SPT3 interacts with TFIID to allow normal transcription in *Saccharomyces cerevisiae*

David M. Eisenmann, Karen M. Arndt, Stephanie L. Ricupero, John W. Rooney, and Fred Winston

Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115 USA

Mutations in the *Saccharomyces cerevisiae* gene *SPT15*, which encodes the TATA-binding protein TFIID, have been shown to cause pleiotropic phenotypes and to lead to changes in transcription in vivo. Here, we report the cloning and analysis of one such mutation, *spt15-21*, which causes a single-amino-acid substitution in a conserved residue of TFIID. Surprisingly, the *spt15-21* mutation does not affect the stability of TFIID, its ability to bind to DNA or to support basal transcription in vitro, or the ability of an upstream activator to function in vivo. To study further the *spt15-21* defect, extragenic suppressors of this mutation were isolated and analyzed. All of the extragenic suppressors of *spt15-21* are mutations in the previously identified *SPT3* gene. Suppression of *spt15-21* by these *spt3* mutations is allele-specific, suggesting that TFIID and SPT3 interact and that *spt15-21* impairs this interaction in some way. Consistent with these genetic data, coimmunoprecipitation experiments demonstrate that the TFIID and SPT3 proteins are physically associated in yeast extracts. Taken together, these results suggest that SPT3 is a TFIID-associated protein, required for TFIID to function at particular promoters in vivo.

[Key Words: Transcription; yeast; TFIID; SPT3; mutants; allele specificity]

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Transcription initiation by RNA polymerase II is a complicated process requiring the formation of a multicomponent, preinitiation complex near the start site of transcription [for review, see Sawadogo and Sentenac 1990; Roeder 1991]. This complex is composed of a large number of proteins, including RNA polymerase II and several general transcription factors. These general factors were originally identified by fractionation of HeLa cell nuclear extracts into a number of activities essential for the accurate initiation of transcription by RNA polymerase II in vitro [Matsui et al. 1980; Samuels et al. 1982; Davison et al. 1983]. At least some of these general factors have been identified in other eukaryotic systems, including *Saccharomyces cerevisiae* [Buratowski et al. 1988; Cavallini et al. 1988; Hahn et al. 1989a], *Drosophila melanogaster* [Wampler et al. 1990], and rat [Conaway and Conaway 1991], indicating that many components of the RNA polymerase II transcription machinery have been evolutionarily conserved.

One of the general factors, TFIID, is believed to begin formation of the preinitiation complex by binding to the TATA box [Sawadogo and Roeder 1985b; Reinberg et al. 1987; Nakajima et al. 1988; Van Dyke et al. 1988; Burtowski et al. 1989]. Subsequent to TFIID binding, the other general factors and RNA polymerase II assemble in an ordered, stepwise fashion, resulting in the formation of a complete preinitiation complex that can initiate transcription in an ATP-dependent manner [Fire et al. 1984; Van Dyke et al. 1988; Buratowski et al. 1989; Maldonado et al. 1990]. In addition to these interactions with other general factors, a number of studies have demonstrated interactions between TFIID and various transcriptional regulatory proteins [for examples, see Sawadogo and Roeder 1985b; Horikoshi et al. 1988; Stringer et al. 1990; Horikoshi et al. 1991; Lee et al. 1991; Workman et al. 1991]. Other factors have also been described that form distinct complexes with TFIID on TATA-containing templates and that have effects on transcription in vitro [Meisterernst and Roeder 1991; Meisterernst et al. 1991b]. TFIID therefore appears to interact with a surprisingly large number of proteins, in addition to interacting with DNA.

Recently, both *Drosophila* and human TFIID fractions have been shown to contain the TATA-binding protein [often referred to as TBP; see Pugh and Tjian 1992] in tight association with several other polypeptides referred to as TBP-associated factors or TAFs [Dynlacht et al. 1991; Pugh and Tjian 1991; Tanese et al. 1991]. The presence of these associated proteins explains the large difference between the observed molecular mass of TFIID activity in these organisms [120–750 kDa]
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1982, Reinberg et al. 1987, Muhich et al. 1990, Timmers and Sharp 1991) and the known molecular mass of the TATA-binding protein (38 kD, Pugh and Tjian 1992). Some of these associated proteins are apparently required for the interaction of activator proteins with TFIID. Unlike HeLa and Drosophila TFIID, yeast TFIID purifies as a single polypeptide with the same molecular mass as predicted by the sequence of the yeast TFIID-encoding gene [Buratowski et al. 1988; Cavallini et al. 1989; Horikoshi et al. 1989]. This result suggests either that yeast TFIID, unlike TFIID from higher eukaryotes, does not associate with other factors or that such factors exist, but their interactions with yeast TFIID are less stable.

We have taken a genetic approach toward the study of TFIID and other transcription factors in S. cerevisiae. Beginning with insertion mutations caused by the retrotransposon Ty, or its long terminal repeat, 6, which perturb transcription of adjacent genes, a large number of suppressor mutations have been identified that restore normal transcription (for review, see Boeke and Sandmeyer 1991; Winston 1992). These mutations have identified >17 SPT [suppressor of Ty] genes that appear to play general roles in transcription. One of these genes, SPT15, encodes yeast TFIID [Eisenmann et al. 1989; Hahn et al. 1989b]. Mutations in SPT15 and in three other genes, SPT3, SPT7, and SPT8, all lead to a common set of phenotypes, including defects in growth, mating, and sporulation, and the ability to suppress many Ty and solo 6 insertion mutations [Winston et al. 1984a, b, 1987; Eisenmann et al. 1989]. These common phenotypes suggest that SPT3, SPT7, and SPT8 gene products may be functionally related to TFIID.

Previous work demonstrated that the SPT15 gene is essential for growth and that one mutation, spt15-21, confers several mutant phenotypes and causes alterations in transcription in vivo [Eisenmann et al. 1989]. Here, we present further studies on spt15-21 and report that the spt15-21 mutation changes a conserved residue of TFIID. This mutation does not alter the ability of TFIID to bind DNA or to interact with other general factors during transcription initiation. Extragenic suppressors of spt15-21 were isolated and shown to be new mutations clustered in the previously identified gene SPT3. We present both genetic and physical evidence that the SPT3 and TFIID proteins interact physically and suggest that SPT3 is a specificity factor required for transcription initiation at particular promoters.

