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# SPT13 (GAL11) of *Saccharomyces cerevisiae* Negatively Regulates Activity of the MCM1 Transcription Factor in Ty1 Elements

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**The Ty transposable elements of *Saccharomyces cerevisiae* consist of a single large transcription unit whose expression is controlled by a combination of upstream and downstream regulatory sequences. Errede (B. Errede, Mol. Cell. Biol. 13:57–62, 1993) has shown that among the downstream control sequences is a binding site for the transcription factor, MCM1. A small restriction fragment containing the Ty1 MCM1-binding site exhibits very weak activation of heterologous gene expression. The absence of SPT13 (GAL11) causes a dramatic increase in activity directed by these sequences. This effect is mediated through the MCM1-binding site itself. MCM1 mRNA and protein levels, as well as its affinity for its binding site, are unchanged in the absence of SPT13. Our results suggest that SPT13 has a role in the negative control of MCM1 activity that is likely to be posttranslational. A role for SPT13 in the negative regulation of the activity of the Ty1 MCM1-binding site is consistent with our previous proposal that *spt13*-mediated suppression of Ty insertion mutations could be attributed to the loss of negative regulation of genes adjacent to Ty elements.**

The Ty (transposable) elements of *Saccharomyces cerevisiae* are a family of retrovirus-like transposons consisting of a unique 5.3-kb (ε) region flanked by 330-bp direct repeats (δ). Transcription of these elements initiates in one δ repeat and terminates in the other (9). In addition to the expected upstream regulatory elements located within δ sequences (9, 29), extensive molecular analysis has revealed additional regulatory elements located inside the transcription unit (13, 37). Several downstream regulatory elements in Ty1 elements that function independently as weak activators of a heterologous reporter gene have been identified and characterized (5–7). One element is located just downstream of the Ty promoter and consists of a STE12-binding site (5). Two other regulatory elements, block I and block II, are located several hundred nucleotides downstream of the Ty transcriptional start site and contain homology to three different regulatory motifs including the simian virus 40 (SV40) enhancer core, the a1-α2 diploid control signal, and the MCM1 recognition sequence or P site (7, 11, 13, 14). The role of these sequences in promoting transcription of Ty elements is unknown. A 28-bp sequence containing block II is weakly capable of activating adjacent-gene transcription (7). Like expression of the Ty element itself, activation directed by these sequences is cell type specific and is not observed in diploid cells (7).

Previous analysis of block II and adjacent sequences revealed the presence of two protein-binding sites (7). The binding site of one complex falls within block II; the second site maps immediately downstream of block II sequences (7). Errede has identified the block II-binding protein as the constitutive factor, MCM1 (11).

We have been analyzing the function of the Ty regulatory elements by the isolation of a series of mutants altered in the expression of Ty-adjacent genes. Naturally occurring insertions of Ty elements into the promoter of a gene may inhibit transcription of that gene (8, 36, 39). This type of insertion mutation can be suppressed by mutations in unlinked *SPT* (suppressor of Ty insertion mutations) genes encoding factors normally involved in the control of Ty expression (16, 43). *spt* mutants that restore transcription to the adjacent gene appear in some cases to allow the use of Ty sequences in place of the displaced natural promoter.

The *SPT13* gene was originally identified as a suppressor of the Ty insertion mutation, *his4-917* (16, 36). Despite the extensive sequence divergence between Ty1 and Ty2 elements (26, 36), *spt13* mutations suppress both types of Ty element insertions. Restored gene transcription in *spt13* mutants is presumably directed by Ty sequences. *spt13* mutations are distinct from other *spt* mutations, which suppress Ty insertion mutations because they fail to suppress solo δ insertions (16). This suggests that *SPT13* might interact with sequences internal to the Ty transcriptional unit rather than with promoter sequences in the δ element. Transcriptional analysis showing that the restoration of adjacent-gene transcription in *spt13* mutants is accompanied by a small increase (not a decrease) in Ty transcription (17) supports this idea.

On the basis of these observations, we previously proposed that SPT13 negatively regulates one or more components of the internal Ty enhancer (17). However, the negative regulatory role of SPT13 in Ty elements is in contrast to its positive role in expression of a large number of other genes (17, 33, 40, 41). For example, the reduced expression of galactose metabolic genes in *spt13* mutants led to the independent identification of the *SPT13* gene as *GAL11* (33, 40).

We show here that the site of action of SPT13 is the Ty1 block II MCM1-binding site. MCM1 activity is negatively controlled by SPT13, and this repression appears to require no other DNA-binding or MCM1-associated proteins. We further found that negative regulation by SPT13 is posttranslational.

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TABLE 1. Yeast strains used in this work

| Strain | Relevant genotype | Source[a] |
|---|---|---|
| JF15 | *MATα ura3-52 leu2-1 his4-917 lys2-128δ* | |
| JF28 | *MATa trp1Δ1 leu2-3,112 his4-917 lys2-128δ* | |
| JF819 | *MATa ura3-52 lys2-128δ his4-917 leu2* | |
| JF903 | *MATα ura3-52 lys2-128δ his4-917 trp1Δ1* | |
| JF1049 | *MATa spt13-201 leu2 ura3-52 his4-917 lys2-128δ trp1Δ1* | |
| JF1050 | *MATα spt13-201 leu2 ura3-52 his4-917 lys2-128δ trp1Δ1* | |
| JF1073 | *MATa ura3-52 lys2-128δ his4-917 leu2 spt13-202* | |
| JF1088 | *MATa ura3-52 lys2-128δ his4-917 trp1Δ1 leu2-3,112 mcm1-1* | JF903 × SY1433 |
| mcm1-ΔDE | *MATα ura3-52 leu2-3,112 his4Δ34 mcm1-ΔDE* | C. Christ and B.-K. Tye |
| SY1433 | *MATa mcm1-1 leu2-3,112 his4Δ537* | G. Sprague |
| Dx817 | *MATa/α his4-917/his4-917 ura3-52/ura3-52 leu2/leu2 trp1Δ1/+ +/lys2-128δ* | |
| Dx813 | *MATa/α spt13-202/spt13-202 his3-11,15/+ +/his4-917 ura3-2/ura3-2 +/lys2-128δ leu2-3,112/leu2 ade2-1/+ trp1-1/+ can1-100/+* | |

[a] When no source is given, the strain is from our strain collection or was constructed for this study.

