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A Genetic Screen for Ribosomal DNA Silencing Defects Identifies Multiple DNA Replication and Chromatin-Modulating Factors

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Transcriptional silencing in *Saccharomyces cerevisiae* occurs at several genetic loci, including the ribosomal DNA (rDNA). Silencing at telomeres (telomere position effect [TPE]) and the cryptic mating-type loci (*HML* and *HMR*) depends on the silent information regulator genes, *SIR1*, *SIR2*, *SIR3*, and *SIR4*. However, silencing of polymerase II-transcribed reporter genes integrated within the rDNA locus (rDNA silencing) requires only *SIR2*. The mechanism of rDNA silencing is therefore distinct from TPE and *HM* silencing. Few genes other than *SIR2* have so far been linked to the rDNA silencing process. To identify additional non-Sir factors that affect rDNA silencing, we performed a genetic screen designed to isolate mutations which alter the expression of reporter genes integrated within the rDNA. We isolated two classes of mutants: those with a loss of rDNA silencing (*lrs*) phenotype and those with an increased rDNA silencing (*irs*) phenotype. Using transposon mutagenesis, *lrs* mutants were found in 11 different genes, and *irs* mutants were found in 22 different genes. Surprisingly, we did not isolate any genes involved in rRNA transcription. Instead, multiple genes associated with DNA replication and modulation of chromatin structure were isolated. We describe these two gene classes, and two previously uncharacterized genes, *LRS4* and *IRS4*. Further characterization of the *lrs* and *irs* mutants revealed that many had alterations in rDNA chromatin structure. Several *lrs* mutants, including those in the *cdc17* and *rfc1* genes, caused lengthened telomeres, consistent with the hypothesis that telomere length modulates rDNA silencing. Mutations in the HDB (RPD3) histone deacetylase complex paradoxically increased rDNA silencing by a *SIR2*-dependent, *SIR3*-independent mechanism. Mutations in *rpd3* also restored mating competence selectively to *sir3Δ MATα* strains, suggesting restoration of silencing at *HMR* in a *sir3* mutant background.

Heterochromatin in eukaryotic chromosomes is usually associated with transcriptional silencing of nearby genes and also the suppression of recombination. In *Saccharomyces cerevisiae*, silencing occurs at several different genetic loci, including the cryptic mating-type loci (*HML* and *HMR*) (for a review see reference 50), and telomeres (34), both of which are generally recognized as the yeast heterochromatin equivalent. Remarkably, silencing of several RNA polymerase II (Pol II)-transcribed reporter genes also occurs within the rDNA locus (11, 70), even though this region of the yeast genome is very actively transcribed by Pol I and III. The ribosomal DNA (rDNA) of *S. cerevisiae* consists of 100 to 200 copies of a 9.1-kb unit organized into a perinuclear tandem array (59, 60), an arrangement reminiscent of the heterochromatin of higher eukaryotes.

Silencing in yeast is mediated by a specialized heterochromatin-like structure that is dependent on a series of *trans*-acting factors, including the proteins encoded by the four silent information regulator (*SIR*) genes. Efficient silencing at the *HM* loci requires all four *SIR* genes, while telomere position effect (TPE) requires *SIR2*, *SIR3*, and *SIR4* (2). *SIR1* contributes to the efficient establishment of silencing only at the *HM* loci (61). TPE and *HM* silencing also share requirements for Rap1, histones H3 and H4, and several other factors (50). As a result of this overlap in required silencing factors, the underlying mechanism of repression is thought to be similar between the *HM*

loci and telomeres, although the mechanism is not yet well understood. Current models indicate that Sir2p, Sir3p, and Sir4p form a multimeric complex which interacts with the hypoacetylated N-terminal tails of histones H3 and H4 within nucleosomes, leading to the formation of a silenced chromatin domain at telomeres and the *HM* loci (35). The Sir proteins may therefore be structural components of yeast heterochromatin, although their exact functions have not yet been identified.

rDNA silencing is distinct from TPE and *HM* silencing in that *SIR2* is the only absolutely required *SIR* gene (11, 70), implying that there are underlying differences in the mechanism of repression. Furthermore, unlike the *HM* and telomere loci, the rDNA is possibly the most transcriptionally active region of the entire genome, making rDNA silencing paradoxical. It is currently unknown whether the Pol I or Pol III transcription of rDNA plays any role in silencing. rDNA silencing is also exquisitely sensitive to alterations in *SIR2* dosage (31, 71), suggesting that Sir2p is a structural component of rDNA chromatin; indeed, Sir2p specifically associates with rDNA by chromatin immunoprecipitation analysis (32). Although *SIR4* function is not directly required for efficient rDNA silencing, it plays a regulatory role, mediating competition between telomeres and the rDNA for limiting amounts of Sir2 protein (71). This is consistent with the cellular localization of Sir2p, which is mostly nucleolar; smaller amounts of Sir2p also localize to perinuclear telomeric foci (32).

There are several potential functions of rDNA silencing in the yeast cell. The first is suppression of mitotic and meiotic recombination within the tandemly repeated rDNA. Deletion of *SIR2* not only causes a loss of silencing in the rDNA (11, 70) but also increases the rate of rDNA recombination (33). The

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second is suppression of a cryptic Pol II promoter in the rDNA that overlaps with the well-characterized Pol I promoter (20). The third is modulation of rRNA transcription by Pol I. Deletion of *SIR2* increases the percentage of rDNA repeats that are actively transcribed by Pol I (70). Fourth, silencing has been linked to the regulation of life span in yeast cells (47, 69). Certain mutations of *SIR4* which promote longevity (47) also strengthen rDNA silencing (71), suggesting that there may be a link between the counteraction of aging and rDNA silencing. However, this link is complex, as aging is associated with redistribution of Sir3p and Sir4p to the nucleolus (46), yet neither of these proteins participates directly in rDNA silencing, as operationally defined by the silencing of Pol II reporter genes placed in the rDNA (70).

Thus far, a few genes other than *SIR2* and *SIR4* have been implicated as rDNA silencing factors. These genes encode topoisomerase I (*TOP1*), the ubiquitin-conjugating enzyme (*UBC2/RAD6*), histones H2A and H2B (11), and most recently Sas10p (42). Using multiple rDNA silencing reporter genes, we have performed a genetic screen that identified numerous non-*SIR* genes with rDNA silencing functions. Interestingly, multiple genes with known roles in DNA replication and/or chromatin modulation were identified. Several of the rDNA silencing genes identified in this screen have similar functions in TPE and *HM* silencing, implying that the rDNA silencing mechanism is distinct yet has some features in common with the other forms of silencing in yeast. We propose a model for rDNA silencing in which multiple cellular processes collaboratively lead to an rDNA chromatin structure that is repressive to Pol II reporter gene expression and recombination.

MATERIALS AND METHODS

Media, plasmids, and yeast strains. Unless stated otherwise, media used were as previously described (63, 70). Pb^{2+}-containing medium (MLA) consisted of 0.3% peptone, 0.5% yeast extract, 4% glucose, 0.02% (wt/vol) ammonium acetate, 0.1% $Pb(NO_3)_2$, and 2% agar. Glucose was the sole carbon source on all media. All yeast strains used (Table 1) were congenic to GRF167 (6, 70). All liquid and plate incubations of yeast strains were performed at 30°C. pJSS70-9 (2μm *TRP1 SIR2*) was constructed by ligating a *Xho*I-*Not*I *SIR2* fragment from pCAR237 (70) into the *Xho*I-*Not*I sites of pRS424 (17).

JS306, JS311, JS314, and JS315 were congenic haploid spores dissected from the diploid JS262 (Table 1). JS262 was constructed as follows. The *mURA3/HIS3* expression cassette ("m" indicates the minimal *TRP1* promoter) was integrated into the 18S rRNA-coding region of yeast strain JS237, using a PCR product generated from pJSS51-9 as a template (70) and oligonucleotides JB1271 (5′ ACATGGTATAACCGTGGTAATTCTAGAGCTAATACATGCT<u>ATACG ACTCACTATAGGGCG</u> 3′), and JB1272 (5′ TATCTAATAAATTCATCTC TTCCAAAGGGTCGAGATTTTA<u>AAGGGAACAAAAGCTGGAGC</u>   3′). The underlined sequences are complementary to regions flanking the multiple cloning site of pRS vectors. Using this PCR product, the expression cassette was inserted between nucleotides 3333 and 3334 of the rDNA repeat (68, 70). The resulting strain was called JS260 (Table 1). JS260 was then mated to JB721 to produce the JS262 diploid. JS262 was sporulated and tetrads dissected to produce haploid spores which were used in this study. Throughout this report, transposon insertion mutations are written in lowercase and deletions are designated by the suffix "Δ." JS401 was created by elimination of the *mURA3/HIS3* and Ty1-*MET15* markers from the rDNA of strain JS400. JS422, JS424, and JS426 were haploids derived from the parental diploids JS420 and JS421 (Table 1). Haploids JS432, JS434, and JS436 were derived from the diploids JS430 and JS431. Haploids JS443 and JS445 were derived from the diploid JS442. Haploids JS556, JS557, and JS558 were derived from the diploid JS555. The haploids JS561, JS562, JS563, and JS564 were derived from the diploid JS560.

Mutagenesis and identification of affected genes. Haploid strains JS306 and JS311 were mutagenized using transposon Tn*3::lacZ::LEU2* as previously described by Burns et al. (13). A yeast genomic DNA library was obtained from Mike Snyder's lab (via Susan Michaelis). This library had been mutagenized in *Escherichia coli* by random Tn*3::lacZ::LEU2* integration events. The mutated genomic DNA inserts were removed from the vector backbone by digestion with *Not*I and transformed into JS306 and JS311 by using a high-efficiency lithium acetate-polyethylene glycol-dithiothreitol procedure. Cells were plated onto leucine-deficient synthetic complete (SC−Leu) medium (approximately 200 to 250 transformants/plate) to select for Leu⁺ mutant colonies in which a transposon-disrupted DNA fragment had integrated into the genome by homologous recombination.

Leu⁺ colonies grown for 3 days were replica plated to SC−Leu, SC−Ura, SC−His, and MLA media to detect changes in rDNA silencing phenotypes. Over a period of 3 days, the replica plates were monitored daily for colonies which significantly differed from other colonies on the same plate. After 3 days, colonies were selected for further study only if they had altered silencing phenotypes on both SC−Ura and MLA plates. Two major classes of mutants, loss of rDNA silencing (*lrs*) and increased rDNA silencing (*irs*), were obtained. The *HIS3* reporter was useful for the increased rDNA silencing screen because *irs* mutant colonies were often phenotypically Ura⁻ but still His⁺, allowing them to be differentiated from colonies which simply lost the *mURA3/HIS3* reporter cassette from the rDNA. Mutant colonies were picked and restreaked for single colonies on SC−Leu medium, grown 3 days, and replica plated to SC−Ura, SC−His, and MLA media to retest the mutant phenotypes (2° screen).

