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Spt6 Is Essential for rRNA Synthesis by RNA Polymerase I

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Spt6 (suppressor of Ty6) has many roles in transcription initiation and elongation by RNA polymerase (Pol) II. These effects are mediated through interactions with histones, transcription factors, and the RNA polymerase. Two lines of evidence suggest that Spt6 also plays a role in rRNA synthesis. First, Spt6 physically associates with a Pol I subunit (Rpa43). Second, Spt6 interacts physically and genetically with Spt4/5, which directly affects Pol I transcription. Utilizing a temperature-sensitive allele, *spt6-1004*, we show that Spt6 is essential for Pol I occupancy of the ribosomal DNA (rDNA) and rRNA synthesis. Our data demonstrate that protein levels of an essential Pol I initiation factor, Rrn3, are reduced when Spt6 is inactivated, leading to low levels of Pol I-Rrn3 complex. Overexpression of *RRN3* rescues Pol I-Rrn3 complex formation; however, rRNA synthesis is not restored. These data suggest that Spt6 is involved in either recruiting the Pol I-Rrn3 complex to the rDNA or stabilizing the preinitiation complex. The findings presented here identify an unexpected, essential role for Spt6 in synthesis of rRNA.

Three eukaryotic RNA polymerases (Pols), Pol I, Pol II, and Pol III, have critical and yet distinct roles in gene expression. Pol II synthesizes mainly mRNAs, which serve as the templates for translation and give rise to the diverse collection of cellular proteins. Pols I and III are responsible for producing the stable RNA components of the translation machinery, rRNA and tRNA, respectively. In *Saccharomyces cerevisiae* (yeast), Pol I transcribes the ribosomal DNA (rDNA) to generate the 35S rRNA precursor within a subcompartment of the nucleus called the nucleolus. This precursor is co- and posttranscriptionally processed into the mature 25S, 18S, and 5.8S rRNAs and assembled into ribosomes. Pol III synthesizes all tRNAs and the 5S rRNA. The highly repetitive nature of the rDNA (~200 tandem copies of the rDNA in yeast) helps cells meet the exceptional demand for protein synthesis and, therefore, for ribosomes. However, only about half of the rDNA repeats are actively transcribed at any given time (1, 2).

There is an intimate connection between ribosome biogenesis, protein synthesis, and cell growth capacity. It was observed over 100 years ago that cancer cells have enlarged nucleoli compared to those of normal host cells (3). We now know that the rate of rRNA synthesis is increased in many forms of cancer (4, 5). A number of laboratories have recently shown that selective inhibition of Pol I transcription is an effective method for impairing tumor cell growth (6–8). Taken together, both the old insights and the new insights agree that Pol I is emerging as a promising chemotherapeutic target.

Despite the central role Pol I plays during cell growth, a detailed understanding of its regulation is lacking. However, a collection of *trans*-acting factors have been shown to influence Pol I at the initiation or elongation step in transcription. One such complex is suppressors of Ty4 and -5 (Spt4/5), which was previously shown to play a role in Pol II promoter-proximal pausing and elongation. Recent studies have revealed that Spt4/5 influences Pol I activity as well (9, 10). Two lines of evidence suggest that another transcription factor, Spt6, might also be involved in the control of Pol I activity. First, Spt6 physically and genetically interacts with Spt4/5 (11). Second, a yeast 2-hybrid analysis performed in the Thuriaux laboratory identified a physical interaction between Spt6 and the Rpa43 subunit of Pol I (12). Together, these data suggest that Spt6 is well positioned to influence transcription of the rDNA.

Spt6 is an essential protein in yeast and is conserved in humans. The Spt proteins were initially identified in a screen for suppressors of the deleterious effects of a Ty insertion at the 5′ end of the *HIS4* gene (13). The screen identified many genes involved in modulation of chromatin structures and Pol II transcription. It is now known that Spt6 influences several steps in mRNA synthesis, including Pol II transcription initiation (14, 15), elongation (16–19), and termination (20, 21). Spt6 also has histone chaperone activity (22) and is involved in the deposition of specific histone modifications (23–26) as well as in modulation of nucleosome occupancy (27).

Spt6 colocalizes with Pol II along mRNA genes (25, 27–31) and directly binds the Pol II C-terminal domain (CTD) (19, 32–36). Transcription initiation at inducible genes typically requires factor-dependent recruitment of Pol II to promoter elements. However, inactivation of Spt6 induces constitutively active expression of *ADH2* and *PHO5*, despite depletion of their respective regulatory factors (14, 37, 38). Mutations in *SPT6* also render upstream activating sequences dispensable for many Pol II-transcribed genes (39). It is speculated that these effects of Spt6 are mediated through alterations in chromatin. Consistent with this hypothesis, Spt6 is a histone chaperone capable of assembling nucleosomes on naked DNA templates in the presence of topoisomerase I (22) and

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is involved in preventing loss of histones through transcribed regions (25, 27, 40–43).

Transcription assays for Pol II that included human cell extracts with and without depletion of Spt6 identified a positive role for Spt6 in Pol II transcription elongation rate and processivity (17). Additionally, knockdown of *SPT6* decreased the Pol II transcription elongation rate 2-fold through *Drosophila* heat shock genes (16). During the elongation phase of transcription, Spt6 is involved in the protein interaction network that recruits Set2 (19, 23, 25, 32), the methyltransferase responsible for histone H3, lysine 36 monomethylation and di- and trimethylation (H3K36me2/3). H3K36me2/3 serves to prevent transcription initiation from cryptic Pol II promoters within the coding regions of genes through recruitment of Rpd3 and subsequent deacetylation of histones (23). With the exception of the *spt6-140* allele, which does not abolish H3K36 methylation (44, 45), mutation of Spt6 leads to the absence of H3K36me2/3 and accumulation of aberrant antisense transcripts as well as transcripts originating from cryptic promoters within many genes (25, 43, 46).

Here, we asked whether Spt6 affects Pol I transcription of the ribosomal DNA. We found that Spt6 associates with actively transcribed ribosomal DNA repeats and that conditional inactivation of Spt6 results in nearly complete loss of Pol I transcription. Despite the presence of all known Pol I initiation factors, Pol I occupancy of the rDNA is lost when Spt6 function is perturbed. These and other data suggest that Spt6 plays a critical role in transcription initiation by Pol I, modulating polymerase occupancy of the rDNA independently of known transcription initiation factors.

MATERIALS AND METHODS

Yeast strains, plasmids, media, and growth conditions. Standard media and conditions were used for yeast growth. When indicated, a 39°C temperature shift at an optical density at 600 nm (OD_{600}) of ~0.2 was performed for 90 min. Yeast strains containing deletions and tags were isolated using standard techniques (47, 48). The strain harboring *spt6-1004* was a gift from Fred Winston, and the mutant allele was incorporated into the wild-type (WT) background using pRS306 integration and counterselection with 5-fluoroorotic acid. All strains and plasmids used are listed in Table S1 in the supplemental material.