Results

The spt15-21 mutation causes an amino acid change in a conserved region of TFIID

Mutations in SPT15 were selected as suppressors of his4-9176, a solo 6 insertion in the promoter of the HIS4 gene that causes a His+ phenotype [Winston et al. 1987]. Mutations in SPT15, such as spt15-21, change this phenotype from His+ to His+ by altering transcription initiation at this promoter insertion mutation [Eisenmann et al. 1989]. Strains that contain spt15-21 have other mutant phenotypes, including poor growth, defects in both mating and sporulation, and reduced levels of wild-type HIS4 transcripts, indicating that the spt15-21 mutation affects transcription at many loci in vivo [Eisenmann et al. 1989].

To determine the change in spt15-21 that causes these mutant phenotypes, the spt15-21 insertion locus was cloned from the genome by the process of gap repair [see Materials and methods]. The mutant gene was sequenced, and a single-base-pair change was found in the open reading frame. This change alters codon 174 from GGG to GAG, resulting in the substitution of glutamic acid for glycine [G174E, see Fig. 4B, below]. Glycine 174 is a residue in the second of the two conserved repeats of yeast TFIID [Pugh and Tjian 1992], and this glycine residue is conserved in all TFIID sequences determined to date.

The TFIID-G174E mutant protein behaves similarly to wild-type TFIID by several assays

To attempt to determine how the spt15-21 mutation alters TFIID function, the activity of the mutant TFIID protein encoded by spt15-21 [hereafter referred to as TFIID–G174E] was compared with that of wild-type TFIID by several in vitro and in vivo assays. Surprisingly, the mutant and wild-type proteins behaved similarly to each other in every assay.

First, the ability of the purified mutant protein to bind to DNA was tested by using a gel-shift assay. The result shows that the binding of wild-type and mutant TFIID proteins to the adenovirus major late promoter (AdMLP) TATA box region is comparable [Fig. 1A, lanes 2, 7]. In addition, both proteins form an identical slower mobility complex when partially purified HeLa TFIIA is added, indicating that they both interact similarly with this general factor [Fig. 1A, lanes 3–6, 8–11]. The same result was obtained by use of a δ-element TATA box and partially purified yeast TFIIA [data not shown]. DNase I protection experiments also showed that the two proteins bind to the TATA box in the AdMLP promoter, as well as to two different TATA boxes in the promoter of the insertion mutation his4-9126, with similar affinity [data not shown]. Therefore, TFIID–G174E is able to bind to DNA and to interact with TFIIA normally in vitro.

Second, the ability of TFIID–G174E to function in an in vitro transcription system was tested, by use of a TFIID-depleted nuclear extract from HeLa cells. The result [Fig. 1B] shows that the mutant protein supports basal transcription as well as wild-type TFIID, suggesting that TFIID–G174E is not impaired in its ability to interact with the other general transcription factors.

Finally, the ability of an acidic transcription activator to function in vivo was compared in yeast strains producing either wild-type TFIID or TFIID–G174E. This experiment was done by measuring the ability of the acidic activator protein GCN4 to stimulate transcription of the wild-type HIS4 gene after amino acid starvation [Hinnebusch 1988]. The results [Fig. 1C] show that although the
probe containing the AdMLP was incubated with the following proteins under the conditions described in Materials and methods: (Lane 1) No additions; (lanes G174E with the AdMLP TATA box and HeLa TFIIA. A DNA retie mobility-shift assay of the interaction of TFIID and TFIID-TFIID; (lanes Figure 1. Analysis of TFIID-G174E function. (A) Electrophoretic mobility-shift assay of the interaction of TFIID and TFIID-G174E. In vitro-translated TFIID and TFIID-G174E were added to transcription reactions containing TFIID-depleted HeLa cell nuclear extract. The G-minus product of the transcription template pML(C~AThgA-50 (Sawadogo and Roeder 1985a) is shown. In vitro-translated TFIID (lanes 2–4) or TFIID-G174E (lanes 5–7) was added as follows: (lanes 2,5) 0.65 ng; (lanes 3,6) 1.3 ng; (lanes 4,7) 2.6 ng. Lane 1 shows the products of a control transcription reaction containing 6.5 µl of an in vitro transcription translation reaction to which no RNA was added. (C) Northern hybridization analysis of HIS4 transcripts in SPT15 + and spt15-21 strains grown under repressing and derepressing conditions. Northern analysis was performed on 4 µg of total RNA prepared from SPT15 + and spt15-21 strains grown in rich media (YPD), supplemented synthetic media (SD), and supplemented synthetic media containing 10 mM 3-aminotriazole (SD + 3AT) as indicated.

absolute level of HIS4 transcripts is decreased in an spt15-21 mutant, the ratio of induced to basal levels of transcription is comparable to that observed in the wild-type strain. Thus, activation by GCN4 is not impaired in a strain producing TFIID-G174E. A second acidic activator, the bovine papillomavirus E2 protein, which can function in yeast, was also shown to be able to stimulate transcription normally in an spt15-21 strain [J. Benson and P. Howley, pers. comm.]. These results indicate that the spt15-21 mutation does not disrupt the ability of acidic activators to stimulate transcription.

Therefore, although the spt15-21 mutation is pleiotropic and results in alterations in transcription in vivo, there is no apparent effect of this mutation on the ability of TFIID to bind to DNA, to interact with other general transcription factors, or to allow stimulation of transcription by acidic activators. We have also observed no effect of the spt15-21 mutation on the level of TFIID in vivo (described in a later section).

Isolation of extragenic and intragenic suppressors of spt15-21

To study the defects caused by spt15-21 by a genetic approach, a selection for suppressors was carried out. The selection for spt15-21 suppressors relied on the phenotypes of an spt15-21 strain carrying the insertion mutations his4-9178 and lys2-173R2. An SPT15 + strain that contains these insertion mutations is His− and Lys+. [Winston et al. 1984b]. The spt15-21 mutation reverses these phenotypes such that an spt15-21 his4-9178 lys2-173R2 strain is His+ and Lys−. [Eisenmann et al. 1989]. To isolate suppressors of spt15-21, Lys+ revertants were selected and then screened for those that had also become His−. Forty-eight independent, spontaneous revertants of spt15-21 were isolated and were classified as either strong [His− Lys+] or weak [His−/Lys+] revertants. Of 48 revertants, 10 were dominant and linked to SPT15 and were assumed to be true revertants, the remaining 38 revertants were recessive.

