SPN1, a Conserved Gene Identified by Suppression of a Postrecruitment-Defective Yeast TATA-Binding Protein Mutant

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ABSTRACT

Little is known about TATA-binding protein (TBP) functions after recruitment to the TATA element, although several TBP mutants display postrecruitment defects. Here we describe a genetic screen for suppressors of a postrecruitment-defective TBP allele. Suppression was achieved by a single point mutation in a previously uncharacterized *Saccharomyces cerevisiae* gene, *SPN1* (*s*uppresses *p*ostrecruitment functions gene *n*umber *1*). *SPN1* is an essential yeast gene that is highly conserved throughout evolution. The suppressing mutation in *SPN1* substitutes an asparagine for an invariant lysine at position 192 ($spn1^{K192N}$). The $spn1^{K192N}$ strain is able to suppress additional alleles of TBP that possess postrecruitment defects, but not a TBP allele that is postrecruitment competent. In addition, Spn1p does not stably associate with TFIID *in vivo*. Cells containing the $spn1^{K192N}$ allele exhibit a temperature-sensitive phenotype and some defects in activated transcription, whereas constitutive transcription appears relatively robust in the mutant background. Consistent with an important role in postrecruitment functions, transcription from the *CYC1* promoter, which has been shown to be regulated by postrecruitment mechanisms, is enhanced in $spn1^{K192N}$ cells. Moreover, we find that *SPN1* is a member of the *SPT* gene family, further supporting a functional requirement for the *SPN1* gene product in transcriptional processes.

INITIATION of RNA polymerase II (Pol II) transcription in eukaryotic organisms requires the well-orchestrated involvement of a large number of proteins at specific sites on the DNA template (Orphanides *et al.* 1996; Hampsey 1998). TFIID, a general transcription factor (GTF) containing TATA-binding protein (TBP) and TBP-associated factors (TAFs), nucleates the preinitiation complex by binding the TATA element in a sequence-specific manner. A competent preinitiation complex also requires other GTFs, including TFIIA, -B, -E, -F, and -H, as well as Pol II. *In vivo*, several of these GTFs and other factors such as Srb/Mediator are thought to preassemble with Pol II in a multicomponent complex (termed the holoenzyme) prior to binding TFIID and DNA (Kim *et al.* 1994; Koleske and Young 1994; Ossipow *et al.* 1995).

TBP occupancy at a promoter correlates strongly with transcriptional output (Kuras and Struhl 1999; Li *et al.* 1999) and recruitment of TBP to the promoter can be a rate-limiting step for transcription initiation (Colgan and Manley 1992; Tansey *et al.* 1994; Chatterjee and Struhl 1995; Klages and Strubin 1995; Xiao *et al.* 1995), which is enhanced by the presence of an activator (Klein and Struhl 1994). Additionally, transcriptional activators function via protein-protein interactions to enhance recruitment to the promoter of other GTFs (Stringer *et al.* 1990; Ingles *et al.* 1991; Lin *et al.* 1991; Choy and Green 1993; Drysdale *et al.* 1998), certain coactivators (Drysdale *et al.* 1998; Utley *et al.* 1998), the holoenzyme (Koleske and Young 1994; Drysdale *et al.* 1998; Natarajan *et al.* 1999), and chromatin remodeling or modifying enzymes (Natarajan *et al.* 1999; Neely *et al.* 1999, 2002). In this way, activators contribute positively to the assembly and stabilization of a productive preinitiation complex (Hahn 1993; Orphanides *et al.* 1996). In addition to stimulating transcription at the initiation step, certain activators can influence subsequent steps in the transcriptional process. Activators can enhance promoter clearance (Kumar *et al.* 1998), aid in release of Pol II pausing (Brown *et al.* 1996), and increase the elongation rate of Pol II (Yankulov *et al.* 1994). Thus, activators appear to exert influence on both recruitment and postrecruitment steps in the transcriptional process.

One way to bypass the requirement for an activation domain is through artificial recruitment of a member of the preinitiation complex. For example, when wild-type TBP is tethered to DNA via a DNA-binding domain (LexA) located upstream of a reporter gene, an activator is no longer required and LexA-TBP activates transcription of the gene directly (Chatterjee and Struhl 1995; Klages and Strubin 1995; Xiao *et al.* 1995).

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Under these artificial recruitment circumstances, interactions that occur prior to TBP binding to the TATA element, such as interaction with an activation domain, can be bypassed. However, functions that are required after TBP is recruited to the promoter (such as the recruitment of Pol II or promoter clearance) cannot be bypassed with this assay. Although activation-defective alleles of TBP that exhibit postrecruitment defects have been described (Stargell and Struhl 1996), little is known about the functions of TBP after it binds the TATA element.

We have focused on one particular postrecruitment-defective allele of TBP, F237D (in which the phenylalanine at position 237 is mutated to aspartic acid). To identify factors important in these postrecruitment functions, the F237D allele was used in a genetic screen in which spontaneous mutations that allow LexA-F237D to activate transcription from a reporter gene containing a LexA operator were isolated. Thus, we targeted directly the postrecruitment defect. Here, we describe the initial cloning and characterization of the first of these genes, termed *SPN1* (*s*uppresses *p*ostrecruitment functions gene *n*umber *1*). *SPN1* is essential in yeast and conserved throughout evolution. Our functional characterization of this gene shows that a mutation in *SPN1* alters transcription by Pol II and confers Spt$^-$ phenotypes. Taken together, these results suggest an important role for *SPN1* in the mechanisms of transcriptional regulation *in vivo*.

MATERIALS AND METHODS

Yeast strains and DNA: Strains are shown in Table 1. Most are derived from the FT5 strain, with genotype *MAT*α *ura3-52 trp1-*Δ*63 his3-*Δ*200 leu*Δ*2::PET56* (Tzamarias and Struhl 1994). A *SPN1* deletion strain JF11 was created using a one-step knockout (via *LEU2*) and a *URA3*-marked cover plasmid with a genomic *Eco*RI-*Hin*dIII fragment containing the *SPN1* gene. Genomic PCR confirmed *LEU2* integration at the appropriate locus. In the yeast strain JF12, the upstream region of the *HIS3* gene was repaired to wild type by recombination with a wild-type fragment of this region. *TRP1*-marked plasmids based on YCp22 were constructed, bearing full-length wild-type or K192N mutant *SPN1*, as well as the N-terminal deletions and C-terminal truncations of *SPN1*. These were made with and without the myc epitope tag at the N terminus. These plasmids were transformed into JF12, followed by growth on 5-fluoroorotic acid (5-FOA) to cure the strain of the *URA3*-marked cover plasmid, yielding JF13 (wild-type *SPN1*), JF14 (*spn1*^{K192N}), JF15 (myc-*SPN1*), and JF16 (myc-*spn1*^{K192N}). Immunoprecipitation studies employed the SK1, JF15, and RYP5 strains. RYP5 is derived from BYΔ2 (Cormack *et al.* 1991). The strains with various activator-binding sites upstream of the *HIS3* gene (VI47, VI48, VI66, and VI64) were derived from KY2002 (Iyer and Struhl 1995a). A one-step genomic knockout of *SPN1* was created in each strain, using the same method as described above. The knockout strains were transformed with the YCp22-based *SPN1* plasmids (wild type or K192N) and then cured of the *URA3*-marked cover plasmid to yield the strains that were used for transcriptional analysis: JF17, JF18, JF19, and JF20 (all wild-type *SPN1*) and JF21, JF22, JF23, and JF24 (all *spn1*^{K192N}). Elongation and inositol phenotypes were assayed using strains derived from BY4741 (ResGen). A one-step genomic knockout of *SPN1* was created in BY4741 as described above for other strains. To make the LZ1 and LZ2 strains, the knockout strain LZ0 was transformed with *HIS3*-marked *SPN1* plasmids (wild type or K192N) and then cured of the *URA3*-marked cover plasmid. The LZ1 and LZ2 strains and the control BY4741 *spt4*Δ strain were made *URA3*$^+$ prior to spotting for growth on 6-azauracil and mycophenolic acid. Spt$^-$ phenotypes were tested using strains FY120 and FY241 (gift of Fred Winston) and the FY120-derived JF25. The JF25 strain was derived from FY120 by making a one-step genomic knockout of *SPN1* using *LEU2* and a *URA3*-based cover plasmid containing *spn1*^{K192N}. The phenotypes of various TBP alleles were tested by making a genomic one-step knockout of TBP via *LEU2* in the SK1 and JF10 strains. The strains were then transformed with various TBP alleles on *TRP1*-marked plasmids and cured of the *URA3*-marked cover plasmid by growth on 5-FOA.

