Evidence that Spt4, Spt5, and Spt6 control transcription elongation by RNA polymerase II in Saccharomyces cerevisiae

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Previous characterization of the Saccharomyces cerevisiae Spt4, Spt5, and Spt6 proteins suggested that these proteins act as transcription factors that modify chromatin structure. In this work, we report new genetic and biochemical studies of Spt4, Spt5, and Spt6 that reveal a role for these factors in transcription elongation. We have isolated conditional mutations in SPT5 that can be suppressed in an allele-specific manner by mutations in the two largest subunits of RNA polymerase II (Pol II). Strikingly, one of these RNA Pol II mutants is defective for transcription elongation and the others cause phenotypes consistent with an elongation defect. In addition, we show that spt4, spt5, and spt6 mutants themselves have phenotypes suggesting defects in transcription elongation in vivo. Consistent with these findings, we show that Spt5 is physically associated with RNA Pol II in vivo, and have identified a region of sequence similarity between Spt5 and NusG, an Escherichia coli transcription elongation factor that binds directly to RNA polymerase. Finally, we show that Spt4 and Spt5 are tightly associated in a complex that does not contain Spt6. These results, taken together with the biochemical identification of a human Spt4–Spt5 complex as a transcription elongation factor (Wada et al. 1998), provide strong evidence that these factors are important for transcription elongation in vivo.

[Key Words: Transcription elongation; Spt4; Spt5; Spt6; NusG; chromatin]

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histones, that it can assemble nucleosomes in vitro, and that spt6 mutations alter chromatin structure in vivo (Bortvin and Winston 1996). In addition to their role in transcription, genetic studies suggest that these proteins are required for normal recombination (Malagon and Aguilera 1996) and chromosome segregation (Basrai et al. 1996), raising the possibility that they are involved in the establishment or maintenance of chromatin states necessary for diverse chromosomal functions.

Previous studies of SPT4, SPT5, and SPT6 involved the analysis of mutant alleles isolated by particular genetic selections (Winston and Carlson 1992). Because SPT5 and SPT6 are essential for growth, these studies may have yielded mutations conferring only a subset of the possible mutant phenotypes that could be acquired. In this work, we have isolated new mutations in SPT5, requiring only that they cause cold-sensitive growth. Our analysis of these mutants and of extragenic suppressors has led us to evidence that Spt4, Spt5, and Spt6 are required for transcription elongation in vivo. Consistent with these genetic data, we have obtained evidence that Spt5 associates with RNA Pol II. Finally, we show that Spt4 and Spt5 are in a complex that does not contain Spt6. These results, in conjunction with those in the accompanying manuscript (Wada et al. 1998) that show that a human Spt4–Spt5 complex affects transcription elongation in vitro, provide strong evidence that these proteins play an important role in transcription elongation throughout eukaryotes.

Results

Identification of cold-sensitive spt5 mutants

Previous genetic analysis of SPT5 relied on spt5 mutations that were selected either as suppressors of insertion mutations or as suppressors of snf–swi mutations (Winston and Carlson 1992). Because SPT5 is essential for growth, such mutations might represent a particular class of spt5 alleles and, hence, only cause a subset of possible spt5 mutant phenotypes. Therefore, we screened for a new class of spt5 mutations—those that cause cold-sensitive (Cs−) growth. Two new spt5 mutations that cause a Cs− phenotype, spt5-242 and spt5-276, were isolated. Both mutations also cause the phenotype observed for the original spt5 mutations, suppression of Ty Δ insertion mutations (Spt− phenotype; Table 1). As shown in subsequent sections, however, these new spt5 alleles have revealed a previously unknown role for Spt5. In both spt5 Cs− mutants, the level of Spt5 protein is unaffected, even when the strains are grown at nonpermissive temperature for 48 hr (data not shown).

To look directly at the effects of the spt5 Cs− mutations on transcription, we examined the steady-state levels of three different mRNAs by Northern hybridization analysis. The results show that the spt5 mutations cause a significantly reduced level of TPI1 and HIS4 mRNAs when cells are shifted to the nonpermissive temperature (13°C) for 48 hr (Fig. 1). Under the conditions of this experiment, there was no loss of viability for the spt5 mutants (data not shown). These results show that the spt5 Cs− mutations cause decreased levels of some, but not all, RNA Pol II-dependent transcripts.

Extragenic suppressors in RPB1 suggest that the spt5 Cs− mutations impair transcription elongation

To identify proteins that might interact with Spt5, we selected for suppressors of the two spt5 Cs− mutants.

Table 1. Phenotypes of spt5 Cs− and rpb1/rpo21 mutants

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Growtha</th>
<th>Other phenotypes</th>
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<tbody>
<tr>
<td></td>
<td>15°C</td>
<td>30°C</td>
</tr>
<tr>
<td>1 wild type</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2 spt5-242</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3 spt5-276</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td>4 rpb1-221</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5 rpb1-244</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6 rpo21-18</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>7 rpb1-5</td>
<td>+f</td>
<td>+</td>
</tr>
<tr>
<td>8 rpb1-244 spt5Cs−</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>9 rpb1-221 spt5 Cs−</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>10 rpo21-18 spt5-242</td>
<td>−</td>
<td>−/+</td>
</tr>
<tr>
<td>11 rpb1-5 spt5-276</td>
<td>−</td>
<td>−/+</td>
</tr>
</tbody>
</table>

aAll phenotypes were scored by replica plating patches of cells to YPD plates and incubating at the indicated temperature. For growth at 15°C, + indicates confluent growth after 3 days, −/+ indicates slightly less growth than +, and − indicates little or no growth after 5 days. For growth at 30°C and 37°C, + indicates confluent growth after 1 day, −/+ indicates that the strain grew nearly as well as wild type, −/+ indicates slow but definite growth after 3 days, and − indicates little or no growth after 3 days. (N.D.) Not done.

bSC−His tests the Spt phenotype. Spt− strains are His−, and Spt+ strains are His+ (Winston et al. 1984). Growth on SC−His was scored at 30°C. + indicates confluent growth after 2 days, −/+ indicates slow but definite growth after 3 days, and − indicates little or no growth after 3 days.