Four weak revertants were linked tightly to spt15-21, as expected for an intragenic revertant. The mutant spt15-21 gene was cloned and sequenced from three of these strains [L809, L811, and L812]; all three mutant genes still contained the spt15-21 mutation and had also acquired a second mutation. All three intragenic revertants contained a TTT → GTT change at codon 237, resulting in the substitution of valine for phenylalanine (P237V). We assume that this second amino acid change, which does not cause an Spt− phenotype on its own [data not shown], must partially compensate for the effects of the G174E change on TFIID function.

Of the remaining 34 revertants, 23 were shown to be unlinked to spt15-21, demonstrating that they are extragenic. Analysis of progeny from these crosses revealed that 16 of these 23 extragenic suppressors caused a mutant Spt− phenotype [suppression of his4-9178] in an SPT15 + background. This phenotype suggested that some of these mutations were in genes functionally related to SPT15.

The extragenic suppressors of spt15-21 are mutations in SPT3

Among the large number of SPT genes identified, only mutations in SPT3, SPT7, SPT8, and SPT15 confer suppression of his4-9178 [Winston et al. 1987; Eisenmann et al. 1989]. We therefore tested whether the extragenic suppressors of spt15-21 might be alleles of SPT3, SPT7,
or SPT8. By a combination of complementation tests and linkage analysis, it was determined that, surprisingly, all 34 extragenic suppressors of spt15-21 were mutations in SPT3. We refer to these 34 spt3 mutations as spt3<sup>sup</sup> alleles. Of the 34 isolated, 18 were strong suppressors and 16 were weak suppressors, according to the strength of their suppression of spt15-21.

To determine whether the spt3<sup>sup</sup> mutations suppress spt15-21 by restoring a wild-type transcription pattern, several transcripts affected by spt15-21 were examined in both spt15-21 spt3<sup>sup</sup> double mutants and in spt3<sup>sup</sup> single mutants. In these experiments, four different spt3<sup>sup</sup> alleles were used: one strong allele, spt3-401, and three weak alleles, spt3-417, spt3-426, and spt3-445. For each case examined, the spt3<sup>sup</sup> mutations suppressed the transcriptional defects caused by spt15-21 (Fig. 2).

For his4-9178, previous work demonstrated that spt15-21 suppresses this insertion mutation by altering transcription start site selection [Eisenmann et al. 1989, diagramed in Fig. 2A]. In SPT15<sup>+</sup> SPT3<sup>+</sup> his4-9178 strains, the his4-9178 transcript produced is longer than the wild-type HIS4 mRNA [Fig. 2B, cf. lanes 1 and 2]. The presence of translational start and stop codons upstream of the HIS4 open reading frame presumably renders this long transcript nonfunctional, leading to the His<sup>−</sup> phenotype associated with his4-9178. In spt15-21 his4-9178 strains, initiation from the wild-type HIS4 transcription start site is restored, resulting in a His<sup>+</sup> phenotype [Fig. 2B, lane 4]. Analysis of spt15-21 spt3<sup>sup</sup> strains shows that the spt3<sup>sup</sup> mutations reverse the effect of spt15-21 on transcription of his4-9178, that is, the pattern of transcripts in spt15-21 spt3<sup>sup</sup> double mutant strains resembles that of an SPT15<sup>+</sup> SPT3<sup>+</sup> strain [Fig. 2B, cf. lane 2 with lanes 5–8].

Effects on transcription were also observed in the two other cases examined. First, in an spt15-21 strain, Ty transcripts are much less abundant and are shorter in length than in a wild-type strain [Fig. 2B, cf. lanes 2 and 4]. This same alteration has been observed in spt3, spt7, and spt8 strains; and in those cases, it has been shown to be the result of a change at the 5′ end, presumably caused by altered initiation [Winston et al. 1984b, 1987]. This defect is present in the context of a complete Ty element. UAS and TATA refer to promoter elements of the HIS4 gene. In an SPT15<sup>+</sup> strain, a transcript is produced that initiates from a site within the δ element. This transcript results in a His<sup>−</sup> phenotype. In an spt15-21 mutant, a transcript that initiates from the normal HIS4 start site is made [Eisenmann et al. 1989]. [B] Northern hybridization analysis of transcripts in spt15-21 and spt15-21 spt3<sup>sup</sup> strains. Total RNA was prepared from the following strains and analyzed by Northern hybridization: [Lane 1] 3618C [HIS4<sup>+</sup>]; [lane 2] FY167; [lane 3] FY293; [lane 4] FY255; [lane 5] L805; [lane 6] FY583; [lane 7] FY584; [lane 8] FY585; [lane 9] L806; [lane 10] FY586; [lane 11] FY587; [lane 12] FY588. All strains except 3618C contain his4-9178. Lane 1 contains 2 μg of total RNA; the remaining lanes contain 12 μg of total RNA. Therefore, the MFA1 and TUB2 messages are not detected as well in lane 1. The same filter was hybridized sequentially with 32P-labeled plasmid probes specific for transcripts from HIS4, Ty elements, MFA1, and TUB2.

**spt3<sup>sup</sup> mutations do not alter TFIID protein levels**

To determine whether spt15-21 or different spt3 mutations alter TFIID levels, several strains were examined by use of Western immunoblot analysis and anti-TFIID antisera. Neither spt15-21 nor an spt3 null mutation affects the level of TFIID [Fig. 3, cf. lanes 2, 3, and 4]. Likewise, the strong spt3<sup>sup</sup> allele spt3-401 shows no obvious effect on TFIID levels in either an SPT15<sup>+</sup> spt3-401 strain or in an spt15-21 spt3-401 strain [Fig. 3, lanes 5, 6]. Identical results were observed in strains containing the weak spt3<sup>sup</sup> mutations spt3-417, spt3-426, and spt3-445 [data not shown]. Therefore, the spt3<sup>sup</sup> mutations do not alter TFIID levels, strongly suggesting that they
suppress spt15-21 by compensating for some defective function of the TFIID–G174E protein.

