Analysis of the yeast *SPT3* gene and identification of its product, a positive regulator of Ty transcription

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ABSTRACT

Previous work has demonstrated that the yeast *SPT3* gene is required for transcription from δ sequences, the long terminal repeats that flank yeast Ty elements. In *spt3* null mutants, transcription fails to initiate in δ sequences and instead initiates farther downstream. Null mutations in *SPT3* cause other mutant phenotypes, including defects in sporulation and diploid formation. In this paper we report further genetic and physical characterization of the *SPT3* gene and protein. By extensive linker insertion mutagenesis, we have delimited the region necessary for *SPT3* function. From DNA sequence analysis, *SPT3* encodes a protein of 337 amino acids. We have identified this protein with an anti-*SPT3* antibody. Finally, we show that overproduction of the *SPT3* gene product does not alter the level of Ty transcription.

INTRODUCTION

Ty elements of the yeast, *Saccharomyces cerevisiae*, are transposable genetic elements, present in 30-35 copies per haploid genome. They are 5.9 kilobases long and consist of 330 bp terminal repeats, δ sequences, flanking the internal ϵ sequence (1). There are also approximately 100 solo δ sequences in the yeast genome.

Ty elements are structurally and functionally similar to a group of eukaryotic transposable elements that includes copia-like elements of *Drosophila* and retroviral proviruses of mammals. They contain two open reading frames analogous to the gag and pol regions of retroviruses (2-4), transpose via an RNA intermediate (5) and appear to encode a reverse transcriptase (6,7). Ty mRNA constitutes 5-10% of the total polyA⁺ mRNA in a haploid yeast cell. It is 5.6 kb in length, extends from δ to δ , and is terminally redundant for 45 bases, similar to retroviral genomic RNA (8).

Insertion mutations caused by Ty elements, copia-like elements and mammalian proviruses can inhibit or otherwise alter adjacent gene expression (see 9-11, for reviews). Trans-acting suppressors of insertion mutations have been identified in yeast (9,13,14), *Drosophila* (15-18) and mice (19). In yeast, Ty and solo δ insertions that inhibit adjacent gene expression are suppressed by mutations in several unlinked genes (*SPT* genes (14; J. Fassler

Fig. 1. Transcriptional effects in *spt3* mutants. For both (a) Ty elements and (b) solo δ insertions a similar effect occurs: in *SPT3* wild type strains, transcription initiates in the δ sequence; in *spt3* mutants, transcription initiates farther downstream.

and F.W., unpublished)). For several different *spt* mutations, experiments have demonstrated that suppression of insertion mutations occurs at the transcriptional level (20,21; C. Clark-Adams, J. Fassler, F.W., unpublished).

Mutations in the *SPT3* gene suppress insertion mutations apparently as a result of a specific alteration of transcription of Ty elements. In *spt3* mutants, there is no full length normal δ - δ Ty mRNA; rather, a less abundant Ty transcript 800 bp shorter at the 5' end is made (21). For solo δ insertions in the 5' noncoding region of *HIS4* a similar result has been seen: transcription initiates from the δ in wild type *SPT3* strains and transcription initiates farther downstream in *spt3* mutants (Fig. 1). These results led to the model that *SPT3* is a factor required for transcription initiation in δ sequences (21). More recent evidence demonstrates that this requirement for *SPT3* results in abolishment of Ty transposition in *spt3* mutants (22).

In addition to suppression of insertion mutations, *spt3* mutants have several other phenotypes. These include defects in diploid formation and sporulation (21). The mutant phenotypes suggest that *SPT3* is important for

Table 1. Yeast strains

Name	Genotype				
FW665	MATa	his4-917 δ	lys2-173R2	ura3-52	spt3-101
L37	MATa	his4-917 δ	lys2-173R2	trp1 Δ 1	
FW948	MAT α	his4-917 δ	lys2-173R2	spt3-202	ura3-52 cry1
FW1149	MATa	his4-917 δ	lys2-173R2	ura3-52	spt3-202/pFW64
FW1150	MATa	his4-917 δ	lys2-173R2	ura3-52/pFW32	
FW1151	MATa	his4-917 δ	lys2-173R2	ura3-52/pFW64	

other transcription in addition to normal transcription initiation in δ sequences.