“N.D.” data were reported in Scafe et al. (1990a).

The spt5 Cs− mutations suppressed the rpb1sup mutations indistinguishably. Therefore, lines 7 and 8 each refer to double mutant combinations of the rpb1 allele with both spt5-242 and spt5-276.
Interestingly, two of the suppressor mutations are in RPBI/RPO21, which encodes the largest subunit of RNA Pol II (see Materials and Methods; hereafter, we will refer to this gene and its mutant forms as RPBI, except for mutant alleles published previously as RPO21). These suppressor mutations have been designated rpb1-221 and rpb1-244 and will be referred to as rpb1sup mutations. In addition to suppressing the Cs− phenotype of the spt5 mutants, the rpb1sup mutations cause two other mutant phenotypes: sensitivity to 6-azauracil (6AUs), and a weak Spt− phenotype in an SPT5+ genetic background (Table 1).

Substantial evidence suggests that a 6AUs phenotype indicates a defect in transcription elongation (Uptain et al. 1997). 6AUs is a drug that reduces UTP and GTP levels in vivo (Exinger and Lacroute 1992), and decreased nucleotide levels have been shown to increase pausing and arrest by RNA Pol II in vitro (Uptain et al. 1997). In addition, particular mutations in RNA Pol II subunits and in the transcription elongation factor TFIIS confer a 6AUs phenotype in yeast (Archambault et al. 1992; Exinger and Lacroute 1992; Powell and Reines 1996). Thus, the 6AUs phenotype caused by the rpb1sup mutations suggests that they impair transcription elongation.

The sequence of the rpb1sup mutations was determined. Each mutant gene contains a single base pair change; rpb1-221 causes the amino acid change H1367D, and rpb1-244 causes the amino acid change E1351K. Both of these amino acid changes occur between regions G and H of Rpb1, two of the eight blocks of sequence homology conserved among the largest subunits of eukaryotic and prokaryotic DNA-directed RNA polymerases (Archambault and Friesen 1993). Interestingly, previous studies have identified other mutations in the same region of RPBI, and some of these mutations also cause a 6AUs and/or an Spt− phenotype (Archambault and Friesen 1993; Berroteran et al. 1994). Two of these 6AUs rpb1 mutations, rpo21-18 and rpo21-24, cause RNA Pol II to have a decreased affinity for the transcription elongation factor TFIIS (Wu et al. 1996). Thus, based on both their phenotypes and position, the rpb1sup mutations may cause a defect in transcription elongation.

The rpb1sup mutations might represent a particular class of rpb1 mutation. If so, we would expect the suppression of spt5 Cs− mutations by rpb1 mutations to be allele specific. To test this possibility, we analyzed double mutants between the spt5 Cs− mutations and two other rpb1 mutations. The results (Table 1) show that suppression of spt5 mutations by rpb1 mutations is indeed allele specific. First, we tested the rpb1 mutation rpo21-18, which, as mentioned, causes a decreased affinity between RNA Pol II and TFIIS, and a 6AUs phenotype. The rpo21-18 mutation behaved in the opposite fashion from the rpb1sup mutations: The rpo21-18 spt5-242 double mutant was extremely sick even at 30°C (Table 1). At 37°C, the temperature at which the spt5-242 mutant grows almost as well as wild type, the double mutant was viable. We also tested rpb1-5, which causes an amino acid change, R335C, distant from the GH region of Rpb1 (Scafe et al. 1990a). This mutation also does not suppress the spt5 Cs− mutations (Table 1). This allele specificity indicates that Spt5 interacts directly with RNA Pol II and/or that only a particular type of defect in RNA Pol II will suppress the spt5 Cs− phenotype. Both of these possibilities are supported by results described in later sections.

As a second way to test whether the rpb1sup mutations cause a distinct type of transcription defect, we analyzed them in double mutants with a deletion of the gene encoding the elongation factor TFIIS. During elongation, after each nucleotide is added to the nascent mRNA, RNA Pol II can either add another nucleotide, pause in an elongation-competent state, or arrest in an elongation-incompetent state (Fig. 2; Uptain et al. 1997). TFIIS acts on arrested RNA Pol II, stimulating an intrinsic nuclease activity of RNA Pol II to digest a few nucleotides from the 3′ end of the nascent mRNA (Uptain et al. 1997). This action resets RNA Pol II into an elongation-competent state, allowing it to make another effort to elongate through the site where it had arrested previously. In Saccharomyces cerevisiae, TFIIS is encoded by the PPR2 gene. In an otherwise wild-type background, deletion of PPR2 causes no detectable defect except to cause a 6AUs phenotype (Table 2; Exinger and Lacroute 1992; Uptain et al. 1997). The lack of a strong ppr2Δ phenotype is presumably because either TFIIS is partially redundant with another factor, or arrest is not a

Figure 1. Northern blot analysis of SPT5+ and spt5 Cs− strains. SPT5+, spt5-242, and spt5-276 strains were grown at 30°C or 13°C for 48 hr prior to isolation of RNA for Northern analysis. The blots were probed with HIS4 or TPI1 as indicated, and also with TUB2 as a normalization control. Note that the order of the two mutants is different in the two experiments shown. The blot on the left contains RNA from strains FY2, FY1672, and FY1673. The blot on the right contains RNA from strains FY120, FY1634, and FY1635.

