An unusual form of transcriptional silencing in yeast ribosomal DNA

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Generalized transcriptional repression of large chromosomal regions in *Saccharomyces cerevisiae* occurs at the silent mating loci and at telomeres and is mediated by the silent information regulator (*SIR*) genes. We have identified a novel form of transcriptional silencing in *S. cerevisiae* in the ribosomal DNA (rDNA) tandem array. Ty1 retrotransposons marked with a weakened *URA3* gene (Ty1-*mURA3*) efficiently integrated into rDNA. The *mURA3* marker in rDNA was transcriptionally silenced in a *SIR2*-dependent manner. *MET15* and *LEU2* were also partially silenced, indicating that rDNA silencing may be quite general. Deletion of *SIR4* enhanced *mURA3* and *MET15* silencing, but deletion of *SIR1* or *SIR3* did not affect silencing, indicating that the mechanism of silencing differs from that at telomeres and silent mating loci. Deletion of *SIR2* resulted in increased psoralen cross-linking of the rDNA in vivo, suggesting that a specific chromatin structure in rDNA down-regulates polymerase II promoters.

[*Key Words:* Transcriptional repression; *S. cerevisiae;* silent mating loci; *SIR* genes; yeast ribosomal DNA]

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Chromatin structure can influence gene expression both positively and negatively (for review, see Kingston et al. 1996). Negative influence over a large region of DNA in the budding yeast *Saccharomyces cerevisiae* is called transcriptional silencing and is known to occur at the silent mating loci and telomeres (Loo and Rine 1994). The chromatin structure associated with these regions is probably analogous to the cytologically defined heterochromatin of more complex eukaryotes such as *Drosophila* (Hecht et al. 1995; Braunstein et al. 1996). Similarities between silenced regions and heterochromatin include condensed chromatin structure, replication late in S phase, perinuclear location, and epigenetic repression of gene activity (Thompson et al. 1994). Little effort has been directed toward defining additional silenced regions in the yeast genome.

Silenced regions such as telomeres often have repetitive DNA sequences. The most repetitive yeast DNA sequence is the ribosomal DNA (rDNA). The *S. cerevisiae* rDNA consists of a tandem array of 9.1 kb units repeated 100–200 times on chromosome XII (Petes and Botstein 1977; Philippsen et al. 1978) and is localized in the nucleolus, which, like telomeres, lies in apposition to the nuclear envelope. Each repeat contains a 5S rRNA gene transcribed by RNA polymerase III (Pol III) and a 35S pre-rRNA gene transcribed by RNA Pol I. Repeated DNA in the genome is subject to recombination, and for essential genes such as the rDNA, excessive recombination could be deleterious. Regulation of such recombination is therefore likely to be a critical process. How rDNA mitotic and meiotic recombination is repressed is unclear, but this suppression requires the silencing gene, *SIR2* (Gottlieb and Esposito 1989).

SIR2, along with *SIR3, SIR4, RAP1,* and histones H3 and H4, are proteins required for silencing at the *HM* loci and telomeres in *S. cerevisiae* (Loo and Rine 1995). Telomeric silencing, also called telomere position effect (TPE), originates at the telomere and "spreads" 3–5 kb toward the centromere, such that genes introduced near chromosome ends are silenced progressively less strongly with distance from the telomere (Gottschling et al. 1990). *SIR1* is necessary for establishment, but not maintenance of *HM* locus silencing (Pillus and Rine 1989), and is not required for TPE (Aparicio et al. 1991), suggesting there may be different forms of silencing. The role of *SIR2* in suppression of rDNA recombination could result from a repressive chromatin structure in rDNA. However, integration of the *LEU2, URA3,* and *ADE2* genes in single copy into rDNA leads to their expression (Szostak and Wu 1980; Keil and McWilliams 1993), indicating that at least some Pol II-transcribed genes can be expressed within rDNA.

Silenced regions have a generally repressive chromatin structure. This is demonstrated by shutdown of pol III transcription (Schnell and Rine 1986) and inaccessibility of *HM* loci and telomeres to *HO* endonuclease and DNA methyltransferase in vivo (Strathern et al. 1982; Kostriken et al. 1983; Gottschling 1992; Singh and Klar

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1992) and to restriction endonucleases in isolated yeast nuclei (Loo and Rine 1994).

In vivo psoralen cross-linking has revealed that the chromatin structures of inactive and actively transcribed rDNA gene copies are different (Dammann et al. 1993). Inactive gene copies are packaged in regular nucleosomal arrays, similar to bulk chromatin, whereas active gene copies are nucleosome-free. The nontranscribed spacer (NTS) of inactive rDNA gene copies is also packaged into a regular nucleosomal array, whereas the NTS of active gene copies shows an unusual cross-linking pattern suggestive of a complex structure (Dammann et al. 1993). These studies suggest that rDNA chromatin structure is highly influenced by Pol I transcription. Accordingly, topoisomerases I (*TOP1*), II (*TOP2*), and III (*TOP3*) appear to be important for pol I transcription and/or suppression of rDNA recombination (Brill et al. 1987; Christman et al. 1988; Kim and Wang 1989; Gangloff et al. 1994).

In this paper we describe a novel form of transcriptional silencing in *S. cerevisiae* rDNA. Ty1 retrotransposons marked with a modified *URA3* gene (*mURA3*) efficiently integrated into rDNA, targeting primarily upstream of the Pol III-transcribed 5S rRNA gene. This *mURA3* marker was found to be silenced. The *MET15* and *LEU2* genes were also silenced, demonstrating that rDNA silencing is not gene-specific. rDNA silencing depended on *SIR2*, consistent with the increased accessibility of rDNA to psoralen cross-linking observed in *sir2* mutants. Deletion of *SIR4* actually enhanced rDNA silencing, but *SIR1* and *SIR3* deletions did not significantly affect silencing, indicating that rDNA silencing differs from classical silencing. Remarkably, deletion of *SIR2* increased the proportion of actively transcribed rDNA gene copies in the tandem array.

Results

Identification of repressed Ty insertions in the rDNA

Ty1 was marked with a *URA3* gene and placed under control of the *GAL1* promoter (Fig. 1A). Galactose induction produced high levels of Ty1–*mURA3* transposition, as with other marked elements (Boeke et al. 1985, 1988; Curcio and Garfinkel 1991). Ura$^-$ parent cells that receive a new chromosomal Ty1–*mURA3* element through transposition become Ura$^+$ and sensitive to 5-fluoro-orotic acid (FOA) (Boeke et al. 1984). Following transposition induction of strain JS50-2 (Table 1), FOA-resistant (FOA^R) colonies were retested for their Ura phenotype, and, as expected, most were Ura$^-$. Surprisingly, ~15% of the FOA^R colonies were weakly Ura$^+$. Among these, four classes of Ura$^+$ growth were observed (+/−, +, ++, and +++; Fig. 1B). No differences in growth were detected on nonselective medium.

A genomic Southern blot of DNA isolated from such FOA^R/Ura$^+$ clones was probed with *URA3* (Fig. 1C). The size of each band represents the distance between the Ty1 *Hin*dIII site to the first flanking genomic *Hin*dIII site, which varies for different integration sites (Boeke et al. 1985). Most of the clones (22 of 27) contained single integration events, and thus the varied repression of Ura$^+$ growth was attributable not to differences in Ty1–*mURA3* copy number, but rather to position effects on