Genetic screen for spontaneous suppression of F237D TBP: SK10 cells, containing a LexA operator in place of the Gcn4-binding site upstream of the *HIS3* gene and harboring the fusion protein LexA-F237D on a *URA3*-marked plasmid, were spread at 10^6 cells/plate onto solid medium containing 15 mM 3-aminotriazole (AT) and incubated at 30° until AT-resistant colonies appeared. Strains were analyzed to determine if growth on AT was due to extragenic suppression. First, the LexA-F237D plasmid was shuffled out of each strain by growth on 5-FOA; strains that retained AT resistance were discarded. The remaining strains were then retransformed with the LexA-F237D plasmid and checked for growth on AT. The strains that remained sensitive to AT were discarded. In addition, the shuffled strains were transformed with a plasmid encoding the LexA DNA-binding domain only, and only the strains that remained AT sensitive were retained. Of 758 strains originally isolated, 11 suppressors fit the above criteria.

Cloning by complementation: The suppressor strain JF10, which has a recessive temperature-sensitive (ts) phenotype, was transformed with a yeast genomic library (no. 95-02A, YCp50 CEN, ATCC). Cells in mid-log growth phase were transformed by the standard lithium acetate method with library DNA (2 μg per 10^8 cells) and plated to medium lacking uracil at 10^8 cells/plate. Plates were left at room temperature for 1 hr and then incubated at 38° until colonies appeared. Calculated transformation efficiency was ~1650 colonies per microgram of DNA. The library was covered at least six times. Five independent plasmids were able to confer temperature resistance when retransformed. These five plasmids were found to contain an overlapping region of genomic DNA, which contained eight open reading frames (ORFs). A series of plasmids was constructed with various sections of the genomic DNA to determine which ORF was responsible for complementation of the suppressor strain phenotype. The ORF found to confer temperature resistance was YPR133c. The plasmid containing the YPR133c ORF was cut with restriction enzymes and transformed into the suppressor strain to recover the genomic allele of YPR133c by gap repair. Phenotypic analysis of several gapped plasmids for their ability to complement the ts phenotype of the suppressing strain served to map the location of the suppressing mutation. This region was sequenced, and the mutation was identified.

Artificial recruitment assay: Plasmids encoding LexA fused to various TBP alleles were transformed into the SK1 and JF10 strains, and the resulting transformants were streaked onto solid medium containing 5–15 mM AT. Growth was generally scored after 5 days of incubation at 30°. Failure to grow on AT indicates that the TBP allele has a postrecruitment defect in transcription.

TABLE 1

Yeast strains used in this study

Strain	Description	Reference/Source
FT5	*MAT*α *ura3-52 trp1-Δ63 his3-Δ200 leuΔ2::PET56*	Tzamarias and Struhl (1994)
SK1	FT5 *LexAOp::HIS3*	This study
SK10	SK1 + pRS316-LexA-F237D (*URA3*)	This study
JF10	SK1 $spn1^{K192N}$	This study
JF11	SK1 *spn1::LEU2* + YCp50-*SPN1* (*URA3*)	This study
JF12	JF11 *HIS3*	This study
JF13	JF12 + YCp22-*SPN1* (*TRP1*) lacking YCp50-*SPN1* (*URA3*)	This study
JF14	JF12 + YCp22-$spn1^{K192N}$ (*TRP1*) lacking YCp50-*SPN1* (*URA3*)	This study
JF15	JF12 + YCp22-myc-*SPN1* (*TRP1*) lacking YCp50-*SPN1* (*URA3*)	This study
JF16	JF12 + YCp22-myc-$spn1^{K192N}$ (*TRP1*) lacking YCp50-*SPN1* (*URA3*)	This study
KY2002	Isogenic with FT5 except *MAT***a**	Iyer and Struhl (1995a)
VI47	KY2002 *basal promoter::HIS3*	Iyer and Struhl (1995a)
VI48	KY2002 *poly(dA:dT)::HIS3*	Iyer and Struhl (1995a)
VI66	KY2002 $Gal4_{UAS}$*::HIS3*	Iyer and Struhl (1995a)
VI64	KY2002 Hsf_{UAS}*::HIS3*	Iyer and Struhl (1995a)
JF17	VI47 + *spn1::LEU2* + YCp22-*SPN1 (TRP1)*	This study
JF18	VI48 + *spn1::LEU2* + YCp22-*SPN1 (TRP1)*	This study
JF19	VI66 + *spn1::LEU2* + YCp22-*SPN1 (TRP1)*	This study
JF20	VI64 + *spn1::LEU2* + YCp22-*SPN1 (TRP1)*	This study
JF21	VI47 + *spn1::LEU2* + YCp22-$spn1^{K192N}$ *(TRP1)*	This study
JF22	VI48 + *spn1::LEU2* + YCp22-$spn1^{K192N}$ *(TRP1)*	This study
JF23	VI66 + *spn1::LEU2* + YCp22-$spn1^{K192N}$ *(TRP1)*	This study
JF24	VI64 + *spn1::LEU2* + YCp22-$spn1^{K192N}$ *(TRP1)*	This study
BYΔ2	*MATa ura3-52 trp1-Δ1 his3-Δ200* +YCp86-*SPT15 (URA3)*	Cormack *et al.* (1991)
RYP5	BYΔ2 + YCp22-myc-*SPT15* (*TRP1*)	Campbell *et al.* (2000)
FY120	*MAT***a** *his4-912δ ura3-52 lys2-128δ leu2Δ1*	Swanson *et al.* (1991)
FY241	*MAT*α *his4-912δ ura3-52 lys2-128δ leu2Δ1spt4-3 trp1Δ63*	Gift of Fred Winston
JF25	FY120 *spn1::LEU2* + YCp50-$spn1^{K192N}$ (*URA3*)	This study
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen
LZ0	BY4741 *spn1::LEU2* + YCp50-*SPN1 (URA3)*	This study
LZ1	BY4741 *spn1::LEU2* + pRS313-*SPN1* (*HIS3*)	This study
LZ2	BY4741 *spn1::LEU2* + pRS313-$spn1^{K192N}$ (*HIS3*)	This study

Phenotypic studies: To assay the Spt$^-$ phenotype, strains FY120, JF25, and FY241 (*spt4-3*) were spotted in 10-fold serial dilutions onto medium lacking histidine or lysine and scored after 3 days of growth. Growth on both types of media indicates suppression of the insertion mutations. Elongation phenotypes were tested with BY4741-derived strains (LZ1, LZ2, and BY4741Δ*spt4*) on synthetic complete medium lacking uracil and supplemented with 75 μg/ml 6-azauracil or 20 μg/ml mycophenolic acid. These strains were also tested on synthetic complete medium lacking inositol.

