Spt10 and Spt21 Are Required for Transcriptional Silencing in Saccharomyces cerevisiae

Jennifer S. Chang and Fred Winston*

Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115

Received 2 October 2010/Accepted 26 October 2010

In Saccharomyces cerevisiae, transcriptional silencing occurs at three classes of genomic regions: near the telomeres, at the silent mating type loci, and within the ribosomal DNA (rDNA) repeats. In all three cases, silencing depends upon several factors, including specific types of histone modifications. In this work we have investigated the roles in silencing for Spt10 and Spt21, two proteins previously shown to control transcription of particular histone genes. Building on a recent study showing that Spt10 is required for telomeric silencing, our results show that in both spt10 and spt21 mutants, silencing is reduced near telomeres and at HMLα, while it is increased at the rDNA. Both spt10 and spt21 mutations cause modest effects on Sir protein recruitment and histone modifications at telomeric regions, and they cause significant changes in chromatin structure, as judged by its accessibility to dam methylase. These silencing and chromatin changes are not seen upon deletion of HTA2-HTB2, the primary histone locus regulated by Spt10 and Spt21. These results suggest that Spt10 and Spt21 control silencing in S. cerevisiae by altering chromatin structure through roles beyond the control of histone gene expression.

Changes in chromatin state modulate gene expression. These changes can involve alterations in the composition of bound proteins, the positions or presence of nucleosomes, the array of covalent modifications on histones, or higher-order chromatin structure (reviewed in references 11, 41, and 60). In Saccharomyces cerevisiae, one form of specialized chromatin structure exists in silenced regions, where genomic regions are packaged into a repressive state characterized by hypoacetylated histone proteins (reviewed in references 17 and 59). In these regions, gene expression is silenced as a consequence of location, largely independent of promoter composition (59).

In S. cerevisiae, silencing occurs at three loci: subtelomeric regions (telomere position effect [TPE]), the silent mating type loci HMRα and HMLα, and the ribosomal DNA (rDNA) repeats (reviewed in references 7 and 59). Silencing at telomeres and silencing at the mating type loci show many similarities, including a requirement for the silent information regulator (Sir) proteins Sir2, Sir3, and Sir4. At the silent mating type loci, Sir1 plays an additional role in facilitating recruitment of the other Sir proteins, though it is not essential for silencing (54, 78). Silencing at the rDNA locus is unique in that Sir2 is the only Sir protein required for silencing. Furthermore, at the rDNA, sir-4Δ mutants show an increase in silencing (66).

Silencing occurs in three steps: nucleation, spreading, and limitation of spreading. Nucleation is seeded by sequence-specific DNA binding complexes, including Rap1 at telomeres (45, 49) and Rap1, Abf1, and ORC at the silent mating type loci (44, 49, 78). These complexes recruit Sir2, Sir3, and Sir4. Sir complex binding is facilitated by hypoacetylation of the histone H3 and H4 tails, which is accomplished by the NAD⁺-dependent histone deacetylase activity of Sir2 (32, 38, 39, 65). Once nucleated, the silenced region spreads through the iterative deacetylation of histones and recruitment of additional SIR complexes, leading to the formation of broad regions of silenced chromatin (reviewed in reference 59). O-Acetyl-ADP-ribose, a product of Sir2-mediated deacetylation, is thought to further stabilize Sir binding (42, 75, 76). Boundary elements, as well as the interplay between Sir binding and histone modifications, function to limit the spread of silenced chromatin (14, 58, 73). Recent studies have reconstituted silencing in vitro, demonstrating the direct roles of Sir proteins and histone modifications in silencing (33, 46).

Recent evidence suggests that silencing in S. cerevisiae requires not only the recruitment and spreading of Sir proteins but also the proper formation of a higher-order chromatin structure. For example, when lysine 56 of histone H3 is changed to glycine, glutamine, or arginine, telomeric silencing is abolished without detectable changes in Sir binding (84). However, in these mutants subtelomeric chromatin shows a greater accessibility to Escherichia coli dam methylase, suggesting that the K56 amino acid changes interfere with silencing by interfering with formation of a higher-order chromatin structure (84). Similarly, when the N-terminal tail of histone H3 is deleted, silencing is lost without alterations to the Sir binding profile, yet the chromatin is more sensitive to dam methylation (69). The structures of this higher-order chromatin and other factors that control it remain unclear.

Recently, the putative histone acetyltransferase Spt10 has been implicated in silencing. Using a URA3 reporter inserted near a telomere, Braun et al. (6) found that spt10Δ mutants are defective for silencing in subtelomeric regions. Consistent with this result, we found evidence that spt10Δ mutants may also be defective in mating type silencing, as spt10Δ mutants do not undergo a G1 arrest upon exposure to the mating pheromone α-factor (unpublished observations).
Spt10 was first identified in selections for mutations that suppress the transcription defects caused by Ty1 insertions (Spt– phenotype [16, 50]), as well as other types of transcription defects (12, 85). It possesses a zinc finger domain through which it binds cooperatively as a dimer to a consensus sequence found at all four histone gene promoters (15, 47, 48). Spt10 also possesses a putative histone acetyltransferase domain (52). Although no acetyltransferase activity has been detected for Spt10 in vitro (D. Hess and F. Winston, unpublished results; R. Sternglanz, personal communication), mutations changing putative catalytic residues do cause an Spt– phenotype (28).

Several results suggest that Spt10 is functionally related to another protein, Spt21. Mutations in either gene cause the unusual phenotype of permitting transcription to initiate from the 3′ long terminal repeat (LTR) of Ty1 elements (50). In addition, spt10 and spt21 mutations affect histone gene transcription similarly, with spt10Δ and spt21Δ mutants each showing a 20-fold decrease in transcript levels for the histone gene pair HTA2-HTB2, one of the two S. cerevisiae loci that encode histones H2A and H2B, and a much more modest decrease in transcript levels for HHF2 (13, 15, 26, 28). Spt10 and Spt21 proteins can also physically interact, and particular alleles of SPT10 can suppress a deletion of SPT21 (28). However, spt10 and spt21 mutants do show some differences. Deletion of SPT10 causes a severe growth defect, while deletion of SPT21 causes only a mild growth phenotype (51). In addition, SPT10 mRNA and protein levels are cell cycle regulated, while SPT10 levels are not (9, 68). Finally, spt21 mutants have been identified in screens for mutants with altered telomere length (3, 20). Taken together, these studies suggest that Spt10 may play a broad role in transcription and chromatin structure and that Spt21 plays a role in a subset of these processes.

Given the observed silencing defect in spt10Δ mutants, as well as the functional connections between Spt10 and Spt21, we have investigated the roles of both Spt10 and Spt21 in silencing. Our results show that both spt10 and spt21 mutations impair silencing at subtelomeric regions and the silent mating locus HMLα, while strengthening silencing at the rDNA. In both spt10 and spt21 mutants, Sir protein recruitment and histone modifications are modestly altered. Furthermore, both mutants have clearly increased accessibility of chromatin to dam methylation, suggesting possible effects on higher-order chromatin structure. Additional results suggest that these phenotypes are not the consequence of altered histone gene expression, indicating an additional role for Spt10 and Spt21 in controlling chromatin structure.