The spt3<sup>sup</sup> mutations are clustered

To determine the sequence changes caused by the spt3<sup>sup</sup> mutations, we isolated and sequenced the entire mutant spt3 gene from each of seven independent spt3<sup>sup</sup> mutants: three strong suppressors, spt3-401, spt3-429, and spt3-441; and four weak suppressors, spt3-417, spt3-426, spt3-443, and spt3-445. The results of this analysis (Fig. 4A) show that six of the seven spt3<sup>sup</sup> mutations are clustered. First, all three strong spt3<sup>sup</sup> mutations, although independent, are the same GAG → AAG change at codon 240, resulting in a substitution of lysine for glutamic acid [E240K]. Second, three weak spt3<sup>sup</sup> mutations change the termination codon of the SPT3 gene. Two of these mutations, spt3-426 and spt3-443, are TAA → GAA substitutions, which add the amino acids EIFV to the end of the SPT3 protein. The third allele, spt3-417, also alters the stop codon (TAA → CAA), adding the residues QIFV to the end of the protein. Finally, the other weak spt3<sup>sup</sup> mutation, spt3-445, is a change in the amino-terminal region of the SPT3 protein, AAA → AAT, encoding the change K74N. This clustering of spt3<sup>sup</sup> mutations in the SPT3 open reading frame suggests that only certain changes in the SPT3 protein can compensate for the TFIID–G174E defect.

Suppression between spt15 and spt3 mutations is allele specific

The clustered sequence changes of the spt3<sup>sup</sup> alleles suggest that their ability to suppress may be allele specific. To test this idea, we constructed double mutant strains containing different combinations of mutations in SPT15 and SPT3. The results of this analysis (Table 1) demonstrate that suppression is allele specific. First, two other alleles of SPT3, spt3-1 and spt3Δ203::TRP1, are not able to suppress spt15-21. Second, spt3-401 and spt3-426, isolated as suppressors of spt15-21, are unable to suppress the Spt<sup>−</sup> phenotype associated with a different spt15 mutation, spt15-301. These double mutant strains grew more slowly and had more severe Spt<sup>−</sup> phenotypes than the parent strains. This behavior of different spt15 spt3 double mutant combinations shows that only certain mutations in these two genes can interact to restore wild-type function and is consistent with the idea that the TFIID and SPT3 proteins directly interact.

**Figure 4.** (A) spt3<sup>sup</sup> mutations are clustered. The box represents the SPT3 protein. The locations and predicted amino acid changes of seven, independent spt3<sup>sup</sup> mutations are shown. Those shown above the box are strong suppressors of spt15-21; those below are weaker suppressors. (B) Mutations in SPT15 that suppress spt3-401. Mutations in the SPT15 gene that can suppress the phenotypes of spt3-401 were isolated and sequenced as described in Materials and methods. Shown are the two repeats of TFIID, aligned with each other. Identical amino acids are indicated by bars; similar amino acids are indicated by colons. The locations and predicted amino acid changes caused by the 12 spt15 mutations are shown. The boxed change, G174E, is the same change that is caused by the spt15-21 mutation. Multiple isolates of the same change are indicated. The mutation found in the first TFIID repeat (top line) is a double mutation causing adjacent amino acid substitutions [V85I, A86T]. When examined in an SPT3<sup>−</sup> genetic background, only the mutations affecting residue 174 cause an Spt<sup>−</sup> phenotype [data not shown].
Table 1. Allele specificity of the spt15-21 spt3-401 interaction

| Strain     | Relevant genotype | Phenotype     |
|------------|-------------------|---------------|
| FY167      | spt15-21          | mutant        |
| FY255      | spt3-401          | mutant        |
| L805       | spt15-21 spt3-401 | mutant        |
| FY810      | spt15-21 spt3A203 | mutant        |
| L815       | spt15-21 spt3-1   | mutant        |
| L813       | spt15-301 spt3-401| mutant        |
| L814       | spt15-301 spt3-426| mutant        |

All strains carry the insertion mutation his4-9178. Phenotype refers to the growth of these strains on plates lacking histidine: [Wild type] No growth (Spt+ phenotype); [mutant] growth (Spt- phenotype).

New spt15 mutations that suppress spt3-401 are also highly clustered

To investigate the idea that only particular mutations in SPT3 and SPT15 can suppress one another, additional mutations in SPT15 that can suppress spt3-401 were isolated. This was done by mutagenizing the SPT15* gene and then identifying suppressors of spt3-401 by a plasmid shuffle scheme (see Materials and methods). From ~11,000 transformants, 12 plasmids were found that contained spt15 mutations able to suppress spt3-401. The mutant spt15 genes from these 12 plasmids were sequenced, and the mutations that they contained were found to be highly localized [Fig. 4B]. Of the 12 mutations, 10 cause several different amino acid substitutions at positions 171, 174, and 177 in TFIID, including the spt15-21-encoded change, G174E, which was reisolated twice. The eleventh mutation, a double substitution, causes adjacent amino acid changes in the equivalent region of the first repeat of TFIID (V85I, A86T). The twelfth mutation encodes the change K239E at the carboxyl terminus of TFIID; this change is only 2 residues away from the intragenic suppressor of spt15-21 described earlier.

The fact that the majority of mutations are within a stretch of only 7 codons centered around the site of spt15-21 strongly hints at some essential role for this region in an SPT3–TFIID interaction. Taken together with the allele specificity of the spt15 spt3 interaction and the clustering of the spt3sup mutations, these data strongly suggest that the TFIID and SPT3 proteins directly interact and that the defect in TFIID–G174E is in its functional or physical interaction with SPT3.

Coimmunoprecipitation of the TFIID and SPT3 proteins

To test directly whether the SPT3 protein physically interacts with TFIID, we performed coimmunoprecipitation experiments using extracts from yeast strains overexpressing both the TFIID and SPT3 proteins. Previous results showed that SPT3 could only be detected by Western analysis when produced from a high-copy-number plasmid, suggesting that it is not an abundant protein (Winston and Minehart 1986). To facilitate detection of SPT3, oligo-directed mutagenesis was used to create a gene encoding SPT3 with a 9-amino-acid epitope from the influenza virus hemagglutinin protein (HA1; Niman et al. 1983) at its amino terminus. This tagged protein, HA1–SPT3, is specifically detected by a monoclonal antibody to the HA1 epitope, and it migrates on SDS–polyacrylamide gels with a molecular mass of ~43 kD [Fig. 5A]. This modified SPT3 gene is fully functional in vivo based on complementation tests (data not shown).