Co-immunoprecipitation: Co-immunoprecipitation experiments were performed essentially as described previously (Moqtaderi *et al.* 1996) with a few modifications. Cultures were grown to an optical density (600 nm) of ~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 plain protein A-sepharose beads (Pharmacia) for 1 hr at 4°. A small sample was taken after the preclear step to provide a load control. Anti-myc antibodies were coupled to protein A-sepharose beads, and the remaining extract was incubated with 50 μl of these coupled beads for 2 hr at room temperature. After six washes, the beads were boiled in loading buffer and 15 μl was loaded for SDS-PAGE, followed by immunoblot analysis.

Acid phosphatase assays: Assays were performed as described (Haguenauer-Tsapis and Hinnen 1984). Cultures of JF13 and JF14 in midlog phase were harvested and resuspended in medium lacking inorganic phosphate ($-P_i$) to induce the phosphatase genes. For the assay at 38°, cultures in standard ($+P_i$) medium were shifted to 38° for 15 min, then allowed to recover at 30° for 1 hr, and finally shifted to 38° for 1 hr. After this hour at 38°, the medium was replaced with $-P_i$ medium, and the assay was otherwise conducted in the same way. All cultures were assayed in duplicate at each time point, and assays were performed on at least three independent cultures.

Transcriptional analyses: S1 nuclease analyses were conducted as described (Iyer and Struhl 1996). When a temperature shift was involved, the cultures were incubated at 38° for 15 min, then 30° for 1 hr, and finally 38° for 1 hr. RNA was prepared by hot phenol extraction and quantitated spectrophotometrically at 260 nm. For activated transcription of the *HIS3* and *HIS4* genes, AT was added to a final concentration of 10 mM and cells were incubated one additional hour. For *PHO5* analysis, cells grown in $+P_i$ medium were resuspended in $-P_i$ medium and incubated for several additional hours. Similarly, for *GAL7* analysis, the cells were grown in raffinose medium, then diluted in galactose medium, and incubated several hours. 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° for 6 hr. For all

analyses at 38°, the temperature shift was done 1 hr prior to supplementing or changing the medium. Hybridizations with excess probe were normally done with 25–30 μg of RNA overnight at 55°. S1 nuclease digestion was performed on the hybridized samples for 30–45 min at 37°. Band intensity was normalized to the intensity of the $tRNA^{w}$ band, which was unaffected by *spn1*^{K192N}. For analysis using the panel of strains with various upstream activator-binding sites, cultures were induced by means appropriate for the activator: Gal4 by culturing in galactose-containing medium for 3 hr and heat-shock factor (Hsf) by shifting to 38° for 15 min. Strains containing poly(dA:dT) or no activator-binding site were grown in rich medium.

RESULTS

Isolation and identification of a suppressor of a postrecruitment-defective TBP allele: The TBP allele F237D was isolated in a previous genetic screen for TBP mutants able to support transcription by RNA polymerase III but defective for some aspect of RNA polymerase II transcription (Stargell and Struhl 1996). Further characterization of the F237D mutant revealed a defect in its response to activators. Moreover, the activation defect of F237D cannot be bypassed through artificial recruitment to the promoter, indicating that this TBP mutant is defective in one or more postrecruitment steps. These observations do not preclude additional defects in recruitment steps, but offer an approach to target the postrecruitment steps *in vivo*.

Therefore, a genetic screen was conducted to obtain extragenic suppressors of the postrecruitment-defective TBP allele F237D (Figure 1). The suppressor screen was performed in yeast strain SK10, containing a LexA operator located upstream of the *HIS3* gene and a *URA3*-marked plasmid encoding the F237D TBP allele fused at its N terminus with a LexA DNA-binding domain. Despite this DNA-binding domain, and unlike wild-type TBP, the F237D allele is unable to activate transcription in this context. Thus, transcriptional output from the *HIS3* reporter gene is insufficient to support viability on medium containing 3-aminotriazole (AT), a competitive inhibitor of the *HIS3* gene product. We selected 758 spontaneous suppressors that formed colonies in the presence of AT. All strains were analyzed to determine if growth on AT was due to extragenic suppression. First, the LexA-F237D plasmid was shuffled out of each strain by growth on 5-FOA, and the cured strains were tested for growth on AT. In the absence of LexA-F237D, 563 strains maintained AT resistance. These were eliminated since they are likely to possess mutations that potentiate basal transcription in a manner that is not dependent on F237D function. The remaining 195 strains were retransformed with the LexA-F237D plasmid and checked for growth on AT. We eliminated 163 strains that remained sensitive to AT, as their suppressing phenotype is likely due to a mutation linked to the original LexA-F237D plasmid. In addition, the shuffled strains were transformed with a plasmid encoding the LexA DNA-binding domain without the F237D fusion. Those 21 strains that grew on AT in the presence of LexA were rejected because the suppression could have been due to mutations that potentiate weak activators. Eleven suppressor strains fit all of the above criteria. Here we describe the cloning and characterization of the gene *SPN1*.

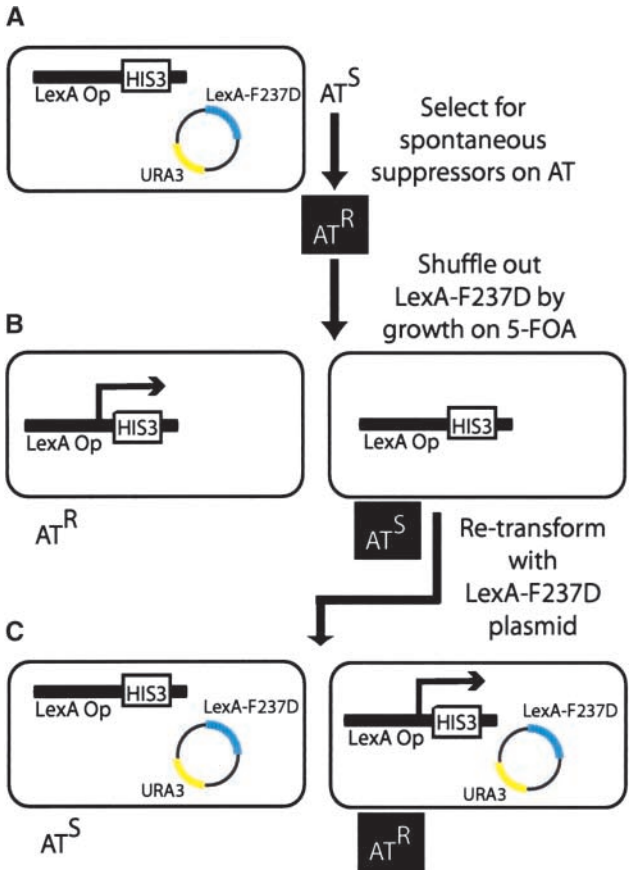

Figure 1.—Genetic screen for spontaneous suppressors of the postrecruitment defects of the TBP allele F237D. (A) The starting strain contained a LexA operator (LexA Op) and the LexA-F237D derivative on a *URA3*-marked plasmid. When plated on media containing AT, a competitive inhibitor of the *HIS3* gene product, the strain fails to grow (AT^S). Spontaneous suppressors were selected that were resistant to AT (AT^R). These were subsequently cured of the LexA-F237D plasmid by growth on 5-FOA. (B) Strains that maintained AT^R in the absence of LexA-F237D were discarded. AT^S strains were retransformed with the LexA-F237D plasmid. (C) Those that remained AT^S were discarded, as their suppressing phenotype was likely due to a mutation on the original LexA-F237D plasmid. Those that regained AT^R in the presence of LexA-F237D were further characterized by transforming the cured strain with LexA alone (not shown) to test for dependence on the TBP portion of the fusion derivative.

