Elongating RNA polymerase II is targeted by macromolecular assemblies that regulate mRNA synthesis and processing. The capping apparatus is the first of the assemblies to act on the nascent pre-mRNA. Although recruitment of the capping enzymes to the transcription complex is dependent on phosphorylation of the C-terminal domain of the Rpb1 subunit of polymerase II (Pol-II), there may be additional levels of control that coordinate capping with elongation. Here we show that the triphosphatase (Pct1) and guanyllytransferase (Pce1) enzymes of the capping yeast capping apparatus bind independently to the elongation factor Spt5. The C-terminal domain of the 990-amino acid Schizosaccharomyces pombe Spt5 protein, composed of repeats of a nonapeptide motif (consensus sequence TPAWNSGSK), is necessary and sufficient for binding to the capping enzymes in vivo (in a two-hybrid assay) and in vitro. As few as four nonamer repeats suffice for Spt5 binding to Pct1 in vitro, whereas six repeats are required for Spt5 binding to Pce1. A 116-amino acid fragment of the guanyllytransferase Pce1 suffices for binding to the Spt5 C-terminal domain (CTD) but not for binding to the Pol-II CTD. Pct1 and Pce1 can bind simultaneously to the Spt5 CTD in vitro. We find that Spt5 is essential for viability of S. pombe and that it interacts in vivo with S. pombe Spt4 via a central domain distinct from the Spt5 CTD. We suggest that Spt5-induced arrest of elongation at promoter proximal positions ensures a temporal window for recruitment of the capping enzymes.

mRNA capping occurs cotranscriptionally by a series of three enzymatic reactions in which the 5’ triphosphate terminus of the pre-mRNA is cleaved to a diphosphate by RNA triphosphatase, then capped with GMP by RNA guanyllytransferase, and methylated at the N7 position of guanine by guanilylttransferase (1). Targeting of cap formation to the pre-mRNA is cleaved to a diphosphate by RNA triphosphatase (2–5). The CTD of the largest subunit of Pol-II (Pol-II), there may be additional levels of control that coordinate capping with elongation. Here we show that the triphosphatase (Pct1) and guanyllytransferase (Pce1) enzymes of the capping yeast capping apparatus bind independently to the elongation factor Spt5. The C-terminal domain of the 990-amino acid Schizosaccharomyces pombe Spt5 protein, composed of repeats of a nonapeptide motif (consensus sequence TPAWNSGSK), is necessary and sufficient for binding to the capping enzymes in vivo (in a two-hybrid assay) and in vitro. As few as four nonamer repeats suffice for Spt5 binding to Pct1 in vitro, whereas six repeats are required for Spt5 binding to Pce1. A 116-amino acid fragment of the guanyllytransferase Pce1 suffices for binding to the Spt5 C-terminal domain (CTD) but not for binding to the Pol-II CTD. Pct1 and Pce1 can bind simultaneously to the Spt5 CTD in vitro. We find that Spt5 is essential for viability of S. pombe and that it interacts in vivo with S. pombe Spt4 via a central domain distinct from the Spt5 CTD. We suggest that Spt5-induced arrest of elongation at promoter proximal positions ensures a temporal window for recruitment of the capping enzymes.

The Pol-II CTD is composed of a tandemly repeated heptad motif (consensus sequence YSPTSPS), which undergoes a cycle of extensive serine phosphorylation and dephosphorylation during the transcription cycle.

In the budding yeast Saccharomyces cerevisiae, the guanyllytransferase and methyltransferase components of the capping apparatus bind independently in vitro and in vivo to the phosphorylated Pol-II CTD (2, 6). The S. cerevisiae RNA triphosphatase does not bind to the Pol-II CTD by itself (7), but it does bind to the guanyllytransferase (8–10). The mammalian capping enzyme (Mce1) is a bifunctional polypeptide composed of an N-terminal RNA triphosphatase domain linked to a C-terminal guanyllytransferase domain. The guanyllytransferase domain per se binds to Pol-II CTD-Po4, but the triphosphatase domain does not (5, 11). The fission yeast Schizosaccharomyces pombe employs a unique strategy of cap targeting whereby the triphosphatase (Pct1) and guanyllytransferase (Pce1) enzymes of the capping apparatus are not associated physically with each other (as they are in budding yeast and metazoans) but instead bind independently to the phosphorylated Pol-II CTD (12).

Although interactions between Pol-II and capping enzymes offer a satisfying explanation for the specific capping of nascent pre-mRNAs, it is conceivable that other factors are also involved in linking capping to transcription. For example, Wen and Shatkin reported that hSpt5, the human homolog of yeast transcription elongation factor Spt5, interacts directly with the mammalian capping enzyme (13). hSpt5 translated in vitro binds independently to the N-terminal triphosphatase and C-terminal guanyllytransferase domains of Mce1. Human immunodeficiency virus (HIV) employs yet another mechanism to recruit the capping enzyme to the HIV transcription unit, whereby the viral Tat protein binds directly and independently to the triphosphatase and guanyllytransferase domains of Mce1 and up-regulates both catalytic activities (14).