To determine whether HA1–SPT3 was associated with TFIID, coimmunoprecipitation experiments were done as described in Materials and methods. These experiments showed that the HA1–SPT3 protein reproducibly coimmunoprecipitated with wild-type TFIID, indicating that these two proteins are physically associated [Fig. 5B, lane 1]. [A low level of TFIID was also precipitated in the absence of anti-TFIID antitsera (Fig. 5B, lane 2), probably as a result of a nonspecific interaction of TFIID with the negatively charged resin coupled to our secondary antibody [see Materials and methods]. However, consistent with the result found in the presence of the primary antibody, this nonspecific precipitation of TFIID also led to a low level of HA1–SPT3 being coprecipitated [Fig. 5B, lane 2].]

Two control experiments were performed to test whether the coimmunoprecipitation of HA1–SPT3 with TFIID indicates a specific interaction between these two proteins. First, we examined whether the amount of HA1–SPT3 that coimmunoprecipitated was dependent on the amount of TFIID present in the yeast extracts. Extracts were prepared from strains overexpressing HA1–SPT3 and either overexpressing or not overexpressing TFIID. Both strains contained the same amount of HA1–SPT3 [Fig. 5C, cf. lanes 1 and 6]. From the extract with a high level of TFIID, we saw coimmunoprecipitation of HA1–SPT3 [Fig. 5C, lane 2]. However, from the extract with a low level of TFIID, only a very small amount of HA1–SPT3 coimmunoprecipitated, consistent with the decrease in TFIID levels [Fig. 5C, lane 4]. This result shows that the presence of HA1–SPT3 in the immune complex is dependent on TFIID and is not the result of a nonspecific interaction. Second, HA1–β-galactosidase was not coimmunoprecipitated with TFIID, demonstrating that the result seen with HA1–SPT3 is specific for SPT3 and is not the result of an interaction caused by the presence of the HA1 epitope [Fig. 5D, lane 1]. These coimmunoprecipitation experiments, then, support our genetic data and strongly suggest that the wild-type SPT3 and TFIID proteins physically interact in the cell.

Our genetic data are consistent with the idea that the spt15-21 mutation alters the interaction between the TFIID and SPT3 proteins. Conceivably, the spt15-21 mutation could disrupt the physical association of these proteins; alternatively, it could result in a mutant TFIID protein that still interacts physically with SPT3 but has lost a functional interaction between these proteins that
is necessary for wild-type activity. To distinguish between these possibilities, we tested whether the TFII-D–G174E mutant protein is also able to interact physically with SPT3. Experiments performed as before showed that HA1–SPT3 also coimmunoprecipitated with TFII-D–G174E (Fig. 5E, lane 3), indicating that these two proteins do interact. However, we found a variability in the amount of HA1–SPT3 that was coimmunoprecipitated; in some experiments, less HA1–SPT3 was coimmunoprecipitated with TFII-D from native yeast extracts. This result may indicate that the interaction between SPT3 and TFII-D–G174E is less stable than that of the wild-type proteins under these conditions. Nevertheless, coimmunoprecipitation indicates that physical association between these two proteins is not completely abolished by the spt15-21 mutation and is consistent with a model in which the primary defect caused by that mutation is a disruption of a functional interaction between SPT3 and TFII-D necessary for wild-type activity.

Discussion

Mutations in SPT15 and SPT3 were originally identified as suppressors of the transcriptional defects caused by Ty and solo 6 insertion mutations in the 5′ region of the yeast HIS4 and LYS2 genes (Winston et al. 1984a, 1987). One mutation in SPT15, spt15-21, causes a number of mutant phenotypes and leads to alterations in transcription in vivo (Eisenmann et al. 1989). The sequence of the spt15-21 mutation predicts a single-amino-acid substitution [G174E] in a highly conserved region of TFII-D. Surprisingly, the mutant product of spt15-21, TFII-D–G174E, shows no obvious defect in protein levels, in DNA binding, or in its ability to interact with other general factors in vitro.

To attempt to determine the defect of TFII-D–G174E that causes its multiple in vivo phenotypes, intragenic and extragenic suppressors of spt15-21 were isolated and analyzed. All 34 independently isolated, extragenic suppressors of spt15-21 are mutations in SPT3. The stronger of these spt3sup alleles suppress all of the phenotypes associated with spt15-21, and in every case examined, suppression occurs by a reversal of the spt15-21 transcriptional defects. Furthermore, the suppression between spt15 and spt3 mutations is allele specific, and these mutations are clustered in each gene. Previous work has shown that allele-specific interactions are strongly indicative of protein–protein interactions (for examples, see Jarvik and Botstein 1975; Adams et al. 1989). These genetic data therefore strongly suggest that the SPT3 protein and TFII-D interact directly. In support of this hypothesis, an epitope-tagged SPT3 protein coimmunoprecipitates with TFII-D from native yeast extracts. Taken together, these results are consistent with the idea that the SPT3 protein is physically associated with TFII-D in vivo and that the primary defect in TFII-D–G174E is its inability to interact functionally with wild-

Figure 5. Coimmunoprecipitation analysis of TFII-D and SPT3. (A) Identification of the HA1–SPT3 protein. Extracts from strain FY294 containing plasmids encoding HA1–SPT3 (lane 1) or SPT3 (lane 2) were analyzed by Western blotting. The positions of molecular mass markers are shown. (B–E) Coimmunoprecipitation experiments. Extracts were prepared from strain FY294 transformed with high-copy-number plasmids encoding the proteins as listed below. TFII-D was immunoprecipitated as described in Materials and methods (+ anti-TFII-D Ab), and a duplicate sample was processed to which no anti-TFII-D antibody was added (− Ab). Each sample was then divided and separated on two 12% polyacrylamide gels. Proteins were transferred to nitrocellulose and incubated with antibodies to detect the HA1-tagged proteins of each protein in 20 μg of total extract (total). (B) FY294 containing high-copy-number plasmids that encode HA1–SPT3 and TFIID. (Lane 1) + anti-TFII-D Ab; (lane 2) − Ab; (lane 3) total. (C) FY294 containing high-copy-number plasmids that encode HA1–SPT3 and TFIID. (Lane 1) + anti-TFII-D Ab; (lane 2) − Ab; (lane 3) total. (D) FY294 containing high-copy-number plasmids that encode HA1–SPT3 and TFII-D. (Lane 1) + anti-TFII-D Ab; (lane 2) − Ab; (lane 3) − Ab. FY294 containing only a high-copy-number plasmid encoding HA1–SPT3; (lane 4) + anti-TFII-D Ab; (lane 5) − Ab; (lane 6) total. These two extracts were done side by side and run on the same gels. To maximize detection of HA1–SPT3, this filter was washed less stringently. (D) FY294 containing high-copy-number plasmids that encode HA1–β-galactosidase and TFII-D. (Lane 1) + anti-TFII-D Ab; (lane 2) + anti-HA1 Ab; (lane 3) total. (E) Extracts from FY294 containing two different pairs of plasmids were used. (Lanes 1,2,5) FY294 containing high-copy-number plasmids that encode HA1–SPT3 and TFII-D; (lanes 3,4,6) FY294 containing high-copy-number plasmids that encode HA1–SPT3 and TFII-D–G174E. (Lanes 1,3) + anti-TFII-D Ab, (lanes 2,4) − Ab, (lanes 5,6) total. We estimate that 10–15% of total TFII-D is immunoprecipitated in these experiments and that 5–10% of the HA1–SPT3 coimmunoprecipitates with TFII-D.
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type SPT3. This model is consistent with previous work that has shown that the phenotypes of an spt3 null mutant are virtually identical to those of an spt15-21 mutant [Winston et al. 1984b, Hirschhorn and Winston 1988; Eisenmann et al. 1989].

