REPORT

Characterization of the *Schizosaccharomyces pombe* Spt5-Spt4 complex

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ABSTRACT

The Spt5-Spt4 complex regulates early transcription elongation by RNA polymerase II and has an imputed role in pre-mRNA processing via its physical association with mRNA capping enzymes. Here we characterize the *Schizosaccharomyces pombe* core Spt5-Spt4 complex as a heterodimer and map a trypsin-resistant Spt4-binding domain within the Spt5 subunit. A genetic analysis of Spt4 in *S. pombe* revealed it to be inessential for growth at 25°C–30°C but critical at 37°C. These results echo the conditional *spt4Δ* growth phenotype in budding yeast, where we find that *Saccharomyces cerevisiae* and *S. pombe* Spt4 are functionally interchangeable. Complementation of *S. cerevisiae spt4Δ* and a two-hybrid assay for Spt4-Spt5 interaction provided a readout of the effects of 33 missense and truncation mutations on *S. pombe* Spt4 function in vivo, which were interpreted in light of the recent crystal structure of *S. cerevisiae* Spt4 fused to a fragment of Spt5. Our results highlight the importance of the Spt4 Zn²⁺-binding residues—Cys12, Cys15, Cys29, and Asp32—and of Ser57, a conserved constituent of the Spt4-Spt5 interface. The 990-amino acid *S. pombe* Spt5 protein has an exceptionally regular carboxyl-terminal domain (CTD) composed of 18 nonapeptide repeats. We find that as few as three nonamer repeats sufficed for *S. pombe* growth, but only when Spt4 was present. Synthetic lethality of the *spt5*¹⁻⁸³⁵ *spt4Δ* double mutant at 34°C suggests that interaction of Spt4 with the central domain of Spt5 overlaps functionally with the Spt5 CTD.

Keywords: elongation checkpoint; mRNA capping; transcription elongation

INTRODUCTION

Eukaryal mRNA processing is coordinated physically and temporally with transcription elongation. The earliest processing step is mRNA capping, which can occur as soon as the 5' triphosphate terminus of the nascent RNA extrudes from the elongating RNA polymerase (Chiu et al. 2002). The capping enzymes are directed to nascent mRNAs by binding to the phosphorylated carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II (Cho et al. 1997; McCracken et al. 1997; Yue et al. 1997; Ho and Shuman 1999; Pei et al. 2001; Fabrega et al. 2003). Capping enzymes also bind to the Pol II elongation factor Spt5 (Wen and Shatkin 1999; Pei and Shuman 2002), which, in conjunction with its binding partner Spt4, elicits an

elongation arrest at promoter-proximal sites (Wada et al. 1998a). The Spt5/Spt4-induced elongation arrest in metazoans is alleviated by the Cdk9 protein kinase, a subunit of positive transcription elongation factor b (P-TEFb) (Wada et al. 1998b). In the fission yeast *Schizosaccharomyces pombe*, the essential Cdk9 kinase exists as a stoichiometric complex in vivo with its cyclin partner (Pch1) and the methyltransferase component of the *S. pombe* capping apparatus (Pei and Shuman 2003; Pei et al. 2006; Guiguen et al. 2007). *S. pombe* Cdk9 also interacts with the RNA triphosphatase component of the capping apparatus (Pei et al. 2003). These and other observations underlie the proposal of a transcription elongation checkpoint that ensures a temporal window for capping of nascent mRNAs (Pei et al. 2003; Mandal et al. 2004; Sims et al. 2004). Evidence is emerging that positive and negative regulation of transcription can occur at the step of capping enzyme recruitment (Chiu et al. 2002; Gao and Gross 2008).

The Spt5-Spt4 complex is the lynchpin of the putative checkpoint. There is ample genetic and biochemical evidence from budding yeast, zebrafish, roundworm, fruitfly, and mammalian systems that Spt5 and Spt4 exert both

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negative and positive effects on transcription elongation (Hartzog et al. 1998; Andrulis et al. 2000; Guo et al. 2000; Renner et al. 2001; Shim et al. 2002; Wu et al. 2003; Jennings et al. 2004). Spt5 is a large polypeptide (~1000–1200 amino acids) composed of multiple domain modules, including an acidic N-terminal domain, central NusG-like NGN (NusG N-terminal homology), and KOW domains (Hartzog et al. 1998; Wade et al. 1998a; Ponting 2002), and a CTD targeted for threonine phosphorylation (Pei and Shuman 2003; Yamada et al. 2006). Spt5 is essential for cell growth in budding and fission yeast (Swanson et al. 1991; Pei and Shuman 2002). *Saccharomyces cerevisiae* Spt5 participates in a vast network of genetic and physical interactions with the Pol II elongation complex and mRNA processing systems (Lindstrom et al. 2003; see also *Saccharomyces Genome Database*, www.yeastgenome.org). By contrast, Spt4 is a small polypeptide (100–120 amino acids) and is nonessential for cell growth in budding yeast (Malone et al. 1993). Yeast *spt4Δ* cells display pleiotropic phenotypes that are probably linked to defects in transcription elongation and RNA processing (Rondón et al. 2003; Lindstrom et al. 2003; Mason and Struhl 2005; Qui et al. 2006; Schneider et al. 2006). Many genetic interactions with *spt4Δ* have been observed in budding yeast (*Saccharomyces Genome Database*, www.yeastgenome.org).

