20), confirm that in wild-type cells, Spt4p and Spt5p play one or more negative roles in Pol I transcription elongation.

Mutations in SPT5 Do Not Reduce rDNA Copy Number—It was shown previously that deletion of *SPT4* reduced the rDNA copy number by ~3-fold (20). To determine whether mutation of *SPT5* similarly affected the rDNA, we measured the size of chromosome XII using contour-clamped homogeneous field electrophoresis gels. After the chromosomes were separated in the gel, we transferred the DNA to a membrane and detected chromosome XII by Southern blot, using an rDNA probe (Fig. 2*A*). We compared the size of chromosome XII in WT and *spt5* mutant cells to reference strains with known rDNA copy numbers. We plotted the migration distance of chromosome XII in the reference strains *versus* the rDNA copy number to generate a linear regression (Fig. 2*B*). From that regression, we estimated the rDNA copy number for the strains included in this study (Fig. 2*C*). We conclude that there is no reduction in the rDNA copy number in either of the *spt5* mutant strains compared with WT. Furthermore, the *rpa49*Δ strain exhibits a reduction in the rDNA copy number relative to WT, but strains carrying *rpa49*Δ and *spt5* mutations do not rescue that reduction. Thus, the mechanism by which *spt5* mutations suppress the *rpa49*Δ phenotype is not mediated by changes in the rDNA array size.

Effects of spt5(C292R) on Pol I Transcription—Our genetic data and growth phenotypes, as well as published data from the Hartzog lab (36), suggest that mutation of the serine at position 324 in Spt5p impairs association with Spt4p. The objective of this study is to characterize Spt5p-specific effects on Pol I transcription; thus, we focused our studies on the *spt5*(*C292R*) strain. This strain grows at ~40% of the WT rate (Fig. 1*D*). To measure rRNA synthesis rates, we isolated RNA after a 5-min pulse/chase with [methyl-^{3}H]methionine from WT and



strain	copy #
NOY396 = WT	256
DAS540 = *spt5(C292R)*	262
DAS541 = *spt5(S324P E427K)*	262
DAS577 = *rpa49* Δ	180
DAS573 = *rpa49* Δ *spt5(C292R)*	154
DAS574 = *rpa49* Δ *spt5(S324P E427K)*	185

FIGURE 2. **rDNA copy number is not reduced by mutation of *SPT5*.** *A*, chromosomes from strains indicated (grown in YEPD at 27 °C) were separated by contour-clamped homogeneous field electrophoresis and transferred to a nylon membrane. Southern blot hybridization using an rDNA probe permitted detection of chromosome XII in the upper part of the blot. Control strains with known rDNA copy number (locked by deletion of *FOB1*) were included (*left four lanes*). The image was processed to delete lanes between the *rightmost three lanes* and the remainder of the gel. Contrast and position were not altered. *B*, rDNA copy number from control strains was plotted as a function of migration distance of chromosome XII. A linear regression was generated from these data, and the equation from the regression is shown. *C*, rDNA copy numbers in WT and mutant strains were estimated according to the equation in *B*.