The *lrs* mutants were classified into (i) those which have an rDNA recombination phenotype measured by hypersectoring of MLA-grown colonies and (ii) those which do not sector. The transposon-disrupted gene in each hypersectoring mutant was identified by plasmid rescue and DNA sequencing. For most *lrs* mutants, the *mURA3/HIS3* reporter gene was first removed from the rDNA by simply plating cells onto YPD, and through replica-plating of the resulting colonies, Ura⁻ His⁻ colonies were easily identified. Ura⁻ versions of each *lrs* mutant were inoculated into 10-ml YPD cultures and grown approximately 16 h. Each mutant was then transformed with approximately 0.5 μg of *Pvu*I-linearized Yip5 vector. Ura⁺ colonies were selected, and genomic DNA was isolated by using a Teeny prep spheroplasting method (6). Recovered DNA was digested with *Nsi*I and circularized by self-ligation overnight at 4°C with T4 DNA ligase. The ligation mixture was transformed into *E. coli* DH5α and selected on LB supplemented with carbenicillin (50 μg/ml). Plasmid DNA was recovered, and transposon recovery was verified by restriction mapping. The genomic DNA flanking the recovered transposon was identified by DNA sequencing.

For the *irs* mutants, the procedure used was similar except that the *mURA3/HIS3* cassette was not removed from the rDNA. Instead, *Sca*I linearized pRS404 (*TRP1*) was used in the plasmid rescue. Recovered yeast genomic DNA was cut with *Spe*I before circularization and transformation into *E. coli*.

Dominance tests. Each mutant was mated to a strain of the opposite mating type which did not contain reporter genes in the rDNA and was Trp⁺. Briefly, mutant strains were streaked out for single colonies on SC−Leu, grown for 3 days, and replica plated onto a YPD plate, along with a lawn of either JS314 (*MAT*α) or JS315 (*MAT*a). The two strains were allowed to mate for 5 h at 30°C and the YPD mating plate was then replica plated to SC−Leu−Trp and grown overnight to select for diploid formation. The resulting diploids were then replica plated to the silencing indicator medium SC−Leu−Trp−Ura or to MLA to test for dominance. The heterozygous *lrs* diploids were also restreaked for single colonies onto MLA to test for dominance by the rDNA sectoring assay. Backcross analysis was used to confirm cosegregation of the mutant phenotype with the Tn*3::lacZ::LEU2* transposon insertion for mutants that were represented by a single isolate in the screen.

PCR-mediated gene disruption. Complete open reading frame (ORF) deletions were made for several genes identified in the screen to confirm the Lrs⁻ or Irs⁻ silencing phenotype of that particular mutant. PCR-mediated gene disruption was performed as described elsewhere (5, 51). The dominant drug resistance marker, *kanMX4* (79), was PCR amplified from pRS400 (8, 71), using oligonucleotide primers containing 39 nucleotides complementary to the 5′ and 3′ ends of the targeted ORF. The resulting PCR fragments were transformed into JS306 or JS311, and gene replacement by *kanMX4* was selected for by growth on YPD medium containing G418 (200 μg/ml).

Silencing growth assays. Strains to be tested were patched onto YPD, or selective medium if they contained a plasmid, and grown overnight. Cells were scraped from the plates and resuspended in 1 ml of sterile water. The cell suspension was normalized to an A_{600} reading of 0.5 and then serially diluted in fivefold increments; 5 μl of each dilution was spotted onto either nonselective or selective SC agar plates, using an eight-channel pipette. Plates were incubated for 2 to 5 days. Polaroid photographs were taken of all plates except SC−Ura after 2 days. Photographs of SC−Ura plates were taken after 4 days unless specified otherwise in the figure legends.

Colony color silencing assays. Strains to be tested were patched onto YPD and grown overnight. Cells were then streaked onto MLA plates with four or six sectors per plate. The plates were wrapped with Parafilm to prevent dehydration and then allowed to grow 5 days. Photographs were taken at day 5, using a Leica stereoscopic microscope equipped with a 35-mm color camera.

Telomere length analysis. Two independent colonies for each mutant were lightly inoculated into 10 ml of YPD cultures and incubated for approximately 16 h. Cells were pelleted, and genomic DNA was isolated as previously described, using a Teeny prep spheroplasting method (6). Nucleic acid pellets were resuspended in 100 μl of Tris-EDTA (TE), and 15 μl (2 to 4 μg of DNA) was digested in a 30-μl reaction with *Xho*I and separated on a 0.7% agarose gel. The DNA was transferred to Genescreen Plus (NEN-Dupont), UV cross-linked, and hybridized with a telomere specific probe in Church and Gilbert hybridization solution (1 mM EDTA, 0.5 M Na_2HPO_4 [pH 7.2], 7% sodium dodecyl sulfate, and 1% bovine serum albumin) at 60°C overnight. The blot was washed once at room temperature and three times (10 min) at 60°C with washing solution (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl

TABLE 1. Yeast strains used

Strain	Genotype[a]
JB721	*MATa his3Δ200 ura3-167*
JS237[b]	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15*
JS260	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3*
JS262	*MATa/MATα his3Δ200/his3Δ200 LEU2/leu2Δ1 MET15/met15Δ0 TRP1/trp1 Δ63 ura3-167/ura3-167 RDN1/RDN1::Ty1-MET15 mURA3/HIS3*
JS306	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3*
JS311	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3*
JS314	*MATa his3Δ200 leu2Δ1 met15Δ0 ura3-167*
JS315	*MATa his3Δ200 leu2Δ1 met15Δ0 ura3-167*
JS343[c]	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 sir2Δ::kanMX4*
JS400	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 cac1Δ::kanMX4*
JS401	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 cac1Δ::kanMX4*
JS418	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 rif1Δ::kanMX4*
JS420	*MATa/MATα his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 met15Δ0/met15Δ0 trp1Δ63/trp1 Δ63 ura3-167/ura3-167 rif1::mTn3/RIF1 SIR3/sir3Δ::kanMX4 RDN1/RDN1::Ty1-MET15*
JS421	*MATa/MATα his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3-167/ura3-167 rif1::mTn3/RIF1 SIR4/sir4Δ::HIS3 RDN1/RDN1::Ty1-MET15*
JS333[c]	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15*
JS337[c]	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 sir4Δ::HIS3*
JS335[c]	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 sir3Δ::kanMX4*
JS422	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 rif1::mTn3*
JS424	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 rif1::mTn3 sir3Δ::kanMX4*
JS426	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 rif1::mTn3 sir4Δ::HIS3*
JS430	*MATa/MATα his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3-167/ura3-167 CDC17/cdc17::mTn3 sir3Δ::kanMX4/SIR3 RDN::Ty1-MET15/RDN1*
JS431	*MATa/MATα his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3-167/ura3-167 CDC17/cdc17::mTn3 sir4Δ::HIS3/SIR4 RDN1::Ty1-MET15/RDN1*
JS432	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 cdc17::mTn3*
JS434	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 cdc17::mTn3 sir3Δ::kanMX4*
JS436	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 cdc17::mTn3 sir4Δ::HIS3*
JS442	*MATa/MATα his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3-167/ura3-167 rfc1::mTn3/RFC1 SIR4/sir4Δ::HIS3 RDN1/RDN1::Ty1-MET15*
JS443	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 rfc1::mTn3*
JS445	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15 rfc1::mTn3 sir4Δ::HIS3*
JS490	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 rpd3Δ::kanMX4*
JS493	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 sin3Δ::kanMX4*
JS495	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-mURA3 rif2Δ::kanMX4*
JS497	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-mURA3 rif2Δ::kanMX4 rif1::mTn3*
JS523	JS306 pRS424
JS524	JS306 pJSS70-9
JS527	M179 pRS424
JS528	M179 pJSS70-9
JS533	M154 pRS424
JS534	M154 pJSS70-9
JS537	JS311 pRS424
JS538	JS311 pJSS70-9
JS541	JS490 pRS424
JS542	JS490 pJSS70-9
JS549	M489 pRS424
JS550	M489 pJSS70-9
JS555	*MATa/MATα his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3-167/ura3-167 RDN1/RDN1::Ty1-MET15, mURA3/HIS3 cac1Δ::kanMX4/CAC1 RPD3/rpd3::mTn3*
JS556	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3*
JS557	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 cac1Δ::kanMX4*
JS558	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 cac1Δ::kanMX4 rpd3::mTn3*
JS560	*MATa/MATα his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3-167/ura3-167 RDN1/RDN1::Ty1-MET15, mURA3/HIS3 rif1::mTn3/RIF1 RPD3/rpd3::kanMX4*
JS561	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3*
JS562	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 rif1::mTn3*
JS563	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 rpd3Δ::kanMX4*
JS564	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 rif1::mTn3 rpd3Δ::kanMX4*
JS566	JS311 made *sir2Δ::kanMX4*
JS568	M480 made *sir2Δ::kanMX4*
JS574	JS306 made *lrs4Δ::kanMX4*
JS576	JS306 made *sir2Δ::kanMX4*
JS625	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 sir3Δ::kanMX4*
JS626	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 sir3Δ::kanMX4*
JS627	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 rpd3::mTn3 sir3Δ::kanMX4*
JS628	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 rpd3::mTn3 sir3Δ::kanMX4*
JS629	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 rpd3::mTn3*
JS633	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 sir4Δ::HIS3*
JS634	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 rpd3::mTn3 sir4Δ::HIS3*
JS635	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 sir4Δ::HIS3*
JS636	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 rpd3::mTn3 sir4Δ::HIS3*
JS639	*MATa his3Δ200 leu2Δ1 met15Δ0 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 rpd3Δ::kanMX4 sir2Δ::HIS3*

[a] mTn3 is an abbreviation for mTn3::lacZ::LEU2 throughout the table, and represents the transposon disruption alleles.
[b] (70).
[c] (71).

sulfate). The probe was a 350-bp *Eco*RI fragment from plasmid pYLPV, which contained a 280-bp TG$_{1-3}$ telomeric repeat (81).