## MATERIALS AND METHODS

**Yeast strains.** All yeast strains used in this work (Table 1) are from our strain collection or were constructed for this study unless otherwise indicated. The *spt13-202* mutation consists of a deletion from *Pvu*II at −512 (where +1 refers to the A of the ATG translational start codon in the published sequence) to *Eco*RV at +2241 and an insertion of the *LEU2* gene at this location (40).

**Media.** The media were prepared as described by Sherman et al. (38) and included minimal medium (SD) with amino acids added as needed, synthetic complete medium (SC) lacking one or more specific amino acids, and rich medium (YPD). Plates for the detection of β-galactosidase activity contained 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml and were prepared as described by Larson et al. (27). All yeast strains were grown at 30°C.

**Genetic methods.** Yeast crosses were performed as described by Mortimer and Hawthorne (32). Yeast transformations were performed by the lithium acetate method of Ito et al. (23). The *SPT13* disruption alleles were introduced into the appropriate strains by transplacement (44) and confirmed by Southern hybridization analysis.

**β-Galactosidase assays.** Yeast protein extracts were prepared by glass bead lysis from cultures harvested at $2 \times 10^7$ cells per ml precisely as described by Company et al. (5). β-Galactosidase activities were calculated in Miller units (m.u.) (31) but are also expressed in Tables 2 and 3 as relative units, which were calculated after normalization to the activity of an *ACT1-lacZ* fusion gene. Relative units were calculated as follows: 1,000 × (m.u. of the pLG670Z derivative measured in a particular genetic background/m.u. of pGY63 measured in JF819).

**Plasmids.** Plasmid pLG670Z, a derivative of pLG669Z (20), was used in all experiments as the reporter for measuring the extent to which restriction fragments from the Ty1 element could activate transcription. These vectors carry a fusion of the *CYC1* promoter and the *Escherichia coli lacZ* gene. pLG670Z lacks a functional upstream activation sequence and directs a low level of β-galactosidase activity in a wild-type strain. A unique *Xho*I site is located 250 bp upstream of the translational start site of *CYC1* (20). One or multiple copies of specific Ty sequences were cloned into the *Xho*I site and tested for activity (Tables 2 and 3). Ty1 fragments were isolated from plasmids (described below) or

TABLE 2. Relative β-galactosidase activity of Ty1 enhancer region restriction fragments[a]

| Insert[b] | β-Galactosidase activity for[c]: | | | | | | DR[d] (*spt13*/+) |
|---|---|---|---|---|---|---|---|
| | *SPT13*+ | | | *spt13-202* | | | |
| | No.[e] | m.u. (σ) | r.u. | No. | m.u. (σ) | r.u. | |
| *ACT1-lacZ* | 9 | 3,597.0 (528.0) | 1,000.00 | 9 | 1,852.0 (577) | | |
| None | 9 | 5.0 (1.4) | 1.36 | 7 | 5.4 (2.4) | 1.39 | 1.0 |
| 1×H | 4 | 6.3 (2.5) | 1.7 | 2 | 39.8 (9.4) | 9.5 | 5.4 |
| 1×H reverse | 2 | 11.4 (1.8) | 2.7 | 2 | 34.9 (4.6) | 8.3 | 3.1 |
| 2×H | 2 | 11.2 (1.9) | 3.8 | 2 | 61.9 (4.7) | 21.5 | 5.5 |
| 3×H | 2 | 22.1 (1.2) | 7.7 | 2 | 132.1 (14.3) | 45.9 | 6.0 |

[a] β-Galactosidase activity for each construct is presented as Miller units (m.u.) and as relative units (r.u.), in which the activity of each construct was normalized to expression from plasmid pGY63 carrying an *ACT1-lacZ* fusion gene. Two or three independent transformants were tested for each construct. Variation between individual transformants in a given experiment was less than 30%. However, because of considerable experiment-to-experiment variability (see the standard deviations [σ]), all values in a given experiment were normalized to an *SPT*+ transformant carrying an *ACT1-lacZ* positive control plasmid. The relative values presented are the average of the ratios of *CYC1-lacZ*/*ACT1-lacZ* activities. Although *spt13 ACT1-lacZ* transformants were also measured in every experiment, all *CYC1-lacZ* values were normalized to the value of the *ACT1-lacZ* plasmid in the *SPT*+ strain. This point is further considered in the Discussion.

[b] Fragments were inserted into the upstream activation sequence position in the *CYC1-lacZ* fusion vector pLG670Z. The number and orientation of the inserted sequences were determined by DNA sequence analysis. 1×H, 2×H, and 3×H constructs contain inserts oriented opposite the *CYC1-lacZ* fusion transcript. This preserves the relationship of these Ty element sequences to the adjacent gene. 1×H reverse contains an insert in the same orientation as the *CYC1-lacZ* fusion transcript.

[c] *SPT13*+, strain JF819; *spt13-202*, strain JF1073.

[d] DR, derepression ratio. DR was obtained by the equation [r.u. (*spt13*)]/[r.u. (*SPT13*+)].

[e] The number of trials is the product of the number of transformants tested and the number of individual assays per transformant.

TABLE 3. Relative β-galactosidase activity of Ty1 enhancer region sequence elements[a]

| Insert[b] | β-Galactosidase activity for[c]: | | | | | | DR (*spt13*/+)[d] |
|---|---|---|---|---|---|---|---|
| | *SPT13*+ | | | *spt13-202* | | | |
| | No.[e] | m.u. (σ) | r.u. | No. | m.u. (σ) | r.u. | |
| *ACT1-lacZ* | 9 | 3,597.0 (528.0) | 1,000.00 | 9 | 1,852.0 (577) | | |
| None | 9 | 5.0 (1.4) | 1.36 | 7 | 5.4 (2.4) | 1.39 | 1.0 |
| P(Ty1) | 3 | 18.0 (1.3) | 4.85 | 3 | 53.9 (29.3) | 14.3 | 2.9 |
| P(pal) | 3 | 94.5 (35.1) | 25.1 | 3 | 464.6 (88.0) | 126.9 | 5.1 |
| Extended P(Ty1) | 7 | 8.8 (2.2) | 3.3 | 7 | 24.8 (13.0) | 10.4 | 2.8 |
| Extended P(pal) | 3 | 71.0 (2.1) | 19.2 | 3 | 189.5 (35.0) | 50.7 | 2.6 |
| Extended P*(Ty1) | 3 | 2.5 (0.8) | 0.58 | 3 | 2.1 (0.1) | 0.55 | 0.8 |

[a] β-Galactosidase values for each construct are presented as outlined in footnote *a* of Table 2.
[b] A single copy of each synthetic double-stranded oligonucleotide (see Fig. 2 for sequences) was inserted into the upstream activation sequence position in the *CYC1-lacZ* fusion vector pLG670Z. The number and orientation of the inserted sequences was determined by DNA sequence analysis. Each fragment was inserted in an orientation opposite that of the *CYC1-lacZ* fusion transcript. This preserves the relationship of these Ty element sequences to the adjacent gene.
[c] *SPT13*+, strain JF819; *spt13-202*, strain JF1073.
[d] DR, derepression ratio (see Table 2, footnote *d*).
[e] The number of trials is the product of the number of transformants tested and the number of individual assays per transformant.

were generated synthetically. Synthetic oligonucleotides were designed with cohesive ends containing *Xho*I restriction sites to allow cloning into pLG670Z.

