Spn1 Regulates the Recruitment of Spt6 and the Swi/Snf Complex during Transcriptional Activation by RNA Polymerase II

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We investigated the timing of the recruitment of Spn1 and its partner, Spt6, to the CYC1 gene. Like TATA binding protein and RNA polymerase II (RNAPII), Spn1 is constitutively recruited to the CYC1 promoter, although levels of transcription from this gene, which is regulated postrecruitment of RNAPII, are low. In contrast, Spt6 appears only after growth in conditions in which the gene is highly transcribed. Spn1 recruitment is via interaction with RNAPII, since an spn1 mutant defective for interaction with RNAPII is not targeted to the promoter, and Spn1 is necessary for Spt6 recruitment. Through a targeted genetic screen, strong and specific antagonizing interactions between SPN1 and genes encoding Swi/Snf subunits were identified. Like Spt6, Swi/Snf appears at CYC1 only after activation of the gene. However, Spt6 significantly precedes Swi/Snf occupancy at the promoter. In the absence of Spn1 recruitment, Swi/Snf is constitutively found at the promoter. These observations support a model whereby Spn1 negatively regulates RNAPII transcriptional activity by inhibiting recruitment of Swi/Snf to the CYC1 promoter, and this inhibition is abrogated by the Spn1-Spt6 interaction. These findings link Spn1 functions to the transition from an inactive to an actively transcribing RNAPII complex at a postrecruitment-regulated promoter.

For a large number of well-characterized genes, the rate-limiting step in the transcription process is the formation of the preinitiation complex at the promoter. At these genes, the recruitment of TATA binding protein (TBP) and RNA polymerase II (RNAPII) to the promoter correlates strongly with transcriptional output (35, 41, 67). Indeed, delivery of TBP and RNAPII appears to be sufficient for gene activation in many contexts (for reviews, see references 63 and 64). However, there are a growing number of genes that have been found to be regulated at a step after the recruitment of RNAPII. Such genes include the yeast CYC1 gene, the Drosophila heat shock genes, and mammalian human immunodeficiency virus type 1 and the c-Myc proto-oncogene (3, 39, 41, 51, 77). Indeed, whole-genome studies suggest that a significant portion of the human genome may be regulated postrecruitment of RNAPII (26, 35). As such, these mechanisms have the potential to impact the expression of thousands of human genes. Our understanding of these mechanisms and the rate-limiting steps involved is incomplete, but these observations suggest that functions critical for a high level of transcription are either inhibited or absent under noninducing conditions. To determine the nature of these functions, further characterization of genes regulated after assembly of the general transcription machinery is imperative.

The yeast CYC1 gene encodes iso-1-cytochrome c, a protein involved in the electron transport chain in the mitochondria (75). In the presence of a fermentable carbon source (such as dextrose), CYC1 gene expression is inhibited and transcriptional levels are extremely low (24, 25). When cells are grown on a nonfermentable carbon source (such as lactate or ethanol), CYC1 is activated and transcriptional output is induced approximately 10-fold. In contrast to these dramatic changes in transcriptional output, the occupancy of TBP and RNAPII at the CYC1 promoter changes very little during the carbon source change (41, 51). Thus, CYC1 gene expression is regulated at a step after the recruitment of these two essential members of the general transcription machinery. Postrecruitment mechanisms of gene regulation have been observed in all organisms, ranging from bacteria to yeasts to flies to humans (3, 35, 39, 41, 51, 67). Thus, knowledge gained regarding postrecruitment regulation of gene expression in the highly amenable yeast system has the potential to reveal universal mechanistic insights.

SPN1, a gene that is essential in yeast and highly conserved throughout evolution, appears to play a critical role in regulating transcription after assembly of the general transcription machinery. Mutation of SPN1 results in elevated levels of transcription from the CYC1 gene under noninducing (dextrose) conditions (19). Moreover, Spn1 genetically interacts with TBP and Spt4 and physically interacts with Spt6, factors with known roles in transcription initiation, elongation, processing, and chromatin remodeling (19, 21, 38, 46). Therefore, we set out to determine the functional requirement for Spn1 at the postassembly-regulated CYC1 gene.

We show that Spn1, like TBP and RNAPII, is constitutively recruited to the CYC1 promoter. Moreover, TFIH, capping enzyme, and serine-5 phosphorylation of the C-terminal domain of Rpb1 are also present at the CYC1 promoter prior to...
induction of transcription. Spn1 is targeted to the promoter via interaction with RNA Pol II, since an spn1 mutant defective for interaction with RNA Pol II fails to occupy CYC1. Spf6 appears at CYC1 only after the gene is activated. In the absence of Spn1 promoter occupancy (in the spn1 mutant strain), Spf6 is no longer recruited to the CYC1 promoter, indicating that Spn1 is necessary for Spf6 recruitment. In addition, the results from a genetic screen reveal that the Swi/Snf chromatin-remodeling complex and Spn1 have strong antagonizing functions and that recruitment of Swi/Snf is also impacted by Spn1. Taken together, these studies indicate that Spn1 is essential for coordinating the recruitment of chromatin-remodeling factors for the proper expression of the postassembly-regulated CYC1 gene.

MATERIALS AND METHODS

Yeast strains. The deletion collection strains (83) and the parental BY4741 strain (MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0) were purchased from Research Genetics. A subset of these deletion strains was chosen to study the genetic interactions between SPN1 and the genes deleted in these strains (see Table S1 in the supplemental material). For phenotypic studies, genomic SPN1 in these strains was deleted and covered by either wild-type SPN1 or spn1Δ1296 on plasmids.