Psoralen cross-linking analysis. In vivo psoralen cross-linking assays were performed as previously described (14, 22, 70), with several minor modifications. Fresh 50-ml YPD cultures were inoculated from saturated YPD cultures to an A_{600} of 0.3 and grown for 6.5 h into log phase. Approximately 2.5×10^8 cells were washed with ice-cold H$_2$O and resuspended in 0.7 ml of cold TE in a 24-well tissue culture plate; 40 μl of a 200-μg/ml solution of 4,5′,8-trimethylpsoralen (Sigma) in 100% ethanol was added to each well, and the plate was UV irradiated for 5 min on ice at a distance of 6 cm five times. The light source was a long-wave UV lamp (model B-100A; Ultraviolet Products, Inc.). Cells were washed, spheroplasted with zymolyase at 37°C, lysed, proteinase K treated, phenol-chloroform extracted, and ethanol precipitated. Total nucleic acid was resuspended in 40 μl of TE and normalized to an A_{260} of 0.04. DNA (4 μl) was digested for 5 h at 37°C with *Eco*RI in a 30-μl reaction containing RNase A (80 ng/μl). DNA was separated on 1.3% agarose gel (14.5 by 24 cm) at 80 V for 17 h. Cross-linking was reversed in a Stratagene Stratalinker at 0.6 J/cm². DNA was transferred to Genescreen Plus in 10× SSC and hybridized with probe A, which is a 2.2-kb *Eco*RI-*Sma*I fragment of the rDNA nontranscribed spacer (NTS).

RESULTS

Identification of rDNA silencing mutants. As a first step toward understanding the molecular mechanism of rDNA silencing, we carried out a genetic screen designed to identify mutations in genes which contribute either positively or negatively to silencing. Strains JS306 (*MAT***a**) and JS311 (*MAT*α) were constructed to contain three different Pol II-transcribed reporter genes in the rDNA, namely, a single *MET15* reporter gene (embedded in a Ty1 element) located within NTS2 of one rDNA repeat and a *mURA3*/*HIS3* expression cassette within the 18S rRNA-coding region of a second repeat (Fig. 1A). Met$^+$ strains produce white colonies on Pb^{2+}-containing (MLA) medium, whereas Met$^-$ strains produce dark brown colonies (21, 58). rDNA silencing of *MET15* results in a characteristic intermediate tan colony color (70). Mutants which weaken rDNA silencing were predicted to produce a lighter colony color than wild type (WT), and mutants which strengthen rDNA silencing were predicted to produce darker colonies. The *mURA3* reporter was also repressed by the rDNA and resulted in a very weak Ura$^+$ phenotype (70). *HIS3* was not observed as silenced in previous studies in a replica plating test (11, 70), and so it was used as a marker for the presence of the tightly linked *mURA3* reporter. The relevant WT phenotypes of these reporter strains were therefore a tan colony color on MLA plates, weakly Ura$^+$, and fully His$^+$. During this study we found that *HIS3* is in fact partially silenced when assayed by a more quantitative colony spotting assay.

Mutations which weakened rDNA silencing, such as *sir2*Δ, had the potential to also increase the amount of mitotic recombination between rDNA repeats (33), making the silencing reporter genes unstable and more difficult to work with. To facilitate cloning the affected genes, we used a transposon-mediated gene disruption strategy developed by Burns et al. (13). The transposon mutagenesis method allows for direct recovery and sequencing of the mutated gene. Complementation cloning using potentially unstable silencing reporters is thus avoided. On the other hand, use of transposon mutagenesis biases toward the recovery of nonessential genes.

Approximately 20,000 Leu$^+$ transformants of JS306 and JS311 were generated by transformation with a collection of yeast genomic library inserts previously mutagenized in *E. coli* by transposon Tn*3::lacZ::LEU2* insertions (13) (Fig. 1B). These Leu$^+$ transformants were then replica plated to MLA to observe changes in colony color, SC−Ura to observe changes in *mURA3* reporter expression, SC−His to track the presence of the *mURA3*/*HIS3* cassette, and to SC−Leu as a nonselective growth control.

Several different classes of mutants were generated. Colonies which were lighter in color than WT on MLA plates, more
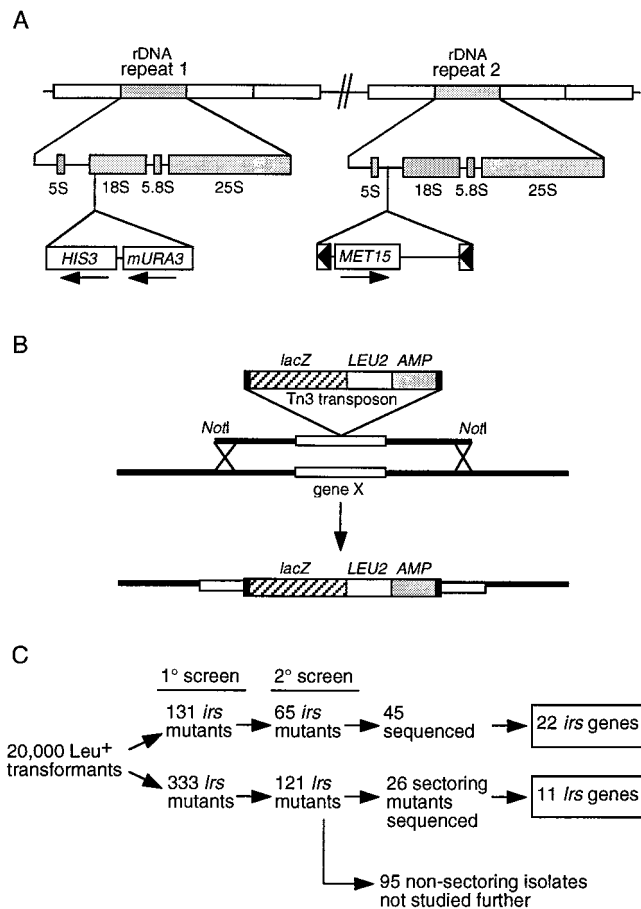

FIG. 1. Genetic screen for rDNA silencing mutants. (A) Schematic representation of the rDNA structure of strains JS306 and JS311, showing the positions of each reporter gene. (B) Schematic drawing showing the transposon mutagenesis strategy used in the screen. *Not*I yeast genomic DNA fragments disrupted with the transposon mTn*3::lacZ::LEU2* (13), were transformed into strains JS306 and JS311. Homologous recombination resulted in the replacement of a specific segment of yeast chromosome (gene X), with an identical DNA fragment which was disrupted with the transposon. Transposon-disruption mutants are selected for the presence of *LEU2* by growth on SC−Leu medium. (C) Flow chart describing the screening procedure and number of isolates at each stage. Sequences for 20 of the 65 of the 2° *irs* isolates were not recovered.

Ura$^+$, and still His$^+$ were classified as *lrs* mutants; those which were darker than WT on MLA and less Ura$^+$ (and sometimes less His$^+$) were classified as *irs* mutants. The screening process is summarized in Fig. 1C.

We identified the disrupted gene in a large subset of the *lrs* isolates, specifically those with phenotypes similar to *sir2* mutants, which had, in addition to the loss of rDNA silencing (Lrs$^-$) phenotypes, a hypersectoring phenotype by the *MET15* color assay, indicative of increased *MET15* marker loss through mitotic rDNA recombination (70). The flanking genomic DNAs were recovered into *E. coli*, and the disrupted genes were identified by DNA sequencing. We identified 11 different *LRS* genes (*LRS1* through *LRS11*) and 22 different *IRS* genes (*IRS1* through *IRS22*). We chose not to analyze the nonsectoring class of *lrs* mutants because most of them were dominant and likely to reflect simple rDNA amplification events that increased reporter gene copy number. However, informative mutants may exist in this collection; this possibility will be addressed in the future. Even though a total of 33 different genes were identified, the screen was not saturated; two known rDNA silencing genes, *SIR2* and *RAD6*, were not recovered.

TABLE 2. Mutants isolated from the genetic screen

Mutation	Gene/ORF name	Isolate no.	Transposon insertion site[a]	Transposon orientation[b]	Gene function	Additional phenotypes
lrs1	*RFC1/CDC44*	M65	Promoter	Plus	DNA replication/processivity factor	Slow growth, long telomeres
lrs4	*YDR439W*	M114	78/347	Plus	Unknown	Slow growth
		M169	78/347	Plus		Slow growth
		M345	78/347	Plus		Slow growth
lrs5	*TOP1*	M122	10/769	Plus	Topoisomerase I	
		M154	267/769	Minus		
		M160	55/769	Plus		
lrs6	*DPB3*	M155	72/201	Plus	DNA polymerase ε/subunits C and C′	Increased spontaneous mutation rate
		M172	34/201	Minus		
		M182	72/201	Plus		
		M286	72/201	Plus		
lrs7	*RIF1*	M98	444/1916	Minus	Telomere length control	Long telomeres
		M158	183/1916	Plus		
lrs8	*CAC1*	M179	272/606	Plus	Nucleosome assembly during replication	UV sensitive
lrs9	*CDC17/POL1*	M326	237/1468	Minus	DNA polymerase α	Long telomeres
irs1	*SIR4*	M87	20/1358	Minus	Silencing factor	Mating defective
irs2	*RPD3*	M480	135/433	Minus	Histone deacetylase	Pleiotropic[c]
irs4	*YKR019C*	M469	69/615	Plus	Unknown	Slow growth on Pb^{2+}
irs8	*YMR263W/SAP30*	M475	107/201	Plus	Sin3-associated protein	Pleiotropic[c]
irs10	*HIR3*	M390	150/1648	Plus	Regulates histone expression	
		M411	702/1648	Plus		
		M487	702/1648	Plus		
		M489	1496/1648	Minus		
irs18	*TUP1*	M419	Promoter	Minus	Transcriptional repressor	Flocculent
		M420	Promoter	Minus		Flocculent
		M432	478/713	Minus		Flocculent

[a] Position of transposon insertion relative to protein sequence in amino acids (insertion site/total amino acids). Exceptions are M65, M419, and M420, which had insertions within the promoter sequences.

[b] Plus orientation means the *lacZ* gene of the transposon is in the same transcriptional orientation as the target ORF.

[c] Cycloheximide sensitive, derepression of *PHO5*, ethidium bromide sensitivity (78), temperature sensitive at 37°C.

