THE TRANSCRIPTION ELONGATION FACTOR SPT5 INFLUENCES
TRANSCRIPTION BY RNA POLYMERASE I POSITIVELY AND NEGATIVELY

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Abstract

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 (rRNA) by RNA polymerase I (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 rpa49Δ mutations together with measurements of rRNA 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.

Introduction

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) by RNA polymerase I (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 (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 (DSIF 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; NELF) 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 NELF 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 spt4Δ 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. Cells were grown in YEPD unless indicated otherwise [e.g. SD-Met; Figure 3A; for recipes see (11)]. Since spt5 strains are sensitive to high temperature and rpa49Δ strains are cold-sensitive, cells were grown at 27° C with aeration for all experiments unless otherwise noted in the figure legend. 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 spt5Δ::HIS3mx6 mutation at 23° C. 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 re-confirmed by sequencing the entire gene. Protein stability/abundance was confirmed by Western blot, by comparison to WT Spt5p (data not shown). Diploid strains were sporulated in liquid sporulation medium (10 g/L potassium acetate and 5mg/L 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.** Cells were grown in SD-Met medium to an A600 = ~0.3. Five ml of culture was then pulse-labeled with 25 µCi/ml [methyl-3H]methionine (Perkin Elmer; Waltham, MA) for 5 minutes and then treated with excess cold methionine (500 µg/ml) for an additional 5 minutes. RNA was then extracted and analyzed as described previously (11).

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

**Chromatin immunoprecipitation (ChIP).** 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 supplementary methods.

**EM analysis of Miller chromatin spreads.** EM analysis was performed essentially as described previously (16), except 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 rRNA genes visualized were photographed, and all genes that could be unambiguously followed from 5’ to 3’ end were analyzed for number of polymerases per gene and for relative polymerase density at 5’ and 3’ ends as described in Figure 5.

### Results

**Characterization of partially active mutations in SPT5.**

SPT4 is not essential for yeast viability, whereas SPT5 is. Thus, 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 (Figure 1 and data not shown). Cells used for the screen were grown at 23° C; however, all 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 (Figure 1A, green spacefill). This residue lies within the conserved NusG-like domain (NGN) 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; Figure 1). The other mutant carried a single point mutation leading to an arginine substitution for cysteine at position 292 [spt5(C292R); Figure 1A shown in red]. This residue also lies within the NGN domain of Spt5p, but was not previously implicated in binding Spt4p. Indeed, C292 is positioned on the opposite face of the domain than 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 spt4A strain or the spt5(S324P) mutant strain (Figure 1D). 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 spt4Δ, whereas the spt5(S324P) spt4Δ double mutant was viable (Figure 1B). 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 negatively, we mated each of the spt5 strains as well as an spt4Δ 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 rpa49Δ mutation, this interaction supports the conclusion that the candidate factor participates (directly or indirectly) in transcription elongation by Pol I. rpa49Δ strains are cold-sensitive, exhibiting almost undetectable growth at 23° C (Figure 1C). We grew haploid segregants representing all 4 genetic possibilities resulting from sporulation of spt rpa49Δ heterozygous diploids at 23° C to test for phenotypic suppression of the rpa49Δ mutation. In each case, we observed robust growth of the spt rpa49Δ double mutant strain whereas the rpa49Δ haploid segregants were not viable (Figure 1C). Thus, we conclude that mutations in SPT4 or SPT5 at least partially suppress the cold sensitivity of the rpa49Δ 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 rpa49Δ growth defect, we measured the exponential growth rate of individual haploid segregants. Cells were grown in YEPD at 27° C, since rpa49Δ mutants are cold-sensitive and spt mutants are high temperature-sensitive. Consistent with the spot test, the observed growth of the spt rpa49Δ double mutants was better than expected based on the growth rates of parental single mutant strains (Figure 1D). We note, however, that suppression of the rpa49Δ growth defect was not complete at this temperature, most likely due to the opposing temperature sensitivities of the candidate mutations. These data, together with previous results using spt4Δ 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 approximately 3-fold (20). To determine if mutation of SPT5 similarly affected the rDNA, we measured the size of chromosome XII using contour-clamped homogeneous field electrophoresis (CHEF) 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 (Figure 2A). 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 (Figure 2B). From that regression we estimated the rDNA copy number for the strains included in this study (Figure 2C). We conclude that there is no reduction in the rDNA copy number in either of the spt5 mutant strains compared to WT. Furthermore, the rpa49Δ strain exhibits a reduction in the rDNA copy number relative to WT, but strains carrying rpa49Δ and spt5 mutations do not rescue that reduction. Thus, the mechanism by which spt5 mutations suppress the rpa49Δ phenotype is not mediated by changes in the rDNA array size.
Effects of \textit{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 \textit{spt5}(C292R) strain. This strain grows at ~40% of the WT rate (Figure 1D). To measure rRNA synthesis rates, we isolated RNA after a 5 minute pulse/chase with [methyl-\textsuperscript{3}H]methionine from WT and \textit{spt5}(C292R) cells. Since 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 \textit{spt5}(C292R) strain compared to WT (Figure 3A; see supplemental methods for detailed discussion of isotopic labeling). Given the obvious growth defect of the \textit{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 \textit{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 \textit{spt5}(C292R) strain. We performed chromatin immunoprecipitation (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 (Figure 3B) and found approximately equal Pol I occupancy of the rDNA in the WT and \textit{spt5}(C292R) strains (Figure 3C). Thus, the rRNA synthesis rate is reduced in \textit{spt5}(C292R) cells despite equal Pol I loading on the rDNA compared to 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 [Figure 1 and (20)].