***In vivo* metabolic labeling.** Synthesis rates were determined using either [^{3}H]methylmethionine or [^{3}H]uridine pulse-chase labeling. For [^{3}H]methylmethionine labeling, yeast cells were grown in synthetic minimal media without methionine (SD-Met) and pulsed during exponential growth. For experiments at 39°C (the nonpermissive temperature for the *spt6-1004* strain), cells were grown to an OD_{600} of ~0.2 at 30°C and were then shifted to 39°C for 90 min prior to labeling. The cells were pulsed for 5 min with 50 μCi/ml of [^{3}H]methylmethionine and then chased with an excess of cold methionine for 5 min. A 1.5-ml volume of each sample was taken both immediately before (P [pulse]) and after (C [chase]) the pulse-chase procedure. RNA was extracted at 65°C using phenol-chloroform followed by precipitation in a solution of 1.25 M ammonium acetate in 95% ethanol at −20°C. The RNA pellet was washed in 70% ethanol and then resuspended in the appropriate volume of RNase-free water in order to normalize to the OD_{600} at the time of the pulse. For example, for a sample taken at an OD_{600} of 0.4, the RNA pellet was resuspended in 40 μl of water. Equal volumes of RNA were loaded onto 0.8% agarose formaldehyde gels. The RNA was then transferred to a nylon membrane using a semidry transfer at 56 mA for 3 to 4 h, UV cross-linked at 1200 μJ, and soaked in Fluoro-Hance (Fisher) for 30 min. The blot was allowed to dry overnight and then exposed to film for the amount of time indicated in the figure legends. Quantification of [^{3}H]methylmethionine incorporation was performed by either cutting the bands out of the blot after development or cutting the bands out of the agarose gel. For bands extracted directly from the agarose gels, the gel slices were melted in water and mixed with scintillation cocktail. Radioactivity from either technique was quantified by liquid scintillation. [^{3}H]uridine incorporation was performed as described in the supplemental material.

ChIP. Yeast cells were grown in 50 ml rich medium and cross-linked with 1% formaldehyde at room temperature with gentle shaking for 6 min for A190 chromatin immunoprecipitation (ChIP) and for 15 min for Spt6-Myc (monoclonal anti-myc 9E10; Pierce), H3 (polyclonal Ab1791; Abcam), H4 (polyclonal Ab7311; Abcam), H3K36me3 (polyclonal Ab9050; Abcam), and Rrn7-3FLAG (monoclonal anti-FLAG M2; Sigma-Aldrich) ChIP. The formaldehyde was quenched with 125 mM glycine for 10 min. The cells were harvested and lysed as described for coimmunoprecipitation (see below), except the cells were washed in cold 1× phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 9.86 mM Na_2HPO_4, 1.76 mM KH_2PO_4) and lysed in cold FA (50 mM HEPES [pH 7.5], 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA) lysis buffer with 2 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor (Pierce protease inhibitor tablet; Thermo Scientific) (one tablet per 50 ml). DNA was sheared at 4°C using a Bioruptor (Diagenode) for 30 cycles of 54 s on and 6 s off per cycle. The total protein concentration of the lysate was measured using a bicinchoninic acid (BCA) kit (Thermo Scientific). The lysates were then diluted to equal protein concentrations using FA lysis buffer. Prior to use, protein G-coupled magnetic beads (Dynabeads; Invitrogen) were washed twice in water and then twice in FA lysis buffer and resuspended to the original volume in lysis buffer (on the basis of the volume of the original suspension as shipped by the manufacturer). Lysate was then incubated at 4°C with gentle rolling overnight with 40 μl of washed and resuspended Dynabeads and 2 μl of the respective antibodies. A 1/10 volume of the lysate used for immunoprecipitation was saved as the input. The Dynabeads were then washed twice with 1 ml for each buffer for 10 min each time with gentle rolling at room temperature using each FA lysis buffer with PMSF and a protease inhibitor tablet, high-salt wash buffer (50 mM HEPES [pH 7.5], 500 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA), deoxycholate wash buffer (10 mM Tris [pH 8.0], 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and 1× TE (10 mM Tris, 1 mM EDTA) (pH 7.5), in that order. Elution was performed at 95°C for 30 min in 1× TE–1% SDS. Input samples were heated as described for the IP procedure. DNAs from both the eluate and input samples were isolated using a PCR cleanup kit (Omega Bio-Tek) or phenol-chloroform extraction. Quantitative PCR (qPCR) was performed as described in reference 49. Signal data were calculated as the percentage of input and were taken from at least two qPCRs. The sequences of the oligonucleotides used are listed in Table S2 in the supplemental material.

Coimmunoprecipitation. Yeast cells were grown in 50 ml medium and harvested during exponential growth and then washed twice in cold lysis buffer (0.1 M potassium acetate, 20 mM Tris [pH 7.9], 20% [vol/vol] glycerol, 0.1% [vol/vol] Tween 20). For the temperature shifts, the WT and *spt6-1004* cultures were grown at 30°C to an OD_{600} of ~0.2 to ~0.25 and then shifted to 39°C for 90 min. The cells were lysed at 4°C using a Fast-Prep instrument and glass beads in lysis buffer with 2 mM PMSF for four cycles (30 s each; 4.5 m/s) with at least a 1-min break between cycles (~300 μl glass beads/500 μl cell suspension). The total protein concentrations of the cleared lysates were determined using a BCA kit (as above for ChIP), and the lysates were diluted to equivalent concentrations using the lysis buffer described above. Protein G-coupled Dynabeads were washed twice in water and twice in lysis buffer and resuspended to the original volume in lysis buffer before use. Equal volumes of lysate were incubated with 40 μl of washed Dynabeads and 2 μl of antibody (as indicated in figure legends) for 1.5 h at 4°C with gently rolling. The beads were then washed four times with 1 ml lysis buffer. Elution was performed at 95°C for 15 min with the addition of 2× protein loading dye containing 5% BME (β-mercaptoethanol). Input and IP samples were loaded onto polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membranes, and probed with the antibody indicated in figure legends. The Western blots were visualized using chemiluminescence (Chemidoc; Bio-Rad).