Figure 2. Model of the RNA Pol II transcription elongation cycle (for review, see Uptain et al. 1997).
Very frequent event during transcription elongation in an otherwise wild-type strain. Previous work has shown that rpo21-18 ppr2Δ mutants are viable, consistent with the fact that rpo21-18 impairs the RNA Pol II–TFIIS interaction (Archambault et al. 1992; Wu et al. 1996). In contrast, rpb1-244 ppr2Δ double mutants are either extremely sick or inviable (Table 2). The striking difference in behavior between the rpb1sup mutations and rpo21-18 strongly supports the idea that these mutations cause two different types of defects in elongation, in turn suggesting that the rpb1sup mutations do not impair RNA Pol II–TFIIS interactions, whereas the rpo21-18 mutation does.

Possible roles for Spt5 in promoting transcription elongation

The suppression of the spt5 Cs− mutations by rpb1 mutations that are likely to cause a transcription elongation defect suggests that Spt5 may play a role in transcription elongation. Therefore, the spt5 Cs− mutations may cause a transcription elongation defect. Such a defect could affect elongation in any of several different ways. We hypothesized that the role of Spt5 might be to promote transcription past potential barriers to elongation. By this model, when RNA Pol II encounters a barrier to elongation, it is much more likely to pause or arrest in an spt5 Cs− mutant, where the essential Spt5 activity is reduced, than it would be in an SPT5+ strain. This leads to two predictions that can be tested genetically. First, if RNA Pol II arrests at a greater frequency in an spt5 Cs− mutant, the cell’s dependence on TFIIS should be greater. Second, if Spt5 activity is reduced, then reducing the rate of elongation by RNA Pol II might provide adequate time for a partially disabled Spt5 to help RNA Pol II transcribe past the barrier, thereby suppressing the spt5 mutant defect. The next two sections present the results of these genetic tests.

Genetic interactions of Spt5 with the elongation factor TFIIS

To test whether the requirement for TFIIS is increased in an spt5 Cs− mutant, we performed double-mutant analysis. As mentioned above, deletion of PPR2, which encodes TFIIS, causes no detectable defect in cell growth except to cause a 6AU− phenotype. When combined with either of the spt5 Cs− mutations, however, ppr2Δ causes a severe growth defect. Both spt5-242 ppr2Δ and spt5-276 ppr2Δ double mutants are inviable at both 15°C and 30°C (Fig. 3; Table 2). At 37°C, the double mutants are viable. These results resemble those obtained previously for the spt5-242 rpo21-18 double mutant (Table 1). Thus, TFIIS becomes an essential factor when Spt5 function is limiting, strongly suggesting that the spt5 Cs− mutations cause RNA Pol II to arrest more frequently.

The spt5 Cs− mutations are suppressed by a decreased rate of transcription elongation

The second genetic test was to determine whether a decreased rate of transcription elongation would compensate for the impaired Spt5 function in the spt5 Cs− mutants. This test was performed in two ways. First, we took advantage of a mutation in RPB2, the gene encoding the second largest subunit of RNA Pol II. This mutation, rpb2-10, causes RNA Pol II to have a decreased elongation rate in vitro and causes a 6AU− phenotype in vivo (Powell and Reines 1996). Consistent with our model, in an spt5-242 rpb2-10 double mutant, rpb2-10 suppressed the Cs− phenotype of the spt5 mutation (Fig. 4A). As a control, we also tested another rpb2 mutation, rpb2-7, which, although it also causes a 6AU− phenotype, does not affect the RNA Pol II elongation rate in vitro (Powell and Reines 1996). This mutation did not suppress spt5-242 (Fig. 4A). Second, we treated cells with a low level of 6AU to decrease the concentration of nucleotides in vivo, thereby presumably decreasing the rate of transcription elongation. This test was possible because the spt5 Cs− mutations, unlike the rpb1sup mutations, do not cause a 6AU− phenotype (see Discussion). Our results show that 6AU suppresses the Cs− phenotype: The spt5 Cs− mutants grew as well as SPT5+ cells at 15°C on the 6AU-containing media (Fig. 4B). Thus, slower elongation by RNA Pol II apparently suppresses the Cs− defect of the spt5 Cs− mutants.

Genetic evidence that spt4, spt5, and spt6 mutations all affect transcription elongation

Previous work has strongly suggested that two other pro-
Expression of GST–Spt4 and GST–Spt6 fusion proteins, Spt4 and Spt6, act with Spt5 to control transcription in vivo (Swanson and Winston 1992). Therefore, we tested whether spt4 or spt6 mutants display defects consistent with an effect on transcription elongation. We also included previously identified mutant phenotypes that had been selected by their Spt− phenotype. First, we constructed and analyzed spt4Δ ppr2Δ, spt5−194 ppr2Δ, and spt6−50 ppr2Δ double mutants. We were able to use an spt4Δ mutation because, unlike Spt5 and Spt6, Spt4 is not essential for growth (Malone et al. 1993). All three double mutants show a strong temperature sensitive growth phenotype not observed for any of the single mutants (Fig. 5). These results show that the requirement for TFIIS is greatly increased in spt4, spt5, and spt6 mutants. spt4, spt5, and spt6 mutations do not generally cause synthetic lethality in combination with mutations in transcription factors (Winston et al. 1984), supporting the idea that the double-mutant phenotypes with ppr2Δ indicate an elongation defect. Second, we tested spt4Δ and several spt5 and spt6 mutants for a 6AU− phenotype. The spt4Δ mutation causes a strong 6AU− phenotype, as severe as that observed in a ppr2Δ mutant (Table 3). Of the three spt6 mutants tested, only one, spt6−50, was 6AU−. These mutant phenotypes strongly suggest that like Spt5, Spt4 and Spt6 play a role in transcription elongation.