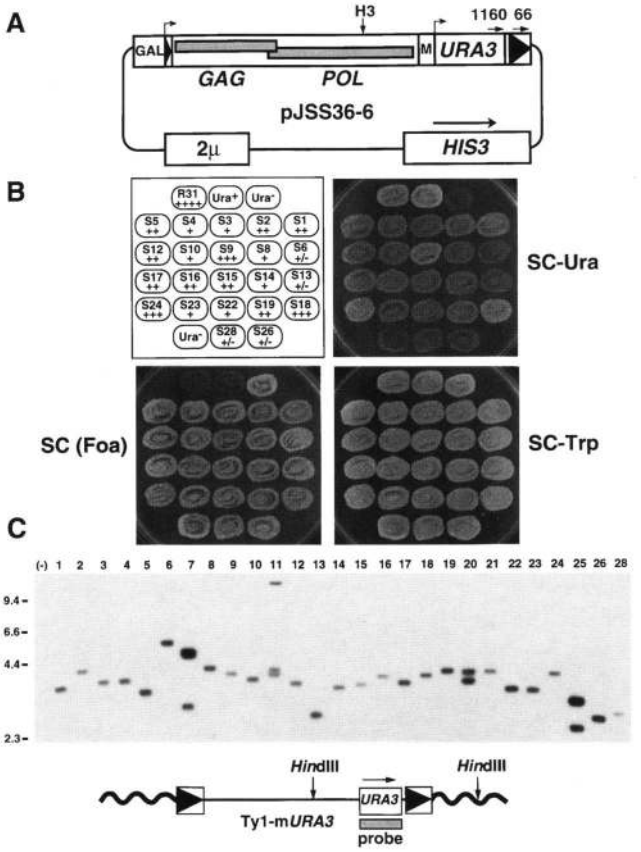

Figure 1. Ty1–*mURA3* transposition and isolation of FOA^R/ura$^+$ (Foa^R/Ura$^+$) integrants. (*A*) Structure of the pGAL–Ty1–*mURA3* overexpression plasmid pJSS36-6. Ty1 is marked with the *URA3* open reading frame (ORF) driven by a minimal *TRP1* promoter (M). Ty1 is fused to the *GAL1* promoter. The directions of Ty1 and *mURA3* transcription are depicted by the bent arrows. The *Hin*dIII site in the Ty1–*mURA3* sequence is indicated (H3). The sites for primers JB1160 and JB66 are shown (horizontal arrows); *GAG* and *POL* are Ty1 protein coding genes. (*B*) FOA^R/Ura$^+$ colonies isolated after Ty1–*mURA3* transposition induction containing single Ty1–*mURA3* insertions were patched on YPD, replica-plated to SC–Ura, nonselective SC–Trp, and SC (FOA) plates, and grown 21 hr at 30°C; Ura$^-$ control is parental strain JB740, URA3$^+$ strain is JS93. R31 is a random Ura$^+$/FOA^s isolate that contains a single non-rDNA Ty1–*mURA3* insertion. Levels of growth on SC–Ura compared with R31 are indicated as +/−, +, ++, or +++. (*C*) Genomic blot analysis of FOA^R/Ura$^+$ Ty1–*mURA3* insertions. *Hin*dIII-digested genomic DNA fragments were separated on 0.7% agarose, transferred to GeneScreen Plus, and hybridized with a *URA3* probe (*bottom* diagram) that recognizes *Hin*dIII fragments containing the 3′ end of Ty1–*mURA3*. Each band represents a single Ty1–*mURA3* insertion. The Ura$^-$ control (−) is parent strain JB740; M_r markers (kb, *left*), and isolate numbers (*top*) are indicated. Chromosomal DNA is represented by thick wavy lines.

Table 1. *Yeast strains*

Strain	Genotype	Parent
BY384[a]	*MATα his3Δ200 leu2Δ1 trp1Δ63 lys2Δ202 ura3-52*	—
GRF167[b]	*MATα his3Δ200 ura3-167*	—
JB740	*MATα his3Δ200 leu2Δ1 ura3-167*	GRF167
JS92	*MATα his3Δ200 leu2Δ1 ura3-167 trp1Δ63*	JB740
JS93	*MATα his3Δ200 leu2Δ1 URA3⁺*	JB740
JS207	*MATα his3Δ200 leu2Δ1 ura3-167 sir2Δ::HIS3*	JB740
JS209	*MATα his3Δ200 leu2Δ1 ura3-167 trp1Δ63 met15Δ1*	JS92
JS50-2	*MATα his3Δ200 leu2Δ1 ura3-167* pJSS36-6	JB740
S1 → S28[c]	*MATα his3Δ200 leu2Δ1 ura3-167 RDN1::Ty1–mURA3*	JS50-2
R31[d]	*MATα his3Δ200 leu2Δ1 ura3-167 ????::Ty1–mURA3*	JS50-2
JS210	*MATα his3Δ200 ura3-167 trp1Δ63 leu2Δ1::pJSS60-2*	JS92
JS215	*MATα his3Δ200 ura3-167 trp1Δ63 leu2Δ1 RDN1::pJSS60-2*	JS92
JS216	*MATα his3Δ200 leu2Δ1 ura3-167 trp1Δ63 met15Δ1* pJSS46-8	JS209
M1[e]	*MATα his3Δ200 leu2Δ1 ura3-167 trp1Δ63 met15Δ1 RDN1::Ty1–MET15*	JS216
JS218	*MATα his3Δ200 leu2Δ1 ura3-167 trp1Δ63 met15Δ1 RDN1::Ty1–MET15 sir2Δ::HIS3*	M1
JS219	*MATα his3Δ200 leu2Δ1 ura3-167 trp1Δ63 met15Δ1 RDN1::Ty1–MET15 sir4Δ::HIS3*	M1
JS220	*MATα his3Δ200 ura3-167 trp1Δ63 leu2Δ1::mURA3/HIS3*	JS92

[a]From Winston et al. (1995).
[b]From Boeke et al. (1985).
[c]Ty1–*mURA3* insertions at various locations of the rDNA.
[d]Ty1–*mURA3* insertion at an unknown non-rDNA locus.
[e]Ty1-*MET15* insertion upstream of the 5S rDNA.

mURA3 expression. Most of the bands clustered between 3 and 4 kb in size, suggesting that the FOA^R/Ura$^+$ Ty1–*mURA3* insertions might be in a common genomic region. DNAs from three isolates were cloned and sequenced. Each element had inserted into rDNA in or near the nontranscribed spacer (Fig. 2A).

Based on the locations of these integration sites, oligonucleotide primers were designed to detect rDNA Ty1–*mURA3* integration events in each FOA^R/Ura$^+$ isolate shown in Figure 1B, using a colony PCR assay. The insertion sites were inferred from PCR product size and confirmed by detailed Southern blot mapping. Most insertions clustered within a 600-bp window upstream of the 5S rRNA gene with Ty1–*mURA3* integrating in both orientations (Fig 2A).

Using plasmid targets in vivo, Ty1 integration has been shown to target upstream of genes transcribed by Pol III, including the 5S rRNA gene (Devine and Boeke 1996). Because the rDNA tandem array contains 100–200 copies of the 5S rRNA gene, it could be a highly preferred site of integration. To determine whether the rDNA region is an over- or under-represented target, 40 random Ura$^+$ isolates were collected following transposition induction and the locations of the rDNA insertions determined as above. Most of the 40 Ura$^+$ isolates contained more than one Ty1–*mURA3* insertion, and of 133 total insertions found in these 40 strains, 19 (14.3%) were rDNA-specific. Minimum estimates for the frequency of Ty1 integration into rDNA compared with other regions of the genome were then calculated (Table 2). The rDNA was approximately equivalent to the rest of the genome in terms of overall targeting frequency. However, because of its repetitiveness, a relatively large percentage of the total genomic Ty1 integration events occurred within rDNA.

To quantitatively determine the differences in Ura$^+$ growth between various Ty1–*mURA3*–rDNA and non-rDNA isolates, growth curves of isolates S2, S3, S6, S9, and S26 in SC–Ura medium were performed (Fig. 2B). Control isolate R31 contains a single Ty1–*mURA3* insertion that is outside the rDNA and, like other non-rDNA insertions, is completely FOA-sensitive (FOA^s). It serves as a control for the strength of the minimal *TRP1* promoter used to drive *mURA3* expression (Fig. 1A). The *URA3*$^+$ strain (JS93) grew approximately three times faster than R31, demonstrating the reduced activity of the *mURA3* promoter. Growth rates of the rDNA isolates tested ranged from ~4-fold (S9+++) to 38-fold (S6+/−) slower than R31.

Ability to grow on both SC–Ura and FOA selection media is unusual. In *S. cerevisiae,* this phenotype is observed when *URA3* is placed adjacent to telomeres (Gottschling et al. 1990); a percentage of cells in the population are FOA^R, representing those in which *URA3* is completely silenced. To determine whether a similar phenomenon was occurring in rDNA, the efficiency of plating on SC–Ura and FOA media was tested for selected Ty1–*mURA3* insertions (Table 3). For rDNA insertions, up to 100% of the cells were FOA^R, even though close to 100% of the cells also produced Ura$^+$ colonies after extended incubation. Good Ura$^+$ growth phenotypes correlated with lower percentages of FOA^R cells in the population. These results are consistent with each isolate having an intermediate level of *mURA3* expression that is not above the threshold of expression to produce FOA sensitivity. Interestingly, the colony sizes

