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Yeast Transcription Elongation Factor Spt5 Associates with RNA Polymerase I and RNA Polymerase II Directly*[S]

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Spt5 is a transcription factor conserved in all three domains of life. Spt5 homologues from bacteria and archaea bind the largest subunit of their respective RNA polymerases. Here we demonstrate that Spt5 directly associates with RNA polymerase (Pol) I and RNA Pol II in yeast through its central region containing conserved NusG N-terminal homology and KOW domains. Deletion analysis of *SPT5* supports our biochemical data, demonstrating the importance of the KOW domains in Spt5 function. Far Western blot analysis implicates A190 of Pol I as well as Rpb1 of Pol II in binding Spt5. Three additional subunits of Pol I may also participate in this interaction. One of these subunits, A49, has known roles in transcription elongation by Pol I. Interestingly, *spt5* truncation mutations suppress the cold-sensitive phenotype of *rpa49*Δ strain, which lacks the A49 subunit in the Pol I complex. Finally, we observed that Spt5 directly binds to an essential Pol I transcription initiation factor, Rrn3, and to the ribosomal RNA. Based on these data, we propose a model in which Spt5 is recruited to the rDNA early in transcription and propose that it plays an important role in ribosomal RNA synthesis through direct binding to the Pol I complex.

The study of ribosome biogenesis is fundamentally important. Multiple studies have connected dysregulation of the factors that affect ribosome biogenesis with cell transformation and cancer (1, 2). Transcription of ribosomal DNA (rDNA)[2] by RNA polymerase (Pol) I is the initial step of ribosome synthesis. In *Saccharomyces cerevisiae* (for simplicity referred to as "yeast"), Pol I produces the 35S rRNA precursor, which is co-transcriptionally and post-transcriptionally processed into the mature 18, 5.8, and 25 S rRNA species that form the bulk of the small and large ribosomal subunits.

Pol I consists of 14 subunits; seven of which are Pol I-specific, whereas the other seven are shared with RNA Pol II and/or RNA Pol III. Pol I transcription initiation *in vivo* requires four additional factors: TATA-binding protein, Rrn3, core factor, and the upstream activating factor. Formation of the Rrn3-Pol I complex is a crucial step in the recruitment of Pol I to the promoter. As such, this step in transcription is tightly regulated. However, the initiation step alone cannot account for the magnitude of regulation of Pol I transcription observed *in vivo* (3, 4). Recent data demonstrate that transcription elongation by Pol I is also regulated and that transcription elongation by Pol I is functionally coupled to rRNA processing (5–7). Thus, factors that influence the elongation step in transcription may play one or more important roles in overall ribosome synthesis.

Although Pol I transcription elongation remains understudied, significantly more is known about factors that affect Pol II transcription elongation. One of these factors is the conserved Spt4/5 complex. In mammalian and *Drosophila* cells, Spt4/5 induces pausing of the Pol II elongation complex in close proximity to the promoter (reviewed in Ref. 8). This pause is thought to facilitate pre-mRNA 5′-capping and can be a target for the regulation of gene expression. After release from the pause, Spt4/5 remains associated with the elongation complex and stimulates downstream transcription elongation (reviewed in Ref. 8). In yeast cells, a similar positive role for Spt4/5 in Pol II transcription has been described (9–11). However, to date no negative role for Spt4/5 in Pol II elongation has been observed in yeast.

In addition to its role in Pol II transcription, the Spt4/5 complex acts as a Pol I transcription elongation factor (12). We showed that Spt4 and Spt5 associate with Pol I and rDNA *in vivo*. Deletion of *SPT4* leads to impairment of rRNA processing and a small increase in Pol I transcription elongation rate relative to WT. We proposed that Spt4/5 inhibits Pol I transcription elongation (12). Further studies using point mutations in *SPT5* support the previous model that Spt4/5 can inhibit Pol I transcription *in vivo* (13). Interestingly, characterization of these mutants also identified a positive role for Spt5 in Pol I transcription elongation that was not evident from characterization of *spt4*Δ strains (13).

SPT5 is an essential gene conserved throughout eukarya (14, 15), and Spt5 homologues share similar domain organization (see Fig. 1*A*). The NusG N-terminal homology (NGN) domain and the KOW (Kyrpides, Ouzounis, Woese) domains of Spt5 display sequence homology and structural similarity to the N-terminal domain and the C-terminal KOW domain of the bacterial transcription factor NusG (16–19). Unlike bacterial NusG with its single KOW motif, eukaryotic Spt5 has multiple KOW domains. For example, there are four KOW motifs in yeast Spt5 (9), whereas human Spt5 (hSpt5) has six.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental text and Tables S1–S3.

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[2] The abbreviations used are: rDNA, ribosomal DNA; Pol, polymerase; NGN, NusG N-terminal homology; rRNA, ribosomal RNA; h, human; RNAP, RNA polymerase; CTR, C-terminal repeat.

Spt5 homologues from eukaryotes and prokaryotes associate with the RNA polymerase elongation complexes (9, 20–23). NusG was shown to bind directly to bacterial RNA polymerase (RNAP) through its N-terminal domain but not through its KOW domain (21). In contrast, the region containing the KOW motifs of hSpt5 was shown to bind to Pol II *in vitro* (22, 23).

In this study, we characterized the interaction of Spt5 with Pol I and Pol II in yeast. We showed that Spt5 associates with Pol I directly *in vitro* and that the central region of Spt5 (including the NGN and the KOW domains of Spt5) mediates this interaction. Identical regions associate with yeast Pol II. Far Western analysis identified four subunits of Pol I that can interact with Spt5: A190, A135, A49, and A34.5. *In vivo* analyses support our biochemical data, demonstrating the importance of the KOW domains in Spt5 function. We also observed that the cold-sensitive phenotype of *rpa49*Δ strains is suppressed by *spt5* mutations. Finally, we identified an unexpected direct association of Spt5 with the Pol I transcription initiation factor Rrn3. Together, these observations support a model in which Spt4/5 plays direct roles early in transcription elongation by Pol I.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions—The strains used in this study are listed in supplemental Table S1. The cells were grown in YEPD (for recipes see Ref. 7) at 30 °C unless otherwise indicated in the text or figure legends.

