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Genetic Identification of Factors That Modulate Ribosomal DNA Transcription in *Saccharomyces cerevisiae*

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ABSTRACT

Ribosomal RNA (rRNA) is transcribed from the ribosomal DNA (rDNA) genes by RNA polymerase I (Pol I). Despite being responsible for the majority of transcription in growing cells, Pol I regulation is poorly understood compared to Pol II. To gain new insights into rDNA transcriptional regulation, we developed a genetic assay in *Saccharomyces cerevisiae* that detects alterations in transcription from the centromere-proximal rDNA gene of the tandem array. Changes in Pol I transcription at this gene alter the expression of an adjacent, modified *URA3* reporter cassette (*mURA3*) such that reductions in Pol I transcription induce growth on synthetic media lacking uracil. Increases in Pol I transcription induce growth on media containing 5-FOA. A transposon mutagenesis screen was performed with the reporter strain to identify genes that play a role in modulating rDNA transcription. Mutations in 68 different genes were identified, several of which were already known to function in chromatin modification and the regulation of Pol II transcription. Among the other classes of genes were those encoding proteasome subunits and multiple kinases and phosphatases that function in nutrient and stress signaling pathways. Fourteen genes were previously uncharacterized and have been named as **r**egulators of **r**DNA **t**ranscription (RRT).

RIBOSOME biogenesis is a complex, tightly regulated process consisting of ribosomal DNA (rDNA) transcription, ribosomal RNA (rRNA) processing, ribosomal protein synthesis, and ribosome assembly. Its regulation is important for the control of cellular growth because it dictates the availability of the number of ribosomes required for efficient protein synthesis. Of particular importance is the synthesis of rRNA by RNA polymerase I (Pol I), which comprises the majority of all transcription in growing yeast (*Saccharomyces cerevisiae*) (Warner 1999). Ribosomal RNA synthesis rates are very high in these cells, but as nutrients in the growth medium are depleted and cells stop growing in stationary phase, the rate of rDNA transcription is greatly diminished (Ju and Warner 1994). One of the common hallmarks shared by rapidly proliferating cancer cells, or by hypertrophic cardiomyocytes in response to hypertension, is an increase in rDNA transcription (Hannan and Rothblum 1995; White 2005). Determining how cells activate and repress rDNA transcription in response to external nutrient and stress signals is therefore imperative for understanding the mechanisms of uncontrolled cell growth in such diseases. Yeast is an excellent model system to address such a problem.

Yeast rDNA consists of a single tandem array on the right arm of chromosome XII containing ~150–200 head-to-tail repeats of the 9.1-kb rDNA gene. Each rDNA gene is transcribed by Pol I toward the centromere-proximal end of the array to produce a large 35S rRNA precursor, which is then co- and post-transcriptionally processed into the 18S, 5.8S, and 25S rRNAs (for review see Nomura 2001). Although only ~50% of the rDNA genes are transcribed in a proliferating yeast cell (Dammann *et al.* 1993), each active gene is highly transcribed by ~50 Pol I enzymes (French *et al.* 2003). Upon entry into stationary phase, the reduction in rate of rDNA transcription is accompanied by a lower percentage of rDNA genes that are actively transcribed and maintained in an open, psoralen-accessible chromatin state (Dammann *et al.* 1993). The histone deacetylase Rpd3 is required for closing the rDNA genes during stationary phase, although the mechanism by which this occurs remains uncertain (Sandmeier *et al.* 2002).

The rDNA in yeast can be transcribed by RNA polymerase II (Pol II) under certain conditions, although this is normally prevented by the Pol I transcription factor UAF through an unknown mechanism (Vu *et al.* 1999). Pol II-transcribed reporter genes inserted into the rDNA are silenced in a Pol I- and UAF-dependent manner (Buck *et al.* 2002; Cioci *et al.* 2003). This type of silencing (known as rDNA silencing) also requires the

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NAD^+-dependent histone deacetylase Sir2 (Bryk *et al.* 1997; Fritze *et al.* 1997; Sandmeier *et al.* 2002). In contrast, mutating *SIR2* has no measurable effect on Pol I transcription (Sandmeier *et al.* 2002). The mechanism of how UAF and Pol I function in silencing Pol II transcription remains unknown, but the involvement of Sir2 implies that environmental changes that alter rRNA synthesis rates could have significant effects on the rDNA chromatin structure.

In yeast, formation of the Pol I preinitiation complex requires four transcription factors: upstream activation factor (UAF), core factor (CF), TATA-binding protein, and Rrn3 (Nomura 2001). Rrn3 directly associates with Pol I, and the differential phosphorylation of Rrn3 regulates Pol I function (Milkereit and Tschochner 1998; Fath *et al.* 2001). Additional phosphorylation sites on Pol I subunits have been proposed to regulate Pol I function (Gerber *et al.* 2008). Rrn3 is equivalent to TIF-IA in mammals (Bodem *et al.* 2000), and CF is analogous to the mammalian SL1 complex (Lalo *et al.* 1996). A mammalian equivalent of UAF has not been identified, but the HMG-box-containing protein, UBF, functions in the initiation of mammalian Pol I transcription and is important for determining the number of active rDNA genes (Sanij *et al.* 2008), a function shared by UAF in yeast (Hontz *et al.* 2008). UBF has also been implicated in Pol I elongation (Stefanovsky *et al.* 2006) and is a target of regulation by phosphorylation and acetylation mechanisms, as is SL1 (for review see Moss *et al.* 2007).

The complex nature by which rDNA transcription, rRNA processing, and ribosomal protein gene transcription are coordinated suggests that researchers have only begun to scratch the surface of understanding the mechanisms involved in regulating these processes. In yeast, several signaling pathways are implicated in the regulation of rDNA transcription. Mutants defective in the secretory pathway respond by repressing the transcription of both rDNA and ribosomal protein genes through a protein-kinase-C-dependent regulatory circuit (Thevelein and de Winde 1999). Ribosomal DNA and ribosomal protein gene transcription also respond to signaling by the TOR (for review see Martin *et al.* 2006 and Tsang and Zheng 2007) and cAMP/PKA (Klein and Struhl 1994; Thevelein and de Winde 1999) pathways upon changes in nutrient availability. The details of how these pathways mediate changes in rDNA transcription, especially at the chromatin level, remain largely unknown. In this study, we developed a genetic assay that detects changes in Pol I transcription and employed it in a genetic screen for mutations that either improve or weaken rDNA transcription. Of the genes identified, several have previously been implicated in the regulation of Pol II transcription via chromatin modification, while others have no prior ties to transcriptional regulation. Importantly, the data provide new insights into the cellular processes that impact on Pol I transcription.

MATERIALS AND METHODS

Yeast strains and growth media: Yeast strains were derived from JB740 (Smith and Boeke 1997). YRH4 and YRH269 contain the *mURA3-HIS3* reporter cassette adjacent to the centromere-proximal rDNA gene, where the NTS1 sequence was replaced with a 361-bp sequence derived from pJSS51-9 (Buck *et al.* 2002). The genetic identifier for this reporter cassette is *nts1Δ::mURA3-HIS3*, where *HIS3* is used for transformant selection. Strains used in this study are listed in the supporting information, Table S1. YSB519 is similar to YRH4, except that the *mURA3-HIS3* reporter replaced the *TRP1* gene (Buck *et al.* 2002). Genes were tagged with 13 copies of the myc epitope (13xMyc) in YRH269 as previously described (Longtine *et al.* 1998), using primers listed in Table S2. Spot-test growth assays were performed by growing strains as patches on YPD plates. Cells were scraped from the patches, resuspended in 1 ml of sterile H_2O, normalized to an OD_{600} of 1.0, and serially diluted fivefold in 96-well plates. Five microliter aliquots of the dilutions were then spotted onto SC, SC−ura, and SC media containing 0.1% 5-fluoro-orotic acid (5-FOA). We use FOA to represent this compound throughout the text. Recipes for SC media are from Burke *et al.* (2000). Plates were incubated for 3 days before photographs were taken using an Alpha Innotech imager system.

Transposon mutagenesis: YRH4 was transformed with *Not*I-digested plasmid pools that contained fragments of yeast genomic DNA with random *mTn3* transposon insertions (Burns *et al.* 1994), as previously described (Smith *et al.* 1999). Transformants were selected on SC−leu plates, grown for 3 days, and then replica plated to SC−leu, SC−leu−ura, and SC−leu+FOA (0.2%) to identify mutants with altered rDNA transcription (Figure 2A). To cut down on background, 0.2% FOA was used. Locations of the *mTn3* insertions were determined using either a plasmid rescue technique with pRS404, as previously described (Smith *et al.* 1999), or inverse PCR from genomic DNA (Burke *et al.* 2000). In both cases, sequencing across the transposon–yeast DNA junction was carried out with primer JS702. Sequences adjacent to the transposon were compared to the *Saccharomyces* Genome Database using BLAST to identify the exact nucleotide location of the insertion.

Reverse transcriptase PCR: Yeast were grown in 50-ml YPD cultures to an OD_{600} of ~1.0. Total RNA was isolated using the hot-acid phenol method (Ausubel *et al.* 2000). cDNA was synthesized using the Invitrogen SuperScript reverse transcriptase II kit following the manufacturer's instructions. Five micrograms of total RNA was used for each cDNA reaction using either primer 1 (JS635) or the *ACT1* primer JS84 at final concentrations of 1 μM. The following oligonucleotides were used for PCR reactions: primer 1 (JS635), primer 2 (JS636), primer 3 (JS639), *ACT1* forward (JS83), and *ACT1* reverse (JS84). Primers for this study are provided in Table S2. The PCR conditions were as follows: 95° for hot start, 94° for 2 min, 35 cycles of 94° for 1 min, 50° (for *ACT1* cDNA) or 55° (for rRNA readthrough cDNA) for 1 min, 72° for 1 min, and 72° for 10 min. The products were run on a 1% agarose/TBE gel.

For the real-time RT–PCR assays, cDNA was produced from 5 μg total RNA using 1 μM primer 4 (JS766) for the rRNA product or 1 μM JS770 for the *ACT1* product. The primer set for the rRNA readthrough product was JS765 and JS766, and the primer set for *ACT1* was JS769 and JS770. Each 20-μl PCR reaction contained 200 nM of each primer, 10 μl 2× SYBR Green PCR Master Mix (Applied Biosystems), and 1 μl of cDNA (from a 1:625 dilution of the cDNA reaction). The manufacturer's instructions were followed. PCR reactions utilized a four-stage profile: stage 1, 50° for 2 min; stage 2, 95° for 10 min; stage 3, 95° for 15 sec, 60° for 1 min (40×); and

stage 4 (dissociation), 95° for 15 sec, 60° for 30 sec, and 95° for 15 sec. Triplicates of each unknown cDNA were run simultaneously on a single 96-well plate, with standard deviation error bars calculated on the basis of the average of three to four independent cultures. Reactions were performed on an Applied Biosystems 7300 real-time PCR machine.

Pulsed-field gel electrophoresis: The procedure was performed as previously described (Gerring *et al.* 1991). Yeast spheroplasts in low-melting-point agarose plugs were incubated in 1 ml LET solution (0.5 M EDTA, pH 8; 0.01 M Tris–HCl, pH 7.5) overnight at 37°, then equilibrated at 4° for 1 hr. The LET solution was replaced with 2 mg/ml proteinase K in NDS solution (0.5 M EDTA, pH 8; 0.01 M Tris–HCl, pH 7.5; 0.5 g Sarkosyl) and incubated for 2 days at 50°. The proteinase K/NDS solution was removed and replaced with 1 ml EDTA/Tris (0.05 M EDTA, pH 8; 0.01 M Tris–HCl, pH 7.5). For *Bam*HI digests, the plugs were preequilibrated in 500 μl 1× *Bam*HI buffer (NEB) + 0.1 mg/ml BSA on ice for 1 hr. The buffer was replaced two times. Following the final incubation, 2 μl *Bam*HI enzyme (NEB; 20,000 units/ml) was added and incubated on ice for 2 hr and then shifted to 37° overnight. Plugs were placed into the wells of a 1% agarose/0.5× TBE gel and run for 68 hr on a Bio-Rad CHEF Mapper electrophoresis system. The running conditions were as follows: 120° angle; linear ramp (default); 3.0 V/cm; initial switch time, 300 sec; final switch time, 900 sec with 0.5 TBE× circulated at 14° as the running buffer. Following Southern blotting, the rDNA array released by *Bam*HI was detected by hybridization with a ^{32}P-labeled probe specific for the transcribed 35S region, followed by autoradiography.