Ty1 fragments were isolated from pJF351, a pUC19 derivative in which the *Eco*RI site of the polylinker had been previously converted to an *Xho*I site. The 112-bp *Hpa*I-*Sau*3A fragment D (14) was isolated from the Ty61 element in pGS5 (17, 39), treated with the large fragment of *E. coli* DNA polymerase to generate a blunt-ended species, and cloned into the *Sma*I site of the modified pUC19. This fragment could be released from pJF351 by digestion with *Xho*I plus *Sal*I.

pGY11 is a derivative of pJF351 in which an internal *Taq*I site was converted to a second *Xho*I site. pJF351 was partially digested with *Taq*I, and *Xho*I linkers were ligated to the isolated linear partial-digestion product. pGY11 was recovered after screening by *Xho*I digestion for candidates containing an appropriate-sized *Xho*I fragment.

pGY63, a derivative of plasmid pYAB1 (a gift of J. Rossi) (27), was constructed to serve as a positive control plasmid in β-galactosidase assays. The *Eco*RI fragment containing the *TRP1* gene and *ARS1* sequences was deleted and replaced with the *Eco*RI fragment from plasmid YEp24 containing 2 μm sequences. The YEp24 *Hin*dIII fragment containing the *URA3* gene was inserted at the *Hin*dIII site (base 105) of the 2 μm insert. On average, this plasmid yielded roughly half as much β-galactosidase activity in *spt13* mutant backgrounds as in wild-type strains (Tables 2 and 3).

For detection of *MCM1* mRNA levels, a 1.1-kb *Eco*RI fragment was isolated from plasmid pGA1761 (a gift of G. Ammerer) (1).

**RNA isolation and Northern hybridization analysis.** Cells were grown to a concentration of $2 \times 10^7$/ml in YPD. RNA was prepared by the method of Carlson and Botstein (3). Electrophoresis, blotting, and hybridization were performed as previously described (16). $^{32}$P-labeled probes were prepared by using random primers (18, 19).

**Western analysis.** Crude protein extracts were prepared as described below for electrophoretic mobility shift assays. Equal microgram amounts of each extract were subject to electrophoresis on sodium dodecyl sulfate (SDS)–10% (acrylamide/bisacrylamide ratio, 30:0.8) polyacrylamide gels. Transfer to nitrocellulose membrane was performed by using a Bio-Rad transfer apparatus with Tris-glycine methanol buffer in the absence of SDS. The relative amount of protein loaded in each lane was visualized by Ponceau S staining. The Amersham ECL Western immunoblotting procedure was used, with the following minor modifications. The membrane was blocked with 5% nonfat milk in 0.1% Tween 20 in phosphate-buffered saline at 37°C for 15 min. Affinity-purified MCM1 antibody (anti-MIP peptide antibody), the generous gift of L. Bruhn and G. Sprague (24), was used at a dilution of 1:1,000. Secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) diluted 1:5,000. Visualization was performed by chemiluminescence (Amersham).

**Cloning of synthetic oligonucleotides.** Oligonucleotides were prepared by C. Olson or J. Ferm with an Applied Biosystems DNA synthesizer. Both strands of the sequence were synthesized for use in cloning experiments. Annealing was accomplished by mixing equimolar amounts of each strand, heating the mixture for 10 min at 65°C in 0.1 M NaCl–0.01 M Tris HCl (pH 7.5), and cooling it slowly to room temperature. When insertion of multiple copies of the oligonucleotide sequence was desired, the duplex was first phosphorylated with T4 polynucleotide kinase and ATP (30). Ligation conditions were those recommended by the enzyme supplier (Promega Biological Research Products). The correct clones were detected by colony hybridization. Colonies were lysed by autoclaving as follows: nitrocellulose filters to which the colonies had been transferred were autoclaved (2 min at 200°F [93.3°C]) and then baked for 2 h at 80°C in a vacuum oven. For use as a hybridization probe, the oligonucleotide duplex was treated with *E. coli* DNA polymerase large fragment and deoxynucleotides including [α-$^{32}$P]dCTP. Hybridization conditions were either aqueous (2) or 50% formamide (16). The copy number and orientation of the insert sequences were verified by sequence analysis with the Sequenase sequencing kit (U.S. Biochemical Corp.). A sequencing primer complementary to sequences 28 bp 3′ to the pLG670Z *Xho*I restriction site was synthesized for this purpose (5′-GTTGCCTGGCCATCCAC-3′). For the sequences of other synthetic oligonucleotides used in these studies, see Fig. 2.

**Plasmid copy number determinations.** The plasmid copy number was determined by Southern hybridization analysis. Yeast DNA was isolated from cultures by using the glass bead lysis method of Hoffman and Winston (22). Electrophoresis, blotting, and hybridization were performed as previously described (16). $^{32}$P-labeled probes were prepared

by random priming by the method of Feinberg and Vogelstein (18, 19). Densitometric analysis was performed on autoradiographic exposures by using preflashed film and a single intensifying screen (28). For analysis of the plasmid copy number, yeast genomic DNA was digested with *Eco*RI. The hybridization probe consisted of the 505-bp *Acc*I fragment from the *URA3* gene isolated from plasmid YIp5 (42). The copy number was quantitated densitometrically by comparing levels of plasmid (*URA3*$^+$) and genomic (*ura3-52*) hybridization in a given lane.

**Gel mobility shift analysis.** Preparation of yeast cell extracts, protein–DNA-binding reactions, and electrophoretic fractionation of complexes were performed essentially as described by Company et al. (5) with a few minor modifications. After disruption of the yeast cell cultures by glass bead lysis and clarification of the supernatant, $(NH_4)_2SO_4$ was added to a saturation of 40%. The supernatant was incubated at 4°C with gentle agitation in the presence of $(NH_4)_2SO_4$ overnight and then centrifuged to obtain the final protein precipitate.