Diploid strains were sporulated in liquid sporulation medium (10 g/liter potassium acetate and 5 mg/liter zinc chloride plus required nutrients) for 5 days with mild agitation at room temperature. Tetrads were dissected using a Zeiss Axioskop 40 tetrad micromanipulator.

Chromosomal deletions leading to C-terminal truncations of Spt5 were constructed using standard PCR-based strategies (24). The nomenclature of the mutant alleles indicates the amino acid length of the corresponding proteins (*e.g. spt5*(*1–930*) expresses a His_7-HA_3-tagged protein that includes the first 930 amino acid residues of Spt5). Spt5 protein abundance was measured by Western blot analysis using an anti-HA antibody (12CA5) and compared with epitope-tagged WT Spt5 (data not shown).

The *spt5*Δ*KOW2* mutant allele carries a deletion of a region of *SPT5* that corresponds to amino acids 515–578. The *spt5*Δ*KOW2* strain bears a chromosomal deletion of *SPT5* complemented by expression of the mutant allele on a single copy CEN plasmid (supplemental Table S2) and was obtained by tetrad dissection of a diploid strain containing a heterozygous deletion of *SPT5* (*spt5*Δ::*HIS3Mx6*/*SPT5*).

Cloning of SPT4 and SPT5 into Bacterial Expression Vectors—We cloned regions of *SPT5* corresponding to the indicated domains into pET41a (Novagen). We used the predicted Spt5 domain structure from European Bioinformatics Institute Data base (EMBL-EBI) and from Ref. 9 to select our domain end points. The plasmids expressing Spt5 and Spt5 domains are described in supplemental Table S2.

For the expression of the Spt4/5 complex, *SPT4* and *SPT5* were cloned into pRSFDuet-1 and pETDuet-1 vectors (Novagen), respectively. We introduced a FLAG-His_6-TEV-HA_3-His_6 epitope tag to the C terminus of Spt5 as described in supplemental Table S2. The sequences of the primers used for the cloning of *SPT5* or *SPT4* and a detailed description of cloning strategy are listed in supplemental Table S3 and the supplemental text.

Protein Purification—Pol I and Pol II were purified using a His_7-HA_3 C-terminal epitope tag on the A135 and Rpb2 subunits, respectively, using the same strategy described previously (25). His_6-Rrn3 was expressed in *Escherichia coli* BL21(DE3) from pNOY3162 plasmid and purified as described previously (25).

Full-length Spt5 and Spt5 truncation constructs were expressed in *E. coli* BL21(DE3) as N-terminal His_6-GST fusion proteins in the presence of 0.5 mM isopropyl β-D-thiogalactopyranoside. The cells were lysed using a French Press (Thermo IEC) in TIB buffer (20 mM HEPES, pH 7.5, 400 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.05% Nonidet P-40, 1 mM DTT, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture; Roche Applied Science). The lysates were cleared by centrifugation at 20,000 × *g* for 15 min at 4 °C. The proteins were purified from the soluble fraction using nickel affinity chromatography followed by glutathione affinity purification and a second nickel affinity purification step. Equal concentrations of the proteins were used when performing GST pulldown assays or far Western blot analysis.

His_6-Spt4 and His_6-Spt5 were co-expressed in *E. coli* BL21(DE3) in the presence of 0.5 mM isopropyl β-D-thiogalactopyranoside. The Spt4/5 complex was purified from the soluble fraction of the cell extract using nickel affinity chromatography followed by anion exchange chromatography (HiTrap Q; GE Healthcare), a second nickel affinity purification step, and a final anion exchange step (Mono Q; GE Healthcare).

GST Pulldown Assay—To test for interactions between Spt5 and Pol I or Pol II, HA-tagged Pol I and Pol II were incubated in binding buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM phenylmethylsulfonyl fluoride, a protease inhibitor mixture (Roche Applied Science), 1% Nonidet P-40 (or an indicated percentage of Nonidet P-40)) with GST-tagged Spt5 (full-length or mutant constructs) immobilized on glutathione resin (GE Healthcare). To test for an interaction between Spt5 and Rrn3 or the Pol I-Rrn3 complex, we immobilized GST-Spt5 on glutathione resin in binding buffer and added either Pol I, Rrn3, or prebound Pol I-Rrn3 complex. The Rrn3-Pol I complex was formed as described previously (25) using an equimolar ratio of Pol I:Rrn3 or with excess of Rrn3 (ratios 1:3.5 and 1:5). The resin was then washed three times with binding buffer and analyzed by Western blot probed with an anti-HA antibody (12CA5) or with a polyclonal anti-Rrn3 antibody.

Far Western Blot Analysis—Far Western blot analysis was performed as described previously (26) with the following modifications. Pure Pol I and Pol II subunits were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking, the blot was incubated with equal concentration of the GST-fused probes in the probe dilution buffer (1× PBS, 0.05% Tween, 2% milk, 1 mM DTT), followed by a series of washings. We then proceeded with the standard Western blot analysis

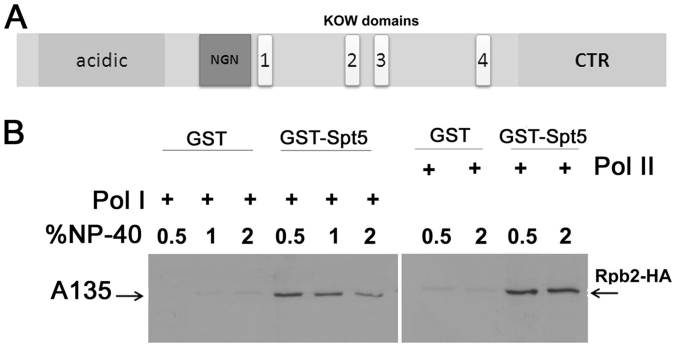

FIGURE 1. **Spt5 associates with Pol I directly.** *A*, yeast Spt5 domain organization. *B*, GST-only or recombinant Spt5 were tested for binding to the pure Pol I or Pol II complexes (as indicated by the *plus signs*). Western blot analysis was performed using anti-HA antibody (12CA5) to detect HA_3-His_7 C-terminal tags on the A135 subunit of Pol I or Rpb2 subunit of Pol II.

protocol using a polyclonal anti-GST antibody (Z-5; Santa Cruz Biotechnology).