Here we conducted a physical and genetic characterization of the *S. pombe* Spt5-Spt4 complex that addressed the following issues: (1) the native size and subunit stoichiometry of a recombinant core Spt5-Spt4 complex; (2) structure probing of the complex by limited proteolysis; (3) whether *S. pombe* Spt4 can function in lieu of *S. cerevisiae* Spt4 in vivo; (4) identification of functionally important amino acids of Spt4 via alanine scanning and conservative substitutions; (5) the effect of genetic ablation of Spt4 in *S. pombe*; and (6) synthetic enhancement of *S. pombe* Spt5 mutations by ablation of Spt4.

RESULTS AND DISCUSSION

Recombinant *S. pombe* Spt5-Spt4 complex

The human Spt5-Spt4 complex was purified initially based on its elongation-arrest activity. Wada et al. (1998a) estimated by gel filtration a native size of >300 kDa and suggested that the Spt5-Spt4 complex is a multimer of the 165-kDa Spt5 protein. Deletion analysis delineated a central Spt4-binding domain on human Spt5 from amino acids 176–313 (Yamaguchi et al. 1999; Ivanov et al. 2000), but the methods employed (GST pull-downs, co-immunoprecipitations) did not reveal the stoichiometry or size of the complex.

Our aim here was to assess the quaternary structure of the core *S. pombe* Spt5-Spt4 complex formed by recombinant subunits. Based on our initial two-hybrid data showing that both the N-terminal 164-amino acid segment

and the C-terminal 190-amino acid nonamer array of *S. pombe* Spt5 are dispensable for binding to Spt4 (Pei and Shuman 2002), we sought to physically characterize the complex of Spt4 with Spt5-(165–800). This was accomplished by coexpressing His-tagged Spt4 with untagged Spt5-(165–800) in bacteria and then purifying His-Spt4 by Ni-agarose chromatography (Fig. 1). The affinity-purified material (Fig. 1B, lane Ni) consisted of the 14-kDa His-Spt4 plus the untagged 70-kDa Spt5-(165–800) polypeptide in near 1:1 stoichiometry. This result proves that Spt4 and Spt5 associate in the absence of other fungal proteins. The Ni-agarose preparation was sedimented in a glycerol gradient with internal markers catalase (a 248-kDa tetramer of a 62-kDa subunit), bovine serum albumin (BSA) (a 68-kDa monomer), and vaccinia topoisomerase (vtopo) (a 36-kDa monomer). Analysis of the gradient fractions by SDS-PAGE showed that Spt5 sedimented as a single discrete component coincident with the BSA peak and clearly heavier than the vtopo peak (Fig. 1A). There was no detectable heavier component of Spt5 between BSA and catalase, arguing against Spt5-(165–800) dimerizing. The fact that the majority of the Spt4 cosedimented with Spt5 suggests that the complex is probably a 1:1 heterodimer, although we cannot exclude the possibility that two copies of the Spt4 polypeptide are associated with Spt5-(165–800). The residual “free” Spt4 sedimented as a lighter shoulder. We subjected the peak glycerol fraction containing Spt5-Spt4 and the markers to another round of Ni-affinity purification, which resulted in efficient recovery of His-Spt4 and untagged Spt5, but not the markers (Fig. 1B, cf. lanes G and G+Ni), thereby attesting to the stability of the reconstituted Spt5-(165–800)-Spt4 complex.

We probed the structure of the isolated Spt5-(165–800)-Spt4 complex by limited proteolysis with trypsin (Fig. 1C). We observed a discrete set of tryptic fragments that persisted at trypsin levels in excess of the amount sufficient to cleave all the input Spt5-(165–800). The N-terminal sequences of the input Spt5 and the tryptic fragments were determined and are indicated in Figure 1C at right (note the Spt5 N-terminus includes 3 amino acids upstream of Asp165 introduced during cloning into the bacterial expression vector). The protease-accessible sites in Spt5 were thereby mapped to the Arg217-Ser218, Lys381-Asn382, and Lys466-Gly467 dipeptides. These sites could demarcate surface-exposed loops or interdomain linkers within the Spt5 tertiary structure. The changes in the spectrum of the proteolytic fragments as a function of trypsin concentration provide insight to the structure of the Spt5-Spt4 complex. For example, at the lowest trypsin concentration, we see a ~55-kDa fragment initiating at Ser218, consistent with initial incision at a single major tryptic site at Arg217-Ser218. The predicted short N-terminal peptide (amino acids 165–217) product of initial cleavage was not seen in Figure 1C, because it would migrate well ahead of the intact Spt4 protein and be compressed at the