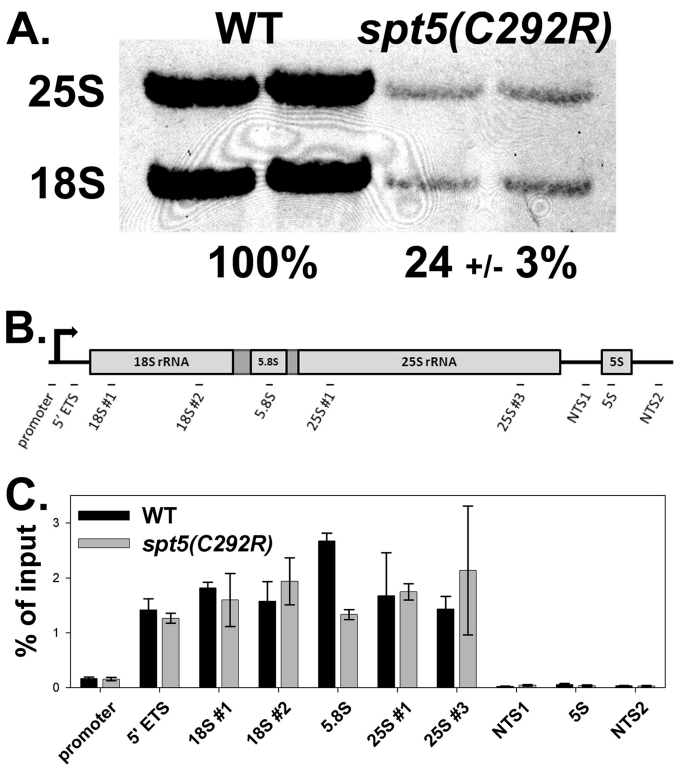


FIGURE 3. **Mutation of *SPT5* reduces rRNA synthesis rate but not Pol I occupancy of rDNA.** *A*, duplicate WT and *spt5*(*C292R*) cultures were grown in SD−Met at 27 °C with aeration to A_{600} = ~0.3. The cells were pulse-labeled for 5 min with 25 μCi/ml [methyl-^{3}H]methionine and chased for 5 min with excess cold methionine (500 μg/ml). Isolated RNA from same number of cell equivalents (normalized to final A_{600} of culture) was subject to electrophoresis in a 1% formaldehyde:agarose gel, transferred to a nylon membrane, and visualized by autoradiography. The film was developed after 24 h of exposure. 25 and 18 S rRNA (and a background band) were excised from the membrane, and ^{3}H incorporation was quantified by a scintillation counter. The counts were averaged and normalized to WT with 1 standard deviation ± shown. *B*, diagram of location of primer pairs used for quantitative PCR analysis of ChIP DNA. *C*, ChIP data demonstrate that Pol I occupancy of rDNA is not reduced in *spt5*(*C292R*)(DAS540) cells compared with WT (NOY396). A polyclonal anti-A190 antibody was used for immunoprecipitation. The data shown are the averages of three DNA dilutions from each of two independent cultures. The *error bars* represent one standard deviation.

spt5(*C292R*) cells. Because rRNA is co-transcriptionally methylated, this experiment is a reliable method for calculating the steady state rRNA synthesis rate (38). We found that rRNA synthesis was reduced ~4-fold in the *spt5*(*C292R*) strain compared with WT (Fig. 3*A*; see supplemental methods for detailed discussion of isotopic labeling). Given the obvious growth defect of the *spt5*(*C292R*) strain, we anticipated a reduction in the rRNA synthesis rate. However, reduced rRNA synthesis would not be expected if Spt5p plays only negative roles in Pol I transcription in WT cells; thus, these data suggest that WT Spt5p positively influences Pol I transcription directly or indirectly.

If mutation of *SPT5* leads to indirect effects on Pol I transcription initiation but not on transcription elongation (*e.g.* through altered expression of essential factors by Pol II), occupancy of the rDNA by Pol I would be reduced in the *spt5*(*C292R*) strain. We performed ChIP experiments using a polyclonal antibody that binds the largest subunit of Pol I (A190). We examined Pol I occupancy at 10 different positions of the rDNA repeat (Fig. 3*B*) and found approximately equal Pol I occupancy of the rDNA in the WT and *spt5*(*C292R*) strains (Fig. 3*C*). Thus, the rRNA synthesis rate is reduced in *spt5*(*C292R*) cells despite equal Pol I loading on the rDNA compared with WT. The simplest interpretation of these data is that WT Spt5p increases Pol I transcription elongation rate, in addition to its previously detected inhibitory roles (Fig. 1 and Ref. 20).