Electrophoretic Mobility Shift Assay—The rRNA oligonucleotide (corresponding to the first 40 nucleotides of 35S rRNA) was labeled with ^{32}P at the 5′ end using T4 polynucleotide kinase (New England BioLabs). EMSA was performed as described previously (27) with modifications described in detail in the supplemental text.

RESULTS

Spt5 Associates with Pol I Directly in Vitro—Spt4 and Spt5 form a heterodimer that influences transcription elongation by Pol I (12). Previously, we observed a physical interaction (co-purification and co-immunoprecipitation) of these proteins with Pol I. To determine whether this association is direct or mediated through a network of other proteins, we tested for binding of Spt5 to Pol I *in vitro*.

We expressed yeast Spt5 with an N-terminal GST tag in *E. coli* and purified the protein using three affinity steps. Endogenously expressed Pol I bearing an HA tag on the A135 subunit was purified from yeast. These proteins were used to perform a GST pulldown assay in which Spt5 was immobilized on glutathione-Sepharose resin and incubated with Pol I. After multiple washes, bound Pol I was detected by Western blot analysis using an anti-HA antibody (12CA5). As a positive control, we included HA-tagged Pol II purified from yeast. Human Pol II has been shown previously to directly bind hSpt5 (22, 23), and as expected (9), yeast-derived Pol II directly associated with yeast Spt5 (Fig. 1*B*). Consistent with our results, the Cramer lab (28) has recently shown that recombinant yeast Spt4/5 binds directly to pure Pol II. As for Pol II, we observed specific association of Pol I with GST-Spt5 but not GST-only (Fig. 1*B*). Thus, Spt5 directly and stably binds to Pol I.

The Central Region of Spt5 Containing the NGN and the KOW Domains Binds to Pol I in Vitro—To identify the domain(s) of Spt5 that bind to Pol I and/or Pol II, we constructed a series of Spt5 variants fused to GST and purified these constructs from *E. coli* (Fig. 2, *A* and *B*). After three affinity purification steps, these proteins were used in GST pulldown assays with either yeast-derived Pol I or Pol II. After multiple washing steps, the bound polymerases were detected by Western blot analysis (Fig. 2, *C* and *E*).

The same regions of Spt5 are required for binding to either Pol I or Pol II (Fig. 2, *C* and *E*). The N-terminal region containing the NGN domain (construct B) binds to Pol I, although the amount of polymerase retained on the beads is less than in the presence of the full-length Spt5 (Fig. 2*F*). Because the first 244 amino acid residues do not bind to polymerases (construct A; Fig. 2, *C–E*), we conclude that the NGN domain mediates the detected association with Pol I and Pol II. This observation supports the model that the interaction of the NGN domains with RNA polymerases is conserved in all three domains of life (20, 21, 28, 29).

The mutant proteins that contained the central region of Spt5 including the KOW domains and related sequences (*e.g.* the linkers between the KOW motifs) but that lacked the NGN domain (constructs D and E) also associated with both polymerases (Fig. 2, *C* and *D*). However, the binding of these constructs to Pol I was not as robust as for the full-length protein (Fig. 2*F*). Indeed, binding of both polymerases to Spt5 was more robust when the immobilized construct contained a larger region including the NGN domain and at least two KOW domains (constructs C, G, H, J, and K). These constructs retained as much Pol I or Pol II as full-length Spt5. Therefore, we conclude that unlike bacteria and archaea, in eukaryotes the NGN domain of Spt5 is not sufficient for robust association with RNA polymerases. Instead, the additional sequences, most likely the KOW domains, appear to enhance the association of Spt5 with both Pol I and Pol II. These data demonstrate that the association of Spt5 with RNA polymerases is conserved but apparently more complex in eukaryotes compared with prokaryotes, consistent with the more complex domain structure of eukaryotic Spt5.

KOW Domains Are Important for Survival—We have shown that the central region including the NGN and at least two KOW domains is important for robust association of Spt5 with both polymerases *in vitro*. Recent data demonstrated that the NGN domain and the KOW domains of Spt5 are essential for survival (30). However, because the presence of all four of the KOW domains is not required for binding to Pol I and Pol II *in vitro*, we predicted that deletion of one or more KOW motifs would not be lethal in yeast.

To test our hypothesis and to verify our biochemical data *in vivo*, we constructed a variety of *spt5* mutant strains carrying either C-terminal truncations or an internal deletion of KOW2 (Fig. 3*A*). These strains were tested for viability under different conditions. We confirmed previously published data that the CTR region of Spt5 is not essential in yeast (31). The mutants *spt5*(*1–930*) and *spt5*(*1–886*) have mild growth defects at permissive temperatures (30 and 25 °C) and cold-sensitive phenotypes (slow growth at 16 °C) (Fig. 3*B* and data not shown). Interestingly, deletion of KOW4 together with the CTR deletion (allele *spt5*(*1–797*)) is viable. This strain also exhibits mild growth defects at 30 °C (Fig. 3*B*) and more severe cold sensitivity than *spt5*(*1–930*) and *spt5*(*1–886*). Spt5 abundance in all three mutant strains was not altered compared with WT (measured by Western blot; data not shown). These results are consistent with our biochemical data showing that the region containing KOW4 and/or CTR is not required for binding of Spt5 to Pol I or Pol II *in vitro* (Fig. 2).