Western blotting: Log-phase yeast cultures (5 ml) were pelleted and then extracted in 0.5 ml 20% trichloroacetic acid (TCA) by vortexing with glass beads as previously described (Tanny *et al.* 1999). TCA precipitates were resuspended in 200 μl sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue), 10 μl β-mercaptoethanol, and 50 μl 2M Tris base. Ten-microliter aliquots of the samples were separated on 10% SDS polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore). The membrane was blocked with 5% milk in PBS/0.1% Tween-20 and then probed with a 1:5000 dilution of anti-myc monoclonal antibody 9E10 (Millipore) or with a 1:5000 dilution of antitubulin monoclonal antibody (B-5-1-2, Sigma) in 25 ml PBS/0.1% Tween for 1 hr and then with a 1:5000 dilution of goat anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase. Proteins were detected by chemiluminescence using ECL (GE Healthcare).

Chromatin immunoprecipitation assays: The chromatin immunoprecipitation (ChIP) procedure was performed as previously described (Dasgupta *et al.* 2005). YPD cultures (100 ml) were grown into log phase and crosslinked with 1% formaldehyde for 6 min at 30°. The washed cell pellets were resuspended in 0.6 ml FA-lysis 140 buffer + protease inhibitors [(50 mM HEPES, 140 mM NaCl, 1.0% Triton X-100, 1.0 mM EDTA, 0.1% sodium deoxycholate), 25 ml 100× protease inhibitor cocktail (Sigma), 24 μl 100 mM benzamidine, 6 μl fresh 500 mM PMSF] and disrupted using a mini-bead beater 8 (Biospec Products) at 4°. Cell extracts were sonicated with eight 10-sec pulses (30% output, 90% duty cycle) on ice, then centrifuged at 4°, and the supernatants were transferred to a new microfuge tube. For immunoprecipitations, 1.5 mg of protein for each extract was incubated in a 0.6-ml microfuge tube with FA-lysis 140 solution in a total volume of 0.4 ml. Prior to antibody addition, an RNase A treatment was performed as previously described (Jones *et al.* 2007). A 1:50 dilution of anti-myc antibody (8 μl) (Millipore; clone 9E10) was added and rotated overnight at 4°. One-tenth of the chromatin extract volume used for the immunoprecipitation (IP) was used as the input control. The tubes were centrifuged at 14,000 rpm for 2 min at 4°, and the supernatant was added to a new 0.6-ml microfuge tube containing 60 μl protein A sepharose beads (50% slurry in FA lysis 140 buffer) and rotated at 4° for 4 hr. Beads were spun at 5000 rpm for 30 sec at room temperature and then washed 4 times with 0.5 ml FA-lysis 140 buffer, 4 times with 0.5 ml FA lysis 500 buffer (50 mM HEPES, 500 mM NaCl, 1.0% Triton X-100, 1 mM EDTA, 0.1% sodium deoxycholate), and 4 times with 0.5 ml LiCl detergent wash buffer (10 mM Tris–HCl, pH 8, 250 mM LiCl, 0.5% NP40, 0.5% sodium deoxycholate, 1.0 mM EDTA). DNA and protein was washed 2 times with 75 μl (5× TE + 1% SDS) and incubated 15 min at 65°. The 150 μl total volume was incubated overnight at 65° to reverse crosslinks. DNA was purified using an Invitrogen PCR purification kit. ChIP DNA was analyzed by real-time PCR. Input DNA was diluted 1:125, and the ± antibody samples were diluted 1:5. Each sample was run in triplicate per plate, with $n = 3$–4. The fivefold standard curve for each sample used YRH269 input DNA, starting with a 1:25 primary dilution. Values are reported as the ratio of IP sample to the input DNA. *P*-values were calculated using the two-tailed Student's *t*-test.

RESULTS

A reporter gene assay that is sensitive to changes in RNA polymerase I transcription: Transcription of yeast rDNA genes terminates within the nontranscribed spacer 1 (NTS1) region at the Reb1-binding site. The 10% of transcription that leaks through this primary terminator is halted by a secondary "fail-safe" termination sequence located downstream of the Reb1 site, also within NTS1 (Reeder *et al.* 1999). At the centromere-proximal rDNA repeat, the termination sequences in NTS1 prevent Pol I from transcribing into the unique chromosome XII sequence flanking the tandem array (Figure 1A, top). The introduction of a modified *URA3* reporter gene driven by a *TRP1* promoter (*mURA3*) into the flanking unique sequence resulted in *SIR2*-dependent silencing of the reporter (Buck *et al.* 2002). Repression of the *mURA3* reporter gene was retained when the NTS1 sequence was deleted (Figure 1A, bottom), but the repression was no longer dependent on *SIR2* (Buck *et al.* 2002). We hypothesized that deleting the Pol I termination sequences in NTS1 allowed Pol I transcription to proceed into the adjacent *mURA3* promoter, thus interfering with its expression.

To demonstrate that Pol I was transcribing into the adjacent *mURA3* reporter gene when NTS1 was deleted, we implemented RT–PCR to detect primary rRNA transcripts from the centromere-proximal rDNA gene that do not properly terminate (Figure 1A, bottom). Total RNA was isolated from a reporter strain that lacks the NTS1 sequence (YRH4; Δ) and a related strain (YSB425; +) that has an intact NTS1 sequence. An antisense *mURA3* promoter primer was used to generate cDNA (Figure 1A, primer 1). When the cDNA was PCR amplified using primer 1 and a 35S-specific return primer (primer 3), a product of the expected 886-bp size was observed only when NTS1 was deleted (Figure

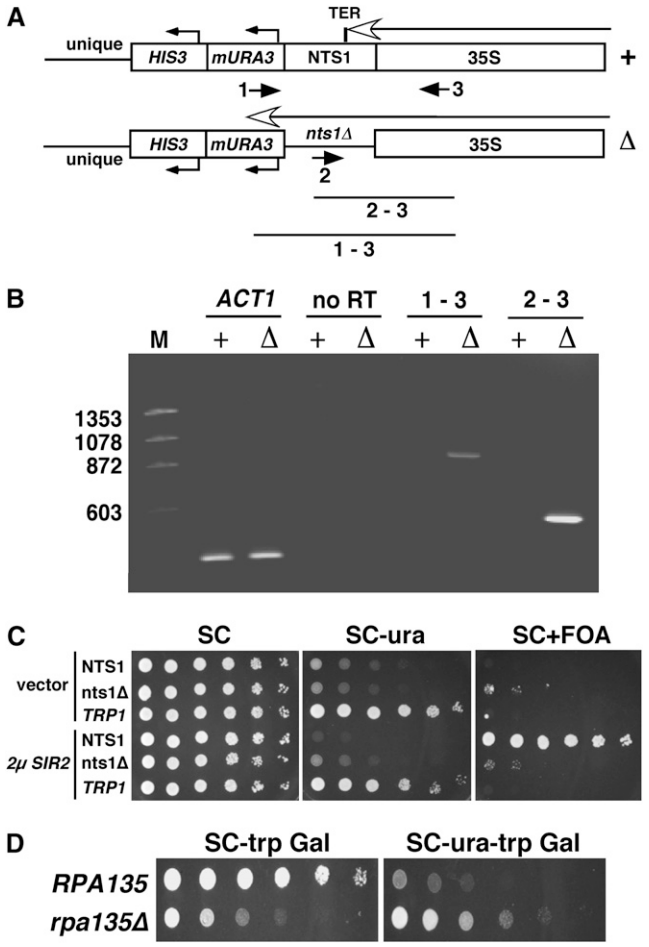

FIGURE 1.—Pol I readthrough transcription inhibits *mURA3* expression. (A) Schematics of the centromere-proximal rDNA repeat when the NTS1 sequence is present (YSB425, +) or replaced with a stuffer fragment (YRH4, Δ). Primer 1 was used for cDNA synthesis. PCR products obtained with primer pairs 1–3 and 2–3 are indicated. (B) Ethidium-bromide-stained agarose gel showing RT–PCR products obtained from YSB425 (+) and YRH4 (Δ). The size markers (M) are *Hae*III-digested ØX174 DNA (New England Biolabs). *ACT1* primers were JS83 and JS84. Without reverse transcription, products were not observed for *ACT1* or the rDNA (no RT). (C) The *nts1Δ::mURA3-HIS3* reporter in YRH4 is insensitive to increased *SIR2* dosage. YSB425 (NTS1), YRH4 (nts1Δ), and YSB519 (*TRP1*) were transformed with pRS425 (empty vector) or pSB766 (2μ *SIR2* plasmid). Fivefold serial dilutions were spotted onto SC, SC−ura, and SC containing 0.1% 5-FOA (SC+FOA). SC plates were incubated for 2 days, while SC−ura and SC+FOA plates were incubated for 3 days. (D) Pol I transcription is required for *mURA3* repression.

1B). Similarly, a 556-bp fragment was observed in the nts1Δ strain when the cDNA was PCR amplified with primer 3 and a nested primer (primer 2) specific for the stuffer fragment that replaced NTS1 (Figure 1B). Equal amounts of RNA were loaded into the cDNA reactions as measured by RT–PCR for the *ACT1* transcript, and no product was observed when the RNA was not reverse transcribed. Pol I is therefore able to read into the *mURA3* reporter when the terminators are removed, leading to poor expression of *mURA3*. This result suggested that the expression level of *mURA3* in the nts1Δ reporter strain (YRH4) could potentially be used as a surrogate marker for the level of Pol I transcription.

To confirm that the expression of *mURA3* in the nts1Δ reporter strain was unaffected by Sir2p-dependent silencing, we overexpressed *SIR2* to test whether *mURA3* repression was improved (Figure 1C). With typical rDNA silencing, repression of the *mURA3* reporter is stronger when *SIR2* is overexpressed, leading to even less growth on SC−ura plates (SMITH *et al.* 1998). While repression of *mURA3* in the *nts1Δ::mURA3* reporter strain (YRH4) causes poor growth on SC−ura media, it is not strong enough to trigger robust growth on SC medium containing 5-FOA (Figure 1C), which is toxic to cells sufficiently expressing *URA3* (BOEKE *et al.* 1984). Improved *mURA3* repression would be expected to weaken growth on SC−ura and improve growth on FOA. As predicted, transformation of the NTS1 strain with a high-copy *SIR2* plasmid induced stronger silencing of *mURA3*, as indicated by a lack of growth on SC−ura and by stronger growth on SC+FOA when compared to the empty vector control (Figure 1C). However, the *SIR2* plasmid had no effect when NTS1 was deleted or when *mURA3* was positioned outside the rDNA at the non-silenced *TRP1* locus (Figure 1C). Promoter interference of *mURA3* by Pol I readthrough transcription is therefore unrelated to *SIR2*-mediated silencing.

To show that *mURA3* repression required Pol I readthrough interference, we deleted the *RPA135* gene, which encodes the second-largest subunit of Pol I. Viability of the *rpa135Δ* mutant was maintained on galactose-containing SC media by the presence of a *TRP1* plasmid (pNOY199) that expressed the 35S rRNA from a galactose-inducible promoter (VU *et al.* 1999). As shown in Figure 1D, repression of *mURA3* was eliminated in the *rpa135Δ* mutant. Together, these results in Figure 1 demonstrate that the *nts1Δ::mURA3-HIS3* reporter strain (YRH4) acts as an indicator of active Pol I transcription from the centromere-proximal rDNA repeat.