It is possible that mutation of *SPT5* leads to overproduction of rRNA (>4-fold) and degradation of the excess RNA by the nuclear exosome. To test this hypothesis, we repeated our pulse labeling in WT, *rrp6*Δ, *spt5*(*C292R*), and *spt5*(*C292R*) *rrp6*Δ double mutants (Fig. 4). Rrp6p is the nuclear exosome subunit that is not essential for growth but is required for efficient degradation of defective or excessive stable RNA species (39, 40). We observed a small increase in the rRNA synthesis rate in the *rrp6 spt5*(*C292R*) double mutant compared with the *spt5*(*C292R*) mutant alone (Fig. 4, *lane 8 versus lane 6*), suggesting that some degradation of rRNA does occur in the *spt5*(*C292R*) mutant. However, deletion of *RRP6* in the *spt5*(*C292R*) mutant does not lead to accumulation of rRNA at WT levels. These data are consistent with the overall model that Spt5p can influence Pol I transcription elongation both positively and negatively.

Electron Microscopy Supports a Positive Role for Spt5p in Pol I Transcription—Robust transcription of tandemly repeated rDNA by Pol I can be visualized by electron microscopy of Miller chromatin spreads (16). This unique feature of Pol I transcription allowed us to quantify polymerase occupancy of the rDNA and the percentage of genes actively transcribed.

We examined Miller chromatin spreads made from WT and *spt5*(*C292R*) strains grown at 27 °C in rich medium. Representative genes from each of these strains are shown in Fig. 5*A*. It is clear that polymerase occupancy of the rDNA was not reduced significantly in the *spt5*(*C292R*) mutant cells despite the ~4-fold reduction in rRNA synthesis rate (Figs. 3*A* and 4). We detected 43 Pol I complexes/gene on average in the mutant cells compared with 50/gene in WT (Fig. 5*B*). These data are consistent with the ChIP data described above (Fig. 3*C*) and support the model that a reduction in transcription initiation rate alone cannot account for the reduced rRNA synthesis rate observed in the *spt5*(*C292R*) strain.

In WT yeast cells, approximately half of the rDNA repeats are maintained in an epigenetically silent state. From EM analyses, we can trace the rDNA and quantify the percentage of actively transcribed genes. For an rDNA repeat to be scored as "inactive," it must be on the same chromatin strand as at least one actively transcribed repeat; thus, our analysis has a slight tendency to overestimate the percentage of active genes. However, because this bias is true for all of the spreads analyzed, this measure is suitable for comparing the percentage of genes transcribed between strains. When we measured this value in the *spt5*(*C292R*) cells relative to WT, we found that 85% of the rDNA repeats were active relative to 70% in WT cells (Fig. 5*B*). From this percentage, together with the average number of polymerases per gene and the rDNA copy number (Fig. 2), we calculate that there are approximately equal numbers of polymerases engaged in transcription in the WT and *spt5* strains