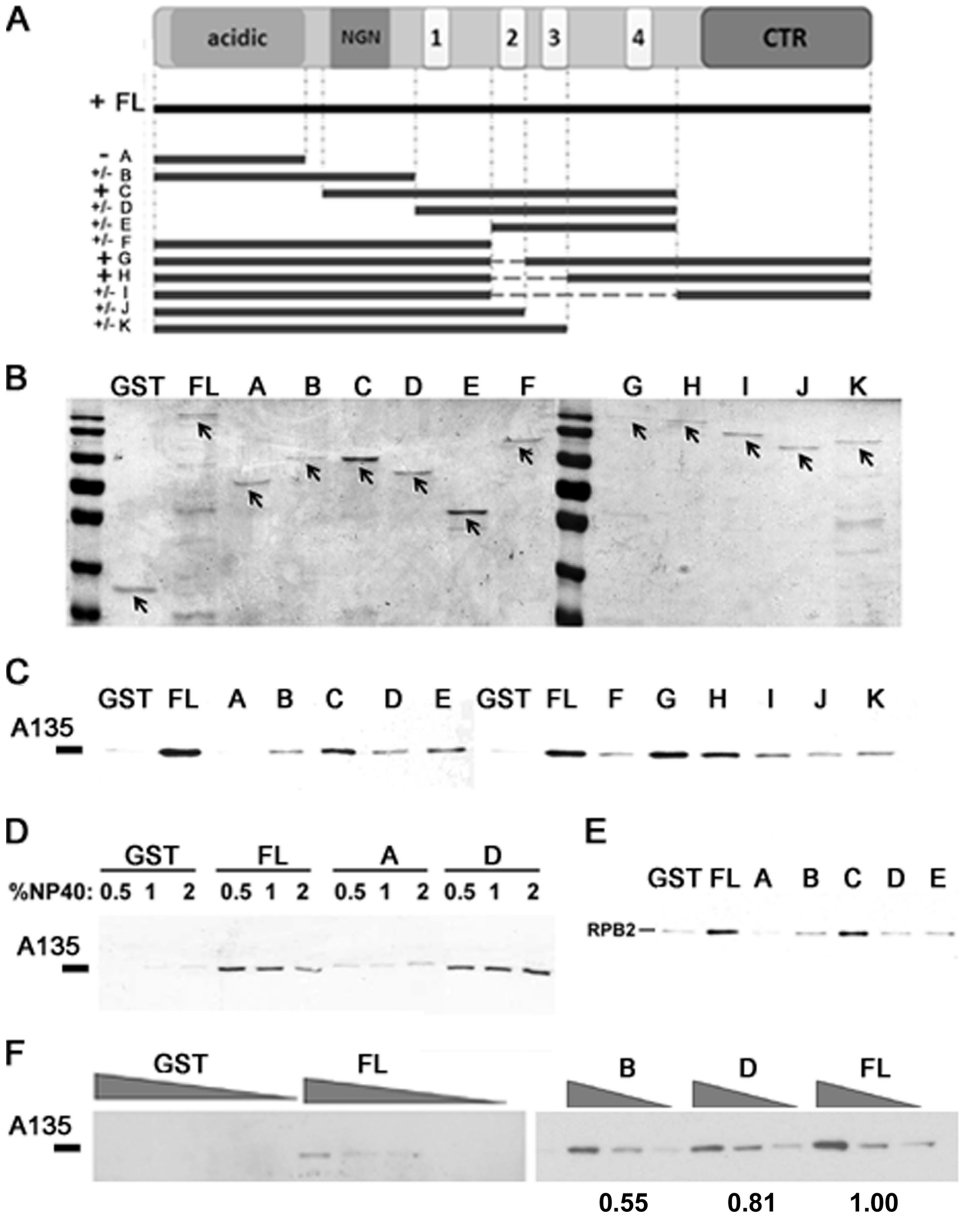

FIGURE 2. **The central region of Spt5 (including the NGN and KOW domains) binds to Pol I *in vitro*.** *A*, a schematic representation of Spt5 constructs used in the study. *Dashed lines* correspond to end points of individual constructs. *Plus signs* indicate binding to Pol I. *B*, the Spt5 constructs were expressed in *E. coli* and purified using a three-step purification. The proteins were visualized with Coomassie Blue. *C*, the protein constructs A–K were tested for the association with Pol I. Bound Pol I was detected using Western blot analysis with anti-HA antibody. *D*, Pol I bound to the construct D, but not to the construct A or GST-only control in variable concentrations of Nonidet P-40. *E*, the Spt5 constructs A–E were tested for the association with Pol II. Western blot analysis with anti-HA antibody was used to detect bound Pol II. Association of constructs F-K with Pol II was similar to that with Pol I (data not shown). *F*, semi-quantitative analysis of Pol I association with Spt5 constructs B and D in comparison with the full-length Spt5. We used 1 μM of either GST-Spt5 (full length and constructs B or D) or GST-only and incubated with 3-fold serial dilutions of Pol I (starting from 0.5 μM). Bound Pol I was detected as in *C* and quantified using Quantity One software (Bio-Rad).

A mutant allele of *SPT5* that contains an internal deletion of the KOW2 region (*spt5*Δ*KOW2*) is also viable. However, this strain has the most severe phenotype among the described non-lethal *spt5* mutants exhibiting very slow growth at 25 °C (Fig. 3*B*) and lethality at 30 °C. Both *spt5*Δ*KOW2* and *spt5*(*1–797*), have one of the KOW domains deleted; however, the phenotype of the *spt5*Δ*KOW2* allele is significantly more severe than that of the *spt5*(*1–797*) mutant. We conclude that KOW2 is more critical for Spt5 function than KOW4 and the CTR combined. The observation that *spt5*(*1–797*) and *spt5*Δ*KOW2* strains are viable supports our biochemical data indicating that fewer than four KOW domains is sufficient for interaction with RNA polymerases (constructs J, H, and K from Fig. 2).