In addition to suppressing LexA-F237D, *spn1* cells exhibit a recessive ts phenotype (data not shown). Cloning by complementation of the ts phenotype was used to identify the suppressing gene as the yeast open reading frame YPR133c. *SPN1* is an essential gene and encodes a 410-amino-acid polypeptide (data not shown). The

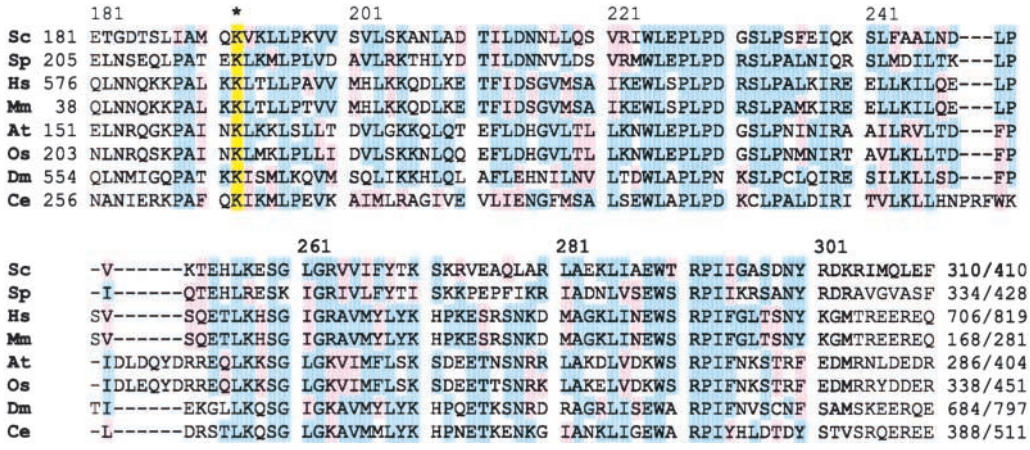

FIGURE 2.—*SPN1* has a highly conserved central domain. Identical amino acids are shown in blue; similar amino acids are shown in pink; and the lysine 192 that is mutated in the suppressor strain is highlighted in yellow and with an asterisk. Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Hs, *Homo sapiens*; Mm, *Mus musculus*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*. Numbers indicate the starting and ending amino acid positions shown for each homolog, followed by the total number of amino acids in the homolog. Alignment was produced with the aid of the BLAST search engine (ALTSCHUL *et al.* 1997).

spn1 allele present in JF10 was recovered by plasmid gap repair. Sequencing revealed a point mutation at residue 192, resulting in the substitution of an asparagine for a lysine (*spn1*^{K192N}). All phenotypes observed for JF10 were recapitulated in a strain with the genomic copy of *SPN1* disrupted by *LEU2* and covered by *spn1*^{K192N} on a plasmid (data not shown), indicating that this single substitution confers the suppressing and ts phenotypes.

***SPN1* is highly conserved throughout evolution:** Yeast *SPN1* has homologs in a wide range of eukaryotic organisms, including human, mouse, plants, fly, and worm (ALTSCHUL *et al.* 1997). Sequence alignment reveals a central conserved region of ~113 amino acids in length, in which the human sequence is 42% identical and 63% similar to the yeast sequence (Figure 2). Notably, the suppressing substitution, K192N, occurs in an invariant residue within this conserved region. N-terminal deletions and C-terminal truncations of *SPN1* were constructed, assayed for expression, and tested for their ability to cover a genomic knockout of the gene (Figure 3). These studies demonstrate that the nonconserved N-terminal and C-terminal regions are dispensable, while the entire conserved core is necessary for the essential functions of *SPN1* in yeast cells. Interestingly, none of the homologs has been functionally characterized, and Spn1 protein (Spn1p) contains no known functional protein motifs. Thus, yeast *SPN1* is the first characterized member of this class of genes.

***spn1*^{K192N} suppresses the postrecruitment defect of two additional TBP alleles:** We next tested whether the suppression of the postrecruitment defect of LexA-F237D by *spn1*^{K192N} is allele specific. The strain harboring the suppressing mutation was transformed with a panel of LexA-TBP derivatives, some of which exhibit a postrecruitment defect in a wild-type *SPN1* background (F237D, E236P, and K201I). As shown, the suppressor enabled growth of all of the postrecruitment-defective TBP alleles (Table 2), which are defective in a variety of interactions (YAMAMOTO *et al.* 1992; LEE and STRUHL 1995; STARGELL and STRUHL 1996). Given that *spn1*^{K192N} is not allele specific in its suppression ability, it is unlikely to represent a compensatory mutant for F237D TBP.

To investigate further the link between *spn1*^{K192N} and TBP function, we looked for suppression of TBP mutant phenotypes by *spn1*^{K192N} outside the context of the artificial recruitment assay. The genomic copy of TBP was knocked out in SK1 (*SPN1*) and JF10 (*spn1*^{K192N}) strains and covered by a variety of TBP alleles. Unfortunately, when not tethered to a DNA-binding domain, the F237D allele of TBP exhibits no mutant phenotypes for *spn1*^{K192N} to suppress (Table 3). The same is true for the E236P derivative. The final postrecruitment-defective allele, K201I, did exhibit mutant phenotypes in this strain background, and we observed that two phenotypes ascribed to the K201I allele were suppressed in the *spn1*^{K192N} background (Table 3). This result parallels the finding that the K201I allele, which is postrecruitment defective in a wild-type *SPN1* strain, is competent for artificial recruitment in the context of *spn1*^{K192N} (Table 2). In contrast, the S118L/S128T allele, which is postrecruitment competent, gains exacerbated phenotypes in the *spn1*^{K192N} context (Table 3). Thus, a mutation in *SPN1* specifically suppresses a TBP mutant with known defects in postrecruitment functions, whereas another TBP mutant, which is functional in a postrecruitment assay, is not suppressed.