The snf2Δ strain (MATa adaΔ adaΔ3 leu2Δ1 ura3Δ0 snf2Δ4) was kindly provided by Caroline Kane (15). The spf6-11 and poxb-7 mutants and their parental strain (MATa trp1 leu2 ura3 his3) were provided by Timothy Formosa (20).

Yeast medium. Yeast media used to analyze phenotypic changes were made as described in the literature (27). 5-Fluoroorotic acid plates were made as described previously (7). YPgal and YPEG plates were made by replacing the dextrose in YPD with 2% galactose, 2% ethanol, and 2% glycerol. Plates containing sorbitol, NaCl, and H2O2 were made by supplementing YPD medium with 1 M sorbitol, 1 M NaCl, and 4 mM H2O2. Medium lacking inositol (Inc−) was made as described (30). Methylotrophic acid (MMA) plates were made by supplementing SC-U plates with 20 μg/ml MPA.

Plasmid construction. A 2.2-kb fragment of the LEU2 gene containing its promoter, coding region, and terminator was amplified from yeast genomic DNA and subcloned into the pJF201 (TRP1 CEN) plasmid (19) to replace the SPN1 open reading frame (ORF). The resulting plasmid, pLT-1, has the LEU2 gene flanked by the SPN1 promoter and terminator and was used to produce the SPN1:LEU2 fragment for genomic SPN1 deletion. An SPN1-carrying plasmid (pUS-1) was created by ligation of the TOA1 promoter, SPN1 ORF, and TOA1 terminator and subcloning into pRS316 (URA3 CEN). Two 1.7-kb fragments containing the SPN1 promoter and terminator and either the wild-type SPN1 ORF or spn1Δ1296 were isolated from pJF201 or pJF202, respectively, and subcloned into pRS313 (HIS3 CEN) to generate pH5-1 (wild type) and pH5-2 (spn1-k192N).

Genetic screen. To combine the spn1-k192N allele with the deletion mutants of various RNAPII transcriptional factors, strains were transformed first with a URA3-marked engineered SPN1-encoding carbox plasmid (pUS-1, which contains the promoter and terminator from TOA1). Subsequently, the genomic SPN1 ORF was deleted by LEU2 replacement using the insert from pLT-1 and homologous recombination with sequences within the promoter and terminator of SPN1. The use of the TOA1 promoter and terminator on the plasmid-borne copy of SPN1 targeted the one-step disruption solely to the genomic copy of SPN1. The deletion of genomic SPN1 in the mutant strains was confirmed by PCR (data not shown). The SPN1 gene (wild type; pH5-1) or the spn1-k192N derivative (pH5-2) on a HIS3-marked plasmid was then introduced by plasmid shuffling. The expression levels of the plasmid-borne wild-type SPN1 and spn1-k192N molecule are comparable to that of genomic SPN1 (data not shown). To assay the genetic interactions of SPN1 with different transcription factors, the strains were grown under 10 different conditions. Phenotypic changes were scored by comparing the growth of strains covered by SPN1 versus spn1-k192N under the following conditions: 30°C, 30°C, and 18°C, 1 M NaCl, 1 M sorbitol, 4 mM H2O2, and Ino− media; glucose versus galactose or ethanol/glycerol as a carbon source; 50 mM aminotriazole (AT); or growth on 20 μg/ml MPA.

Transcriptional assays. S1 nuclease assays were conducted as described previously (19). For CYC1 induction, cultures grown overnight in rich medium containing 2% glucose were washed three times in medium lacking glucose, diluted into medium containing 3% ethanol, and cultured at 30°C for 6 h. For uninduced samples, cells were grown in YPD for 6 h at 30°C until the optical density reached 0.8 to 1.0. Yeast cells were then harvested, and total RNA was isolated by hot-phenol extraction. Hybridizations with excess probe were normally done with 25 to 30 μg of RNA samples hybridized with excess 32P-labeled probe overnight at 55°C. S1 nuclease digestion was performed on the hybridized samples for 30 to 45 min at 37°C. Band intensity was normalized to the intensity of the RNA A band.

Chimnopenetratin and ChIP analyses. Chimnopenetratin experiments were performed with the indicated strains (Table 1) as described previously (79) with a few modifications. Cells were grown to an optical density (600 nm) of about 1.0 in rich medium containing 2% dextrose. Cell extracts (300 μg) were used immediately following preparation and were precleared by incubation with 50 μl protein A-Sepharose beads (Pharmacia) for 1 h at 4°C. A small sample was taken after the preclear step to provide a load control. Antihemagglutinin (anti-HA) (Santa Cruz), polyclonal anti-Spn1, and anti-Rpb1 (8WG16; Covance, Inc.) antibodies were coupled to protein A-Sepharose beads, and the remaining extract was incubated with 50 μl of these coupled beads for 2 h at room temperature with occasional stirring. After six washes, the beads were boiled in loading buffer and 15 μl was loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblot analysis.