We were not able to isolate cells carrying deletions of two or more KOW domains. Spores carrying *spt5*(*1–582*), which lacks the C-terminal region including CTR, KOW4, and KOW3, do not grow at 30 or 25 °C (Fig. 3*B*). Even expansion of the C-terminal deletion to include the 188-amino acid residue linker between KOW3 and KOW4 (allele *spt5*(*1–610*)) is lethal (Fig. 3*B*). These data are consistent with previously observed lethality of an *spt5*(*1–640*) allele (30). To determine whether these lethal *spt5* constructs could still associate with Pol I, we exam-

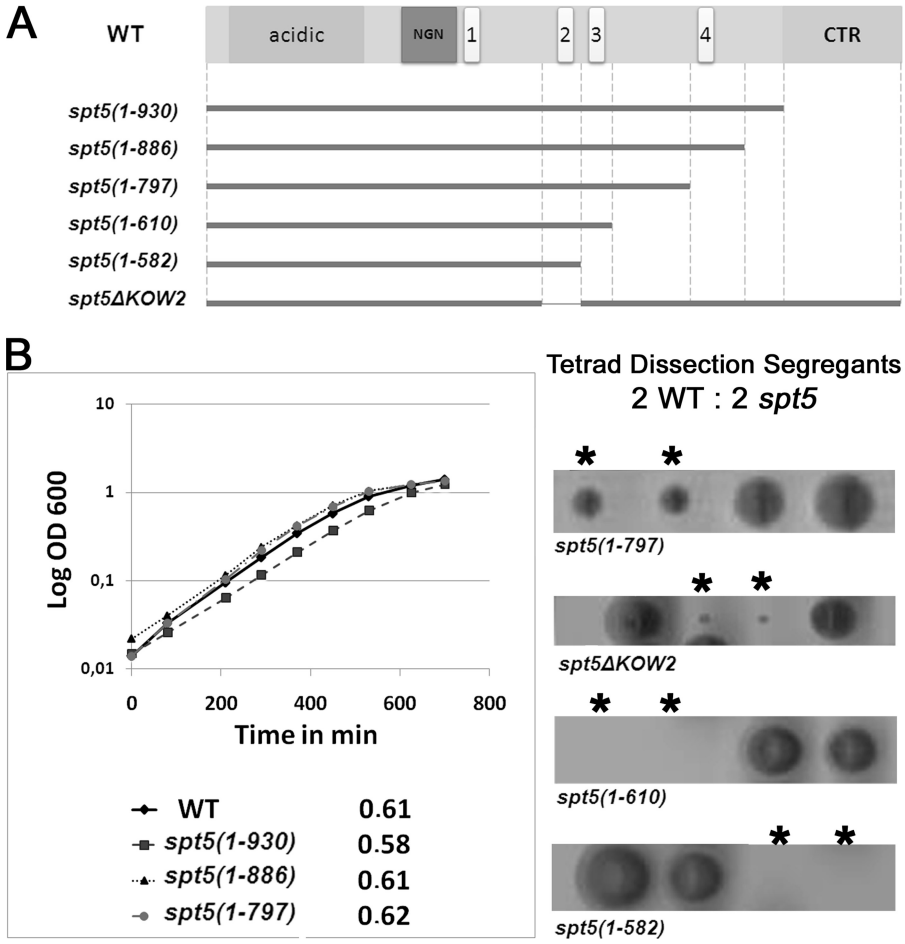

FIGURE 3. **KOW domains are important for survival.** *A*, schematic representation of the *spt5* mutant alleles constructed for *in vivo* studies. *B*, growth curves for WT, *spt5*(*1–930*), *spt5*(*1–886*), and *spt5*(*1–797*) strains at 30 °C are shown with the doubling time indicated (*below plot*). Individual haploid segregants from heterozygous *spt5/SPT5* diploid strains are shown on the *right*. Each tetrad yielded two WT spores and two *spt5* mutants (indicated by an *asterisk*). The *spt5ΔKOW2* strain was incubated at 25 °C, and three other strains were grown at 30 °C. The pictures were taken on the fourth day for the *spt5*(*1–797*) strain and on the seventh day for the rest of the strains.

ined their expression and co-immunoprecipitation with the polymerase in heterozygous diploid cells. We did not detect any significant difference in the abundance of these truncated Spt5 proteins compared with the full-length protein when expressed in heterozygous diploids (*spt5*(*1–582*)/*SPT5* or *spt5*(*1–640*)/*SPT5*; measured by Western blot analysis; data not shown). Moreover, truncated Spt5 expressed in the *spt5*(*1–640*)/*SPT5* diploid strain was still able to associate with Pol I in co-immunoprecipitation experiments (data not shown), consistent with our *in vitro* pulldown assays (Fig. 2). These data demonstrate that the lethality of the *spt5*(*1–582*) and *spt5*(*1–610*) alleles is most likely due to defects in Spt5 function rather than impaired protein folding or defects in the binding of Spt5 to the polymerases.

Our analysis of *spt5* deletion mutants shows that cells with minor disruption of the region containing KOW domains can survive. However, larger deletions within the KOW domains are lethal. These results support the functional importance of the KOW domains *in vivo* and are consistent with our *in vitro* data.

Multiple Subunits of Pol I Associate with Spt5 in Vitro—Because we have shown that Spt5 physically interacts with Pol I, we wished to determine which subunits of Pol I are involved in the interaction. To do this, we used far Western blot analysis. Blots carrying purified Pol I and Pol II were probed with three different Spt5 constructs fused to GST (Fig. 4*A*) and with purified GST as a negative control. Binding of the probes was detected using an anti-GST antibody (Fig. 4, *B* and *C*). A duplicate lane from the original SDS-PAGE gel was stained with silver nitrate to determine the identity of the polymerase subunits. We detected binding of all three Spt5 probes to the largest subunits of both Pol I and Pol II: A190 and Rpb1 (Fig. 4, *B* and *C*). The A190 subunit of Pol I is homologous to Rpb1 and to the β' subunit of prokaryotic RNA polymerases (32). The NGN-like domains in bacteria and archaea are known to bind to the β' subunit or its homologues (20, 28, 29, 33). Rpb1 of human Pol II was also shown to bind to human Spt5 (22), and we confirmed that interaction for yeast Pol II (Fig. 4*B*). Thus, the observed interaction between Spt5 and the largest subunit of Pol I is conserved.

Two of three Spt5 probes (probes C and D) also associated with three additional Pol I subunits: A135, A49, and A34.5. The third probe (probe F) associated with A135 and A34.5 in addition to A190. Binding of the probe F to the A49 subunit was not detected. Thus, the NGN domain is not sufficient for association with A49.