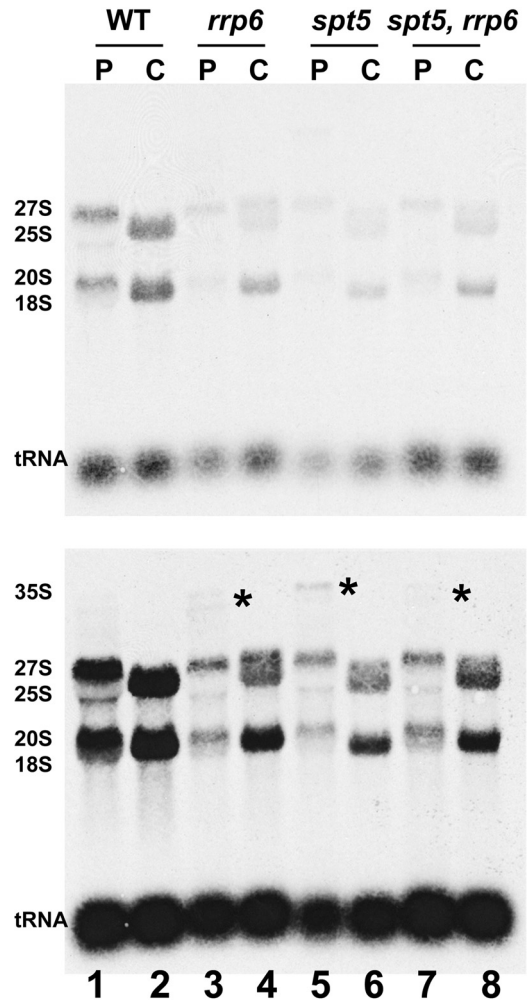


FIGURE 4. **rRNA is not overproduced in *rrp6*Δ *spt5*(*C292R*) double mutants.** NOY396, DAS208, DAS570, and DAS604 were grown and labeled as described for Fig. 3, except that cells were harvested after a 4-min pulse (without chase) and after a 5-min pulse and a 5-min chase with cold methionine. *P* indicates pulse samples, and *C* indicates pulse-chase samples. RNA was loaded for equal A_{600} of the culture. Precursor and mature RNA species in the gel are labeled. The *upper panel* is a 24-h exposure of the film, and the *lower panel* is a 4-day exposure of film with the same blot.

(Fig. 5*B*). Thus, there is a ~4-fold reduction in the rate of rRNA synthesis per Pol I complex in the mutant strain. Taken together, these data demonstrate that WT Spt5p plays a positive role in transcription elongation by Pol I. Because a positive role was not seen for Spt4p when identical experimental approaches were used to characterize rRNA synthesis in *spt4*Δ strains (20), we conclude that the positive effect of Spt4/5 on Pol I transcription elongation does not require Spt4p.

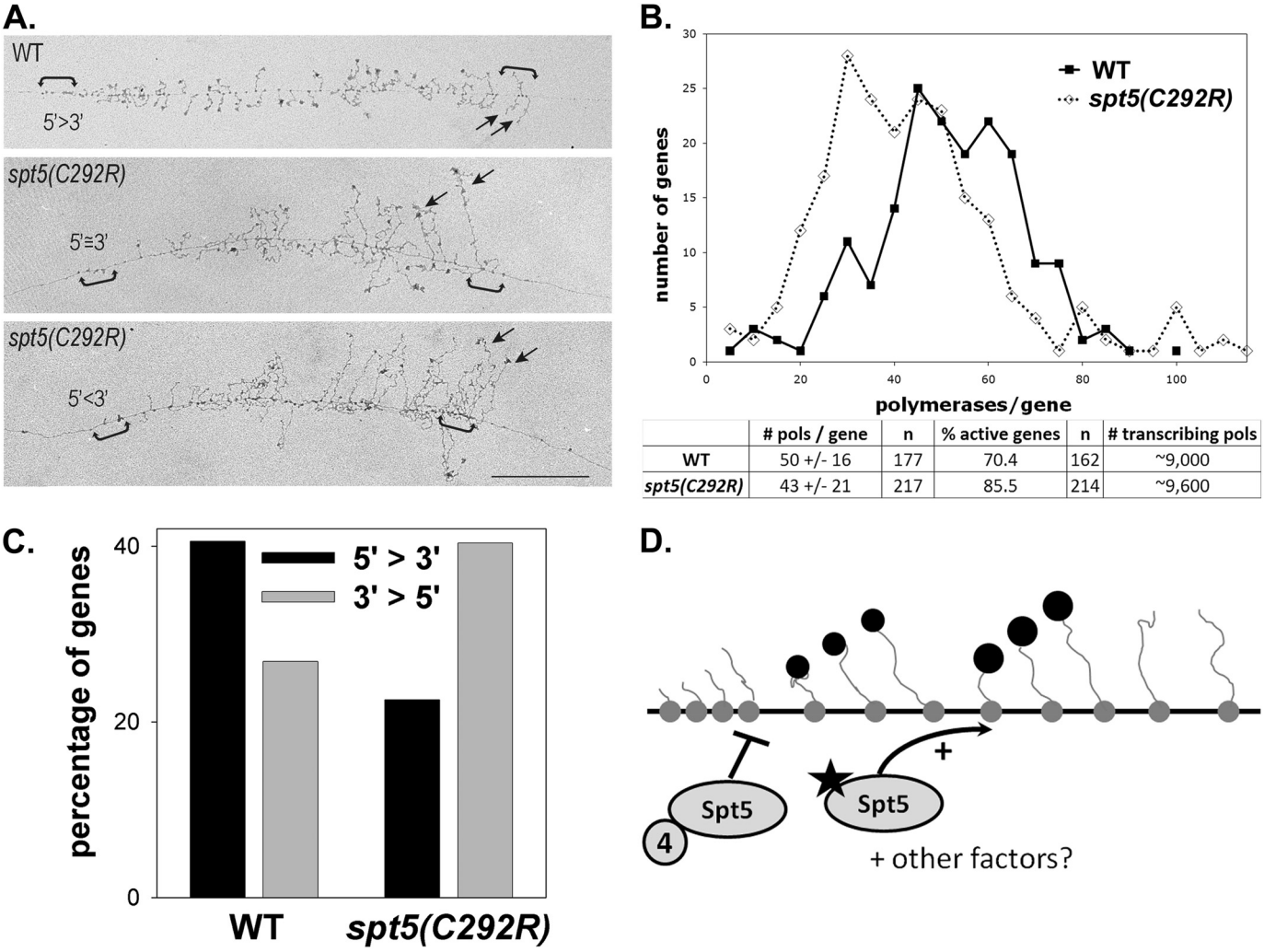
Spt5p Influences Co-transcriptional Processing of rRNA—In viewing the representative genes in Fig. 5*A*, the most obvious difference between WT and *spt5*(*C292R*) is the length of the transcripts at the 3′ ends of the genes (*arrows*). It has been shown that most nascent rRNA molecules in yeast undergo co-transcriptional cleavage (41, 42), which separates pre-rRNA into precursors for small and large ribosomal subunits (20 and 27 S RNA species, respectively). The short transcripts seen at the 3′ end of the WT gene in Fig. 5*A* are evidence that this cleavage has occurred (42). However, this was not the case in the *spt5* mutant. Rather, we detected robust accumulation of long unprocessed nascent RNAs in the 3′ end of active rRNA genes in the *spt5*(*C292R*) spreads (Fig. 5*A*). Consistent with this observation, we observed accumulation of uncleaved 35S pre-rRNA in the *spt5*(*C292R*) mutant after pulse labeling with [methyl-^{3}H]methionine (Fig. 4, *lower panel*, *asterisks*).