A genetic screen for modulators of Pol I transcription: Because deleting *RPA135* eliminated *mURA3* repression in the *nts1Δ* strain, we hypothesized that smaller effects on Pol I transcription that do not cause lethality would also be detectable. For example, mutations in genes that promote Pol I transcription would cause less readthrough interference of *mURA3* and an improved Ura^+ phenotype, whereas mutations in Pol I repressors would cause more transcriptional readthrough and improved growth on FOA. On the basis of these predictions we carried out a screen in which YRH4 (the *nts1Δ::mURA3-HIS3* reporter strain) was mutagenized by transformation with a library of yeast genomic DNA fragments that harbored random artificial transposon insertions (BURNS *et al.* 1994; SMITH *et al.* 1999) (Figure 2A). Insertion mutations were selected by growth on SC

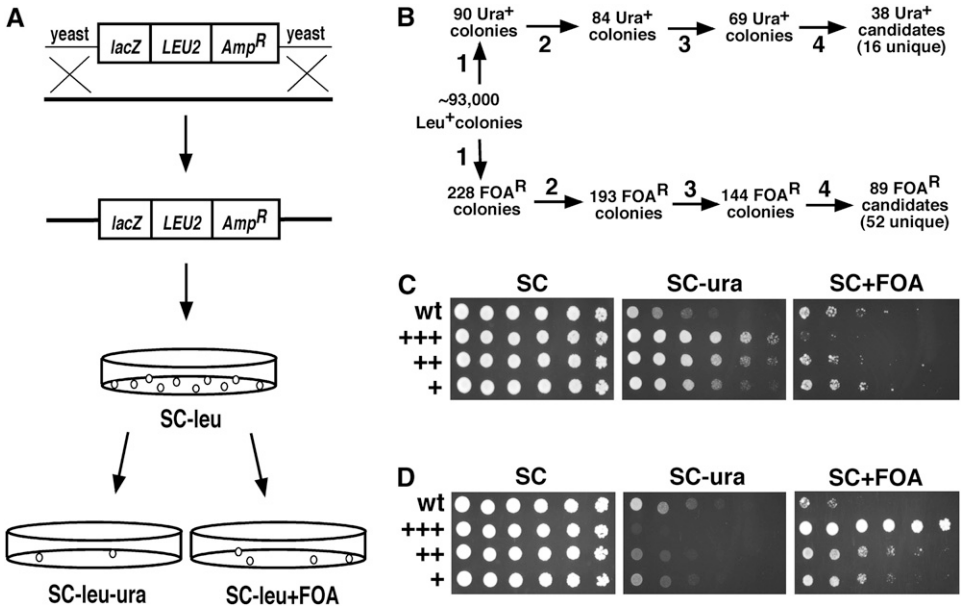

FIGURE 2.—Experimental design for the transposon mutagenesis screen. (A) A schematic of the transposon mutagenesis and screening procedure. Yeast genomic DNA fragments containing random mTn3 transposon insertions were transformed into YRH4, and transformants were selected on SC−leu plates. Colonies were then replica plated to SC−leu−ura and SC−leu+0.2% FOA plates to identify relevant mutants. (B) Numbers of mutant candidates remaining after multiple steps of screening. Step 1: initial screening of transformants. Step 2: first single-colony isolation and replica plating. Step 3: second single-colony isolation and replica plating. Step 4: backcross test. (C) Examples of phenotype strength from Ura$^+$ mutants isolated from the screen after 1 day (+++), 2 days (++), or 3 days (+) incubation after replica plating. The mutants shown are *uaf30-Tn* (PS1-174; +++), *rrn5-Tn* (PS1-212; ++), and *nts1-Tn* (PS1-170; +). (D) Examples of FOA^R mutants isolated after day 1, 2, or 3 of the screen. The mutants shown are *rpn8-Tn* (PS1-7; +++), *bre1-Tn* (PS1-26; ++), and *ira2-Tn* (PS1-117; +).

media lacking leucine (SC−leu). We obtained ~93,000 Leu$^+$ colonies that were replica plated to SC−leu−ura and SC−leu+0.2% FOA plates (Figure 2A). Initially, 90 Ura$^+$ and 228 FOA^R colonies were chosen for analysis (Figure 2B, step 1). Following two successive rounds of restreaking for single colonies and replica plating to verify the phenotypes, 69 Ura$^+$ and 144 FOA^R candidates remained (Figure 2B, steps 2 and 3). The phenotypic strength for each mutant was classified on the basis of the number of days following replica plating that they were identified: day 1 (+++), day 2 (++), and day 3 (+). Examples for the Ura$^+$ mutants are shown in Figure 2C and the FOA^R mutants in Figure 2D. As expected, increased growth on SC−ura correlated with decreased growth on FOA, and vice versa.

From 213 candidates, we were able to pinpoint the site of transposon insertion for 181 (85.0%). The transposon insertions in several mutants occurred at loci such as Ty elements or the rDNA locus that were not expected to cause a phenotype, suggesting that some mutants had secondary mutations or genomic rearrangements unrelated to the transposon insertion. To determine if the *mURA3* growth phenotype was linked to the transposon insertion, we crossed each *MAT*α haploid mutant to a *MAT***a** strain that did not contain the *mURA3* reporter (JS579). Through tetrad dissection of the resulting diploids and replica plating of the spores, we were able to reduce the final number of sequenced candidates with phenotype–transposon (*LEU2*) linkage down to 38 Ura$^+$ (16 unique) and 89 FOA^R (52 unique) (Figure 2B, step 4). The unique genes are listed in Table 1 (Ura$^+$) and in Table 2 (FOA^R). All uncharacterized ORFs were renamed **r**egulators of **r**DNA **t**ranscription (RRT). An expanded list describing the transposon insertion site for each mutant and whether the mutation is recessive or dominant is shown in Table S3. Not surprisingly, some of the FOA^R mutants contained a transposon insertion within a gene involved in uracil biosynthesis and metabolism, and these likely affect growth on FOA independently of any changes in Pol I transcription. The effects of the mutations on *mURA3* expression were predicted to rely on the NTS1 sequence being absent, thus allowing Pol I readthrough. We therefore picked 10 Ura$^+$ and 10 FOA^R mutants and crossed them to a reporter strain that retained the NTS1 sequence, followed by tetrad dissection and retesting. As shown in Table S4, the presence of NTS1 usually prevented any effect of the mutations on the *mURA3* expression phenotype when compared to the nts1Δ counterparts. Sometimes there were even opposite effects.

Mutants isolated from the genetic screen alter transcription of the centromere-proximal rDNA gene: Two Ura$^+$ mutants had transposon insertions in *UAF30* and *RRN5*, both of which encode subunits of the Pol I transcription factor complex UAF (KEYS *et al.* 1996; SIDDIQI *et al.* 2001). Isolating these genes suggested that many of the Ura$^+$ mutants would have Pol I transcription defects. Because severe Pol I defects could cause general slow growth or lethal phenotypes, the changes in rDNA expression were predicted to be mild. We utilized a quantitative real-time RT–PCR (qRT–PCR) assay to measure changes in rRNA production from the centromere-proximal rDNA gene. A cDNA product was synthesized from total RNA using primer 4 (JS765), which is specific for the "stuffer" fragment that replaced NTS1. PCR was then performed on the cDNA using

TABLE 1

Genes from Ura$^+$ screen

Gene	ORF	Function
	Pol I transcription	
RRN5	*YLR141W*	UAF subunit
UAF30	*YOR295W*	UAF subunit
	Proteasome	
RPN7	*YPR108W*	Regulatory subunit
	Miscellaneous	
ATG2	*YNL242W*	Autophagy
BNI4	*YNL233W*	Targeting subunit for Glc7 phosphatase
GAP1	*YKR039W*	Amino acid permease
HMS1	*YOR032C*	myc family txn factor
MNN1	*YER001W*	Mannosyltransferase
MSS4	*YDR208W*	Phosphatidylinositol kinase
SEY1	*YOR165W*	Vesicular trafficking
	Unknown	
RRT11	*YBR147W*	Uncharacterized
RRT12	*YCR045C*	Uncharacterized
RRT13	*YER066W*	Uncharacterized
RRT14	*YIL127C*	Uncharacterized, nucleolar
RRT15	*YLR162W-A*	Uncharacterized
RRT16	*YNL105W*	Dubious ORF, overlaps with *INP52*
	Intergenic	
VTS1/PDE2	*YOR359W/YOR360C*	

primers 4 (JS765) and 5 (JS766), which amplify a segment of the stuffer transcribed by Pol I (Figure 3A). This rRNA product was then compared to an *ACT1* mRNA loading control to indicate the relative changes in rRNA. As expected, the rRNA signal was greatly reduced in mutants or growth conditions that were known to reduce Pol I transcription, including the *rpa135*Δ mutant (Figure 3B), a wild-type strain grown to stationary phase (Figure 3C), and a wild-type strain treated with rapamycin (Figure 3D). We next tested the effects of various mutants from the screen. As shown in Figure 3E, each Ura$^+$ mutant that we initially tested, including the *top1*Δ positive control, showed a reduction in rRNA compared to the wild-type strain (YRH4), indicating that the Ura$^+$ phenotype is a good indicator of decreased rRNA transcription from the centromere-proximal repeat. Among these genes was *HMS1*, which encodes a helix-loop-helix protein with similarity to Myc-family transcription factors that has previously been implicated in regulation of pseudohyphal filamentous growth (Lorenz and Heitman 1998). Hms1 also physically interacts with Cdc14 (Ho *et al.* 2002), a phosphatase that is a subunit of the nucleolar RENT complex (Shou *et al.* 1999). Moreover, deletion of *HMS1* is synthetically lethal with deletion of topoisomerase I (*TOP1*) (Tong *et al.* 2004), which functions in Pol I transcriptional elongation (Schultz *et al.* 1992; Hontz *et al.* 2008).

We next tested the effects of several FOA^R mutants using the qRT–PCR assay. These mutants were predicted to have increased levels of rDNA transcription at the left repeat. As shown in Figure 3F, the *hos2-Tn*, *gcn5-Tn*, and *bre1-Tn* mutants each resulted in a modest increase in rRNA signal compared to wild type. Several FOA^R mutants affected subunits of the large RPD3 histone deacetylase complex Rpd3L (Table 1). Similarly, deleting *RPD3* from YRH4 caused a modest increase in rRNA product. One of the exceptions that did not cause an increase in rRNA signal in the qRT–PCR assay was *ira1-Tn*. Both *IRA1* and *IRA2*, which were isolated from the screen multiple times, are required for proper entry into stationary phase (Russell *et al.* 1993). Since the qRT–PCR assays were performed with cells growing in YPD media where rDNA transcription is active, it was possible that the *ira1-Tn* mutant and perhaps other FOA^R mutants would have greater effects in the qRT–PCR assay in stationary phase where the rDNA is repressed. We retested several mutants from Figure 3F after growth for 24 hr into late diauxic shift/early stationary phase. The *ira1-Tn* mutant now showed higher rRNA signal than wild type, and the effects with *rpd3*Δ and *hos2-Tn* mutants were more severe than in log phase (compare Figure 3G with Figure 3F). The FOA^R phenotype with the *nts1*Δ*::mURA3-HIS3* reporter is, therefore, an effective indicator of increases in rDNA synthesis from the centromere-proximal rDNA repeat.

Altering rDNA transcription by changing the tandem array size: Prior to the backcrossing that eliminated a large number of the mutant candidates, three of the FOA^R class had a transposon insertion in *ZIP2*, *RED1*, or *MER1*, genes that specifically function in the formation of the meiotic synaptonemal complex. Each of these mutants produced large increases in centromere-proximal rDNA gene expression in the qRT–PCR assay (Figure 4A). However, the size of their rDNA tandem array was clearly reduced compared to wild type, which had an array size estimated as ~175 copies (Figure 4B). The reduced rDNA copy number in these mutants could explain the increase in expression of the centromere-proximal rDNA repeat, because a previous study showed that rDNA genes in a 42-copy strain were more highly transcribed than rDNA genes in a 143-copy strain due to compensation (French *et al.* 2003). To test if that was the case here, we deleted *ZIP2*, *RED1*, or *MER1* from YRH4 and retested their array size (Figure 4B) and the effects on FOA^R growth (Figure 4C). The deletion mutants no longer had short arrays and their FOA and SC−ura phenotypes were the same as wild type, indicating that the effects on rDNA expression were due to the change in array size. As expected, the FOA^R phenotype also did not cosegregate with the transposon insertions after tetrad dissections (data not shown), suggesting that the mutants that passed the backcross test (Table 2) are unlikely to have a significant change in array size.

TABLE 2

Genes from FOA^R screen

Gene	ORF	Function
	Chromatin-related	
ADA1	*YPL254W*	SAGA subunit
ADA2	*YDR448W*	SAGA subunit
GCN5	*YGR252W*	SAGA subunit
SPT20	*YOL148C*	SAGA subunit
PHO23	*YNL097C*	RPD3 subunit
RXT3	*YDL076C*	RPD3 subunit
SAP30	*YMR263W*	RPD3 subunit
SIN3	*YOL004W*	RPD3 subunit
UME1	*YPL139C*	RPD3 subunit
BRE1	*YDL074C*	H2B ubiquitin ligase
HOS2	*YGL194C*	Histone deacetylase
SIR1	*YKR101W*	*HM* silencing factor
DPB11	*YJL090C*	DNA Pol ε subunit
	Proteasome	
ECM29	*YHL030W*	Bridging subunit
PRE9	*YGR135W*	20S subunit
RPN8	*YOR261C*	Regulatory subunit
	Miscellaneous	
CHS6	*YJL099W*	Exomer subunit
CIN1	*YOR349W*	Tubulin folding factor D
IMP2′	*YIL154C*	Transcription factor
MLC2	*YPR188C*	Myosin regulatory light chain
MLP1	*YKR095W*	Myosin-like protein
PAU7	*YAR020C*	Seripauperin gene family
NTE1	*YML059C*	Serine esterase
REV3	*YPL167C*	DNA polymerase ζ
SBA1	*YKL117W*	Hsp90-binding chaperone
	Intragenic	
BMH2/TVP15	*YDR099W/YDR100W*	
MRP13/RPL 11B	*YGR084C/YGR085C*	
	Signaling	
CMP2	*YML057W*	Calcineurin A isoform
GPB1	*YOR371C*	cAMP/PKA signaling
GPG1	*YGL121C*	cAMP/PKA signaling
INM1	*YHR046C*	Inositol monophosphatase
IRA1	*YBR140C*	RAS/cAMP signaling
IRA2	*YOL081W*	RAS/cAMP signaling
PPH22	*YDL188C*	PP2A catalytic subunit
PRR1	*YKL116C*	ser/thr protein kinase
PSK2	*YOL045W*	PAS domain, ser/thr kinase
PXL1	*YKR090W*	Cdc42 and Rho1 signaling
SAC7	*YDR389W*	GAP for Rho1 signaling
YPK1	*YKL126W*	ser/thr kinase, Sphingolipids
	Metabolism	
AAP1′	*YHR047C*	Arg/Ala aminopeptidase
AGC1	*YPR021C*	Nitrogen metabolism
GPM3	*YOL056W*	Phosphoglycerate mutase
GUT2	*YIL155C*	Glycerol utilization
PIG2	*YIL045W*	Glycogen synthesis
	Unknown	
RRT1	*YBL048W*	Dubious ORF/next to *MOH1*
RRT2	*YBR246W*	Two-hybrid with Rrt4
RRT3	*YDR020C*	Recently named *DAS2*
RRT4	*YDR520C*	Two-hybrid with Rrt2
RRT5	*YFR032C*	Uncharacterized
RRT6	*YGL146C*	Uncharacterized
RRT7	*YLL030C*	Dubious ORF/next to *GPI13*
RRT8	*YOL048C*	Uncharacterized
	Pyrimidine synthesis	
FUR4	*YBR021W*	Uracil permease
URA5	*YML106W*	*De novo* pyrimidine synthesis
URA6	*YKL024C*	*De novo* pyrimidine synthesis