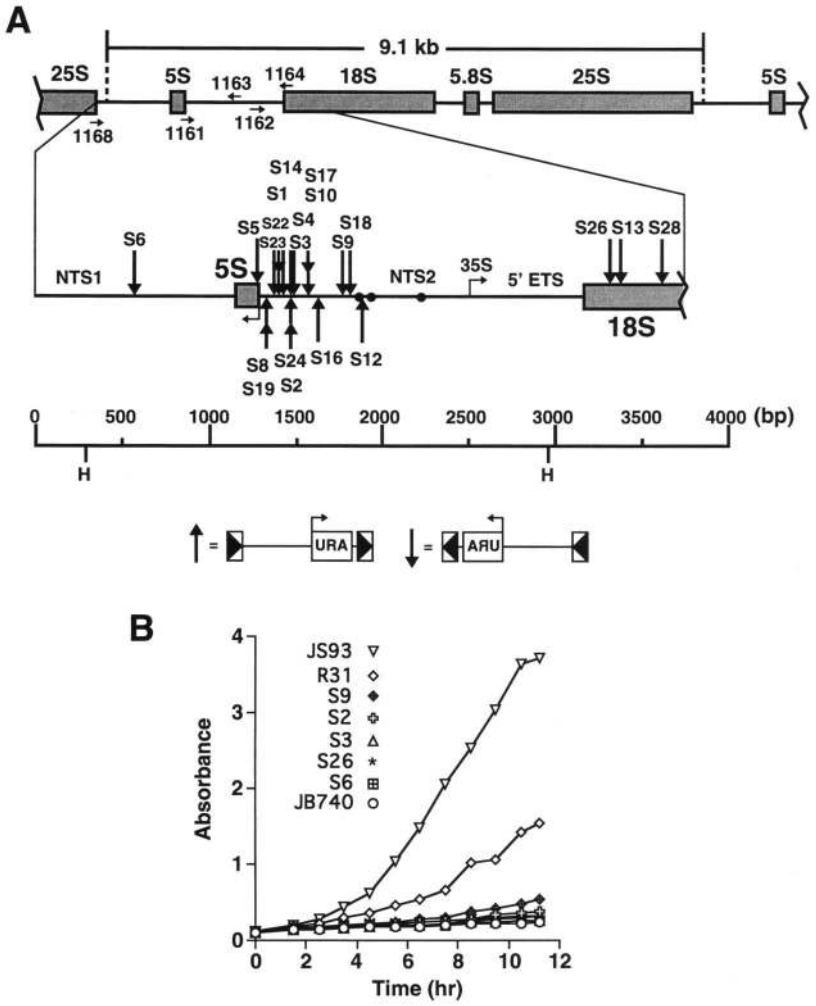

Figure 2. Targeting of Ty-1-*mURA3* integration to rDNA. (*A*) Organization of the *S. cerevisiae* 9.1-kb rDNA repeat, embedded within a tandem array. The 5S rRNA gene is represented by 5S. The 18S, 5.8S, and 25S rRNAs are processed from the 35S precursor RNA. The NTS between the 25S of one repeat and 18S of another repeat is interrupted by a 5S gene. Locations of PCR primers used for detection of Ty1 insertions within rDNA are indicated (horizontal arrows). Integration sites and orientations of Ty1–*mURA3* insertions (Fig. 1B) are shown as vertical arrows (*inset*). Orientations of Ty1–*mURA3* insertions relative to the rDNA sequence are shown for each arrow direction. The three ARS near-consensus elements in nontranscribed spacer 2 (NTS2) are shown as solid circles. Thin bent arrows represent 5S and 35S transcription start sites. Nucleotide scale for the *inset* is based on EMBL accession no. X00486 (Skryabin et al. 1984); *Hin*dIII sites are indicated (H). (*B*) Growth of rDNA insertion isolates was measured in liquid SC–Ura medium. Overnight YPD cultures were pelleted, washed with H_2O, diluted to A_{600} of 0.1 in SC–Ura, and growth monitored over time. Symbols for each strain are indicated, listed in descending order of Ura$^+$ growth.

on SC–Ura medium were quite variable (data not shown), indicating that this level of intermediate expression can vary within a certain range, occasionally over the threshold of Foa sensitivity. However, this assay cannot differentiate between the above model and one in which switching occurs very quickly between two alternative states.

Silencing of mURA3 *is independent of Ty1 sequences*

The reduction in growth on SC–Ura medium observed for the above isolates is consistent with a form of silencing within rDNA. Whereas the degree of silencing varied between isolates, there was no obvious correlation between integration location or orientation in the repeat unit with the extent of *mURA3* expression. Because the *mURA3* marker is located at the 3′ end of Ty1, the distance between the *mURA3* start codon and any rDNA sequence differs by 4619 bp depending on the orientation of Ty1–*mURA3* integration. Ty1 sequences upstream of the *mURA3* promoter potentially could influence the level of *mURA3* silencing by acting as a buffer between the rDNA and the promoter, making differences in expression between isolates difficult to interpret.

It has been proposed that TPE is a result of the continuous spreading of a heterochromatin-like structure outward from the telomere toward the centromere (Renauld et al. 1993; Hecht et al. 1995). To determine whether there is a similar gradient of silencing in the rDNA, a dual-marker silencing reporter lacking Ty1 sequences was developed that can be integrated at any position within the rDNA sequence by transformation of a *mURA3–HIS3* PCR product with 39 nucleotides of predetermined rDNA sequence at each end. *mURA3* and *HIS3* are adjacent to each other and in tandem, with *HIS3* transcription directed away from *mURA3* (Fig. 3A). *HIS3* is not noticeably repressed within the rDNA, which allows for selection of His$^+$ transformants containing the reporter without bias. The reporter was integrated in both orientations at four locations within the rDNA: NTS1, NTS2, 5′ end of 18S, and in the middle of the 25S (Fig. 3A). The levels of Ura$^+$ growth for each integration site shown in Figure 3A and in both possible orientations were approximately equal (+/−), consistent

Table 2. *Ty1 rDNA integration targeting frequency*

Assumed number of rDNA units	rDNA cluster size (kb)[a]	rDNA targeting frequency (insertions/kb)[b]	Non-rDNA targeting frequency (insertions/kb)[c]
100	910	2.08×10^{-2}	0.94×10^{-2}
150	1365	1.39×10^{-2}	0.94×10^{-2}
163	1485[d]	1.28×10^{-2}	0.94×10^{-2}
200	1820	1.04×10^{-2}	0.94×10^{-2}

[a]Based on 9.1 kb for each rDNA unit.
[b]Nineteen rDNA insertions were detected in a total of 133 integration events from 40 Ura$^+$ colonies, using each primer shown in Fig. 2A, in combination with JB1160. In the population of cells studied, 50% were Ura$^+$.
[c]The remaining 114 Ty1–*mURA3* insertions were not detected within the NTS and are assumed to be outside the rDNA, assuming non-rDNA consists of 12,500 kb.
[d]Size of rDNA in the strain S288C (Rustchenko and Sherman 1994).

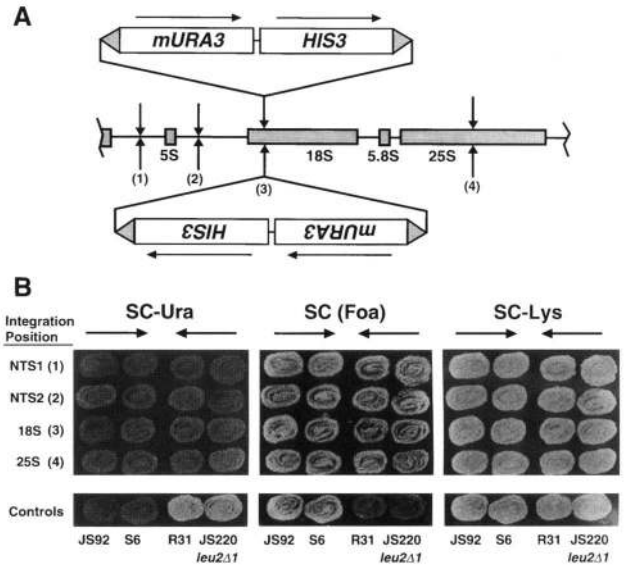

Figure 3. Ty1-independent rDNA silencing of *mURA3*. (*A*) Schematic diagram of *mURA3–HIS3* dual marker reporter, generated as a PCR cassette with adjacent rDNA sequences (shaded triangles). Using eight different sets of primers, the cassette was integrated at four different rDNA sequences in both orientations by transformation into JS92 and selection for His$^+$ colonies. Integration locations were confirmed by colony PCR as described for Ty1–*mURA3* integration events. Vertical arrows indicate integration sites, represented as 1, 2, 3, and 4. The diagram is not to scale. (*B*) Comparison of Ura$^+$ growth at each rDNA position. Two transformants at each location were patched onto YPD medium and grown 24 hr at 30°C. This plate was replica-plated to SC–Ura, SC+FOA, and nonselective SC–Lys plates and grown 21 hr at 30°C. Transcriptional direction of each cassette (*top*) and positions of insertion (*left*) are indicated. Controls are indicated.

with silencing of the *mURA3* marker throughout the rDNA repeat sequence (Fig. 3B). Each transformant was also FOA^R, as was observed for the Ty1–*mURA3* insertions. However, when the dual-marker reporter was integrated at the non-rDNA locus *LEU2*, Ura$^+$ growth was strong and fully sensitive to FOA, as observed previously for R31. Selection for both markers simultaneously did not increase Ura$^+$ growth (data not shown), indicating that *HIS3* transcription does not affect *mURA3* silencing. Thus the observed *mURA3* silencing does not require *cis*-acting sequences within Ty1.