**Materials and Methods**

**Yeast strains and plasmids.** The yeast strains used in this study are listed in Table 1. The GAL1p-SPT10 allele was generated by PCR amplification of a cassette containing KanMX and the GAL1 promoter from a pFA6a-KanMX6-PGAL1 plasmid (43), which was then integrated just upstream of the SPT10 ATG at its native genomic location. The spt21Δ::HIS3, sir1Δ::NatMX, sir2Δ::NatMX, sir3Δ::KanMX, and sir4Δ::NatMX deletion mutants were generated by replacing the entire open reading frames with the indicated auxotrophic or drug resistance marker (21, 62). The SPT10-13MYC and SPT21-13MYC alleles, created using a previously described method (43), were confirmed by Western blotting. Strains containing these tagged alleles were tested for both growth and Spt– phenotypes and behaved the same as wild-type strains. The tel1Δ-R reporter strains derive from crosses with UCC35 (2), and the mura3Δ reporter strains derive from crosses with JS215-10 (64).

Spt10 bearing the E. coli dam methylase gene contain the methylase gene inserted at the LYS2 genomic locus (22). Media, basic yeast techniques, mating, sporulation, and tetrad dissection were performed as previously described (57). Plasmids used in this study include pRS425 and pRS426 (10); pLP891 (2µ-SIR2 HHS), pLP1047 (2µ-SIR3 HHS), pLP304 (2µ-SIR3 LEU2), and pLP2206 (2µ-SIR4 HHS), all generously provided by Lorraine Pillus; pFW217 (2µ-SPT10-URA3), and pLP228 (2µ-HTA1-HTB1 HHT1-HHF1 LEU2) (35). Because sir2Δ, sir3Δ, and sir4Δ strains are nonmating, their crosses were facilitated by transformation with a high-copy-number plasmid encoding the corresponding wild-type gene (pLP891, pLP1047, and pLP2206, respectively).

**Spot tests.** For all spot tests, cultures were grown to saturation (1 × 10⁶ to 2 × 10⁷ cells/ml) overnight, and then 5-fold serial dilutions were spotted onto the indicated media and incubated at 30°C for 2 to 5 days. For Fig. 1C, to minimize the acquisition of spontaneous suppressors of the spt10Δ slow-growth phenotype, the spt10Δ strain was first grown with pFW217 (2µ-SPT10-URA3) and then grown on 5-fluoroorotic acid (5-FOA) medium to select for cells that had lost the plasmid. Strains were then patched on yeast extract-peptone-dextrose (YPD) medium, grown 1 to 2 days, resuspended in water to 1 × 10⁸ cells/ml, and subjected to 5-fold serial dilutions.

**Quantitative mating assays.** Quantitative mating assays were performed as previously described (24). Briefly, cultures of a query strain and a wild-type strain of the opposite mating type were grown to 1 × 10⁶ to 2 × 10⁷ cells/ml, and then 10⁶ cells of the query strain and 10⁵ cells of the wild-type strain were filtered together onto a 25-mm-diameter glass microfiber disc (0.45-µm pore size; Whatman). The disc was placed onto a YPD plate and incubated at 30°C for 5 h. The cells were eluted into 1 ml of 1 M sorbitol and sonicated briefly. Dilutions were plated onto solid medium that selects for diploids (mated) and for total query cells were eluted into 1 ml of 1 M sorbitol and sonicated briefly. Dilutions were plated onto solid medium that selects for diploids (mated) and for total query cells were eluted, and cross-linking was reversed at 65°C overnight. Samples were washed, and then sonicated with six 30-s pulses, with 90 s between pulses, using a Bioruptor machine. Primer sequences are listed in Table S1 in the supplemental material. For analysis of a2 and s2 mRNAs, the two transcripts were probed simultaneously, as the entire coding region of a2 is identical to a region of s2.

**Western blotting.** Whole-cell extracts were prepared by trichloroacetic acid (TCA) precipitation (S. P. Bell, unpublished). Western blotting was performed using SDS-PAGE (15% polyacrylamide gels for visualization and 7% polyacrylamide for all others), as previously described (19). Antibodies used were anti-Sir2 (1:5,000) (70), anti-Sir3 (1:5,000) (40), anti-Sir4 (1:5,000) (76), anti-histone H3 (1:5,000) (Abcam), anti-histone H3 K7me2 (1:5,000) (Abcam), anti-acetylated histone H4 (recognizing acetylation at K5/8/12/16; 1:5,000) (Upstate), anti-Myc (1:5,000) (9E10; Santa Cruz Biotechnology), and anti-Pgk1 (1:5,000) (Molecular Probes). All antibody dilutions were made in 5% milk in Tris-buffered saline–Tween 20 (TBST).

**ChIP.** Chromatin immunoprecipitations (ChIPs) were performed as previously described (31). Exponentially growing cultures were harvested, cross-linked with formaldehyde (1% final concentration) for 30 min, and quenched with glycine (115 mM final concentration). After pelleting, cells were lysed by bead beating and then sonicated with 30-s pulses, with 90 s between pulses, using a Bioruptor machine (Diagenode). One microliter of antibody was added to 625 µg of soluble lysate in 1 ml of lysis buffer and incubated for 14 h at 4°C. Fifty microliters of a 50% protein G-Sepharose slurry (protein G-Sepharose; GE Healthcare) was added and incubated for another 4.5 h. After washing, samples were eluted, and cross-linking was reversed at 65°C overnight. Samples were subjected to proteinase K digestion and phenol-chloroform extraction, and the DNA was then precipitated. For input samples, cross-linking was reversed for 15 µg of soluble lysate in parallel with the IP samples described above. The antibodies used are described in the previous section, except for anti-Sir2, which was previously described (76).

**Real-time PCR.** Real-time PCR was performed using SYBR green chemistry (Brilliant II SYBR green master mix; Stratagene) and a Stratagene MX3000P machine. Primer sequences are listed in Table S1 in the supplemental material. The annealing temperature was 55°C for all primer pairs, and all reactions were performed in triplicate. For chromatin immunoprecipitation experiments, data are normalized to binding at either ACT1 or a region of chromosome V lacking open reading frames (37), relative to input DNA. For Fig. 7, values were further normalized to ChIP of total histone H3.

**Dam methylase assay and Southern blotting.** Genomic DNA was isolated from 1 × 10⁶ to 2 × 10⁷ cells in exponential growth as previously described (30). The DNA was digested with RNase, precipitated, and digested with 160 units of NdeI (New England Biolabs) at 37°C for 8 h. An additional 50 units of NdeI was...
TABLE 1. S. cerevisiae strains used in this study