FIGURE 1. Characterization of a recombinant fission yeast Spt5-Spt4 complex. (A) Zonal velocity sedimentation of the HisSpt4/Spt5-(165-800) complex with internal standards was performed as described in Materials and Methods. Aliquots of the odd-numbered glycerol gradient fractions were analyzed by SDS-PAGE; the Coomassie blue-stained gel is shown. (B) Comparison by SDS-PAGE of the polypeptide compositions of the Ni-affinity purified HisSpt4/Spt5-(165-800) complex that was applied to the glycerol gradient (Ni), glycerol gradient fraction 15 containing a mixture of HisSpt4/Spt5-(165-800), BSA, and vtopo (G), and the imidazole eluate obtained by subjecting an aliquot of glycerol gradient fraction 15 to a second round of Ni-affinity purification (G + Ni). The Coomassie blue-stained gel is shown. (C) Limited proteolysis and automated Edman sequencing were performed as described (Lehman et al. 1999). Affinity-purified HisSpt4/Spt5-(165-800) was treated with 3.1, 6.2, 12.5, 25 or 50 ng of trypsin. Trypsin was omitted from the control mixture in lane -. The digests were resolved by SDS-PAGE. Polypeptides were transferred electrophoretically to a polyvinylidene difluoride membrane and visualized by staining the membrane with Coomassie blue dye. Membrane slices containing intact Spt5-(165-800) and individual proteolytic products denoted by arrowheads were excised and sequenced. The N-terminal peptide sequences are indicated on the right. (D) The amino acid sequence of *S. pombe* (Spo) Spt5 from residues 205–468 is aligned to the sequences of the homologous human (Hsa) and *S. cerevisiae* (Sce) Spt5 proteins. Positions of side-chain identity or similarity in all three proteins are indicated by •. The secondary structure elements of the NGN domain of *S. cerevisiae* Spt5 are displayed below the alignment (with β -strands depicted as arrows and α -helices as cylinders). The mapped sites of tryptic cleavage of Spt5 in the recombinant *S. pombe* Spt4-Spt4 complex are denoted by ▼.

electrophoretic front. The apparent size of the largest tryptic fragment was less than the value of 64 kDa calculated for Spt5-(218-800), which suggests that trypsin also cleaved near the C terminus to yield one or more small fragments that are not visualized in the experiment. Also present at low trypsin concentrations were an ~37-kDa polypeptide initiating at Asn382 and an ~18-kDa species that retained the N-terminal Ser218 of the initial 55-kDa tryptic fragment. The size of the smaller species agrees with the calculated size of the Spt5-(218-381) fragment. The 37-kDa and 18-kDa fragments persisted as trypsin concentration increased and all of the initial 55-kDa species decayed (Fig. 1C). In turn, the 37-kDa species was converted at the highest trypsin concentrations to an ~29-kDa derivative initiating at Gly467.

The small number of discrete tryptic cleavage sites in Spt5-(165-800), which has 81 lysine and arginine residues,

suggests that the protein is well folded in the complex with Spt4. The locations of the three trypsin-sensitive sites in *S. pombe* Spt5 are denoted by arrowheads above the aligned amino acid sequences of the *S. pombe*, human, and *S. cerevisiae* Spt5 proteins (Fig. 1D). The Spt4 binding domain of *S. pombe* Spt5 had previously been localized by two-hybrid assays to the segment from amino acids 165–400 that includes two of the tryptic sites, Arg217 and Lys381 (Pei and Shuman 2002). Coexpression in bacteria of His-Spt4 and Spt5-(218-800) resulted in a stable complex of the two polypeptides after Ni-affinity chromatography (data not shown), suggesting that the entire polypeptide segment preceding the tryptic site at Arg217 is not strictly necessary for interaction with Spt4. The segment between the two most sensitive tryptic sites (Arg217 and Lys381) in *S. pombe* Spt5 corresponds to amino acids 147–329 in human Spt5, which embraces tightly the mapped binding domain for human Spt4

(Yamaguchi et al. 1999; Ivanov et al. 2000). The apparent resistance of this segment of the *S. pombe* Spt5-Spt4 complex to trypsin cleavage suggests that it comprises a folded domain.

This inference is underscored by the recently published crystal structure (Guo et al. 2008) of a chimeric protein composed of *S. cerevisiae* Spt4 fused to the homologous segment of *S. cerevisiae* Spt5 (amino acids 285–375). The structure revealed that the yeast Spt5 fragment adopts a compact tertiary structure composed of a central anti-parallel β sheet flanked by three α helices. The Spt5 secondary structure elements are depicted below the aligned sequences in Figure 1D.