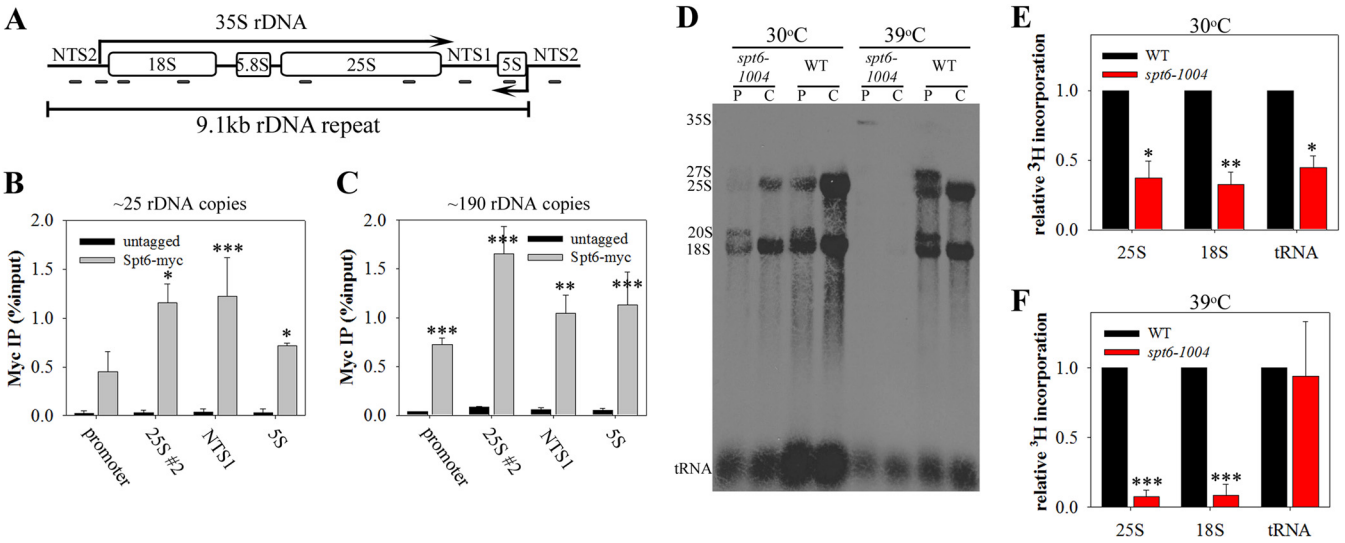


FIG 1 Spt6 localizes to the rDNA, and the mutant allele causes drastic reduction in rRNA synthesis at the nonpermissive temperature. (A) Schematic depicting the yeast rDNA repeat and the locations of the oligonucleotides used for ChIP analyses in this and subsequent figures. NTS, nontranscribed spacer. (B) ChIP analysis was used to detect whether or not Spt6 associates with the rDNA. Data represent Spt6-myc versus untagged Spt6 in a strain containing ~25 copies of the rDNA. IP was done using an anti-myc antibody (9E10; Pierce). Enrichment was calculated as percent input. (C) Same as panel B, except that the experiments were performed in a strain containing ~190 copies of the rDNA. The data in panels B and C are representative of experiments from 2 independent trials. Standard deviations and significance were calculated from at least two technical replicates. (D) WT and *spt6-1004* cells were grown in SD-Met medium and pulse-labeled (P) using [^{3}H]methylmethionine for 5 min followed by a 5-min chase (C) during exponential growth at the permissive temperature (30°C) and after a 90-min shift to the nonpermissive temperature (39°C). The representative autoradiographic image of labeled rRNA and tRNA species was obtained by denaturing agarose gel electrophoresis followed by transfer to a nylon membrane and exposure for 5 days. (E) Quantification of tRNA, 25S, and 18S rRNA synthesis from [^{3}H]methylmethionine labeling experiments for the WT and *spt6-1004* strains at 30°C ($n = 2$). (F) Same as described for panel E, except that quantification of the results of the experiments was performed at 39°C ($n = 4$). Error bars show standard deviations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

RESULTS

Spt6 associates with rDNA and is essential for rRNA synthesis. Evidence in the literature suggests that Spt6 might have a role in Pol I transcription (11, 12). To determine if Spt6 could have a direct effect on Pol I transcription, we performed chromatin immunoprecipitation (ChIP) analysis of a myc-tagged version of Spt6 in cells carrying ~190 copies of the rDNA repeat (a schematic of an rDNA repeat and of the oligonucleotides used during all ChIP experiments is shown in Fig. 1A) and in cells with a reduced rDNA copy number (~25 repeats). Previous studies have shown that ~50% of rDNA genes are actively transcribed in cells carrying a high repeat number whereas all of the genes are active in the reduced-copy-number strain (2, 50). We found that Spt6 localized to the rDNA in both strains (Fig. 1B and C). In order to control for the possibility that Spt6 nonspecifically interacts with DNA, we tested the association of Spt6-Myc with the mitochondrial DNA (mtDNA). Indeed, tagged Spt6 localizes to the rDNA but not to the mtDNA (see Fig. S1 in the supplemental material). Thus, Spt6 associates with actively transcribed rDNA and is poised to influence Pol I transcription directly.

Spt6 is an essential protein in *S. cerevisiae*; therefore, we used a temperature-sensitive allele that was initially isolated and characterized in the Winston laboratory. The *spt6-1004* allele contains an in-frame internal deletion of a helix-loop-helix motif (residues 931 to 994) (43). Strains carrying *spt6-1004* have a mild growth defect at 30°C but are nonviable above 37°C. We measured rRNA synthesis rates in WT and *spt6-1004* strains growing at the permissive temperature (30°C) and after a 90-min shift to the nonpermissive temperature (39°C). These experimental conditions mimic those used for previous characterization of the *spt6-1004* mutation (40, 43) and were used for all analyses in this study.

Metabolic labeling of the rRNA was used to quantify Pol I transcription in WT and *spt6-1004* cells. Briefly, cells were pulsed with [^{3}H]methylmethionine and then chased with cold methionine. Samples were collected before and after the chase. Since rRNA is methylated cotranscriptionally, this labeling strategy provides a sensitive and accurate measure of the rate of rRNA synthesis (51). Samples collected before the chase provide insight into pre-rRNA accumulation and processing (as evident by the appearance of 35S, 27S, and 20S precursor rRNA in the pulse lanes only), whereas one can accurately quantify the incorporation of radioactive signal into the mature rRNA products (25S and 18S) after the chase. Using this technique, we observed that the *spt6-1004* allele caused a 2-fold reduction in the amount of rRNA produced at 30°C (Fig. 1D and E). A drastic reduction (~92%) in rRNA synthesis relative to the WT results was observed after the 39°C temperature shift, but tRNA synthesis was largely unaffected (Fig. 1D and F).

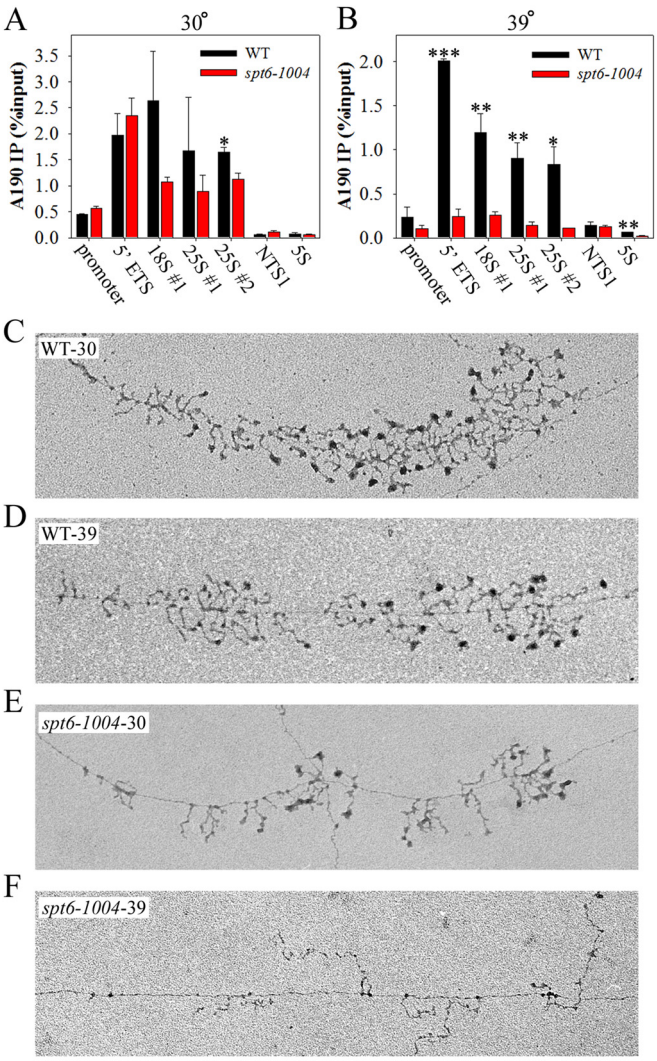
We also measured rRNA synthesis by pulse-labeling cells with [^{3}H]uridine (see Fig. S2 in the supplemental material). After labeling, RNA was extracted and ^{3}H incorporation into 18S and 25S rRNA was measured by liquid scintillation. This strategy also showed that rRNA synthesis was essentially lost after the shift to the nonpermissive temperature, confirming our observations obtained using [^{3}H]methylmethionine and ruling out any specific effect on rRNA methylation (described further in the supplemental material). Specifically, we observed an ~90% reduction in rRNA synthesis in the *spt6* mutant compared to the WT at 30°C