Protein (20 μg) and 1 μg of poly(dI-dC) (nonspecific competitor) were routinely added to binding-reaction mixtures. The reaction mixtures were incubated at 30°C for 20 min to allow protein-DNA complex formation. Specific competitor DNAs were added to a 100-fold molar excess.

DNA probes were isolated as *Xho*I or *Xho*I-*Sal*I restriction fragments (see above) or were synthetic oligonucleotides containing *Xho*I ends. Radiolabeled DNA fragments were generated for use as probes by treatment with *E. coli* DNA polymerase large subunit and deoxynucleotides including [α-$^{32}$P]dCTP. Approximately 0.5 to 1.0 ng of DNA was used in each binding reaction.

Gels for resolving protein-DNA complexes were 6% polyacrylamide gels (polyacrylamide/bisacrylamide ratio, 29:1) in 1× filtered TBE (89 mM Tris, 89 mM borate, 2.4 mM EDTA [pH 8.0]). The gels were preelectrophoresed for 1 to 4 h at 180 V (22 mA). After loading, electrophoresis conditions were 150 V (10 mA) for a maximum of 2 h.

## RESULTS

The phenotypes of *spt13* mutants strongly suggest that the *SPT13* gene product functions formally as a negative regulator of enhancer activity in Ty elements (16, 17). The role of *SPT13* in negative regulation of Ty-mediated adjacent gene expression is distinct from other *SPT13*-mediated effects. Several *GAL4*-regulated genes and *RAP1*-regulated genes exhibit reduced levels of steady-state transcript in *spt13* mutants, suggesting a role for *SPT13* as a positive regulator in many cases (17, 33, 34, 40, 41).

To elucidate the regulatory role of *SPT13* in Ty1-mediated gene expression, we sought to define the SPT13 site of action. Here we consider the region of Ty1 sequence containing homology to the SV40 enhancer core as a candidate for the SPT13 site of action. Two such sites, block I and block II, have been identified in Ty1 elements (6, 13); we have chosen the better-characterized block II for these studies (7). Within block II, the SV40 enhancer core homology overlaps an MCM1-binding site (P site) (11, 13) and the P site overlaps an **a**1-α2-binding site. A second **a**1-α2-binding site adjoins the first (Fig. 1).

**Fragment H contains a weak transcriptional activator.** Block II is contained on a 61-bp restriction fragment that we have designated H to correspond to nomenclature used in previous work on this region of Ty1 elements (6). Our fragment H differs from the originally designated fragment H by 2 bp because of our use of the *Taq*I rather than the *Hin*fI site as the division point (see Fig. 1 for clarification). The transcriptional activity of fragment H in wild-type (*SPT13*$^+$) cells was measured by its ability to stimulate β-galactosidase expression when inserted into the promoter of pLG670Z (see Materials and Methods). Throughout the text, β-galactosidase activities are reported as relative units; absolute values were normalized to expression of a control *ACT1-lacZ* plasmid in a wild-type (*SPT*$^+$) background, measured in each experiment. Absolute values are also provided (Table 2 and 3). Although one copy of H (1×H) conferred minimal activity (1.7 r.u.) three copies of H (3×H) directed 7.7 r.u. of β-galactosidase activity. These results (Table 2) were consistent with those previously reported (7).

**Block II sequences are under the control of SPT13.** A three- to fivefold increase in β-galactosidase expression from plasmids containing fragment H was observed in *spt13* mutants. The increase in β-galactosidase activity observed in *spt13* mutants is almost certainly due to changes in transcription, because no plasmid copy number differences were observed between *SPT*$^+$ and *spt13* hosts.

**SPT13 has no apparent role in a1/α2 diploid control.** Ty expression is known to be cell type regulated (10, 12); Ty is highly expressed in haploids but poorly expressed in diploids. Fragment H contains several copies of the same **a**1/α2 diploid control sequence present in front of other haploid-specific genes, and H-mediated activation has been shown to be haploid specific (7). To test whether SPT13 has any role in **a**1/α2 diploid control, we transformed *spt13*/*spt13* and *SPT13*$^+$/*SPT13*$^+$ homozygous diploids with the 3×H reporter plasmid and tested them for β-galactosidase activity. β-Galactosidase activity was decreased 25-fold in the *SPT*$^+$ diploid relative to the *SPT*$^+$ haploid, whereas *spt13* mutant diploids had 11-fold-decreased β-galactosidase activity relative to the *spt13* haploid. We conclude that strains lacking *SPT13* are still functional for **a**1/α2 diploid control.

**MCM1-binding-site-mediated activation is under SPT13 control.** Previous experiments show one protein-binding site mapping to sequences in fragment H that exhibits homology to the MCM1-binding site (P site) (7). The accompanying paper shows that the P site in fragment H is indeed occupied by the MCM1 protein (11). To examine the effect of *SPT13* mutations on the P site present within block II sequences, a synthetic 34-bp oligonucleotide [called extended P(Ty1)] containing the Ty1 P site and the adjacent 10 bp was introduced in single copy into pLG670Z. β-Galactosidase levels increased 2.9-fold in the *spt13* mutant (Table 3) relative to the level observed in an *SPT13*$^+$ background. To verify that the P site and not adjacent sequences which include the SV40 enhancer core homology was responsible for this effect, extended P(Ty1) was resynthesized with a point mutation in the P site [extended P*(Ty1)] and introduced in single copy into pLG670Z. Consistent with results of previous studies (7), extended P*(Ty1) showed basal levels of β-galactosidase expression in *SPT13*$^+$ strains. Expression was not increased in the *spt13* mutant (Table 3). These results indicate that SPT13 is a negative regulator of P-site-mediated activation.

To examine whether the specific P site and adjacent sequences within Ty constitute a unique regulatory context or whether SPT13 is a general regulator of MCM1, we synthesized and tested two P-site oligonucleotides: P(pal), which contains a perfect palindromic P site, and P(Ty1), which contains the P-site sequence as it appears in the Ty1 element (Fig. 2). The P(Ty1) and P(pal) oligonucleotides are 10 bases shorter than the extended P(Ty1) oligonucleotide.