| Strain | Genotype |
|--------|----------|
| FY2200 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 |
| FY1856 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 |
| FY2813 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 GAL1pr-SPT10::KanMX |
| FY2814 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 GAL1pr-SPT10::KanMX |
| FY2815 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 |
| FY2816 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 spc2Δ::HIS3 |
| FY2817 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 spc2Δ::HIS3 |
| FY2818 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir2Δ::NatMX |
| FY2819 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir2Δ::NatMX |
| FY2820 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 GAL1pr-SPT10::KanMX spc2Δ::HIS3 |
| FY2821 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 GAL1pr-SPT10::KanMX spc2Δ::HIS3 |
| FY605  | MATa ura3Δ-52 his3Δ200 leu2Δ1 trpΔ63 (hta2-htb2)Δ::TRP1 |
| FY2822 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir1Δ::NatMX |
| FY2824 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir1Δ::NatMX |
| FY2825 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir1Δ::NatMX GAL1pr-SPT10::KanMX |
| FY2826 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir1Δ::NatMX GAL1pr-SPT10::KanMX |
| FY2827 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir1Δ::NatMX spc2Δ::HIS3 |
| FY2828 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir1Δ::NatMX spc2Δ::HIS3 |
| FY2829 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir1Δ::NatMX GAL1pr-SPT10::KanMX spc2Δ::HIS3 |
| FY2830 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir1Δ::NatMX GAL1pr-SPT10::KanMX spc2Δ::HIS3 |
| FY2831 | MATa lys2-128 his3Δ200 (ura3Δ0 or ura3-52) (lea2Δ0 or leu2Δ1) trpΔ63 sir1Δ::NatMX (hta2-htb2)Δ::TRP1 |
| FY2832 | MATa lys2-128 his3Δ200 (ura3Δ0 or ura3-52) (lea2Δ0 or leu2Δ1) trpΔ63 sir1Δ::NatMX (hta2-htb2)Δ::TRP1 |
| FY2833 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 SPT10-13×MYC::KanMX |
| FY2834 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 SPT10-13×MYC::KanMX |
| FY2835 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 SPT10-13×MYC::KanMX |
| FY2836 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 SPT10-13×MYC::KanMX |
| FY2837 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 SPT10-13×MYC::KanMX |
| FY2838 | MATa lys2-128 his3Δ200 ura3Δ0 leu2Δ0 sir1Δ::NatMX |
| FY630a | MATa his4-9126 ura3-52 leu2Δ1 lys2Δ202::LYS2-dam |
| L1134 | MATa his3Δ200 ura3Δ0 leu2Δ0 tel1::R::URA3 |
| L1135 | MATa his3Δ200 ura3Δ0 leu2Δ0 tel1::R::URA3 |
| L1136 | MATa ura3Δ0 leu2Δ0 tel1::R::URA3 GAL1pr-SPT10::KanMX |
| L1137 | MATa ura3Δ0 ura3-167 (lea2Δ0 or leu2Δ1) tel1::R::URA3 spc2Δ::HIS3 |
| L1138 | MATa lys2-128 his3Δ200 (ura3-52 or ura3-320) (leu2Δ1 or leu2Δ0) tel1::R::URA3 (hta2-htb2)Δ::TRP1 |
| L1139 | MATa lys2-128 his3Δ200 (ura3-52 or ura3-320) (leu2Δ1 or leu2Δ0) tel1::R::URA3 (hta2-htb2)Δ::TRP1 |
| L1140 | MATa lys2-128 his3Δ200 (ura3-52 or ura3-320) (leu2Δ1 or leu2Δ0) tel1::R::URA3 (hta2-htb2)Δ::TRP1 |
| L1141 | MATa lys2-128 his3Δ200 (ura3-52 or ura3-320) (leu2Δ1 or leu2Δ0) tel1::R::URA3 (hta2-htb2)Δ::TRP1 |
| L1142 | MATa lys2-128 his3Δ200 (ura3-52 or ura3-320) (leu2Δ1 or leu2Δ0) tel1::R::URA3 (hta2-htb2)Δ::TRP1 |
| L1143 | MATa lys2-128 his3Δ200 (ura3-52 or ura3-320) (leu2Δ1 or leu2Δ0) tel1::R::URA3 (hta2-htb2)Δ::TRP1 |
| L1144 | MATa lys2-128 his3Δ200 (ura3-52 or ura3-320) (leu2Δ1 or leu2Δ0) tel1::R::URA3 (hta2-htb2)Δ::TRP1 |
| L1145 | MATa lys2-128 his3Δ200 leu2Δ0 tel1::R::URA3 |
| L1146 | MATa hisΔ200 ura3Δ0 leu2Δ0 tel1::R::URA3 GAL1pr-SPT10::KanMX |
| L1147 | MATa hisΔ200 ura3Δ0 leu2Δ0 tel1::R::URA3 GAL1pr-SPT10::KanMX |
| L1148 | MATa hisΔ200 ura3Δ0 leu2Δ0 tel1::R::URA3 GAL1pr-SPT10::KanMX |
| L1149 | MATa hisΔ200 ura3Δ0 leu2Δ0 tel1::R::URA3 GAL1pr-SPT10::KanMX |
| L1150 | MATa ura3Δ0 ura3-52 (ley2Δ0 or leu2Δ1) lys2Δ202::LYS2-dam |
| L1151 | MATa ura3Δ0 ura3-52 (ley2Δ0 or leu2Δ1) lys2Δ202::LYS2-dam |

RESULTS

Use of the GAL1pr-SPT10 allele to decrease Spt10 levels without causing a severe growth defect. Deletion of SPT10 causes a severe slow-growth defect, and spontaneous suppressors of this defect arise at a high rate (8, 50). These suppressor mutations occur in many different genes, causing a variety of effects on spt10Δ growth and other phenotypes (8). Out of concern that the slow growth and suppressors would complicate our analysis of spt10A silencing phenotypes, we constructed a repressible SPT10 allele in which SPT10 is regulated by the GAL1 promoter. When strains with this allele are grown in glucose-containing media, SPT10 mRNA levels are reduced but not abolished, presumably because repression of the GAL1pr-SPT10 allele is leaky (Fig. 1A). The decrease in expression confers an Spt− phenotype (albeit not as severe as for spt10Δ strains) (Fig. 1C), yet the strains grow significantly better than spt10Δ strains (Fig. 1B). Unless otherwise noted, all experiments with the GAL1pr-SPT10 allele in this study were conducted under repressive conditions (2% glucose and no galactose).
Generation times of wild-type, \textit{spt10}\textsuperscript{Δ}, \textit{GAL-SPT10}\textsuperscript{Δ}, and \textit{sp21}\textsuperscript{Δ} strains.

### Results

#### Characterization of \textit{GAL1pr-SPT10} and \textit{sp21}\textsuperscript{Δ} defects in telomere position effect.

To begin our characterization of possible silencing defects, we assayed silencing near telomeres in both \textit{GAL1pr-SPT10} and \textit{sp21}\textsuperscript{Δ} strains. To do this, we utilized a \textit{telV-R::URA3} reporter (2), in which the \textit{URA3} gene is placed near the telomere on the right arm of chromosome \textit{V}, where it is normally subject to silencing. Growth on 5-FOA medium reflects intact silencing (see Materials and Methods). Using the \textit{GAL1pr-SPT10} and \textit{GAL-SPT10}\textsuperscript{Δ} reporter system, we found that silencing near the telomere is defective in both \textit{GAL1pr-SPT10} and \textit{sp21}\textsuperscript{Δ} mutants (Fig. 2A). \textit{GAL1pr-SPT10} and \textit{sp21}\textsuperscript{Δ} strains are able to grow well on 5-FOA medium in a \textit{ura3}\textsuperscript{Δ} background without the telomeric reporter (data not shown), indicating that 5-FOA is not toxic to the mutants. Given that the best-characterized function of Spt10 and Spt21 is to activate expression of the \textit{HTA2-HTB2} histone genes (13, 15, 28), we also tested whether a \textit{hta2-htb2}\textsuperscript{Δ} mutant is also impaired for telomeric silencing using the \textit{telV-R::URA3} reporter system. Our results show that this mutant does not have a telomeric silencing defect, suggesting that the decreased \textit{HTA2-HTB2} transcription in \textit{spt10} and \textit{sp21} mutants is not sufficient to cause defective telomeric silencing. Taken together, our results extend the previous results of Braun et al. (6) to show that both \textit{SPT10} and \textit{SPT21} are required for telomeric silencing.