Previous studies that linked impaired transcription elongation by Pol I with inefficient co-transcriptional cleavage led to a model in which early steps in rRNA processing and transcription elongation by Pol I are functionally coupled in optimal growth conditions (20, 21). The observation of impaired co-transcriptional cleavage in the *spt5*(*C292R*) mutant, similar to that seen in *spt4*Δ cells (20), lends additional support to this model. We note, however, that the overall effect on rRNA processing (as assessed by comparing precursor rRNA abundance to mature rRNAs in pulse lanes; Fig. 4) is modest. Thus, the observed effect of the *spt5*(*C292R*) mutation on rRNA synthesis rate is not an indirect consequence of a large impairment of rRNA processing.

Spt5p Influences the Distribution of Pol I on the rDNA—Previous work in higher eukaryotes has shown that Spt5p participates in the establishment of a promoter-proximal pause in elongating Pol II complexes (28, 30, 31, 43). To determine whether Spt5p may play a similar role at the rDNA in yeast, we compared the polymerase density in the 5′ end *versus* the 3′ end of individual rDNA genes in WT and *spt5*(*C292R*) strains because emerging evidence indicates a higher density of Pol I in the 5′ region of rDNA genes as compared with downstream regions (Ref. 49 and data not shown). Our EM data showed that more genes in WT cells had a higher density of Pol I complexes in the 5′ 10% of the transcribed region than in the 3′ 10% (Fig. 5*C*), supporting the model that a transient kinetic block to transcription elongation may occur early in transcription elongation. However, in the *spt5*(*C292R*) cells, most genes no longer showed a typical high polymerase density at the 5′ end, resulting in many genes with a greater number of polymerases occupying the 3′ end of the gene than the 5′ end. For this analysis, ~200 genes were mapped for both WT and mutant strains; the fraction of genes in the different categories varied significantly between WT and *spt5*(*C292R*) cells (chi-squared test, $p < 0.001$). This observation supports a potential role for Spt5p in establishment of a short-lived, 5′ pause event in Pol I transcription elongation complexes.