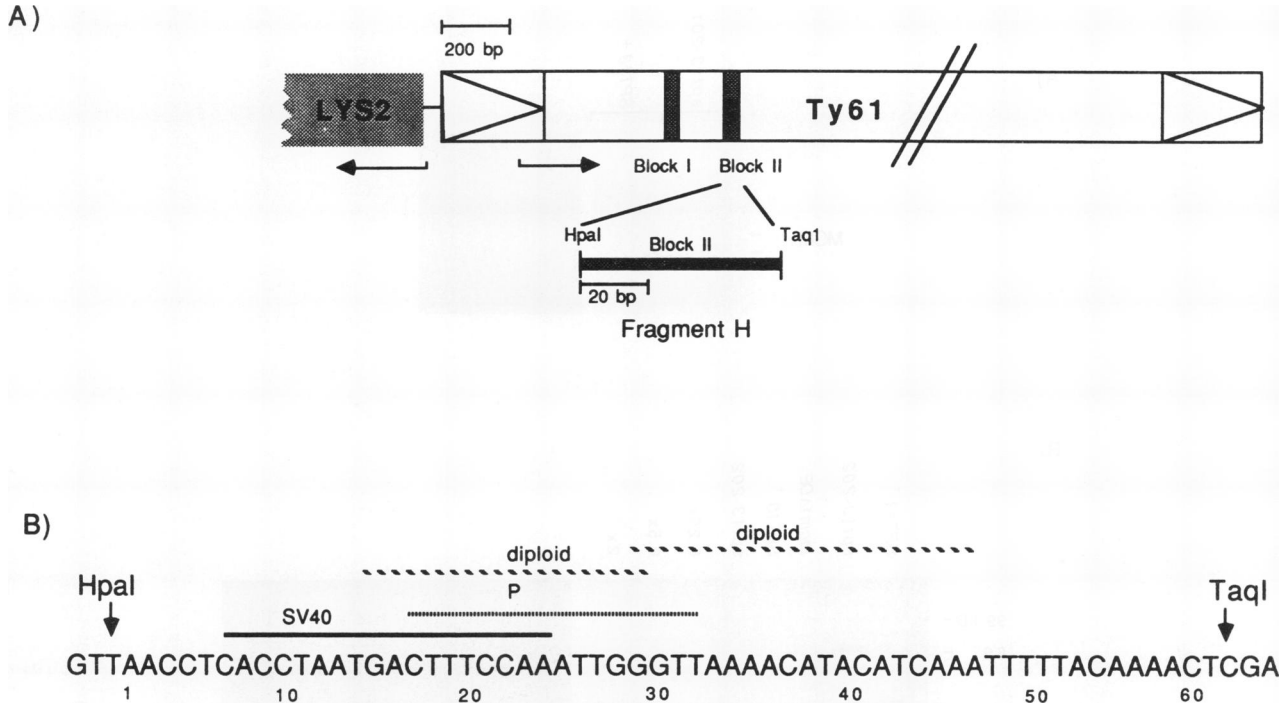


FIG. 1. Details of the block II region of the Ty61 element. (A) Location of the block II sequences within the Ty element. The arrows drawn in the terminal repeats (δ) indicate the direction of transcription of the element. The promoter for Ty transcription is located in the leftmost repeat. The arrows drawn beneath the Ty element and the adjacent *LYS2* gene indicate the direction of Ty and *LYS2* transcription, respectively. The first 1,000 bp of the element are drawn to scale; the remainder of the element is condensed. The 61-bp *Hpa*I-*Taq*I fragment H is drawn below to illustrate the location of the block II sequences. (B) Sequence of the *Hpa*I-*Taq*I fragment H. Sequence similarities are indicated. Abbreviations: SV40, similarity to the enhancer core region from within the SV40 72-bp repeats; P, similarity to the consensus MCM1-binding site; diploid, similarity to the a1/α2 diploid control sequence or its complement.

Activity directed by both P-site oligonucleotides remained under the negative control of SPT13 (Table 3). As expected, the palindromic P site was considerably more active than the Ty1-derived P-site sequence, presumably because of the increased affinity of MCM1 for the palindromic P site relative to its affinity for the imperfect Ty1 P site. Interestingly, we also found that the activity of the palindromic P site was better repressed than that of the isolated Ty1 P site (Table 3).

**SPT13 affects neither *MCM1* expression nor MCM1 DNA-binding activity.** Since many of the phenotypes of *spt13* mutants can be explained by alterations in transcript levels of relevant genes (17, 33), it seemed possible that SPT13 regulates MCM1 activity by decreasing the expression of the *MCM1* gene. Northern (RNA) hybridization analysis showed no significant changes in total *MCM1* mRNA levels in *spt13* mutants (Fig. 3A). The *MCM1* gene is normally expressed as multiple transcripts because of 3′ heterogeneity (35). We noted a small but reproducible difference in the relative abundance of *MCM1* transcripts in *SPT13*$^+$ and *spt13* strains; however, the significance of this modest change is unclear. Furthermore, Western blot analysis showed no differences in MCM1 protein levels in *SPT13*$^+$ and *spt13* mutants (Fig. 3B). We conclude that the effect of *spt13* mutations on MCM1 activity is posttranslational.

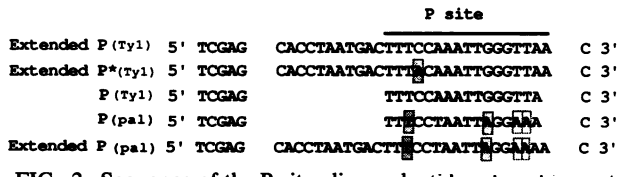


FIG. 2. Sequence of the P-site oligonucleotides cloned in vector pLG670Z for expression studies. These oligonucleotides were also used in electrophoretic mobility shift assays. The first four bases in each synthetic oligonucleotide allow cloning into *Xho*I restriction sites. Complementary strands were synthesized for each sequence; only the 5′-3′ sequence is indicated here. Sequences corresponding to the P site are indicated by overlining. Bases differing from the Ty1 P site are shaded.