To begin to understand its function, we have further characterized SPT3. In this paper we present analysis of linker insertion mutagenesis of SPT3, the DNA sequence of SPT3, identification of the SPT3 mRNA and gene product, and analysis of the effects of overproduction of SPT3 on the level of Ty transcription.

MATERIALS AND METHODS

Yeast strains and general genetic methods. All yeast strains are listed in Table 1 and were constructed for these studies. Strains that contain plasmids are indicated by a slash, followed by the name of the plasmid. Standard yeast genetic procedures of crossing, sporulation, and tetrad analysis were followed (23,24). All media were made as described previously (24). Transformations were done by the lithium acetate procedure (25). The mutation spt3-202 is an internal deletion of SPT3, removing 698 base pairs from the coding region between the BamHI linker insertions spt3-302 and spt3-382 (see below). This deletion was constructed in vitro and recombined into the genome, replacing the wild type SPT3 allele.

Enzymes. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase and E. coli DNA polymerase I were purchased from New England Biolabs. S1 nuclease, M. luteus DNA polymerase and nucleotide triphosphates were purchased from P-L Biochemicals. E. coli DNA polymerase I Klenow fragment was purchased from Amersham. All enzymes were used as recommended by the vendors.

Plasmids and M13 vectors. Plasmids containing spt3 BamHI linker insertions were derived from plasmid pFW32 (Fig. 2a). This plasmid contains an EcoRI-BglII restriction fragment carrying the SPT3 gene in the E. coli-yeast shuttle vector, pCGS42. Plasmid pFW64 is the same as pFW32 except that it contains the spt3 mutation spt3-202. The M13 vectors mp10, mp11, mp18, and mp19 (26,27) were used for DNA sequencing. For production of an SPT3- β -galactosidase hybrid protein, plasmid pFW59 was used. This plasmid (Fig. 2b)

Fig. 2. Plasmid restriction maps. Thin lines, pBR322 sequences; hatched segments, SPT3 sequences; open segments, 2 μ circle sequences in pFW32 and lacZ in pFW59; solid black segment, URA3 sequences. R, EcoRI; M, MluI; X, XhoI; S, SalI; P, PstI; B, BamHI. In pFW59, 'SPT3' indicates that DNA encoding the first 51 amino acids of SPT3 is missing, resulting in a protein fusion between lacZ and SPT3.

was constructed by subcloning a BamHI-PstI fragment from the pFW32 derivative containing the BamHI linker mutation spt3-302 into plasmid pUR292 (28). This construction creates an in-frame fusion between lacZ and SPT3 that encodes 286 amino acids of SPT3 fused onto the carboxy-terminus of β -galactosidase. Plasmids used as probes were as follows: for SPT3, pFW42, carrying the SPT3 EcoRI-XhoI fragment in pBR322 (29); for Ty, B161, carrying a Ty BglII restriction fragment in pBR322 (R. Surosky, B.-K. Tye, and G.R. Fink, unpublished) and for PYK (pyruvate kinase), pFR2, a 6.7 kb HindIII fragment in pBR322 (provided by Dr. P. Sinha).

DNA preparations. *E. coli* plasmid mini-preps were done by the boiling method of Holmes and Quigly (30). Large scale *E. coli* plasmid preps were prepared and purified on ethidium bromide-cesium chloride gradients (31). *E. coli* strain HB101 (32) was the host for all plasmids unless otherwise noted.

Linker insertion mutagenesis of SPT3. Linker mutagenesis of plasmid pFW32 was performed in several steps. Supercoiled pFW32 was nicked with DNAase I in the presence of 0.15 mg/ml ethidium bromide. The nicks were extended into short single stranded gaps with *M. luteus* DNA polymerase (33). The gapped molecules were linearized by treatment with S1 nuclease and the ends were made blunt by treatment with T4 DNA polymerase in the presence of dATP, dCTP, dGTP and dTTP at 100 μ M. Phosphorylated BamHI linkers (purchased from New England Biolabs) were ligated onto the linear molecules. The linears were purified on a 1% agarose gel to separate the plasmid molecules from the excess unligated linker molecules. The linears were digested with BamHI to generate BamHI sticky ends and were then ligated at a concentration of 2 μ g/ml overnight, concentrated and transformed into *E. coli* strain DB6507, a pyrF74::Tn5 derivative of HB101 (34). Transformants were selected for ampicillin resistance and screened for a Ura⁺ phenotype to eliminate any plasmids that contained linker insertions in the URA3 gene.