Multiple chromatin-modifying factors function in Pol I transcriptional regulation: A large number of FOA^R candidate genes were previously characterized as chromatin related and/or associated with Pol II transcription. These include Bre1, the E3 ubiquitin ligase for histone H2B (Hwang *et al.* 2003; Wood *et al.* 2003); Hos2, an H3/H4 histone deacetylase that is part of the SET3 complex (Pijnappel *et al.* 2001; Wang *et al.* 2002); and several subunits each from the SAGA/SLK/ADA histone acetyltransferase complexes and the Rpd3L/RpdS histone deacetylase complexes. Each of these has been previously linked to Pol II transcriptional elongation. We therefore deleted other genes with connections to these factors in elongation, including *RAD6*, the E2 ubiquitin-conjugating enzyme for H2B; *SET1*, the H3-K4 methyltransferase; *SET2*, the H3-K36 methyltransferase; and *PAF1*, which is a subunit of the conserved PAF complex that functions in Pol II elongation (Squazzo *et al.* 2002; Krogan *et al.* 2003; Chu *et al.* 2007). As shown in Figure 5A, the *rad6*Δ mutant had an FOA^R phenotype similar to the *bre1-Tn* mutant, as one might have expected. Deletion of *SET1* had no effect (Figure 5A), which was interesting because H3-K4 methylation was previously shown to function in rDNA silencing of Pol II-transcribed reporter genes (Briggs *et al.* 2001). In contrast, deletion of *SET2* did cause an increase in growth on FOA (Figure 5A) and caused a

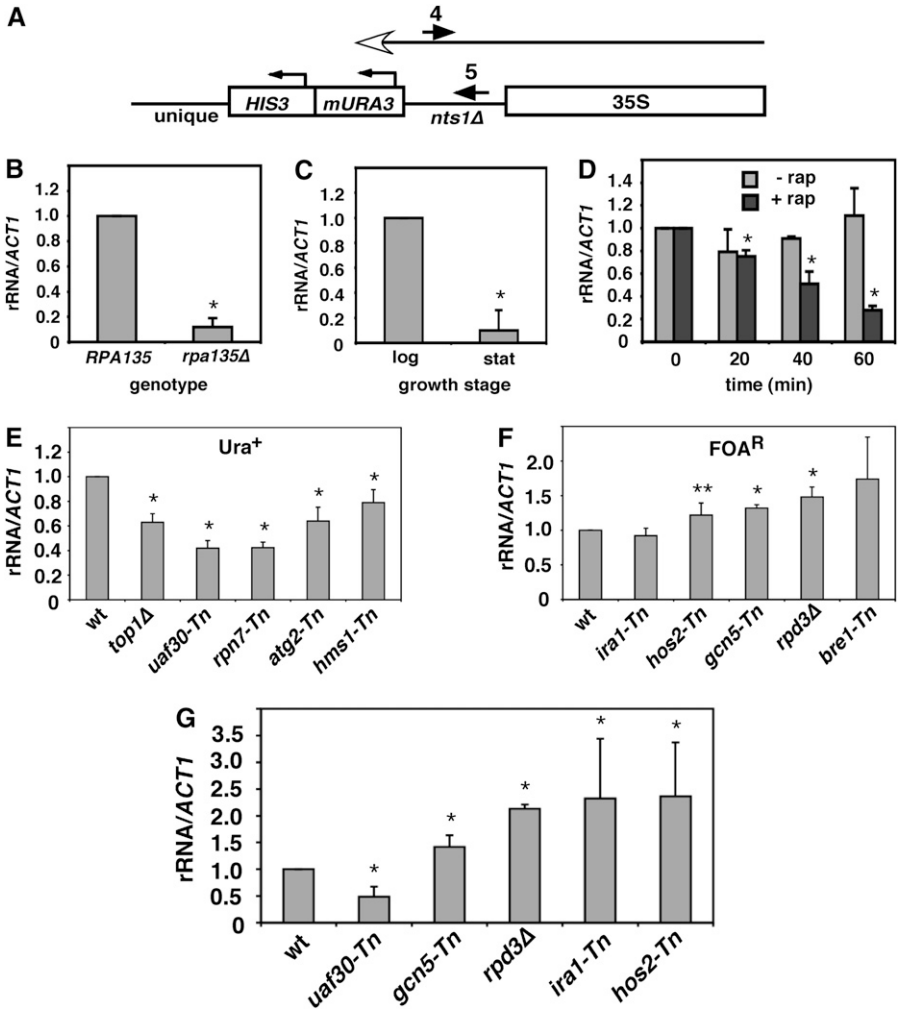

FIGURE 3.—qRT–PCR assay for measuring changes in Pol I transcription from the centromere-proximal rDNA gene. (A) Schematic of the *mURA3* reporter gene showing two PCR primers (4 and 5) used to specifically detect transcription of the nts1Δ stuffer fragment sequence. (B) Deletion of *RPA135* causes a decrease in Pol I transcription. The *RPA135* strain was YRH92 and the *rpa135*Δ strain was YRH95. (C) qRT–PCR assay of YRH4 grown into log phase or early stationary phase (stat). (D) YRH4 was treated with 0.2 mg/ml rapamycin during log-phase growth and analyzed by qRT–PCR at 20-min intervals. (E) Examples of four Ura$^+$ mutants from the screen: *uaf30-Tn* (PS1-174), *rpn7-Tn* (PS1-152), *atg2-Tn* (PS1-184), and *hms1-Tn* (PS1-186). A deletion mutant for *TOP1* was used as a positive control for reduction in Pol I transcription. (F) Examples of four FOA^R mutants from the screen: *ira1-Tn* (PS1-56), *hos2-Tn* (PS1-120), *gcn5-Tn* (PS1-72), and *bre1-Tn* (PS1-26). A deletion mutant for *RPD3* (YRH5) was also tested. (G) Examples of five mutants tested by qRT–PCR in stationary phase (4 days at 30°). In A–G, the ratio of rRNA qRT–PCR product to *ACT1* RNA qRT–PCR product was calculated and normalized to 1.0 for wild type at time 0. All experiments were performed in triplicate with standard deviation error bars. A single asterisk indicates a *P*-value of < 0.05, and a double asterisk indicates $P < 0.1$.

higher rRNA signal in the qRT–PCR assay (Figure 5C). Deleting *PAF1* resulted in strong growth on FOA (Figure 5A) and in a 2.5-fold increase in the rRNA qRT–PCR assay (Figure 5C). Because loss of *PAF1* causes both H2B ubiquitination and H3-K36 methylation defects (Wood *et al.* 2003; Chu *et al.* 2007), we tested a *set2*Δ *bre1-Tn* double mutant and found similar results to those of the *paf1*Δ mutant by spot growth assay and qRT–PCR (Figure 5, A and C), consistent with the *paf1*Δ mutation causing a defect in both pathways.

Genetic dissection of histone deacetylase complexes that function in Pol I regulation: Each of the Rpd3-associated genes isolated (*SIN3*, *PHO23*, *SAP30*, *RXT3*, and *UME1*) encode subunits of the Rpd3L complex, which represses Pol II transcription at target genes by locally deacetylating histones at the promoter (Kadosh and Struhl 1997). The Rpd3S (small) complex is targeted to the transcribed regions of genes by interactions with histone H3 methylated at K36, a modification catalyzed by Set2 (Carrozza *et al.* 2005; Keogh *et al.* 2005). Rpd3S shares some core subunits with the Rpd3L complex such as Sin3 and Ume1, but also contains the unique subunits Eaf3 and Rco1 (Carrozza *et al.* 2005). To test whether the Rpd3S complex functioned in Pol I transcription repression, we deleted *RCO1*. As shown in Figure 5B, the *rco1*Δ mutant had a moderate FOA^R phenotype that was not as strong as the Rpd3L-specific mutant, *pho23-Tn*. Neither reduced growth on SC−ura as well as the *rpd3*Δ mutant, suggesting that both complexes contribute to Pol I regulation, with most of the activity coming from Rpd3L. The Ume6 subunit of Rpd3L specifically targets deacetylation to certain meiosis-related genes (Kadosh and Struhl 1997). This mutant did not induce FOA resistance (Figure 5B), indicating that the repressive effects are independent of Ume6-regulated genes.

Other histone deacetylases were tested to see if there were general deacetylase effects on rDNA transcription. While the *hos2-Tn* mutant produced a strong phenotype, deleting genes for other related deacetylases, *HOS1* or *HOS3*, had little effect (Figure 5B), despite their known roles in deacetylating histones in the rDNA locus (Robyr *et al.* 2002). This result suggested that the Hos1- and Hos3-mediated histone deacetylation likely

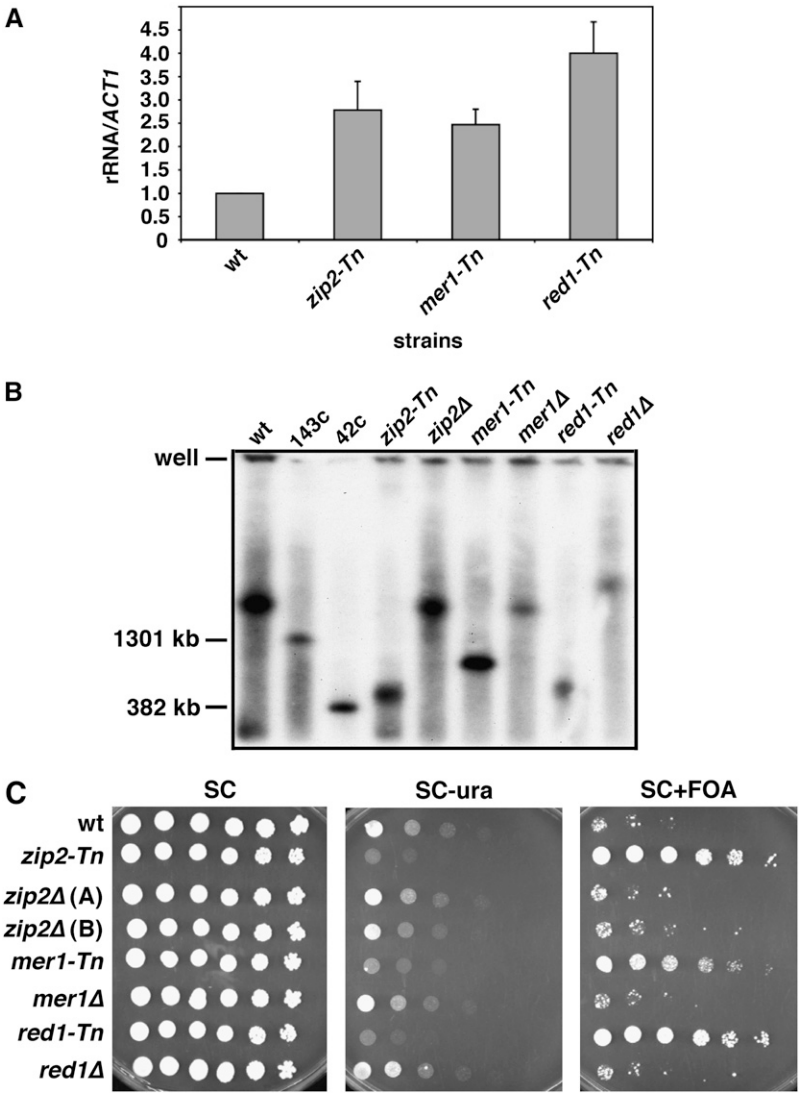

FIGURE 4.—Some primary mutants from the genetic screen affect Pol I transcription levels by changing the rDNA tandem array size. (A) qRT–PCR analysis of transposon mutants of *ZIP2* (*zip2-Tn*; PS1-13), *MER1* (*mer1-Tn*; PS1-14), and *RED1* (*red1-Tn*; PS1-115). Values were calculated relative to wild type (YRH4) normalized to 1 ($n = 3$). (B) Pulsed-field gel electrophoresis on transposon mutants and direct knockouts of *ZIP2* (YRH244), *MER1* (YRH253), and *RED1* (YRH254). Controls for rDNA array size are wild type (YRH4), a 42-copy strain (NOY886), and a 143-copy strain (NOY1051). (C) The same strains from B were tested for effects on the *mURA3* reporter using the spot test assay. SC plates were grown for 2 days, and SC−ura and SC+FOA plates for 3 days. *P*-values for changes were all < 0.01.

has another function. To confirm that the chromatin-related mutants do not affect rDNA expression by changing the array size, we measured the rDNA in the *rpd3*Δ, *hos2-Tn*, and *bre1-Tn* mutants by pulsed-field gel electrophoresis and found no difference from the wild-type YRH4 strain (Figure 5D). This is consistent with mutants having altered array sizes being excluded by the backcrossing procedure. It is not clear why the rDNA became degraded in the *hos2-Tn* mutant. Additional genes deleted from the YRH4 reporter strain during the course of this study, along with their *mURA3* growth phenotypes, are listed in Table S5. Among these mutants was *rsc1*Δ, which produced an FOA^R phenotype but also affected *mURA3* expression when located at the non-rDNA location *TRP1* (Figure S1). Therefore, *RSC1* directly affects expression of the Pol II-driven reporter gene. Importantly, *rpd3*Δ, *hos2*Δ, and *set2*Δ mutations had no effect on Ura^+ growth when *mURA3* was positioned at the *TRP1* locus (Figure S1), which is consistent with their effects on this reporter being specific to changes in Pol I transcription.