Description of *lrs* mutants and their silencing phenotypes. The *LRS* class of genes were predicted to encode proteins which contributed structurally or were positive regulators of rDNA silencing. In this paper we describe *LRS1* (*RFC1*), *LRS4* (*YDR439W*), *LRS5* (*TOP1*), *LRS7* (*RIF1*), *LRS8* (*CAC1*), and *LRS9* (*CDC17*) (Table 2). The remaining LRS genes will be described elsewhere. Figure 2 shows the effects of a subset of these mutants on *mURA3*, *HIS3*, and *MET15* silencing in the rDNA as measured by quantitative growth assays, which were previously shown to correlate with reporter gene expression levels (71). Figure 3 shows the effect of the mutations on *MET15* expression as measured by a qualitative colony color assay. Each *lrs* mutant was more Ura$^+$ and His$^+$ than WT (Fig. 2A) and produced white colonies with a hyperrecombination sectoring phenotype on Pb^{2+} medium (Fig. 3). The *lrs5* insertions were in the topoisomerase I gene (*TOP1*), a known rDNA silencing factor (11, 18), and therefore validated the specificity of the *lrs* screen.

LRS1 was identified as a single transposon insertion into the promoter region of the gene encoding the large subunit of replication factor C (*RFC1*), which acts as a processivity factor for DNA polymerases δ and ε. *RFC1* is an essential gene, and this mutation likely alters its expression level. The *LRS9* gene was identified as encoding the essential catalytic subunit of DNA polymerase α (*CDC17/POL1*). The single *lrs9* mutation consisted of a transposon integrated within the N-terminal portion of the ORF. We confirmed that no WT copy of *CDC17* was present in the strain by PCR and backcross analysis (data not shown), indicating that this mutation of *cdc17* is indeed viable. This suggests that there may be a cryptic yeast promoter in the 5′ end of the promoterless *lacZ* gene of the transposon, which can transcribe a 5′ truncated version of the *CDC17* ORF.

LRS7 was identified as the *RIF1* gene. *RIF1* (Rap1-interacting factor 1) was originally isolated from a two-hybrid screen

for proteins which interact with the C terminus of the essential silencing factor Rap1p (38). Deletion of *RIF1* increases telomere length, strengthens TPE and weakens silencing at *HMR*, probably due to a shift in the balance of Sir3 and Sir4 proteins between the *HM* locus and telomeric chromatin compartments (12, 38).

LRS8 was identified as *CAC1* (chromatin assembly complex), which encodes the large subunit of yeast chromatin assembly factor I (yCAF-I). yCAF-I is composed of three protein subunits (Cac1p [p90]; Cac2p [p60], and Cac3p [p50]) and preferentially assembles newly synthesized histones H3 and H4 with a deposition-competent acetylation pattern into nucleosomes on newly replicated DNA (45), similar to the activity of human CAF-I (hCAF-I) (72). The *CAC* genes are not essential, but their deletion causes modest UV sensitivity and weakens TPE and *HM* silencing (28, 45). While the exact function of yCAF-I in silencing is not known, it was recently shown to be required for the stable maintenance of repressed chromatin at telomeres and *HM* loci (27, 54). Both the original *lrs8* (*cac1*) mutant and a *cac1* deletion mutant derepressed all three rDNA reporter genes (Fig. 2A and 3). Furthermore, deletion of *CAC2* or *CAC3* resulted in an Lrs$^-$ phenotype (data not shown), indicating that the yCAF-I complex as a whole contributes to rDNA silencing.

LRS4 was identified as the previously uncharacterized ORF *YDR439W*. This gene is not highly homologous to any genes of known function and encodes a positively charged protein (pI = 10.34). It does appear to encode a coiled-coil protein with limited homology to myosin and other coiled-coil proteins. Its function in rDNA silencing is not known.

Description of *irs* mutants and their phenotypes. The *IRS* class of genes was predicted to include negative regulators of rDNA silencing. *IRS1* was identified as *SIR4*, deletion of which was previously shown to increase rDNA silencing (70). Isola-
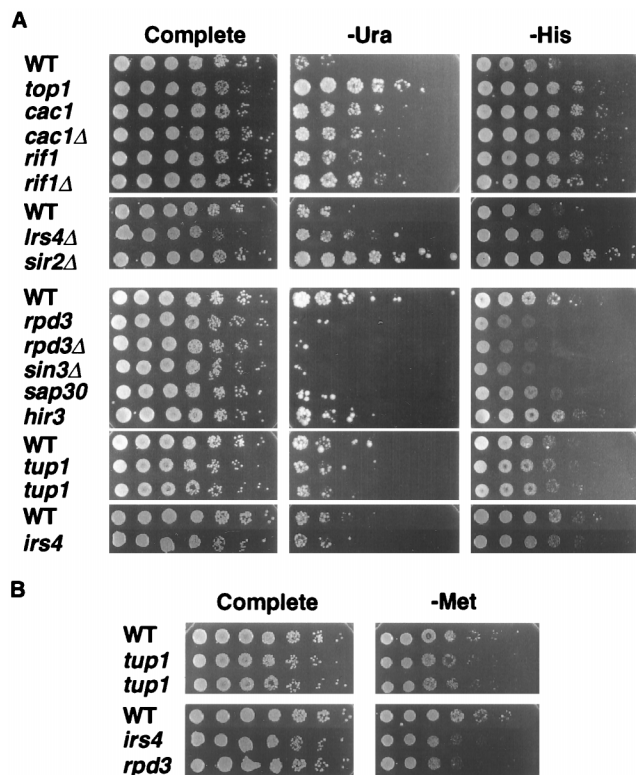
A

	Complete	-Ura	-His
WT			
top1			
cac1			
cac1Δ			
rif1			
rif1Δ			
WT			
lrs4Δ			
sir2Δ			
WT			
rpd3			
rpd3Δ			
sin3Δ			
sap30			
hir3			
WT			
tup1			
tup1			
WT			
irs4			

B

	Complete	-Met
WT		
tup1		
tup1		
WT		
irs4		
rpd3		

FIG. 2. rDNA silencing phenotypes of selected mutants. (A) Quantitative growth assays measuring the silencing of *mURA3* and *HIS3* within the rDNA. Fivefold serial dilutions of freshly grown cells were plated onto either SC (Complete), SC−Ura (−Ura), or SC−His (−His) medium. Strains shown are WT (JS306 or JS311), *top1* (M122), *cac1* (M179), *cac1Δ* (JS400), *rif1* (M158), *rif1Δ* (JS418), *lrs4Δ* (JS574), *sir2Δ* (JS576), *rpd3* (M480), *rpd3Δ* (JS490), *sin3Δ* (JS493), *sap30* (M475), *hir3* (M489), *tup1* (M419 and M432), and *irs4* (M469). The photographs were taken for the SC−Ura plates at day 3 for the *lrs* mutant series (top of panel) and day 4 for the *irs* series (bottom of panel). (B) Quantitative growth assay measuring silencing of *MET15* in NTS2. Fivefold serial dilutions of cells were plated onto SC and SC−Met −Cys medium. Strains were the same as for panel A.

tion of a *sir4* mutant therefore validated the specificity of this screen for *irs* mutants. Other *IRS* genes described in this study are *IRS2* (*RPD3*), *IRS4* (*YKR019C*), *IRS8* (*SAP30*), *IRS10* (*HIR3*), and *IRS18* (*TUP1*) (Table 2). Each of these genes (except *IRS4*) has previously been shown to have a chromatin-related function (26, 39, 44, 64, 73, 84). Other *IRS* genes will be described elsewhere.

IRS2 was identified as the histone deacetylase gene *RPD3*. Rpd3p is part of a larger multiprotein complex called histone deacetylase B (HDB) (64), which also contains the transcriptional corepressor Sin3p (41, 43). Physical interactions between Sin3p and specific DNA binding proteins target HDB to various promoters which causes local histone deacetylation and transcriptional repression (41, 65). It was therefore surprising to isolate a mutation in *rpd3* that caused a strong increased rDNA silencing (Irs⁻) phenotype (Fig. 2 and 3). Identical Irs⁻ phenotypes were observed when *RPD3* or *SIN3* were deleted (Fig. 2A and 3), suggesting that the defect of the *irs2* mutant was a loss of activity by the HDB complex. While unexpected, this phenotype was fully consistent with previous work showing that *rpd3* or *sin3* mutations also strengthen TPE and *HM* silencing (23, 64, 77).

Another recently identified member of HDB, called *SAP30* (84), was isolated from our screen as *IRS8*. Similar to *rpd3* and

sin3Δ mutants, the *irs8* (*sap30*) mutant had an Irs⁻ phenotype (Fig. 2A and 3). However, its increase in silencing was not as strong as the *rpd3* or *sin3Δ* mutants as measured by each rDNA silencing assay (Fig. 2A and 3). Since the loss of HDB activity alters the expression of multiple genes, the effects of HDB mutants on silencing could potentially be indirect.

IRS10 was identified as the histone regulator gene *HIR3*, which, like *HIR1* and *HIR2*, encodes a transcriptional corepressor required for a feedback control system that regulates the expression of the *HTA1-HTB1* genes in response to cellular histone H2A and H2B levels (73). *hir* mutations derepress histone transcription during the entire cell cycle, rather than normally in late G₁ or early S phase (67). Interestingly, *hir* mutations exacerbate the telomeric silencing defects of *cac* mutants but have little or no TPE phenotype on their own (44, 62). Compared to *rpd3* mutants, *hir3* mutants were relatively mild in their strengthened rDNA silencing (Fig. 2A and 3).