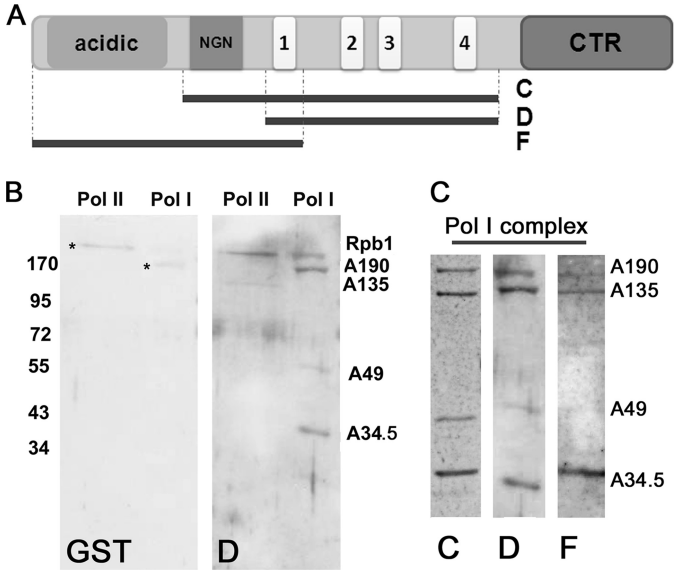

FIGURE 4. **Spt5 associates with four Pol I subunits *in vitro*.** *A*, schematic representation of the Spt5 constructs (constructs C, D, and F, the same as in Fig. 2*A*) used as probes in far Western blot analysis. *B*, binding of construct C or the GST-only probe (*GST*) to Pol I and Pol II subunits was tested by far Western blot analysis. The bands detected after the incubation with the probe C correspond to the Rpb1 subunit of Pol II and to the A190, A135, A49, and A34 subunits of Pol I. The *asterisks* show weak nonspecific binding of the GST-only probe to Rpb1 and A135. *C*, association of the probes C, D, and F with Pol I subunits was tested by far Western blot analysis as in Fig. 4*B*.

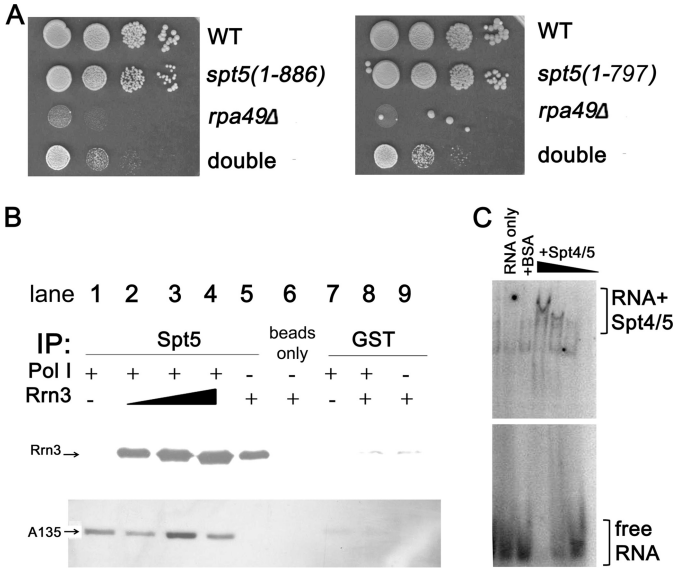

FIGURE 5. **Spt5 interacts physically and genetically with Pol I transcription machinery.** *A*, *spt5*(*1–886*) (on the *left*) and *spt5*(*1–797*) (on the *right*) alleles partially suppress *rpa49*Δ cold-sensitive phenotype. The strains were plated on YEPD medium as serial 10-fold dilutions and incubated at 25 °C. The pictures were taken on the sixth day. Several spontaneous suppressors can also be observed as bigger sized colonies for *rpa49*Δ but not for the double mutants. *B*, Spt5 (*lanes 1–5*) or GST-only (*lanes 7–9*) immobilized on glutathione resin were tested for interaction with either Pol I (*lane 1*), Rrn3 (*lane 5*), or Pol I-Rrn3 (*lanes 2–4*) complex (as indicated by the *plus signs above* the *lanes*). Rrn3 was incubated with the glutathione resin without GST or Spt5 as an additional negative control (*lane 9*). Bound Rrn3 and Pol I were detected by Western blot analysis using anti-Rrn3 or anti-HA antibody, respectively. *C*, Spt4/5 binding to an rRNA oligonucleotide was tested using EMSA. Free RNA and RNA-Spt4/5 complexes visualized by autoradiography are indicated. *IP*, immunoprecipitation.

A135 is the second largest subunit of Pol I and, together with A190, forms the catalytic core of the polymerase. The A34.5 and A49 subunits associate with one another and act as an intrinsic elongation factor (34). Although all three probes associated with the A34.5 subunit, the binding to A34.5 might be a false positive. A34.5 bears a lysine-rich region at its C terminus, which can lead to robust nonspecific interactions *in vitro*.

Our data suggest that Spt5 is capable of binding to the largest subunits of Pol I, as well as subunits that specifically participate in transcription elongation. Thus, it is likely that Spt5 associates with Pol I through multiple contacts, mediating its direct effect on transcription elongation.

*Mutations in SPT5 Suppress the rpa49*Δ *Phenotype*—Mutations in *SPT5* have been shown previously to interact with mutations in genes encoding Pol II subunits or Pol II transcription factors (9, 11, 31). We therefore tested for genetic interactions of *spt5* alleles with mutations in Pol I. *RPA49* encodes the A49 subunit of Pol I and is not an essential gene (35). Pol I lacking the A49 subunit is defective in transcription initiation and elongation (34, 36). We observed that Spt5 has affinity for A49 in far Western blot analysis (Fig. 4). Because Spt4/5 can affect Pol I transcription elongation (12, 13), we tested for genetic interactions between *rpa49*Δ and multiple *spt5* alleles.