rDNA-mediated repression occurs only within the tandem array

It was possible that *cis*-acting sequences in the rDNA could simply be negatively affecting *mURA3* expression locally, rather than silencing being mediated globally by the rDNA array. If this were the case, similar repression would occur if an rDNA gene copy containing the *mURA3* marker was positioned elsewhere in the genome, outside of the array. A *LEU2*-integrating vector (pJSS60-2) was constructed that contains a single 9.1-kb rDNA gene copy with *mURA3* inserted in the 25S region (Fig. 4A). The entire rDNA::Ty1–*mURA3* construct was integrated at the *leu2Δ1* locus or into the rDNA tandem array to compare the levels of *mURA3* expression at each location (Fig. 4B). When targeted to *leu2Δ1*, all transformants were completely Ura$^+$ and FOA^s, indicating there are no *cis*-acting sequences within a single rDNA gene copy that can silence *mURA3* when located outside the tandem array. In contrast, when targeted into the rDNA array, *mURA3* was silenced as expected. Surprisingly, even the *LEU2* gene of pJSS60-2 was also partially silenced as judged by reduced colony size on SC–Leu medium when targeted to rDNA (Fig. 4B). Silencing of *LEU2* was unexpected because it was integrated into rDNA previously without any report of repression (Szostak and Wu 1980). We conclude that

Table 3. *Efficiency of plating*

Strain	Ura phenotype	SC–Ura[b]	SC (FOA)
JB740	–	0.00	1.10
R31	++++	1.04	0.00
S2	++	0.99	0.67
S3	+	0.93	0.82
S26	+/–	0.01	0.91
S6	+/–	0.04	1.09

[a]Strains were grown as patches for 24 hr at 30°C on a YPD plate. The patches were scraped from the plate and diluted in sterile water. Two hundred microliters (~150–200 cells) was spread on three plates each of SC–Trp glucose, SC–Ura glucose, and SC (FOA) glucose. All visible colonies were counted after 4 days growth. Efficiency of plating was calculated as the number of SC–Ura or SC (FOA) colonies divided by the number of SC–Trp colonies.
[b]Additional small colonies will eventually grow for strains S3, S6, and S26 on SC–Ura plates after incubations >4 days.

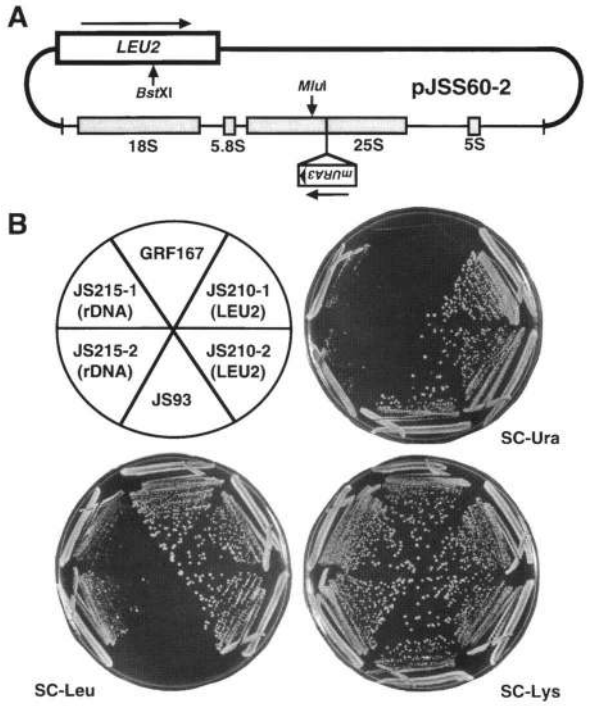

Figure 4. Integration of a single rDNA::*mURA3* unit outside the tandem array. (*A*) Schematic of plasmid pJSS60-2, which contains a single rDNA unit with *mURA3* inserted in the 25S region. Transformation of *Bst*XI-linearized plasmid into JS92 results in integration of the plasmid to *leu2Δ1* (JS210-1,2); transformation of *Mlu*I-linearized plasmid results in integration in the rDNA tandem array (JS215-1,2). The 5′ end of this repeat unit is downstream of the Pol I transcriptional start site. The Ty1 3′ LTR is used as the terminator for *mURA3* transcription. (Thick lines) Plasmid sequences; (thin lines) rDNA sequences. The diagram is not to scale. (*B*) Two transformants at each locus were streaked out for single colonies on either SC–Ura, SC–Leu, or nonselective SC–Lys plates and incubated for 2 days at 30°C. GRF167 and JS93 are *LEU2*$^+$ and *URA3*$^+$ controls, respectively. Strain numbers are indicated at *top left*, along with the integration site.

rDNA-mediated silencing is specific to the tandem array and, furthermore, is not restricted to *mURA3*.

SIR2 *is required for silencing of* mURA3 *in rDNA*

To determine whether the *SIR* genes were required for silencing of *mURA3* in the rDNA, we deleted *SIR1*, *SIR2*, *SIR3*, or *SIR4* from the Ty1–*mURA3* isolates S2, S3, and S6. Deletion of *SIR1*, *SIR3*, or *SIR4* did not increase Ura$^+$ growth, indicating that these genes are not required for silencing of *mURA3* (Fig. 5A). However, a *sir4* mutant appeared to reduce the Ura$^+$ growth of isolate S2. The *sir2* mutants resulted in a dramatic increase in Ura$^+$ growth (Fig. 5A). The increased growth was comparable to that observed for control non-rDNA isolate R31. Because of the increased levels of mitotic unequal crossing-over within rDNA in *sir2* mutants (Gottlieb and Esposito 1989), it was possible that the increased Ura$^+$ growth rate resulting from a *sir2* mutation was caused by amplification of the *mURA3* marker rather than derepression of silencing. If this were the case, then the Ura$^+$ growth increase would not be reversed by reintroduction of *SIR2*$^+$ back into the *sir2* mutant. To test this, we transformed the *sir2* mutant strains with a *SIR2*

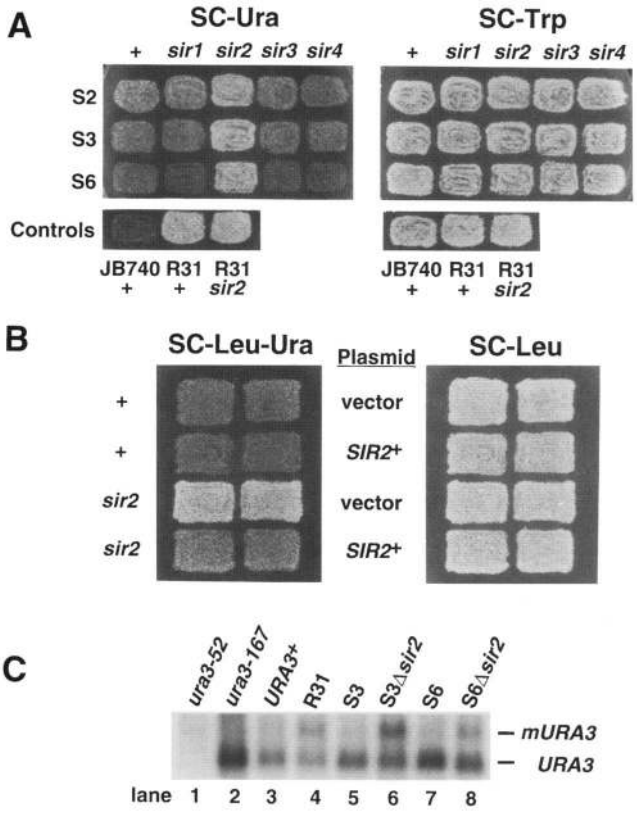

Figure 5. Effect of *SIR* gene deletions on rDNA silencing of Ty1–*mURA3* insertions. (*A*) The four individual *SIR* genes were deleted from Ty1–*mURA3* isolates S2, S3, and S6 by replacement with *HIS3*. Parental insertion strains are indicated (+). *sir1Δ::HIS3*, *sir2Δ::HIS3*, *sir3Δ::HIS3*, or *sir4Δ::HIS3* strains were patched on YPD plates, replica-plated to SC–Ura and nonselective SC–Trp plates, and grown for 20 hr. Controls are JB740 (*ura3-167*), R31 (non-rDNA insertion), and *sir2Δ::HIS3* in the R31 background. Controls were grown on the same plates as S2, S3, and S6. (*B*) The S6 and S6*Δsir2* strains shown in *A* were transformed with either control vector pRS415 or a *SIR2 CEN* plasmid (pCAR237). *SIR2* genotype is indicated. Two independent transformants for each combination were patched onto a SC–Leu plate and grown for 24 hr at 30°C, followed by replica-plating to SC–Leu–Ura and SC–Leu. Photos were taken after 20 hr growth at 30°C. Ura$^+$ growth is indicated on the SC–Leu–Ura plates. (*C*) RNA blot analysis of *mURA3* transcriptional silencing. Total RNA isolated from strains BY384 (*ura3-52*; lane *1*), JB740 (*ura3-167*; lane *2*), JS93 (*URA3*$^+$; lane *3*), R31 (lane *4*), S3 (lane *5*), S3 *Δsir2* (lane *6*), S6 (lane *7*), and S6*Δsir2* (lane *8*) was separated on formaldehyde-containing 1.2% agarose, transferred to GeneScreen Plus, and hybridized with an antisense 3′ *URA3* probe. The *ura3-167* and *URA3*$^+$ messages are the same size and their locations are indicated by *URA3*. The location of the *mURA3* message is also indicated.