Chimnopenetratin. Chromatin immunoprecipitations (ChIPs) were performed with the indicated strains (Table 1) as described previously (79), with a few modifications. Cells (150 ml) were grown to an optical density of 600 nm of 0.8 to 1.0. Cells were treated with a final concentration of 1% formaldehyde for 15 min, with occasional swirling of the flasks at intervals of 5 min. Glycine was added to a final concentration of 125 mM at room temperature and left for 5 min to stop cross-linking. Cells were collected and washed twice in ice-cold Tris-buffered saline. Cells were resuspended in FA-lysis buffer (500 μl of FA-lysis buffer for a total of 50 ml of cell culture). Chromatin was sheared by sonication using a Branson W-350 Sonifier (10 times at 10 seconds each on continuous pulse at a microtip power setting of 6). A portion (10%) of the chromatin material used for the immunoprecipitation was processed as the input after reversing the cross-links and purifying the DNA. About 500 μl of the chromatin material was incubated with approximately 5 μl of either anti-Spn1, anti-HA (Santa Cruz), anti-Myc (Upstate), anti-RNA Pol II (8WG16; Covance, Inc.), or anti-serine 5-phosphorylated C-terminal domain (CTD) (H14; Covance, Inc.) antibodies by rotation overnight at 4°C. A 50-μl portion of protein-A-Sepharose beads (Pharmacia; prepared as a slurry as per the manufacturer’s directions) was further incubated with the chromatin material for 2 h at 4°C. The beads were spun down, and the antigen-antibody complexes bound to the beads were recovered and further treated with Trit-IDTA-sodium dodecyl sulfate buffer for 15 min at 65°C to elute the complexes. Protein-DNA cross-links were reversed by incubation overnight at 65°C, and the DNA was purified by phenol-chloroform extraction and used for the PCR analysis.

PCR were carried out in a total volume of 25 μl. Each reaction mixture contained 1 μl of a 1:10 dilution of 10-mM Tris Cl 1251-labeled dATP. Different dilutions of each input or immunoprecipitated samples were used to determine the linear range of the PCR. The PCR products were run on 5% native polyacrylamide gels in 0.5× Tris-borate-EDTA buffer. The gels were dried and exposed to a phosphorimage screen. Images were scanned by STORM and quantified using ImageQuant software analysis to detect the strengths of various signals. The primers used to amplify the promoter region of CYC1 (−234 to +79)
were 5'AGGCGTGTATATATAGCGTGGAT3' and 5'CCACGGTGTGGCATTTGTAGACAT3'. The signal strength ratio between the immunoprecipitated sample and the input after subtracting the signal of a no-antibody control was used as an indication of the occupancy of the protein. Each experiment was repeated (from independent cultures of cells) a minimum of three times.

RESULTS

Spn1 is found in association with Spt6 (21, 38), a factor with important functions for transcription through chromatin (2, 8, 31). To examine the mechanistic relationship between Spn1 and Spt6 at a promoter that is regulated postassembly of TBP and RNAPII, we examined their occupancy levels on the CYC1 gene via ChIP analysis. Occupancy levels of Spn1 on the CYC1 promoter are comparable under uninduced (partial repression) and induced (activation) conditions (Fig. 1A and C). Thus, Spn1 is constitutively recruited to CYC1, like TBP and RNAPII (41, 51). In contrast to the case for Spn1, TBP, and RNAPII, occupancy levels of Spt6 are lower under uninduced conditions and increase more than fivefold under inducing conditions. Thus, Spn1 and Spt6 have separable functions prior to activation of CYC1, and the appearance of Spt6 correlates with the transition to an actively transcribing RNAPII complex.

**Diminished Spn1 occupancy results in failure to recruit Spt6 to CYC1.** In the spn1-K192N mutant (where lysine at position 192 is replaced with asparagine), CYC1 transcription levels are elevated under noninducing conditions and are also abnormally high under inducing conditions (19). These alterations in CYC1 expression suggest that RNAPII is in a transcriptionally more active state at CYC1 in the mutant background and that a rate-limiting step due to Spn1 functions has been removed. Occupancy levels of Spn1 and Spt6 were thus determined in the spn1-K192N background. Strikingly, the occupancy level of Spn1 is diminished under noninducing conditions in the spn1-K192N mutant, and this low level of occupancy is unchanged under fully inducing conditions (Fig. 1B and C). Occupancy levels of Spt6 are also diminished in the spn1 mutant background under both noninducing and inducing conditions. This indicates that the recruitment of Spt6 is dependent on the presence of Spn1 and that neither Spn1 nor Spt6 is required to activate transcription of CYC1. Indeed, transcription levels of CYC1 are elevated under noninducing conditions in the spn1 mutant background (19). This suggests that Spn1 is required to maintain the diminished activity of RNAPII at the postassembly-regulated CYC1 gene.

**Distinct functions of Spn1 and Spt6 during RNAPII transcription.** To investigate the requirement for Spn1 at CYC1, we utilized a well-characterized mutant allele of SPT6, spt6-1004 (31), and examined CYC1 transcription. We found that activation of CYC1 in a spt6-1004 mutant was decreased by 30% (Fig. 2A). A decrease in CYC1 transcription in the spt6-1004 strain suggests a positive role of Spt6 in regulating RNAPII transcription at CYC1. This is in striking contrast to the fivefold increase in CYC1 transcription observed in a strain containing the spt6-K192N mutation (19).