DISCUSSION

New Model for the Roles of Spt5p in Pol I Transcription—Spt4/5 plays multiple important roles in Pol II transcription elongation. Our data suggest that Spt4/5 also has complex roles in Pol I transcription (Fig. 5*D*). Because *spt4* and *spt5* mutations suppress growth defects induced by deletion of *RPA49*, we conclude that WT Spt4/5 can inhibit Pol I transcription elongation. This conclusion is additionally supported by previous EM and rRNA synthesis rate experiments (20). However, the observation that rRNA synthesis is reduced in the *spt5*(*C292R*) mutant despite approximately equal polymerase occupancy of the rDNA indicates that Spt5p can also positively influence Pol I transcription. Thus, Spt5p plays dual roles in Pol I transcription elongation.



	# pols / gene	n	% active genes	n	# transcribing pols
WT	50 +/- 16	177	70.4	162	~9,000
spt5(C292R)	43 +/- 21	217	85.5	214	~9,600

FIGURE 5. **EM analysis of rRNA gene transcription supports a role for Spt4/5 in pause and release of Pol I transcription elongation.** *A*, representative rDNA repeats from WT (NOY396) and *spt5*(*C292R*) (DAS540) analyzed by EM of Miller chromatin spreads are shown. The 5′ end of each gene is oriented to the *left*. The *straight arrows* indicate individual transcripts near the 3′ end of the genes that are characteristic of the transcript processing status for that strain (cleaved at A2 for WT and uncleaved for *spt5*(*C292R*)) (41, 42). *Bracketed arrows* at the 5′ and 3′ ends of each gene indicate gene regions quantified for polymerase density for *C*. *Scale bar*, 0.5 μm. *B*, the frequency of detection of polymerase density was plotted as a function of the number of polymerases per gene for WT and *spt5*(*C292R*) spreads. The data were averaged with errors indicated (n = number of active genes analyzed). Analysis of "on" *versus* "off" rDNA repeats was performed as described previously (22), and the data are shown (n = number of rDNA repeats analyzed). The error in each case equals one standard deviation. By multiplying the rDNA copy number (Fig. 3), the average number of polymerases per gene, and the percentage of active genes, we calculated the approximate number of polymerase engaged in transcription in the WT and *spt5*(*C292R*) strains. *C*, polymerase density in the first and last 10% of each rDNA repeat (as shown by *brackets* in *A*) was quantified. For WT (NOY396) and *spt5*(*C292R*) (DAS540) strains, the frequency at which the density was greater at the 5′ end *versus* the 3′ end on individual genes was plotted as well as the frequency at which the density was lesser at the 5′ end *versus* the 3′ end. *D*, a model for the positive and negative effects of Spt4/5 on Pol I transcription is depicted using an idealized EM view of one rDNA repeat. *Gray circles* on the *straight line* indicate transcribing Pol I on rDNA. Increased polymerase density near 5′ end of the gene indicates a proposed Spt4/5-mediated pause of the transcription elongation complex. One or more modifications of Spt5p (depicted by *star*) led to pause release and enhancement of Pol I transcription elongation rate in the remaining portion of the gene. *Black circles* at the ends of rRNA transcripts represent formation of mature processomes, which are cleaved from nascent transcripts after compaction of the pre-18 S rRNA into the processome (42).

Based on these data and by reference to models for the role of Spt4/5 in Pol II transcription, we propose the relatively simple model that Spt4p and Spt5p are required for early pause events or a general reduction in the Pol I transcription elongation rate (Fig. 5*D*). Then, perhaps after covalent modification of Spt5p (*e.g.* by Bur1/2 or Ctk1), the complex enhances Pol I transcription through the rDNA. Although aspects of this model remain to be tested and refined, it is supported by EM analysis of Pol I distribution on active rDNA repeats (Fig. 5*C*).

Differences between spt4Δ and spt5(C292R)—Many previous genetic studies have employed *spt4*Δ strains as models for "partial impairment" of Spt4/5 function (20, 34, 44). This assumption may be true, but the data presented here suggest that not all of the robust roles for Spt4/5 are impaired by deletion of *SPT4*.