Spt5 and Tat are intimately connected to the regulation of HIV gene expression. hSpt5 (a 1087-amino acid polypeptide) and its binding partner hSpt4 (a 117-amino acid polypeptide) comprise the transcription elongation regulatory factor DSIF (DRB sensitivity inducing factor) (15, 16). DSIF binds to Pol-II and, in concert with the negative elongation factor (NELF), represses elongation at promoter-proximal positions in the transcription unit (17, 18). Escape from the repressive effect of DSIF/NELF requires the action of P-TEFb (Positive Transcription Elongation Factor b), a DRB-sensitive protein kinase that phosphorylates both the CTD of the largest subunit of RNA polymerase II and the Spt5 subunit of DSIF (19–23). P-TEFb is composed of two subunits, Cdk9 and cyclin T, and it binds to the HIV Tat protein via the cyclin T subunit (20). In the case of HIV, it has been suggested that: (i) Spt5-induced arrest at promoter-proximal sites prolongs the opportunity for recruitment of the mammalian capping enzyme to the elongation...
complex through a multiplicity of Mce1 interactions with Tat, Spt5, and the Pol-II CTD and (ii) Tat directly enhances the efficiency of capping of the HIV pre-mRNA (14).

It is not yet clear whether and how the capping apparatus fits into the Spt5 regulatory axis during gene expression in yeast. By studying the macromolecular interactions of the \textit{S. pombe} capping apparatus \textit{in vivo} using a two-hybrid approach, we have identified the essential \textit{S. pombe} Spt5 protein as a binding partner of the triphosphatase (Pct1) and guanylyltransferase (Pce1) components of the fission yeast capping machinery. We show that the C-terminal nonapeptide repeat domain of \textit{S. pombe} Spt5 binds avidly and independently to Pct1 and Pce1 \textit{in vitro}. A discrete domain of Pce1 suffices for binding to Spt5. We hypothesize that the purpose of the Spt5/capping enzyme interaction is to ensure timely capping of the nascent pre-mRNA before committing Pol-II to processive elongation.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—The screen was performed as described previously using binding domain (BD)-Pce1 and BD-Pct1 as the bait (12). Plasmid DNA recovered from the strains that tested positive for both an AD and a BD expression was utilized for PCR amplification of the \textit{S. pombe} DNA insert with flanking primers specific for the activation domain (AD) fusion plasmid. The PCR products were gel-purified and then sequenced. The AD plasmid clones were recovered after transformation into \textit{E. coli} DH5a.

**Spt5, Pct1, and Pce1 Truncation Mutants**—Gene fragments encoding N-terminal-truncated versions of \textit{S. pombe} Spt5 were generated by PCR amplification using sense primers that introduced a \textit{BamH}I site at the 5′ end of the truncated Spt5 coding region and an antisense primer that introduced an \textit{XhoI} site immediately downstream of the stop codon. Gene fragments encoding C-terminal-truncated versions of Spt5 were generated by PCR amplification using antisense primers that introduced stop codons in lieu of the codons for amino acids 961, 925, 899, and 801, and an \textit{XhoI} site immediately 3′ of the stop codon. The PCR products were digested with \textit{BamH}I and \textit{XhoI} and then inserted into the two-hybrid AD fusion vector pGAD-GH.

**Gene fragments encoding** N-terminal-truncated versions of Pce1 were generated by PCR amplification using sense primers that introduced an \textit{NcoI} site at the 5′ end of the truncated coding region and an antisense primer that introduced a \textit{BamH}I site immediately downstream of the stop codon. Gene fragments encoding C-terminal-truncated versions of Pce1 were generated by PCR amplification using antisense primers that introduced stop codons in lieu of the codons for amino acids 351, 301, and 251 and a \textit{BamH}I site immediately 3′ of the stop codon. The PCR products were digested with \textit{NcoI} and \textit{BamH}I and then inserted into the two-hybrid BD fusion vector pAS2–1.

**Gene fragments encoding** N-terminal-truncated versions of Pct1 were generated by PCR amplification using sense primers that introduced an \textit{NdeI} site at the codons for amino acids 21, 41, 51, 61, or 77. The antisense primers introduced a \textit{BamH}I site immediately downstream of the stop codon. The PCR products were digested with \textit{NdeI} and \textit{BamH}I and then inserted into pAS2–1.

The full-length \textit{S. pombe} SPT4 gene was PCR-amplified from genomic DNA using primers that introduced an \textit{NdeI} site at the start codon and a \textit{BamH}I site immediately 3′ of the stop codon. The PCR product was digested with \textit{NdeI} and \textit{BamH}I and then inserted into pAS2–1. All of the inserts were sequenced to ensure that the truncated SPT5, PCT1, or PCE1 genes were fused in-frame to BD or AD and that no unwanted coding changes had been introduced during amplification and cloning.