Although Spt6 can stimulate elongation in vitro on naked DNA (16), it also binds histones and has critical functions for maintaining and modifying chromatin during transcription elongation in vivo (2, 8, 31, 56). One molecular aspect of these chromatin-related functions of Spt6 is in reestablishing chromatin structure after transcription (31). For example, loss of the proper chromatin structure in the spt6-1004 mutant results in transcription initiation by RNAPII from cryptic TATA sites within the coding regions of certain genes, such as FLO8 (31).
To determine if Spn1 is involved in this process, we tested the spn1-K192N strain for short transcripts at FLO8. Unlike wild-type (WT) and spn6 mutant strains grown under partially repressed (in medium containing 2% dextrose) and activated (in medium containing 3% ethanol) conditions were analyzed by S1 nuclease assay using 32P-labeled CYC1 and tryptophan tRNA probes. tRNA* signal was used as a loading control to normalize the signal of CYC1 transcripts. A representative gel is shown. For quantification, the transcription level of CYC1 in the wild-type strain was set to 100%, and the values for the spn6 mutant strain from three independent experiments (±4%) are indicated. (B) Effect of an spn1 mutation on FLO8 transcription. Total RNA from wild-type and spn1 or spn6 mutant strains, grown at 30°C or after an 80-minute shift to 37°C, was subjected to Northern blot analysis for FLO8 RNA. The position of the transcript generated from the cryptic TATA element is indicated.

To determine if Spn1 is involved in this process, we tested the spn1-K192N strain for short transcripts at FLO8. Unlike the spn6 mutant strain, the spn1 mutant strain showed no changes in FLO8 transcription (Fig. 2B). Thus, although the Spn1/Spt6 complex is involved in regulation of CYC1 gene expression, these two proteins are also likely to have additional and separable functions in overall regulation of gene expression.

**Spn1 is recruited to the CYC1 gene via interaction with RNAPII.** How is Spn1 recruited to the CYC1 promoter? Likely candidates for recruitment of Spn1 are TBP and RNAPII, since both are constitutively recruited to the CYC1 gene even when it is partially or fully repressed (41, 51). Although SPN1 genetically interacts with TBP, Spn1 was not found to coimmunoprecipitate with TBP or TFIIID (19), Thus, we tested the hypothesis that Spn1 is recruited to CYC1 by interacting with RNAPII. Indeed, we found that Spn1 coimmunoprecipitates with RNAPII (Fig. 3). These results are consistent with other studies that demonstrate an interaction between RNAPII and Spn1 by coimmunoprecipitation and by footprinting experiments (38, 46). Strikingly, we found that the mutant form of Spn1, Spn1-K192N, fails to coimmunoprecipitate with RNAPII (Fig. 3). This loss of interaction with RNAPII, coupled with the observation that Spn1 is no longer present at the CYC1 promoter in the spn1-K192N strain, indicates that the Spn1-RNAPII interaction is essential for targeting Spn1 to the CYC1 promoter.

Additional members of the preinitiation complex are present on the CYC1 promoter under noninducing conditions. To investigate the potential rate-limiting step impacted by Spn1, we further characterized the promoter-bound complex found at the CYC1 promoter. As previously noted (51), RNAPII occupies the CYC1 promoter prior to induction, with very little change upon transcriptional activation (Fig. 4). Typically, the CTD of Rpb1 of RNAPII is hypophosphorylated prior to initiation and becomes hyperphosphorylated on serine 5 during the transition from initiation to elongation (13, 37). Thus, this phosphorylation event may be a good candidate for a rate-limiting step impacted by Spn1 functions. Rpb1 specifically phosphorylated on serine 5 can be immunoprecipitated with the monoclonal antibody H14 (9, 59). Strikingly, ChIP assays using H14 antibodies show that the CTD of Rpb1 is phosphorylated under noninducing conditions at the CYC1 promoter (Fig. 4). Interestingly, this occupancy level drops when transcriptional output is high (under inducing conditions), which may reflect the recruitment of hypophosphorylated Rpb1 with Spn1.
lated RNAPII during multiple rounds of transcription initiation. The presence of serine 5 phosphorylation on the CTD suggests that TFIIH also occupies the promoter, since it possesses CTD kinase as well as helicase activities (18, 48, 69, 73, 74). Indeed, tagged derivatives of two different subunits of TFIIH (Rad3 and Ssl2, the two helicases) were clearly detected at the CYC1 promoter prior to activation of gene expression (Fig. 4). A subsequent step in the transcription process is the capping of the transcript (33, 37). Thus, we next tested for the occupancy of Ceg1, a subunit of the yeast mRNA capping enzyme that has been shown to directly contact the phosphorylated form of the CTD (70, 71). Ceg1 was also found to occupy the promoter prior to activation, and upon induction, Ceg1 occupancy increased slightly (less than twofold) (Fig. 4). Taken together with the previous observations that RNAPII and TBP occupy the CYC1 promoter prior to transcriptional activation, these results with serine 5 phosphorylation, TFIIH, and capping enzyme occupancy indicate a remarkably complete preinitiation complex at the CYC1 promoter. Moreover, we must expand the screen for potential candidates for the rate-limiting step impacted by Spn1 function.