BamHI linkers in the EcoRI-XhoI SPT3 restriction fragment were detected by triple digests of mini-prep DNA with EcoRI, XhoI and BamHI. Those plasmids that contained a BamHI site in the SPT3 fragment were saved for further study. By this method, insertion of the linker is generally accompanied by a small deletion of approximately 20-25 base pairs.

Preparation of M13 DNA. M13 single stranded phage DNA for sequencing reactions was prepared as described in the Amersham booklet, "M13 Cloning and Sequencing Handbook." A 1.5 ml lysate usually yielded around 10 μ g of single stranded DNA. To prepare M13 RF DNA, *E. coli* strain JM101 was grown to OD₆₀₀ = 0.1. Five mls of culture were infected with a single plaque and grown at 37° for 4-5 hours. Cells were pelleted in the table top centrifuge and DNA was isolated by the boiling mini-prep method (30).

DNA sequence determination. DNA was sequenced by the dideoxy method of Sanger (35) using reagents and instructions provided in the Amersham sequencing kit. ³²P-dATP was purchased from Amersham. The subclones used in sequence reactions were constructed in mp10 and mp11. Into mp10 we subcloned XhoI-BamHI fragments from pFW32 derivatives containing the following spt3 BamHI linker insertion mutations: spt3-302, spt3-349, spt3-379 and spt3-382. We also cloned the SPT3 XhoI-EcoRI restriction fragment into mp10 for sequencing. For sequencing the other strand we subcloned EcoRI-BamHI restriction fragments into mp11 from pFW32 derivatives containing the following spt3 linker insertion mutations: spt3-302, spt3-332, spt3-343, spt3-348, spt3-374 and spt3-379. Using these subclones, we sequenced 1673 basepairs on both strands.

Genetic mapping of an *spt3* ochre mutation. The region of *SPT3* containing the ochre mutation *spt3-271* was determined by marker rescue transformation experiments using linearized gapped plasmids missing different portions of *SPT3*. Gaps were generated in derivatives of pFW32 containing different *Bam*HI linker mutations. Plasmids were digested with *Bam*HI and either *Xho*I or *Mlu*I. Both sets of double digests generate linear fragments whose ends are within *SPT3* DNA. Transformation of these linear fragments into an *spt3-271* mutant and selection for Ura⁺ results in repair of the plasmids using the chromosomal copy as template (36). If the gap covers *spt3-271*, we expect most Ura⁺ transformants to have an Spt⁻ phenotype; if the gap does not cover *spt3-271*, most transformants will have a wild type phenotype.

Preparation of antiserum. Antiserum against *SPT3* was made following the general strategy of Shuman *et al.* (37): construction of a fusion between the *E. coli lacZ* gene and the gene of interest followed by generation of antibody against the hybrid protein. The *lacZ-SPT3* hybrid protein is encoded on plasmid pFW59 (Fig. 2b). Expression of the fusion is under *lac* control. To obtain sufficient quantities of the hybrid protein for immunization of rabbits, *E. coli* cultures containing pFW59 were induced with IPTG when at a concentration of 5×10^7 cells/ml and grown to 2×10^8 cells/ml. Cells were concentrated 40-fold in sample buffer (38) and frozen in 400 μ l aliquots at -70°C until used. To prepare the hybrid protein for each injection, eight 400 μ l samples were run on two 3 mm thick SDS-polyacrylamide gels (38). The gels were stained with Coomassie Brilliant Blue, destained and the hybrid protein was excised. It was homogenized with incomplete Freund's adjuvant and injected subcutaneously into adult New Zealand white rabbits. Each injection contained approximately 500 μ g of fusion protein. The rabbits were given initial injections on the first day, a booster after two weeks and a second booster after three weeks. Rabbits were bled one week after the final injection and the sera were screened for anti- β -galactosidase activity. Preimmune sera, taken one week before the initial injection, were used as a control. Serum from one of two rabbits showed anti- β -galactosidase activity and this was then tested for anti-*SPT3* activity.