To determine the effect of SPT13 on MCM1 binding to DNA, we used fragment H (data not shown) and shorter synthetic oligonucleotides containing the Ty1 P site as DNA probes in electrophoretic mobility shift assays. Using the radiolabeled extended P(Ty1) oligonucleotide (Fig. 2 and 4) to monitor protein binding, we observed three electrophoretically distinct complexes with wild-type protein extracts (Fig. 4, lane 2). Complex 2 (NSII), the least abundant of the three complexes, appeared to be nonspecific since it was observed even in the presence of a variety of unlabeled competitor DNA molecules (Fig. 5). Complex 1 (I) was dependent on the MCM1 protein; extracts prepared from an *mcm1-1* point mutant (35) (the fact that *MCM1* is an essential gene prevented our using a null mutant) formed greatly reduced levels of this complex (Fig. 4, lane 4). That MCM1 is a component of this complex was supported by our observation that the amount of complex 1 was greatly reduced when the extended P*(Ty1) oligonucleotide, containing a single-base-pair change in the P site, was used as a labeled DNA probe (Fig. 4, lanes 6 and 7). Complex 3 (III)

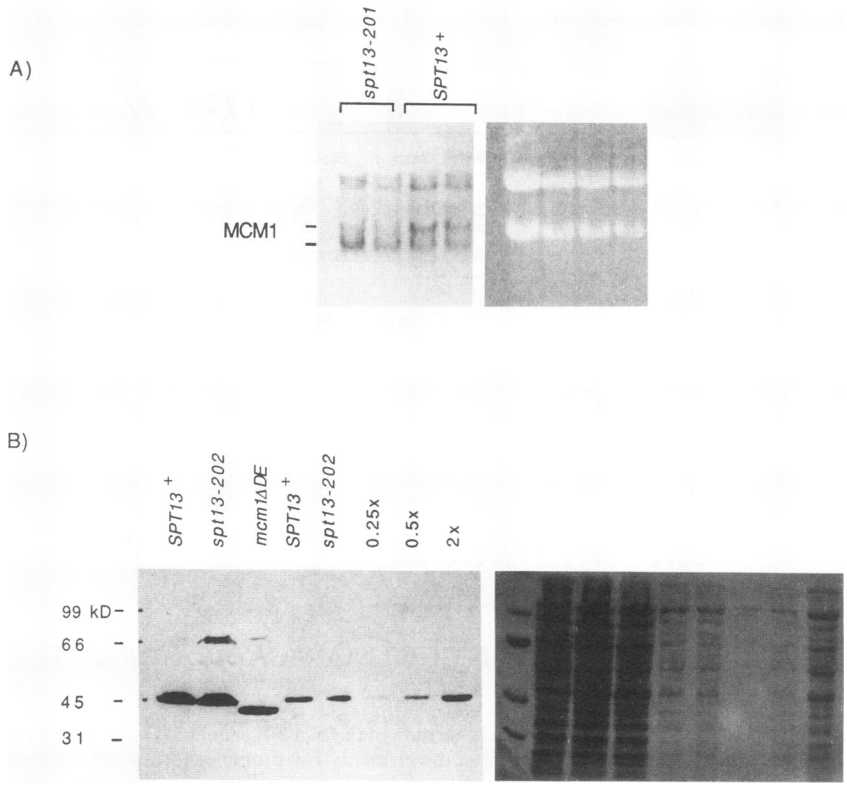


FIG. 3. Effect of *spt13* mutation on *MCM1* mRNA and protein levels. (A) Northern hybridization analysis of *spt13-201* deletion (JF1049 and JF1050) (lanes 1 and 2) and *SPT13*$^+$ (JF15 and JF28) (lanes 3 and 4) strains. Hybridization probe was prepared from plasmid pGA1761, which contains the coding region of the *MCM1* gene. Multiple transcripts are seen. By comparison with the migration of rRNA, the smaller bands are probably the 1.6- and 1.2-kb transcripts previously observed (35). The larger transcripts have not been previously noted. The ethidium bromide-stained gel is shown to confirm loading of equivalent microgram amounts of RNA in each lane. (B) Western blot analysis of the MCM1 protein in the isogenic strains, JF819 (*SPT13*$^+$) and the deletion derivative JF1073 (*spt13-202*). *SPT13*$^+$ and *spt13-202* extracts were prepared and loaded in pairs (lanes *SPT13*$^+$ and *spt13-202*). Extract prepared from a truncation mutant (mcm1-ΔDE) (4) was also loaded. MCM1 migrates at ~45 kDa on this gel. The Ponceau S-stained filter is shown to confirm that equal microgram amounts of each pair of extracts were loaded. In the three right hand lanes, the wild-type extract was loaded at 0.25×, 0.5×, and 2× levels relative to the amount in lane 4.

exhibited exceptionally high mobility and was insensitive both to the presence of functional MCM1 in the extract (Fig. 4, lane 4) and to the P-site mutation in the P*(Ty1) oligonucleotide (Fig. 4, lanes 6 to 8). As described below, we believe that this complex reflects the binding of a small unidentified protein interacting with sequences immediately 5′ to the P site.

Competition analysis provided additional support for the conclusion that complex 1 contained the MCM1 protein bound to the P site. Formation of complex 1 could be inhibited largely by unlabeled P(pal) and by extended P(Ty1) oligonucleotides but less well by the identical oligonucleotide containing a mutation in the P site [extended P*(Ty1)] (Fig. 5).

It is apparent from Fig. 4 that the *spt13* mutation has no effect on levels of MCM1-containing complex 1 formed (lanes 2 and 3). Competition analysis (Fig. 5) showed no differences in the ability of different P-site oligonucleotides to compete for binding to Ty1 P site sequences in the presence and absence of SPT13. This suggests that the effect of *spt13* mutations on MCM1 activity is not due to altered affinity of MCM1 for the P site. To further examine the affinity of MCM1 for the P site in the presence and absence of SPT13, we added an increasing amount of unlabeled P(pal) competitor (the molar ratio of competitor to probe was varied from 4:1 to 16:1) to binding-reaction mixtures containing 20 μg of *SPT*$^+$ or *spt13-202* extracts. The amount of competitor required to reduce by 50% the amount of probe retained in the MCM1 complex was not significantly changed by the absence of SPT13 in the extract (data not shown). From this analysis we conclude that the negative effect of SPT13 on MCM1 activity was not attributable to a reduced MCM1 affinity for the P site. Furthermore, we found no evidence for the independent binding of SPT13 directly to the DNA. Both complex 1 and complex 3 were as abundant in binding reactions lacking SPT13 as they were in reactions containing it (Fig. 4 and 5).

**An unidentified factor binds to sequences adjacent to or overlapping the P site.** Formation of complex 3 on the extended P(Ty1) probe was inhibited by unlabeled extended P(Ty1) and by extended P*(Ty1) oligonucleotides but not by P(pal) oligonucleotide (Fig. 5). This suggests that the binding site for the complex 3 protein is not the P site but, rather, the sequences in Ty1 elements immediately adjacent to the P site. Adjacent to and overlapping with the P site is homology to the SV40 enhancer core motif (13). We tentatively designate this binding activity MCM1-neighboring factor (MNF). This factor has no apparent role in *SPT13*-mediated regulation of MCM1 activity since the activity of P-site oligonucleotides which do not allow the formation of complex 3 nevertheless remain *SPT13* regulated.