and barely detectible levels of rRNA synthesis at 39°C in the *spt6-1004* cells. The reduction in rRNA synthesis at the permissive temperature in the *spt6-1004* mutant compared to the WT observed using [^{3}H]uridine incorporation was much more drastic than that seen when [^{3}H]methylmethionine was used. Taking the growth rate of the *spt6-1004* strain (~75% of the WT growth rate) into consideration, the ~90% reduction in rRNA synthesis shown using [^{3}H]uridine is implausible. The most likely explanation for this discrepancy is defective uridine uptake in the *spt6* mutant. Irrespective of the magnitude of the defect, these data clearly support the conclusion that Spt6 is required for efficient rRNA synthesis.

Spt6 was shown to act as a Pol II transcription elongation factor both *in vitro* (17, 19) and *in vivo* (16) and plays a role in the release of promoter-proximally paused Pol II at heat shock loci (28). Given Spt6's known involvement in transcription by Pol II, it is feasible that the defect observed in rRNA synthesis results from a perturbation of Pol II activity. To test this model, we performed [^{3}H]methylmethionine labeling in a strain carrying a deletion of a nonessential Pol II subunit (*rpb9*Δ) under the temperature shift conditions previously described for comparison of the *spt6-1004* strain to the WT. The *rpb9*Δ and *spt6-1004* strains share two key features. First, growth of both strains leads to an alteration of Pol II start site selection (52). Second, the kinetics of growth inhibition observed in the *rpb9*Δ strain are almost identical to those observed in the *spt6-1004* strain (see Fig. S3A in the supplemental material). Based on these similarities, we reasoned that the *rpb9*Δ strain could serve as an appropriate control for potential Pol II-mediated effects on rRNA synthesis. We tested this idea by performing [^{3}H]methylmethionine labeling and saw that the rate of rRNA synthesis in the *rpb9*Δ strain was approximately 40% of the WT level at permissive and nonpermissive temperatures (30°C and 39°C) (see Fig. S3B in the supplemental material). We conclude that the reduced rate of rRNA synthesis at the permissive temperature was a result of a feedback response due to the lower growth rate of the *rpb9*Δ cells. In direct contrast to *spt6-1004*, this steady-state defect is not exacerbated by the shift to the nonpermissive temperature. Thus, direct perturbation of Pol II activity through deletion of *RPB9* does not eliminate rRNA synthesis at 39°C. These data support the model that drastically impaired rRNA synthesis in the *spt6-1004* strain at 39°C is not an effect of perturbed Pol II activity.

The *spt6-1004* mutation reduces Pol I occupancy of the rDNA. To determine whether Spt6 affects Pol I occupancy of the rDNA, we performed ChIP assays for Pol I in WT and mutant cells. Consistent with the observed loss of rRNA synthesis, the Pol I ChIP signal at the rDNA was reduced to background levels in the mutant at 39°C but not at 30°C (Fig. 2A and B; compare the coding region to the background in the nontranscribed spacer 1 [NTS1] and 5S regions).

For a more detailed view of active Pol I transcription, we analyzed electron micrographs (EM) of Miller chromatin spreads from WT and *spt6-1004* strains grown at the permissive and nonpermissive temperatures. Miller spreading allows one to obtain an *in vivo* "snapshot" of active rDNA genes and can provide information on the number of polymerases per gene, Pol I processivity, and percentage of active rDNA repeats. Since the rDNA is an array of tandem repeats and typically carries a high number of engaged polymerases, the rDNA is readily distinguished from other genomic DNA on the EM grid. Under permissive growth conditions, Pol I occupancy of the rDNA in the *spt6-1004* cells was slightly reduced compared to that of the WT (averages of 47 Pols/gene for the WT and 36 Pols/gene for *spt6-1004*; Fig. 2G). The percentages of actively transcribed repeats were nearly identical (Fig. 2G). However, after the 90-min shift to 39°C, rDNA repeats were barely recognizable in the mutant strain due to the low number of polymerases per gene (Fig. 2F; see Fig. S4 in the supplemen-



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	polymerases/gene	% repeats active
WT-30°	47 +/- 13	54%
WT-39°	36 +/- 12	46%
spt6-1004 -30°	36 +/- 11	53%
spt6-1004 -39°	<5	n/a

FIG 2 Pol I is lost from the rDNA in the *spt6-1004* strain at the nonpermissive temperature. (A) Pol I occupancy at the rDNA at 30°C was determined via ChIP analysis using a polyclonal anti-A190 antibody. ETS, external transcribed spacer. (B) Same as described for panel A, except that experiments were performed at 39°C. For panels A and B, the results of representative experiments are shown from 4 biological replicates. (C to F) Electron microscopy of Miller chromatin spreads showing examples of single rDNA genes under the conditions labeled. (G) A table summarizing the information obtained from analysis of Miller chromatin spreads. *n* (for polymerase count per gene) = 62 genes counted for the WT at 30°C, 68 genes for the WT at 39°C, and 70 genes for the *spt6-1004* strain at 30°C. n/a, not applicable (for *spt6-1004* at 39°C). Error bars show standard deviations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

tal material), whereas the WT was affected only mildly (Fig. 2D). Given that one criterion for identification of a transcribed region as rDNA in Miller spreads is the observation of multiple highly transcribed genes in a row, we were unable to confidently calculate the number of active repeats or assign a firm number of polymerases per gene in the *spt6-1004* strain at 39°C. Taken together, the ChIP data and EM data consistently demonstrated that Pol I occupancy of the rDNA was nearly lost in the *spt6-1004* strain after the temperature shift, accounting for the observed drastic reduction in rRNA synthesis (Fig. 1).

Pol I initiation complex assembly is impaired by *spt6-1004*. These data suggest that *spt6-1004* severely impairs rRNA synthesis by preventing loading of Pol I onto the rDNA. We therefore sought to determine which step in transcription initiation by Pol I is inhibited. The Pol I preinitiation complex is simpler than that of Pol II and consists of four factors: upstream activating factor (UAF), core factor (CF), TATA-binding protein (TBP), and Rrn3 (53). The UAF-TBP-CF complex assembles at the rDNA and serves as a platform for interaction between the preassembled Pol I-Rrn3 complex and the rDNA promoter (54, 55). To determine whether the reduced Pol I occupancy after inactivation of Spt6 is due to perturbation of transcription initiation factors at the rDNA, we used ChIP to measure occupancy of CF on the rDNA using a FLAG-tagged version of the Rrn7 subunit of CF. Since CF is essential for even basal levels of Pol I transcription (56), the presence or absence of CF is indicative of preinitiation complex assembly. Indeed, CF is properly recruited to the rDNA in the *spt6-1004* strain at both 30°C and 39°C (see Fig. S5 in the supplemental material). These data suggest that Spt6 function is not required for efficient assembly or for the stability of the basal transcription initiation factors on the rDNA promoter.