Immunoblotting. Reactivity of the antibody to yeast proteins was determined by Western blotting (39). Total protein from yeast was isolated by TCA extraction (40), subjected to electrophoresis on 1.5 mm thick 10% polyacrylamide gels and transferred onto nitrocellulose using a Biorad Trans-blot Cell. The Vectastain ABC immunoperoxidase system was used to detect specific binding of primary antisera to yeast proteins using procedures provided by the manufacturer (Vector Labs, Burlingame, California).

Northern hybridization analysis. Northern hybridization analysis was done as previously described (21) with the modification that after blotting, the Genescreen filters were exposed to short wave ultraviolet light for 2 minutes at 1200 $\mu\text{W}/\text{cm}^2$ (41), instead of baking in a vacuum oven for two hours. Autoradiograms were analyzed by scanning densitometry. The intensity of the bands on the autoradiograms corresponding to the Ty transcripts were normalized to the bands corresponding to the yeast pyruvate kinase gene (42), whose expression is unaffected by *spt3* mutations.

RESULTS

Analysis of linker mutations in *spt3*. To define the physical boundaries of *SPT3*, we constructed *Bam*HI linker insertion mutations in a restriction fragment containing the *SPT3* gene. The positions of the linker insertion mutations within the *SPT3* *Eco*RI-*Xho*I fragment were determined by measuring the fragment sizes produced in *Eco*RI-*Bam*HI and *Xho*I-*Bam*HI double digests.

We determined the effect of each linker insertion on *SPT3* activity by transforming each plasmid containing an insertion into yeast strain FW665 (*MATa spt3-101 his4-917 δ lys2-173R2*). This strain has a *His*⁺ *Lys*⁻ phenotype because of the effect of the *spt3* mutation on the insertions *his4-917 δ* and *lys2-173R2* (14,21). Since *spt3* mutations are recessive, if a transforming plasmid contains a functional *SPT3* gene, the *Ura*⁺ transformants will be *His*⁻ *Lys*⁺; if the linker insertion resulted in a nonfunctional *SPT3* gene,

Fig. 3. Linker insertion mutations in *SPT3*. The arrows show the physical positions of the *Bam*HI linker insertion mutations in the *Xho*I-*Eco*RI restriction fragment that contains *SPT3*. Arrows above the line indicate linker insertions that reduce or abolish *SPT3* function (see text for details). Linker insertion mutations in parenthesis confer a leaky *spt3* phenotype. Arrows below the line indicate linker insertions that have no apparent effect on *SPT3* function. The linker insertion *spt3-355* contains a deletion whose boundaries are indicated by the black bar.