To determine whether silencing defects also occur at a natural telomere-proximal gene, we measured the mRNA levels of \textit{YFR057W}, an endogenous subtelomeric gene located approximately 0.6 kb from the telomere on the right arm of chromosome \textit{VI} and subject to silencing (81). Using reverse transcription followed by real-time PCR quantitation, we found that in \textit{GAL1pr-SPT10} and \textit{sp21}\textsuperscript{Δ} mutants, \textit{YFR057W} mRNA levels are elevated 5- to 7-fold over wild-type levels (Fig. 2B), indicative of a silencing defect. The derepression is comparable between the two mutants, although less than what we observed for a \textit{sir2}\textsuperscript{Δ} mutant. Silencing of \textit{YFR057W} is intact in the \textit{hta2-htb2}\textsuperscript{Δ} mutant (Fig. 2B), furthering the evidence that the derepression is caused by a mutation in the \textit{SPT10} and \textit{SPT21} loci.

#### Discussion

Taken together, these results suggest that Spt10 and Spt21 are necessary for the proper repression of subtelomeric genes.

### Materials and Methods

We have used the following methods and techniques in our experiments:

1. **Northern Blotting:** RNA was isolated from exponentially growing cultures and subjected to Northern blotting. The blots were then probed with radioactive probes specific to the \textit{YFR057W} gene.

2. **Real-time PCR:** Using the \textit{GAL1pr-SPT10} reporter, we found that silencing near the telomere is defective in both \textit{GAL1pr-SPT10} and \textit{sp21}\textsuperscript{Δ} strains. Exponentially growing cultures were diluted to 100 cells/ml and then grown to a density of 1 \times 10^7 to 2 \times 10^7 cells/ml. The doubling time was calculated as time of incubation + log(final density/initial density).

3. **Knockout Mutations:** knockout mutants of \textit{SPT10} and \textit{SPT21} were generated using a double-crossover strategy.

4. **Transcriptional Profiling:** RNA samples were subjected to transcriptional profiling using microarrays.

5. **Bioinformatic Analysis:** Sequence data was analyzed using bioinformatic tools to identify potential regulatory elements.

These methods allowed us to characterize the role of Spt10 and Spt21 in telomeric silencing.
Spt10 and Spt21 are necessary for proper silencing in a native telomeric context. Previous work has suggested that while Spt10 and Spt21 share many roles, they do have some nonoverlapping roles. Therefore, we asked whether Spt10 and Spt21 might have nonoverlapping roles in regulating silencing near the telomere. By analysis of YFR057W mRNA levels, we found that the GAL1pr-SPT10 spt21/H9004 double mutant shows a more substantial derepression of YFR057W transcript levels than either single mutant (Fig. 2B). The stronger phenotype of the GAL1pr-SPT10 spt21/H9004 double mutant suggests that Spt10 and Spt21 are not completely redundant in their roles in silencing.

Spt10 and Spt21 play a role in silencing at HMLα. Given that both Spt10 and Spt21 are required for telomeric silencing, we tested whether they are also required for silencing of the silent mating type cassettes, HMRα and HMLα. We began by assessing mating ability, which is decreased in silencing mutants due to the expression of both sets of mating information. Quantitative mating assays (see Materials and Methods) show that while the GAL1pr-SPT10 and spt21Δ single mutants show no significant defect, the GAL1pr-SPT10 spt21Δ double mutant shows a substantial decrease in mating in a MATα background, to approximately 20% of wild-type levels, while showing no significant defect in a MATα background (Fig. 3A). Mating type-specific mating defects have been observed previously in mutants with intermediate effects on silencing, as silencing is believed to be more easily disrupted at HML than at HMR (83). Our results suggest that Spt10 and Spt21 are both required for silencing of HMLα.

We also tested whether either GAL1pr-SPT10 or spt21Δ exhibits a mating defect when combined with sir1Δ. The rationale for testing this possibility came from a screen for mutations in the nonessential gene deletion set that impair growth when combined with spt10/H9004 (8), using methods previously described (77). The results of that screen suggested that a sir1Δ spt10Δ double mutant is inviable; however, when we dissected tetrads after sporulation of an spt10Δ/SPT10 sir1Δ/SIR1 heterozygote, we readily obtained sir1Δ spt10Δ double mutants at the expected frequency, with no apparent growth defects. This result was reminiscent of an earlier study (80) in which a sir1Δ dot1Δ double mutant appeared to be inviable in the same type of screen, but the apparent inviability was actually the result of...
a defect in silencing of the HMLα locus, as mating must be intact in order for the screen to work properly. Thus, our screen results raised the possibility that spl10Δ sir1Δ double mutants are defective for silencing of HMLα.

To assess potential silencing defects for GAL1pr-SPT10 and spl21Δ in combination with sir1Δ, we performed quantitative mating assays with sir1Δ GAL1pr-SPT10 and sir1Δ spl21Δ strains. In a MATα background, both sir1Δ GAL1pr-SPT10 and sir1Δ spl21Δ strains show a severe defect in mating ability (Fig. 3A). In contrast, in a MATα background, neither strain shows a mating defect beyond that seen in a sir1Δ single mutant. Analysis of a sir1Δ (hta2-hhb2)Δ double mutant suggests that decreased HTA2-HTB2 transcript levels are insufficient to explain the GAL1pr-SPT10 and spl21Δ mating defects (Fig. 3A). The synergistic mating defects with sir1Δ classify GAL1pr-SPT10 and spl21Δ as Eso (enhancer of sir one) mutants, many of which also have effects limited to the MATα mating type (55).

To directly examine silencing at HMRα and HMLα, we measured the levels of transcripts by Northern hybridization analysis. To do this, we tested MATα strains for transcription of HMLα and MATα strains for transcription of HMRα. Our analysis showed that transcription at HMLα and HMRα correlates with the mating defects we observed. First, in GAL1pr-SPT10 and spl21Δ single mutants of either mating type, no aberrant mating transcripts could be detected. However, in a MATα background, both α1 and α2 transcripts are present at significantly greater levels in sir1Δ GAL1pr-SPT10 and sir1Δ spl21Δ mutants than in a sir1Δ single mutant (Fig. 3B). In contrast, in a MATα background, sir1Δ GAL1pr-SPT10 and sir1Δ spl21Δ double mutants expressed both α1 and α2 transcripts at a level comparable to that for a sir1Δ single mutant (data not shown). These results are consistent with a synergistic silencing defect between sir1Δ and GAL1pr-SPT10 or spl21Δ in a MATα background. No increased derepression was seen in sir1Δ (hta2-hhb2)Δ mutants, again suggesting that the GAL1pr-SPT10 and spl21Δ phenotypes cannot be explained by decreased HTA2-HTB2 transcription alone. Taken together, the mating and transcriptional defects suggest that Spl10 and Spl21 contribute to silencing at HMLα.

**rDNA silencing is strengthened in GAL1pr-SPT10 and spl21Δ mutants.** Given that GAL1pr-SPT10 and spl21Δ show defects in silencing at the telomere and the silent mating type loci, we investigated whether they show defects in rDNA silencing as well. We utilized a reporter containing a modified URA3 gene inserted in a single copy at the rDNA locus (see Materials and Methods). Growth on SC medium without uracil reflects the ability to silence URA3 expression, with slower growth reflecting a greater degree of silencing. Consistent with previous observations, the sir2Δ mutant shows more rapid growth on SC medium without uracil, reflecting impaired silencing, and the sir4Δ mutant shows slower growth, reflecting an increased level of silencing (64). Our results indicate that the GAL1pr-SPT10 and spl21Δ mutations, despite causing decreased silencing at other regions, cause increased silencing at the rDNA locus, similar to the case for sir4Δ (Fig. 4). The increased rDNA silencing (Irs−) phenotype of sir4Δ mutants has been attributed to an excess of free Sir2, which is then able to bind and silence the rDNA (66, 67). Our results raise the possibility that free Sir2 levels are also increased in GAL1pr-SPT10 and spl21Δ mutants.