The data observed using the *spt5*(*C292R*) mutant were strikingly different from those observed previously using an *spt4*Δ strain. One obvious difference was observed in the effect of the mutations on the size of the rDNA array. We used standard Southern blots (20) and contour-clamped homogeneous field electrophoresis gels (data not shown) to demonstrate that deletion of *SPT4* reduced the rDNA copy number. No such reduction was observed in these *spt5* mutant strains (Fig. 2). Previous work has shown that *spt4*Δ mutants have a hyperrecombination phenotype (45), and our results suggest that this phenotype

may be specific to the *spt4*Δ mutation. If Spt4p participates in pausing Pol I transcription, perhaps overproduction of rRNA in the *spt4*Δ strain would be sufficient to select for a reduction in the rDNA copy number.

The *spt5*(*C292R*) strain grows ~2.5-fold slower than WT, whereas deletion of *SPT4* only affects growth by ~12% (Fig. 1*D*). Consistent with the defect in growth rate, we observe a large (~4-fold) decrease in Pol I transcription of the rDNA that cannot be accounted for by rRNA degradation or reduced Pol I transcription initiation in the *spt5*(*C292R*) strain. This large difference between results observed using *spt4*Δ and *spt5* mutants supports the model that Spt5p has important cellular functions that it can perform in the absence of Spt4p at the rDNA and likely elsewhere in the cell.

Direct versus Indirect Effects of Spt5p—Spt5p directly or indirectly influences transcription by Pol I both positively and negatively. Because Spt5p directly binds Pol I and Spt5p has affinity for the Pol I transcription initiation factor Rrn3p (20, 52), we propose that Spt5p is recruited to the rDNA during transcription initiation (or early elongation) and that Spt5p directly mediates both pausing and pause-release/activation. However, we cannot exclude more complicated indirect models. For example, Spt4/5 has been shown previously to influence recruitment of the Paf1 complex to Pol II genes. The Hinnebusch lab (44) found that deletion of *SPT4* reduced Paf1C recruitment to the *ARG1* gene, whereas the Hahn lab (33) showed that deletion of the C-terminal domain of Spt5p reduced Paf1C recruitment. We have shown previously that Paf1C enhances Pol I transcription elongation rate *in vivo* and *in vitro* (11, 22). Thus, the *spt5*(*C292R*) mutation could potentially reduce Paf1C recruitment to the rDNA, inhibiting Pol I transcription elongation. However, multiple lines of evidence suggest that this is not a primary mechanism by which WT Spt5p influences Pol I. We and others have shown that mutations in *SPT5* are lethal when combined with *paf1*Δ mutations (Ref. 46 and data not shown). Furthermore, *spt5* mutations suppress phenotypes associated with the *rpa49*Δ mutation (Fig. 1), whereas *paf1*Δ mutations are lethal when combined with *rpa49*Δ mutations (11). Thus, it is unlikely that these factors perform redundant positive functions (at the rDNA or elsewhere). Indeed, we see no significant reduction in Paf1C occupancy of the rDNA in the *spt5*(*C292R*) strain compared with WT (data not shown). Future *in vitro* studies will definitively test whether Spt4/5 can directly increase and/or decrease transcription elongation efficiency of Pol I.

Transcription Elongation versus Initiation—ChIP analysis and EM studies demonstrated that Pol I occupancy of the rDNA is not significantly reduced in *spt5*(*C292R*) cells compared with WT. Thus, defects in transcription initiation alone could not account for the observed 4-fold reduction in rRNA synthesis rate in the mutant cells; hence, we conclude that Spt5p influences transcription elongation by Pol I. However, if it were possible to slow transcription elongation while maintaining normal fast initiation, one would observe an increase in the Pol I occupancy of the rDNA. We did not observe such an effect. Thus, the initiation rate in the *spt5*(*C292R*) cells was reduced by approximately the same magnitude as the effect on the elongation rate. There are at least two potential explanations for this observation. First, Spt5p could affect the efficiency of transcription initiation apart from its roles in elongation. Alternatively, the defects induced by mutation of *SPT5* may also affect promoter escape, which could be rate-limiting for transcription initiation. We favor the latter model, because we did not observe genetic interactions between mutations in Pol I transcription initiation factors (*e.g. rrn3(S213P)*) and mutations in *SPT5* (data not shown and Ref. 52).