1	TATCTCAGCCCCCTGGGACTCTGATGCCGTTACACCAACGCTGCTGTGATTGGAGTGAAATATCTGGATACTAATAGA	80
81	AATATTACGATAAAAAAGAGTTATCTACAGTTTGTCTGATTATTTTTCGGTTATCGGTCAATCAACTTTAAGAGATCACC	160
161	GTACATTTCCGGCAGCATTAGCGGGCGTCTCTTAGTTTCTTTGCAATACAGTAGGAACCTCCAATTTTTTGTTCGTCTCCT	240
241	CGCTACATTACAGATTCACCTGCAATCTCGAAAAAAGTTTTTATGCCGCCAAAAATAGGTATACATTGCGTTCAATGCAT	320
321	TCTCATTCATCTCTATCGCAAGAAGCTCTTGTTCAAAGCTGCCCTTTCTTGAGGCTTCAAGCGCAGCGCACAGAAAAGAA	400
401	GAAAAATCCGAACCATGTAATATAATCGGCGGAAACGAAAAAGTAAAAAGTAAGGTTGAAGACACTCTGAACCCGGGGGAAAG	480
481	AGTGATACAAAGGTTACCGTGCCAAAATTCAAGAGATTAGGGCAGGA	526
527	ATG ATG GAC AAG CAT AAG TAT CGT GTG GAG ATT CAA CAG ATG ATG TTT GTC TCT GGT GAA Met Met Asp Lys His Lys Tyr Arg Val Glu Ile Gln Gln Met Met Phe Val Ser Gly Glu	586
587	ATT AAC GAC CCA CCC GTA GAA ACC ACA TCA CTG ATA GAA GAT ATA GTG AGG GGT CAA GTG Ile Asn Asp Pro Pro Val Glu Thr Thr Ser Leu Ile Glu Asp Ile Val Arg Gly Gln Val	646
647	ATA GAA ATT CTT TTA CAG TCA AAC AAA ACG GCG CAT CTT AGG GGA AGT AGG AGC ATT CTC Ile Glu Ile Leu Leu Gln Ser Asn Lys Thr Ala His Leu Arg Gly Ser Arg Ser Ile Leu	706
707	CCT GAA GAC GTC ATT TTC TTG ATC AGA CAC GAC AAG GCC AAA GTC AAT CGT TTG AGA ACA Pro Glu Asp Val Ile Phe Leu Ile Arg His Asp Lys Ala Lys Val Asn Arg Leu Arg Thr	766
767	TAT CTG TCA TGG AAG GAT TTG CGT AAA AAC GCC AAG GAC CAA GAT GCT AGT GCC GGT GTA Tyr Leu Ser Trp Lys Asp Leu Arg Lys Asn Ala Lys Asp Gln Asp Ala Ser Ala Gly Val	826
827	GCG AGT GGC ACT GGA AAT CCT GGG GCA GGT GGT GAA GAT GAT TTG AAA AAA GCA GGT GGT Ala Ser Gly Thr Gly Asn Pro Gly Ala Gly Gly Glu Asp Asp Met Lys Lys Ala Gly Gly	886
887	GGC GAG AAA GAC GAA AAA GAT GGT GGA AAC ATG ATG AAG GTC AAG AAA TCC CAA ATT AAG Gly Glu Lys Asp Glu Lys Asp Gly Gly Asn Met Met Lys Val Lys Lys Ser Gln Ile Lys	946
947	CTG CCA TGG GAA TTG CAG TTT ATG TTC AAT GAA CAT CCT TTA GAA AAT AAT GAC GAC AAT Leu Pro Trp Glu Leu Gln Phe Met Phe Asn Glu His Pro Leu Glu Lys Asn Asn Asp Asp Asn	1006
1007	GAT GAT ATG GAT GAG GAT GAA CGA GAA GCT AAT ATA GTC ACT TTG AAA AGG CTG AAA ATG Asp Asp Met Asp Glu Asp Glu Arg Glu Ala Asn Ile Val Thr Leu Lys Arg Leu Lys Met	1066
1067	GCT GAC GAT AGA ACA CGA AAC ATG ACT AAA GAG GAG TAC GTG CAT TGG TCC GAT TGT CGA Ala Asp Asp Arg Thr Arg Asn Met Thr Lys Glu Glu Tyr Val His Trp Ser Asp Cys Arg	1126
1127	CAG GCA AGT TTT ACA TTT AGG AAG AAT AAA AGG TTC AAG GAC TGG TCT GGA ATT TCG CAA Gln Ala Ser Phe Thr Phe Arg Lys Asn Lys Arg Phe Lys Asp Trp Ser Gly Ile Ser Gln	1186
1187	TTA ACT GAG GGG AAA CCC CAT GAT GAT GTG ATT GAT ATA CTG GGG TTT CTA ACT TTT GAG Leu Thr Glu Gly Lys Pro His Asp Asp Val Ile Asp Ile Leu Gly Phe Leu Thr Phe Glu	1246
1247	ATT GTC TGT TCT TTG ACG GAA ACA GCT CTG AAA ATC AAA CAA AGA GAA CAG GTA TTA CAG Ile Val Cys Ser Leu Thr Glu Thr Ala Leu Lys Ile Lys Gln Arg Glu Gln Val Leu Gln	1306
1307	ACT CAA AAG GAC AAA TCC CAG CAA TCT AGC CAA GAT AAT ACT AAC TTT GAA TTT GCA TCA Thr Gln Lys Asp Lys Ser Gln Gln Ser Ser Gln Asp Asn Thr Asn Phe Glu Phe Ala Ser	1366
1367	TCC ACA TTA CAT AGA AAG AAA AGA TTA TTT GAT GGA CCT GAA AAT GTT ATA AAC CCG CTC Ser Thr Leu His Arg Lys Lys Arg Leu Phe Asp Gly Pro Glu Asn Val Ile Asn Pro Leu	1426
1427	AAA CCA AGG CAT ATA GAG GAA GCC TGG AGA GTA CTA CAA ACA ATT GAC ATG AGG CAT AGG Lys Pro Arg His Ile Glu Glu Ala Trp Arg Val Leu Gln Thr Ile Asp Met Arg His Arg	1486
1487	GCT TTG ACC AAC TTT AAA GGT GGT AGA CTC AGT TCT AAA CCA ATT ATC ATG TAA ATTTTT Ala Leu Thr Asn Phe Lys Gly Gly Arg Leu Ser Ser Lys Pro Ile Ile Met End	1546
1547	GTATAATTTTCATCGAGGTGTCAGGGTTTCCTTCTGGTATGATAAATGTGAGCCAAATGAATTGAAAAATACAAATAA	1626
1627	AGATTATGTACGTAGATACGGTTATAAAGGAGCTATCTGATCATT	1673