Chromatin-related factors isolated from the screen physically associate with the rDNA: Changes in rDNA transcription could occur through direct or indirect mechanisms, so it became important to ask whether several of our candidate proteins physically associated with the rDNA locus. Rpd3, Hos2, Ada2, Sap30, Rad6, Paf1, and Chd1 were C-terminally tagged with 13 copies of the Myc epitope at the endogenous chromosomal locus. Each tagged protein was expressed at the predicted size in a Western blot (Figure 6A) and had growth properties similar to the untagged parental strain (YRH269; genotypically identical to YRH4) (Figure 6B). Quantitative ChIP assays were then performed to determine if the tagged proteins associated with four different locations on the rDNA genes (Figure 6C). To increase the chances of detecting a signal at the rDNA locus, we treated each crosslinked extract with RNaseA prior to immunoprecipitation to reduce the massive amount of rRNA associated with the active genes. This technique was previously used to demonstrate that Chd1 is enriched on the transcribed regions of the

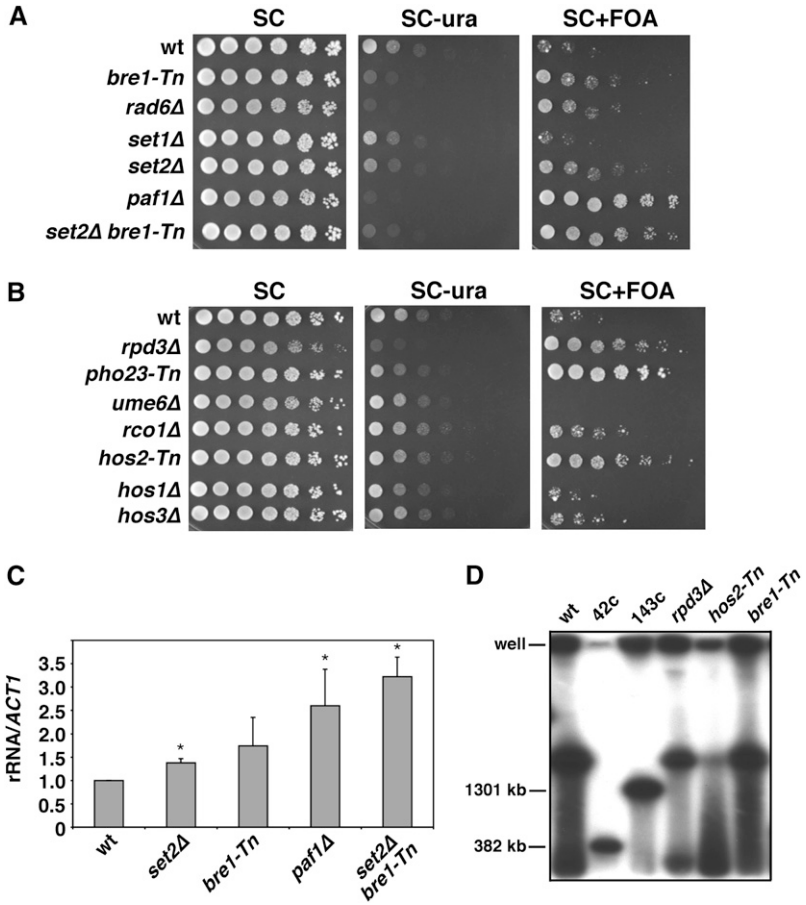

FIGURE 5.—Effects of several chromatin-related genes on transcription of the centromere-proximal rDNA gene. (A) Reporter gene assay with wild type (YRH4), *bre1-Tn* (PS1-26), *rad6*Δ (YRH698), *set1*Δ (YRH132), *set2*Δ (YRH134), *paf1*Δ (YRH164), and a *set2*Δ *bre1-Tn* double mutant (YRH265). (B) Reporter gene assay with wild type (YRH4), *rpd3*Δ (YRH5), *pho23-Tn* (PS1-76), *ume6*Δ (YRH51), *rco1*Δ (YRH152), *hos2-Tn* (PS1-120), *hos1*Δ (YRH142), and *hos3*Δ (YRH143). (C) qRT–PCR results of several candidates highlighted in A. Wild type was normalized to 1.0. (D) Pulsed-field gel electrophoresis analysis of the rDNA array size in wild type (YRH4), 42-copy rDNA control (NOY886), 143-copy rDNA control (NOY1051), *rpd3*Δ (YRH5), *hos2-Tn* (PS1-120), and *bre1-Tn* (PS1-26) strains. An asterisk indicates a *P*-value > 0.05.

rDNA genes (JONES *et al.* 2007). On the basis of this previous study, we used the Myc-tagged Chd1 as a positive control for rDNA association (Figure 6C). *CHD1* mutations cause an FOA^R phenotype in YRH4. All of the tagged proteins were detected on the rDNA to varying degrees, each with a signal greater than that for the untagged negative control. While some of the increases for the transcribed region and NTS1 were not significant ($P > 0.05$), all of the promoter interactions at NTS2 were significant (Figure 6C, primer pair 1). All rDNA interactions were significant for Chd1 and Paf1. These results raise the possibility that some factors isolated from the screen, and not previously linked to chromatin, may also directly associate with the rDNA to modulate its transcription. This will be a focus of future studies.

DISCUSSION

Possible mechanisms for mutants to affect Pol I transcription: The genetic screen performed in this study takes advantage of an inhibitory effect of Pol I when it transcribes into an adjacent Pol II-transcribed reporter gene (*mURA3*). While we have yet to decipher the exact mechanism of this repression, it is likely that the large number of engaged Pol I molecules can displace Pol II-specific transcription factors. This is similar to the phenomenon of promoter interference (occlusion) that occurs for Pol II-transcribed genes when termination of the upstream gene is defective (EGGERMONT and PROUDFOOT 1993). It is clear that an increase or decrease in rDNA transcription from the centromere-proximal repeat affects *mURA3* expression, as shown, respectively, by strains with a small array size (Figure 4) or with an *rpa135* deletion (Figure 1). In theory, the transcription rate of this single repeat could be affected by changes in Pol I initiation or elongation, the rDNA array size, or the percentage of active genes, as we have recently shown for *uaf30* mutants (HONTZ *et al.* 2008). It is also possible that the mutations affect Pol I termination, because there is evidence that some rRNA transcripts manage to terminate without NTS1, as exhibited by a reduction in RT–PCR product when the cDNA is primed from the *mURA3* promoter instead of the stuffer fragment (Figure 1B). If some termination does take place in the nts1Δ reporter strain, then some of the effects caused by the chromatin-related mutants could be through an effect on termination. For example, Chd1 has been implicated in Pol I termination via its chromatin-remodeling activity (JONES *et al.* 2007). Another possibility is that due to the coordination between rRNA processing and transcription, a defect in processing could in turn affect transcription. In any of these scenarios, the screen is still able to identify the

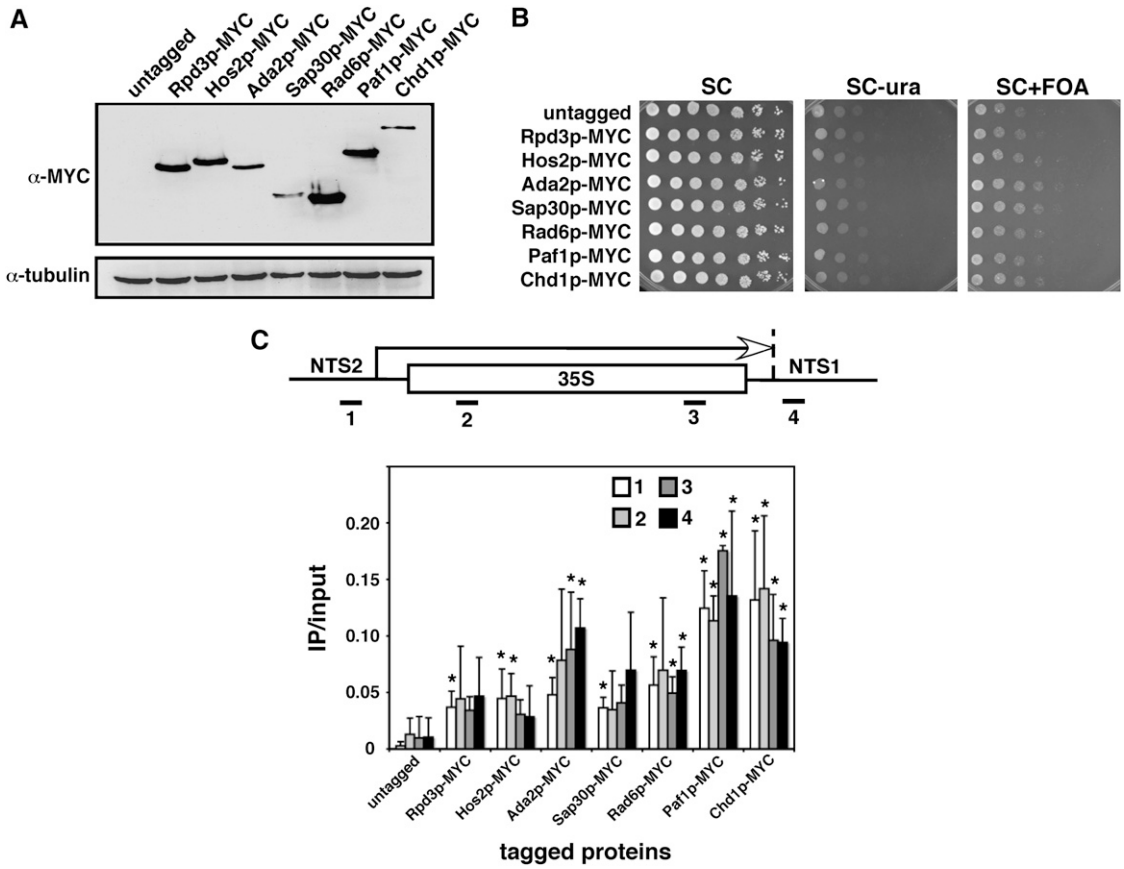

Figure 6.—Chromatin immunoprecipitations by real-time PCR reveal that several chromatin-related candidate proteins associate with the rDNA. (A) Western blot of steady-state protein levels for each 13xMYC epitope-tagged protein. Tubulin is the loading control. The strains used were as follows: untagged/wild type (YRH269), Rpd3p-MYC (YRH902), Hos2p-MYC (YRH901), Ada2p-MYC (YRH912), Sap30p-MYC (YRH915), Rad6p-MYC (YRH905), Paf1p-MYC (YRH914), and Chd1p-MYC (YRH911). (B) The Myc-tagged proteins do not alter expression of the *nts1Δ::mURA3-HIS3* reporter gene using the spot test assay. (C) Real-time PCR assay showing varying levels of rDNA association for each of the tagged proteins. (Top) Locations of the primer pairs are indicated as 1–4. The arrow represents the direction of Pol I transcription. The ratio of the PCR signal from the immunoprecipitated samples (IP) compared to the input chromatin sample (input) are graphed and compared to the untagged control strain. Ratios with a *P*-value >0.05 are indicated with an asterisk.

mutant as being involved in rRNA synthesis at some level. We did not expect to identify many known positive regulators of Pol I transcription since they are typically essential, although, interestingly, the only nonessential transcription factor (Uaf30) and a viable mutant allele of an essential factor (Rrn5) from the UAF complex were found. Recovering such large numbers of mutants suggests that Pol I transcription is a finely tuned process.