Recently, Pol I assembly was shown to be regulated by ubiquitination and degradation of the largest subunit, Rpa190 (57). As an additional control, we measured the levels of Rpa190 and its association with the second largest subunit, Rpa135, after inactivation of Spt6. Similar levels of hemagglutinin (HA)-tagged A135 were recovered after A190 IP in the WT and *spt6-1004* strains at both 30°C and 39°C (see Fig. S6 in the supplemental material). Therefore, gross defects in Pol I assembly do not account for the reduced Pol I occupancy of the rDNA observed in the *spt6-1004* strain.

Rrn3 is an essential protein, and assembly of the Pol I-Rrn3 complex precedes Pol I recruitment to the rDNA promoter (55). Given the pivotal role Rrn3 plays during rRNA synthesis, we measured the protein levels in the WT and *spt6-1004* strains under the permissive and nonpermissive growth conditions. Remarkably, Rrn3 levels were drastically reduced in the *spt6-1004* strain at 39°C (Fig. 3A). Low levels of Rrn3 could explain the failure to recruit Pol I to the rDNA promoter in the *spt6-1004* strain and, likewise, the low levels of rRNA synthesis at 39°C. We also tested whether or not the *spt6-1004* mutation affects the association of Rrn3 with Pol I. An anti-A190 polyclonal antibody was used for IP of Pol I, and Western blots of both input and IP material were probed for Rrn3-FLAG using an anti-FLAG antibody. Indeed, Rrn3 association with Pol I was reduced in the mutant strain at 39°C (Fig. 3C).

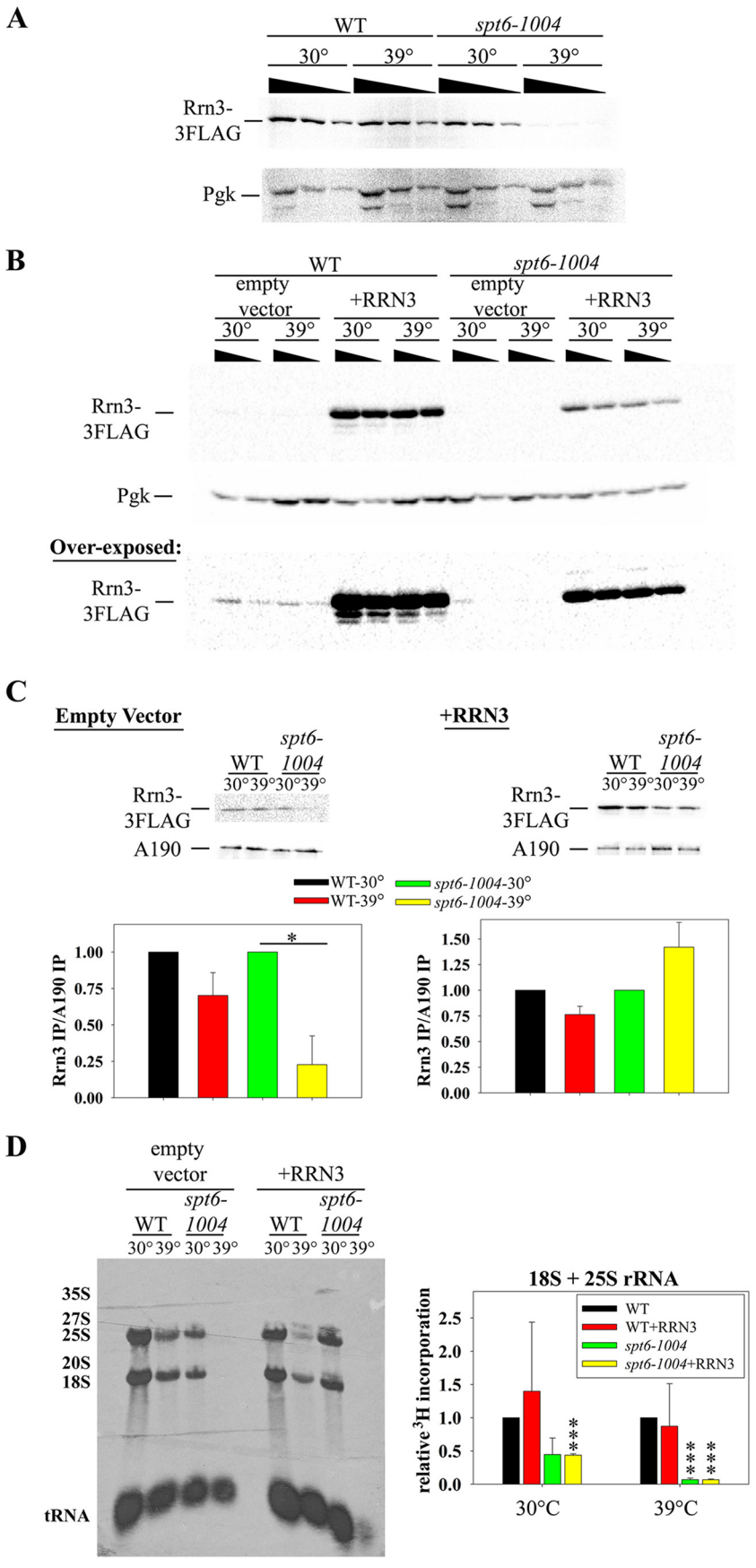
Rrn3 protein levels could be modulated at the level of mRNA synthesis or protein stability. Previously published microarray data (43; data were received via correspondence with Craig Kaplan, Texas A&M University) quantified mRNA abundance in both the WT and *spt6-1004* strains at 30°C and after a 90-min temperature shift to 39°C. Analysis of these data showed that Rrn3 mRNA levels in the *spt6-1004* cells were twice the WT levels at 39°C. Additionally, a subsequent publication reported levels of mRNA abundance in WT and *spt6-1004* strains at 30°C and after an 80-min shift to 37°C (46). The authors used microarrays with probes directed to sequences throughout the coding regions of genes to detect the occurrence of cryptic transcripts. Their data identified an ~2-fold reduction in the amount of full-length *RRN3* mRNA in the mutant cells as well as the accumulation of cryptic transcripts. Even though the results of these two microarrays are not in complete agreement, these data suggest that the severity of the reduction of Rrn3 abundance in the *spt6-1004* mutant was not entirely due to decreased mRNA levels; rather, they implicate posttranscriptional regulation.

Overexpression of Rrn3 rescues Pol I-Rrn3 association but not rRNA synthesis. Degradation of Rrn3 could result from impaired association with Pol I. We can envision two potential roles for Spt6. First, Spt6 could direct Rrn3 association with Pol I. In support of this idea, the A43 subunit of Pol I is the primary docking site for Rrn3 (58), and previous data also identified a physical interaction between A43 and Spt6 (12, 58). Alternatively, Pol I and Rrn3 may still form a complex in the absence of Spt6, but recruitment of the complex to the rDNA promoter may be dependent on Spt6.

To differentiate between these scenarios, we overexpressed Rrn3 using a *TEF1* promoter on a multicopy plasmid (pRS425). If Spt6 is required for association of Pol I with Rrn3, then overexpression of Rrn3 will not rescue this interaction. However, if formation of the Pol I-Rrn3 complex is rescued but rRNA synthesis is not, then the data suggest that Spt6 is required for recruitment of Pol I-Rrn3 to the rDNA promoter or during early transcription elongation.