**Recombinant Capping Enzymes—\textit{S. pombe} guanylyltransferase Pce1 and RNA triphosphatase Pct1 were produced in \textit{E. coli} as N-terminal His\textsubscript{6}-tagged fusions and purified from soluble bacterial lysates by nickel-agarose chromatography as described previously (12, 24). Recombinant Pce1 without an affinity tag was produced as follows. A DNA fragment containing the complete PCE1 cDNA with a \textit{BamH}I restriction site at each end was inserted into the bacterial expression vector PET-28, which carries a polyhistidine (His\textsubscript{10}) tag. The His\textsubscript{10}-tagged fusion protein was then purified free of the tag. The Spt5(801–880) and Spt5(845–898) were PCR-amplified using primers that introduced an \textit{NcoI} site at the 5′ end and an \textit{XhoI} site immediately 3′ of the stop codon. The PCR products were digested with \textit{NcoI} and \textit{XhoI} and inserted into the pAS2–1 vector. The resulting plasmid was introduced into \textit{E. coli} BL21(DE3).

**Protein Affinity Chromatography—** GST and the GST-Spt5 fusion proteins were purified from soluble lysates by affinity chromatography on a glutathione-Sepharose 4B resin according to the instructions of the vendor (Pharmacia). GST and the GST–Spt5 fusion proteins were eluted from the resin with 50 mM glutathione, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol, and 0.05% Triton X-100. The fusion protein was then digested with purified recombinant Ulp1 (25) at a Ulp1/GST-Spt5 ratio of 1:200 (w/w) for 2 h on ice. The digested material was applied to a 1-ml column of Ni-NTA-agarose that had been equilibrated with buffer B. Native Pce1 (1 mg) was recovered in the flow-through fraction. Protein concentrations were determined by using the BioRad dye reagent with bovine serum albumin as the standard.

**Recombinant GST-Spt5 Fusion Proteins and Native Spt5 CTD**—The open reading frames encoding Spt5(801–990), Spt5(801–888), Spt5(845–898), and Spt5(845–880) were PCR-amplified using primers that introduced an \textit{NcoI} site at the 5′ end and an \textit{XhoI} site immediately 3′ of the stop codon. The PCR products were digested with \textit{NcoI} and \textit{XhoI} and inserted into the pGEX-KG vector. The resulting plasmids were transformed into \textit{E. coli} BL21(DE3). Single transformants were grown at 37 °C in 100 ml of Luria-Bertani medium containing 100 μM/ml ampicillin until the \(A_{600}\) reached 0.5. The cultures were placed on ice for 30 min and then adjusted to 0.5 mM isopropyl-1-thio-β-galactopyranoside and 2% (v/v) ethanol. Incubation was continued for 20 h at 18 °C with constant shaking. Cells were harvested by centrifugation.

**Protein Affinity Chromatography—** 20 μg of purified His-Pct1 or His-Pce1 was adsorbed to 50 μl of Ni-NTA-agarose beads (Qiagen) during a 1-h incubation at 4 °C in 500 μl of binding buffer 1 (50 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 8.0, 300 mM NaCl, 20 mM imidazole, and 0.005% Tween 20). The beads were washed once with 0.5 μl of binding buffer 1 to remove any unbound protein. The beads were then mixed for 1 h at 4 °C with 5 μg of purified GST or GST-Spt5 in 300 μl of binding buffer 1. The beads were then centrifuged, and the supernatant was withdrawn. The beads were resuspended in 0.5 μl of binding buffer 2 and subjected to two rounds of concentration and washing. After the second wash, the bound proteins were eluted with 50 μl of binding buffer 1 containing 250 mM imidazole. Aliquots (25 μl) of the input and the bead-bound fractions were analyzed by SDS-PAGE.

**Protein Affinity Chromatography—** 20 μg of purified GST or GST-Spt5 fusion protein was adsorbed to 50 μl of GSH-Sepharose beads (Amersham) during a 1-h incubation at 4 °C in 500 μl of binding buffer 2 (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5% glycerol, 1 mM DTT, 0.03% Triton X-100). The beads were then washed twice with 1 μl of binding buffer 2 to remove unbound protein. Then the beads were mixed with 5 μg of purified His-Pct1 or His-Pce1 for 1 h at 4 °C in 50 μl of binding buffer 2. The beads were then centrifuged by microcentrifugation, and the supernatant was withdrawn. The beads were resuspended in 1 μl of binding buffer 2 and subjected to two rounds of concentration and washing.
Interactions between mRNA Capping Enzymes and Spt5

RESULTS

*S. pombe* Triphosphatase and Guanylyltransferase Interact with *S. pombe* Spt5 in Vivo—A two-hybrid screen of ~200,000 transformants for guanylyltransferase-interacting proteins using a Gal4 DNA-BD-Pce1 fusion as bait yielded 11 His+ isolates, two of which contained plasmids encoding the Gal AD fused in-frame to C-terminal fragments of a predicted 990-amino acid S. pombe polypeptide segment of the fusion protein. Robust growth on selective media was scored as +++ (Fig. 2A). Strains that grew no better than the BD-Pce1 plus AD controls (see Fig. 2A) were scored as +. Intermediate levels of growth were scored as + on the basis of His+ colony size.