We created double mutants bearing *rpa49*Δ combined with either *spt5*(*1–930*), *spt5*(*1–886*), or *spt5*(*1–797*) alleles. Deletion of *RPA49* results in cold sensitivity with limited or no growth at 25 °C (37). However, when *spt5* mutations were combined with *rpa49*Δ, the double mutants supported growth at 25 °C (Fig. 5*A* and data not shown). We found that all of the *spt5* alleles partially suppress the cold-sensitive phenotype of *rpa49*Δ. Furthermore, other recently isolated *spt5* alleles and *spt4*Δ suppressed the cold sensitivity of *rpa49*Δ (13). Thus, genetic interactions between *RPA49* and *SPT5* or *SPT4* support the model that Spt4/5 influences Pol I transcription elongation. Suppression of the *rpa49*Δ phenotype by *spt5* alleles is consistent with previously characterized negative roles for the Spt4/5 complex in Pol I transcription (12, 13). Although the molecular mechanisms of the observed suppression remain to be discovered, they are likely to be direct and may involve physical interactions of Spt5 with A49.

Spt5 Directly Associates with Rrn3 and rRNA—Spt5 is known to associate with both the 35S rRNA coding region and its promoter region (12). We hypothesize that Spt5 is recruited to the Pol I complex soon after transcription initiation occurs. Although Spt5 directly binds to Pol I, the Pol I initiation factors or the RNA transcript could also participate in the early recruitment of Spt5 to the rDNA. To test this hypothesis, we examined the interaction of Spt5 with the Pol I initiation factor Rrn3 and with rRNA.

Rrn3 associates with Pol I and escorts the polymerase to the promoter. After initiation of transcription, Rrn3 is released from the complex (3, 38, 39). Co-immunoprecipitation experiments showed that Spt5 and Rrn3 interact with each other *in vivo* (data not shown). Because both Spt5 and Rrn3 are known to bind to the Pol I complex, we tested whether there is a direct association between recombinant Rrn3 and Spt5 *in vitro*. We observed direct binding of recombinant Rrn3 to recombinant Spt5 in a GST pulldown assay (Fig. 5*B*, *lane 5*).

Because we detected a physical interaction of Spt5 with Rrn3, we tested for genetic interactions between *spt5* and *rrn3* muta-

tions. We created double mutants carrying either *spt5*(*1–886*) or *spt5*(*1–797*) allele with *rrn3*(*S213P*). The *rrn3*(*S213P*) mutation reduces the Pol I transcription initiation rate under non-permissive conditions (37 °C) (3). The double mutants showed an additive phenotype under all conditions tested (30, 25, 33, or 37 °C and media with addition of 6-azauracil), indicating an absence of epistatic interactions between the alleles tested (data not shown).

Thus, Spt5 directly associates with Rrn3; however, we do not detect a significant affect of Spt5 on Pol I transcription initiation (no genetic interactions between *RRN3* and *SPT5* and data from Refs. 12 and 13). We hypothesize that the observed physical interaction of Spt5 with Rrn3 contributes to the recruitment of Spt5 to the Pol I complex at the rDNA promoter rather than to the participation of Spt5 in Pol I transcription initiation.

The Spt4/5 complex was shown to require a nascent transcript longer than 18 nucleotides for efficient recruitment to the reconstituted *Drosophila* Pol II elongation complex *in vitro* (40). We hypothesized that the synthesis of the nascent transcript by Pol I might also contribute to Spt5 recruitment. We tested for binding of recombinant Spt4/5 complex to an rRNA oligonucleotide using an EMSA. An oligonucleotide corresponding to the first 40 bp of 35S rRNA labeled with ^{32}P at the 5′ end was used in the assay. To separate the bound RNA complexes from free RNA, we performed native PAGE. We detected a slow migrating RNA band in the presence of the Spt4/5 but not in the presence of BSA control. The association of the rRNA oligonucleotide with the Spt4/5 complex was concentration-dependent (Fig. 5*C*). These data confirm that Spt4/5 is an RNA-binding complex, and the rRNA transcript could aid recruitment of Spt4/5 to the early elongation complex. Taken together, we have identified three factors present in the early Pol I elongation complex that directly bind to Spt5 and might contribute to recruitment of Spt5 to the rDNA: Pol I, Rrn3, and rRNA.

DISCUSSION

We have shown that Spt5 interacts with Pol I directly *in vitro* through its central region containing conserved NGN and KOW domains and can associate with four subunits of Pol I: A190, A135, A49, and A34. Moreover, we confirmed our biochemical data using *spt5* mutations and genetic interactions of *SPT5* with *RPA49*. We also detected direct binding of Spt5 to Rrn3 and to rRNA, leading to the hypothesis that these factors together with Pol I might participate in recruitment of Spt5 to the rDNA. All of these data support the model that Spt5 is recruited to the rDNA early in transcription and that it plays important direct roles in rRNA synthesis.

The Direct Association of Spt5 with Pol I—The first evidence for a physical interaction between Spt5 and Pol I was demonstrated in a mass spectrometry screen for proteins that co-purified with Spt5 (11). Later we confirmed that Spt4 and Spt5 co-purified with the Pol I complex from growing yeast cells (12). Whether this interaction was direct or mediated by a network of other proteins remained undefined.

In this study, we have shown that Spt5 directly binds to Pol I *in vitro*. This is the first demonstration of a direct interaction of Spt5 with Pol I. Based on our previous data and the results obtained here, we propose that direct association of Spt4/5 with Pol I is important for transcription of rDNA *in vivo*.

Interestingly, the prokaryotic homologue of Spt5, NusG, is known to participate in the control of rRNA operon transcription elongation and anti-termination in *E. coli* through direct binding to the RNAP elongation complex (41). Although the overall mechanisms by which NusG and Spt5 control synthesis of rRNA are quite different, they certainly share some common biochemical properties.

The Roles of the NGN and the KOW Domains of Spt5 in Polymerase Binding—The binding of hSpt5 to Pol II was shown to be mediated by the central region of hSpt5 containing the KOW motifs (22, 23). In our study, we also observed that the region containing KOW domains and related sequences (*e.g.* the linker regions between the KOW motifs) of yeast Spt5 is sufficient for its interaction with Pol I and Pol II. The most likely interpretation of these data is that the conserved KOW domains are necessary for direct interaction with the polymerases; however, we cannot exclude the possibility that other motifs from the central region are crucial for this binding.