IRS18 was identified as the global transcriptional repressor gene, *TUP1*. Tup1p and its partner, Ssn6p, are recruited to many yeast genes by interactions with specific DNA binding proteins to repress transcription through a histone H3- and H4-dependent mechanism (39). Direct interactions of Tup1p
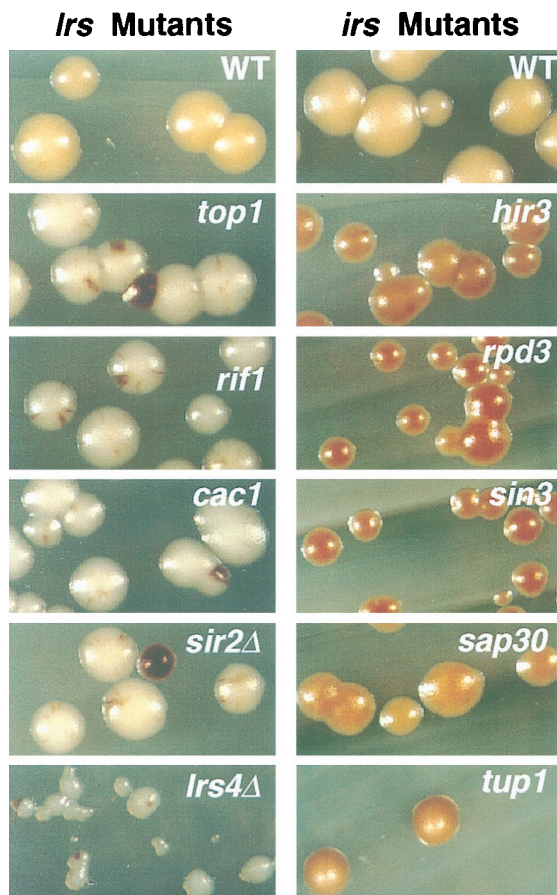
lrs Mutants   **irs Mutants**

lrs	*irs*
WT	WT
top1	*hir3*
rif1	*rpd3*
cac1	*sin3*
sir2Δ	*sap30*
lrs4Δ	*tup1*

FIG. 3. Qualitative colony color assay showing the *lrs* and *irs* phenotypes of selected mutants. Freshly grown cells were scraped from YPD medium and streaked onto MLA medium for single colonies. Cells were grown 5 days before photographs were taken. *lrs* mutants produce white colonies and often display a hypersectoring phenotype; *irs* mutants produce a darker colony color, indicative of reduced *MET15* expression compared to WT. *lrs* strains shown are WT (JS306), *top1* (M122), *rif1* (M158), *cac1* (M179), *sir2Δ* (JS576), and *lrs4Δ* (JS574); *irs* strains shown are WT (JS311), *hir3* (M487), *rpd3* (M480), *sin3Δ* (JS493), *sap30*, M475, *irs4* (M469), and *tup1* (M419).

A

Strain	plasmid	-Trp -Ura	-Trp -His	-Trp

B

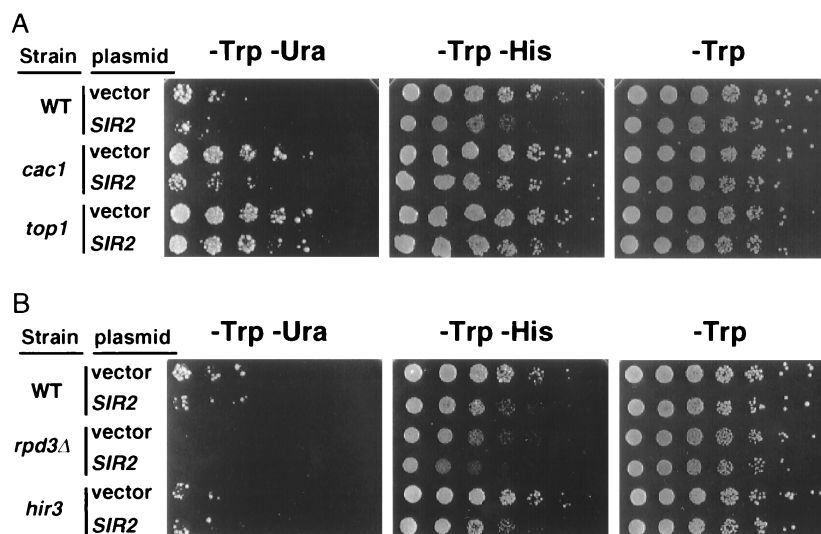| Strain	plasmid	-Trp -Ura	-Trp -His	-Trp

FIG. 4. Effect of *SIR2* overexpression on rDNA silencing mutants. (A) The high-copy-number empty vector pRS424 or the *SIR2* vector pJSS70-9 was transformed into the WT strain JS306, several *lrs* mutants including *cac1* (M179), and the *top1* mutant (M154). Fivefold serial dilutions were spotted onto SC−Trp, which selects for the plasmid, SC−Trp−Ura to measure silencing of *mURA3*, and SC−Trp −His to measure silencing of *HIS3*. (B) Fivefold serial dilutions of *irs* mutants containing a high-copy-number empty vector or a high-copy-number *SIR2* vector.

with the N-terminal tails of histones H3 and H4 contribute to repression (26). Even though *irs18* isolates M419 and M420 each contained mTn*3* insertions within the *TUP1* promoter, they had silencing phenotypes similar to M432, in which mTn*3* disrupted the ORF. The *tup1* mutants appeared to have only modest effects on silencing of *mURA3* and *HIS3* (Fig. 2A) but had a more pronounced Irs⁻ phenotype for the *MET15* reporter (Fig. 2B and 3). This is the first report of mutations in *tup1* having effects on *SIR*-mediated silencing. However, it is likely that this effect is indirect, because Tup1p represses the expression of many genes, yet the mutants increase the strength of silencing.

IRS4 was identified as ORF *YKR019C*. Its major distinguishing characteristic was a C-terminal Eps15 homology (EH) domain, a recently discovered protein-protein interaction domain (24). The prototypical yeast EH protein is Pan1p, which is involved in actin organization and endocytosis (75). Interestingly, Irs4p also contains a DNA polymerase B signature motif (YDDTDS) at amino acid positions 390 to 396. Similar to the *tup1* mutants, the *irs4* mutant had only modest effects on the *mURA3* and *HIS3* reporters (Fig. 2A). A more dramatic Irs⁻ phenotype (approximately 25-fold increase in silencing) was observed for the *MET15* reporter positioned in NTS2 (Fig. 2B).

Importantly, the expression of *mURA3* or *MET15* reporters located outside the rDNA was not significantly altered by the *lrs* or *irs* mutations that we tested (data not shown). These mutations included *cac1Δ*, *rpd3Δ*, *rif1Δ*, *lrs4Δ*, and *sin3Δ*. These results suggested that most of the effect of these mutations on *mURA3* and *MET15* expression in the rDNA is due to the general influence of rDNA chromatin and not to specific effects on the individual promoters used.

rDNA silencing in *lrs* and *irs* mutants is controlled by Sir2p levels. rDNA silencing is exquisitely sensitive to *SIR2* dosage (31, 71). We were therefore interested in determining whether the *lrs* and *irs* mutants remained susceptible to *SIR2* dosage effects. If any of these genes were downstream of *SIR2* in a silencing pathway, then their Lrs⁻ or Irs⁻ phenotypes should become resistant to changes in *SIR2* dosage. To test this hypothesis, we overexpressed *SIR2* in several mutant backgrounds and tested rDNA silencing strength in Ura⁺ and His⁺

growth assays. *SIR2* overexpression increased the silencing strength of the *cac1* (*lrs8*) mutant, as measured by less Ura⁺ growth, compared to a strain containing an empty vector (Fig. 4A). However, less of an effect was observed in the His⁺ assay, consistent with the *HIS3* reporter being more resistant to rDNA silencing in this mutant. The effect of *SIR2* overexpression was less pronounced for the *top1* mutant in the Ura⁺ and His⁺ assays, suggesting that *top1* mutants become partially resistant to increased *SIR2* dosage. *rif1* and *lrs4* mutants were fully sensitive to *SIR2* dosage (data not shown). *SIR2* overexpression also further strengthened rDNA silencing in all *irs* mutants tested, including *tup1*, *sin3Δ*, *sap30* (not shown), *rpd3Δ*, and *hir3* (Fig. 4B). These results indicated that most *lrs* and *irs* mutants are fully responsive to elevations in *SIR2* dosage, and they localized these genes either upstream of SIR2 or in different genetic pathways to rDNA silencing.

Several mutants with long telomeres also weaken rDNA silencing. We previously proposed that rDNA silencing strength could be modulated by telomere length due to a competition between the rDNA and telomeres for a limited pool of Sir2p (71). Similarly, extra telomeres weaken silencing of a telomeric reporter gene by titrating an unidentified silencing factor (82). Certain mutations in the DNA replication genes *cdc17* (*pol1*) and *rfc1*, and null mutations of *rif1*, cause telomeres to lengthen compared to WT strains (1, 38). Lrs⁻ insertion alleles were isolated for each of these genes (Table 2; Fig. 5A). The Lrs⁻ phenotype of these mutants might therefore be due to long telomeres sequestering Sir2p and depleting it from the rDNA. As predicted by this model, the viable *rfc1* (*lrs1*) and *cdc17* (*lrs9*) mutants indeed had significantly longer telomeres than the parent strains (Fig. 5B). The *rif1* (*lrs7*) mutant had longer telomeres which produced a qualitatively different banding pattern than the other mutants (Fig. 5B). This unusual banding pattern was specific to the *rif1* mutation because it cosegregated in backcrosses.

Each rDNA repeat contains an origin of replication (74). It was therefore possible that in addition to their effect on telomere length, Cdc17p and Rfc1p may have a more direct role in rDNA silencing related to their DNA replication functions. Furthermore, Top1p and Dpb3p, two other DNA replication
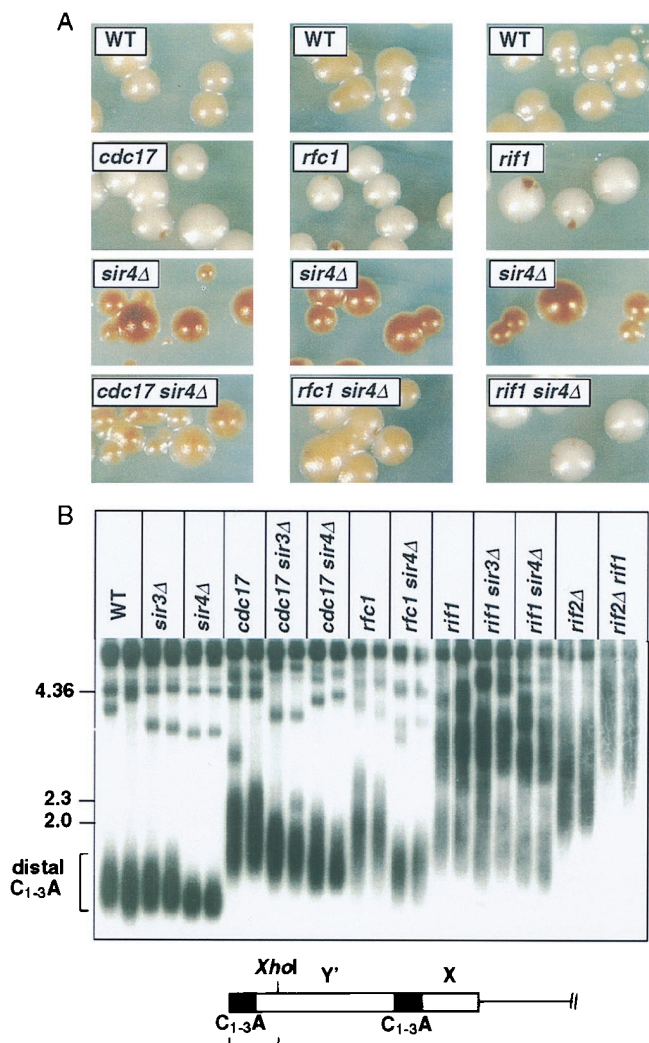
FIG. 5. A class of rDNA silencing mutants with abnormally long telomeres. (A) The DNA replication mutants *cdc17* and *rfc1*, and the telomere regulation mutant *rif1*, were each isolated as *lrs* mutants. Each mutant was crossed to a *sir4Δ::HIS3* mutant of the opposite mating type to produce a heterozygous diploid which was sporulated and dissected for tetrads. The resulting haploid strains were grown on MLA medium and assayed for loss of silencing of *MET15* as measured by colony color. Strains shown are WT (JS333), *sir4Δ* (JS337), *cdc17* (JS432), *cdc17 sir4Δ* (JS436), *rfc1* (JS443), *rfc1 sir4Δ* (JS445), *rif1* (JS422), and *rif1 sir4Δ* (JS426). (B) Steady-state telomere length of *lrs* mutants in combination with *sir3Δ* and *sir4Δ* mutations. Genomic DNA was isolated from two isolates of each mutant listed, digested with *Xho*I, and separated on a 0.7% agarose gel. The transferred DNA was detected with a $C_{1-3}A$-specific DNA probe, which on this blot will detect typical Y′ telomeres (shown schematically at bottom). The variable-length telomeres are visualized as a smear (brackets). In addition to the mutants described in panel A, there are congenic *sir3Δ* (JS335), *cdc17 sir3Δ* (JS434), *rif1 sir3Δ* (JS424), *rif2Δ* (JS495), and *rif2Δ rif1* (JS497) strains.