Identification of an Spt4–Spt5 complex

Previously identified mutant phenotypes, including double-mutant lethality and unlinked noncomplementation, strongly suggested that Spt4, Spt5, and Spt6 might physically interact in a complex. Indeed, coimmunoprecipitation experiments detected a weak physical interaction between Spt5 and Spt6 (Swanson and Winston 1992). We have now examined the physical interactions between all three proteins, using three different methods.

First, we expressed GST–Spt4 and GST–Spt6 fusion proteins in vivo to assay their physical association with the other Spt proteins. For each fusion, we assayed its interactions with Spt5 and with Spt6. We observed that

Table 3. Growth of spt4, spt5, and spt6 mutants on media containing 6AU

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Growtha</th>
<th>Other phenotypes</th>
</tr>
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<tbody>
<tr>
<td>spt4−14 + + Spt +</td>
<td>−/− +</td>
<td>+ 6AU +</td>
</tr>
<tr>
<td>spt5−242 + + Spt +</td>
<td>+</td>
<td>+ 6AU +</td>
</tr>
<tr>
<td>spt5−276 + + Spt +</td>
<td>−/− +</td>
<td>+ 6AU +</td>
</tr>
<tr>
<td>spt5−194 + + Spt +</td>
<td>−/− +</td>
<td>+ 6AU +</td>
</tr>
<tr>
<td>spt6−14 + + Spt +</td>
<td>−/− +</td>
<td>+ 6AU +</td>
</tr>
<tr>
<td>spt6−140 + + Spt +</td>
<td>−/− +</td>
<td>+ 6AU +</td>
</tr>
<tr>
<td>spt6−50 + + Spt +</td>
<td>−/− +</td>
<td>+ 6AU +</td>
</tr>
</tbody>
</table>

aAll phenotypes were scored by replica plating patches of cells to the indicated media at 30°C. (+) Confluent growth after 1–2 days; (−/+ slow but definite growth after 3 days; (−) little or no growth after 3 days. All spt4, spt5, and spt6 mutations cause an Spt− phenotype.
Spt5 binds strongly to GST–Spt4 and only weakly to GST–Spt6 (Fig. 6A). There was no detectable interaction of Spt5 with GST alone. A control experiment showed that the Spt5/GST–Spt4 interaction was stable even after washing the bound complexes with up to 1M ammonium acetate (data not shown). In contrast to Spt5, Spt6 did not detectably bind to either GST–Spt4 or GST–Spt6. These results indicate a strong association between Spt4 and Spt5, a weak association between Spt5 and Spt6, and no detectable association between Spt4 and Spt6.

Second, we performed coimmunoprecipitation experiments to test for interactions among Spt4, Spt5, and Spt6. For these experiments, we used an HA1-epitope-tagged version of Spt4. Coimmunoprecipitation of Spt4 and Spt5 was tested in both directions, and the results show that the two proteins coimmunoprecipitate (Fig. 6B,C). In contrast, Spt6 did not detectably coimmunoprecipitate with either HA1–Spt4 (data not shown) or Spt5 (Fig. 6B).

Finally, we used a Flag-epitope-tagged version of Spt5 to partially purify Spt5 and associated proteins. When extracts made from Spt5–Flag-containing cells were applied to beads conjugated to anti-Flag antibody, both Spt5–Flag and Spt4 bound, whereas Spt6 did not (Fig. 7). Spt4 remained bound even after extensive washing in buffer containing 1M KCl (data not shown). As a control, extracts of wild-type cells, in which Spt5 was not epitope-tagged, were used for immunoprecipitations with either HA1–Spt4 (data not shown) or Spt5 (Fig. 6B).

Spt5 binds to RNA Pol II

Two results prompted us to test whether Spt5 physically associates with RNA Pol II. First, the extensive spt5–rpb1 genetic interactions suggested an Spt5–RNA Pol II interaction. Second, analysis of silver-stained SDS gels of the proteins that associate with Spt5-Flag showed the copurification, albeit substoichiometrically, of a protein of ∼220 kD, the size of Rpb1. Therefore, we performed Western analysis with anti-Rpb1 antibodies and found that Rpb1 copurifies specifically with the Spt4–Spt5 complex (Fig. 7). Furthermore, the association of Rpb1 with the anti-Flag beads was stable to extensive washes of buffer containing 0.5M KCl (data not shown). Thus, in addition to its genetic interactions with Rpb1 and Rpb2,
Spt5 appears to associate with RNA Pol II in vivo. Interestingly, computer searches have revealed a weak homology between a conserved portion of the Spt5 family of proteins and the bacterial elongation factor NuG (Fig. 8; Wada et al. 1998), which is also known to interact with E. coli RNA polymerase (Condon et al. 1995; Morige et al. 1995).

Discussion

In vivo and in vitro studies suggest an important role for Spt4, Spt5, and Spt6 in transcription elongation.

Our studies have provided evidence that the transcription factors Spt4, Spt5, and Spt6 are required for transcription elongation in vivo. This conclusion is supported by four sets of results presented in this paper. First, we have shown both a genetic and a physical interaction between Spt5 and RNA Pol II. Second, we have shown that spt4, spt5, and spt6 mutations all cause phenotypes that strongly suggest elongation defects in vivo. These phenotypes include allele-specific suppression of spt5 mutations by rpb1 and rpb2 mutations, including one rpb2 mutation that has been characterized as causing an elongation defect in vitro. In addition, spt4, spt5, and spt6 mutations all show conditional lethality in combination with ppr2Δ, a mutation that abolishes the function of the elongation factor TFIIS, and many spt4, spt5, and spt6 mutations cause a 6AU* phenotype. Third, Spt5 shows some sequence similarity to the E. coli transcription elongation factor NuG. Finally, we have shown that Spt4 and Spt5 form a complex; this result fits extremely well with the results in Wada et al. (1998), in which a human Spt4–Spt5 complex has been shown to affect transcription elongation in vitro and to interact with RNA Pol II.