A C-terminal Spt5 Domain Suffices for Interaction with *S. pombe* Capping Enzymes in Vivo—The AD-Spt5 fusions that interacted in vivo with Pce1 or Pct1 contained at their C termini 18 tandem copies of a nonapeptide repeat (consensus sequence TPANNSGSK), plus a variable segment of the Spt5 protein upstream of the start of the repeats (Figs. 1 and 4A). To gauge the role of the non-reiterative protein segment, we engineered a series of AD-Spt5 clones in which the fusion junction was moved incrementally toward the start of the repeat region. These clones were tested in a directed two-hybrid assay paired with BD-Pce1 and BD-Pct1. We found that the Spt5 interaction with both capping enzymes persisted when the AD fusion started from position 801 and contained little more than the terminal 190-amino acid segment containing all of the nonamer repeats, i.e. Spt5(772–990) was isolated once (Fig. 1). Control experiments confirmed that HIS3 and lacZ expression required cotransformation with the BD-Pct1 and AD-Spt5(628–990) plasmids (not shown).

Interactions between mRNA Capping Enzymes and Spt5. Two different AD-Spt5 fusion clones were isolated: AD-Spt5(628–990) was recovered three times and AD-Spt5(772–990) was isolated once (Fig. 1). Control experiments confirmed that HIS3 and lacZ expression required cotransformation with the BD-Pct1 and AD-Spt5(628–990) plasmids (not shown).

Interaction of Proximal and Distal Nonamer Repeats with Spt5—The AD-Spt5 fusion that interacted in vivo with Pce1 or Pct1 contained at their C termini 18 tandem copies of a nonapeptide repeat (consensus sequence TPANNSGSK), plus a variable segment of the Spt5 protein upstream of the start of the repeats (Figs. 1 and 4A). To gauge the role of the non-reiterative protein segment, we engineered a series of AD-Spt5 clones in which the fusion junction was moved incrementally toward the start of the repeat region. These clones were tested in a directed two-hybrid assay paired with BD-Pce1 and BD-Pct1. We found that the Spt5 interaction with both capping enzymes persisted when the AD fusion started from position 801 and contained little more than the C-terminal repeats per se (Fig. 1). Thus, the entire N-terminal 900-amino acid segment was dispensable for interaction with the capping enzymes. On the other hand, deletion of the C-terminal 190-amino acid segment containing all of the nonamer repeats, i.e. in the fusion clone AD-Spt5(165–800), completely abolished the two-hybrid interaction with Pce1 and Pct1 (Fig. 1).

FIG. 1. *S. pombe* capping enzymes interact with *S. pombe* Spt5 in vivo. Plasmids encoding the indicated AD-Spt5 fusions were transformed into *S. cerevisiae* Y190 cells bearing the BD-Pce1 or BD-Pct1 plasmids. The limits of the Spt5 polypeptide segment of the fusion protein are indicated and drawn to scale as horizontal lines. The nonamer repeats are depicted as vertical bars. The AD-Spt5 fusions that were recovered in the two-hybrid screen of the *S. pombe* cDNA library are indicated by check marks.

A two-hybrid screen of ~100,000 transformants for triphosphatase-interacting proteins using a BD-Pct1 fusion as bait yielded 16 His+ isolates, of which four contained plasmids encoding the AD fused in-frame to C-terminal fragments of S. pombe Spt5. Two different AD-Spt5 fusion clones were isolated: AD-Spt5(628–990) was recovered three times and AD-Spt5(772–990) was isolated once (Fig. 1). Control experiments confirmed that HIS3 and lacZ expression required cotransformation with the BD-Pct1 and AD-Spt5(628–990) plasmids (not shown).
nonamer repeats in Spt5(899–990) and Spt5(917–990), respectively (Fig. 1). Apparently, the C-terminal twelve repeats sufficed for Spt5 binding to Pet1 in vivo, but the C-terminal eight and six repeats did not. From the opposite direction, we found that elimination of three, five, or eight nonamers from the C terminus of Spt5 had no effect on its interaction with Pet1 (Fig. 1). Thus, the first ten repeats (upstream of amino acid 898) were sufficient for Pet1 binding in vivo. This analysis indicates that the proximal (801–898) and distal (863–990) nonamer arrays of the Spt5 CTD are functionally redundant with respect to Pet1 binding.

The two-hybrid interaction of Spt5 with the guanylyltransferase Pce1 displayed a more stringent requirement for Spt5 CTD length. Binding of Pce1 was abolished by deletion of as few as three upstream nonamers (in Spt5(836–990)) indicating that the remaining 15 C-terminal nonamers were insufficient. Pce1 binding persisted, albeit with diminished strength, upon elimination of three, five, or eight nonamers from the C terminus of Spt5 (Fig. 1). Thus, with respect to Pce1 binding the proximal and distal repeats are functionally distinct. Note that the proximal nonamers adhere more closely to the consensus sequence than do the distal repeats (Fig. 4A).

* S. pombe Spt5 Is Essential for Cell Viability and Interacts with S. pombe Spt4—There have been no antecedent studies of Spt5 from fission yeast. Thus, we asked two questions. (i) Is Spt5 essential in *S. pombe*? (ii) Does *S. pombe* Spt5 interact with a homolog of Spt4? To address the first issue, we constructed a deletion allele of Spt5 from *S. pombe* Spt5—i.e., none contained the sp5::kanMX allele. We conclude that the sp5<sup>+</sup> gene, which we isolated in the two-hybrid screen against the capping enzymes, is essential for cell growth in *S. pombe*.