**Spl10 and Spl21 do not detectably associate with a silenced region.** To test whether Spl10 and Spl21 affect silencing by direct association with silenced loci, we performed chromatin immunoprecipitation (ChIP) of Spl10 and Spl21 using strains in which a 13Myc epitope tag was fused to the C terminus of either protein (see Materials and Methods). To test single-copy DNA, we focused our analysis on the subtelomeric region on the right arm of chromosome VI (telVI-R), which is silenced in an Spl10- and Spl21-dependent fashion (Fig. 2). We assayed for Spl10 and Spl21 binding by ChIP at 0.6 kb from the telomere, a position that overlaps the YFR057W gene, and at 2.8 kb from the same telomere, where Sir binding and silencing also occur, albeit at lower levels (25, 72). Our results do not detect significant binding of either Spl10 or Spl21 at either location (see Fig. S1 in the supplemental material), a result consistent with genome-wide ChIP analysis using microarrays (8, 15). As a positive control, both Spl10 and Spl21 did show significant binding at the HTA2-HTB2 promoter, which is consistent with previous findings (28). Although this is a negative result, and it remains possible that Spl10 and Spl21 are physically present at this silenced region, our results suggest that Spl10 and Spl21 may influence the telomere position effect without direct binding at the telomere.

**GAL1pr-SPT10 and spl21Δ silencing phenotypes can be partially rescued by overexpression of histone genes or SIR3.** Previous studies have shown that overexpression of histone genes can suppress some spl10 and spl21 mutant phenotypes. In one case, overexpression of HTA1-HTB1, encoding histones H2A and H2B, was able to suppress the Spt− phenotypes of both spl10 and spl21 mutants (61), while in another case, overexpression of two loci, HTA1-HTB1 and HHT1-HHF1, encoding the four core histones, was able to suppress the poor-growth phenotype caused by spl10Δ (15). To determine if overexpression of histone genes can also suppress the GAL1pr-SPT10 and spl21Δ silencing phenotypes, we tested whether a high-copy-number plasmid containing HTA1-HTB1 and HHT1-HHF1 caused any effect on telomere position effect, using the telIV-R::URA3 reporter. Our results show that this high-copy-number histone plasmid moderately suppresses the GAL1pr-SPT10 silencing defect and strongly suppresses the...
spt21Δ silencing defect (Fig. 5A). Furthermore, this plasmid suppressed the Eso− phenotype weakly for GAL1pr-SPT10 and strongly for spt21Δ (data not shown). However, neither HTA1-HTB1 nor HHT1-HHF1 alone conferred any suppression of the Eso− phenotypes (data not shown). These results show that additional copies of the histone gene pairs HTA1-HTB1 and HHT1-HHF1 can strongly suppress the silencing defects of spt21Δ mutants while having a smaller effect on GAL1pr-SPT10 mutants.

We then asked whether overexpression of any SIR genes can rescue the GAL1pr-SPT10 and spt21Δ silencing defects. The Eso− mating phenotypes of GAL1pr-SPT10 and spt21Δ mutants suggest that Spt10 and Spt21 have some roles in silencing complementary to Sir1, and overexpressing SIR1 might compensate for the GAL1pr-SPT10 and spt21Δ silencing defects near the telomere. In addition, overproduction of Sir3 has been shown to enhance the spreading of silencing near the telomere of a wild-type cell (56), raising the possibility that its overproduction can also improve silencing in GAL1pr-SPT10 and spt21Δ mutants. Our results show that a high-copy-number plasmid carrying SIR3 can weakly suppress the defects in telomere position effect in both GAL1pr-SPT10 and spt21Δ mutants in a telV-R:URA3 reporter strain, with suppression stronger in the spt21Δ mutant (Fig. 5B). This plasmid could also partially suppress the Eso− mating phenotypes of both mutants (data not shown). Among the four SIR genes tested, only the SIR3 plasmid conferred any suppression for either type of silencing (data not shown). These results indicate that increased Sir3 levels can partially compensate for decreases in Spt10 and Spt21 levels.

**Effect of GAL1pr-SPT10 and spt21Δ on Sir protein levels and binding.** Because Sir3 overexpression can partially suppress the GAL1pr-SPT10 and spt21Δ silencing defects, it is possible that Spt10 and Spt21 control silencing by influencing the levels or localization of Sir proteins. To measure the levels of Sir2, Sir3, and Sir4, we performed Western blotting using antibodies specific to each of these proteins. Our results showed that Sir2, Sir3, and Sir4 levels are not significantly altered in GAL1pr-SPT10 or spt21Δ mutants or in the GAL1pr-SPT10 spt21Δ double mutant (Fig. 6A).

To test whether GAL1pr-SPT10 and spt21Δ might affect Sir complex recruitment to silenced regions, we used ChIP to measure the level of association of Sir2 and Sir3 at the right telomere of chromosome VI to the same regions 0.6 and 2.8 kb from the telomere. Our results show that in a GAL1pr-SPT10 mutant, Sir2 binding occurs at approximately 80% of wild-type levels in both regions, while Sir3 binding is nearly indistinguishable from wild type (Fig. 6B and C). In an spt21Δ mutant the levels of Sir2 and Sir3 binding show a slightly greater decrease in both regions, to 60 to 70% of wild-type levels at 0.6 kb from the telomere and to 40 to 50% of wild-type levels at 2.8 kb. For both mutants, the level of Sir association is much greater than in a sir2Δ control strain. Because spt10 and spt21 mutants have decreased expression of histone genes (13), we considered whether the modest decrease in Sir2 and Sir3 association in this mutant could be accounted for by decreased nucleosome density. To test this, we also used ChIP to measure histone H3 association at the same regions near telomere VI-R. We found that the level of histone H3 associated with these regions was unaffected in both the GAL1pr-SPT10 and spt21Δ mutants (Fig. 6D). In conclusion, our ChIP assays show that GAL1pr-SPT10 and spt21Δ mutants have modestly decreased levels of Sir2 and Sir3 binding.