plasmid. As shown in Figure 5B for S6, the *SIR2* plasmid reversed the phenotype of the S6 *sir2* mutant to the silenced state, whereas an empty vector did not. Subsequent loss of the *SIR2* plasmid resulted in a further reversal in phenotype to the expressed state. Amplification of *mURA3* was also ruled out by a quantitative Southern blot, which showed that the copy number of *mURA3* in the *sir2* mutant population of S6 had not significantly increased over that of the *SIR2* parent population. We therefore conclude that the effect of the *sir2* mutation on the expression of *mURA3* does not result from recombinational amplification.

mURA3 *silencing is transcriptional*

The effect of a *sir2* deletion on *mURA3* expression levels for rDNA insertions S3 and S6 was tested directly by RNA blot analysis. The ~1-kb endogenous *URA3* transcript is present in the *ura3-167* background and provides a convenient RNA-loading control for RNA blots (Fig. 5C, lane 2). The *mURA3* transcript produced by Ty1–*mURA3* is terminated by the Ty1 3′ long terminal repeat (LTR) and is expected to be ~1165 nucleotides long before polyadenylation. Non-rDNA insertion R31 produced a *mURA3* transcript of about that size (Fig. 5C, lane 4). In contrast, this *mURA3* transcript was undetectable in rDNA isolates S3 and S6 (lanes 5 and 7, respectively). Deletion of *SIR2* in S3 and S6 resulted in the appearance of *mURA3* RNA (lanes 6 and 8, respectively). These results directly demonstrate the transcriptional inactivity of *mURA3* within the rDNA, and the requirement of *SIR2* for this silencing.

A colony–color assay for analyzing rDNA-mediated silencing

Transcriptional silencing of *ADE2* placed near telomeres results in reversible switching between on and off states of expression that produces easily visible red and white sectors in colonies (Gottschling et al. 1990). To determine whether switching occurs in rDNA-mediated silencing, a colony–color assay based on the *MET15* gene was adapted for use in the rDNA. Growth of *met15* mutant yeast strains on Pb^{2+}-containing medium results in dark brown colonies, whereas colonies of *MET15*$^+$ strains remain white (Ono et al. 1991; Cost and Boeke 1996). *MET15* was introduced into the rDNA by 5S rDNA-targeted transposition of a *MET15*-marked Ty1 (Fig. 6A). Random insertions of Ty1–*MET15* into non-rDNA genomic regions resulted in white colonies on Pb medium (isolate M9), indicating that Ty1–*MET15* can fully complement a *met15* deletion in single copy (Fig. 6B). However, Ty1–*MET15* insertions into rDNA resulted in colonies with an intermediate tan color (isolate M1), indicating that *MET15* is also silenced in the rDNA. The darkness of the tan color varied among different Ty1–*MET15* rDNA isolates and intensified with time for all isolates (data not shown). Occasional dark brown sectors were observed, but these represented recombination events in the colony leading to permanent loss of *MET15*, not reversible switching (data not shown). The frequency of these dark brown sectors dramatically increased and the background colony color turned white when *sir2* was deleted (Fig. 6B), indicating loss of silencing and increased recombination. The intermediate color phenotype of rDNA::Ty1–*MET15* insertions differs from that observed for telomeric silencing, in which switching results in either white or red sectors but not an intermediate pink color (Gottschling et al. 1990).

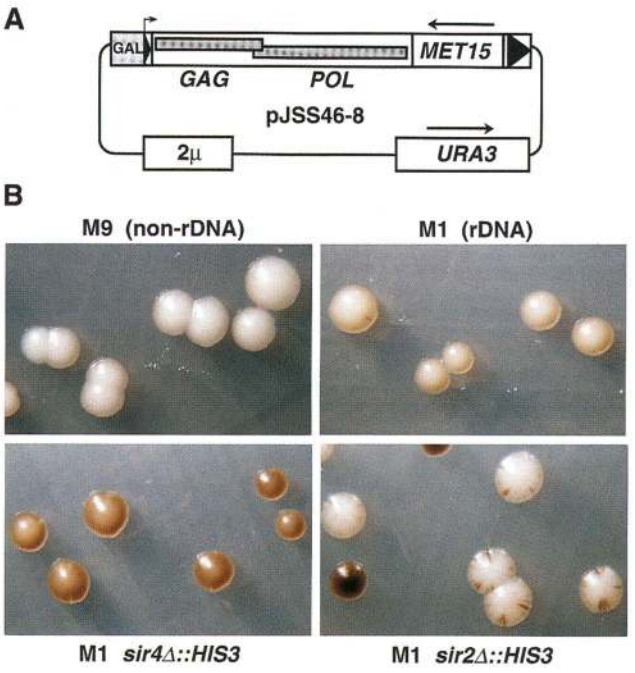

Figure 6. Colony color assay for rDNA silencing. (*A*) Schematic diagram of *GAL1*–Ty1–*MET15* vector pJSS46-8. Ty1 is marked at the 3′ end with *MET15* in the reverse orientation. *MET15* expression is driven by its own promoter. (*B*) *SIR2* or *SIR4* were replaced with *HIS3* in Ty1–*MET15* rDNA isolate M1. The parent (M1) and its *sir2* and *sir4* deletions were streaked for single colonies onto Pb-containing rich medium and incubated for 5 days at 30°C, when photos were taken at 6× magnification. Strain M9 is a non-rDNA Ty1–*MET15* insertion used as a Met$^+$ control that produces white colonies. The uniform dark brown color of Met$^-$ colonies can be seen in the *sir2* mutant. These colonies originated from single cells that had already lost *MET15* through recombination at the time of plating.

The only *SIR* gene deletion that eliminated *mURA3* silencing in Figure 5A was *sir2Δ::HIS3*. To determine whether deletion of any of the other *SIR* genes might enhance silencing, *SIR1*, *SIR3*, or *SIR4* were deleted in isolate M1, and assayed for colony color. Deletion of *SIR4* greatly enhanced silencing, as measured by a significantly darkened colony color (Fig. 6B), and confirmed initial observations with the Ty1–*mURA3* insertions. Deletion of *SIR1* or *SIR3* had little if any affect on silencing strength (data not shown). This result suggests that Sir4p is involved in the regulation of rDNA silencing. Two of the known *SIR* genes have now been implicated in rDNA silencing, although *sir4* mutations have

opposite phenotypes in the rDNA as compared with *HM* loci and telomeres.

rDNA chromatin structure depends on SIR2

Silencing of Pol II transcripts in the rDNA could result from a repressive chromatin structure. Inactive and active rDNA gene copies in a yeast cell population can be differentiated by their different chromatin structures and accessibility to UV cross-linking by psoralen (Dammann et al. 1993). These differences are visualized by separation on agarose gels; the more heavily cross-linked DNA migrates more slowly. Actively transcribed gene copies lack nucleosomes and are therefore more accessible to psoralen, and migrate more slowly (Dammann et al. 1993). If *SIR2* was responsible for a repressive rDNA structure, deletion of *SIR2* should make rDNA chromatin more accessible to psoralen cross-linking, resulting in even slower migration.

Log-phase cultures of JB740 (*SIR2*$^+$) and JS207 (*sir2Δ::HIS3*) were photoreacted with psoralen. *Eco*RI-digested genomic DNA was then separated on agarose and hybridized with probe B (35S-specific), which detects 1.9- and 2.8-kb 35S fragments from non-cross-linked samples (Fig. 7A, lanes 1,2). Cross-linking shifted these into two separate bands, inactive gene copies (f) and actively transcribed gene copies (s). Deletion of *SIR2* caused the inactive (f) band to supershift to an even slower migration, indicating that this subset of rDNA copies became more accessible to psoralen. Supershifting of the actively transcribed (s) band was minimal. Supershifting of the NTS species detected by probe A (NTS-specific) in the *sir2* mutant was even more dramatic, indicating that the NTS is affected by Sir2p more than the transcribed region. The non-rDNA 1.5-kb *TRP1* fragment did not display any *sir2*-dependent supershift, indicating specificity for rDNA. Remarkably, the ratio of active and inactive rDNA gene copies was also different in the *SIR2*$^+$ and *sir2* strains in log phase cultures. Loss of Sir2p function increased the ratio of actively transcribed to inactive gene copies from 40:60 to 50:50 on average (Fig. 7B), suggesting that *SIR2* and the silencing it mediates may also influence pol I transcription. We conclude that rDNA chromatin is more accessible to psoralen cross-linking when Sir2p function is lost, consistent with the loss of a chromatin structure required for transcriptional silencing.