**Genetic interactions of Spn1 with RNAPII transcription factors.** To investigate the functional relationship between Spn1 and other proteins involved in the regulation of gene expression, a targeted genetic screen was designed to identify transcription factors that genetically interact with Spn1. Eighty different strains were chosen from the yeast deletion collection (see Table S1 in the supplemental material). These strains were selected due to the annotated functional activity of the deleted gene product in some stage of the transcriptional process. The factors include subunits of chromatin-remodeling complexes (SWI/SNF, ISW, FACT, RSC, and CHD1) and -modifying complexes (SAGA, NuHAT, and histone deacetylases), Mediator complex, and elongation factors, activators, and additional SPT genes. Since these factors have defined functions in the transcription process, combining this subset of mutants with the *spn1-K192N* allele has the potential to reveal Spn1 functions during RNAPII transcription. Each of the strains containing a unique deletion was combined with the *spn1-K192N* allele. Since *Spn1* is essential for viability and *spn1-K192N* is a recessive allele, introduction of *spn1-K192N* into each deletion strain was accomplished via a genomic knockout of *Spn1* and plasmid shuffling (for details, see Materials and Methods). Strains with a deleted gene and *spn1-K192N* were compared to the parent deletion strain containing wild-type *Spn1* or a strain containing *spn1-K192N*. Eleven different conditions, including various temperatures, different carbon sources, and a number of stress-inducing agents, were assayed for phenotypic changes. A phenotypic change indicates a genetic interaction between *Spn1* and the deleted gene. Of the 81 deletion strains tested, 6 showed genetic interactions with *spn1-K192N* (Table 2 and Fig. 5).

*spn1-K192N* is synthetically lethal with a deletion in *SPT4* and exacerbates the phenotypes of deletions in *RTF1* or *DST1*. As has been previously reported for another allele of *Spn1* (46), deleting *SPT4* results in cell inviability when combined with the mutation in *Spn1* (Table 2). Since Spt4 and the essential gene product Spt5 are known to interact genetically and physically with Spt6 (29), this result is not unexpected. Human Sppt4 and its binding partner Spt5 comprise the positive transcription elongation factor DBF3 sensitivity-inducing factor, which binds directly to RNAPII (80, 84). Two other deletion strains showed synthetic growth defects when combined with the *spn1-K192N* allele. Deletion of either *RTF1* or *DST1* when combined with the *spn1-K192N* allele resulted in mild growth defects under a variety of conditions (Fig. 5). For the *RTF1* deletion strain these conditions included growth on ethanol/glycerol-containing media and Ino– media. Similar to the case for *Spn1* (19), *RTF1* was originally identified in a screen for suppressors of a TBP mutant (78). Rtf1 is a member of the Pafl complex (57). Exacerbation of several observable growth defects was also observed when *spn1-K192N* was combined with a deletion in *DST1* (Fig. 5). *DST1* encodes the general transcription factor TFIIS (66). In contrast to these effects for *SPT4, RTF1*, and *DST1*, none of the other SPT genes, elongation factors, or Pafl complex members tested exhibited genetic interactions with *Spn1*. Thus, the specific genetic interactions between *Spn1*, *SPT4*, *RTF1*, and *DST1* suggest an important linkage to later steps in the initiation process and the early steps in elongation.

*spn1-K192N* synthetically rescues the effects of deletions in Swi/Snf subunits. In contrast to the genetic effects observed

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* Deletion strains (as indicated) were combined with the K192N derivative of *Spn1*, and alterations in growth properties under 11 different conditions were assayed.

**Table 2. Observed synthetic interactions between *Spn1* and specific gene products involved in RNAPII-mediated transcription**

To determine the potential role of Spn1 in transcription initiation, we used a genetic screen to identify genes that genetically interact with Spn1. Eighty different strains were chosen from the yeast deletion collection, and these strains were selected based on their annotated functional activity of the deleted gene product in some stage of the transcriptional process. The factors include subunits of chromatin-remodeling complexes (SWI/SNF, ISW, FACT, RSC, and CHD1) and -modifying complexes (SAGA, NuHAT, and histone deacetylases), Mediator complex, and elongation factors, activators, and additional SPT genes. Since these factors have defined functions in the transcription process, combining this subset of mutants with the *spn1-K192N* allele has the potential to reveal Spn1 functions during RNAPII transcription. Each of the strains containing a unique deletion was combined with the *spn1-K192N* allele. Since *Spn1* is essential for viability and *spn1-K192N* is a recessive allele, introduction of *spn1-K192N* into each deletion strain was accomplished via a synthetic knockout of *Spn1* and plasmid shuffling (for details, see Materials and Methods). Strains with a deleted gene and *spn1-K192N* were compared to the parent deletion strain containing wild-type *Spn1* or a strain containing *spn1-K192N*. Eleven different conditions, including various temperatures, different carbon sources, and a number of stress-inducing agents, were assayed for phenotypic changes. A phenotypic change indicates a genetic interaction between *Spn1* and the deleted gene. Of the 81 deletion strains tested, 6 showed genetic interactions with *spn1-K192N* (Table 2 and Fig. 5).

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*spn1-K192N* synthetically rescues the effects of deletions in Swi/Snf subunits. In contrast to the genetic effects observed
with the above-described strains, in which double mutants were less healthy (or dead), combining the spn1-K192N allele with deletions in three other genes resulted in cells that grow significantly better (Table 2 and Fig. 5). Enhanced growth/synthetic rescue is seen upon combining spn1-K192N with a deletion for genes encoding three different subunits of the Swi/Snf complex: SNF2, SNF5, and SNF6. Swi/Snf is an ATP-dependent chromatin-remodeling complex (for a review, see reference 52). Deletion of SNF2 alone causes severe growth defects under all assay conditions, while snf5Δ and snf6Δ mutants are similar to each other and exhibit growth defects on galactose or ethanol/glycerol media, Ino− plates, or plates containing hydrogen peroxide, AT, or MPA. Importantly, all of these growth defects are suppressed by spn1-K192N. More strikingly, these three Swi/Snf mutants also suppress the temperature-sensitive phenotype of spn1-K192N (Fig. 5). We did not observe genetic interactions with four other genes that encode products found in this complex. However, SNF2 encodes the enzymatic ATPase subunit of the complex (60), and Snf2 requires both Snf5 and Snf6 for proper function (12, 22, 43). As such, SPN1 genetically interacts with the critical gene products of the Swi/Snf complex but not with other related factors (see Table S1 in the supplemental material), such as ATP-dependent chromatin-remodeling factors (such as ISW1, ISW2, CHD1, RAD26, ITC1, etc.), or chromatin-modifying complexes (such as SAGA, other HATS, or histone deacetylases).