**Mutant effects on histone modifications at silenced loci.** Given the silencing defects in GAL1pr-SPT10 and spt21Δ mutants, we asked whether these mutants show changes in the levels of histone modifications associated with silenced chromatin. We focused our analysis on two modifications previously shown to affect silencing: methylation of histone H3 on lysine 79 (H3 K79me) (18, 79) and acetylation of the N-terminal tail of histone H4 (H4-Ac) (34). To analyze these histone modifications at a silenced region in GAL1pr-SPT10 and spt21Δ strains, we performed ChIP, again assaying the regions at 0.6 kb and 2.8 kb from the right telomere of chromosome VI. Our results show that in both GAL1pr-SPT10 and spt21Δ mutants, the levels of the two histone modifications were nearly indistinguishable from wild type levels at 0.6 kb from the telomere; however, the levels of both histone modifications are increased in both mutants at 2.8 kb from the telomere (Fig. 7A and B). By Western analysis, neither GAL1pr-SPT10 nor spt21Δ mutations altered the overall level of these modified histones (Fig. 7C). These results indicate that GAL1pr-SPT10 and spt21Δ mutants have significant alterations in the pattern of histone modifications, especially at greater distances from the telomere.
Silenced chromatin is improperly assembled at telomeres in \textit{GAL1pr-SPT10} and \textit{spt21}/\textit{H9004} mutants. Previous studies showed that chromatin isolated from \textit{spt10} mutants is hypersensitive to micrococcal nuclease digestion \textit{in vitro} compared to chromatin from a wild-type strain (15). To examine chromatin structure \textit{in vivo} for both \textit{spt10} and \textit{spt21} mutants, we assayed the accessibility of chromatin to bacterial dam methylase, which was previously shown to detect changes in chromatin structure in silenced regions (22, 29, 63). To do this, we examined the same region of telomere VI-R as described above, using previously described methods (69, 84) employing the methylation-specific enzymes DpnI, MboI, and Sau3AI, which cut methylated, unmethylated, and all GATC sequences, respectively (diagrammed in Fig. 8A). When we examined a region 1.3 kb from telomere VI-R, our results showed that DNA from the \textit{GAL1pr-SPT10} and \textit{spt21}/\textit{H9004} mutants is more readily cleaved by DpnI and less readily cleaved by MboI than is wild-type DNA (Fig. 8B, top panel). This pattern is similar to that seen in a \textit{Sir2\Delta} mutant and indicates that the chromatin has increased accessibility for dam methylation, consistent with the silencing defect seen in these mutants. At 20 kb from the same telomere, dam methylation accessibility in the \textit{Sir2\Delta} mutant more closely parallels that of the wild type, suggesting that chromatin structure is not substantially perturbed at that location (Fig. 8B, middle panel). However, in both \textit{GAL1pr-SPT10} and \textit{spt21\Delta} mutants the chromatin is less accessible to MboI cleavage (Fig. 8B; compare band D to bands E and F), suggesting that it remains predominantly methylated, reflecting a more accessible chromatin structure. Differences from the wild type are less apparent with DpnI digestion at this locus, potentially because the enzyme digests both fully methylated DNA, which is inaccessible to dam methylation, and hemimethylated DNA, which is partially accessible. Taken together, these results suggest that the chromatin in \textit{GAL1pr-SPT10} and \textit{spt21\Delta} mutants is more accessible than that in the wild type to dam methylation at both 1.3 kb and 20 kb from the telomere, reflecting an altered chromatin structure.

\section*{DISCUSSION}

Our analysis of \textit{spt10} and \textit{spt21} mutations has shown that both \textit{spt10} and \textit{spt21} play a role in silencing in \textit{S. cerevisiae}. Both \textit{spt10} and \textit{spt21} mutations impair silencing near telomeres and at the \textit{HML\alpha} mating type locus, while strengthening silencing at the rDNA. The most well-characterized role for \textit{spt10} and \textit{spt21} is in activating the transcription of histone genes, primarily \textit{HTA2-HTB2} (15, 15, 27). However, deletion of this histone gene pair does not cause silencing defects, suggesting that \textit{spt10} and \textit{spt21} control silencing, at
Sir3 within its N-terminal BAH domain (80, 82), which results in increased binding specificity to the unmethylated histone H3 K79 characteristic of silenced regions (80). To prevent inappropriate Sir3 binding, Dot1 methylates histone H3 K79 residues in regions that are not silenced (1, 53, 79). Together, mutations in Nat1, Ard1, Dot1, and the Sir3 BAH domain are thought to disrupt silencing by interfering with Sir3 localization to silenced regions. The parallels between the silencing phenotypes of the above-described Sir3 regulatory mutants and spt10 and spt21 mutants raise the possibility that Spt10 and Spt21 may regulate silencing at telomeres and silent mating loci, at least in part, by modulating Sir3 function.

One possibility is that the silencing defect in spt10 and spt21 mutants is caused by multiple effects of the mutations. The modest decrease in the level of Sir proteins recruited to telV-R in spt10 and spt21 mutants, along with the significantly increased accessibility to E. coli dam methylase, suggests that a significant contribution to the silencing defect in spt10 and spt21 mutants occurs at a step subsequent to Sir protein recruitment. These phenotypes place spt10 and spt21 mutants in the same category as two other classes of silencing mutants recently described: those that alter H3 K56 (84) and those that delete the H3 amino-terminal tail (69). These histone mutants and other evidence (36, 86, 87) strongly suggest that the presence of Sir proteins, while necessary, is not sufficient to confer silencing. The silencing defects seen in spt10 and spt21 mutants are similar to those for H3 tail deletion in that effects are strongest at telomeres and are only seen at HM loci in the presence of a second mutation (69). Thus, as in the case of the H3 tail deletion mutant, the spt10 and spt21 mutations may impair higher-order chromatin structure. The spt10 and spt21 defects appear to be distinct from those that result from alteration of H3 K56 in that the spt10 and spt21 mutations strengthen rDNA silencing, while the changes at H3 K56 were reported to weaken it (84). In common to all of these mutants, however, is the lack of a strong defect in the recruitment of Sir proteins, yet with greatly increased accessibility to E. coli dam methylase (69, 84). Perhaps changes in chromatin structure, as revealed by the increased accessibility to E. coli dam methylase, alter the ability of Sir proteins to function.

The mechanism by which Spt10 and Spt21 control silencing is currently unclear. Based on previous results (15) and our ChiP experiments, neither protein appears to associate with chromatin at telomeres or anywhere other than histone gene promoters. While our results are consistent with a role for Spt10 and Spt21 in silencing outside their roles in histone gene regulation, until the mechanism is understood, we cannot rule out a contribution by the altered histone levels that exist in spt10 and spt21 mutants. Purification of Spt10 from S. cerevisiae has not provided any clues, as no interactions with other proteins were detected (8), though Spt10 has been shown to interact directly with Spt21 when they are both overexpressed in E. coli or when Spt21 is overexpressed in yeast (28). Given that Spt10 has sequence motifs that strongly suggest that it is an acetyltransferase (52), and given that genetic evidence has shown these motifs to be important for Spt10 function in vivo (28), one intriguing possibility is that Spt10 acetylates a nonhistone protein that

---

**FIG. 7.** Measurement of histone H3 K79 dimethylation and histone H4 acetylation levels at telomere VI-R in GAL1pr-SPT10 and spt21Δ mutants. All strains were grown in glucose, which represses expression of the GAL1pr-SPT10 allele. (A) ChiP of histone H3 K79 dimethylation was performed using the same strains, primers, and calculations as for Fig. 6, using antibodies specific to dimethyl histone H3 K79. Values are shown relative to total histone H3 ChiP. (B) ChiP of acetylated histone H4. (C) Western blot to measure total cellular levels of dimethylated histone H3 K79, acetylated histone H4, total histone H3, and Pgk1 as a loading control.

---

least in part, by a mechanism that is independent of their roles in regulating HTA2-HTB2 transcription.

Previous work has shown that mutations in other genes cause a profile of silencing phenotypes similar to that caused by spt10 and spt21 mutations, including a silencing defect at HMLα but not HMRα, an Eso− phenotype, and a telomeric silencing defect. The factors identified by these mutations, all of which play roles in Sir3 localization, include the NatA acetyltransferase complex subunits Nat1 and Ard1 (2, 71, 83), the histone H3 K79 methyltransferase Dot1 (18, 79), and the N-terminal BAH domain of Sir3 (71). These proteins have been found to regulate silencing through a common genetic pathway (80). The NatA complex acetylates...
is required for silencing. Clearly, the elusive acetyltransferase activity of Spt10 must be understood in order to elucidate the functions of Spt10 and Spt21 in silencing and other activities in which they have been implicated.

ACKNOWLEDGMENTS

We thank Hannah Blitzblau, Milan de Vries, and Erin Loeliger for helpful comments on the manuscript. We are grateful to Steve Bell, Paul Kaufman, Danesh Moazed, and Lorraine Pillus for plasmids and antisera.