the transformants will remain His⁺ Lys⁻. Although we expected that most linker insertion mutations in SPT3 would result in a null phenotype (21), leaky mutations are also possible if the correct reading frame of SPT3 is maintained and the insertion is not in a region essential for function. We expected that a leaky spt3 mutation might be His⁺ Lys⁺ based on the phenotype of the leaky spt3 allele, spt3-1 (14; A. Happel and F.W., unpublished).

The linker insertion mutations allow us to determine the functional regions of the SPT3 gene. The results of this analysis (Fig. 3) show the map positions and phenotypes of the 22 linker mutations studied. A cluster of mutations, spanning approximately 1 kb, cause an spt3 mutant phenotype and are within the SPT3 gene based on DNA sequence analysis (described in the following section). Surprisingly, one mutation in the middle of the gene, spt3-332 is phenotypically wild type and two others, spt3-330 and spt3-349 are both leaky. At the 3' end of the SPT3 coding region, the insertion spt3-382 has a wild type phenotype. The spt3-348 insertion, also at the 3' end of the gene, has a leaky phenotype. Insertion of a 3 kb DNA fragment that contains the *E. coli* lacZ gene into spt3-382 creates a complete mutant phenotype; this suggests that the portion of SPT3 3' to spt3-382 is necessary for function.

One linker insertion mutation, spt3-355, produced a large deletion of approximately 300 base pairs in the SPT3 5' noncoding region (Fig. 3). This mutation abolishes SPT3 function, suggesting that a region over 300 base pairs from the coding region is necessary for SPT3 expression.

DNA sequence of SPT3. We sequenced 1673 base pairs on both strands of the SPT3 gene, beginning 75 base pairs from the XhoI site (Fig. 3). Analysis of the sequence revealed one large open reading frame of 1011 base pairs that coincided with the large cluster of linker insertions that cause an spt3 mutant phenotype (Fig. 4). The sequence does not definitively indicate where translation initiates because the beginning of the open reading frame encodes two tandem AUG triplets. The first falls within the context of GNNAUGA, an infrequently found sequence context for initiator AUG triplets in eukaryotic mRNAs (43). The second is within the greatly preferred context of ANNAUGG. A presumed TATA region occurs at base pairs 417-423 (Fig. 4).

To verify the identity of this open reading frame as SPT3, we mapped

Fig. 4. DNA sequence and predicted amino acid sequence of the SPT3 gene. The DNA sequence is numbered from nucleotides 1 to 1637. The SPT3 sequence begins at nucleotide 527 and is translated in the standard three letter amino acid code. The spt3-271 mutational change is shown above the wild type base at position 641. Linker insertion mutations that do not abolish SPT3 function are also shown.