Previous results, along with the results presented in this paper, suggest that SPT3 is required for normal transcription initiation for a subset of the yeast genes that require TFIID. This idea is consistent with the fact that unlike TFIID, SPT3 is not essential for growth. Experiments to date have not suggested an obvious biochemical function for SPT3. The predicted sequence of the 337-amino-acid SPT3 protein has no significant similarity to other protein sequences or to characterized functional motifs [Winston and Minehart 1986]. Although SPT3 is nuclear localized (C.S. Hoffman and F. Winston, unpubl.), in vitro-synthesized SPT3 has no detectable DNA-binding activity [J. Hirschhorn and F. Winston, unpubl.]. Finally, SPT3 has little ability to activate transcription in vivo when fused to the DNA-binding domain of GAL4 [D.M. Eisenmann and F. Winston, unpubl.]. Therefore, it seems unlikely that SPT3 functions as a transcriptional activator protein.

One possible function for SPT3 is as a transcriptional adaptor or coactivator at certain genes, being required for the productive interaction of upstream activator proteins with the preinitiation complex [for review, see Pugh and Tjian 1992]. The promoters that are affected in spt3 mutants are not known to share a common transcription activator, suggesting that if SPT3 functions in this role, it must be capable of interacting with several different activator proteins. Because two acidic activator functions normally in an spt15-21 mutant, SPT3 is unlikely to be an adaptor for that class of transcriptional activator.

We favor a general model in which SPT3 is required for TFIID to function at particular promoters, perhaps by being required for the binding of TFIID to certain TATA boxes or for the interaction of TFIID with other members of the preinitiation complex. One component of this model is that in vivo, all TATA boxes are not equivalent and that other factors besides TFIID, such as SPT3, may be important for promoter recognition. A number of previous observations have also suggested this idea. For example, several studies have shown that different TATA boxes do not function similarly in the context of a given promoter, suggesting that the primary sequence of the TATA box and/or flanking sequences is important [Harbury and Struhl 1989; Simon et al. 1990, Taylor and Kingston 1990; Wefald et al. 1990; Li and Sherman 1991].

Results from our laboratory have shown a difference between the use of TATA boxes in vivo and their binding by TFIID in vitro. In the case of the insertion mutation his4-9128, there are two TATA elements, ~200 bp apart. Previous studies have shown that in SPT+ strains, only the upstream TATA box directs transcription initiation, but that in spt3 null and spt15-21 mutants, the downstream TATA directs transcription initiation [Hirschman et al. 1988, Eisenmann et al. 1989]. However, in vitro TFIID binds to both TATA boxes with similar affinity [Arndt et al. 1992]. Taken together, these results indicate that TFIID alone may be unable to discriminate in vitro between several TATA sequences that are functionally distinct in vivo. We propose that SPT3 may be one of a number of factors that modulates promoter specificity in vivo by interacting with TFIID.

Recently, both the human and Drosophila TATA-binding proteins have been found to be tightly associated with a number of other proteins, termed TAFs [Dynlacht et al. 1991; Pugh and Tjian 1991; Tanese et al. 1991]. Our work suggests that SPT3 may be a yeast counterpart of one of these TAFs. Given the conservation of the components of the RNA polymerase II transcription machinery found to date, it seems likely that yeast TFIID, like its Drosophila and human homologs, is also associated with several other proteins in vivo, in addition to SPT3. Given the differences observed between the biochemical properties of TFIID from S. cerevisiae and from higher eukaryotes, if yeast TFIID is part of a complex, then the biochemical identification of other proteins in this complex may prove to be more difficult than for their Drosophila and human functional counterparts. However, the ability to combine genetic and biochemical approaches in yeast should facilitate the identification and study of such factors. In the case of SPT3, further characterization of its interaction with wild-type and mutant TFIID and with other transcription factors, both in vivo and in vitro, will allow us to begin to test specific models of SPT3 function.

Materials and methods

Strains, genetic methods, and media

The S. cerevisiae strains used (Table 2) are derived from S288C (MATa gal2) and were constructed in this laboratory by standard methods (Rose et al. 1990), except for strains 3618C [from Gerald Fink, M.I.T., Boston, MA] and 4202-15-3 [from Duane Jenness, University of Massachusetts Medical Ctr., Boston, MA]. The spt15 alleles spt15Δ101:LEU2 and spt15-301 are described in Arndt et al. [1992]. The leaky allele spt3-1 and the null alleles spt3-101, spt3Δ202, and spt3Δ203:TRP1 have been described [Winston et al. 1984a, Winston and Minehart 1986; Happel 1989]. Escherichia coli strains HB101 and TB1 were used as plasmid hosts [Ausubel et al. 1988]. Mating, sporulation, and tetrad analysis were done by standard methods (Rose et al. 1990). Yeast strains were transformed by the lithium acetate procedure [Ito et al. 1983]. Rich (YPD), minimal [SD], synthetic complete (SC), and sporulation media were prepared as described in Rose et al. [1990]. Cells that had lost URA3-containing plasmids were isolated on SC media containing 5'-fluoro-orotic acid [5'-FOA] [Boeck et al. 1987].