proteins for which we isolated *lrs* mutant alleles, did not have lengthened telomeres (data not shown). To differentiate this replication model from the telomere competition model, we combined the *rfc1*, *cdc17*, and *rif1* mutations with *sir3Δ* and *sir4Δ* mutations and tested whether the Lrs⁻ phenotype was reversed in the double mutants. Deletion of *SIR3* or *SIR4* in the *lrs* mutants was predicted to prevent telomeric titration of Sir2p to telomeres and away from the rDNA. Sir2p exclusively localizes in the nucleolus of *sir4Δ* mutant strains (32). For *rfc1* and *cdc17*, double-mutation combinations with *sir4Δ* indeed reversed the Lrs⁻ phenotype to WT or slightly stronger than

WT levels (Fig. 5A, bottom row). However, the full Lrs⁻ phenotype expected of a *sir4Δ* mutant was not observed. These results suggest that much, but perhaps not all, of the effect on rDNA silencing caused by *rfc1* and *cdc17* mutations was due to telomere competition. Similar results were observed when *rfc1* and *cdc17* were combined with a *sir3Δ* mutation (data not shown). Telomere length for these double-mutant strains was also intermediate between WT and single-mutant lengths (Fig. 5B). Furthermore, telomere length did not change during prolonged strain passage (data not shown).

In contrast, the *rif1 sir4Δ* double mutant had weakened rDNA silencing compared to WT, similar to the *rif1* single mutant (Fig. 5A). This result suggested that Rif1p may have a direct role in rDNA silencing that is independent of telomere competition. Rif1p physically interacts with another Rap1-interacting factor called Rif2p (83), which also functions in telomere length regulation. Unexpectedly, deletion of *RIF2* had no effect on the strength of rDNA silencing (data not shown), even though these strains had long telomeres (Fig. 5B). Furthermore, a *rif1 rif2Δ* mutant had extremely long telomeres (Fig. 5B) but did not weaken rDNA silencing more than a *rif1* single mutant (data not shown). These results suggest that the telomere lengthening effects of *rif1* mutants may be unrelated to their Lrs⁻ phenotype.

***lrs* and *irs* mutants alter the structure of rDNA chromatin.** To determine whether mutations which weaken rDNA silencing (*lrs*) also disrupt rDNA chromatin structure, we tested each *lrs* mutant in an in vivo psoralen cross-linking assay (22). This assay measures the intracellular accessibility of the rDNA to psoralen cross-linking. The Lrs⁻ phenotype of *sir2* mutants was previously shown to be associated with an increase in psoralen cross-linking at the rDNA, reflecting a more open chromatin structure (70). Increased psoralen cross-linking was detected by the slower mobility of isolated rDNA fragments on a native agarose gel. The accessibility of the NTS in a subset of the *lrs* mutants is shown in Fig. 6A. The 2.5-kb NTS fragment released by *Eco*RI digestion displayed a slower gel mobility for each *lrs* mutant, similar to the *sir2Δ* control. This result indicated that the NTS had a more open chromatin structure in most *lrs* mutants, not just those carrying *sir2Δ*.

Since the *lrs* mutants had an open rDNA chromatin structure, we anticipated that the *irs* mutants might have a more closed chromatin structure which would be consistent with stronger silencing. To test this hypothesis, the psoralen cross-linking assay was repeated for a subset of *irs* mutants (Fig. 6B). The only mutant tested which produced an NTS fragment with moderately faster gel mobility than WT (less cross-linking) was *irs18* (*tup1*). The *sir4Δ* (data not shown) and *hir3* mutants had no detectable effect on the rDNA chromatin in this assay. Since this assay measures the average state of the rDNA chromatin, we cannot rule out that the chromatin associated with the marker genes has not been altered in these cases. Unexpectedly, the *rpd3Δ*, *sin3Δ*, and *sap30* (HDB) mutants each produced NTS fragments with slower gel mobility, indicative of a more open chromatin structure (Fig. 6B). Histone acetylation is thought to increase the access of transcription-associated factors to DNA. The more open chromatin structure of the *rpd3Δ*, *sin3Δ*, and *sap30* mutants was therefore fully consistent with histone hyperacetylation in the rDNA caused by a lack of histone deacetylation, and it suggested that HDB may directly deacetylate rDNA histones. However, this result was again inconsistent with the paradoxical Irs⁻ phenotype of the HDB mutants.

The Irs⁻ phenotype of an *rpd3* mutant is partially reversed by *cac1*, *rif1*, and *sir2* mutations. The opposing silencing and chromatin accessibility phenotypes of *rpd3*, *sin3*, and *sap30*
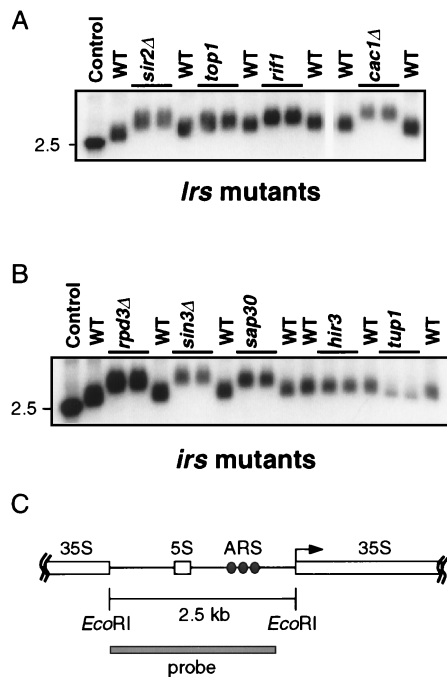
FIG. 6. rDNA chromatin accessibility of mutants as measured by in vivo psoralen cross-linking. (A) Log-phase cultures of *lrs* mutants were UV cross-linked with psoralen in vivo. Isolated DNA was digested with *Eco*RI and separated on a 1.3% agarose gel, and the transferred DNA was detected with a probe specific for the NTS of the rDNA (C). The *sir2*Δ strain (JS343) acted as a positive control for increased accessibility to psoralen cross-linking. Other strains shown are WT (JS306), *top1* (M122), *rif1* (M158), and *cac1*Δ (JS400). (B) An identical cross-linking procedure using the same NTS-specific probe was carried out on a subset of the *irs* mutants. The strains tested are WT (JS311), *rpd3*Δ (JS490), *sin3*Δ (JS493), *sap30* (M475), *hir3* (M411), and *tup1* (M419). The control lanes show the 2.5-kb *Eco*RI fragment which is observed when the strains are not cross-linked. (C) Schematic drawing of the *Eco*RI rDNA fragment detected from this assay. ARS, autonomously replicating sequence.

mutants prompted a more in-depth examination of the genetic interactions between *RPD3* and the *LRS* genes. We asked whether the Irs⁻ phenotype caused by *rpd3* mutations depends on any of the *LRS* genes, specifically *SIR2*, *CAC1*, or *RIF1*. Double-mutant combinations were generated and tested in an epistasis analysis for rDNA silencing strength using the Ura⁺ and His⁺ growth assays and the colony color assay (Fig. 7). We obtained different epistasis results that correlated with the location of the silencing reporter gene in the rDNA repeat. For example, with the *mURA3/HIS3* cassette readouts, *rpd3* mutations fully overrode the effects of the *cac1*Δ and *rif1* mutations (Fig. 7A). However, for the *MET15* reporter in the same strains, the double mutants produced Met⁺ (data not shown) and colony color phenotypes that were intermediate between those of the two single mutants (Fig. 7B). Thus, *rpd3*Δ partially overrode the *cac1*Δ and *rif1* Lrs⁻ phenotypes. Therefore, *mURA3* and *HIS3*, both located within the 18S coding region, appeared to be influenced by the *rpd3* mutant effect more than *MET15*, which was located within NTS2. Alternatively, these differences could be caused by simple promoter differences.