The strong genetic interaction between Spn1 and components of the Swi/Snf complex prompted us to explore the role of Swi/Snf in CYC1 transcription regulation. Using S1 nuclease assays, CYC1 transcripts were measured in the parental, spn1-K192N, snf5Δ, and snf6Δ strains, as well as spn1-K192N snf5Δ and spn1-K192N snf6Δ double mutant strains. Consistent with our previous studies, the K192N mutation in SPN1 results in an additional fivefold increase in CYC1 transcription under inducing conditions (19). Deletion of SNF5 or SNF6 results in a 50 or 35 percent decrease, respectively, in CYC1 transcription compared to that in the wild-type parental strain (Fig. 6A). This indicates that the function of the Swi/Snf complex is required for normal levels of CYC1 transcription. Mutating SPN1 in these Swi/Snf deletion strains restored CYC1 transcription to normal levels. These effects were not due to changes in Spn1 protein levels in the Swi/Snf mutant backgrounds, as immunoblot analysis indicates no changes in Spn1 levels in the different strain backgrounds (Fig. 6B). Taken together, the results support our genetic observations and imply a counteracting effect between Spn1 and the Swi/Snf complex at a molecular level.

**Snf2, the ATPase subunit of Swi/Snf, is recruited to CYC1 during activated transcription.** Since deleting components of the Swi/Snf complex resulted in transcriptional defects in CYC1 expression, we next investigated whether this was a direct effect of the functional activity of Swi/Snf at the CYC1 promoter. An epitope-tagged version of Snf2 (the ATPase subunit) was created and used in a ChIP assay to determine the occupancy of Snf2 at the CYC1 promoter. We found that Snf2 shows a significant increase (fivefold) in occupancy at CYC1 under inducing conditions (Fig. 7). Thus, like for Spt6, the appearance of Snf2 at the postassembly-regulated CYC1 gene correlates with the transition to a highly active RNApII state.

**Diminished Spn1 occupancy leads to autonomous recruitment of Snf2 at CYC1.** The genetic suppression between mutants of Spn1 and the Swi/Snf complex and their opposite

### Table: Phenotypes assayed

<table>
<thead>
<tr>
<th>Strain</th>
<th>YPD 30</th>
<th>YPD 38</th>
<th>YPEG</th>
<th>Ino minus</th>
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</thead>
<tbody>
<tr>
<td>BY4741 WT</td>
<td>MT</td>
<td>MT</td>
<td>MT</td>
<td>MT</td>
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<tr>
<td>rtf1Δ</td>
<td>WT</td>
<td>MT</td>
<td>MT</td>
<td>MT</td>
</tr>
<tr>
<td>dst1Δ</td>
<td>WT</td>
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<td>MT</td>
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<tr>
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<td>snf6Δ</td>
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</tbody>
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**FIG. 5.** SPN1 genetically interacts with SNF2, SNF5, SNF6, RTF1, and DST1. Yeast cells of the indicated strains were diluted serially and plated onto the indicated medium. Pictures were taken after 2 to 3 days of growth. The growth defects of the rtf1Δ and dst1Δ strains are exacerbated by the mutation in SPN1. The temperature-sensitive phenotype of the spn1 mutant (spn1-K192N) is suppressed by snf2Δ, snf5Δ, and snf6Δ, and the growth defects of the Swi/Snf mutants are suppressed by spn1-K192N. WT, wild type; MT, mutant.
effects on transcription of the CYC1 gene suggest a simple model whereby Spn1 antagonizes the function of Swi/Snf. One way to accomplish this would be for Spn1 to inhibit Swi/Snf recruitment to the gene until inducing conditions are present. If correct, then this model predicts that in the absence of Spn1, the Swi/Snf complex would be constitutively recruited to the CYC1 promoter. We tested this hypothesis by performing ChIP assays in a spn1-K192N background, in which Spn1 fails to occupy the CYC1 promoter. In contrast to the case for wild-type cells, which showed low levels of Snt2 during partial repression, we found high occupancy levels of Snt2 at the CYC1 gene in the mutant strain (Fig. 7). The autonomous increase in recruitment of this component of Swi/Snf in the mutant SPN1 strain is in good accord with the elevated levels of transcription also observed in this strain (19). Taken together, these results suggest that Spn1 plays an inhibitory role in the recruitment of the Swi/Snf complex.

Spn6 is recruited before Swi/Snf during CYC1 activation. If Spn1 has an inhibitory effect on the recruitment of the Swi/Snf complex during partial repression, then how is this relationship altered under inducing conditions, considering that Spn1 still occupies the promoter? One possibility is that the direct interaction of Spn1 with Spn6 abrogates the inhibitory activity. If this model is correct, then Spn6 would appear at the promoter prior to Swi/Snf. CYC1 transcription reaches maximum levels at approximately 6 h of growth in medium containing ethanol (Fig. 8A). One hour after activation, Spn6 occupancy levels reach over 70% of the maximum level (Fig. 8B and D). In contrast, occupancy levels of the Swi/Snf complex increase at around 2 h after activation and reach the maximum at the 4-h time point (Fig. 8C and D). As such, Swi/Snf occupancy correlates well with CYC1 transcription levels. The fact that Spn6 is recruited earlier than Swi/Snf upon activation supports a model whereby the interaction of Spn6 with Spn1 relieves the inhibitory effect of Spn1 on the recruitment of the Swi/Snf complex.