We also concluded that both polymerases associate with the NGN domain of Spt5 in yeast. These data are consistent with the known association of the homologous N-terminal domain of NusG and of the NGN domain of archaeal Spt5 with their respective RNA polymerases (20, 21, 28, 29). We propose that the association of the NGN domain of hSpt5 with Pol II is also conserved, although it was not defined in previous studies (22, 23). The most likely explanation for this potential discrepancy is that the homology of NusG and Spt5 through the KOW motifs was clear upon initial characterization of Spt5 (9, 22, 23), whereas the homology of the N-terminal domain of NusG and Spt5 through the NGN domain was defined later (16). Thus, the constructs containing the N-terminal region of hSpt5 with the full-sized NGN domain were absent in the pulldown assays with human Pol II in both previous studies (22, 23).

The most robust association of Spt5 with Pol I or Pol II *in vitro* was observed with constructs containing the central region of Spt5 including the NGN domain and at least two KOW domains. In addition, these regions (the NGN domain, the KOW domains as well as the intervening amino acids) are important for cell viability (Fig. 3 and Ref. 30). We propose that the interaction of the NGN domain with RNA polymerases is conserved between prokaryotes and eukaryotes. Based on the known roles of the N-terminal domain of NusG in RNAP elongation, we speculate that the homologous NGN domain of Spt5 participates in the direct modulation of transcription elongation by Pol I and Pol II. However, unlike prokaryotes, the binding of the eukaryotic Spt5 proteins to RNA polymerases is additionally stabilized by interactions with the multiple KOW domains. These domains may also play critical roles in recruitment of multiple other eukaryotic transcription elongation and RNA biogenesis factors.

Our model for Spt5-Pol I interaction is consistent with the predicted Spt5-Pol II association recently published by the Cramer lab (28). Cramer and co-workers predicted that the NGN domain of eukaryotic Spt5 contacts Pol II above the active center cleft, and the flexible KOW1 domain is located between the top of the clamp and the wall. The details of association

between full-length Spt5 and Pol II are still not known, but the extended eukaryote-specific region including the rest of the KOW domains and the CTR could reach any position on the Pol II surface (28).

Interaction of Spt5 with RNA Polymerase Subunits—NusG homologues in bacteria and archea were shown to interact with the largest subunits of their cognate RNA polymerases (20, 28, 29, 33). The homologues of the largest subunit of prokaryotic RNAP in eukaryotic RNA polymerases are A190, Rpb1, and Rpc1 (for Pol I, Pol II, and Pol III, respectively) (32). Rpb1 was shown to associate with hSpt5 in far Western blot analysis (22). Using the same approach, we confirmed these data for yeast Spt5 and Rpb1. We further observed that Spt5 associates with the A190 subunit of Pol I. Surprisingly, we also detected association of Spt5 with three additional Pol I-specific subunits: A135, A49, and A34.5. In agreement with these data, three of the four Pol I subunits identified here (A190, A135, and A49) were also previously identified in the mass spectrometric analysis of the Spt5-associated proteins (11). As mentioned, the A49 and A34.5 subunits, which form a subcomplex within the Pol I complex, have intrinsic elongation activity (34). If the observed interaction of Spt5 with these two subunits occurs *in vivo*, Spt5 could directly modulate the effect of A49/A34.5 on Pol I transcription elongation. In support of this model, we detected genetic interactions between *RPA49* and *SPT5*.

A Proposed Model for the Roles of Spt5 in Transcription by Pol I—We have previously shown that Spt4/5 plays a role in Pol I transcription elongation and rRNA processing (12). We observed negative effects of Spt4/5 on Pol I transcription previously (12); however, recent studies revealed dual (negative and positive) roles for Spt4/5 in rRNA synthesis (13).

Based on the multiple functions of Spt4/5 in cells, it is hard to differentiate direct *versus* indirect effects of Spt4/5 on Pol I transcription. This study demonstrates that Spt5 binds to Pol I *in vitro*, supporting a direct role for the Spt4/5 complex in rRNA synthesis *in vivo*.

Spt4 and Spt5 are associated with the rDNA coding sequence as well as the promoter (12). In this study, we have shown that Spt5 binds the initiation factor Rrn3. However, none of the previous studies identified significant Spt4/5-mediated effects on Pol I transcription initiation *in vivo* (12, 13). In support of these data, we also did not detect any epistatic interactions between *SPT5* and *RRN3*. Thus, we speculate that the interaction of Spt5 with Rrn3 facilitates the recruitment of Spt5 to the rDNA near the promoter.

Based on our previous data and the results of this study, we propose a model for the interactions of Spt4/5 with the rRNA synthesis apparatus. Pol I transcription initiation occurs when a Pol I-Rrn3 complex is recruited to the rDNA promoter; soon after initiation, Rrn3 is released from the Pol I complex. During the transition from initiation to elongation (while Rrn3 is still bound to Pol I), Spt4/5 is recruited to the rDNA by virtue of its interactions with Rrn3, the nascent rRNA transcript, and Pol I. Thus, it appears Spt5 is engaged by the transcription machinery during the initiation or early elongation step. This model is supported *in vivo* by co-immunoprecipitation and ChIP data showing Spt4/5-Rrn3 interaction and co-localization of these factors at the rDNA promoter (3, 12, 36). After the release of Rrn3, Spt4/5 stays associated with the elongation complex to modulate rRNA synthesis.

Spt5 can inhibit as well as promote Pol I transcription elongation. Our studies of *spt5* mutants and observed genetic interactions confirm the importance of Spt5 functions for efficient rRNA synthesis and proper rRNA processing *in vivo* (12, 13). Based on the recent structural data for the archeal Spt5-RNAP complex (28, 29), Spt5 is thought to lock the RNA-DNA hybrid of the transcription bubble in the cleft of the polymerases increasing the elongation complex stability. Taking the homology between the multisubunit polymerases as well as between various Spt5/NusG homologues into account, this function of Spt5 is very likely to be true in the Pol I system as well. The exact mechanisms by which Spt4/5 affects of Pol I elongation remain to be elucidated.

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Yeast Transcription Elongation Factor Spt5 Associates with RNA Polymerase I and RNA Polymerase II Directly

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