The *rpd3 sir2*Δ combination of mutations produced a significantly different result. The double mutant again resulted in an intermediate rDNA silencing phenotype but in this case had an Lrs⁻ phenotype relative to WT in each assay (Fig. 7). This result indicated that *SIR2* was required for the Irs⁻ phenotype of the *rpd3* mutant but that *sir2*Δ was not completely epistatic

to *rpd3*. For the *rpd3 sir2*Δ combination, there was also no differential effect on silencing based on the reporter gene position in the rDNA repeat sequence. To determine whether the other *SIR* genes contributed to the Irs⁻ phenotype of an *rpd3* mutant, we generated *rpd3 sir3*Δ double mutants through backcrossing. Deletion of *sir3* in an *rpd3* mutant did not reverse the Irs⁻ phenotype (data not shown), indicating that the Irs⁻ phenotype of an *rpd3* mutant occurs through a *SIR3*-independent, *SIR2*-dependent mechanism.

rpd3 mutations partially restore mating competence to *sir3*Δ mutants. In the process of analyzing the interactions of *rpd3* and *sir3*Δ mutations, we made some surprising observations about effects on *HM* locus silencing. Previous studies have noted enhancement of silencing effects by *rpd3* mutations on a weakened *HMR*Δ*A* silencer (77). We observed behavior suggesting that *rpd3* mutations can restore silencing to the native *HMR* silencer in *sir3*Δ mutants. All of the 17 *MAT*α *rpd3 sir3*Δ spores resulting from a cross of *rpd3* and *sir3*Δ strains efficiently mated to a *MAT*a tester strain, indicating that the *rpd3* mutation partially reversed the silencing defect caused by the *sir3*Δ mutation. This effect was specific for *MAT*α cells, suggesting that *HMR*a was affected but *HML*α was not affected
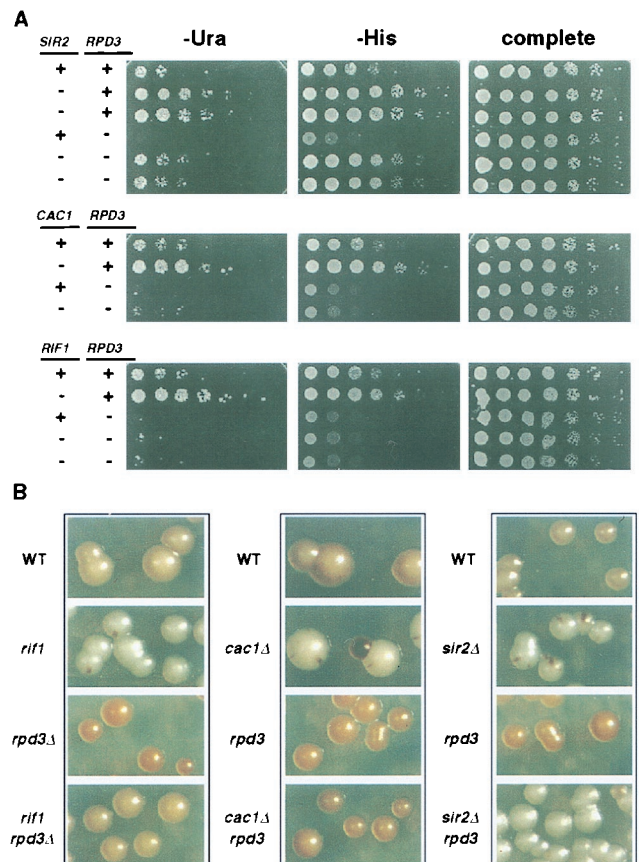

FIG. 7. Epistasis analysis between *rpd3* and *lrs* mutations for rDNA silencing phenotypes. (A) Silencing reporter strains were generated with combinations of *rpd3* mutations with either *sir2*Δ, *cac1*Δ, or *rif1* mutations. The resulting strains were assayed for rDNA silencing strength in Ura⁺ and His⁺ growth assays. Fivefold serial dilutions of each haploid strain were spotted on indicator plates. A plus sign indicates the strain is WT for a particular gene, and a minus sign indicates a mutation of a particular gene. (B) The same strains were plated onto MLA medium and tested for silencing strength in the colony color assay. Each column represents strains grown on the same plate.
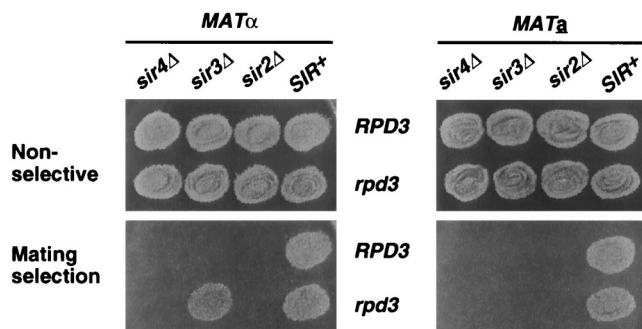
FIG. 8. Mating phenotypes of *rpd3 sir* double-mutation combinations. The *rpd3::*mTn*3* mutation was combined with either *sir2*Δ, *sir3*Δ, or *sir4*Δ mutation to determine the effect on mating ability. Strains were patched onto YPD, grown for 24 h, and then mated for 6 h with a lawn of *MAT***a** or *MAT*α tester strains. Diploids were selected on SD minimal medium by growth for 18 h. The original YPD master plate was replica plated to SC medium as a nonselective growth control. The double-mutation combinations were tested in both *MAT*α and *MAT***a** genetic backgrounds. Mating is measured by the efficiency of diploid formation on SD medium.

(Fig. 8). In contrast, the *rpd3* mutation did not rescue the mating defect of *MAT*α *sir2*Δ or *MAT*α *sir4*Δ strains (Fig. 8).

DISCUSSION

Multiple genetic pathways to rDNA silencing. An unanticipated outcome from this screen was the absence of genes involved in transcription by RNA Pol I or III, especially since this specialized transcription is intimately associated with the rDNA. It was possible that rDNA silencing of Pol II reporter genes was caused by promoter interference from Pol I or III. Instead, we recovered multiple genes involved with DNA replication or chromatin modulation. The DNA replication genes included *POL1*, *RFC1*, and *DPB3*. The chromatin modulating class included *RPD3*, *SIN3*, *SAP30*, *TUP1*, *HIR3*, *SIR4*, and *RIF1*. The *TOP1* and *CAC1* genes could be placed in either DNA replication or chromatin modulating classes, since they participate in both processes. These results, coupled with the intermediate phenotypes of several double-mutation combinations, are strong evidence for several independent pathways which converge on rDNA silencing (Fig. 9). The *SIR2* pathway is extremely important, but it appears that all the pathways must be functional to achieve full silencing strength.

There are several reasons why a role for Pol I or Pol III transcription in rDNA silencing cannot be ruled out by this study. First, the screen was not carried out to saturation, since we did not recover transposon insertions into *SIR2* or *RAD6*, two genes already known to be required for rDNA silencing (11, 70). Second, many genes involved in Pol I and Pol III transcription are essential and thus are more difficult to recover with this mutagenesis method than nonessential genes. Third, a bias was placed on the *lrs* mutant selection when we chose to study the hypersectoring class. Pol I or Pol III mutants might lack rDNA recombination phenotypes. Future work will address whether there is any role of specialized rRNA transcription in rDNA silencing.

Telomere length effects on rDNA silencing. Sir4p mediates competition between the rDNA and telomeres for a limiting amount of Sir2 protein (71). Variations in telomere length are therefore predicted to influence the strength of rDNA silencing by changing the balance of Sir2p between the rDNA and telomere compartments. Indeed, three different *lrs* mutants with significantly longer than WT telomeres were isolated. Also consistent with this competition model, introducing a

*sir4*Δ mutation into *rfc1* or *cdc17* mutant strain backgrounds partially reversed their Lrs⁻ phenotypes back to at least WT silencing strength and also significantly reduced telomere length. This was an important result because in the absence of Sir4p, all cellular Sir2p is localized in the nucleolus and not sequestered at telomeres (32). Taken together, these two findings suggest that *rfc1* and *cdc17* mutations weaken the Irs⁻ phenotype of a *sir4*Δ mutant through a telomere length-independent mechanism, perhaps related to their replication functions.

This model was complicated by our finding that *rif2*Δ mutants had long telomeres but did not weaken rDNA silencing. Furthermore, unlike the case for *cdc17* and *rfc1* mutants the Lrs⁻ phenotype of *rif1* mutants was not dramatically reversed by introduction of *sir4*Δ or *sir3*Δ mutations. This result suggested that *rif1* plays a direct role in rDNA silencing, which partially uncouples telomere length control from rDNA silencing. Rif1p and Rif2p interact with each other and with Rap1p (83). They also have recently been shown to interact with telomeric DNA sequences in a one-hybrid assay (7). Deletion of *RIF2* causes telomeres longer than those in WT strains and improves TPE but has little or no effect on *HMR* silencing (83). Since deletion of *RIF2* also has little or no effect on rDNA silencing, *rif2*Δ mutant telomeres probably contain normal amounts of Sir2p, even though they are longer.

In addition to causing loss of rDNA silencing (this study), *rif1* mutations shorten the life span of yeast (4). The short life span of *rif1*Δ mutants was proposed to result from sequestration of the Sir2p-Sir3p-Sir4p silencing complex by longer telomeres (4). However, our data suggest that a *rif1* mutation has negative effects on rDNA silencing that are telomere length independent. Introduction of *sir4* mutations which lengthen life span (47), and increase rDNA silencing (71), into a *rif1*Δ mutant background only partially reverses the short life span phenotype (4). The remaining short life span effect observed in
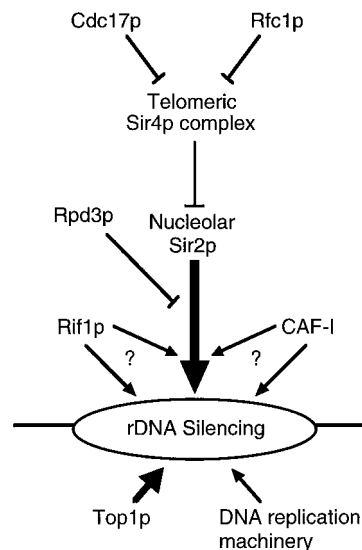

FIG. 9. Model of multiple pathways to rDNA silencing. Sir2p is a central factor in rDNA silencing. Cdc17p and Rfc1p inhibit formation of extended telomeres, limiting the impact of telomere-rDNA competition for Sir2p, which is mediated by Sir4p. Cdc17p and Rfc1p may also positively act on rDNA silencing through their DNA replication functions. Silencing in most *lrs* and *irs* mutants can be increased by overexpression of Sir2p. CAF-I and Rif1p could be upstream of Sir2p, act in cooperation with Sir2p, or be completely separate inputs. Rpd3p counteracts rDNA silencing mostly through a Sir2p-dependent mechanism but also through a *SIR*-independent mechanism. Topoisomerase I may influence rDNA silencing by a mechanism that is partially independent of Sir2p.

those double mutants is therefore also consistent with a direct role for Rif1p in rDNA silencing. A final piece of evidence for a direct Rif1p role in rDNA silencing is that Rif1p directly interacts with Sir2p in 2-hybrid and biochemical assays (67a).