It is possible that mutation of \textit{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, \textit{rrp6Δ}, \textit{spt5}(C292R) and \textit{spt5}(C292R) \textit{rrp6Δ} double mutants (Figure 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 \textit{rrp6 spt5}(C292R) double mutant compared to the \textit{spt5}(C292R) mutant alone (Figure 4, lane 8 vs. lane 6), suggesting that some degradation of rRNA does occur in the \textit{spt5}(C292R) mutant. However, deletion of \textit{RRP6} in the \textit{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 \textit{spt5}(C292R) strains grown at 27 °C in rich medium. Representative genes from each of these strains are shown in figure 5A. It is clear that polymerase occupancy of the rDNA was not reduced significantly in the \textit{spt5}(C292R) mutant cells despite the ~4-fold reduction in rRNA synthesis rate (Figure 3A and Figure 4). We detected 43 Pol I complexes per gene on average in the mutant cells compared to 50 per gene in WT (Figure 5B). These data are consistent with the ChIP data described above (Figure 3C) and support the model that a reduction in transcription initiation rate alone cannot account for the reduced rRNA synthesis rate observed in the \textit{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, since this bias is true for all spreads analyzed, this measure is suitable for comparing the percentage of genes transcribed between strains. When we measured this value in the \textit{spt5(C292R)} cells relative to WT, we found that 85% of the rDNA repeats were active relative to 70% in WT cells (Figure 5B). From this percentage, together with the average number of polymerases per gene and the rDNA copy number (Figure 2), we calculate that there are approximately equal numbers of polymerases engaged in transcription in the WT and \textit{spt5} strains (Figure 5B). 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. Since a positive role was not seen for Spt4p when identical experimental approaches were used to characterize rRNA synthesis in \textit{spt4\Delta} 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 Figure 5A, the most obvious difference between WT and \textit{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 \cite{41,42}, which separates pre-rRNA into precursors for small and large ribosomal subunits (20S and 27S RNA species, respectively). The short transcripts seen at the 3’ end of the WT gene in Figure 5A are evidence that this cleavage has occurred \cite{42}. However, this was not the case in the \textit{spt5} mutant. Rather, we detected robust accumulation of long unprocessed nascent RNAs in the 3’ end of active rRNA genes in the \textit{spt5(C292R)} spreads (Figure 5A). Consistent with this observation, we observed accumulation of uncleaved 35S pre-rRNA in the \textit{spt5(C292R)} mutant after pulse-labeling with [methyl-\textsuperscript{3}H]methionine (Figure 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 \cite{20,21}. The observation of impaired co-transcriptional cleavage in the \textit{spt5(C292R)} mutant, similar to that seen in \textit{spt4\Delta} cells \cite{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; Figure 4) is modest. Thus, the observed effect of the \textit{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 \cite{28,30,31,43}. To determine if 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 \textit{spt5(C292R)} strains since emerging evidence indicates a higher density of Pol I in the 5’ region of rDNA genes as compared to downstream regions (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% (Figure 5C), supporting the model that a transient kinetic block to transcription elongation may occur early in transcription elongation. However, in the \textit{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, approximately 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-square 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 (Figure 5D). Since 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 ~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.