Discussion

Transcriptional repression within rDNA is surprising, because transcription by Pol I and Pol III occurs efficiently at this locus. These RNA polymerases normally act within rDNA, whereas Pol II does not. However, Pol II is clearly present in the nucleolus, as several Pol II-driven genes such as native *URA3* and *ADE2* are expressed when inserted into rDNA (Keil and McWilliams 1993), raising the question of how and why *mURA3*, *MET15*, and *LEU2* are silenced. Several lines of evidence from this study support the hypothesis that the transcriptional repression we observe in rDNA is actually a novel form of silencing. (1) Three out of four genes we inserted into the rDNA were repressed, indicating a non-gene-specific mechanism. (2) Repression depends on the silent information regulator gene, *SIR2*, which is also required for other forms of transcriptional silencing. (3)

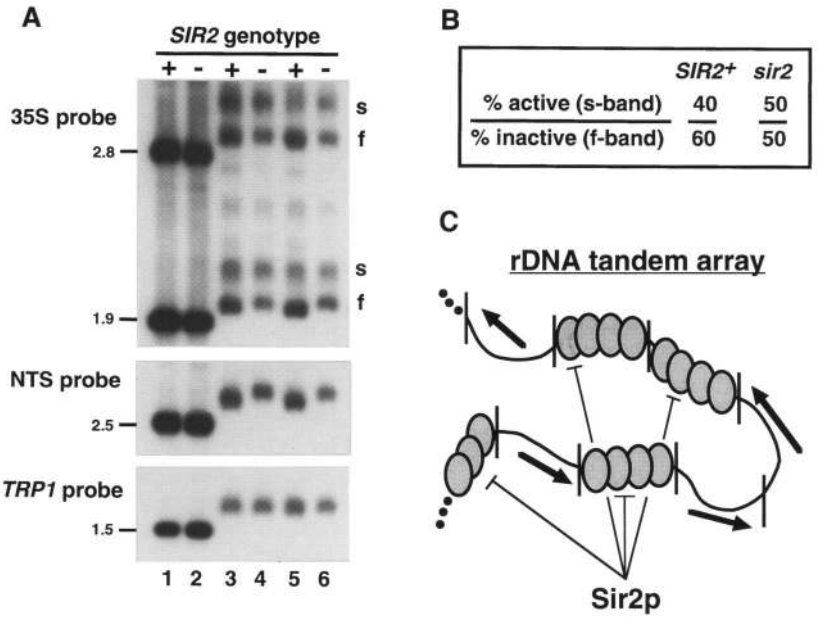

Figure 7. The rDNA is more accessible to psoralen cross-linking in the absence of *SIR2*. (*A*) *Eco*RI-digested DNA from either noncross-linked cells (lanes *1,2*) or cross-linked cells (lanes *3–6*) were separated on 1.3% agarose, transferred to GeneScreen Plus, and hybridized with either a 35S-specific probe in the *top* panel, an NTS-specific probe in the *middle* panel, or a *TRP1*-specific probe in the *bottom* panel. The *SIR2* genotype is indicated at the *top*; lane numbers are at the *bottom*. For the 35S-probed panel, slow (s) and fast (f) migrating cross-linked bands are indicated. Non-cross-linked fragment sizes are indicated to the *left*. JB740 is the *SIR2*$^+$ strain, and JS207 is the *sir2* strain. DNA from the cross-linked cells was loaded in duplicate. Each panel represents the same filter stripped and rehybridized. (*B*) The ratio of actively transcribed gene copies (s bands) to inactively transcribed gene copies (f bands) of the above experiment was determined by PhosphorImager quantitation of each band. The average ratios, including both the 2.8- and 1.9-kb bands, are indicated. (*C*) Model of proposed mechanism of silencing that depends on the transcriptional status of a given rDNA gene copy within the tandem array. The distribution of active gene copies (arrows) is random, with the activity status of each gene copy not stable from generation to generation. Sir2p would be required for silencing in any of the inactive gene copies (shaded ovals). A marker in an active rDNA gene copy would also be active.

Another silent information regulator gene, *SIR4*, appears to limit the degree of repression. (4) A specific chromatin structure was detected at the rDNA that requires *SIR2* activity.

Strength of rDNA silencing depends on promoter properties

The degree of telomeric silencing depends on promoter strength. The efficiency of *URA3* silencing at telomeres is markedly increased by reducing the strength of the *URA3* promoter through deletion of the activator *PPR1* (Renauld et al. 1993). Similarly, *TRP1* lacking activation sequences was the most silenced marker tested near telomeres (Gottschling et al. 1990). The minimal *TRP1* promoter driving *mURA3* expression also lacks complete activation sequences (Kim et al. 1986). *MET15* and *LEU2* both had intact promoters, as did the Ty1 element studied by Bryk et al. (1996). *HIS3*, which was not detectably silenced by rDNA, was also poorly silenced at telomeres (Gottschling et al. 1990). rDNA silencing is therefore not as potent as telomeric silencing but, like silencing at telomeres, depends on the strength of the particular promoter tested.

SIR *gene involvement in regulation of rDNA silencing*

SIR2 has never before been implicated in silencing independent of *SIR3* or *SIR4*. Interestingly, immunofluorescence has revealed that a large fraction of Sir2p normally localizes to the nucleolus, suggesting that its major function is associated with rDNA (S. Gasser, pers. comm.). Given the large amount of rDNA, the yeast cell may use another set of factors that, along with *SIR2*, form and maintain repressive rDNA chromatin. This would prevent competition with the telomeres and *HM* loci for other silencing factors such as Sir3p. Competition between the telomeres and *HMR* has been demonstrated previously (Buck and Shore 1995). Genes homologous to *SIR2* (*HST1*, *HST2*, *HST3*, and *HST4*) were identified recently and shown to have roles in silencing and are candidates for functioning in rDNA silencing (Brachmann et al. 1995; Derbyshire et al. 1996).

Deletion of *SIR4* significantly enhances rDNA silencing of *MET15*, suggesting a *SIR4* regulatory function. Sir3p, Sir4p, and Rap1p have been shown by immunofluorescence to colocalize with telomeres as foci within the nuclear periphery of *S. cerevisiae* (Palladino et al. 1993; Gotta et al. 1996; Maillet et al. 1996). It has been shown recently that loss of *SIR4* function delocalizes the telomeric foci, with Sir3p relocalizing to the nucleolus (S. Gasser, pers. comm.), where it could have an as yet unknown function in rDNA silencing, perhaps in cooperation with Sir2p. Sir3p and Sir2p have been shown recently to interact (Holmes et al. 1997). Rap1p could also be released to act elsewhere. A Rap1p-binding site exists in the rDNA that has been shown in vitro to be efficiently bound by Rap1p (Buchman et al. 1988). However, it is unknown whether *RAP1* has any function in rDNA silencing. In a *sir4* mutant, Sir2p is no longer detectable at telomeres by either cross-linking or immunofluorescence, whereas the amount detected on rDNA increases (S. Gasser, pers. comm.). This could also explain the observed enhancement of rDNA silencing in a *sir4* deletion strain. Consistent with this possibility, increases in *SIR2* gene copy number enhance rDNA silencing (J.S. Smith and J.D. Boeke, in prep.).

Sir4p was shown recently to interact biochemically with Sir2p, Sir3p, Ubp3p, and a 69-kD protein (Moazed and Johnson 1996). Ubp3p is a deubiquitinating enzyme (Baker et al. 1992) that in the above study was shown to regulate telomeric and *HM* loci silencing. Deletion of *UBP3* results in improved silencing at both locations. If Ubp3p were also involved in regulation of rDNA silencing, then loss of Sir4p could alter this regulation through the loss of Sir4p–Ubp3p interaction. In Bryk et al. (this issue), deletion of the *UBC2* (*RAD6*) ubiquitin-conjugating enzyme gene resulted in loss of rDNA silencing, implicating a possible ubiquitin-mediated regulatory mechanism involved in rDNA silencing. Possible mechanisms of rDNA silencing regulation by *SIR4* described above can now be tested.

Chromatin and rDNA silencing

Silencing of *mURA3* caused by rDNA occurs only within the tandem array, not when an rDNA gene copy containing *mURA3* is positioned at the *LEU2* locus, suggesting that there may be a repressive chromatin structure specifically associated with the rDNA array. Inactive rDNA gene copies and the NTS were more accessible to psoralen cross-linking in a *sir2* mutant, whereas active gene copies were minimally affected, consistent with a *SIR2*-dependent chromatin structure repressive to transcription. In a topoisomerase I mutant, the chromatin of actively transcribed rDNA gene copies was more accessible to psoralen, but the inactive gene copies were unaffected (Cavalli et al. 1996). Altered supercoiling was proposed to increase the accessibility of transcribed gene copies (Cavalli et al. 1996). Topoisomerase I and Sir2p therefore affect rDNA chromatin structure differently. The largest *SIR2* affect on chromatin structure was at the NTS, where the regulatory elements for Pol I and Pol III transcription, as well as an origin of replication, are located. *SIR2*, therefore, potentially could influence all of these processes.