The fission yeast gene encoding a 104-amino acid homologue of human Spt4 (GenBank<sup>®</sup> accession no. AL157918) was fused to the Gal4 DNA binding domain, and the resulting BD-Spt4 construct was tested in a directed two-hybrid assay for interaction with *S. pombe* Spt5. Their interaction in vivo was evinced by histidine prototrophy and *lacZ* expression in yeast cells cotransformed with the BD-Spt4 and AD-Spt5(165–990) plasmids (Fig. 2B). Neither plasmid alone was able to activate *HIS3* or *lacZ*. An additional experiment showed that the C-terminal deletion variant Sp5(165–800), which lacks the entire nonamer repeat array, retains its interaction with Sp4 in the two-hybrid assay as does the even shorter fragment Sp5(165–400) (not shown). Thus, there are distinct and non-overlapping binding sites for Sp4 and the mRNA capping enzymes on the *S. pombe* Spt5 protein.

* Capping Enzymes Have Distinct Structural Requirements for Binding to Spt5 and the Pol-II CTD—The *S. pombe* guanylyltransferase Pce1 is a 402-amino acid monomeric protein (26). The active site of nucleotidyl transfer is composed of six motifs (I, III, IIIa, IV, V, and VI) that are conserved in order and spacing in the guanylyltransferases of all eukaryotes and several families of eukaryotic DNA viruses (1). RNA guanylyltransferase consists of two structural domains (27). The larger N-terminal domain (domain 1) includes motifs I, III, IIIa, and IV. The smaller C-terminal domain 2 includes motif VI. Motif V comprises the linker segment connecting domains 1 and 2. To gauge whether Pce1 contains a discrete functional domain for binding to Sp5, we engineered a series of Pce1 deletion variants as BD fusions and tested them for two-hybrid interaction with AD-Spt5(165–990) (Fig. 3A). Pce1(235–402), consisting of motif V plus domain 2, retained full activity in the two-hybrid binding assay with Sp5; thus, domain 1 is not required for guanylyltransferase binding to Sp5. Further deletion of the segment from amino acids 235–260, which eliminates motif V,
results in the loss of in vivo binding to Spt5, suggesting that motif V may comprise part of the Spt5 binding site. Spt5 binding was retained upon deletion of the C-terminal segment of Pce1 from residues 351–402 but was abolished by a more extensive deletion embracing residues 301–402 (Fig. 3A). A 116-amino acid fragment of the guanylyltransferase extending from residues 235–350 sufficed for the interaction with Spt5 (Fig. 3A). In stark contrast, the two-hybrid interaction of Pce1 with the C-terminal domain of Rbp1, the largest subunit of Sp. pombe Pol-II, was abrogated by every one of the Pce1 deletions tested (Fig. 3A). We surmise that the structural requirements for Pce1 binding to Pol-II CTD are more complex than those for its binding to Spt5.

The Sp. pombe RNA triphosphatase Pct1 is a 303-amino acid polypeptide with a homodimeric quaternary structure (24). The phosphohydrolase active site includes two glutamate-containing motifs (A and C) that comprise the binding site for the essential divalent cation cofactor. Motifs A and C are widely separated in the primary structure (Fig. 3B) but are closely positioned in the enzyme’s tertiary structure (28). Fungal RNA triphosphatases display considerable variability in the length and amino acid sequence of the segments upstream of motif A. To assess the role of this region in the binding of Pct1 to Spt5, we constructed a series of N-terminal Pct1 deletions and tested them in the two-hybrid assay (Fig. 3B). Removal of 20, 40, or 50 amino acids from the N terminus of Pct1 did not affect its in vivo binding to Spt5, but a further deletion of residues 51–60 abolished Spt5 binding. The in vivo interaction of Pct1 with the Pol-II CTD was unaffected by deletion of 20 or 40 amino acids but was abolished by removal of amino acids 41–50 (Fig. 3B).

**FIG. 3.** Effects of Pce1 and Pct1 deletion mutations on their binding interactions in vivo. *A*, the 402-amino acid Pce1 polypeptide is depicted as a horizontal line with the six nucleotidyl transferase motifs (I, III, IIIa, IV, V, and VI) shown as boxes. *B*, the 301-amino acid Pct1 polypeptide is depicted as a horizontal line with the metal-binding motifs A and C shown as boxes. The limits of the truncated derivatives of Pce1 and Pct1 are indicated and drawn to scale as horizontal lines. Plasmids encoding the BD fusions were introduced into yeast cells containing plasmids encoding AD fusions to Sp. pombe Spt5(165–990) or the Pol-II CTD, Rpb1(1324–1752). The strengths of the pairwise two-hybrid interactions were scored as described in Fig. 1.