Spn1 protein does not stably associate with the TFIID complex *in vivo*: It is possible that direct interaction of Spn1p with the TFIID complex could mediate suppression of postrecruitment-defective TBP alleles. Coimmunoprecipitation experiments were performed to determine if Spn1p is associated with TFIID *in vivo*. Whole-cell extracts were prepared from strains expressing myc-tagged versions of TBP or Spn1p and from a control strain. Antibodies against the myc epitope were used for immunoprecipitation from these extracts. The precipitated complexes were analyzed by immunoblot,

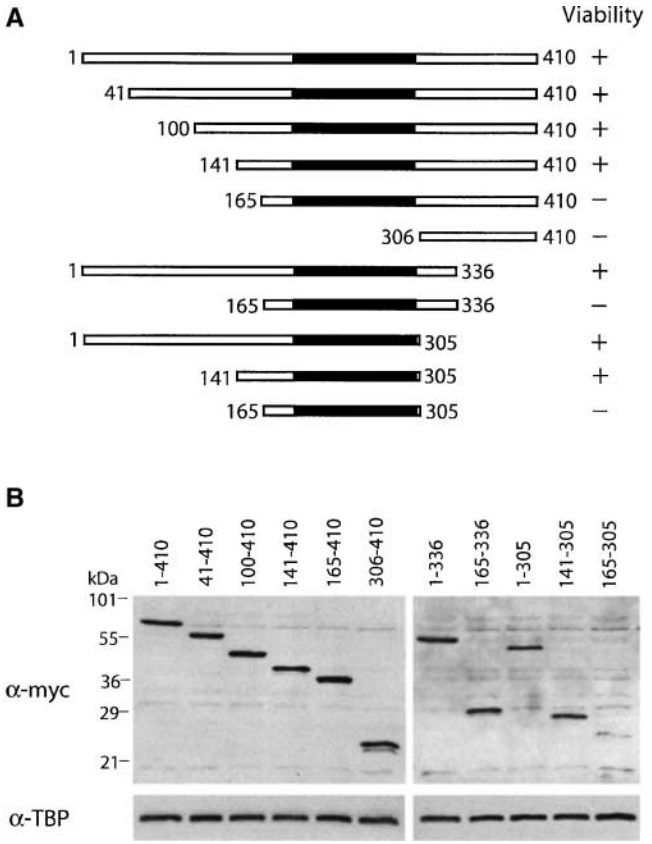

FIGURE 3.—The central conserved region of *SPN1* is necessary for the essential functions of *SPN1*. (A) N- and C-terminal residues of each truncation construct are labeled. The conserved region is denoted by the solid box. Viability denotes the growth (+) or no growth (−) phenotype of cells when the indicated derivative is tested for its ability to cover a genomic knockout of the *SPN1* gene in a plasmid shuffle experiment. (B) Immunoblot probed with α-myc antibodies showing protein expression of myc-tagged truncations corresponding to the constructs in A. The residues included in each construct are shown above each lane. The blot was stripped and reprobed for TBP as a load control.

employing polyclonal antibodies against TBP, Spn1p, and several TAFs (Figure 4). Immunoprecipitation of myc-TBP shows association of TBP with TAFs, indicating the presence of intact TFIID, but Spn1p was not detected. Moreover, in the reciprocal experiment, when myc-Spn1p was immunoprecipitated, TBP was not detected. These data indicate that Spn1p does not stably associate with the TFIID complex *in vivo*. This result is not surprising given the ability of *spn1*^{K192N} to suppress the postrecruitment defects of multiple TBP alleles, each of which may possess unique defects in postrecruitment functions.

***spn1*^{K192N} cells are mildly defective for constitutive transcription:** To analyze the effect of a mutation in *SPN1* on transcription, RNA was isolated from cultures of *SPN1* and *spn1*^{K192N} strains at the permissive and restrictive temperatures, and S1 nuclease experiments were performed. When transcript levels from the constitutively expressed genes *HIS3*, *DED1*, *HTA2*, *RPS4*, and *ENO2* were measured, the mutant strain was observed to have only mild reductions in RNA levels compared to wild type (Figure 5). At 30°, we observed 15–35% reductions in transcriptional output of the *spn1*^{K192N} strain. At 38°, levels of transcripts from *spn1*^{K192N} show changes similar to those observed at 30°, with the exception that *DED1* exhibits a more significant reduction. Thus, the *spn1*^{K192N} allele has modest effects on the expression of constitutively transcribed genes.

***SPN1* plays an important role in activated transcription at a subset of inducible genes:** Transcriptional output from a constitutively expressed gene *in vivo* is likely to reflect the maintenance of activated transcription, which might differ on a mechanistic level from the induction of transcription. Hence, we tested whether the *spn1*^{K192N} mutant is competent for inducible gene transcription. Several different inducible systems were tested at their endogenous responsive genes. Total RNA was isolated from cells grown under inducing conditions appropriate for activation of *GAL7* (growth on galactose), *SSA4* (heat shock), *PHO5* (absence of phosphate), or *HIS3* and *HIS4* (growth on aminotriazole). The results reveal defects that are dependent on the temperature and the particular gene examined. *GAL7* transcription displayed no significant reduction in the mutant strain at either 30° or 38° (Figure 6A). Similarly, *SSA4* transcription in the *spn1*^{K192N} background upon induction by heat shock was also robust (Figure 6B). In contrast, *PHO5* in the *spn1*^{K192N} cells induced nearly as well as wild type at 30° but failed to activate at 38° compared to a two- to threefold induction in wild type (Figure 6C). Thus, transcriptional induction of *PHO5* is temperature dependent. The results for both temperatures were confirmed with acid phosphatase colorimetric assays on wild-type and *spn1*^{K192N} strains (data not shown). In contrast, the induction of *HIS3* at 30° was significantly affected by *spn1*^{K192N}, with induced transcription levels in the mutant strain at one-half of those observed in the wild-type strain (Figure 6D). This effect is not due to reduced levels of Gcn4 in the *spn1*^{K192N} strain, since the *HIS4* gene, which is also regulated by Gcn4, induces with equal robustness in wild-type and mutant strains at 30°. Thus, the *HIS3* transcriptional defects are not likely to be due to a reduction of Gcn4 in the mutant strain. Like the *PHO5* gene, both *HIS3* and *HIS4* induce poorly at 38° in the *spn1*^{K192N} strain, reaching only 25 and 15% of wild-type induced levels, respectively. As determined by immunoblot analyses, the amount of Spn1p in wild-type and mutant strains does not vary with temperature (data not shown), suggesting that temperature-dependent effects on transcription are likely to be due to loss of activity of Spn1p rather than to a reduction in protein level.

Various factors could account for the gene-specific differences in transcriptional output seen under inducing conditions in the *spn1*^{K192N} strain. A gene-specific

TABLE 2

***spn1*^{K192N} can suppress the postrecruitment defects of multiple TBP alleles**

LexA fusion[a]	TBP functional defect	Artificial recruitment[b] *SPN1*	Artificial recruitment[b] *spn1*^{K192N}	Reference
S118L/S128T	Activation defective	+	+	Lee and Struhl (1995)
K201I	DNA-binding defective	−	+	Yamamoto *et al.* (1992)
E236P	Activation defective	−	+	Stargell and Struhl (1996)
F237D	Activation defective	−	+	Stargell and Struhl (1996)

[a] LexA fusions were created with the indicated TBP alleles on *URA3*-marked plasmids.
[b] Plasmids encoding the LexA-TBP fusions were transformed into the wild-type and *spn1*^{K192N} strains. Transformants were streaked onto solid medium containing AT. If cells grew on AT, they were scored as +.

effect may reflect dependence either on a given activator or on a particular gene context (*i.e.*, promoter sequence, chromatin structure, and/or the transcribed region of the gene). To investigate this further, we assayed *HIS3* transcription from a set of strains in which different sequence elements were cloned upstream of the promoter (Iyer and Struhl 1995a). As such, each element must function in the same gene context. As expected for constitutive transcription, in this case due to intrinsic DNA structure (Iyer and Struhl 1995b), the poly(dA:dT) upstream sequence stimulated transcription of *HIS3* equally well in *spn1*^{K192N} and in wild-type cells (Figure 7). We next tested Gal4- and Hsf-binding sites using inducing conditions appropriate for each activator. We observed robust activation of the *HIS3* gene in wild-type cells driven from the Gal4- and Hsf-binding sites, whereas *SPN1* mutant cells exhibited reproducible 30 and 50% reductions from these promoters, respectively (Figure 7). Thus, in the context of the *HIS3* gene, Hsf and, to a lesser extent, Gal4, show dependence on Spn1p for normal levels of induced transcription. And yet, no dependence on Spn1p was observed in the context of their native responsive promoters (Figure 6). Thus, the *HIS3* gene is particularly sensitive to perturbations in normal Spn1p function, since activators that are *SPN1* independent at their normal sites become dependent in the *HIS3* gene context. Whether this is due to promoter sequence, chromatin structure, and/or the transcribed region of the *HIS3* gene remains to be determined.