When Rrn3 was overexpressed (Fig. 3B), immunoprecipitation of Rrn3 with Pol I in the *spt6* mutant cells at 39°C was fully rescued (Fig. 3C). Therefore, Spt6 is not required for stable association of Rrn3 with Pol I. Metabolic labeling experiments showed that rRNA synthesis in both WT and *spt6-1004* cells is lower with overexpression of Rrn3 than without at 30°C. Most importantly, rRNA synthesis is not rescued at 39°C in the mutant strain with overexpression of Rrn3 (Fig. 3D), even though association of Rrn3 with Pol I is rescued under these conditions. These data show that Spt6 is required for a step in Pol I transcription other than formation of the Rrn3-Pol I complex and that loss of Spt6 function results in defective transcription initiation or Pol I-DNA complex stability. To further test this idea, we performed ChIP experiments to measure the level of Pol I occupancy at the rDNA in the presence of overexpression of Rrn3 under the same conditions as those used for analysis of synthesis rates and co-IPs. The results are in agreement with our model and show that, even though Pol I and Rrn3 association is rescued by overexpression of Rrn3, recruitment of Pol I to the rDNA is not (see Fig. S7 in the supplemental material). Our findings identify a new, essential role for the conserved Spt6 protein in the first step of ribosome biosynthesis, and this role appears to be independent of previously identified transcription initiation factors for Pol I.

Alterations in the rDNA chromatin do not explain the paucity of Pol I on the rDNA. Spt6 participates in shaping the chromatin structure of Pol II-transcribed genes. Mutation of *SPT6* can lead to depletion of histones H3 and H4 in actively transcribed regions and the inability to di- and trimethylate lysine 36 on his-


A
WT
spt6-1004
30°
39°
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39°
Rrn3-3FLAG
Pgk
B
WT
spt6-1004
empty vector
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empty vector
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30°
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Rrn3-3FLAG
Pgk
Over-exposed:
Rrn3-3FLAG
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Empty Vector
+RRN3
WT
spt6-1004
WT
spt6-1004
30°39°30°39°
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Rrn3-3FLAG
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Rrn3-3FLAG
A190
WT-30°
WT-39°
spt6-1004-30°
spt6-1004-39°
Rrn3 IP/A190 IP
1.00
0.75
0.50
0.25
0.00
*
Rrn3 IP/A190 IP
1.50
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empty vector
+RRN3
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spt6-1004
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30°39°30°39°
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35S
27S
25S
20S
18S
tRNA
18S + 25S rRNA
relative 3H incorporation
2.5
2.0
1.5
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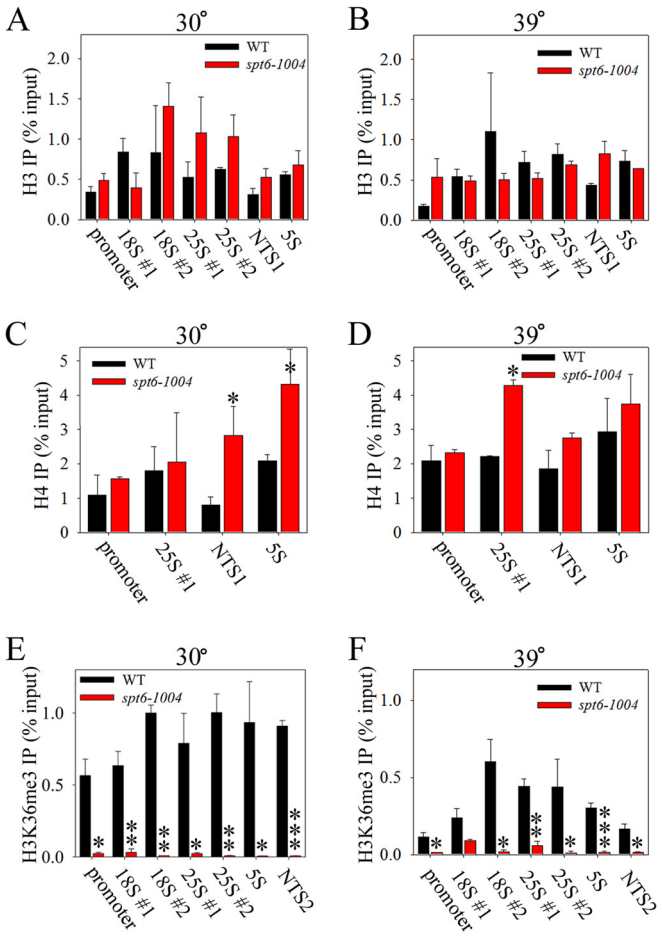

FIG 4 Core histone levels are not drastically different at the rDNA in *spt6-1004*. ChIP experiments were performed as described for Fig. 1B. H3 (A and B), H4 (C and D), and H3K36me3 (E and F) occupancy of the rDNA was measured using an anti-H3 antibody (Ab1791; Abcam), anti-H4 (Ab7311; Abcam), and anti-H3K36me3 (Ab9050; Abcam), respectively, at both 30°C and 39°C. The results of representative experiments from 3 independent trials each are shown. Standard deviations and significance were calculated from at least two technical replicates per trial. Error bars show standard deviations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

tone H3 (14, 27, 40, 41, 43, 59). Therefore, we asked whether changes in chromatin structure might account for the observed effect of Spt6 on Pol I transcription initiation.

To test this model, we probed the chromatin environment at the rDNA using ChIP. The histone H3 and H4 occupancy of the rDNA seen with the WT cells was similar to that seen with *spt6-1004* cells at 30°C (Fig. 4A and C). Minor differences between the WT and *spt6-1004* cells were observed at 39°C. For example, H3 occupancy was slightly elevated at the promoter in the *spt6-1004* strain compared to the WT (Fig. 4B). To further examine H3 occupancy of the rDNA, we analyzed previously published H3 chromatin immunoprecipitation sequencing (ChIP-seq) data comparing the WT and an *spt6* degron allele (41). The *spt6* degron was repressed at 37°C with doxycycline for 1 h, conditions that substantially reduced Spt6 protein levels. Under those conditions, the patterns of H3 occupancy across Pol II-transcribed genes were significantly altered (41); however, the levels of H3 at the rDNA in the Spt6-depleted and WT states were similar (see Fig. S8 in the supplemental material). Previous studies have shown a similar alteration of H3 occupancy at Pol II-transcribed genes between the *spt6* degron and the same allele as that used here, *spt6-1004* (27), indicating that the *spt6* degron phenotypically mimics *spt6-1004*.

We also observed a modest increase in histone H4 occupancy in the 25S region in the *spt6-1004* strain at 39°C, but the H4 levels at the promoter were the same (Fig. 4D). Given that the minor differences in ChIP signal for H3 and H4 did not overlap one another, we conclude that these data are not reflective of intact nucleosome occupancy. Taking the results together, the ChIP data for H3 and H4 certainly did not reveal systematic changes in histone occupancy across the rDNA that could explain the extreme changes in rDNA transcription observed. Although the finding that H3 occupancy of the rDNA is not reduced drastically by *spt6* mutation is in disagreement with previous observations within protein-coding genes (43), our result is in agreement with a study performed in the Lis laboratory in which *SPT6* knockdown in *Drosophila melanogaster* reduced production of Hsp70 mRNA independently of reduction in H3 levels (16). We also note that one cannot discriminate whether the minor changes in histone occupancy at the rDNA are a cause or an effect of reduced Pol I occupancy at 39°C.