Role for Rpa49p in Pol I Transcription Elongation—Genetic and biochemical data suggest that the A49 subunit of Pol I is an intrinsic, positively acting transcription elongation factor (37, 47). Deletion of *RPA49* results in reduced growth rate at 30 °C and cold sensitivity. We observed suppression of the *rpa49*Δ phenotypes when we combined that mutation with *spt4*Δ or *spt5* mutations. Thus, we concluded that Spt4/5 plays at least one negative role in Pol I transcription elongation in WT cells. However, we also observed a ~4-fold reduction in the synthesis rate of rRNA per transcribing Pol I complex in the *spt5*(*C292R*) strain relative to WT, suggesting that in WT cells Spt5p plays a critical positive role in transcription elongation by Pol I. If this model is true, why does this mutation suppress the phenotype of the *rpa49*Δ strain rather than exacerbate it?

Previous evidence demonstrated that disruption of the positively acting Paf1 complex (by deletion of either *PAF1* or *CTR9*) was lethal in combination with *rpa49*Δ (11). Thus, Paf1C apparently enhances a step in transcription elongation that is rate-limiting in the *rpa49*Δ strain. Conversely, the simplest interpretation of the genetic data presented here is that Spt4/5 induces one or more barriers to transcription elongation by Pol I, and deletion of *RPA49* renders those pauses rate-limiting for transcription (especially at low growth temperatures). Mutation of *SPT4* or *SPT5* reduces the efficiency of that pause, ameliorating the phenotype of the *rpa49*Δ mutation. Furthermore, from these genetic data we can conclude that positive roles for Spt5p and A49 in Pol I transcription in WT cells are not redundant. Transcription elongation by Pol I (as for Pol II and bacterial RNA polymerase) is likely nonuniform throughout the gene, confronted with a variety of intrinsic and extrinsic kinetic barriers.

A Role for Spt4/5 in rRNA Processing—Previous studies in *Drosophila* have shown that Spt4/5 participates in the induction of promoter-proximal stalling of RNA polymerase II (28, 31). This stall is thought to function as a checkpoint to ensure proper processing of the 5′ end of the messenger RNA (30, 31, 48). To date, no similar strong pause for Pol II has been detected in yeast cells. Indeed, an important mediator of this pause, the negative elongation factor, is apparently absent from the yeast proteome. Based on these observations, one could conclude that promoter-proximal pausing is not conserved between yeast and higher eukaryotes. Alternatively, this pause/quality control step in Pol II transcription may be kinetically fast in yeast, rendering its detection less likely.

Our EM studies revealed a similar defect in co-transcriptional cleavage of rRNA in both *spt4*Δ and *spt5*(*C292R*) strains (Fig. 5 and Ref. 20). We have confirmed this observation biochemically (Fig. 4 and Ref. 20). Perhaps Spt4/5 plays a role in Pol I transcription similar to its described role in pausing Pol II in higher eukaryotes. It is clear that efficient processing of rRNA is

coupled to transcription elongation by Pol I (21) and that rRNA processing and modification occur co-transcriptionally (41, 42). Thus, we propose that Spt4/5 may also mediate a quality control checkpoint for rRNA processing. Emerging evidence suggests that there are one or more "hot spots" for transcriptional pausing of Pol I within the rDNA (Refs. 22 and 49 and data not shown), and future studies will determine whether Spt4/5 influences the efficiency of pausing at these or other sites.

Conclusions—Spt5p is the only transcription factor that is conserved throughout all of the kingdoms of life. This observation has led to speculation that control of transcription elongation rate may have existed prior to mechanisms that control transcription initiation rates (50). Our data confirm that within eukaryotic cells, Spt5p plays important, apparently conserved functions in at least two of the three nuclear RNA polymerase systems.

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