***spn1*^{K192N} aids in transcription from the postrecruitment regulated *CYC1* promoter:** At the majority of promoters, recruitment of the transcription machinery is the primary mechanism by which transcription is regulated (Kuras and Struhl 1999; Li *et al.* 1999). However, the *CYC1* promoter is occupied by both TBP and Pol II when in a transcriptionally inactive state (Lue *et al.* 1989; Chen *et al.* 1994; Kuras and Struhl 1999; Martens *et al.* 2001). Activation in this case is not limited by TFIID or Pol II recruitment but is regulated in a postrecruitment step. One would predict that if Spn1p functions to regulate *CYC1* expression, then transcription should be enhanced in *spn1* mutant cells. After a 6-hr incubation at 30° in 2% glucose (partially repressing) or 3% ethanol (activating), total RNA was isolated

TABLE 3

Mutant phenotypes of TBP alleles in *SPN1* and *spn1*^{K192N} backgrounds

TBP allele	Growth[a] *SPN1*	Growth[a] *spn1*^{K192N}
Wild type	None	ts
S118L/S128T	ino$^{\pm}$	ts, ino$^{\pm}$, cs
K201I	gly^{-}, cs	ts
E236P	None	ts
F237D	None	ts

[a] The indicated strains were scored as sensitive if they failed to grow at 38° (ts) or at 15° [cold sensitive (cs)] or on medium containing glycerol (gly^{-}) or lacking inositol (ino^{-}). A score of ino$^{\pm}$ indicates weak growth on medium lacking inositol.

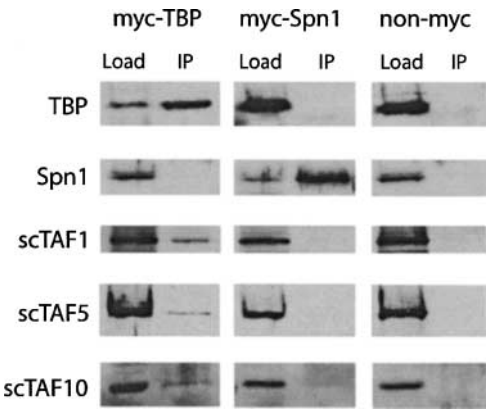

Figure 4.—Spn1p is not stably associated with TFIID *in vivo*. Co-immunoprecipitation was performed on cell extracts from strains expressing myc-TBP, myc-Spn1p, or untagged cells (non-myc). Immunoprecipitated complexes (IP) were resolved by SDS-PAGE and probed by immunoblot analysis using polyclonal antibodies specific to TBP, Spn1p, scTAF1, scTAF5, and scTAF10. Input from each extract (Load) is indicated.

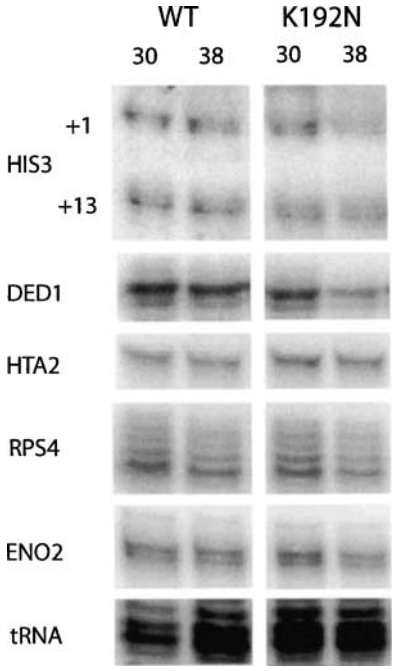

FIGURE 5.—Constitutive transcription is mildly affected in *spn1*^{K192N} cells. Mutant (K192N) and wild-type (WT) *SPN1*-containing cells were grown under steady-state conditions and harvested in log phase at 30° or shifted to the restrictive temperature (38°) for 1 hr. Total RNA harvested before and after the temperature shift was hybridized with a 100-fold excess of the indicated probe, treated with S1 nuclease, and resolved on a denaturing gel; tRNA^{w} served as a load control.

from wild-type and *spn1*^{K192N} cells, and *CYC1* transcription was analyzed by S1 nuclease assay. The *spn1*^{K192N} cells had *CYC1* transcript levels significantly greater than the corresponding wild-type levels, under both partially repressing and activating conditions (Figure 8). This result indicates that transcription at a postrecruitment regulated promoter is facilitated by *spn1*^{K192N} and further supports our previous data suggesting that Spn1p acts at a postrecruitment step in transcription.

***SPN1* is a member of the *SPT* gene family:** The TBP gene, *SPT15*, was cloned using a genetic screen for suppressors of Ty insertions in the 5′ regions of *HIS4* and *LYS2* (EISENMANN *et al.* 1989; HAHN *et al.* 1989). The *SPT* family of genes, each capable of suppressing Ty insertions when mutated, represents a large collection of genes involved in an array of transcriptional functions. In addition to TBP, this family includes histones and histone modifying or remodeling complexes, members of the Pol II mediator complex, and proteins involved in elongation steps of transcription (WINSTON 1992). In addition to the genetic interactions described herein between *SPN1* and *SPT15* (TBP), a further link to the *SPT* gene family arises from the recent observation that Spn1p was found to associate with Spt6p, using a large-scale proteomics approach (GAVIN *et al.* 2002). Spt6p has been shown to bind histones and assemble nucleosomes *in vitro* and to remodel chromatin *in vivo*