Histone hypoacetylation has been implicated in transcriptional silencing in *S. cerevisiae*, with histones H3 and H4 packaging the *HM* silent cassettes displaying the same acetylation patterns as found in metazoan heterochromatin (Braunstein et al. 1996). *SIR2* overexpression was shown previously to cause a decrease in histone acetylation (Braunstein et al. 1993). Regulation of nucleosome acetylation states within rDNA chromatin therefore could play a role in maintaining the Pol II-silenced/recombination-silenced state.

Model for intermediate rDNA silencing phenotypes

The most striking phenotype of rDNA-mediated

mURA3 silencing is that colonies are FOA^R yet still Ura^+, similar to TPE. Switching between on and off transcriptional states was demonstrated by the red and white sectors of colonies that contain a telomeric *ADE2* gene (Gottschling et al. 1990). Large sector sizes in this assay indicate that switching occurs infrequently, which is phenotypically similar to classic position effect variegation caused by heterochromatin spreading in *Drosophila*. Using the *MET15*-based colony–color assay, we were unable to detect large reversible sectors, indicative of slow switching, in colonies containing Ty1–*MET15* in the rDNA. An intermediate colony color was observed, which is more similar to centromeric and telomeric silencing in *Schizosaccharomyces pombe* (Allshire et al. 1994; Nimmo et al. 1994), where intermediate colored colonies were observed.

The chromatin structure of a single rDNA gene copy, including both the NTS and 35S coding regions, changes dramatically depending on its Pol I transcription state (Dammann et al. 1993). In addition, the distribution of active gene copies in the array is random with the activity state of a specific gene copy not stably inherited (Dammann et al. 1995). This provides a potential stochastic on/off switch for controlling associated processes such as rDNA silencing. A model for rDNA silencing therefore can be developed in which *mURA3* or *MET15* expression rapidly alternates between two different states depending on the Pol I activity of the particular rDNA gene copy that the marker was inserted into (Fig. 7C). For example, active Pol I transcription could produce higher *mURA3* expression, whereas an inactive gene copy would lack *mURA3* expression. A *SIR2*-dependent repressive chromatin structure could silence the marker gene when its host rDNA gene copy is inactive. Such fast alternation between the two states would produce the phenotypic equivalent of a stably maintained intermediate expression level, and is highly consistent with all of our data.

Biological functions of rDNA silencing

Why should the rDNA, which is a long uninterrupted stretch of DNA designed to be transcribed by Pol I and Pol III, contain a mechanism to silence transcription? A likely function could be to provide a chromatin state that excludes the recombinational machinery. Recombination rates within the rDNA are normally low relative to non-rDNA. *sir2* mutations increase both meiotic and mitotic recombination rates in rDNA (Gottlieb and Esposito 1989), consistent with the loss of a protective chromatin environment. Other mutations that eliminate rDNA silencing also increase rDNA recombination rates (Bryk et al., this issue).

Another potential function of rDNA silencing could be to discourage the integration of foreign invaders like transposable elements into the array. The loss of one or a few rDNA gene copies by transposon insertion probably has no measurable effect on host viability, and therefore rDNA represents a potential "safe haven" for transposons. Indeed, the R1–R4 elements of invertebrates are highly specialized for insertion into rDNA (Jakubczak et al. 1991; Burke et al. 1995). Ty1 targeting upstream of Pol III-transcribed genes provides Ty1 with a mechanism of integrating within the rDNA (Devine and Boeke 1996). The targeting of Ty1 to the rDNA and its subsequent silencing is intriguing because another yeast retrotransposon, Ty5, targets its integration to silenced genomic regions (Zou et al. 1996). Interestingly, Ty3 can utilize the 5S rRNA gene as a target on an episomal plasmid, yet integration events into the tandem array do not occur (Chalker and Sandmeyer 1990), consistent with selective exclusion of retrotransposons. At the homologous recombination level, Pol II silencing could be a means of preventing the amplification of a Ty1 element that happened to integrate into rDNA. Bryk et al. (this issue) show that Ty1 transposition is indeed reduced when Ty1 integrates into rDNA.

rRNAs are coordinately synthesized in most organisms, including *S. cerevisiae*. The levels of these RNA species change with different growth conditions such as nutrient availability and growth rate (Warner 1989). *S. cerevisiae* activates or inactivates rDNA gene copies as needed in response to growth conditions (Warner 1989; Dammann et al. 1993). *SIR2* appears to affect this regulatory mechanism. We have shown that the proportion of transcriptionally active rDNA gene copies consistently increases in the absence of *sir2*. Loss of a *SIR2*-dependent rDNA chromatin structure may increase the probability that a particular rDNA gene copy is actively transcribed, which potentially could have dramatic effects on the overall levels of rRNA synthesis. The possible effects on 5S Pol III transcription or rDNA replication has not yet been addressed.

Bryk et al. (this issue) demonstrate that transposition of Ty1 embedded in the rDNA is repressed in a *UBC2*-, *SIR2*-, and *TOP1*-dependent manner. This repression was a result of silencing of transcription from the Ty1 promoter. Ubc2p is an E2 ubiquitin-conjugating enzyme that can polyubiquitinate the core histones H2A and H2B, in vitro. Consistent with a role for chromatin structure, they also show that changing the stoichiometry of H2A and H2B by deleting one gene pair also relaxes rDNA silencing. These findings by Bryk et al. (this issue) provide independent evidence for an unusual chromatin structure in the rDNA tandem array that is repressive to Pol II transcription. Finally, the *mURA3* and *MET15* marker systems described here provide versatile and sensitive reporters for the identification of other genes that are involved in the formation and maintenance of the repressive chromatin structure within yeast rDNA, which could provide new insights into the formation and function of yeast "heterochromatin."

Materials and methods

Plasmids

mURA3-marked GAL–Ty1 plasmids were constructed as follows. pJEF1114 is a 2μ, *TRP1* vector that contains an unmarked Ty1 element fused to the *GAL1* promoter (Natsoulis et al. 1989). The *Bam*HI site between the 3′ LTR and *TRP1* marker

was filled in, producing pJEF1712. The 3′ end of Ty1 in pJEF1712 was replaced with a *Sal*I–*Nco*I fragment from pD123 (Ji et al. 1993), containing a *Bam*HI site at nucleotide 5561 in Ty1, producing pJSS19. A promoterless *URA3* fragment was PCR-amplified using primers JB1006 (5′-CGCGGATCCATGTCGAAAGCTACATAT-3′) and JB1007 (5′-CGCAGATCTTAGTTTTGCTGGCCGCAT-3′), cut with *Bam*HI and *Bgl*II (sites underlined), and ligated into the *Bam*HI site of pJSS19, producing pJSS20-1. A *TRP1* promoter [nucleotides −209 to −1 (Kim et al. 1986)] was PCR-amplified from pRS404 (Sikorski and Hieter 1989), using primers JB1003 (5′-CGCGGATCCTATGACGCCAGATGGCA-3′) and JB930 (5′-GCGAGATCTCCAAGCTGCCTTTG-3′), digested with *Bam*HI and *Bgl*II, and ligated into the *Bam*HI site of pJSS20-1, producing pJSS22-7. The 3′ end of unmarked Ty1 in pJEF1695 (*HIS3* plasmid marker, J.D. Boeke, unpubl.) was replaced with the 3′ end of *mURA3*-marked Ty1 from pJSS22-7 by swapping *Sal*I–*Nco*I fragments, producing pJSS36-6.

MET15-marked Ty1 plasmid pJSS46-8 was constructed by inserting a *MET15* PCR fragment consisting of nucleotides −333 to +1332 amplified from plasmid pGC3 (Cost and Boeke 1996), using primers JB1266 (5′-CGCGGATCCTTGAGGTCACATGATCGC-3′) and JB1265 (5′-CGCAGATCTCATGGTTTTTGGCCAGCG-3′) into the *Bam*HI site of pD123.