Spt4 and Spt5 form a complex that does not contain Spt6.

Although our previous genetic results were most consistent with a model in which Spt4, Spt5, and Spt6 form a complex, the biochemical data presented here show clearly that Spt4 and Spt5 form a tight complex that, at most, associates only weakly with Spt6. We observed the tight association of Spt4 and Spt5 in three different assays (Figs. 6 and 7). In contrast, both our past (Swanson and Winston 1992) and current works show that Spt5 and Spt6 interact weakly at best, and in no case was any association detected between Spt4 and Spt6. Consistent with these findings, in preliminary experiments in which we have biochemically fractionated yeast extracts, Spt4 and Spt5 cofractionate, whereas Spt6 separates away from the Spt4–Spt5 complex (data not shown). Consistent with our findings, Wada et al. (1998) purified a human Spt4–Spt5 complex that does not contain human Spt6. Thus, any model for a common function for these three proteins must account for them working in physically distinct complexes. Given the genetic evidence that Spt4, Spt5, and Spt6 work together, an Spt4–Spt5 complex may interact with Spt6 in a dynamic, but biochemically unstable fashion in vivo.

Spt4, Spt5, and Spt6 are conserved regulators of transcription elongation.

Our results suggest that Spt5 serves a positive role for transcription elongation in vivo. Although we have not yet been able to obtain direct biochemical evidence that spt5 mutants are defective for elongation, Wada et al. (1998) provided a clear biochemical demonstration of a direct role for Spt4–Spt5 in transcription elongation in vitro. The sequence conservation between the yeast and human Spt4 and Spt5 proteins (Chiang et al. 1996a; Hartzog et al. 1996; Stachora et al. 1997) and the fact that human Spt4 functions well in yeast (Hartzog et al. 1996) suggest that these proteins are functionally conserved. Thus, it is likely that at a fundamental level, Spt4 and Spt5 carry out similar roles in yeast and humans.

Our past genetic studies of Spt4, Spt5, and Spt6 have strongly suggested that these genes also play a negative role in transcription initiation (Swanson and Winston 1992; Winston and Carlson 1992). These results fit well with the results of Wada et al. (1998), showing both negative and positive effects of human Spt4–Spt5 in vitro. Our previous conclusions about a role for Spt4, Spt5, and Spt6 in initiation were based on the ability of many spt4, spt5, and spt6 mutations to suppress loss of the Snf–Swi complex (Niegelborn et al. 1987; Swanson and Winston 1992), the deletion of a UAS element (Prelie and Win-

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**Figure 8.** A repeated element in Spt5 is homologous to a region of NuG. Alignments of a conserved, repeated element in Spt5 proteins along with the consensus sequence developed by the program MEME. This consensus is similar to a motif of unknown function, KOW (Kyrpides et al. 1996), found in NuG, ribosomal protein L24, and their homologs. For comparison, the homologous sequences of the highest scoring NuG and L24 proteins are included. The accession number for S. cerevisiae Spt5 is M62882; for human, U56402; for Caenorhabditis elegans, Z60316; for Schizosaccharomyces pombe, Z99753; for Tetrahymena thermophila, NusG, P39572; and for Mycoplasma capricolum, L24, P10141.
mutations in positive activators (Denis and Malvar 1990), and the negative effects of insertion mutations in the promoter regions of genes (Swanson and Winston 1992). Although we believed these effects to occur at the level of transcription initiation, we must now consider that some of them could occur at the level of elongation. Consistent with this reinterpretation of our data, recent studies suggest a role for Snf-Swi in elongation (Brown et al. 1996) and that promoter-bound transcription factors can modify the processivity of RNA Pol II (Bentley 1995; Krumm et al. 1995).

Spt5 and RNA Pol II interact physically and functionally to promote elongation

The allele-specific interactions we have observed between spt5 and rpb1 mutations have suggested two types of interactions between Spt5 and RNA Pol II. Allele-specific interactions between mutations in different genes are often believed to indicate a physical interaction of their protein products. The demonstration of an Rpb1–Spt5 physical interaction (Fig. 7) supports this interpretation of the genetic data. The spt5 Cs− mutations, however, are also suppressed in an allele-specific manner by rpb2 mutations, as well as by treatment with 6AU. These results suggest that the spt5 Cs− mutations are suppressed by a particular type of defect in RNA Pol II that is conferred by the rpb1sup and rpb2-10 mutants, as well as by treatment with 6AU. Given that the rpb2-10 mutation causes RNA Pol II to have a decreased elongation rate in vitro and that 6AU treatment is believed to decrease the rate of transcription elongation in vivo (Powell and Reines 1996; Uptain et al. 1997), it is likely that the common defect that causes suppression of the spt5 Cs− mutations is a decrease in the processivity of RNA Pol II. Thus, the allele-specific nature of the rpb1–spt5 interactions likely reflects both a functional and physical interaction between Spt5 and RNA Pol II. Additional genetic and biochemical analysis will be required to determine which RNA Pol II subunits are actually in direct contact with Spt4–Spt5.