Use of budding yeast for structure-function analysis of *S. pombe* Spt4

Whereas Spt4 is not essential for growth of budding yeast at 25°C, deletion of the *spt4* gene prohibits growth at 37°C (Malone et al. 1993; Basrai et al. 1996). The available structure-function information for yeast Spt4 concerns the role of an N-terminal tetracysteine motif that was predicted to comprise a zinc-binding site. Malone et al. (1993) had shown that *S. cerevisiae* Spt4 function in vivo was abolished by single serine substitutions for Cys7, Cys24, or Cys27. Basrai et al. (1996) identified a spontaneous C10Y mutation in yeast Spt4 that also abolished its function in vivo. By contrast, a double-mutation of human Spt4 in which Cys16 and Cys19 were replaced by serine did not have any apparent effect on binding of human Spt4 to human Spt5 or on the reconstitution of a transcriptionally active Spt5-Spt4 (DSIF) complex (Kim et al. 2003). Thus, there are discordant results with respect to the effect of disrupting the putative Zn-binding site of yeast versus human Spt4. Wenzel et al. (2008) verified by optical emission spectrometry that purified human Spt4 contains an equimolar amount of Zn^{2+} . *S. pombe* Spt4 is notable for its deviation from the tetracysteine motif (Fig. 2B). *S. pombe* Spt4 has three of the conserved cysteines (Cys12, Cys15, and Cys29), but the fourth cysteine is replaced by an aspartate (Asp32).

Previous studies had shown that the *ts* growth defect of an *S. cerevisiae* *spt4* Δ strain could be rescued by expressing human or *Kluyveromyces lactis* Spt4 (Hartzog et al. 1996; Hikkel et al. 1998). Here we constructed an *S. cerevisiae* *spt4* Δ strain and demonstrated rescue of the *ts* growth defect by expres-

sion of *S. pombe* Spt4 from a *CEN* plasmid under the control of the yeast *TPI1* promoter (Fig. 3A). This allowed us to exploit budding yeast as a surrogate system for structure-function analysis of the 105-amino acid *S. pombe* Spt4 protein. We introduced Ala-mutations at 18 residues of *S. pombe* Spt4, including the four putative Zn^{2+} ligands and 14 other amino acids that are conserved in human and *S. cerevisiae* Spt4 (Fig. 2B). We found that alanine mutations at nine of the conserved residues had no apparent impact on *spt4* Δ complementation: Arg10, Gly28, Thr41, Ser42, Gly47, Trp58, Arg61, Trp62, and Gly71 (Fig. 3A). Alanine changes at nine other conserved positions eliminated or reduced *spt4* Δ complementation at 37°C: Cys12, Cys15, Phe24, Cys29, Asn31, Asp32, Phe45, Ser57, and Tyr73 (Fig. 3A).

The *S. pombe* Spt4-Ala mutants were tested for interaction with *S. pombe* Spt5 in the yeast two-hybrid assay, with dual reporter readouts (His^+ and lacZ^+) of a positive interaction between a DNA binding domain (BD) fused to *S. pombe* Spt4 and a transcription activation domain (AD) fused to Spt5-(165-990) or Spt5-(165-400) (Table 1). Whereas all 18 of the Spt4-Ala proteins retained interaction with the larger Spt5-(165-990) protein (Supplemental Fig.

FIGURE 2. Conserved amino acids targeted for alanine scanning in *S. pombe* Spt4. (A) Location of targeted residues in the Spt4 tertiary structure. The figure (rendered in Pymol from PDB coordinates 2exu) shows a stereo view of a ribbon trace of *S. cerevisiae* Spt4 (with β -strands colored magenta and α -helices colored cyan) in complex with the NGN domains of Spt5 (colored gray). The nonglycine side-chains of Spt4 that were targeted for alanine scanning are shown, numbered according to their position in *S. pombe* Spt4. The Zn^{2+} ion is depicted as a green sphere. Pertinent atomic contacts are depicted as dashed lines. (B) The amino acid sequence of *S. pombe* (Spo) Spt4 from residues 8–81 is aligned to the sequences of the homologous human (Hsa) and *S. cerevisiae* (Sce) Spt4 proteins. The secondary structure elements of *S. cerevisiae* Spt4 are displayed below the alignment (colored as in panel A). The 18 positions targeted for alanine scanning are indicated by ●. The nine residues of *S. pombe* Spt4 that were required for *spt4* Δ complementation in *S. cerevisiae* are highlighted in gray boxes.

FIGURE 3. Mutational effects on the function of *S. pombe* Spt4 assayed by complementation of *S. cerevisiae* *spt4Δ*. *CEN TRP1* plasmids expressing wild-type *SceSpt4*, wild-type *SpoSpt4*, or mutated *SpoSpt4* were transformed into *S. cerevisiae* *spt4Δ* cells. Control cells were transformed with the empty *CEN TRP1* vector. Growth of the indicated *spt4Δ* strains was assessed by spotting serial dilutions to SD-Trp agar medium. The plates were photographed after incubation for 3 d at 30°C or 37°C as specified. (A) Effects of alanine mutations. (B) Effects of conservative mutations. (C) Effects of C-terminal truncations.