Plasmids

Plasmids were constructed, maintained, and isolated from E. coli by standard methods [Ausubel et al. 1988]. DNA modification and restriction enzymes were purchased from New England Biolabs [Beverly, MA] and Boehringer Mannheim Biochemicals [Indianapolis, IN]. Plasmid pDE38-9 consists of a 6.6-kb ClaI–ScaI restriction fragment containing SPT15 [Eisenmann et al. 1989] in vector pRS316 [Sikorski and Hieter 1989] and was used to clone spt15-21 by gap repair, resulting in plasmid p1259-1. A 2.4-kb EcoRI–BamHI piece from p1259-1 was subcloned into plasmid pRS424 [Sikorski and Hieter 1989].
Table 2. Yeast strains

| Strain    | Description                                                                 |
|-----------|-----------------------------------------------------------------------------|
| FW1259    | MATa spt15-21 his4-9178 lys2-173R2 trp1Δ1 ura3-2                            |
| FY3       | MATa ura3-2                                                                |
| FY167     | MATa his4-9178 lys2-173R2 trp1Δ1 leu2Δ1 ura3-2                            |
| FY255     | MATa spt15-21 his4-9178 lys2-173R2 trp1Δ1 leu2Δ1 ura3-2                  |
| FY293     | MATa spt3Δ202 his4-9178 lys2-173R2 ura3-52                                |
| FY294     | MATa spt3Δ202 his4-9178 lys2-173R2 trp1Δ63 ura3-52                       |
| FY567     | MATa spt15-21 his4-9178 lys2-173R2 leu2Δ1 ura3-52 ade8                   |
| FY583     | MATa spt15-21 spt3-417 his4-9178 lys2-173R2 leu2Δ1                       |
| FY584     | MATa spt15-21 spt3-426 his4-9178 lys2-173R2 leu2Δ1 ura3-52               |
| FY585     | MATa spt15-21 spt3-445 his4-9178 lys2-173R2 trp1Δ63 ura3-52              |
| FY586     | MATa spt15-21 spt3-417 his4-9178 lys2-173R2 trp1Δ1 ura3-52               |
| FY587     | MATa spt3-426 his4-9178 lys2-173R2 trp1Δ1 ura3-52                        |
| FY588     | MATa spt3-445 his4-9178 lys2-173R2 trp1Δ63 ura3-52                       |
| FY647     | MATa spt15-21 spt3-417 his4-9178 lys2-173R2 leu2Δ1 ura3-52 ade8         |
| FY648     | MATa spt15-21 spt3-426 his4-9178 lys2-173R2 leu2Δ1 ura3-52 ade8          |
| FY649     | MATa spt15-21 spt3-429 his4-9178 lys2-173R2 trp1Δ1 leu2Δ1 ura3-52        |
| FY650     | MATa spt15-21 spt3-441 his4-9178 lys2-173R2 trp1Δ1 leu2Δ1 ura3-52        |
| FY651     | MATa spt15-21 spt3-443 his4-9178 lys2-173R2 trp1Δ1 leu2Δ1 ura3-52        |
| FY652     | MATa spt15-21 spt3-445 his4-9178 lys2-173R2 trp1Δ1 leu2Δ1 ura3-52        |
| L662      | MATa spt15Δ101::LEU2 spt3-401 his4-9178 lys2-173R2 trp1Δ1 leu2Δ1 ura3-52 |
| L706      | MATa spt3-101 his4-9178 asp5                                             |
| L707      | MATa spt3-101 his4-9178 asp5                                             |
| L708      | MATa spt15-21 his4-9178 asp5                                             |
| L709      | MATa spt15-21 his4-9178 asp5                                             |
| L805      | MATa spt15-21 spt3-401 his4-9178 lys2-173R2 trp1Δ1 ura3-52               |
| L806      | MATa spt3-401 his4-9178 lys2-173R2 ura3-52                                |
| L809      | MATa spt15-21 spt3-401 his4-9178 lys2-173R2 trp1Δ1 ura3-52               |
| L810      | MATa spt15-21 spt3Δ203::TRP1 his4-9178 lys2-173R2 trp1Δ1 leu2Δ1 ura3-52 |
| L811      | MATa spt15-21 spt3Δ203::TRP1 his4-9178 lys2-173R2 trp1Δ1 leu2Δ1 ura3-52 |
| L812      | MATa spt15-21 spt3Δ203::TRP1 his4-9178 lys2-173R2 trp1Δ1 leu2Δ1 ura3-52 |
| L813      | MATa spt15-301 spt3-401 his4-9178 lys2-173R2 leu2Δ1                       |
| L814      | MATa spt15-301 spt3-426 his4-9178 lys2-173R2 ura3-52 leu2Δ1               |
| L815      | MATa spt15-21 spt3-1 his4-9178 lys2-173R2 trp1Δ1                         |
| L861      | MATa spt3-401 his4-9178 lys2-173R2 leu2Δ1 ura3-52                       |
| 3618C     | MATa his3-532                                                             |
| 4202-15-3 | MATa bar1-1 ade2-1 his4-580 lys2 trp1 tyr SUP4-3(ts)                    |

Isolation of suppressors of spt3-21

To select for suppressors of spt3-21, 50 cultures each of strains FY255 and FY567 were inoculated with different single colonies and grown to saturation in 2 ml of YPD. These cultures were washed twice in H2O, diluted in H2O, and 5 × 10^6 cells (0.2 ml) of each culture were plated on SC-Lys plates. After several days, all plates contained ~100 small Lys+ colonies, and most plates had 1–10 larger Lys+ colonies. The larger colonies were regrown on a YPD plate and then replica plated to check their His and Lys phenotypes. Twenty-six independent His+ Lys+ revertants were isolated from FY255, and 20 were isolated from FY567. Two additional revertants, spt3-401 and the intragenic spt15-21R7, were isolated separately as spontaneous His+ Lys+ revertants of strain FW1259 after plating on SC-Lys plates. These 48 revertants were categorized as strong [His+ Lys+] or weak [His+/- Lys+] revertants. All revertants were tested for dominance or recessiveness by mating to spt15-21 lawns and for the presence of spt3 mutations by complementation tests using spt3 lawns. Ten dominant revertants and 27 of 38 recessive revertants were crossed to wild-type (SPT+) strains to test for linkage of the new mutation to the SPT15 locus. The progeny were scored with respect to His and Lys phenotypes, and all progeny were tested for complementation of spt15 and spt3 strains. Two cloned and sequenced spt3^ts alleles, spt3-401 and spt3-445, were reintegrated into the genome at the SPT3 locus and were shown to confer suppression of spt15-21.