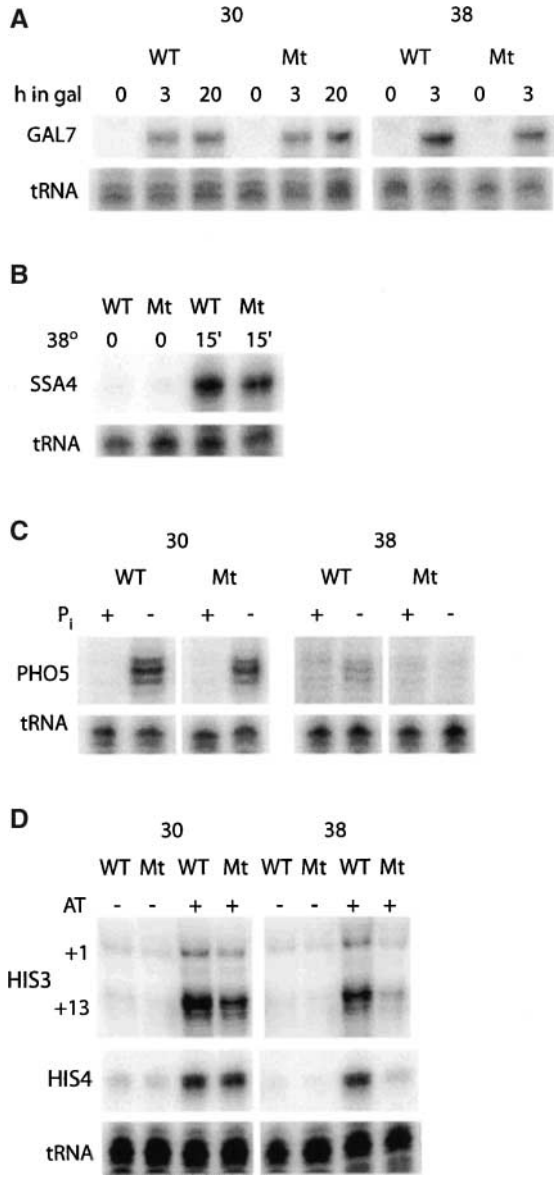

FIGURE 6.—Analyses of inducible gene expression in *spn1*^{K192N}. (A) To examine the response to the transcriptional activator Gal4, cells were grown to log phase in raffinose-containing medium and then transferred into galactose-containing medium for the time indicated and probed for expression of *GAL7*. Strains contained either wild-type *SPN1* (WT) or *spn1*^{K192N} (Mt). (B) Response to heat-shock factor was assayed by transferring cells to 38° for 15 min prior to harvest and probing for expression of *SSA4*. (C) Activation by Pho4 was induced by growing cells to log phase and then transferring cells into medium lacking inorganic phosphate for 6 hr (30°) or 4 hr (38°) prior to harvest, as indicated. (D) Gcn4 responsiveness was determined by treating cells with 10 mM AT for 1 hr prior to harvest and by examining the levels of *HIS3* (with +1 and +13 start sites indicated) and *HIS4* transcripts. For all treatments, 25 μg of total RNA was hybridized with excess probe, treated with S1 nuclease, and resolved on a denaturing gel.

(BORTVIN and WINSTON 1996). In addition, Spt6p, in association with Spt4p and Spt5p, is thought to function in elongation (HARTZOG *et al.* 1998; ANDRULIS *et al.*

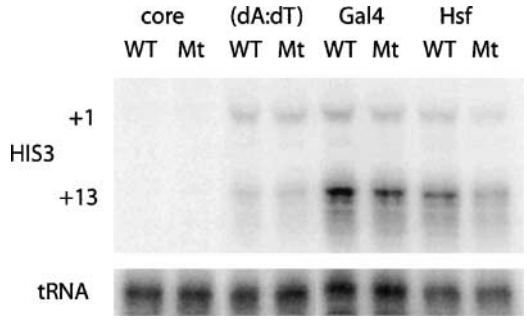

FIGURE 7.—Transcriptional output from a variety of activators at *HIS3* in wild-type and *spn1*^{K192N} cells. RNA from strains containing various activator-binding sites upstream of the *HIS3* locus was subjected to quantitative S1 analysis. Cells were grown under conditions appropriate for the activator of interest and harvested in log phase. For Gcn4, 10 mM AT was added for 1 hr. For Gal4, cells were grown in galactose-containing medium for 3 hr. For Hsf, cells were exposed to 38° for 15 min. Other strains were grown in rich medium under normal conditions. Strains contained either wild-type *SPN1* (WT) or *spn1*^{K192N} (Mt). Thirty micrograms of total RNA was hybridized with excess probe, treated with S1 nuclease, and resolved on a denaturing gel. tRNA^{w} is a load control.

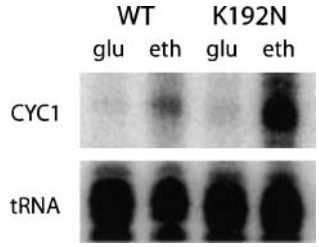

FIGURE 8.—Transcription is enhanced by *spn1*^{K192N} at a postrecruitment-regulated promoter. RNA was isolated from wild-type (WT) and *spn1*^{K192N} (K192N) strains grown in medium containing either 2% glucose (glu) or 3% ethanol (eth) for 6 hr. Glucose causes partial repression of *CYC1*, while ethanol causes activation. The RNA was subjected to S1 nuclease analysis using a probe for *CYC1*. tRNA^{w} is the load control.

2000; KAPLAN *et al.* 2000). These connections to *SPT* genes prompted us to test directly whether the *SPN1* mutant suppresses Ty insertions. We constructed a one-step genomic knockout of *SPN1* in the yeast strain FY120, which possesses the insertion mutations *his4-912*δ and *lys2-128*δ (SWANSON *et al.* 1991). The genomic knockout of *SPN1* was covered by *spn1*^{K192N} and assayed on medium lacking histidine or lysine (Figure 9A). Like the *spt4-3* control cells, the *spn1*^{K192N} cells grow well on these media, indicating the ability to suppress the insertion mutations. Thus, *SPN1* falls under the umbrella of the *SPT* gene family.

SPT6 has been implicated in elongation processes, along with *SPT4* and *SPT5* (HARTZOG *et al.* 1998; ANDRULIS *et al.* 2000; KAPLAN *et al.* 2000). Since elongation is clearly a postrecruitment function, we tested the *SPN1* mutant for sensitivity to the drugs 6-azauracil (6-AU) and mycophenolic acid (MPA). Such sensitivity generally indicates the presence of a defect in elongation, presumably due to a decrease in the pools of free nucleotides, which results in increased pausing and arrest by Pol II (EXINGER and LACROUTE 1992; UPTAIN *et al.* 1997). In contrast to *spt4*Δ cells, which are sensitive to 6-AU and MPA (HARTZOG *et al.* 1998), *spn1*^{K192N} cells grow robustly on media containing these agents (Figure 9B). Inositol auxotrophy is another phenotype commonly shared by mutations in *SPT* genes (WINSTON and CARLSON 1992; GRANT *et al.* 1998). Unlike *spt4*Δ cells, which display impaired growth in the absence of inositol, again the *spn1*^{K192N} cells grow robustly (Figure 9C). As such, although a mutation in *SPN1* confers some phenotypes consistent with an *SPT* designation, the observed differences in growth on media with 6-AU, MPA, and minus inositol suggest a role that may be, at least in part, functionally distinct from that of *SPT4-SPT5-SPT6*.

DISCUSSION

***SPN1* is involved in postrecruitment functions of TBP:** A spontaneous suppressor screen of a postrecruitment-defective allele of TBP (F237D) has identified *SPN1*. *SPN1* is an essential gene in yeast that is conserved across species. A single substitution of a conserved lysine to an asparagine (*spn1*^{K192N}) is able to suppress the postrecruitment defects of F237D. The fact that the *spn1*^{K192N} mutant can suppress additional postrecruitment-defective TBP alleles, with presumably a range of mechanistic defects, indicates that *spn1*^{K192N} is not a compensatory mutant for F237D. However, in the case of S118L/S128T, a postrecruitment competent TBP allele, mutant phenotypes are not suppressed but rather exacerbated by *spn1*^{K192N}. Thus the *spn1*^{K192N} mutant is not a general suppressor of TBP defects. Although a transient or DNA-dependent association cannot be ruled out, Spn1p is not detected in association with TFIID by co-immunoprecipitation. Taken together, the data suggest a broader involvement of Spn1p in the transcription process, rather than an involvement via direct association with TBP.