Spt4, Spt5, and Spt6 may help RNA Pol II transcribe past nucleosome barriers

The synthetic conditional phenotypes we observed for spt prp2-1 double mutants suggest that, in the absence of Spt4, Spt5, and Spt6 function, RNA Pol II encounters conditions that cause it to arrest. Interestingly, a DNA-bound protein can block elongation in vitro and impose a requirement for TFIIIS function (Reines and Mote 1993). Previous studies have shown that nucleosomes form a barrier to elongating RNA Pol II in vitro, suggesting that they also form a barrier in vivo (Chang and Luse 1997; Uptain et al. 1997). In addition, recent work has shown that the nucleosome remodeling activity Snf–Swi (Brown et al. 1996), and another activity, FACT (Orphanides et al. 1998), are important for elongation on chromatin templates. Spt4, Spt5, and Spt6 are distinct from these activities, as Spt5 and Spt6 are not part of either Snf–Swi (B. Cairns, pers. comm.) or FACT (D. Reinberg, pers. comm.). We propose that one role for Spt4, Spt5, and Spt6 in transcription elongation in vivo is to help RNA Pol II elongate past nucleosomes. The results in this work and those in Wada et al. (1998) have shown an interaction of Spt4–Spt5 with RNA Pol II. In addition, these investigators have shown that Spt4–Spt5 affects transcription elongation in vitro on a nonchromatin template. These results suggest that Spt4–Spt5 interacts directly with RNA Pol II to control its processivity. Past biochemical analysis has shown a direct interaction of Spt6 with histones (Bortvin and Winston 1996). In addition, past genetic analyses have shown that spt4, spt5, and spt6 mutations share many phenotypes with mutations in histone genes (Hirschhorn et al. 1992; Winston and Carlson 1992; Bortvin and Winston 1996). These results suggest that Spt4, Spt5, and Spt6 may mediate interactions of RNA Pol II with chromatin. In helping RNA Pol II elongate past nucleosomes, Spt4, Spt5, and Spt6 could conceivably be required for either of two hypothetical steps: (1) nucleosome remodeling or disassembly to allow the passage of RNA Pol II, or (2) the reassembly of nucleosomes into a normal chromatin structure after the passage of RNA Pol II. Our results do not yet distinguish between these possibilities. Previous studies of Spt6 have shown that it has nucleosome assembly activity in vitro (Bortvin and Winston 1996); whether or not an Spt6 nucleosome assembly activity is relevant to the role Spt6 plays in elongation in vivo remains to be determined. Given the known biochemical interactions so far established, one possibility is that Spt4–Spt5 is primarily associated with the transcriptional apparatus, whereas Spt6 is primarily associated with nucleosomes.

The suppression of the spt5 Cs− mutations by a decreased rate of transcription elongation also supports a model in which the spt5 mutations impair the ability of RNA Pol II to elongate past nucleosomes. In this model, the rate of Spt4–Spt5 function must be appropriately coupled to the rate of RNA Pol II elongation to avoid pausing. In an spt5 Cs− mutant grown under nonpermissive conditions, Spt4–Spt5 function is reduced such that RNA Pol II has an increased probability of pausing or arresting when it encounters a nucleosome. The pause is caused by the inability of Spt4–Spt5 to act at a normal rate to remove the nucleosome barrier. A decreased rate of elongation by RNA Pol II, then, would allow adequate time for the weakened Spt4–Spt5 activity to remove the barrier, thereby avoiding a stalled RNA Pol II. Previous studies have suggested that Cs− mutations can impair protein–protein interactions and/or protein activity (see discussion in Noble and Guthrie 1996). Thus, the specific defect caused by the spt5 Cs− mutations could be a weakened interaction with RNA Pol II, histones, or some other transcription elongation factor. Alternatively, the spt5 Cs− mutations may interfere with an intrinsic activity of Spt5. Because the spt5 Cs− mutations do not cause a 6AU sup phenotype and are actually suppressed by 6AU, the elongation defect that they cause is very likely to be distinct from that caused by rpb1,
rpB2, or other spt mutations that cause a 6AU− phenotype and are believed to impair elongation.

In conclusion, our work has provided strong genetic evidence for a role in transcription elongation for yeast Spt4, Spt5, and Spt6. Biochemical experiments on the human Spt4 and Spt5 proteins also strongly suggest a role in elongation (Wada et al. 1998). Future experiments will be aimed to discover other proteins that physically associate with both the Spt4–Spt5 complex and with Spt6, to address how these factors might play both positive and negative roles in transcription elongation in vivo, and to develop in vitro assays for these factors on chromatin templates. It will be interesting to determine whether Spt4, Spt5, and Spt6 are required for the function of transcriptional activators that work by overcoming a transcriptional pause, such as Drosophila hsp70 (Lis and Wu 1993) or HIV Tat (Jones 1997).

Materials and methods

Media and genetic methods

The S. cerevisiae strains used in this study (Table 4) are isogenic to S288C and are GAL2+ (Winston et al. 1995). Strain constructions and other genetic manipulations were carried out by standard methods. Yeast media, including rich media (YPD), minimal media (SD), and synthetic complete media lacking uracil (SC − Ura) were made as described previously (Rose et al. 1990). Strains containing the rpB1Δ187::HIS3 and rpB2Δ297::HIS3 deletions were made as described previously (Nonet et al. 1987; Scafe et al. 1990b). 6AU sensitivity was scored on SC − Ura plates with 50 µg/ml of 6AU. The ppR2::hisG mutation was used to integrate plasmid pG3. Strains carrying the plasmidborne rpB1 and rpB2 mutations were created by plasmid shuffle methods (Guthrie and Fink 1991) by use of host strains with either rpB1Δ187::HIS3 or rpB2Δ297::HIS3. Preliminary experiments utilized a derivative of SPT5 that had been tagged with the Myc epitope. Although this epitope was not used, several of the strains listed do carry Spt5–Myc. All epitope-tagged alleles of SPT4, SPT5, and SPT6 used in this work were phenotypically indistinguishable from the wild-type genes. Double-mutant strains that are inviable at 30°C were isolated by germination of dissected tetrads at 37°C.