Spt6 is essential for the deposition of H3K36me3 in the wake of transcription by Pol II (23, 25, 26, 32). This effect is mediated by a series of protein interactions between the Ser2-P form of Pol II's CTD, Spt6, and Set2, the methyltransferase responsible for catalysis of H3K36me2/3 (32). To test whether H3K36 methylation is affected at the rDNA, we used ChIP with an antibody specific to trimethyl H3K36. We found that the mark was not present at the rDNA in the *spt6-1004* mutant strain at either 30°C or 39°C (Fig. 4E and F), whereas it was readily detected at the rDNA in WT cells under both growth conditions.

Since rRNA synthesis was abolished in the *spt6-1004* strain only at 39°C but H3K36me3 was absent at both 30°C and 39°C, either H3K36me3 is not essential for rRNA synthesis or the modification is required only during heat shock. To test the depen-

FIG 3 Overexpression of Rrn3 partially rescues Pol I-Rrn3 association but does not rescue rRNA synthesis. (A) Rrn3-3FLAG levels were measured in WT and *spt6-1004* strains grown at 30°C or 39°C using an anti-FLAG antibody (M2; Sigma-Aldrich). Equal amounts of total protein were loaded in each lane. Dilutions are 1×, 1/2×, and 1/4×. Pgk abundance was used as a loading control and was determined using an anti-Pgk antibody (Life Technologies). A representative image is shown ($n = 4$). (B) Abundance of Rrn3-3FLAG was determined in strains carrying either pRS425 (empty vector; WT indicates DAS996 and *spt6-1004* indicates DAS1000) or pRS425 with a *TEF1* promoter driving overexpression of Rrn3-3FLAG (+RRN3; WT indicates DAS998 and *spt6-1004* indicates DAS1002). $n = 2$. (C) Association of Pol I with Rrn3 was determined via coimmunoprecipitation in the same strains as those used as described for panel B. An anti-A190 polyclonal antibody was used for IP, and the eluate was probed with an anti-FLAG antibody (M2; Sigma-Aldrich) to detect Rrn3-3FLAG. Samples in which Rrn3 was overexpressed were run separately from empty vector due to the chromosomally encoded Rrn3 signal being masked by the overexpressed signal. A representative image is shown. $n = 2$. (D) *In vivo* rRNA synthesis rates were measured as described for Fig. 1D except that a 10-min pulse was used instead of a 5-min pulse. The same strains as those processed as described for panels B and C were used and were grown in SD-Met-Leu. Only chase samples were collected and are shown here. The representative blot shown was exposed for 3 days. Error bars show standard deviations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. $n = 2$.

dence of Pol I activity on H3K36me, we used a strain expressing an H3K36A mutant as the sole source of H3. The H3K36A mutant grew similarly to the WT strain at both the permissive (30°C) and nonpermissive (37°C) temperatures (see Fig. S9 in the supplemental material). These data show that methylation of H3K36 is not essential for Pol I transcription and that loss of this mark is not responsible for the observed loss of rRNA synthesis at 39°C in the *spt6-1004* cells.

The idea of an alteration of the chromatin environment at the rDNA seemed to be a plausible hypothesis to explain the defect in recruitment of the Pol I-Rrn3 complex to the promoter. However, levels of intact nucleosomes did not change in response to *spt6-1004* mutation or depletion of Spt6. Our results also showed that, even though the presence of *spt6-1004* led to loss of H3K36me at the rDNA, this effect was not the cause of defective recruitment of Pol I. On the basis of these observations, we propose that Spt6 influences Pol I activity independently of histone modification or positioning and that Spt6 instead plays a direct positive role in transcription initiation by Pol I.

DISCUSSION

Potential roles for Spt6 in Pol I transcription. Several lines of evidence in the literature suggested that Spt6 may influence Pol I transcription in yeast. Here, we showed that Spt6 associates with the rDNA and is required for transcription by Pol I. When Spt6 is perturbed, Pol I is lost from the rDNA almost completely despite normal occupancy of the known transcription initiation factors. Although the abundance of a critical Pol I transcription factor, Rrn3, is diminished by inactivation of Spt6, restoration of Rrn3 association with Pol I did not rescue rRNA synthesis at the nonpermissive temperature. We suggest two potential mechanisms that could explain this dramatic defect. Either Spt6 plays a direct role in formation of a stable Pol I transcription complex or Spt6 is required for expression of at least one previously unidentified, essential transcription factor for Pol I.

The role of Rrn3 as a master regulator of Pol I transcription. The observation that inactivation of Spt6 led to a drastic reduction in Rrn3 levels suggested that the lack of the Pol I-Rrn3 complex was the reason for the rRNA synthesis defect. However, when the Pol I-Rrn3 complex was restored with overexpression of Rrn3, rRNA synthesis was not rescued (Fig. 3C and D). These findings suggest that the loss of Rrn3 is not causative but could instead be a consequence of the presence of an unstable or underutilized Pol I-Rrn3 complex. In this model, Spt6 (potentially together with other factors) might be required to recruit the Pol I-Rrn3 complex to the rDNA promoter. Consistent with this idea, Rrn3 mRNA levels do not change markedly in *spt6-1004* cells after a temperature shift to 39°C (43). Furthermore, it has been suggested that levels of Rrn3 and the abundance of the Pol I-Rrn3 complex are key regulators of rRNA synthesis (60). However, in the same study, the authors noted that the kinetics of inhibition of rRNA synthesis occurred much faster than the reduction in the Pol I-Rrn3 complex in response to rapamycin treatment. These observations support a model in which availability of the Pol I-Rrn3 complex is not always rate-limiting for Pol I transcription initiation. Rather, subsequent steps are used as targets for regulation under extreme conditions such as rapamycin treatment. Given the short time used for the temperature shift and the persistence of rRNA synthesis upon selective perturbation of Pol II (see Fig. S3 in the supplemental material), the idea of a direct role for Spt6 in Pol I activity is favored; however, we cannot rule out indirect effects.

Potential role for Spt6 in transcription elongation by Pol I. In our study, we showed that Spt6 localizes to active rDNA repeats (Fig. 1B). Interestingly, a recent study by Bentley and colleagues (41) examined the association of Spt6 in a genome-wide manner using chromatin immunoprecipitation with microarray technology (ChIP-chip) and concluded that Spt6 occupancy was not enriched at tRNA or rRNA genes. However, their data identified a slight increase in Spt6 binding in the promoter-proximal region of 35S rDNA compared to the transcription start site. Additionally, they observed enrichment over the input level of Spt6 in the sequence upstream of the 35S gene, which includes the rDNA promoter. Thus, our ChIP data are, at least in part, consistent with the published study, and both studies support a potential role for Spt6 in early steps of Pol I transcription.

Our ChIP data identified enrichment of Spt6 across the entire active rDNA repeat. This observation would be consistent with the model that Spt6 remains associated with Pol I past transcription initiation and therefore is poised to influence transcription elongation by Pol I. An effect on transcription elongation could explain the minor reduction in rRNA synthesis observed at 30°C. In principle, taking the rDNA copy number, the percentage of actively transcribed genes, the Pol I occupancy, and the rRNA synthesis rate into consideration, one can calculate potential effects on transcription elongation by Pol I *in vivo* (49). However, if the *spt6-1004* allele caused an elongation defect at the permissive temperature, the effect was too small to measure in this study given the error associated with each measurement. Additionally, Spt6 could serve to enhance Pol I processivity; however, our EM data did not identify a processivity defect in the *spt6-1004* strain at 30°C, and the lack of identifiable active repeats at 39°C made such an analysis impossible at the nonpermissive temperature. We also observed a slight reduction in the number of polymerases per gene at 30°C in the *spt6* mutant strain, which suggests that transcription initiation was mildly impaired. However, this defect is not large enough to explain the ~50% reduction in rRNA synthesis. In principle, Spt6 may affect both the initiation and elongation steps in transcription, but since polymerase occupancy of the rDNA is essentially lost after the temperature shift, detailed analysis of transcription elongation is difficult. Whether Spt6 potentially affects multiple steps in the cycle of transcription by Pol I is a topic for continued investigation.