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**Interactions between mRNA Capping Enzymes and Spt5**

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We used affinity chromatography to analyze the interaction of capping enzymes with Spt5 in vitro. Fusion proteins containing GST linked to the complete CTD Spt5(801–990), to the first ten nonamer repeats Spt5(801–898), and to an internal segment Spt5(845–898) containing six consensus repeats were produced in bacteria and purified. The GST fusions were mixed with purified recombinant His$_{10}$-Pct1 protein that had been immobilized on nickel-agarose beads (Fig. 4B). The material that adsorbed to the resin, remained bound after extensive washing, and was subsequently eluted with 250 mM imidazole (bound fraction B) was analyzed by SDS-PAGE in parallel with the input GST fusion protein (load fraction L). Virtually all of the input GST-Spt5(801–990) was adsorbed to the nickel-agarose/His-Pct1 beads and recovered in the imidazole eluate along with the His-Pct1 protein (Fig. 4B). In contrast, purified GST failed to bind to the nickel-agarose/His-Pct1 beads. Thus, we attribute the binding to the Spt5(801–990) component of the
fusion protein. Additional control experiments verified that none of the input GST-Spt5(801–990) protein was retained on nickel-agarose beads alone (not shown). The GST-Spt5(801–898) and GST-Spt5(845–898) proteins also bound nearly quantitatively to the nickel beads containing His-Pct1 (Figs. 4B and 5B) but not at all to nickel-agarose beads alone (not shown). An even shorter fusion protein, GST-Spt5(845–880), composed of just four nonamers also bound to the beads containing His-Pct1; however, the extent of binding was diminished by about half compared with the fusion protein with six nonamers. We conclude that: (i) four consensus nonamer repeats are sufficient for direct binding of Pct1 to Spt5 and (ii) phosphorylation of the Spt5 CTD is not required for Pct1 binding.

Similar nickel-agarose chromatography experiments were performed using recombinant His_{10}-Pce1 (Fig. 4C). Although the GST-Spt5(801–990) fusion protein is not well separated from the 47-kDa His-Pce1 polypeptide during SDS-PAGE, it was still apparent that nearly all of the input GST-Spt5(801–990) adsorbed to the nickel-agarose/His-Pce1 beads, whereas purified GST did not bind to the beads (Fig. 4C). The GST-Spt5(801–898) protein also bound nearly quantitatively to the nickel beads containing His-Pce1 (Fig. 4C), but there was an obvious decrease in the extent of binding by the GST-Spt5(845–898) fusion protein, which contains only six consensus nonamers (Figs. 4C and 5C). We observed no binding of Pce1 to the GST-Spt5(845–880) fusion protein containing four nonamer repeats (Fig. 5C). These data show that binding of Spt5 to Pce1 is direct and confirm the inference from the two-hybrid analysis (Fig. 1) that the Spt5 CTD length requirements are more stringent for Spt5 binding to Pce1 than for its binding to Pct1. The direct nature of the Spt5 interaction with the capping enzymes in vitro was confirmed by reciprocal affinity chromatography experiments that exploited the GST domain as the affinity tag. GST and the GST-Spt5 fusions were immobilized on glutathione-Sepharose beads, which were then mixed with purified His-Pct1. The input His-Pct1 (Fig. 6A, lane 1) was analyzed by SDS-PAGE along with the material that bound to the GSH resin and was subsequently stripped off with gluta-

**Fig. 4.** The C-terminal nonamer repeat domain of Spt5 binds to *S. pombe* capping enzymes in vitro. A, the Spt5 CTD from amino acids 801 to 990 is displayed with the nonamer repeats aligned vertically. The consensus sequence TPAWNSGSK is shown below the alignment. Only modules containing a proline at position 2 are counted as CTD repeats. B and C, aliquots (5 µg) of GST, GST-Spt5(801–990), GST-Spt5(801–898) or GST-Spt5(845–898) were subjected to affinity chromatography using nickel-agarose beads containing either His-Pct1 (B) or His-Pce1 (C). Aliquots comprising 50% of the input GST or GST-Spt5 fusion (lanes L) and 50% of the bead-bound material (lanes B) were analyzed by SDS-PAGE. The polypeptides were visualized by staining with Coomassie Blue dye. His-Pct1 (B) and His-Pce1 (C) are denoted by arrows on the right. The positions of various GST-Spt5 fusion proteins are denoted by dots (●) next to the stained polypeptides in lanes B. The positions and sizes (in kDa) of marker proteins are indicated on the left.
thione (bead-bound fractions, lanes 2–4). We found that His-Pct1 bound avidly to the GSH beads containing the GST-Spt5(801–990) fusion, but did not bind at all to GSH beads alone or to GSH beads containing just GST (Fig. 6A). Fig. 6B shows that the guanylyltransferase His-Pce1 bound to beads containing the GST-Spt5(801–898) fusion but did not bind at all to the beads alone or to beads containing GST.

The binding of GST-Spt5(801–990) to Pce1 had no effect on its guanylyltransferase activity in vitro; GST-Spt5(801–980) binding also had no effect on the triphosphatase activity of Pct1 (data not shown).