S1), four of the Spt4 mutations (C12A, C15A, C29A, and Y73A) affected Spt4 binding to the smaller Spt5-(165-400) fragment that suffices for binding to wild-type Spt4 and 14 other alanine mutants (Table 1). That the *S. pombe* Spt4-Ala mutants retained activity in one or both two-hybrid assays indicates that the mutant polypeptides are indeed produced in budding yeast.

Structure-activity relationships at the essential residues were gleaned by testing the effects of 13 conservative substitutions on *spt4Δ* complementation (Fig. 3B). The C12S, C15S, and C29S mutations abolished Spt4 activity, suggesting that zinc coordination is essential for the fission yeast protein, as it is in budding yeast Spt4. The instructive finding was that replacing the deviant Asp32 residue of *S. pombe* Spt4 with either glutamate or cysteine fully restored complementation activity, whereas serine and asparagine did not (Fig. 3B). We surmise that (1) either a cysteine sulfur or a carboxylate oxygen can serve as the fourth metal ligand and (2) there is no tight steric constraint on distance from the main-chain to the metal-binding atom of the side-chain at position 32. Replacing Tyr73 by phenylalanine resulted in loss of function (Fig. 3B), suggesting that the hydroxyl group was essential. Spt4 was active in vivo when Phe24 was substituted by leucine, indicating that the aromatic ring was not essential. By contrast, the aromatic quality of Phe45 was essential, insofar as the F45L mutant failed to

complement *spt4Δ* (Fig. 3B). The essential function of Asn31 could not be performed by aspartate or glutamine, indicating that the amide group is critical and/or there is a steric constraint on the distance from the main-chain to the amide.

We also tested the effects of serial deletions of 10, 20, 30, 40, or 50 amino acids from the C terminus of *S. pombe* Spt4. The CΔ10 mutant supported slow growth of *spt4Δ* cells at 37°C; the CΔ20 and CΔ30 versions were inactive (Fig. 3C), notwithstanding that the CΔ20 and CΔ30 polypeptides retained their interactions with *S. pombe* Spt5-(165-990) and Spt5-(165-400) in the two-hybrid assay (Table 1). We infer that the segment of Spt4 from amino acids 86–95 (⁸⁶VESLRRRGIN⁹⁵) embraces functional group(s) necessary for biological activity at a step other than mere binding to Spt5. Note that Spt5 binding was abolished by the Spt4 CΔ40 and CΔ50 deletions (Table 1). These results indicate that the Spt4 segment from amino acids 66–75 (⁶⁶DTFTPGIYAT⁷⁵) includes function groups necessary for Spt5 binding, one of which is likely to be Tyr73 (Table 1).

Interpretation of mutational results in light of an Spt4 crystal structure

A stereo image of the crystal structure of *S. cerevisiae* Spt4 in complex with a fragment of Spt5 (Guo et al. 2008) is shown in Figure 2A. The conserved amino acid side-chains that were subjected to mutational analysis in the present study are highlighted; for convenience, they are renumbered

TABLE 1. Mutational effects on Spt4 two-hybrid interaction with Spt5

BD-SpSpt4	AD-SpSpt5-(165-990)	Ad-Spt5-(165-400)
WT	+	+
R10A	+	+
C12A	+	–
F24A	+	–
G28A	+	+
C29A	+	–
N31A	+	+
D32A	+	+
T41A	+	+
S42A	+	+
F45A	+	+
G47A	+	+
S57A	+	+
W58A	+	+
R61A	+	+
W62A	+	+
G71A	+	+
Y73A	+	–
CΔ10	+	+
CΔ20	+	+
CΔ30	+	+
CΔ40	–	–
CΔ50	–	–

is available for fission yeast. Instead, we used high-copy vectors that are standard in the field (Forsburg 1993). Therefore, mutations that cause functional deficits at low gene dosage in budding yeast might be missed at high gene dosage in fission yeast, e.g., because increased production of the mutant Spt4 results by mass action in sufficient Spt5-Spt4 complex to support cell growth at 37°C. We attempted to down-regulate transcription of the plasmid-borne *nmt1-spt4⁺* cassette by inclusion of thiamine in the growth medium (Forsburg 1993) and found that the plasmid still complemented growth of the *spt4Δ* strain at 37°C (data not shown), implying that Spt4 is indeed produced in functional excess from the high copy plasmid.

An alternative explanation for the discrepancy is that the two genetic complementation assays rely on Spt4 interaction with different Spt5 binding partners. The budding yeast complementation assay is contingent on *S. pombe* Spt4 forming a functional complex with a heterologous *S. cerevisiae* Spt5. The cross-species subunit interaction might be more sensitive to certain Spt4 mutations than the intraspecies interaction. Note that whereas we have shown here that wild-type *S. cerevisiae* and *S. pombe* Spt4 proteins are functionally interchangeable in budding yeast, this is not the case for the Spt5 proteins. Specifically, we constructed a *S. cerevisiae* *spt5Δ* p(*CEN URA3 SPT5*) strain suitable for assay of essential Spt5 functions by the plasmid shuffle method. We found that a plasmid-borne *S. cerevisiae* *SPT5* gene complemented the null mutant. By contrast, a plasmid expressing full-length *S. pombe* Spt5 under the control of a constitutive *S. cerevisiae* promoter did not complement in this assay, even when cotransformed with a plasmid expressing *S. pombe* Spt4 (data not shown).