Plasmids

pRP187 and pRP112 are described in Nonet et al. (1987), pRP212, pRP2–7(U), and pRP2–10(U) are described in Scafe et al. (1990b). pRPB1−S is described in Scafe et al. (1990a), pJA509 and pJA483 are described in Archambault et al. (1992), pFW45 (Winston et al. 1984), pYS138 (Som et al. 1988), and pHBS9 (a gift of Henry Baker, University of Florida, Gainesville) were used to make the HIS4, TUB2, and PHO1 probes for Northern blots. pHG25 is a derivative of pMALcRI (New England Biolabs) fused in-frame to the 3′ EcoRI site of SPT5. This results in expression of a fusion of MBP to amino acids 807–1063 of Spt5, pHG123 contains full-length SPT5 fused in-frame to MB in pMALcRI. The HA1 epitope-tagged allele of SPT5 was cloned by subcloning the HindIII fragment of pBM56 (Malone et al. 1993), containing HA1–SPT4, into pRS306 (Sikorski and Hieter 1989) to create pHG90. The SPT5–Flag allele was constructed by site-directed mutagenesis of pHG17, a derivative of pBluescript–IKS+ containing the KpnI–Xhol fragment of SPT5, by use of oligonucleotide OGH52, 5′-CTCCTTCTTTCTTTGATTCATTTTG-3′. Spt4, Spt5, Spt6 and transcription elongation

CATCGTCTCTTTGTAGCTACCTCCCATGTAC-3′. The mutagenized KpnI–Xhol SPT5–Flag fragment was cloned into pRS506, creating plasmid pGH184. pSM4 is described in Swanson et al. (1991), and pBM24 contains the SPT5 genomic DNA fragment from pMS4 in pBM453, a TRP1 CEN shuttle vector. pGH84 contains full-length SPT5 fused in-frame to GST in pEMBL–GST (Kranz et al. 1994), pGH85 contains full-length SPT6 fused in-frame to GST in pEMBL–GST. GST was expressed from plasmid pYBS305 (pEML–GST derivative generously provided by E. Elion, Harvard Medical School, Boston, MA). pGH189 is a derivative of pRP112 that lacks the Aattl–BstEII fragment upstream of RPBI. pGH188 is a derivative of pRP112 that lacks an internal EcoRI–XbaI fragment of RPBI.

Isolation of spt5 Cs− mutants

The spt5 Cs− mutations were isolated by mutagenizing plasmid pBM24 with hydroxylamine as described (Rose et al. 1990). The mutagenized plasmid was used to transform strain FY849 and the Trp+ transformants were replica plated to 5FOA plates to select for loss of the wild-type SPT5 plasmid pMS4. 5FOA-resistant colonies were then replica plated to YPD plates that were incubated at 15°C and 30°C to identify colonies defective for growth at 15°C. Each candidate was restested by isolating the mutant plasmid and using it to retransform strain FY849 to determine if the Cs− phenotype was plasmid linked. Two spt5 mutations conferring a Cs− growth defect were identified and recombined into the S. cerevisiae genome, replacing SPT5+. When integrated, these spt5 mutations, spt5−242 and spt5−276, still cause a Cs− phenotype and also confer a weak Spt− phenotype. Linkage analysis and plasmid complementation were used to verify that these mutant phenotypes were caused by the spt5 mutations.

Isolation of suppressors of the spt5 Cs− mutations and identification of these suppressors as rpB1 mutations

To isolate extragenic suppressors of the spt5 Cs− mutations, the spt5−242 strain FY1635 and the spt5−276 strain FY1634 were mutagenized with 300 ergs/mm2 of UV light, allowed to grow into colonies, and then replica plated to YPD and incubated at 15°C. Colonies that grew at 15°C were purified and restested for a Cs− phenotype. The detailed analysis of most of these suppressors will be described elsewhere. Two of the suppressor mutations were shown to be mutations in RPBI by several steps. These mutations suppressed the Cs− phenotype of the spt5 mutants and also caused a mild Spt− phenotype in an SPT5+ genetic background. To clone the gene corresponding to this complementation group, strain FY1637 was transformed with a plasmid library of yeast genomic DNA (Rose et al. 1987) Transformants were screened for an Spt− phenotype (His− and Lys−). Candidate plasmids were isolated and used to retransform strains FY1637 and FY1636 to determine whether all of the mutant phenotypes could be complemented by the plasmid. The one plasmid that passed these tests was shown to contain RPBI by DNA sequence analysis. Experiments with plasmid pGH189, which contains only RPBI, confirmed that RPBI was sufficient to complement all of the mutant phenotypes of the spt5 Cs− suppressors. In addition, pGH188, which lacks the internal EcoRI–XbaI fragment of RPBI, could not complement the suppressors. Finally, both suppressors were shown to be allelic to RPBI by linkage analysis.

Sequence analysis of rpB1+mutations

The two rpB1+ mutations were cloned from strains FY1637 and FY1638 into plasmid pRP112, by use of the method of gap genescshlp.org on October 16, 2017 - Published by Cold Spring Harbor Laboratory Press
Based on the gap-repair results, the 2.7-kb XbaI–SnaBl fragment of RP1 was sequenced for each mutant at the Biopolymer Facility in the Department of Genetics, Harvard Medical School. In each case, a single base-substitution mutation was identified. To verify that this mutation caused the suppressor phenotype, the XbaI–SnaBl fragment of each mutant was subcloned into the otherwise wild-type RP1 gene in pRP112. For both rpbl-244 and rpbl-221, these reconstructed plasmids, pGH202 and pGH203, were phenotypically indistinguishable from the original mutant isolates.