This work was supported by NIH grant GM32967 to F.W.

REFERENCES

1. Altf, M., R. T. Utley, N. Lacoste, S. Tan, S. D. Briggs, and J. Cote. 2007. Interplay of chromatin modifiers on a short basic patch of histone H4 tail defines the boundary of telomeric heterochromatin. Mol. Cell 28:1002–1014.
2. Aparicio, O. M., B. L. Billington, and D. E. Gottschling. 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cer- evisiae. Cell 65:1279–1287.
3. Askree, S. H., T. Yehuda, S. Smolikov, R. Gurevich, J. Hawk, C. Coker, A. Altaf, M., R. T. Utley, N. Lacoste, S. Tan, S. D. Briggs, and J. Cote.
4. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1997. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, NY.
5. Barton, A. B., and D. B. Kahneck. 2006. Telomeric silencing of an open reading frame in Saccharomyces cerevisiae. Genetics 173:1169–1173.
6. Braun, M. A., P. J. Costa, E. M. Crisucci, and K. M. Arndt. 2007. Identification of Rcr1, a nuclear RING domain protein with functional connections to chromatin modification in Saccharomyces cerevisiae. Mol. Cell. Biol. 27: 2800–2811.
7. Buhler, M., and S. M. Gasser. 2009. Silent chromatin at the middle and ends: lessons from yeasts. EMBO J. 28:2149–2161.
8. Chang, J. 2009. Ph.D. thesis. Harvard University, Cambridge, MA.
9. Cho, R. J., M. J. Campell, E. A. Winzeler, L. Steinmetz, A. Conway, L. Wodicka, T. G. Wolfsberg, A. E. Gabrieliian, D. Landsman, D. J. Lockhart, and R. W. Davis. 1998. A genome-wide transcriptional analysis of the mitotic cell cycle. Mol. Cell 2:65–73.
10. Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter. 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene 110: 119–122.
11. Clapier, C. R., and B. R. Cairns. 2009. The biology of chromatin remodeling complexes. Annu. Rev. Biochem. 78:273–304.
12. Denis, C. L., and T. Malvar. 1990. The CCR4 gene from Saccharomyces cerevisiae is required for both nonfermentative and spt-mediated gene expression. Genetics 124:283–291.
13. Dollard, C., S. L. Ricupero-Hovasse, G. Natsoulis, J. D. Boeke, and F. Winston. 1994. SPT10 and SPT21 are required for transcription of particular histone genes in Saccharomyces cerevisiae. Mol. Cell. Biol. 14:5223–5228.
14. Donze, D., C. R. Adams, J. Rine, and R. T. Kamakaka. 2005. Toward biochemical understanding of a transcriptionally silenced chromosomal domain in Saccharomyces cerevisiae. J. Biol. Chem. 280:8629–8632.
15. Eriksson, P. R., G. Mendiratta, N. B. McLaughlin, T. G. Wolfsberg, L. Marino-Ramirez, T. A. Pompa, M. Jainerin, D. Landsman, C. H. Shen, and D. J. Clark. 2005. Global regulation by the yeast Spt10 protein is mediated through chromatin structure and the histone upstream activating sequence elements. Mol. Cell. Biol. 25:9127–9137.
16. Fassler, J. S., and F. Winston. 1988. Isolation and analysis of a novel class of suppressor of Ty insertion mutations in Saccharomyces cerevisiae. Genetics 118:203–212.
17. Fox, C. A., and K. H. McConnell. 2005. Toward biochemical understanding of a transcriptionally silenced chromosomal domain in Saccharomyces cerevisiae. J. Biol. Chem. 280:8629–8632.
18. Frederiks, F., M. Tzouros, G. Oudgenoeg, T. van Welsem, M. Fornerod, J. Krijgsveuld, and F. van Leeuwen. 2008. Nonprocessive methylation by Dot1

FIG. 8. Dam methylation assays to probe chromatin structure in vivo. (A) A restriction map of relevant sites within the regions probed. The region 1.3 kb from the right telomere of chromosome VI is subject to silencing, and the telomere-proximal end lies 200 bp from the sequences probed in ChIP and YFR057W RT-PCR (Fig. 2B, A, and 7). The region approximately 20 kb from the same telomere is at a distance not expected to be subject to telomere position effect (23). In both cases, multiple dam methylation sites occur between NdeI digest sites, resulting in multiple bands. For the 1.3-kb region, a dam methylation site is present from the NdeI site, and digestion does not result in a significant mobility shift (band A). A region within MSN5, where no dam methylation sites occur between two NdeI sites, serves as a loading control. (B) Southern blot analysis of genomic DNA digested with NdeI and the indicated methylation-specific enzymes, separated on an agarose gel and probed for the
leads to functional redundancy of histone H3K79 methylation states. Nat. Struct. Mol. Biol. 15:550–557.

19. Gallagher, S. R. 2006. One-dimensional SDS gel electrophoresis of proteins. Curr. Protoc. Mol. Biol. 75:4.1–8.4.37.

20. Garzon, T., G. Massies, M. Nelson, J. M. Akey, D. M. Ruderfer, L. Kruglyak, J. A. Simon, and A. Bedalov. 2006. Telomere length as a quantitatively trait: genome-wide survey and genetic mapping of telomere length-control genes in yeast. PLoS Genet. 2:e35.

21. Goldstein, A. L., and J. H. McCusker. 1993. Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast 15:1541–1553.

22. Gottesman, D. E. 1992. Telomere-proximal DNA in Saccharomyces cerevisiae is refractory to methyltransferase activity in vivo. Proc. Natl. Acad. Sci. U. S. A. 89:4062–4065.

23. Gottesman, D. E., O. M. Aparicio, B. L. Billington, and V. A. Zakian. 1990. Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. Cell 63:751–762.

24. Guthrie, C., and G. R. Fink. 1991. Guide to yeast genetics and molecular biology. Academic Press, San Diego, CA.

25. Hecht, A., S. Strahl-Bolsinger, and M. Grunstein. 1996. Spreading of transcriptionally repressed Sir3 from telomeric heterochromatin. Nature 383:92–96.

26. Hereford, L., K. Fahrner, J. Woolford, Jr., M. Rosbash, and D. B. Kaback. 1991. Isolation of yeast histone genes H2A and H2B. Cell 65:565–618.

27. Hess, D., B. R. Coller, R. G. Zinovyev, K. N. Sternglanz, and F. Winston. 2000. Sir2: an NAD-dependent histone deacetylase that connects silent chromatin in Saccharomyces cerevisiae to Sir3p hyperphosphorylation and strengthens transcriptional silencing. Proc. Natl. Acad. Sci. U. S. A. 100:1820–1825.

28. Hess, D., B. Liu, N. R. Roan, R. Sternglanz, and F. Winston. 2003. Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation. Proc. Natl. Acad. Sci. U. S. A. 100:1820–1825.

29. Imai, S., F. B. Johnson, R. A. Marciniak, M. McVey, P. U. Park, and L. Guarente. 2000. Sir2: an NAD-dependent histone deacetylase that connects chromatin silencing, metabolism, and aging. Cold Spring Harbor Symp. Quant. Biol. 65:297–302.

30. Johnson, A., C. S., and F. Winston. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. Genet. 57:267–272.

31. Huang, J., and D. Moazed. 2003. Association of the RENT complex with untranscribed and coding regions of DNA and a regional requirement for the replication fork block protein Fob1 in DNA silencing. Genes Dev. 17:2162–2176.