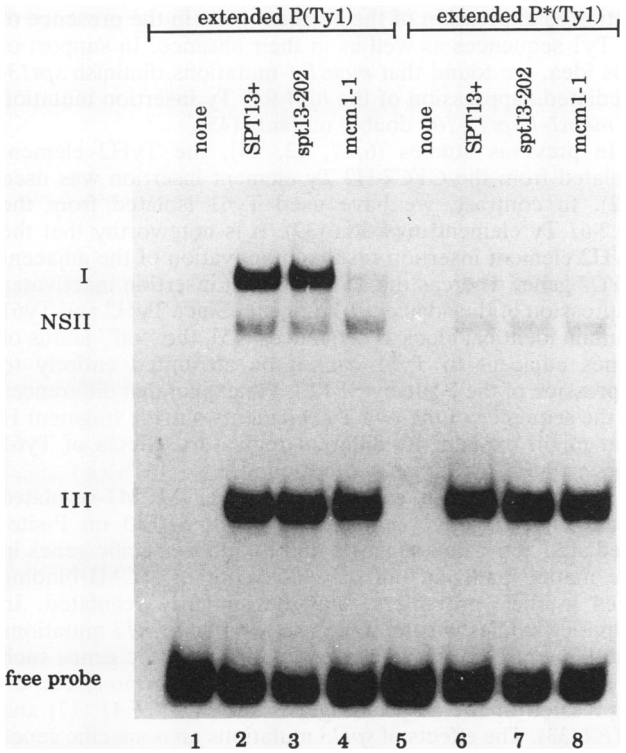


FIG. 4. Factor binding to block II sequences containing either a wild-type [extended P(Ty1)] (lanes 1 to 4) or mutant [extended P*(Ty1)] (lanes 5 to 8) P site. Binding-reaction mixtures contained 20 μg of protein from wild-type (JF819) (lanes 2 and 6), *spt13-202* (JF1073) (lanes 3 and 7), or *mcm1-1* (JF1088) (lanes 4 and 8) strains. Positions of free probe, complex 1 (I), nonspecific complex 2 (NSII), and complex 3 (III) are indicated to the left of the autoradiogram. In lanes 1 and 5 the extract was omitted.

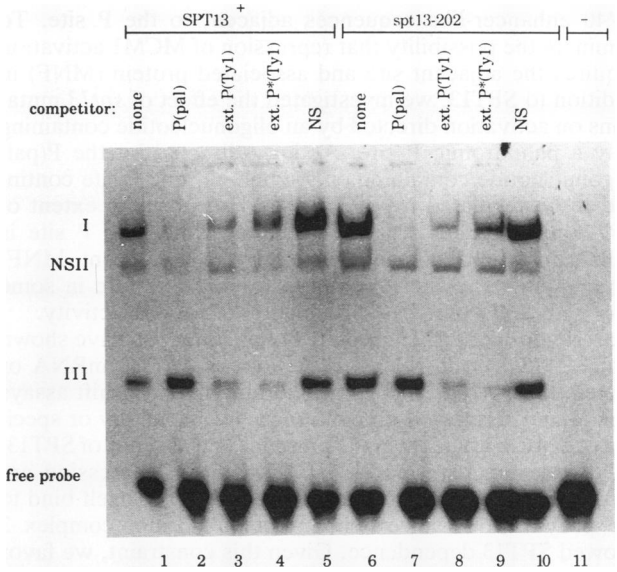


FIG. 5. Competition analysis of factor binding detected by electrophoretic mobility shift assays. Binding-reaction mixtures contained 20 μg of protein prepared from isogenic strains carrying either a wild-type (JF819) (lanes 1 to 5) or deletion (JF1073) (lanes 6 to 10) allele at the *SPT13* locus. Probe DNA was 0.5 to 1 ng of extended P(Ty1) oligonucleotide duplex labeled by filling in the *Xho*I ends with deoxynucleoside triphosphates including [α-$^{32}$P]dCTP. Positions of free probe, complex 1 (I), nonspecific complex 2 (NSII), and complex 3 (III) are indicated to the left of the autoradiogram. Binding reactions were performed in the absence of competitor (none) or with a 100-fold molar excess of P(pal), (lanes 2 and 7) extended P(Ty1) (lanes 3 and 8), extended P*(Ty1) (lanes 4 and 9), or nonspecific (NS) (lanes 5 and 10) oligonucleotides as competitor. In lane 11 the extract was omitted.

## DISCUSSION

**Ty1 block II activation.** One component of a complex network of regulatory signals within Ty1 elements has been referred to as block II (13) and contains homology to three different regulatory motifs including the SV40 enhancer core, the a1/α2 diploid control signal, and the MCM1 recognition sequence site (P site). The precise role of these sequences and a similar block of sequences (block I) just upstream in promoting the transcription of Ty elements is unknown. It was previously shown, however, that a 28-bp sequence from within block II (contained on restriction fragment H) is capable of activating adjacent-gene transcription (7). These sequences form a protein complex with the transcription factor MCM1 (11). Our studies, which involved a different reporter construct and sequences isolated from a different Ty1 element, confirm these observations.

**Negative regulation of the P site by SPT13.** We have shown here that the P-site sequences in Ty1 elements are subject to negative control by SPT13. The molecular basis for this regulation is not yet clear. *spt13*-mediated P-site derepression varied from 2.6- to 6-fold (Tables 2 and 3). For unknown reasons, the magnitude of the effect appeared to depend both on the P-site sequence and on the sequence environment of the P site.

We believe that the reported derepression ratio is likely to underestimate the actual effect of SPT13. This is because the β-galactosidase values reported in Tables 2 and 3 are normalized to expression of an *ACT1-lacZ* fusion construct in an *SPT13*$^+$ strain. Expression of the *ACT1-lacZ* control gene in *spt13* mutants was twofold lower than in the *SPT*$^+$ strain. Northern hybridization analysis showed that the reduction in *ACT1-lacZ* β-galactosidase activity was not transcriptional. *ACT1-lacZ* mRNA levels are actually higher in *spt13* mutants (data not shown). It is likely that the reduced levels of β-galactosidase activity recovered from the *ACT1-lacZ* construct in *spt13* mutants is due to undefined posttranscriptional differences in the two strains. These differences may lead to underestimates of the extent of *SPT13*-mediated repression.