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 (Figure 5D). 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 (Figure 5C).

**Differences between spt4Δ and spt5(C292R)**

Many previous genetic studies have employed spt4Δ strains as models for “partial impairment” of Spt4/5 function [e.g. (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 than those observed previously using an spt4Δ strain. One obvious difference was observed in the mutations’ effects on the size of the rDNA array. We used standard Southern blots (20) and CHEF 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 (Figure 2). Previous work has shown that spt4Δ mutants have a hyper-recombination phenotype (45), and our results suggest this phenotype may be specific to the spt4Δ mutation. If Spt4p participates in pausing Pol I transcription, perhaps over-production of rRNA in the spt4Δ 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% (Figure 1D). 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 spt4Δ 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. Since Spt5p directly binds Pol I, and Spt5p has affinity for the Pol I transcription initiation factor Rrn3p [(20) and Viktorovskaya, et al. this issue], 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 found that deletion of SPT4 reduced Paf1C recruitment to the ARG1 gene (44) whereas the Hahn lab showed that deletion of the C-terminal domain of Spt5p reduced Paf1C recruitment (33). 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 paf1Δ mutations (46); and data not shown. Furthermore, spt5 mutations suppress phenotypes associated with the rpa49Δ mutation (Figure 1), whereas paf1Δ mutations are lethal when combined with rpa49Δ 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 to 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 to 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, since 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 Viktorovskaya et al.).

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 rpa49Δ phenotypes when we combined that mutation with spt4Δ 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 rpa49Δ 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 rpa49Δ (11). Thus, Paf1C apparently enhances a step in transcription elongation that is rate-limiting in the rpa49Δ 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 rpa49Δ 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 non-uniform 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, NELF, 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 spt4Δ and spt5(C292R) strains [Figure 5 and (20)]. We have confirmed this observation biochemically [Figure 4 and (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 [(22,49) and data not shown], and future studies will determine if Spt4/5 influences the efficiency of pausing at these or other sites.

Conclusions

Spt5p is the only transcription factor that is conserved throughout all 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.
References Cited

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Figure 1. Isolated mutations in SPT5 map to different faces of the protein and suppress cold sensitivity of rpa49Δ strains. (A) Ribbon diagram adapted from the published structure of Spt4p-Spt5(NGN) fusion protein [2EXU.pdb; (36)]. Spt4p is colored yellow and the NGN domain of Spt5p is pink. Residues in Spt5p implicated in binding Spt4p, Glu338 and Ser324, are shown in spacefill and colored blue and green respectively. Cys292 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 relevant genotypes of individual haploid strains indicated. The spt4 spt5 double mutants are indicated by “4,5”. Plates were incubated 5 days at 27˚ C. No viable spt4Δ 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.