DISCUSSION

SPN1 encodes a highly conserved protein with an essential role in transcription by RNAPII (19, 38, 46). To further define how Spn1 regulates RNAPII transcription, a targeted genetic
screen was designed to identify transcription factors that genetically interact with SPN1. We found that SPN1 interacts with three Swi/Snf genes (SNF2, SNF5, and SNF6), and SPT4, RTF1, and TFIIIS (DST1). A number of lines of evidence suggest that the genetic interactions we identified are biologically significant. First, the genetic interactions observed were highly specific: only five transcription factors from over 80 strains tested show an interaction with SPN1. We did not observe phenotypic changes upon combining spn1-K192N with other deletion mutations of the transcription machinery, such as Mediator components, RNAPII-associated factors, and different chromatin-remodeling and -modifying factors. Second, we observed that SPN1 genetically interacts with only one ATP-dependent chromatin-remodeling complex, Swi/Snf, and not with other ATP-dependent chromatin-remodeling or -modifying factors such as Isw1, Isw2, FACT, RSC, or SAGA. Third, mutations in SPT6 also suppress the transcription defects of Swi/Snf mutant strains (28, 44, 82) and are synthetically lethal with a DST1 (TFIIIS) null mutant (15, 29). Fourth, synthetically lethal interactions have been observed between a deletion of SPT4 and deletions of RTF1 (14, 42) or DST1 (29). Taken together, these results indicate an extensive set of genetic and biochemical interconnections between Spn1, Spn6, Swi/Snf, Spt4, Rtf1, and TFIIIS (DST1).

To further characterize the functional aspects of SPN1, we examined occupancy of a number of components of the transcription machinery to arrive at a working model for CYC1 gene activation (Fig. 9). Like TBP and RNAPII (41, 51), we find that Spn1 occupies the CYC1 promoter under noninducing conditions. In addition, TFIIH and capping enzyme also appear to occupy the promoter prior to activation, and consistent with this, serine 5 of the CTD of Rpb1 is also phosphorylated. Spn1 appears to be recruited to the promoter via interactions with RNAPII, since the SPN1 mutant (spn1-K192N), which is defective for interaction with RNAPII, does not occupy the CYC1 promoter. Under inducing conditions for CYC1, Spn6 promptly occupies the promoter. Spn6 recruitment is most likely via interaction with Spn1, since a loss of Spn1 at CYC1 also results in the loss of Spn6 in the spn1-K192N background. After Spn6 is recruited, the Swi/Snf complex occupies the CYC1 promoter. Swi/Snf recruitment correlates the best with transcriptional output, suggesting that this is an important step in CYC1 gene expression. Indeed, in the absence of Spn1 (as well as Spn6), Swi/Snf is constitutively recruited to CYC1, indicating that Spn1 negatively regulates Swi/Snf recruitment.

The model described above indicates that Spn1 and Spn6 have distinct but dependent functions for CYC1 regulation. Are Spn1 and Spn6 functions always codependent? Most likely they are not, since we found that Spn1 does not appear to play a role in cryptic start site formation, whereas Spn6 does (31). In addition, others have shown that mutations in SPN1 have no effect on Spn6-mediated chromatin reassembly at PHO5 (2) and that patterns of gene perturbations in microarray studies are fairly distinct for mutations in SPN1 compared to SPT6 (10). All of the above suggests that although Spn1 and Spn6 cooperate for regulation of CYC1 gene expression, they also have additional and separable functions in overall regulation of gene expression.

Since Swi/Snf is a chromatin-remodeling complex (for a review, see reference 52), it is likely to be needed at CYC1 to perturb histone-DNA interactions. Like the majority of yeast promoters (6, 45, 72), CYC1 is “open” and devoid of histones (51), and thus it is unlikely that Swi/Snf is required for promoter chromatin remodeling. In contrast to the promoter re-

**FIG. 8.** Time course of transcription and occupancy levels of Spn6 and Swi/Snf at CYC1. (A) S1 nuclease assay showing the time course of CYC1 activation. CYC1 is fully activated at 6 h of induction (medium containing 3% ethanol); tRNA was used as a loading control. (B) ChIP analysis showing the increase of Spn6 occupancy on the CYC1 promoter during 0 to 5 h of activation. Spn6 occupies the CYC1 promoter within 2 h after activation. (C) ChIP analysis showing the increase of the Swi/Snf complex occupancy on the CYC1 promoter during 0 to 5 h of activation. Swi/Snf occupancy parallels that of CYC1 transcription output. (D) Line graph showing the time course of Spn6 and Swi/Snf occupancy levels on the CYC1 gene upon activation. The levels of Spn6 and Swi/Snf occupancies at 6 h of activation were set as 100%. The occupancy levels of both factors at each time point were converted to the percentage of their maximum occupancy levels and graphed (n = 3; P < 0.005). Error bars indicate standard deviations.
gion, the ORF of CYC1 has detectable levels of histone H3, and deletion of SNF5 results in a two- to threefold increase in histone occupancy at the CYC1 gene. In addition, serine 5 of the CTD of Rpb1 is phosphorylated. Spn1 occupancy prevents Swi/Snf interaction with the CYC1 promoter. (B) Under inducing conditions, Spn6 is recruited to the CYC1 promoter via interaction with Spn1. (C) Spn6 recruitment is followed by the recruitment of the Swi/Snf complex, which correlates with induced levels of gene expression (arrow).