Fig. 5. Northern hybridization analysis of SPT3 mRNA. PolyA⁺ RNA was prepared from strains, subjected to electrophoresis on a 1% formaldehyde-agarose gel, blotted to Genescreen and probed with ³²P-labeled SPT3 DNA. Lane 1, SPT3⁺ (strain L37); lane 2, spt3-202 (strain FW948).

and sequenced an spt3 ochre mutation, spt3-271. The ochre mutation was isolated after growth of an SPT3-containing plasmid in an E. coli mutD strain (C.D. Clark-Adams and F.W., unpublished results). Strains containing

spt3-271 and the ochre suppressor SUP4-o have an Spt⁺ phenotype. Using marker rescue experiments with gapped linear plasmids (as described in Materials and Methods) we localized spt3-271 to the 5' end of the gene. A BclI-KpnI fragment of 230 bp from the 5' end of spt3-271 was sequenced. The ochre mutation was determined to be a GC→AT transition, changing codon 39 encoded by the large open reading frame from CAA (glutamine) to UAA. This confirms that this open reading frame encodes SPT3.

By computer analysis we searched the SPT3 gene for several consensus sequences that have been demonstrated or suggested to be involved in various types of regulation of yeast genes. We found no homology to the consensus sequences for general amino acid control (44-46), MATα2 regulation (47), MATα1/MATα2 regulation (48,49), or cell cycle control at "start" of the cell cycle (50). We also compared the amino acid sequence predicted for SPT3 with all of the amino acid sequences in the National Biomedical Research Foundation (NBRF) Protein Sequence Database and found no significant homologies. The codon usage in SPT3 is not strongly biased towards preferred codons in yeast, a characteristic of genes expressed at low levels (51).

Identification of the SPT3 mRNA. The SPT3 mRNA has been identified by Northern hybridization analysis of wild type and spt3-202 mutant strains (Fig. 5). Hybridization of total polyA⁺ RNA from a wild type SPT3 strain with ³²P-labeled pFW42 (containing the SPT3 EcoRI-XhoI fragment) reveals a prominent band of 1.3 kb and a faint band of about 1.6 kb. When polyA⁺ RNA from an spt3-202 mutant, which carries a large internal deletion in SPT3, is analyzed, the 1.3 kb band is not present. Instead, we see a shorter band whose length is consistent with the size of the deletion. The 1.6 kb band is unaltered by spt3-202, demonstrating that this transcript is unrelated to SPT3. These results identify the 1.3 kb band as the SPT3 mRNA. This same band is the only one that hybridizes to a probe internal to the SPT3 gene (not shown). The estimated 1.3 kb size of the SPT3 mRNA correlates well with the size of the coding region.

Identification of the SPT3 gene product. Using antiserum from a rabbit immunized with a β-galactosidase-SPT3 fusion protein, we have identified the SPT3 gene product in protein extracts from yeast cells. The results of Western immunoblotting analysis identifies a protein that migrates at a molecular weight of 40,000 and is easily detectable in strains containing the SPT3 high copy number plasmid pFW32 (Fig. 6, lane 3). This protein is not detectable in strains that contain a high copy number plasmid with the spt3 deletion mutation, spt3-202, regardless of the genomic SPT3 allele

Fig. 6. Immunoblot analysis of the SPT3 gene product. Total protein extracts were prepared, subjected to electrophoresis on a 10% SDS-polyacrylamide gel, and electroblotted to nitrocellulose. Binding of the primary antiserum raised against a lacZ-SPT3 hybrid protein was visualized using the Vectastain ABC immunoperoxidase system. Lane 1, preimmune serum was used against an extract from strain FW1150 (high copy number SPT3). Lanes 2-4, immune serum was used against extracts from FW1151 (single copy number SPT3), lane 2; FW1150 (high copy number SPT3), lane 3; and FW1149 (spt3-AZ02), lane 4. Numbers on the left side indicate the position of size markers.

(Fig. 6, lanes 2 and 4). This protein is also not recognized by the preimmune sera (Fig. 6, lane 1). We conclude that this protein is SPT3. The estimated molecular weight of the SPT3 protein correlates well with the predicted molecular weight of 38,748. These results show that the high copy number of the SPT3 gene results in an increased level of the SPT3 gene product and suggests that SPT3 is not under autogenous negative control or regulated by a limiting positive regulatory element.