Genetic interactions between Spt4 and the Spt5 CTD

S. pombe Spt5 is distinguished by the orderly primary structure of its CTD, which extends from amino acids 801–990 and consists of a tandem array of nonapeptide repeats of the consensus sequence TPAWNSGK (Fig. 4B). The unmodified Spt5 CTD binds avidly to the triphosphatase and guanylyltransferase components of the *S. pombe* capping apparatus (Pei and Shuman 2002). The Spt5 CTD is also a substrate for threonine phosphorylation by *S. pombe* Cdk9 (Pei and Shuman 2003). Here we interrogated genetically the role of the Spt5 CTD nonamer array, by constructing mutant strains of *S. pombe* in which *spt5* alleles encoding incrementally truncated Spt5 proteins were integrated at the chromosomal *spt5* locus in lieu of the wild-type gene. The Spt5-(1-898), Spt5-(1-862), and Spt5-(1-835) proteins contain rump CTDs with ten, six, and three nonamer repeats, respectively (Fig. 4B). Full-length Spt5 and each of the three Spt5-CTDΔ proteins were expressed in *S. pombe* at comparable steady-state levels, as gauged by Western blotting of whole-cell lysates with purified polyclonal Spt5 antibody (Supplemental Fig. S3).

The immunoreactive Spt5 proteins displayed the expected incremental changes in electrophoretic mobility (Supplemental Fig. S3). We found that haploid *S. pombe* strains bearing any of the three truncated *spt5* alleles grew as well as the *spt5⁺* strain at 30°C, 34°C, and 37°C, as gauged by colony size (Fig. 4A). We conclude that three nonamer repeats (containing two Cdk9 Thr-Pro phosphorylation sites) suffice for viability under laboratory growth conditions. Complete deletion of all nonamer repeats causes an overt growth defect that will be described in detail elsewhere (S Schneider and B Schwer, in prep.).

The instructive finding here was that combining the serial Spt5 CTD truncations with the *spt4Δ* mutation resulted in slowed growth at temperatures normally permissive for the *spt4Δ* strain (Fig. 4A, *spt5¹⁻⁸⁶² spt4Δ*). This synthetic enhancement culminated in the lethality of the *spt5¹⁻⁸³⁵ spt4Δ* double mutant at 34°C and a strong synthetic sick phenotype at 30°C (Fig. 4A). We surmise that a longer Spt5 CTD becomes essential when Spt4 function is ablated. The implication is that interaction of Spt4 with the central domain of Spt5 overlaps functionally with the Spt5 CTD.

Concluding remarks

The present study extends our knowledge of the fission yeast core Spt5-Spt4 complex, which we characterize as a heterodimer. Fission yeast Spt5 has an exceptionally regular CTD nonapeptide array that mediates Spt5 interactions with mRNA capping enzymes. Here we showed that as few as three nonamer repeats sufficed for cell growth, but only when Spt4 was present. A genetic analysis of Spt4 in *S. pombe* revealed it to be inessential for growth at 25°C–30°C, but critical at 37°C. These results echo the temperature-sensitive *spt4Δ* growth phenotype in budding yeast, where *S. cerevisiae* and *S. pombe* Spt4 are functionally interchangeable. The *S. cerevisiae* complementation assay provided a sensitive readout of mutational effects on *S. pombe* Spt4 function in vivo. The targets for the Spt4 alanine scan were chosen based on conservation among eukaryal and archaeal Spt4 homologs. After the mutational study was completed, the coordinates of the crystal structure of *S. cerevisiae* Spt4 bound to a fragment of Spt5 were deposited (RCSB Protein Databank ID code 2exu). The structure, now published (Guo et al. 2008), provided an invaluable guide to interpret the mutational data we obtained for *S. pombe* Spt4. The genetic tools described here can assist further dissection of the postulated transcription elongation checkpoint in fission yeast.