**P-site-mediated activation is MCM1 and SPT13 dependent.** Molecular analysis showed that two distinct protein factors interact with the block II sequences within fragment H. MCM1 was bound to the P site, and a second, unidentified factor (MNF) was bound to sequences immediately upstream of the P site. It is clear that MCM1 and not MNF was responsible for activation from fragment H, since mutant P-site oligonucleotide [extended P*(Ty1)] directed virtually no activity yet allowed the formation of normal levels of complex 3. These results suggest that MCM1 is responsible for activation from Ty1 fragment H. Furthermore, since the activity of the extended P*(Ty1) oligonucleotide was not increased in the absence of SPT13, we also conclude that *SPT13*-mediated repression of these sequences requires MCM1.

**The P site is necessary and sufficient for repression.** Both fragment H and the extended P(Ty1) oligonucleotide contain

SV40 enhancer-like sequences adjacent to the P site. To eliminate the possibility that repression of MCM1 activation requires the adjacent site and associated protein (MNF) in addition to SPT13, we investigated the effect of *spt13* mutations on activation directed by an oligonucleotide containing only a palindromic P site. Activity directed by the P(pal) oligonucleotide containing only a palindromic P site continued to be regulated by SPT13. We note that the extent of *SPT13*-mediated repression is greatest when the P site is palindromic and unaccompanied by the adjacent MNF-binding site. The MNF-binding site may interfere in some way with *SPT13*-mediated regulation of MCM1 activity.

**Mechanism of *SPT13*-mediated repression.** We have shown that SPT13 has no role in regulation of *MCM1* mRNA or protein levels (Fig. 3). Electrophoretic mobility shift assays (Fig. 4 and 5) showed no differences in the affinity or specificity of MCM1 binding in the presence and absence of SPT13.

Possible mechanisms of *SPT13*-mediated repression are further limited by the fact that SPT13 does not itself bind to the p(Ty1) DNA since neither complex 1 nor complex 3 showed SPT13 dependence. Given this constraint, we favor one of two mechanisms. SPT13 may function like GAL80 (24), interacting with MCM1 to somehow mask its activation domain. Alternatively, repression may be due to posttranslational modifications of MCM1 that result in its inactivation. In these examples, repression is defined as interference with activation. In the case of the SSN6-TUP1 repressor, it is apparent that repression can also be an active process (25). SSN6 and TUP1 are required for transcriptional repression mediated by α2-MCM1 and **a**1/α2 operators. Keleher et al. (25) have shown that SSN6 is capable of independently repressing the activity of an unrelated activator when it is artificially brought to the promoter, for example by fusion to the LexA DNA-binding domain. Although similarities in mutant phenotype and protein sequence between *SSN6* and *SPT13* have been noted (17, 40), it is unlikely that SPT13 uses an SSN6-like mechanism for repression because SPT13 is a strong activator of transcription when joined to the LexA DNA-binding domain (21).

There is currently no evidence to support the idea that SPT13 might associate with MCM1 to regulate its activity. We observed no convincing changes in the mobility of complex 1 in the absence of SPT13. In preliminary experiments, addition of SPT13 antibody (a gift of H. Himmelfarb and M. Ptashne) to binding-reaction mixtures did not result in any specific changes in the mobility of complex 1 (45). Likewise, preliminary experiments in which labeled synthetic SPT13 was added to binding-reaction mixtures containing unlabeled synthetic MCM1 and unlabeled P-site probe failed to reveal any SPT13-containing complexes (45). Hence, SPT13 may not be physically associated with MCM1.

Less direct mechanisms for the *SPT13*-mediated change in MCM1 activity are also possible. In light of the predominantly positive role of SPT13 elsewhere in the cell, it remains possible that the true role of SPT13 with respect to Ty-mediated gene expression is that of a positive regulator of a negative factor.

**Role of MCM1 and SPT13 in regulating Ty and adjacent gene expression.** Our experiments establish a negative regulatory role for SPT13 with respect to *MCM1*-mediated activation within Ty1 elements. This is consistent with our previous models of *spt13*-mediated suppression of Ty insertion mutations (17), in which we proposed that SPT13 negatively regulates a Ty1 activator that is capable, on derepression, of driving adjacent-gene expression. We speculate that the derepressed P site makes a significant contribution to expression of the adjacent gene in the presence of 5′ Ty1 sequences as well as in their absence. In support of this idea, we found that *mcm1-1* mutations diminish *spt13*-mediated suppression of the *his4-917* Ty insertion mutation in *mcm1-1 spt13-101* double mutants (45).

In previous studies (6, 7, 13, 14), the TyH2 element isolated from the *CYC7*-H2 Ty element insertion was used (12). In contrast, we have used Ty61 isolated from the *lys2-61* Ty element insertion (39). It is noteworthy that the TyH2 element insertion results in activation of the adjacent *CYC7* gene, whereas the Ty61 element insertion inactivates expression of the adjacent *LYS2* gene. Since TyH2 and Ty61 contain identical block II sequences (45), the "off" status of genes adjacent to Ty61 cannot be attributed entirely to repression of the P site by SPT13. We expect that differences in the sequence of the two Ty1 elements outside fragment H that might explain the different regulatory effects of Ty61 versus TyH2 insertions will be found.

**Role of SPT13 in expression of other MCM1-regulated genes.** The negative regulatory role of SPT13 on P-site-mediated activation suggests that **a**- and α-specific genes in the mating pathway, all of which contain MCM1-binding sites in their promoters, may be similarly regulated. In previous studies we and others showed that *spt13* mutations had large effects on the expression of α-specific genes such as *MFα1* (17, 33) and *STE3* (33) and smaller or no effects on expression of the **a**-specific genes such as *MFA1* (17) and *STE2* (33). The effects of *spt13* mutations on α-specific genes have been attributed to reduced expression of the *MATα1* gene, which is required for their expression. We have recently found that the *MFA2* gene appears to be more sensitive to the absence of SPT13 than other previously measured **a**-specific genes are (15, 17). The effect of *spt13* mutations on the expression of **a**-specific genes cannot be attributed to reductions in *MATα* expression. On the basis of the work reported here, a direct effect of the SPT13 protein on MCM1-mediated activation of **a**- and α-specific gene promoters would be predicted to be negative, leading to increased expression of these genes. It will be interesting to determine whether the observed net decrease in expression of these genes is the sum of a negative effect on MCM1 activity and a positive effect on a second DNA-binding protein, STE12 for example, or whether SPT13 is capable of both negative and positive regulation of MCM1 activity.

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