The relationship between Pol II elongation and Pol I transcriptional repression: The chromatin-related genes that we isolated as FOA^R mutants were previously linked physically or genetically to the Pol II elongation process. For example, SAGA-dependent histone acetylation in the transcribed regions of genes helps to promote elongation by evicting nucleosomes (Govind *et al.* 2007). SAGA also interacts with the Pol II elongation factor TFIIS (Wery *et al.* 2004). Paf1 has recently been reported to destabilize nucleosomes (Marton and Desiderio 2008), suggesting that Paf1-mediated histone modifications may also be involved in this process. When nucleosomes are reassembled behind the transcribing RNA Pol II, they are methylated on H3-K36 by Set2 (Workman 2006). This mark provides a binding site for the Rpd3S complex via the combined affinity of the Eaf3 chromodomain and Rco1 PHD motif (Li *et al.* 2007). Rpd3S-mediated histone deacetylation resets the chromatin to a repressive state that prevents spurious transcription from within the open reading frame (Carrozza *et al.* 2005), a process that also involves Paf1 (Chu *et al.* 2007). However, Set2 has not been reported to interact with Pol I or with the rDNA, and H3-K36 methylation does not appear to occur in the rDNA (Xiao *et al.* 2003), suggesting that the effects of Set2 on Pol I transcription may be indirect. *SET2* deletion could alter the expression of the Pol II-transcribed ribosomal protein genes, which would then affect the coordination between ribosomal protein gene expression and Pol I transcription/rRNA processing. Another possibility is that chromatin-level defects in Pol I

transcription could result in an increase in the number of open rDNA repeats, such as occurs with mutations in Rpd3 (Sandmeier *et al.* 2002), resulting in an FOA^R phenotype as shown here.

Paf1 association with the rDNA was relatively strong in our ChIP assay (Figure 6), and it had a large effect in the qRT–PCR and *mURA3* growth assays. A previous study using immunofluorescence microscopy found that when *PAF1* was deleted, other subunits of the PAF complex localized to the nucleolus (Porter *et al.* 2005). The wild-type complex was not detected, but our data indicate that the complex does normally associate with the rDNA. In the same study, it was reported that deleting *PAF1* caused an rRNA-processing defect that resulted in the accumulation of unprocessed rRNA precursors. More recent investigations of yeast Paf1 found that it was required for silencing of Pol II genes in the rDNA (Mueller *et al.* 2006). While our article was being prepared, the PAF complex was also implicated in Pol I elongation (Zhang *et al.* 2009). Interestingly, deleting the Ctr9 subunit of the PAF complex resulted in an increase in the percentage of rDNA genes in the array that were transcribed (Zhang *et al.* 2009). This would increase the frequency at which the centromere-proximal rDNA gene was transcribed, perhaps explaining why our assays detected greater transcription of the centromere-proximal rDNA gene. Alternatively, Paf1 could be required for limiting the number of rDNA genes that are transcribed, thus acting in a repressive capacity. It will be interesting to further dissect the role of Paf1 in Pol I transcriptional regulation.

Interestingly, there are several Pol II-transcribed genes within the yeast rDNA, including *TAR1* (Coelho *et al.* 2002). Pol II also produces cryptic unstable transcripts from the nontranscribed spacer that may have roles in regulating rDNA silencing and recombination (Kobayashi and Ganley 2005; Li *et al.* 2006; Houseley *et al.* 2007; Vasiljeva *et al.* 2008). Additionally, the rDNA genes have the capacity to be transcribed by Pol II in mutant strains defective for the Pol I transcription factor UAF (Vu *et al.* 1999; Oakes *et al.* 2006). Mutations in certain chromatin-modifying enzymes could alter Pol II transcription within the rDNA, which could in turn influence rRNA synthesis by Pol I. The Nomura lab previously demonstrated that there is a reciprocal relationship between Pol I and Pol II transcription within the rDNA (Cioci *et al.* 2003). Of course it is also possible that some of the chromatin factors from the screen directly repress Pol I transcription through an uncharacterized mechanism, which would be consistent with their rDNA association.

Signaling pathways impact rDNA transcription: Multiple signaling factors were identified from the FOA^R portion of the screen (Table 2). *IRA1*, *IRA2*, *GPB1*, and *GPG1* all function in the negative regulation of the cAMP/PKA signaling pathway, which works in parallel with TOR to coordinate the expression of genes required for cell growth (Zurita-Martinez and Cardenas 2005). *IRA1* and *IRA2* encode GTPase-activating proteins (GAPs) that negatively regulate RAS by converting it from the GTP- to the GDP-bound inactive form, and mutations in these genes result in constitutive activation of RAS and, therefore, of the cAMP/PKA pathway (Broach 1991). *GPB1* encodes a protein that inhibits the cAMP/PKA pathway by binding and stabilizing Ira1 and Ira2 (Harashima *et al.* 2006; Peeters *et al.* 2006), and *GPG1* encodes the gamma subunit required for Gpb1 or Gpb2 to interact with Gpa2 as a heterotrimeric G-protein complex (Harashima and Heitman 2002). Constitutive activation of RAS and the cAMP/PKA pathway prevents the rapamycin-induced repression of Pol I and Pol III transcription (Schmelzle *et al.* 2004) and the starvation-induced repression of ribosomal protein genes (Klein and Struhl 1994). Furthermore, yeast with constitutively high PKA activity have been shown to upregulate ribosomal protein gene expression by approximately twofold (Klein and Struhl 1994). The identification of these four genes as repressors of rDNA transcription is fully consistent with these findings.

Another intriguing signaling gene from the FOA^R mutants is *YPK1*, which encodes a serine/threonine protein kinase that is part of the sphingolipid long-chain base (LCB)-mediated signaling pathway. LCBs are intracellular signaling molecules in yeast (see Dickson *et al.* 2006 for review). Their concentrations rapidly, but transiently, increase in response to cellular stress. Ypk1 can be activated by LCBs, but upstream kinases Pkh1 and -2 are also activated by LCBs and can then activate Ypk1. Ypk1 and its paralog, Ypk2, then regulate cell-wall integrity, actin dynamics, translation, endocytosis, and cell growth. It is also important to note that Ypk1 and Ypk2 are downstream of the TOR-signaling kinase TORC2. Taken together, the data from our screen implicate multiple, diverse signaling pathways in the regulation of Pol I transcription, many of which require further investigation.

Novel genes that influence rDNA transcription: Fourteen previously uncharacterized genes, designated as RRTs, were identified from our screen. Transposon insertions in *RRT1–RRT8* caused an FOA^R phenotype, whereas insertions in *RRT11–RRT16* caused a Ura$^+$ phenotype. Little is known about most of the Ura$^+$ *rrt* mutants, although *RRT14* (*YIL127C*) encodes a nucleolar protein that interacts with Utp5, a subunit of the nucleolar tUTP complex that functions in linking rRNA processing with transcription of the rDNA (Gallagher *et al.* 2004). Among the FOA^R mutants, the Rrt2 and Rrt4 proteins have been shown to interact in a two-hybrid assay (Ito *et al.* 2001), and deletion of *RRT3* (*YDR020C*, also known as *DAS2*) suppresses the 6-azauracil and mycophenolic acid sensitivity of a TFIIS mutant (S. Chavez, personal communication). TFIIS is a Pol II elongation factor (Wind and Reines 2000). Other genes isolated

from the *DAS2* screen were connected with the regulation of ribosomal protein genes (S. Chavez, personal communication). Finally, the transposon insertions for three of the *rrt* mutants occurred in dubious open reading frames that may not encode functional genes. In these cases, it is possible that the transposon insertions instead affected the expression of an adjoining gene. In conclusion, this genetic screen has identified many characterized and uncharacterized genes that affect the level of rDNA transcription from the centromere-proximal rDNA gene. Future analysis will focus on determining the mechanisms of action.

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GENETICS

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Genetic Identification of Factors That Modulate Ribosomal DNA Transcription in *Saccharomyces cerevisiae*

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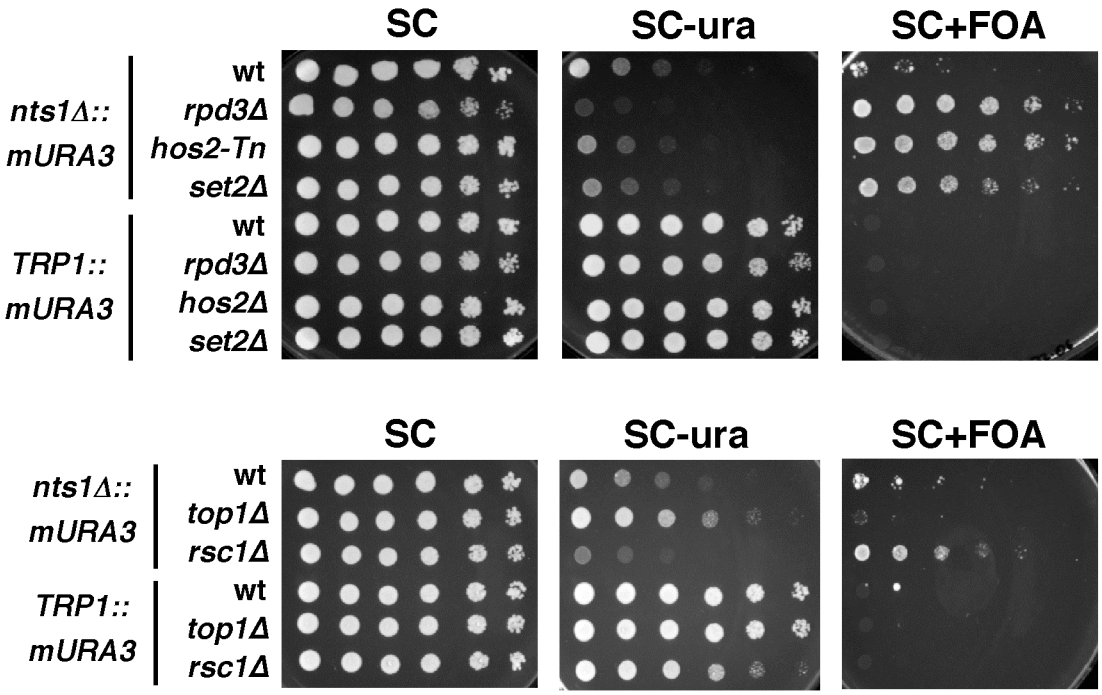

FIGURE S1.—rDNA-specific effects of chromatin factors on *mURA3* expression. The *mURA3-HIS3* cassette was integrated either at the rDNA (nts1Δ) or at the *TRP1* locus (non-rDNA location). Note that only the *rsc1*Δ mutation affected Ura$^+$ growth at the *TRP1* location.