MATERIALS AND METHODS

Recombinant *S. pombe* Spt5-Spt4 complex

The fission yeast Spt4 and Spt5-(165-800) ORFs were inserted into the pETDuet expression vector (Novagen) so that the Spt4 ORF

was fused in-frame to a leader sequence encoding a His-tag. The dual-expression plasmid was transformed into *Escherichia coli* BL21(DE3). Expression of the *S. pombe* proteins was induced by IPTG, and the His-tagged Spt4 and associated Spt5-(165-800) were purified from a soluble bacterial lysate by Ni-agarose chromatography, following protocols developed previously for other recombinant His-tagged *S. pombe* proteins (Pei et al. 2001). The affinity-purified HisSpt4/Spt5-(165-800) complex was mixed with marker protein catalase, BSA and vtopo. The mixture was applied to a 4.8-mL 15%–30% glycerol gradient containing 50 mM Tris-HCl (pH 8.0), 125 mM NaCl, 1 mM EDTA, and 0.05% Triton-X100. The gradient was centrifuged in an SW50 rotor at 50,000 rpm for 18 h at 4°C. Fractions (0.2 mL) were collected from the bottom of the tube. An aliquot of glycerol gradient fraction 15 containing the putative HisSpt4/Spt5-(165-800) heterodimer was subjected to a second round of Ni-affinity purification as described (Pei and Shuman 2002).

Media and genetic manipulations

We followed standard protocols for yeast growth, transformations, mating, sporulation, and tetrad dissection, as described by Guthrie and Fink (1991) and Schiestl and Gietz (1989) for *S. cerevisiae* and on the Forsburg laboratory Pombe Technology website (www.rcf.usc.edu/~forsburg/index.html) for *S. pombe*.

Genetic assay of Spt4 function in *S. cerevisiae*

The *S. cerevisiae* SPT4 open reading frame (ORF) was replaced in a diploid W303 strain with a *spt4::kanMX* gene disruption cassette (Güldener et al. 1996). Integrants were selected on YPD agar plates containing geneticin (150 mg/L, Invitrogen). Correct gene targeting was confirmed by diagnostic Southern blotting of restriction endonuclease-digested genomic DNA. The SPT4 *spt4Δ* diploid was sporulated, and tetrads were dissected. We thereby obtained a haploid *spt4Δ* strain (*Mata spt4::kanMX ura3 ade2 trp1 his3 leu2 can1*) that grew well at 30°C but failed to form colonies on YPD agar at 37°C. A complementing plasmid, p358-SceSpt4, was generated by inserting a genomic NheI-EcoRI fragment (2.3 kb) containing the wild-type *S. cerevisiae* SPT4 gene into pSE358 (*CEN TRP1*). For expression of *S. pombe* Spt4 in budding yeast, a DNA fragment spanning the 318-bp *spt4*⁺ ORF was amplified from chromosomal DNA by PCR with primers designed to introduce an NdeI site at the ATG codon and a BamHI site downstream of the stop codon. The DNA fragment was restricted and inserted into pYN132 (*TRP1 CEN*), a shuttle vector (modified from pYX132, Novagen) in which expression of *S. pombe* Spt4 is under the transcriptional control of the constitutive *TPII* promoter. Alanine mutations were introduced into the wild-type *spt4*⁺ gene by two-stage overlap extension PCR with mutagenic primers and pYN132-spt4 as the template. The mutated alleles were restricted and inserted into pYN132. The inserts of all plasmids were sequenced to verify that no undesired coding changes were introduced during amplification and cloning. The *CEN TRP1* plasmids expressing wild-type SceSpt4, wild-type SpoSpt4, or mutated SpoSpt4 were transformed into *S. cerevisiae spt4Δ* cells. Growth of Trp⁺ transformants was assessed by spotting 3 μL aliquots of serial 10-fold dilutions of yeast cultures (grown to mid-log phase in synthetic dropout [SD] broth lacking tryptophan at 30°C and adjusted to A₆₀₀ of 0.1) to SD-Trp agar medium and then incubating the plates at 30°C and 37°C.

Two-hybrid assay of Spt4-Spt5 interaction

The two-hybrid assays were performed as described previously (Pei et al. 2001; Pei and Shuman 2002). Briefly, aliquots of *S. cerevisiae* Y190 cells (Clontech Laboratories) harboring plasmids for expression of Gal4(AD)-Spt5-(165-990) or Gal4(AD)-Spt5-(165-400) plus wild-type or mutated Gal4(BD)-Spt4 proteins were tested for histidine prototrophy by spotting to SD(-Trp, -Leu, -His) agar medium containing 25 mM 3-amino-1,2,4-triazole (Sigma). Growth was scored after 4 d of incubation at 30°C. Expression of the *lacZ* reporter was scored by blue color in the β-galactosidase colony-lift filter assay.

Deletion of *spt4* in *S. pombe*

The entire *spt4*⁺ ORF was replaced in a diploid strain with either the *kanMX* or *natMX* gene conferring antibiotic resistance. To construct the disruption cassettes, DNA segments spanning 490 bp upstream and 410 bp downstream of the *spt4* ORF were amplified by PCR from chromosomal DNA and inserted into modified pTOPO vectors to flank the *kanMX* (Güldener et al. 1996) or the *natMX* (Goldstein and McCusker 1999) cassette. DNA fragments with the *spt4::kanMX* (2.4 kb) and *spt4::natMX* (2.1 kb) disruption cassettes were transformed into a *S. pombe* diploid. Transformants were selected on YES (yeast extracts with supplements) agar medium containing geneticin (150 mg/L, Invitrogen) or nourseothricin (100 mg/L clonNAT, Werner Bioagents). Correct gene targeting was confirmed by diagnostic colony PCR and Southern blotting. The heterozygous diploids were then sporulated, and tetrads were dissected to obtain *spt4Δ* haploids used for complementation assays.