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 4 lanes). 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 rates 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 A600 = ~0.3. Cells were pulse labeled for 5 min with 25µCi/ml [methyl-3H]methionine and chased for 5 min with excess cold methionine (500µg/ml). Isolated RNA from same number of cell equivalents (normalized to final A600 of culture) was subject to electrophoresis in a 1% formaldehyde:agarose gel, transferred to a nylon membrane and visualized by autoradiography. Film was developed after 24 hour exposure. 25S and 18S rRNA (and a background band) were excised from the membrane and 3H incorporation was quantified by a scintillation counter. Counts were averaged and normalized to WT with 1 standard deviation +/- shown. (B) Diagram of location of primer pairs used for qPCR analysis of ChIP DNA. (C) ChIP data demonstrate that Pol I occupancy of rDNA is not reduced in spt5(C292R)(DAS540) cells compared to WT (NOY396). A polyclonal anti-A190 antibody was used for immunoprecipitation. Data shown are the average of 3 DNA dilutions from each of 2 independent cultures. Error bars represent 1 standard deviation +/-.
Figure 4. rRNA is not overproduced in *rrp6Δ spt5(C292R)* double mutants. NOY396, DAS208, DAS570 and DAS604 were grown and labeled as described for Figure 3, except that cells were harvested after a 4 minute 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 hour exposure of the film and the lower panel is a 4 day exposure of film with the same blot.

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. 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, uncleaved for *spt5(C292R)*; (41,42)]. Bracketed arrows at the 5’ and 3’ end 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. Data were averaged with error 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). Error in each case equals one standard deviation. By multiplying the rDNA copy number (Figure 3), the average number of polymerases per gene and the percentage of active genes, we calculated the approximate number of polymerases 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. Grey 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) lead 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-18S rRNA into the processome (42).
Table 1 Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>NOY396</td>
<td>MAT(a) ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100</td>
<td>(11)</td>
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<tr>
<td>NOY886</td>
<td>Same as NOY396, except rpa135Δ::LEU2 fob1Δ::HIS3 pNOY117 [CEN, RPA135, TRP1] rDNA copy number = ~40</td>
<td>(16)</td>
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<tr>
<td>NOY1051</td>
<td>Same as NOY886, except rDNA copy number = ~140</td>
<td>(16)</td>
</tr>
<tr>
<td>NOY1064</td>
<td>Same as NOY396, except MAT(a) fob1Δ::HIS3 rDNA copy number = ~190</td>
<td>(51)</td>
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<tr>
<td>NOY1071</td>
<td>Same as NOY1064, except rDNA copy number = ~25</td>
<td>(51)</td>
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<td>NOY2167</td>
<td>MAT(a) ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100 spt4Δ::HIS3mx6</td>
<td>(20)</td>
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<tr>
<td>DAS50</td>
<td>MAT(a) ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100 rpa49Δ::LEU2</td>
<td>(11)</td>
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<tr>
<td>DAS208</td>
<td>Same as NOY396, except rrp6Δ::HIS3</td>
<td>this study</td>
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<td>DAS540</td>
<td>Same as NOY396, except spt5(C292R)</td>
<td>this study</td>
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<td>DAS541</td>
<td>Same as NOY396, except spt5(S324P, E427K)</td>
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<tr>
<td>DAS570</td>
<td>Same as NOY396, except spt5(C292R)-(HA)(_3)-his7::URA3</td>
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<tr>
<td>DAS573</td>
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<tr>
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<td>this study</td>
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<tr>
<td>DAS576</td>
<td>Same as DAS50, except MAT(a)</td>
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<tr>
<td>DAS577</td>
<td>MAT(a) haploid segregant of DAS578 rpa49Δ::LEU2 SPT5</td>
<td>this study</td>
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<tr>
<td>DAS578</td>
<td>MAT(a)/(\alpha) diploid resulting from cross of DAS50 x DAS540</td>
<td>this study</td>
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<tr>
<td>DAS579</td>
<td>MAT(a)/(\alpha) diploid resulting from cross of DAS50 x DAS541</td>
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<td>DAS581</td>
<td>MAT(a)/(\alpha) diploid resulting from cross of DAS576 x NOY2167</td>
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<tr>
<td>DAS604</td>
<td>MAT(a) ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100 spt5(C292R)-(HA)(_3)-his(_7)::URA3 rrp6Δ::HIS3</td>
<td>this study</td>
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</tbody>
</table>
### Figure 1