TABLE S1

Strain List

Strain	Genotype	Reference	Figure(s)
NOY886	MATa *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rpa135Δ::LEU2 fob1D::HIS3 pNOY117* (42 rDNA copies)	French et al., 2003	4B, 5D
NOY1051	MATa *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rpa135Δ::LEU2 fob1D::HIS3 pNOY117* (143 rDNA copies)	French et al., 2003	4B, 5D
PS1-7	YRH4 *rpn8-Tn*	This study	2D
PS1-13	YRH4 *zip2-Tn*	This study	4
PS1-14	YRH4 *mer1-Tn*	This study	4
PS1-26	YRH4 *bre1-Tn*	This study	2D, 3F, 5A, 5C, 5D
PS1-56	YRH4 *ira1-Tn*	This study	3F, 3G
PS1-72	YRH4 *gcn5-Tn*	This study	3F, 3G
PS1-76	YRH4 *pho23-Tn*	This study	5B
PS1-115	YRH4 *red1-Tn*	This study	4
PS1-117	YRH4 *ira2-Tn*	This study	3B
PS1-120	YRH4 *hos2-Tn*	This study	3F, 5B, 5D
PS1-152	YRH4 *rpn7-Tn*	This study	3E
PS1-170	YRH4 *nts1-Tn*	This study	2C
PS1-174	YRH4 *uaf30-Tn*	This study	2C, 3E, 3G
PS1-184	YRH4 *atg2-Tn*	This study	3E
PS1-186	YRH4 *hms1-Tn*	This study	3E
PS1-212	YRH4 *rrn5-Tn*	This study	2C
YRH4	MATa *his3D200 leu2Δ1 trp1Δ63 ura3-167 nts1D::mURA3/HIS3*	This study	1B-C, 2, 3C-G, 4, 5
YRH5	MATa *his3Δ200 leu2Δ1 trp1Δ63 ura3-167 nts1Δ::mURA3/HIS3 rpd3Δ::kanMX*	This study	3F-G, 5B, 5D
YRH51	YRH4 *ume6Δ::kanMX*	This study	5B
YRH92	MATa *his3D200 leu2D1 trp1D63 ura3-167 nts1D::mURA3/HIS3 pNOY199*	This study	1D, 3B
YRH95	MATa *his3D200 leu2D1 trp1D63 ura3-167 nts1D::mURA3/HIS3 rpa135D::kanMX pNOY199*	This study	1D, 3B
YRH132	YRH4 *set1D::kanMX*	This study	5A
YRH134	YRH4 *set2D::kanMX*	This study	5A, 5C
YRH142	YRH4 *hos1D::kanMX*	This study	5B
YRH143	YRH4 *hos3D::kanMX*	This study	5B
YRH152	YRH4 *rco1D::kanMX*	This study	5B
YRH160	YRH4 *top1D::kanMX*	This study	3E
YRH164	YRH4 *paf1D::kanMX*	This study	5A, 5C

YRH244	YRH4 *zip2D::kanMX* (A)	This study	4
YRH253	YRH4 *mer1D::kanMX*	This study	4
YRH254	YRH4 *red1D::kanMX*	This study	4
YRH257	YRH4 *zip2D::kanMX* (B)	This study	4
YRH265	PS1-26 *set2D::kanMX*	This study	5A, 5C
YRH269	MATa *his3D200 leu2D1 trp1D63 ura3-167 nts1D::mURA3/HIS3*	This study	7
YRH698	YRH269 *rad6D::kanMX*	This study	5A
YRH901	YRH269 Hos2p-13xMYC-*kanMX6*	This study	7
YRH902	YRH269 Rpd3p-13xMYC-*kanMX6*	This study	7
YRH905	YRH269 Rad6p-13xMYC-*kanMX6*	This study	7
YRH911	YRH269 Chd1p-13xMYC-*kanMX6*	This study	7
YRH912	YRH269 Ada2p-13xMYC-*kanMX6*	This study	7
YRH914	YRH269 Paf1p-13xMYC-*kanMX6*	This study	7
YRH915	YRH269 Sap30p-13xMYC-*kanMX6*	This study	7
YSB425	MAT<u>a</u> *his3Δ200 leu2Δ1 trp1Δ63 ura3-167 50L::mURA3/HIS3*	Buck et al., 2002	1B-C
YSB519	MAT<u>a</u> *his3Δ200 leu2Δ1 trp1Δ63 ura3-167 TRP1::mURA3/HIS3*	Buck et al., 2002	1C

TABLE S2

Oligonucleotides used in the study

Name	Sequence	Usage and Location
JS17	5'-CGCTTGAACGAAACGTTCGTAAAAATATTTATAATTCTTAATACGACTCACTATAGGGCG-3'	*nts1 mURA3/HIS3* replacement primer 1 (pJSS51-9 plasmid)
JS59	5'-CTGCTGAGATTAAGCCTTTGTTGTCTGATTTGTTTTTTATGTTCCTGGCCTTTTGCTGGC-3'	*nts1 mURA3/HIS3* replacement primer 2 (pJSS51-9 plasmid)
JS83	5'-CCAATTGCTCGAGAGATTTC-3'	*ACT1* forward primer, reverse-transcriptase PCR
JS84	5'-CATGATACCTTGGTGTCTTG-3'	*ACT1* reverse primer, reverse-transcriptase PCR
JS542	5-'ATATGAATTAATAAACACCTGTCCATTTTAGAAAACGCTGATTGTACTGAGAGTGCACC-3'	*HOS1 kanMX* knockout primer 1
JS543	5'-GCATTATTAATTTGTATTCAAACGACTAATTAAAACTATCCTGTGCGGTATTTCACACCG-3'	*HOS1 kanMX* knockout primer 2
JS544	5'-TACGTTAAAATCAGGTATCAAGTGAATAACAACACGCAACGATTGTACTGAGAGTGCACC-3'	*HOS2 kanMX* knockout primer 1
JS545	5'-AAAAAAAAACGGGAGATTAACCGAATAGCAAACTCTTAAACTGTGCGGTATTTCACACCG-3'	*HOS2 kanMX* knockout primer 2
JS546	5'-AAGGGCTCTGGAAGTAAACAGAGAAATTCGACGATATAATGATTGTACTGAGAGTGCACC-3'	*HOS3 kanMX* knockout primer 1
JS547	5'-CCACCACTTCTTGTTGTATGTTTTCTTGAAACATGAGAAACTGTGCGGTATTTCACACCG-3'	*HOS3 kanMX* knockout primer 2
JS635	5'-CTCTCCTTTTCTTTTTTCGACCGA-3'	reverse transcriptase PCR; *mURA3* (primer 1)
JS636	5'-ATTGCGTTGCGCTCACTGCCCGCT-3'	reverse transcriptase PCR; *nts1* (primer 2)
JS639	5'-TGGATTATGGCTGAACGCCTCTAA-3'	reverse transcriptase PCR; 35S (primer 3)
JS683	5'-GAGACTACCGCACTCAAACCATTTGCATGGACCTTAACTCGATTGTACTGAGAGTGCACC-3'	*UME6 kanMX* knockout primer 1
JS684	5'-TAATAATAGTAACAATATCTCTTTTTTTTTTTTCAGTGAGCCTGTGCGGTATTTCACACCG-3'	*UME6 kanMX* knockout primer 2

JS721	5'-AAGTACTAAAGCGTTCGTTGACAGCTTTCTTTGCGTTGCCGATTGTACTGAGAGTGCACC-3'	*UAF30 kanMX* knockout primer 1
JS722	5'-ACAACACAAATTTCAACGCCTTGAAATTTTCATGATATCCCTGTGCGGTATTTCACACCG-3'	*UAF30 kanMX* knockout primer 2
JS739	5'-AGAAATGGAAAAAGAAAACAGTGGATTGATTGCCTTTTCACGGATCCCCGGGTTAATTAA-3'	Hos2p-MYC F1 primer
JS740	5'-AAAAAAAAACGGGAGATTAACCGAATAGCAAACTCTTAAAGAATTCGAGCTCGTTTAAAC-3'	Hos2p-MYC R2 primer
JS745	5'-TGCGAGGGACCTACATGTTGAGCATGACAATGAATTCTATCGGATCCCCGGGTTAATTAA-3'	Rpd3p-MYC F1 primer
JS746	5'-TCACATTATTTATATTCGTATATACTTCCAACTCTTTTTTGAATTCGAGCTCGTTTAAAC-3'	Rpd3p-MYC R2 primer
JS748	5'-GATGGCAATGTACGACAAGATAACAGAGTCTCAAAAGAAGCGGATCCCCGGGTTAATTAA-3'	Chd1p-MYC F1 primer
JS749	5'-GGGGAAGGAACAATGGAAAATGTGGTGAAGAAAAATTGTTGAATTCGAGCTCGTTTAAAC-3'	Chd1p-MYC R2 primer
JS765	5'-ATACGCAAACCGCCTCTCC-3'	qRT-PCR; product forward
JS766	5'-TGTCGTGCCAGCTGCATTA-3'	qRT-PCR; product reverse
JS769	5'-ATGTGTAAAGCCGGTTTTGCC-3'	qRT-PCR; *ACT1* forward
JS770	5'-TGGGAAGACAGCACGAGGAG-3'	qRT-PCR; *ACT1* reverse
JS800	5'-TTCTTCAGCATATAACATACAACAAGATTAAGGCTCTTTCGATTGTACTGAGAGTGCACC-3'	*IRA1 kanMX* knockout primer 1
JS801	5'-GGAGCACGACATTCTTGCCAGTATCATTGTTGCTAATCTTCTGTGCGGTATTTCACACCG-3'	*IRA1 kanMX* knockout primer 2
JS806	5'-TCGAGTAATTGAATCAATTTAGAGAAATAGATCXATATTACGATTGTACTGAGAGTGCACC-3'	*ZIP2 kanMX* knockout primer 1
JS807	5'-CCATTCTAAGGTTAATAACTTCTTCAGGCGAGTCATTTGTCTCGGTATTTCACACCG-3'	*ZIP2 kanMX* knockout primer 2
JS822	5'-CGAGAATAATCACCATAGAAAAACCTATAAAAGCTTCGATTGTACTGAGAGTGCACC-3'	*RCO1 kanMX* knockout primer 1
JS823	5'-GGTGTTCACGTTCCTGATTTATTCTTTATGTATGTACGCCCTGTGCGGTATTTCACACCG-3'	*RCO1 kanMX* knockout primer 2
JS825	5'-CCTTATTTGTTGAATCTTTATAAGAGGTCTCTGCGTTTAGGATTGTACTGAGAGTGCACC-3'	*SET1 kanMX* knockout primer 1
JS826	5'-GCTGGAAAGCAACGATATGTTAAATCAGGAAGCTCCAAACCTGTGTATTTCACACCG-3'	*SET1 kanMX* knockout primer 2

JS828	5'-GTGCTGTCAAACCTTTCTCCTTTCCTGGTTGTTGTTTTACGATTGTACTGAGAGTGCACC-3'	*SET2 kanMX* knockout primer 1
JS829	5'-GACAGAAAACGTGAAACAAGCCCCAAATATGCATGTTGGCTGTGCGGTATTTCACACCG-3'	*SET2 kanMX* knockout primer 2
JS834	5'-GGAACAGTGTGGTATTAGCGAAGGGAAATCTGTGAAGTGGATTGTACTGAGAGTGCACC-3'	*RSC1 kanMX* knockout primer 1
JS835	5'-GTGCGTTTTGAAAGGCAACAAAACGATTGAGGTCTATCGTCTGTGCGGTATTTCACACCG-3'	*RSC1 kanMX* knockout primer 2
JS837	5'-CTAAAGGGAGGGCAGAGCTCGAAACTTGAAACGCGTAAAAGATTGTACTGAGAGTGCACC-3'	*TOP1 kanMX* knockout primer 1
JS838	5'-GAACTTGATGCGTGAATGTATTTGCTTCTCCCCTATGCTGCTGTGCGGTATTTCACACCG-3'	*TOP1 kanMX* knockout primer 2
JS859	5'-CGTTGTTAATTATAGACAGAAATGTATTCAGTACAATAGAGATTGTACTGAGAGTGCACC-3'	*PAF1 kanMX* knockout primer 1
JS860	5'-CCAAACAAATGTAAAAAGAACTACAGGTTTAAAATCAATCCTGTGCGGTATTTCACACCG-3'	*PAF1 kanMX* knockout primer 2
JS918	5'-GGGTTAATTTGATTACGCGTCACAGCTACTAATAAAATAAGATTGTACTGAGAGTGCACC-3'	*MER1 kanMX* knockout primer 1
JS919	5'-CGATCAAATAAGCTTACAATCTGGTATCTTCCAACACCATCTGTGCGGTATTTCACACCG-3'	*MER1 kanMX* knockout primer 2
JS921	5'-CAGTGAGGACCACAAAGGGACAGCAAATACGGTGATAAGAGATTGTACTGAGAGTGCACC-3'	*RED1 kanMX* knockout primer 1
JS922	5'-CGATTGCCAGAGTACTAGCTCTGCGAGAAAAAAAAGATTCCTGTGCGGTATTTCACACCG-3'	*RED1 kanMX* knockout primer 2
JS962	5'-GGCACTCCGGGGTAGCCGGAGTCGAAAGCTGG-3'	*RAD6 kanMX* knockout primer 1 (from *rad6* BY4741 strain)
JS963	5'-CGGATCGATAGAGAAAGAAACTCACGATGAAGCCC-3'	*RAD6 kanMX* knockout primer 2 (from *rad6* BY4741 strain)
JS1096	5'-CGTTCATAGCGACATTGCTT-3'	MYC-tag CHIP; NTS2 forward
JS1097	5'-GGGTGAACAATCCAACGCTT-3'	MYC-tag CHIP; NTS2 reverse
JS1100	5'TGTTAGTGCAGGAAAGCGGG-3'	MYC-tag CHIP; NTS1 forward
JS1101	5'-CTACACCCTCGTTTAGTTGC-3'	MYC-tag CHIP; NTS1 reverse
JS1102	5'-GTATGTGGGACAGAATGTCG-3'	MYC-tag CHIP; 3' 35S forward