For expression of Spt4 in fission yeast, DNA fragments encoding wild-type or mutant Spt4 proteins were excised from the respective pYN132-spt4 plasmids and inserted into pREP81x, wherein Spt4 is expressed under the transcriptional control of a weakened *nmt1* promoter (Forsburg 1993). Leu⁺ transformants were selected and the growth of the various pREP81x-*spt4* strains was compared to *spt4Δ* cells that had been transformed with the empty pREP81x plasmid. Aliquots (3 μL) of serial 10-fold dilutions of *S. pombe* cultures (grown to mid-log phase in SD broth lacking leucine at 30°C and adjusted to A₆₀₀ of 0.1) were spotted on to agar medium and the plates were incubated at 30° and 37°C.

Truncations of the Spt5 CTD array in *S. pombe*

We constructed a pUC19-based plasmid harboring the *ura4*⁺ gene upstream of a 534-bp fragment of *S. pombe* genomic DNA located immediately 3' of the stop codon of the *spt5*⁺ gene. We then inserted upstream of *ura4*⁺ a series of *spt5* gene fragments extending from an NdeI site (at nucleotide +1736 within the *spt5* ORF) to either the stop codon at position +2971 (the natural stop codon) or newly created stop codons at positions +2695, +2587, or +2506 that result in truncations of the Spt5 CTD. Linearized *spt5*^{CTD+/Δ-ura4}⁺-*spt5*^{3'} cassettes were transformed into haploid *S. pombe* cells. Ura⁺ transformants were selected and targeted insertions were verified by diagnostic Southern blotting.

Anti-Spt5 antibodies

Polyclonal antibodies were raised in rabbits immunized with recombinant His₁₀Smt3-Spt5-(1-990) at the Pocono Rabbit Farm

and Laboratory (Canadensis, PA). Anti-Spt5 antibodies were affinity purified from whole serum by affinity-chromatography by adsorption to His₁₀Smt3-Spt5-(1-990) coupled to Affigel-10 (Bio-Rad).

Western blot analysis

The haploid (h^-) *spt5*^{CTD+/Δ}-*ura4*⁺-*spt5*^{3'} strains *spt5*-(1-990)::*ura4*⁺, *spt5*-(1-898)::*ura4*⁺, *spt5*-(1-862)::*ura4*⁺, and *spt5*-(1-835)::*ura4*⁺ were grown in YES broth at 30°C. Aliquots of cells from exponentially growing cultures (equivalent to 6 A₆₀₀ units) were harvested by centrifugation. The cell pellets were suspended in 200 μL SDS-sample buffer (2% SDS, 50 mM Tris-HCl at pH 6.8, 100 mM DTT, 0.1% bromophenol blue, 10% glycerol), and acid-washed glass beads (~100 μL) were added. Cells were disrupted by five 30-sec cycles of vigorous vortexing. The samples were heated for 5 min at 100°C and centrifuged to remove macroaggregates and glass beads. Aliquots (25 μL) of the whole-cell lysates were analyzed by 6% SDS-PAGE and stained with Coomassie brilliant blue to verify that similar amounts of total protein were present in each of the lysates. For Western blot analysis, aliquots (2 μL) of the lysate were resolved by 6% SDS-PAGE, and the polypeptides were transferred to a PVDF membrane (NEN Research Products) with a semi-dry transfer apparatus (semi-phor, Hoefer Scientific Instruments). The membrane was probed with affinity purified anti-Spt5 antibodies, and the immune complexes were visualized with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Cell Signaling Technology) using an enhanced chemiluminescence system (ECL, Amersham).

Mutational synergy between *spt4Δ* and *spt5*^{CTDΔ}

Haploid *spt4Δ*::*natMX* (h^+) cells were mated to each of the following haploid (h^-) *spt5*^{CTD+/Δ}-*ura4*⁺-*spt5*^{3'} strains: *spt5*-(1-990)::*ura4*⁺, *spt5*-(1-898)::*ura4*⁺, *spt5*-(1-862)::*ura4*⁺, and *spt5*-(1-835)::*ura4*⁺. Diploids were selected on synthetic medium lacking uracil and containing nourseothricin. The diploids were sporulated and tetrads dissected. The genotypes of haploid progeny were confirmed by diagnostic colony PCR. Growth of the *spt5*^{CTD+/Δ} and the *spt5*^{CTD+/Δ} *spt4Δ* strains was assessed by spotting 3 μL aliquots of serial 10-fold dilutions of logarithmic cultures grown at 30°C onto YES agar medium and then incubating the plates at 30°C, 34°C, or 37°C.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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