Isolation of suppressors of spt3-401

Mutations in the SPT15 gene that could suppress spt3-401 were isolated using the plasmid shuffle technique [Boeke et al. 1987]. Plasmid pDE75-2 was mutagenized in vitro with hydroxylamine [Rose et al. 1990]. Strain L662 (which contains plasmid pDE38-9) was transformed with the mutagenized pDE75-2 DNA, and Trp+ Ura+ transformants were selected. Approximately 11,000 colonies were replica plated to 5'-FOA plates and screened for any that had become His+ (indicating suppression of spt3-401); 24 such strains were found. Plasmids from these strains were rescued into E. coli [Hoffman and Winston 1987].
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Retransformation into L662 showed that 12 of the plasmids carried mutations in SPT15 that suppressed spt3-401. These 12 alleles and the corresponding amino acid changes are spt15-201[R171C], spt15-202[G174W], spt15-203[G174E], spt15-204[R171C], spt15-205[V85L, A86T], spt15-206[K239E], spt15-207[R171C], spt15-208[R171H], spt15-209[R171H], spt15-211[F177L], spt15-212[G174E], and spt15-213[G174R].

Gap repair and DNA sequencing

To clone the spt15-21 gene by gap repair [Orr-Weaver et al. 1983], plasmid pDE38-9 was digested with EcoRI, deleting a 2.4-kb fragment within the yeast sequences. Strain FW1259 was transformed with this gapped plasmid DNA, and Ura + colonies were selected. Plasmids were recovered by transformation of E. coli. This method was also used to rescue spt15-21 intragenic revertants from strains L809, L811, and L812. To clone spt3 alleles, plasmid pDE105-6 was digested with MluI and XhoI, deleting a 1.7-kb fragment covering the SPT3 gene. This gapped DNA was transformed into the following strains: L805 [spt3-401], FY647 [spt3-417], FY648 [spt3-426], FY649 [spt3-429], FY650 [spt3-441], FY651 [spt3-443], FY652 [spt3-445], and FY167 [SPT3]. Plasmid DNA was recovered from these strains into E. coli. The complete open reading frame was sequenced on both strands of DNA for all alleles isolated except for spt15-204, spt15-207, and spt15-212. For these genes, ~200 bp surrounding the base change (on both strands) was sequenced. Both SPT15+ and SPT3+ were also cloned by gap repair and sequenced alongside the mutant genes.

Production of TFII D proteins

Induction of TFII D synthesis in E. coli was done as described [Arndt et al. 1992], except that cells were grown to an OD600 of 1.0. Cells were harvested and washed in two cell-pellet-volumes of buffer A [50 mM Tris-Cl at pH 8.0, 100 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, and 10% [vol/vol] glycerol]. Cells were lysed by incubation [15 min at 4°C] in one cell-pellet-volume of buffer A containing 0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride [PMSF], followed by sonication. Extracts were loaded on DEAE-cellulose [Whatman DE52] columns equilibrated with buffer A containing 1 mM EDTA and 1 mM PMSF. The pass-through and wash fractions that contained TFII D activity were pooled and dialyzed against buffer B [50 mM TrisHC1 at pH 7.5, 250 mM KAc, 2 mM MgAc, 2 mM PMSF], transferred to a microcentrifuge tube, and spun down, and the pellets were incubated in a dry ice/ethanol bath for 10 min. Cells were resuspended in 0.25 ml of lysis buffer and broken open by vortexing with glass beads four times for 20 sec, with 1 min on ice between each vortexing. The bottom of each tube was placed with a hot 19-gauge needle, and the lysate was spun through this hole into another tube by spinning at 2000 rpm for 2 min. The glass beads were then washed with another 0.25 ml of lysis buffer. The combined extract was spun for 10 min at 14,000 rpm, and the supernatant was transferred to a new tube and spun again. Protein concentrations of the supernatant were determined using a Bio-Rad Protein Assay Kit.

For communoprecipitations, 500 µg of extract was diluted into immunoprecipitation buffer [IPB] 50 mM Tris-HCl at pH 7.5, 250 mM KAc, 0.1% [vol/vol] Triton X-100, 0.5 mg/ml of BS A] to a volume of 0.5 ml and incubated at 30°C for 30 min. One micro-liter of anti-yeast TFII D rabbit polyclonal antiserum [gift of Steve Buratowski, M.L.T., Boston, MA] was added, and reactions were incubated for 2 hr on a rocking platform at room temperature. Following this incubation, 50 µl of secondary antibody was added. The secondary antibody was goat anti-rabbit IgG antibody coupled to agarose beads [Sigma], washed twice in IPB. After a 60-min incubation with rocking at room temperature, immune complexes were collected by spin ning at 14,000 rpm for 30 sec, washed four times in IPB plus 1 µl urea, and suspended in 25 µl of protein sample buffer (60 mM Tris-HCl at pH 6.8, 2% SDS, 8% glycerol, 2% b-mercaptoethanol, 0.4% bromophenol blue). Samples were boiled for 3 min, spun in a microcentrifuge at 14,000 rpm for 1 min, and loaded onto two 12%
acrylamide gels. Typically, one-quarter of the total immunoprecipitate was loaded on one gel to measure TFII D levels, and the remainder on a second gel to measure HA1–SPT3 levels. We found the interaction between TFII D and SPT3 to vary with temperature and KAc concentration and to be sensitive to the presence of chloride ions [D. Eisenmann and F. Winston, un- publ.]. A low level of TFII D was precipitated in experiments done without the addition of primary antisera and is likely the result of the known affinity of TFII D for negatively charged resins [see references in Pugh and Tjian 1992] such as the agarose beads coupled to the secondary antibody, because it was seen with uncoupled beads alone and with TFII D purified from E. coli.

Western blotting was performed as described in Swanson and Winston (1992). For the detection of TFII D, the anti-TFII D antiserum was used at a 1 : 1000 dilution. For detection of HA1–SPT3 and HA1–β-galactosidase, ascites fluid containing the anti-HA1 epitope mouse monoclonal antibody 12CA5 [Niman et al. 1983] was used at a dilution of 1 : 666. Anti-IgG secondary antibodies coupled to alkaline phosphatase [Promega] were used at a 1 : 7500 dilution.

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