Functional role of Spn1p: Functional studies indicate that *spn1*^{K192N} has little effect on constitutive transcription at the permissive and restrictive temperatures. However, significant transcriptional defects are observed in the induction of activated transcription, although these defects depend on the gene assayed. Whereas induction of the *GAL7* (via Gal4) and *SSA4* (via Hsf) genes is unaltered in the *SPN1* mutant background, induced transcription of *HIS3* (via Gcn4) is significantly reduced at both temperatures. Thus, the *HIS3* gene is highly dependent on Spn1p for maximum transcriptional output. On the basis of the robust activation of *HIS4* (via

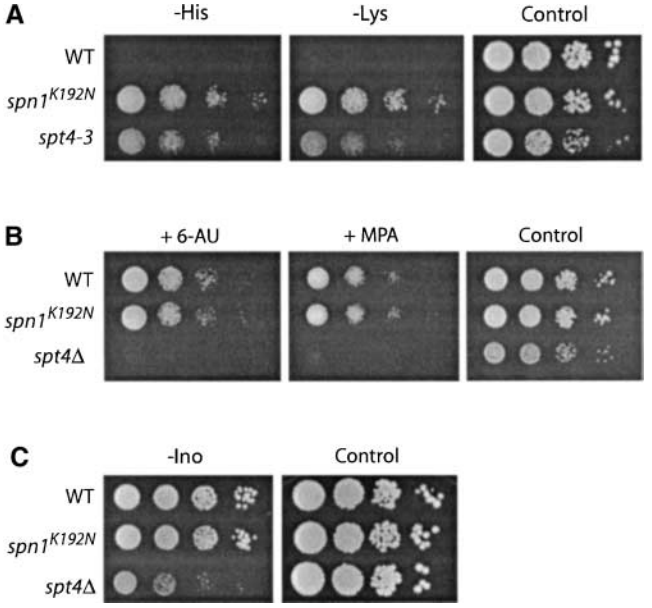

Figure 9.—The *spn1*^{K192N} strain has Spt$^-$ phenotypes. (A) The indicated strains were spotted onto medium lacking histidine (−His), medium lacking lysine (−Lys), or complete medium (Control) to assay for an Spt$^-$ phenotype. The *spn1*^{K192N} strain was derived from the FY120 strain (WT), which contains *his4-912*δ and *lys2-128*δ insertion mutations. The strain harboring the *spt4-3* allele has the same insertions. Growth was scored after 3 days. A Spt$^-$ phenotype corresponds to growth on media lacking histidine and on media lacking lysine, indicating suppression of the insertion mutations. (B) BY4741-derived wild-type (WT), *spn1*^{K192N}, or *spt4*Δ strains were spotted onto medium containing 75 μg/ml 6-azauracil (+6-AU), 20 μg/ml mycophenolic acid (+MPA), or control medium (Control). Growth was examined after 3 days. (C) Strains were also spotted onto synthetic complete medium lacking inositol (−Ino) or rich medium (Control). Growth was examined after 3 days.

Gcn4), a reduction in the activator level is not likely to be the cause of the *HIS3* defects. Moreover, when Gal4 and Hsf act upstream of the *HIS3* gene at engineered binding sites, they become dependent on Spn1p function, whereas they are independent at their native promoters. As such, the negative effect of Spn1p^{K192N} on activation appears to be more dependent on the context of the gene (promoter sequence, chromatin structure, or transcribed region of the gene) and less dependent on the particular activator.

Analysis of transcription at the *CYC1* promoter supports a postrecruitment function for Spn1p. Both Pol II and TBP have been shown to occupy the transcriptionally inactive form of this promoter (Lue *et al.* 1989; Chen *et al.* 1994; Kuras and Struhl 1999; Martens *et al.* 2001). Thus, quite atypically, the recruitment of the transcription machinery is not the primary means of regulation at *CYC1*. The finding that *CYC1* transcription in the *spn1*^{K192N} strain is significantly enhanced as compared to the wild-type strain suggests that Spn1p^{K192N} aids transcription by overcoming some postrecruitment barrier to transcription of *CYC1*. Clearly, the sum of Spn1p^{K192N} functional defects, together with the genetic and transcriptional evidence that *SPN1* suppresses postrecruitment functions, indicates that Spn1p plays an important role in transcription.

Classification of *SPN1* as a member of the *SPT* gene family: The identification of *SPN1* as an *SPT* gene places it in a broad family of genes, which includes chromatin components and remodeling complexes, general transcription factors and mediator/holoenzyme subunits, and elongation factors. Recently, a large-scale proteomics approach has detected an association between Spn1p and Spt6p (Gavin *et al.* 2002). Spt6p has been shown to bind histones, assemble nucleosomes and remodel chromatin (Bortvin and Winston 1996), and function in elongation (Hartzog *et al.* 1998; Andrulis *et al.* 2000; Kaplan *et al.* 2000). Our characterizations of *SPN1* are supportive of a role in transcription that appears to mesh well with the types of influence and regulation likely to be exhibited by Spt6p-associated proteins. However, *spn1*^{K192N} cells do not exhibit growth defects associated with inositol metabolism [whereas *SPT* mutants often display these phenotypes (Winston and Carlson 1992; Grant *et al.* 1998)], nor do they show an elongation defect by phenotypic testing. Thus, it seems likely that Spn1p, in concert with Spt6p or other proteins, has additional or distinct roles in transcriptional processes.

Potential mechanisms of Spn1p in transcriptional processes: The emergence of *spn1*^{K192N} in a screen for suppressors of a postrecruitment-defective TBP allele and the ability of *spn1*^{K192N} to enhance transcription from *CYC1* suggests that wild-type Spn1p functions negatively. However, such a role appears to be at odds with our results demonstrating that activated transcription at a subset of genes is diminished in the *spn1*^{K192N} background, implying a positive role for Spn1p. We can reconcile these opposing effects by considering that Spn1p may function in both postrecruitment and recruitment steps of transcription. In the genetic screen and at *CYC1*, TBP is already recruited. As such, the focus is on interactions centered around the core promoter (*i.e.*, the TBP-binding site and the transcriptional initiation site) and on functions that are important after TBP is recruited (postrecruitment functions). Thus, wild-type Spn1p may have negative effects in the context of core promoter and/or postrecruitment functions. Activated transcription is a complex process in which multiple steps, including recruitment, are influenced by activators bound at upstream promoter elements. In this context, Spn1p appears to positively affect transcription in a gene-specific manner. Thus, the positive and negative effects of Spn1p might reflect differing functions of the factor depending on the step in the transcriptional process (*i.e.*, recruitment *vs.* postrecruitment). A growing body of evidence supports the idea that certain factors can influence multiple steps of the

transcriptional process. For example, the Paf1/Pol II complex (Mueller and Jaehning 2002), the FACT complex (Orphanides *et al.* 1998; Formosa *et al.* 2001), and the Spt4-5-6 complex (Swanson and Winston 1992; Hartzog *et al.* 1998) have each been implicated in initiation (recruitment) and elongation (postrecruitment) steps of transcription. These observations serve to underscore the complexity and interplay between the components of the Pol II machinery.

In summary, we describe the cloning and initial characterization of Spn1p, a new player involved in transcriptional mechanisms *in vivo*. The *SPN1* gene, an essential gene conserved throughout evolution, was identified in a screen for suppressors of a TBP mutant. The *SPN1* gene also shares phenotypes with the *SPT* family of transcription factors, of which TBP is a member. In addition, *SPN1* function is required for normal gene expression at a subset of genes. Taken together, these results support a functional conservation of the role(s) of Spn1p in the mechanism of transcription *in vivo*.

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