DNA replication and rDNA silencing. Connections between DNA replication and silencing are not unprecedented. First, establishment of silencing at the *HM* loci requires progression through S phase (30, 53). Second, the six-subunit origin recognition complex (ORC) is required for silencing at *HML* and *HMR* (29, 52). One role of ORC in silencing at the *HM* loci appears to be recruitment of the Sir-silencing complex through interactions with Sir1p (16, 76). However, ORC is also required for silencing at telomeres in a *SIR1*-independent manner (30). Interestingly, the silencing function of one ORC subunit (Orc5p) at the *HMR-E* silencer can be genetically separated from its replication initiation function, implying that replication initiation is not required for silencing (25). Silencing at the rDNA is qualitatively different from *HM* and telomere silencing, and so it is currently unknown whether ORC functions in rDNA silencing.

Each rDNA repeat contains an origin of DNA replication, which is located upstream of the 5S rRNA in NTS2 (68). It was therefore possible that DNA replication could play a role in rDNA silencing. Indeed, we have shown that relatively mild mutant alleles of *rfc1* (*lrs1*) and *cdc17* (*lrs9*) weakened rDNA silencing. Part of this effect was likely due to lengthened telomeres (see above), but not all of this effect could be explained by this model. The remainder of the Lrs⁻ phenotype could be caused by replication defects. Consistent with this hypothesis, another *lrs* mutant gene (*lrs6*) suffered a Tn*3* insertion into a nonessential subunit of DNA polymerase ε (*DPB3*). Unlike the *cdc17* and *rfc1* mutants, the *dpb3* mutants had normal-length telomeres, indicating that replication mutants can affect rDNA silencing independent of telomere length control.

Finding a DNA polymerase ε (Pol ε) subunit in the *lrs* screen is intriguing because DNA Pol ε is required not only for chromosomal replication but also for base excision repair (80). RFC-1 is also involved in base excision repair. Furthermore, the large subunit of Pol ε (Pol2) is required for replication and DNA damage checkpoints in S phase (57). *dpb3Δ* strains have an elevated spontaneous mutation rate, suggesting it is involved in maintaining replication fidelity (3). Taken together, an alternative model to explain the role of the DNA replication genes in rDNA silencing could be a contribution from the DNA repair machinery.

Histone balancing act. Nucleosomes are formed from two molecules of each of the core histones H2A, H2B, H3, and H4 which tightly associate with 146 bp of DNA. Histones H3 and H4 have been shown to contribute directly to silencing at the *HM* and telomere loci (35). Furthermore, histones H2A and H2B are important for rDNA silencing. Deletion of *HTA1-HTB1*, one of two gene pairs producing H2A and H2B, results in a loss of rDNA silencing (11), suggesting that histone stoichiometry within the nucleosome may be critical. We recovered four independent mutations in the *HIR3* gene from our screen as *irs10* mutants. Mutations in *HIR1*, *HIR2*, or *HIR3* cause deregulation of a transcriptional feedback mechanism which controls *HTA1-HTB1* expression (73). The Irs⁻ phenotype of *irs10* (*hir3*) mutants may therefore be due to increased H2A H2B expression levels. Consistent with this model, telomeric silencing is also slightly increased in a *hir1* mutant strain (44).

The Lrs⁻ phenotype of *cac1*, *cac2*, and *cac3* mutants may also result from altered histone stoichiometry. hCAF-I and yCAF-I (chromatin assembly complexes) assemble newly synthesized histones H3 and H4 onto newly replicating DNA (45,

72). The *CAC* genes are not essential in yeast, but mutations of these genes weaken silencing at all silenced loci (28, 45). It is therefore possible that CAF-I is important for assembly of a silencing-competent nucleosome structure. Indeed, the *HM* and telomere silencing defects of *cac* mutants are caused by poor maintenance of the silent chromatin states (27, 54). In the absence of CAF-I activity, an alternative nucleosome assembly activity may take over and deposit silencing-incompetent nucleosomes that lack the proper histone acetylation pattern or are assembled improperly for efficient silencing. Another straightforward hypothesis for the effect of *cac* mutants on rDNA silencing is that a certain minimal nucleosome density is required to build silencing competent chromatin. The *cac* mutants may fail to deposit nucleosomes at a sufficient density to maintain silencing, even if histone levels are normal. Epigenetic switching between silent and active chromatin states has not been demonstrated for the rDNA, suggesting that the Lrs⁻ phenotype of *cac* mutants is due to a direct structural defect of the rDNA chromatin. Indeed, the structure of rDNA chromatin is altered in a *cac1Δ* mutant as measured by an in vivo psoralen accessibility assay (Fig. 6), consistent with a silencing maintenance defect.

Histone deacetylation and rDNA silencing. Transcriptionally silenced regions of eukaryotic genomes are generally hypoacetylated. In *S. cerevisiae*, the *HM* loci and telomeres are hypoacetylated on histones H3 and H4, with an acetylation pattern similar to that in higher eukaryotes (10). The yeast *RPD3* gene product is the proposed catalytic component of a large multiprotein histone deacetylase complex (HDB) which acts in targeted transcriptional repression (40, 64). Sin3p, another subunit of HDB, acts to target the complex to specific Pol II promoters through association with specific DNA binding proteins (41). Sap30p is also a member of this complex (84). Paradoxically, *rpd3* and *sin3* mutations increase silencing at *HM* loci (77), telomeres (23, 64), and the rDNA (this study). Similarly, *Drosophila* TPE is enhanced by null mutations of its *RPD3* homolog (23). In another study, *sap30* mutants strengthened all types of silencing in yeast, including the rDNA (37). These findings were completely consistent with our results for the *irs8* (*sap30*) mutation using a different strain background and different rDNA silencing reporters. In both studies, these results are paradoxical because loss of histone deacetylase function is expected to result in hyperacetylation of histone N-terminal tails, resulting in derepression of transcription.

Two separate hypotheses have been proposed to explain the *rpd3Δ* effect on silencing (23, 64, 77). The first is that the effect is indirect due to the increased expression of a critical, dosage-dependent silencing factor. This is unlikely to be *SIR2* itself because increased *SIR2* expression does not increase silencing at the *HM* loci but actually slightly derepresses it (19). The second hypothesis is that loss of *rpd3* function causes an increase in the acetylation of an N-terminal lysine residue on one of the core histones which correlates with increased silencing. Lysine 12 of histone H4 has been proposed to be a critical lysine, because it is specifically acetylated in the chromatin of the silent *HM* loci compared to the expressed *MAT* locus (10). This modification was suggested to enhance the interaction between H4 and the Sir3 and Sir4 proteins, thus strengthening silencing (10). If this were true, then it was possible that Sir3p and/or Sir4p were being recruited to the rDNA by increased histone H4 Lys¹² acetylation to increase silencing in an *rpd3* mutant. However, this does not appear to be the case for rDNA silencing, because deletion of *SIR3* had no effect on the Irs⁻ phenotype of an *rpd3* mutant. Furthermore, *SIR4* is also not required for the Irs⁻ phenotype of an *rpd3* mutant (37).

Whatever the mechanism, it is clear that *SIR2* is required for the Irs$^-$ phenotype of the HDB mutants.

A third possible explanation for the increased silencing effect caused by an *rpd3* mutation is that the activity of an unidentified silencing factor is modulated by acetylation on internal lysine residues. Perhaps the HDB activity is required to regulate this potential silencing factor through deacetylation. Loss of deacetylase activity would then hyperactivate the silencing factor, leading to stronger silencing. Several nonhistone proteins including p53 and HMG-I(Y) are known to be acetylated in mammalian cells by the histone acetyltransferases CBP/p300 and P/CAF (36, 56, 66). However, acetylation of a nonhistone protein by a yeast histone acetyltransferase has not been demonstrated.

During the course of these experiments, we made the unexpected observation that *rpd3* mutations efficiently suppress the mating defect of *sir3Δ* strains, but only in the *MATα* background. No such mating was seen in *rpd3 sir2Δ* or *rpd3 sir4Δ* strains. Thus, this effect appears to be *SIR3* specific. We interpret this restoration of mating in *sir3Δ* mutants as an *HMR*-specific restoration of silencing, although other more complex interpretations are possible (e.g., *RPD3* could be required for **a**1/α2 mediated repression of α-specific genes). The latter type of interpretation seems unlikely because it does not explain the *sir3* specificity of the *rpd3* mutation. A direct effect of the *rpd3* mutation on *HMR* silencing could be explained by a direct effect of the histone deacetylase at *HMR*. Increased acetylation of histone H4 Lys^{12} in an *rpd3* mutant might render *HMR* silenceable in the absence of *SIR3*. Alternatively, an *RPD3*-regulated gene encoding a redundant function with *SIR3* could be activated in the *rpd3* mutant and substitute for *SIR3* at *HMR*. In either case, there is clearly a differential effect of the *rpd3* mutation on the α mating type, presumably indicating differential silenceability of *HMR* versus *HML*.

A similar pattern of *HMR*-specific suppression was seen in a *sir2Δ* mutant when the related gene *HST1* was overexpressed (9). Furthermore, the *SUM1-1* mutation suppresses all *sir* mutations, also in an *HMR*-preferential manner (15, 48, 49). However, the situation with an *rpd3* mutation is distinct, in that it suppresses only the *HMR* silencing defects of *sir1* (77) and *sir3* mutations (this study). The basis for specific suppression of the *HMR* silencing defect is not known, but it could be related to the observation that *HMR* silencing is more resistant than *HML* silencing to mutations in non-*SIR* silencing genes such as *nat1* and *ard1* (55). Silencing at *HMR* therefore appears to be more robust than silencing at *HML*, which could be due to the redundancy of the HMR-E silencer (15). In the absence of Sir3p, there may be a residual form of silencing at *HMR* which becomes detectable in an *rpd3Δ* genetic background through mating assays.

Although rDNA silencing is different from TPE and *HM* silencing in that *SIR1*, *SIR3*, and *SIR4* are not required, this and other recent studies have drawn some interesting correlations. There are now several shared factors required for all types of silencing, including Sir2p, Cac1p, and Rad6p. In addition, mutation of *rif1* weakens silencing at both *HMR* and the rDNA. On the other hand, the *RPD3*, *SIN3*, and *SAP30* genes counteract all forms of silencing. All of these correlations further solidify the notion that rDNA silencing is mediated by a specialized repressive chromatin structure that is distinct from yet related to the heterochromatin of telomeres and the *HM* loci.

ACKNOWLEDGMENTS

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