**Simultaneous Binding of Pct1 and Pce1 to the Spt5 CTD**—An important mechanistic question is whether the two cap-forming enzymes bind in a mutually exclusive fashion to the CTD of Spt5 or whether they can bind simultaneously to a single molecule of Spt5 CTD. To test these scenarios, we established an indirect affinity chromatography assay in which the binding of the guanylyltransferase to an affinity resin containing the triphosphatase would be strictly contingent on the action of Spt5 CTD as a molecular bridge (Fig. 7). The success of this assay requires that: (i) Pct1 be the only macromolecule that contains an affinity tag for linkage to the beads (in this case, a His tag) and (ii) the bridging component Spt5 cannot be fused to GST because GST will inherently homodimerize and thereby confound the issue of whether the assay readout reflects the binding of Pct1 and Pce1 to the same molecule of Spt5 (the model being tested) or to two different molecules of Spt5 connected via the GST domain.

Thus, recombinant Pce1 and Spt5(801–990) were produced in bacteria as His6-Smt3 fusion proteins and purified from soluble bacterial extracts by nickel-agarose chromatography. The His6-Smt3 domains were then removed by treatment of the recombinant protein with purified His-tagged Ulp1, a Smt3-specific protease that hydrolyzes the polypeptide chain at the junction between His6-Smt3 and the fused downstream protein (25). Re-chromatography of the digests on nickel-agarose resulted in purification of the “tag-free” Pce1 and Spt5(801–990) proteins, which were recovered in the flow-through and resolved from the His6-Smt3 and His-Ulp1 proteins, which were retained on the resin (data not shown). The native sizes of the
purified proteins were then investigated by sedimentation through glycerol gradients. Marker proteins catalase (248 kDa), bovine serum albumin (66 kDa), and cytochrome c (13 kDa) were included as internal standards. After centrifugation, the polypeptide compositions of the odd-numbered gradient fractions were analyzed by SDS-PAGE. The Spt5(801–990) polypeptide (calculated size 20 kDa) sedimented as a discrete peak coincident with cytochrome c (Fig. 7A). We surmise that the native non-tagged Spt5(801–990) protein is a monomer. (Note that the migration of the Spt5(801–990) polypeptide during SDS-PAGE was aberrantly slow, perhaps because of the presence of reiterated proline-containing motifs. Also, the Spt5(801–990) polypeptide migrated as a doublet composed of a predominant “fast” species and a minority “slow” species. Because Ulp1 processing of the tagged recombinant His<sub>6</sub>-Smt3 fusion proteins is highly site-specific (25), we doubt that the doublet arises from heterogeneity at the N terminus of Spt5(801–990). We suspect that the doublet may reflect conformational heterogeneity of the prolines.) The 47-kDa Pce1 protein sedimented as a discrete peak just slightly behind the MW marker the Spt5 protein.
The alignment are indicated by dashes (-). Positions of side chain identity/similarity in all four Spt5 proteins are denoted by dots (·). Series of transferase activity, evinced by the transfer of [32P]GMP from PAGE; the eluate fractions were also assayed for guanylyl-polypeptide compositions of the eluates were analyzed by SDS-

- **Spo**
  - GSISTA-DGPVPNWAGQARTPAVANGSRTPAW-----NTGRTPAW-NSGKTPAWNS-GSRTPAWS--GNKTPAWN

- **Hsa**
  - GGMSTTY-----------GRTPMY--GSQTMY-----CGSTFPMY--GSOTPDLQ--GSOTPPLHD

- **Cel**
  - GSMTAYDGGRTPAVEGCTRTPA--GYRTAYGDLEHSSRTAYCDSSRTAYGSADEGRTAYGSTEGERRTAPG

- **Dre**
  - GRSSTH----------LRTTPM--GSQTHY--------GTSRTPMY--GSOTPPLHD--GSOTPPLHD

|     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|     |     |     |     |     |     |     |     |     |     |

**FIG. 8.** Comparison of the fission yeast and metazoan Spt5 carboxyl repeat domains. The amino acid sequence of *S. pombe* Spt5 (Spo) from residues 799 to 990 is aligned to the sequences of the *Spt5* polypeptides of Homo sapiens (Hsa), C. elegans (Cel), and D. rerio (Dre). Gaps in the alignment are indicated by dashes (-). Positions of side chain identity/similarity in all four Spt5 proteins are denoted by dots (·). Series of conserved (Thr/Ser)-Pro dipeptides are highlighted in shaded boxes.

bovine serum albumin peak (Fig. 7B), consistent with a monomeric structure, as suggested previously (26).

To measure ternary complex formation, His-Pct1 (a homodimer) was mixed with Spt5(801–990) and adsorbed to nickenel-agarose beads. After the removal of unbound material, the beads were incubated with Pce1 and then washed again to remove unbound protein. The material retained on the beads was eluted under native conditions with 250 mM imidazole. The polypeptide compositions of the eluates were analyzed by SDS-PAGE; the eluate fractions were also assayed for guanylyltransferase activity, evinced by the transfer of [32P]GMP from [α-32P]GTP to the Pce1 polypeptide to form the covalent Pce1-GMP reaction intermediate (Fig. 7C).