FIG. 9. A model for CYC1 gene regulation. (A) Under uninduced conditions, Spn1, TBP, RNAPII, TFIIH, and the capping enzyme subunit, Ceg1, are constitutively recruited to the CYC1 gene. In addition, serine 5 of the CTD of Rpb1 is phosphorylated. Spn1 occupancy prevents Swi/Snf interaction with the CYC1 promoter. (B) Under inducing conditions, Spn6 is recruited to the CYC1 promoter via interaction with Spn1. (C) Spn6 recruitment is followed by the recruitment of the Swi/Snf complex, which correlates with induced levels of gene expression (arrow).

The ORF of CYC1 has detectable levels of histone H3, and deletion of SNF5 results in a two- to threefold increase in histone occupancy in this region (data not shown). Thus, these results are consistent with a role of the Swi/Snf complex in mobilizing histones in the transcribing region. This could enhance the transcriptional elongation process. Indeed, Swi/Snf has established roles in directing remodeling of large chromatin domains encompassing coding regions (36).

It is interesting to note that Swi/Snf has also been implicated in promoter clearance by RNAPII (23). These results, coupled with ours demonstrating that Swi/Snf recruitment correlates with active RNAPII, makes one wonder whether Swi/Snf may facilitate “remodeling” of some other component in the system in addition to nucleosomes. Perhaps RNAPII itself may need to alter its conformation to achieve an active transcribing and/or elongating state at CYC1. The high-resolution structures of the preinitiated and elongating polymerase suggest that conformational changes must occur to accommodate specific promoter recognition, DNA melting, RNA chain extension, etc. (11, 32, 81, 85). Moreover, a large number of poised RNAPIIs (65), as well as partial preinitiation complexes (87), have been detected at various locations in the yeast genome without corresponding transcriptional activity. It is unknown how these inactive complexes are converted into active ones, but it is interesting to speculate that chromatin-remodeling complexes may have other fundamental targets besides nucleosomes and that these remodeling events may play a role in the transition to competent elongation complexes.

We also found that spn1-K192N is synthetically lethal with a deletion in SPT4 (this was also observed by Lindstrom et al. [46]) and exacerbates the phenotypes of deletions in DST1 (TFIIS) or RTFI. Interestingly, each of these gene products plays a potential role in the transition to a competent elongation complex. Spn4 (in combination with Spt5) is implicated in regulating the elongation process (46). The human homologues of Spn4 and Spt5 comprise the positive transcription elongation factor DRB sensitivity-inducing factor, which binds directly to RNAPII and plays a role in release from pausing of RNAPII (80, 84). TFIIH (DST1) plays a role in the initiation of transcription (62) and promoter escape (49) and also rescues arrested RNAPII at pause sites by stimulating the RNAPII to cleave and realign the nascent transcript (1, 4, 40). All of these observations place Spn4 and TFIIH firmly in a multifunctional role that is consistent with the initiation-to-elongation transition. Likewise, RTFI has been implicated in a number of stages in the RNAPII-mediated transcription process, including transcript start site selection, elongation, processing, and histone modifications (14, 29, 50, 58, 76, 78). Like for these other multifunctional factors with which Spn1 genetically interacts, our data strongly suggest that Spn1 negatively regulates the transition to a productive elongation complex. Release of this inhibition, either by lack of recruitment (in the spn1-K192N mutant) or via interaction with Spn6, allows for productive transcription. In addition, Spn1 colocalizes with RNAPII along the entire ORFs of a number of constitutively active genes (34, 38). Also, Spn1 associates with RNAPII phosphorylated on serine 5 and serine 2 residues of the CTD of the largest subunit of RNAPII (46). Phosphorylation of the CTD is thought to correlate with stages of the transcription process in that hypophosphorylated RNAPII binds to promoters, serine 5 phosphorylation occurs during initiation, and serine 2 phosphorylation occurs during elongation (for reviews, see references 54 and 61). Consistent with a novel and negative role in the elongation process, the spn1-K192N mutant does not display 6-azauracil or MPA sensitivity (19), two common phenotypes shared by many mutants with mutations in factors with positive roles in elongation (29, 62, 68). These compounds alter the elongation rate and processivity of RNAPII in vivo (53), due to depletion of nucleotide pools (17).

The extensive primary amino acid sequence identity between yeast and human Spn1 (19), as well as the existence of homologues for the other transcription players involved, suggests the potential for conservation of Spn1 function in higher eukaryotes. As in yeast, mammalian Spn1 (also known as Iws1) is essential for cell viability (47). Moreover, expression of post-recruitment-regulated human immunodeficiency virus type 1 requires functional Spn1 and Spn6, as knockdown and mutational analyses demonstrate defects in transcript production and processing (86). In addition, human Spn1 is involved in the expression of the c-Myc proto-oncogene (86), another gene that is regulated after recruitment of the preinitiation complex (5, 39). Thus, the exciting and emerging picture is one in which Spn1 plays a central role in post-recruitment mechanisms in humans as well as in Saccharomyces cerevisiae.

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