To construct pJSS51-9, containing the *mURA3–HIS3* reporter, an amino-terminal *Bam*HI–*Nco*I fragment of *mURA3* from pJSS22-7 and a carboxy-terminal *Nco*I–*Hin*dIII fragment of *URA3* from YEp24 were ligated together into *Bam*HI–*Hin*dIII linearized pRS425 (Christianson et al. 1992), to regenerate *mURA3* with its native transcription terminator (pJSS42-1). *HIS3* was inserted downstream of *mURA3* by ligating a *HIS3* PCR cassette with *Xho*I termini amplified from pRS403 (Sikorski and Hieter 1989), using primers JB1307 (5′-CGCCTCGAGATTGTACTGAGAGTGCACC-3′) and JB1308 (5′-CGCCTCGAGCTGTGCGGTATTTCACACCG-3′) into the *Xho*I site downstream of *mURA3* in JSS42-1.

pJSS60-2 was constructed by ligating the 5′ and 3′ *Bgl*II fragments of an rDNA unit into the *Bam*HI site of pRS405 to reconstruct a full gene copy in pJSS49-5. A *Bam*HI fragment from pJSS36-6 consisting of *mURA3* and the Ty1 3′ LTR was ligated into the *Bgl*II site of the 25S rDNA of pJSS49-5 in the opposite orientation as rRNA transcription.

pCAR237 was constructed by inserting a *Hin*dIII genomic fragment containing *SIR2* into the *Hin*dIII site of pRS415 (Sikorski and Hieter 1989). The pBS–URA template for producing an antisense *URA3* probe for Northern analysis was constructed by ligating a promoterless *URA3* PCR product into the *Bam*HI site of pBluescript II KS(−).

Media and strains

Unless stated otherwise, media used were as described previously (Rose et al. 1990). Ty1 transposition induction medium (YNB/CAA+Trp galactose) consisted of yeast nutrient broth (YNB) supplemented with 2% casamino acids, 2% galactose, 160 μM adenine, 800 μM tryptophan. SC (FOA) medium contained 5-FOA at 2 mg/ml and glucose at 2%. Pb^{2+}-containing media (MLA) was described previously (Cost and Boeke 1996). Yeast strains are listed in Table 1.

Ty1 transposition and selection

JS50-2 was patched onto SC–His glucose and incubated for 1 day at 30°C. JS216 was patched on SC–Ura glucose. These patches were replica-plated to induction medium (YNB/CAA+Trp galactose) and incubated for 5 days at 22°C. These patches were then replica-plated to SC–His glucose for JS50-2 and SC–Ura glucose for JS216 and incubated for 2 days at 30°C to stop transposition, followed by replica-plating to YPD to allow for donor Ty1 plasmid loss for 1 day at 30°C. JS216 patches were then replica-plated to SC (FOA). JS50-2 cells were scraped from the YPD patches, diluted and spread on SC (FOA) plates and incubated for 3 days at 30°C. FOA^R colonies were replica-plated to SC–Ura glucose to identify FOA^R/Ura^+ isolates. JS216 cells were scraped from the FOA patches, diluted in water, spread on Pb-containing MLA plates, and incubated for 5–10 days at 30°C for color analysis. Transposition induction generally results in 50%–70% of cells receiving one or more transposition event for Ty1–*mURA3* and Ty1–*MET15*.

*Cloning of Ty1–*mURA3 *genomic integration events*

Genomic DNA of FOA^R/Ura^+ clones was isolated (Boeke et al. 1985) and 15 μl was digested with *Hin*dIII and separated on 0.7% agarose. *Hin*dIII cleaves once in Ty1–*mURA3* and at an unknown site in flanking genomic DNA (Fig. 1C). Regions of the gel containing the Ty1–*mURA3* fragment were excised. A minilibrary was constructed by ligating the purified DNA into the *Hin*dIII site of pBluescript II KS(−), and transforming into *E. coli* strain MH6 (which has a *pyrF* mutation). Carbenicillin resistant colonies were replica-plated to M9 medium lacking uracil to select for Pyr^+ (Ura^+) transformants. Integration sites of the cloned insertions were sequenced using primer JB66 (5′-GAGGAGAACTTCTAGTATAT-3′), which reads out from Ty1 into flanking DNA (Fig. 1A).

RNA blot analysis

Saturated YPD cultures were diluted to an A_{600} of 0.2 in YPD and grown at 30°C to mid-log phase (A_{600} of 1.4–1.6). Forty micrograms of total RNA, isolated as described by Chapman and Boeke (1991), was separated on a formaldehyde-containing 1.2% agarose gel and transferred to Genescreen Plus (NEN–Dupont) in 10× SSC. The filter was probed with an antisense *URA3* RNA probe labeled with [α-^{32}P]UTP (800 Ci/mmole). The *URA3* RNA probe was transcribed by T7 RNA polymerase from pBS–URA3 linearized with *Stu*I, producing a 363-nucleotide antisense probe. The filter was prehybridized in 10 ml of 5× SSPE, 5× Denhardt's solution, 1% SDS, 50% formamide (wt/vol), and 100 μg/ml boiled herring sperm DNA at 60°C for 3 hr, followed by hybridization in the above solution without herring sperm DNA at 60°C for 16 hr. The filter was washed twice at room temperature in 2× SSC/0.1% SDS, twice at room temperature in 0.2× SSC/0.1% SDS, and twice at 42°C in 0.2× SSC/0.1% SDS. The filter was imaged on a Molecular Dynamics PhosphorImager.

Identification and mapping of Ty1 insertions in rDNA

Colony PCR reactions contained 10 mM Tris-HCl at pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 19 pmoles of each primer, and 1 unit Amplitaq (Perkin Elmer). Approximately 3.5×10^5 cells from a YPD grown colony were added to each 20 μl reaction. PCR was done in a GeneAmp 9600 (Perkin Elmer; 5 min, 94°C, 1 cycle; 1 min, 94°C, 1 min, 57°C, 1 min, 72°C, 30 cycles). Products were separated on 1% agarose and the insertion points were inferred from product sizes. The Ty1–*mURA3* specific primer was JB1160 (5′-CATATTTGAGAAGATGCGGCCAGC-3′) and the Ty1-*MET15* specific primer was JB1309 (5′-TGCCATTTTGCGATCATGTGACCTC-3′). rDNA specific primers were JB1161 (5′-GATAGTTTAACGGAAACGCAGGTG-3′), JB1162 (5′-GTAGTTGGGAGGTACTTCATGCGA-3′), JB1163 (5′-ATGCTACTTGCAAAATATCATACC-

3′), JB1164 (5′-CTTAAAAGAAGAAGCAACAAGCAG-3′), and JB1168 (5′-CTTTCTAAGTGGGTACTGGCAGGA-3′).

PCR-mediated deletion of SIR *genes and integration of dual marker into rDNA*

SIR1, SIR2, SIR3, and *SIR4* open reading frames (ORFs) were precisely deleted by PCR-mediated gene disruption as described (Baudin et al. 1993; Lorenz et al. 1995), amplifying *HIS3* using primers containing 5′ flanking sequences corresponding to the *SIR* genes. Sir⁻ colonies were identified by a mating test.

To integrate the dual-marker reporter into specific positions in rDNA, primers were designed to amplify the pRS vector polylinker, and any sequences inserted there. For example, to integrate the reporter downstream of the 5S rDNA, primers JB1269 (5′-GCAGTTTCTAGGGAATGATGATGGCAAGTTCCAGAGAG**G**ATACGACTCACTATAGGGCG-3′) and JB1270 (5′-CCTCTTTCTTCTTCCCAGTAGCCTGTTCCTTTTACGCT**G**AAGGGAACAAAAGCTGGAGC-3′), were used for amplification. Underlined sequences are complementary to nucleotides 3102–3121 and 3250–3231 of pR5425, respectively. The bold sequences represent nucleotides 590 and 591 of the rDNA (Skryabin et al. 1984), respectively, indicating the location of integration. This integrates the reporter without deleting or duplicating rDNA sequence. Other rDNA primer pair sequences are available upon request.

Psoralen cross-linking

Fresh 100-ml yeast extract, peptone, dextrose (YPD) cultures were inoculated from saturated cultures YPD cultures to an A_{600} of 0.3 and grown for 6 hr at 30°C into log phase. Cross-linking with 4, 5′, 8-trimethylpsoralen (Sigma) using a long-wave UV lamp (model B-100A; Ultraviolet Products, Inc.) was as described previously (Dammann et al. 1993). Approximately 5×10^8 cells were washed with ice-cold H_2O and resuspended in 1.4 ml cold 10 mM Tris-HCl (pH 7.6), 1 mM EDTA (TE) in a six-well tissue culture plate. Seventy microliters of 200 µg/ml psoralen in 100% ethanol was added to each well and UV-irradiated for 5 min on ice at a distance of 6 cm five times. 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 TE and normalized to A_{260} of 0.1. DNA (10 µl) was digested overnight at 37°C with *Eco*RI in a 30 µl reaction containing 80 ng/µl of RNase A. DNA was separated on 1.3% agarose (14.5 × 24 cm) at 60 V for 24 hr. Cross-linking was reversed in a Stratagene Stratalinker at 0.6 J/cm^2. DNA was transferred to GeneScreen Plus in 10× SSC and hybridized with rDNA-specific probes A (NTS) or B (35S), or a *TRP1*-specific probe.

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