Overproduction of SPT3 does not alter the level of Ty transcription.
If the level of SPT3 protein in the cell determines the frequency or effi-

Fig. 7. Ty transcription in SPT3 overproducing strains. Total RNA was prepared, subjected to electrophoresis on a 1% formaldehyde-agarose gel, blotted to Genescreen and probed with ^{32}P -labeled Ty DNA. Lane 1, strain FW1151 (single copy number SPT3); lane 2, strain 1150 (high copy number SPT3); lane 3, strain 1149 (spt3- Δ 202).

ciency of transcription initiation in δ sequences, then overproduction of SPT3 could result in elevated levels of Ty transcription. We have previously demonstrated that in an spt3 null mutant, transcription initiation in δ sequences occurs at a greatly reduced level (21). To test the effect of overproduction of the SPT3 gene product on Ty transcription, levels of Ty mRNA were compared in strains with 0, 1 or approximately 5-10 copies of the SPT3 gene, to determine the level of Ty transcription. From the Western analysis done on these same strains (Fig. 6), we know that the SPT3 product is overproduced at least 10 fold in a strain with a high copy number plasmid carrying SPT3. The Northern hybridization analysis (Fig. 7) demonstrates that overproduction of SPT3 protein does not cause an increase in the level of Ty transcription. Densitometric scanning of the film shows that there is no significant difference (less than 1.4 fold) in the level of Ty mRNA between the strains that contain a single copy and a high copy of SPT3 (Fig. 7, lanes 1 and 2). This suggests that other factor(s) may be limiting for the level of Ty transcription. SPT3 overproducing strains also have no mutant phenotype with respect to suppression of insertion mutations.

DISCUSSION

Mutations in the *S. cerevisiae* SPT3 gene can suppress Ty and δ insertions mutations in the 5' non-coding regions of genes. Suppression of insertion mutations correlates with a specific transcriptional defect in spt3 mutants: failure to initiate transcription in the δ sequences of Ty elements. In this paper we have further characterized SPT3 genetically and biochemically to begin to understand its function.

The DNA sequence of SPT3 shows that it contains an open reading frame of 1011 base pairs, that would encode a protein of 337 amino acids. The sequence of an spt3 ochre mutation confirms this reading frame as SPT3. The length of the SPT3 mRNA, approximately 1.3 kb, correlates well with the size of the gene.

One model for the function of the SPT3 protein is that it mediates transcription initiation in δ sequences by binding to a region near or at the TATA element in δ sequences. By computer analysis we have found no significant homology at the amino acid level between the SPT3 coding region and other sequenced genes, including those that contain the helix-turn-helix structure common to many DNA-binding proteins (52-54). Therefore, if SPT3 does bind to δ sequences, it probably does so by a different type of protein-DNA interaction than is used by these proteins.

Analysis of 22 linker insertion mutations at the SPT3 locus has yielded information on the coding and non-coding regions important for expression and activity of SPT3. We have identified several linker insertion mutations within the coding region that do not completely abolish SPT3 activity. Presumably these linker insertion mutations have created small in-frame deletions that allow a nearly full length protein to be made. The three linker insertion mutations in the middle of the gene that do not destroy activity may define a region of the protein that is tolerant to alteration.

We have identified the SPT3 gene product using antiserum raised against a β -galactosidase-SPT3 fusion protein. The molecular weight of SPT3 as measured on SDS-polyacrylamide gels is around 40,000 kd; the molecular weight of SPT3 as predicted by the DNA sequence is 38,748. The antibody has allowed us to measure the level of the SPT3 gene product. By Western analysis we have shown that a high gene dosage of SPT3 results in a greatly elevated level of the gene product.

The demonstration of overproduction of the SPT3 protein allowed us to ask if Ty transcripts are present at a greater level. In yeast, overproduction of a positive activator may result in overproduction of the gene product whose

expression it regulates. This result has been demonstrated for both GAL4 and GCN4 (55,56). However, SPT3 overproduction has no significant effect on the level of Ty transcription suggesting that SPT3 is not the only limiting factor for determination of the level of Ty transcription.

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