Indirect effects are unlikely to account for the rRNA synthesis defect in the *spt6-1004* strain. The synthesis of rRNA is a central component in molecular biology, but the coordination of mRNA, rRNA, and tRNA synthesis is complex and incompletely understood. However, it is clear that the activities of Pol I and Pol III are connected (61). Given the cell's demand for each of these RNA components, indirect effects of *spt6-1004* on Pol I activity via changes in Pol II activity could be a reasonable explanation for the drastic reduction in rRNA synthesis.

We have addressed this possibility in two ways. First, we used a strain carrying a deletion of *RPB9*, a nonessential subunit of Pol II. Deletion of *RPB9* renders yeast temperature sensitive, with growth kinetics similar to those seen with the *spt6-1004* strain (see Fig. S3 in the supplemental material), and leads to alteration of Pol II start site selection (52). These similarities between the *spt6-1004* and *rpb9*Δ strains suggest that the *rpb9*Δ strain is a reasonable control to test whether rRNA synthesis is lost upon selective perturbation

of Pol II; however, deletion of *RPB9* did not lead to near-complete loss of rRNA synthesis like the *spt6* mutation did.

Given Spt6's previously identified role in splicing (19) and the fact that many ribosomal protein mRNAs must be spliced in *S. cerevisiae*, one might suspect that *spt6-1004* could indirectly disrupt Pol I activity via a reduction in ribosomal protein abundance. Such an effect is unlikely, since several laboratories have shown that depletion of ribosomal proteins does not inactivate transcription of the rDNA (see, e.g., reference 62). However, the possibility remains that *spt6-1004* affects expression of one or more genes with previously unidentified essential roles in Pol I activity whereas *rpb9*Δ might not.

Second, we examined previously published data in which rRNA synthesis was measured after complete inactivation of Pol II (using the *rpb1-1* allele [63]). *RPB1* is an essential gene which encodes the largest subunit of Pol II. Unlike *rpb9*Δ, *rpb1-1* causes rapid and robust degradation of Rpb1 after the shift to the nonpermissive temperature and complete loss of Pol II activity (64). Strains containing the *rpb1-1* allele are also severely temperature sensitive, to an extent greater than that seen with either the *rpb9*Δ or *spt6-1004* strain. In a study performed by Fath et al. (63), rRNA synthesis was measured by comparing the amount of newly synthesized 35S rRNA precursor to the mature 25S rRNA amount after a shift to 37°C (63). They also used a strain containing a mutation in a gene that encodes an essential Pol I subunit, *RPA43* (*rpa43-ts*), as a positive control. After 150 min at 37°C, the *rpa43-ts* strain had an rRNA synthesis rate of ~15% of the WT level, whereas rRNA synthesis in the *rpb1-1* strain was ~50% of the WT level. These data suggest that Pol II-mediated indirect effects on rDNA transcription are unlikely in the *spt6-1004* mutant strain.

Spt6 recruitment of histone modifiers. Spt6-dependent deposition of H3K36me2/3 and subsequent inhibition of cryptic Pol II initiation was described at protein-coding genes (26). Interactions between Spt6 and Pol II's CTD facilitate recruitment of Set2, the histone methyltransferase responsible for methylation of H3K36 (32). Our data confirm that H3K36me3 is found at the rDNA (65), and we show that, as seen with Pol II-transcribed genes (23), the modification is dependent on Spt6. It is unlikely that recruitment of Spt6 to the rDNA is dependent on the presence of Pol II, since Spt6 occupancy is observed even when all rDNA repeats are actively transcribed by Pol I (Fig. 1; see also references 2, 66, and 67). We favor a model in which Spt6 associates with Pol I to mediate Set2 recruitment to the rDNA. The previously observed interaction between the A43 subunit of Pol I and Spt6 suggests that direct recruitment of Spt6 by Pol I is feasible (12).

The role of H3K36me at the rDNA is unknown. It is reasonable to speculate that the modification may prevent cryptic Pol II activity within the rDNA, though it is unlikely that infrequent occupancy of the rDNA by Pol II could have a major effect on Pol I transcription given the high rDNA copy number and high Pol I density on active genes. Whatever the role of H3K36me at rDNA, it appears to be nonessential since substitution of H3 lysine 36 to an alanine does not inhibit Pol I activity (as inferred from cell growth). This substitution is thought to mimic unmodified H3K36; however, an alanine may not be a perfect mimic and residual interactions could still facilitate rRNA synthesis. With this caveat in mind, we cannot exclude the possibility that modification of H3K36, or other modifications not tested in this study, plays a role in regulation of Pol I transcription initiation.

Chromatin at ribosomal DNA loci. The repetitive and heterogeneous nature of rRNA genes presents a unique opportunity to study the characteristics of chromatin states and the mode by which "open" or "closed" conformations regulate gene expression. It is clear that a fraction of rDNA repeats is inactive (1) and that this repression is maintained through heterochromatin (68). However, the presence and nature of nucleosomes within the actively transcribed repeats are debated. EM analysis of active yeast rDNA repeats reveals a dense population of Pol I along the coding region, leaving little space within most areas of the gene for intact nucleosomes. Under conditions favoring retention of nucleosomes on yeast chromatin, regions within rDNA genes where distances between polymerases are sufficiently large to accommodate nucleosomes appear devoid of nucleosome-like particles (69). DNase and MNase sensitivity experiments also showed that the coding sequence of the rRNA genes is depleted of histones (70–72). However, ChIP experiments performed in the reduced-copy-number strain where all of the rDNA repeats were active revealed a reduced but detectible association of histones with the rDNA-coding region (73). These and other findings have led to the idea that Pol I traverses "dynamic" nucleosomes throughout the coding region. Despite the uncertainty surrounding the nature of chromatin in the active rDNA repeats, it is known that chromatin remodeling complexes help to regulate rRNA synthesis (69, 74). Our data cannot exclude the possibility that Spt6 facilitates Pol I transcription initiation through modulation of nucleosome positioning at the rDNA promoter; however, the ChIP-seq data presented in Fig. S8 in the supplemental material do not identify any obvious alterations in H3 positioning.

Spt6 in human rDNA transcription. The connection between cell growth and rRNA synthesis rate has been appreciated for many years. Not surprisingly, upregulation of rRNA synthesis and ribosome biogenesis is well documented in cancers (4, 5). Despite Pol I's essential role in regulation of cell growth and further implications for disease states, we have an incomplete picture of how Pol I activity is coordinated. This study and others aimed at understanding the basic mechanisms of Pol I activity are crucial to provide a framework for future therapies. Spt6 is conserved between yeast and humans, and knockdown of hSpt6 alters the expression of many tumor suppressor and proto-oncogenes (75). Interestingly, hSpt6 has also been shown to physically interact with SIRT7 (76), which in turn binds Pol I. Future studies will be aimed at determining whether human Pol I activity is also dependent on Spt6 and if so, at identifying the mechanism of its effect.

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