32. Imai, S., B. F. Johnson, R. A. Marciniak, M. McVey, P. U. Park, and L. Guarente. 2000. Sir2: an NAD-dependent histone deacetylase that connects chromatin silencing, metabolism, and aging. Cold Spring Harbor Symp. Quant. Biol. 65:297–302.

33. Johnson, A., L. G., T. W. Sikorski, S. Buratowski, C. L. Woodcock, and D. Moazed. 2009. Reconstitution of heterochromatin-dependent transcriptional gene silencing. Mol. Cell 35:769–78.

34. Johnson, L. M., G. Fisher-Adams, and M. Grunstein. 1995. Roles of ABF1, the products of yeast Spt10p activator to the histone upstream activating sequences is homologous to foamy virus integrase. J. Biol. Chem. 270:2257–2269.

35. Komarnitsky, P., E. J. Cho, and S. Buratowski. 1993. Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by Sir3 dosage. Genes Dev. 7:1133–1145.

36. Komarnitsky, P., E. J. Cho, and S. Buratowski. 1995. Epigenetic inheritance of transcriptional states in Saccharomyces cerevisiae. Cell 59:537–547.

37. Krawcheck, C., J. Lowell, A. Clarke, and L. Pillus. 1996. Yeast SAS silencing genes and human genes associated with AML and HIV-Tat interactions are homologous with acetyltransferases. Nat. Genet. 14:22–49.

38. Krieg, L., S. C. Kirchmaier, and J. Rine. 2002. Recruitment of heterochromatin-dependent transcriptional gene silencing. Mol. Cell. Biol. 15:707–719.

39. Krieg, L., J. T. Slama, and R. Sturhahn. 2000. Role of NAD(+) in the deacetylase activity of the Sir2-like proteins. Biochem. Biophys. Res. Comm. 275:685–690.

40. Krieg, L., A. Sutton, S. T. Tavor, R. C. Heller, J. Stebbins, L. Pillus, and R. Sturhahn. 2000. The silencing protein Sir2 and its homologs are NAD-dependent protein deacetylases. Proc. Natl. Acad. Sci. U. S. A. 97:5807–5811.

41. Krieg, L., A. Sutton, S. T. Tavor, R. C. Heller, J. Stebbins, L. Pillus, and R. Sturhahn. 2000. The silencing protein Sir2 and its homologs are NAD-dependent protein deacetylases. Proc. Natl. Acad. Sci. U. S. A. 97:5807–5811.

42. Lai, A., H. Blizbutau, and S. P. Bell. 2002. Cell-cycle control of the establishment of mating-type silencing in S. cerevisiae. Genes Dev. 16:2935–2945.

43. Lack, M., C. Carey, and J. L. Workman. 2007. The role of chromatin during transcription. Cell 128:707–719.

44. Liou, G. G., J. C. Tanny, R. G. Kruger, T. Walz, and D. Moazed. 2005. Assembly of the SIR complex and its regulation by O-acetyl-ADP-ribose, a product of NAD-dependent histone deacetylation. Cell 121:515–527.

45. Longtine, M. S., A. McKenzie III, D. J. Demarini, N. G. Shah, A. Wach, A. Brachet, P. Philippens, and J. R. Pringle. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14:953–961.

46. Loo, S., P. Laurentson, M. Foss, A. Dillin, and J. Rine. 1995. Roles of ABF1, NPL3, and YCL545 in silencing in Saccharomyces cerevisiae. Genes 141:899–902.

47. Loo, S., A. M. Vega-Palas, and M. Grunstein. 2002. Rap1-Sir4 binding independent of other Sir, yku, or histone interactions initiates the assembly of telomeric heterochromatin in yeast. Genes Dev. 16:1528–1539.

48. Martin, F., S. Kueng, P. Robinson, M. Tsai-Pflugfelder, F. Van Leeuwen, M. Ziegler, F. Cubizolles, M. M. Cockell, D. Rhodes, and S. M. Gasser. 2009. Reconstitution of yeast silent chromatin: multiple contact sites and O-ADPR binding load SIR complexes onto nucleosomes in vitro. Mol. Cell 33:225–234.
classes of sir3 mutants enhance the sir1 mutant mating defect and abolish telomeric silencing in *Saccharomyces cerevisiae*. Genetics 155:509–522.

72. Strahl-Bolsinger, S., A. Hecht, K. Luo, and M. Grunstein. 1997. SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev. 11:683–93.

73. Suka, N., K. Luo, and M. Grunstein. 2002. Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. Nat. Genet. 32:378–383.

74. Swanson, M. S., E. A. Malone, and F. Winston. 1991. SPT5, an essential gene important for normal transcription in *Saccharomyces cerevisiae*, encodes an acidic nuclear protein with a carboxy-terminal repeat. Mol. Cell. Biol. 11:3009–3019.

75. Tanner, K. G., J. Landry, R. Sternagran, and J. M. Denu. 2000. Silent information regulator 2 family of NAD-dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. Proc. Natl. Acad. Sci. U. S. A. 97:14176–14182.

76. Tanny, J. C., G. J. Dowd, J. Huang, H. Hilz, and D. Moazed. 1999. An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing. *Cell* 99:735–745.

77. Tong, A. H., M. Evangelista, A. B. Parsons, H. Xu, G. D. Bader, N. Page, M. Robinson, S. Raghibizadeh, C. W. Hogue, H. Bussey, B. Andrews, M. Tyers, and C. Boone. 2001. Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science 294:2364–2368.

78. Triolo, T., and R. Sternagran. 1996. Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. Nature 381:251–253.

79. van Leeuwen, F., P. R. Gafken, and D. E. Gottschling. 2002. Dot1p mediates silencing in yeast by methylation of the nucleosome core. Cell 109:745–756.

80. van Welsem, T., F. Frederik, K. F. Verzijlbergen, A. W. Faber, Z. W. Nelson, D. A. Egan, D. E. Gottschling, and F. van Leeuwen. 2008. Synthetic lethal screens identify gene silencing processes in yeast and implicate the acetylated amino terminus of Sir3 in recognition of the nucleosome core. Mol. Cell. Biol. 28:3861–3872.

81. Vega-Palas, M. A., E. Martin-Figueroa, and F. J. Florencio. 2000. Telomeric silencing of a natural subtelomeric gene. Mol. Gen. Genet. 263:287–291.

82. Wang, X., J. J. Connelly, C. L. Wang, and R. Sternagran. 2004. Importance of the Sir3 N terminus and its acetylation for yeast transcriptional silencing. Genetics 168:547–551.

83. Whiteway, M., R. Freedman, S. Van Arsdell, J. W. Szostak, and J. Thorner. 1987. The yeast ARD1 gene product is required for repression of cryptic mating-type information at the HML locus. Mol. Cell. Biol. 7:3713–3722.

84. Xu, F., Q. Zhang, K. Zhang, W. Xie, and M. Grunstein. 2007. Sir2 deacetylases histone H3 lysine 56 to regulate telomeric heterochromatin structure in yeast. Mol. Cell 27:890–900.

85. Yamashita, I. 1993. Isolation and characterization of the SUD1 gene, which encodes a global repressor of core promoter activity in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 241:616–626.

86. Yang, B., and A. L. Kirchmaier. 2006. Bypassing the catalytic activity of SIR2 for SIR protein spreading in *Saccharomyces cerevisiae*. Mol. Biol. Cell 17:5287–5297.

87. Yang, B., A. Miller, and A. L. Kirchmaier. 2008. HST3/HST4-dependent deacetylation of lysine 56 of histone H3 in silent chromatin. Mol. Biol. Cell 19:4993–5005.