**Northern analysis**

For Northern analysis, cells were grown in SD media supplemented with necessary amino acids to a density of 0.5 × 10⁷ to 2.0 × 10⁷ cells/ml. RNA was isolated from cells by a hot phenol...
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Antibodies and immunobilots

The anti-Spt4 and -Spt5 antibodies were directed against maltose-binding protein (MBP) fusion proteins produced from plasmids pGH123 and pGH25, respectively, with the MBP protein fusion and purification system from New England Biolabs (Beverly, MA), according to their instructions. Rabbits were immunized with the MBP–Spt4 and MBP–Spt5 fusion proteins by Covance Biomedical (Researchtown, PA). The Spt5 antibody was affinity purified by coupling MBP alone and MBP–Spt5 to Affigel (Bio-Rad), precluding the crude antiserum against the MBP-affigel, and then purifying the Spt5 specific antibodies on the MBP–Spt5-affigel as described previously (Harlow and Lane 1988). The anti-HA1 antibody was produced by BabCo (Berkeley, CA). The anti-Rpb1 antibody is a mouse monoclonal antibody, BWG16, directed against the carboxy-terminal domain (CTD) of RNA Pol II (Thompson et al. 1989). All immunobilots were performed by separating the proteins of interest on 7.5%–16% or 7.5%–20% gradient SDS–polyacrylamide gels and transferring them to Immobilon-NC membranes (Millipore). Western blots were probed under standard conditions (Harlow and Lane 1988). HRP-coupled secondary antibodies were obtained from Bio-Rad. Blots were developed with the Amersham ECL kit according to their instructions.

Computer searches

Homology searches, by use of BLAST (Altschul et al. 1990), identified a repeated, conserved sequence element among Spt5 proteins. With the program MEME (Bailey and Elkan 1994), a consensus for this sequence element was developed. The consensus sequence was then used to search a nonredundant protein database by use of MAST (Bailey and Elkan 1994). For each potential match to the motif, MAST calculates the probability that a random sequence would match the motif as well as the potential match. MAST then calculates the number of expected occurrences of a match to the motif at that probability level as if it were searching a random database of the same size as the nonredundant database; this is termed the E value. Of the 52 proteins with matches to the motif with an E value of less than 1, 41 occurred in Spt5 proteins, ribosomal protein L24 proteins or in NusG proteins.

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method (Ausubel et al. 1988), quantitated with a spectrophotometer, and 10 µg of each sample was separated on an agarose-formaldehyde gel, blotted to Genescreen, and probed as described in Swanson et al. (1991). 32P-Labeled probes were made by nick translation with a kit purchased from Boehringer Mannheim Biochemicals.

Analysis of protein–protein interactions

Strains were grown to a density of 1 × 10^12 to 4 × 10^12 cells/ml, and extracts were prepared by the bead-beating method (Eisenmann et al. 1992). Cells were lysed in buffer containing 25 mM NaPO4, 0.1 mM KAc, 2 mM MgAc, and 10% glycerol. Unless otherwise noted, all manipulations of protein were carried out at 4°C and in the presence of the following protease inhibitors: aprotonin (1.72 µg/ml); pepstatin A (1 µg/ml); chymotatin (0.1 µg/ml); E64 (7.2 µg/ml); phosphoramidon (1.1 µg/ml); and PMSF (1 mM).

For the GST-binding experiments, strain FY1639 was transformed with pYBS305 (GST) and strain FY1640 was transformed with pGH84 (GST–Spt4). All GST fusion proteins were functional in vivo. The FY1639 and FY1640 transformants, as well as strain FY1641 (expressing GST–Spt6 from pGH85), were grown in SC–ura with 2% raffinose as the carbon source to 1 × 10^7 to 4 × 10^7 cells/ml. Galactose was then added to 2%, and cells were grown for 3 hr at 30°C and then processed for extracts as described above. GST pull-down experiments were performed by mixing 500 µg of protein extracts with 200 µl of glutathione-Sepharose (Pharmacia) in a volume of 500 µl in the presence of 0.5% Triton X-100. After gentle mixing for 1 hr at 4°C, the beads were pelleted, the supernatant was removed, and the beads were washed in five volumes of lysis buffer adjusted to 0.5% Triton and 0.4 M ammonium acetate. After washing, 40 µl of beads was removed and mixed with SDS-loading buffer for polyacrylamide gel electrophoresis.

Spt5–Flag was purified by mixing ~6 mg of an extract from strain FY1642 with ~50 µl of M2 anti-Flag beads (Kodak) and mixing gently for 6 hr. The beads were pelleted, the unbound material was removed, and the beads were washed six times in wash buffer (25 mM HEPES at pH 7.4, 1 mM EDTA, 0.02% NP-40, 3 mM MgCl2, 10% glycerol, 1 mM DTT, 0.2% KCl) with 5 min of gentle mixing between washes. The bound proteins were eluted by adding 50 µl of wash buffer containing 2 mg/ml of the Flag peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Lys (Research Genetics), and incubating for 30 min before pelleting the beads gently and removing the eluate. This elution step was repeated two to three times. A control experiment was carried out in parallel in which the anti-Flag beads were mixed with an extract of FY267 cells, in which Spt5 lacks the Flag epitope, to determine the level of nonspecific binding of proteins to the beads.

Immunoprecipitations were performed by mixing 250 µg of protein extract (presum for 10 min in 4°C in a microcentrifuge to remove protein aggregates) in lysis buffer plus 0.5 M ammonium acetate and 0.1% Tween 20 followed by 5 min of gentle rocking. After the final wash, the beads were resuspended in SDS-sample loading buffer for gel electrophoresis.

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