The salient findings were as follows: (i) neither Spt5(801–990) nor Pce1 were bound to the beads in the absence of His-Pct1; (ii) Spt5(801–990) bound to the beads in the presence of His-Pct1; and (iii) Pce1 did not bind to the beads in the absence of His-Pct1 unless Spt5(801–990) was also present in the binding reaction mixture (Fig. 7C). Although the amount of Pce1 protein retained on the beads was much less than the total amount of His-Pct1 present on the beads, it was in the same range (based on staining) as the amount of Spt5(801–990), indicating that the binding of the guanylyltransferase was limited principally by the interaction of the monomeric Spt5 CTD with His-Pct1. We conclude that binding of the capping enzymes to the Spt5 CTD is not mutually exclusive but can occur simultaneously.

**DISCUSSION**

The present study contributes to an emerging picture of how transcription elongation control is connected to cotranscriptional mRNA processing via physical interactions between Pol-II elongation factors and RNA modifying enzymes. We show that fission yeast RNA triphosphatase and RNA guanylyltransferase interact independently with the elongation factor Spt5. These findings provide a rationale for the arrest and subsequent reactivation of Pol-II elongation at promoter proximal sites.

Advantage accrues from a mechanism whereby the commitment of Pol-II to productive elongation is contingent on prior acquisition of a cap. The cap promotes downstream mRNA processing steps, especially the splicing of the 5'–3' proximal intron (29, 30), and it protects mRNA from exonucleolytic decay (31). If Pol-II commits prematurely to traversing the entire transcription unit (covering megabase distances in some metazoan genes) without the benefit of a cap, it runs the risk of failing to excise the first intron in a timely fashion or perhaps at all, a process that would yield a nonfunctional transcript.

Accelerated 5'-3' decay of unguanylated nascent RNAs would also result in a futile round of transcription.

Wasteful commitment of Pol-II is apparently avoided by the imposition of an elongation checkpoint, whereby Spt5/Spt4 (DSIF) plus other negative factors arrest the elongation complex shortly after promoter clearance. This step is especially relevant to the control of HIV gene expression, but recent studies highlight the general localization of Spt5 on actively transcribed cellular genes (32, 33). They also suggest a role for Spt5 in arresting Pol-II at promoter-proximal sites on uninduced heat-shock genes (33). The importance of elongation arrest in Spt5 action *in vivo* is underscored by the finding that a missense mutation in zebrafish Spt5 that specifically prevents its action as a negative elongation factor elicits a phenotype of aberrant neuronal development (34). It is conceivable that the mutant animals are defective in down-regulating elongation in certain Pol-II genes or in developing neurons or that uncapped (and therefore nonfunctional) transcripts are generated from certain Pol-II genes in the absence of Spt5-induced elongation arrest.

Spt5 had not been studied previously in fission yeast. Here we identified *S. pombe* Spt5 in a library screen for proteins that bind to the fission yeast capping enzymes. We showed that Spt5 is essential for viability of *S. pombe*, as it is in budding yeast (35, 36), and that it interacts *in vivo* with the previously uncharacterized *S. pombe* equivalent of Spt4. Although our *in vitro* studies show that Spt4 is not required for Spt5 binding to the capping enzymes, we suspect that Spt5 interacts with the capping enzymes *in vivo* as part of an Spt5/Spt4 complex because the binding sites for Spt5 and the capping enzymes are located on distinct functional domains of the 990-amino acid Spt5 polypeptide. We mapped the Spt4 binding site to the segment of Spt5 from amino acids 165–244. This region corresponds to the Spt4 binding site in human Spt5 (16).

Wen and Shatkin (13) isolated hSpt5 in a two-hybrid screen using mammalian capping enzyme as bait. Our findings suggest that the nexus between capping enzymes and Spt5 is conserved across a wide evolutionary landscape. Nonetheless, there are differences in how the mammalian and fission yeast capping enzymes interact with Spt5. Wen and Shatkin found that the C-terminal domain hSpt5(767–1087) was essential for binding in the two-hybrid assay and that a second segment hSpt5(111–197) near the amino end is also involved. Yet, in the *S. pombe* system, only the Spt5 CTD is required for binding to the capping enzymes in the two-hybrid assay and *in vitro*.

An alignment of the primary structure of the CTD of *S.
and Caenorhabditis elegans Spt5 to that of human Spt5 and the Spt5 orthologs of *pombe* Spt5 proteins contain a series of Thr-Pro and Ser-Pro dipeptides—noteable differences (Fig. 8). The fission yeast and metazoan in...occurs on the nascent chains within the arrested Pol-II complexation and promoter clearance. We posit that cap formation occurs on the nascent chains within the arrested Pol-II complexes. It remains to be discovered if and how the presence of the capping enzymes in the elongation complex, or the presence of cap on the nascent transcript, is implicated in the conversion of the Spt5-arrested yeast transcription complex into a processive elongation complex. By analogy with the mammalian system (20), the proper positioning and/or activation of a P-TEFb-like factor might trigger its phosphorylation of the Spt5 CTD as well as further phosphorylation of the Pol-II CTD, thereby releasing the elongation complex from the arrest. The P-TEFb equivalent has yet to be identified and characterized in fission yeast.

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