#### A.

- spt5(C292R)
- spt5(S324P, E427K)

#### B.

- spt5(C292R)
- spt5(S324P, E427K)

#### C.

<table>
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<th>Strain</th>
<th>Growth Rate</th>
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<th>Expected</th>
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<td>WT</td>
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<tr>
<td>spt5(C292R)</td>
<td>0.22</td>
<td>42.75</td>
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<tr>
<td>rpa49Δ</td>
<td>0.23</td>
<td>44.75</td>
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<td>0.17</td>
<td>33.14</td>
<td>19.13</td>
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<td>spt5(S324P, E427K)</td>
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<td>68.99</td>
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<td>rpa49Δ</td>
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<td>40.98</td>
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<tr>
<td>spt4Δ</td>
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<td>0.29</td>
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Figure 2

A. rDNA copy #

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<tr>
<td>40</td>
<td>396 540 541 577 573 574</td>
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<tr>
<td>25</td>
<td></td>
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<tr>
<td>140</td>
<td></td>
</tr>
<tr>
<td>190</td>
<td></td>
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</table>

B. y = -3.19(x) + 371

C. Strain copy #

<table>
<thead>
<tr>
<th>strain</th>
<th>copy #</th>
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<tbody>
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<td>NOY396 = WT</td>
<td>256</td>
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<tr>
<td>DAS540 = spt5(C292R)</td>
<td>262</td>
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<tr>
<td>DAS541 = spt5(S324P E427K)</td>
<td>262</td>
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<tr>
<td>DAS577 = rpa49 Δ</td>
<td>180</td>
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<tr>
<td>DAS573 = rpa49 Δ spt5(C292R)</td>
<td>154</td>
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<tr>
<td>DAS574 = rpa49 Δ spt5(S324P E427K)</td>
<td>185</td>
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</table>
Figure 3

A. WT  spt5(C292R)

25S
18S

100%  24 +/- 3%

B.

18S rRNA  5.8S  25S rRNA

promoter  5 ETS  18S #1  18S #2  5.8S  25S #1  25S #3  NTS1  5S  NTS2

C.

% of input

WT  spt5(C292R)
Figure 5

A. Diagrams showing gene expression patterns for WT, spt5(C292R), and spt5(C292R) with 5' > 3', 5' = 3', and 5' < 3' labeling.

B. Graph showing the number of genes versus polymerases per gene for WT and spt5(C292R). The graph is bimodal with two peaks.

C. Bar chart illustrating the percentage of genes with 5' > 3' and 3' > 5' for WT and spt5(C292R).

D. Diagram depicting the interaction of Spt5 with other factors, possibly suggesting a role in gene expression regulation.

<table>
<thead>
<tr>
<th></th>
<th># polys / gene</th>
<th>n</th>
<th>% active genes</th>
<th>n</th>
<th># transcribing polys</th>
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<tr>
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<td>177</td>
<td>70.4</td>
<td>162</td>
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<tr>
<td>spt5(C292R)</td>
<td>43 +/- 21</td>
<td>217</td>
<td>85.5</td>
<td>214</td>
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The transcription elongation factor Spt5 influences transcription by RNA polymerase I positively and negatively
Susan J. Anderson, Martha L. Sikes, Yinfeng Zhang, Sarah L. French, Shilpa Salgia, Ann L. Beyer, Masayasu Nomura and David A. Schneider

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