JS1103	5'-GACTTACGTTTGCTACTCTC-3'	MYC-tag CHIP; 3' 35S reverse
JS1108	5'-TGATGATGATGATGACGACGACGACGACGACGAAGCAGACCGGATCCCCGGGTTAATTAA-3'	Rad6p-MYC F1 primer
JS1109	5'-ATCGGCTCGGCATTCATCATTAAGATTCTTTTGATTTTTCGAATTCGAGCTCGTTTAAAC-3'	Rad6p-MYC R2 primer
JS1114	5'-ACAAAAACCAGAGGAAGAAAAGGAAACTTTACAAGAAGAACGGATCCCCGGGTTAATTAA-3'	Paf1p-MYC F1 primer
JS1115	5'-CTACAGGTTTAAAATCAATCTCCCTTCACTTCTCAATATTGAATTCGAGCTCGTTTAAAC-3'	Paf1p-MYC R2 primer
JS1117	5'-AAAAAACCAGAAGAAGAAATTCAAGATGGAATTTCGGGGTCGGATCCCCGGGTTAATTAA-3'	Sap30p-MYC F1 primer
JS1118	5'-TTACATAACTTATACACAAAAGGGCTGCCTCATCGTTTGAGAATTCGAGCTCGTTTAAAC-3'	Sap30p-MYC R2 primer
JS1120	5'-GAATAGAATATACGATTTTTTTCCAGAGCCAGAATTGGATGCGGATCCCCGGGTTAATTAA-3'	Ada2p-MYC F1 primer
JS1121	5'-AACTAGTGACAATTGTAGTTACTTTTCAATTTTTTTTTTTGGAATTCGAGCTCGTTTAAAC-3'	Ada2p-MYC R2 primer
JS1140	5'-GCTTGCGTTGATTACGTCCC-3'	MYC-tag CHIP; 5' 35S forward
JS1141	5'-CACTAAGCCATTCAATCGGT-3'	MYC-tag CHIP; 5' 35S reverse

TABLE S3

List of mutants isolated and confirmed from the screen[a]

FOA^R Candidates	ORF	Reference Number[b]	Strength of Phenotype[c]	Summary of Function	Location of Insertion[d]	Dominant/ Recessive[e]
Unique Genes						
AAP1'	*YHR047C*	75	+++	Arg/Ala aminopeptidase; glycogen metabolism	341 / 2571	SD
ADA2	*YDR448W*	48	+++	SAGA/SLIK/ADA component	505 / 1305	R
ADA2	*YDR448W*	49	+++	SAGA/SLIK/ADA component	+172	R
ADA2	*YDR448W*	54	+++	SAGA/SLIK/ADA component	+172	R
AGC1	*YPR021C*	131	++	mitochondrial transporter (L-Asp/L-Glu)	916 / 2709	SD
BRE1	*YDL074C*	26	++	Rad6p E3 ubiquitin ligase	1116 / 2103	R
CHS6	*YJL099W*	42	++	chitin biosynthesis; vesicle transport	1440 / 4241	R
CIN1	*YOR349W*	139	+++	b-tubulin folding factor D	1353 / 3045	SD
CMP2	*YML057W*	122	+++	type 2B Ser/Thr phosphatase; calmodulin binding	418 / 1815	SD
CMP2	*YML057W*	128	+++	type 2B Ser/Thr phosphatase; calmodulin binding	418 / 1815	SD
DPB11	*YJL090C*	39	+++	DNA Pol e subunit	+10	SD
ECM29	*YHL030W*	10	++	proteosome subunit	425 / 5607	D
FUR4	*YBR021W*	136	+++	uracil permease	+43	SD
GCN5	*YGR252W*	72	+++	SAGA/SLIK/ADA component; non-essential H3 histone acetyltransferase	1127 / 1320	R
GCN5	*YGR252W*	88	+++	SAGA/SLIK/ADA component; non-essential H3 histone acetyltransferase	1054 / 1320	R
GCN5	*YGR252W*	104	+++	SAGA/SLIK/ADA component; non-essential H3 histone acetyltransferase	1215 / 1320	R
GPB1	*YOR371C*	2	++	b subunit of heterotrimeric G-protein	293 / 2694	R
GPG1	*YGL121C*	38	+++	g subunit of heterotrimeric G-protein	64 / 381	R
GPG1	*YGL121C*	40	+++	g subunit of heterotrimeric G-protein	80 / 381	D
GPM3	*YOL056W*	116	+++	phosphoglycerate mutase	+9	SD
GUT2	*YIL155C*	62	+++	mitochondrial G3P dehydrogenase	9 / 1950	SD

HFI1	*YPL254W*	65	+++	SAGA/SLIK component	1001 / 1467	R
HOS2	*YGL194C*	4	++	SET3 component; H3/H4 histone deacetylase	963 / 1359	R
HOS2	*YGL194C*	8	++	SET3 component; H3/H4 histone deacetylase	1155 / 1359	R
HOS2	*YGL194C*	120	+++	SET3 component; H3/H4 histone deacetylase	361 /1359	R
IMP2'	*YIL154C*	125	+++	transcription factor for glucose gene derepression	119 / 1041	SD
INM1	*YHR046C*	24	++	inositol monophosphatase; signaling factor	278 / 888	SD
IRA1	*YBR140C*	37	++	RAS GTPase activator; RAS/cAMP signaling	8185 / 9279	R
IRA1	*YBR140C*	56	+++	RAS GTPase activator; RAS/cAMP signaling	8486 / 9279	R
IRA1	*YBR140C*	101	+++	RAS GTPase activator; RAS/cAMP signaling	8751 / 9279	R
IRA1	*YBR140C*	111	++	RAS GTPase activator; RAS/cAMP signaling	8185 / 9279	R
IRA2	*YOL081W*	20	+++	RAS GTPase activator; RAS/cAMP signaling	2872 / 9240	SD
IRA2	*YOL081W*	22	++	RAS GTPase activator; RAS/cAMP signaling	2777 / 9240	R
IRA2	*YOL081W*	32	+++	RAS GTPase activator; RAS/cAMP signaling	5059 / 9240	R
IRA2	*YOL081W*	61	++	RAS GTPase activator; RAS/cAMP signaling	1267 / 9240	R
IRA2	*YOL081W*	74	++	RAS GTPase activator; RAS/cAMP signaling	866 / 9240	R
IRA2	*YOL081W*	102	+++	RAS GTPase activator; RAS/cAMP signaling	4922 / 9240	R
IRA2	*YOL081W*	108	+++	RAS GTPase activator; RAS/cAMP signaling	5061 / 9240	R
IRA2	*YOL081W*	109	+++	RAS GTPase activator; RAS/cAMP signaling	5061 / 9240	R
IRA2	*YOL081W*	117	+	RAS GTPase activator; RAS/cAMP signaling	1504 / 9240	R
IRA2	*YOL081W*	137	++	RAS GTPase activator; RAS/cAMP signaling	1649 / 9240	R
MER1	*YNL210W*	14	++	meiotic chromosome synaptonemal complex	571 / 813	D
MLC2	*YPR188C*	33	+++	type II myosin light chain	304 / 492	R
MLP1	*YKR095W*	140	+++	telomere length regulator; nuclear import protein	5207 / 5628	SD
NTE1	*YML059C*	118	++	serine esterase; phospholipid metabolite	4703 / 5040	SD
PAU7	*YAR020C*	9	+++	seripauperin gene family; function unknown	+100	SD
PHO23	*YNL097C*	21	+++	Rpd3p-Sin3p complex component	31 / 993	R
PHO23	*YNL097C*	76	+++	Rpd3p-Sin3p complex component	96 / 993	R
PHO23	*YNL097C*	144	+++	Rpd3p-Sin3p complex component	209 /993	R
PIG2	*YIL045W*	89	+++	type I phosphatase subunit	184 / 1617	R
PPH22	*YDL188C*	70	+++	protein phosphatase 2A (PP2A) catalytic subunit	45 / 1134	SD

PRE9	*YGR135W*	90	+++	20S b-type proteosome subunit	164 / 777	R
PRE9	*YGR135W*	123	+++	20S b-type proteosome subunit	517 / 777	R
PRR1	*YKL116C*	60	+++	MAP kinase signaling pathway	455 / 1557	R
PSK2	*YOL045W*	77	+++	PAS-domain Ser/Thr kinase	3262 / 3306	R
PXL1	*YKR090W*	121	++	LIM domain; signaling by Cdc42p & Rho1p	385 / 2121	R
REV3	*YPL167C*	78	+++	DNA Pol z subunit	3948 / 4515	R
RPN8	*YOR261C*	7	+++	proteosome subunit; endopeptidase activity	889 / 1017	SD
RXT3	*YDL076C*	30	+++	Rpd3p-Sin3p complex component	483 / 885	SD
RXT3	*YDL076C*	95	+++	Rpd3p-Sin3p complex component	615 / 885	R
SAC7	*YDR389W*	124	+++	Rho1 GAP	1399 / 1965	D
SAP30	*YMR263W*	69	+++	Rpd3p-Sin3p complex component	322 / 606	R
SBA1	*YKL117W*	110	++	Hsp90 binding chaperone protein	535 / 651	R
SIN3	*YOL004W*	45	++	Rpd3p-Sin3p complex component	4271 / 4611	SD
SIR1	*YKR101W*	46	++	transcription silencer at MAT loci	1082 / 2037	R
SPT20	*YOL148C*	50	++	SAGA component; maintains structural integrity	1122 / 1815	R
UME1	*YPL139C*	23	++	Rpd3p-Sin3p complex targeting component	210 / 1383	D
YPK1	*YKL126W*	53	+++	Ser/Thr kinase	+17	SD
Intergenic						
BMH2 / TVP15	*YDR099W / YDR100W*	92	+++	n/a	n/a	R
RPL11B / MRP13	*YGR085C / YGR084C*	16	+++	n/a	n/a	SD
Uracil Biosynthesis						
URA5	*YML106W*	34	+++	fifth enzymatic step in de novo pyrimidine biosynthesis	232 / 681	SD
URA5	*YML106W*	36	+++	fifth enzymatic step in de novo pyrimidine biosynthesis	309 / 681	SD
URA5	*YML106W*	83	+++	fifth enzymatic step in de novo pyrimidine biosynthesis	232 / 681	SD

URA5	*YML106W*	100	+++	fifth enzymatic step in de novo pyrimidine biosynthesis	617 / 681	R
URA6	*YKL024C*	29	+++	seventh enzymatic step in de novo pyrimidine biosynthesis	+308	D
Uncharacterized						
YBL048W (RRT1)	*YBL048W*	79	+++	uncharacterized; downstream of Moh1p	36 / 312	R
YBR246W (RRT2)	*YBR246W*	12	+++	uncharacterized	854 / 1164	SD
YDR020C (RRT3)	*YDR020C*	119	+++	uncharacterized; nuclear; interacts with Urk1p	568 / 699	SD
YDR020C (RRT3)	*YDR020C*	134	+++	uncharacterized; nuclear; interacts with Urk1p	421 / 699	R
YDR520C (RRT4)	*YDR520C*	68	++	uncharacterized; GAL4-like DBD	1681 / 2319	R
YDR520C (RRT4)	*YDR520C*	85	++	uncharacterized; GAL4-like DBD	476 / 2319	R
YDR520C (RRT4)	*YDR520C*	103	+++	uncharacterized; GAL4-like DBD	544 / 2319	R
YFR032C (RRT5)	*YFR032C*	81	++	uncharacterized; interacts with Nop6p	261 / 870	D
YGL146C (RRT6)	*YGL146C*	80	++	uncharacterized; complex with nucleolar proteins	490 / 936	SD
YLL030C (RRT7)	*YLL030C*	71	+++	uncharacterized	275 / 342	R
YOL048C (RRT8)	*YOL048C*	52	+++	uncharacterized	124 / 1107	SD

URA$^+$ Candidates	ORF	Reference Number[b]	Strength of Phenotype[c]	Summary of Function	Location of Insertion[d]	Dominant/ Recessive[e]
Unique Genes						
ATG2	*YNL242W*	184	++	involved in autophagy and peroxisome degradation	3185 / 4779	R
BNI4	*YNL233W*	205	++	targeting subunit for Glc7p	1535 / 2679	R
GAP1	*YKR039W*	172	++	amino acid permease	+397	R
HMS1	*YOR032C*	186	+	zinc finger protein; similar to myc family of	1001 / 1305	R

				transcription factors; synthetic lethal with Top1		
MNN1	*YER001W*	206	+++	mannosyltransferase; membrane glycoprotein	2151 / 2289	R
MSS4	*YDR208W*	154	+++	actin organization	-187	R
RPN7	*YPR108W*	152	++	proteosome subunit	-89	R
RRN5	*YLR141W*	212	++	essential Pol I UAF subunit (c-term)	-300	R
SEY1	*YOR165W*	202	++	membrane biogenesis and organization	61 / 2331	R
UAF30	*YOR295W*	174	+++	non-essential Pol I UAF subunit	-47	SD
Intergenic						
VTS1 / PDE2	*YOR359W / YOR360C*	155	++	n/a	n/a	R
Uncharacterized						
YBR147W (RRT11)	*YBR147W*	178	++	uncharacterized; upregulated by rapamycin/during stationary phase	665 / 891	R
YCR045C (RRT12)	*YCR045C*	193	+++	uncharacterized	486 / 1476	SD
YER066W (RRT13)	*YER066W*	183	+++	uncharacterized; homologous to Cdc4p	+103	R
YIL127C (RRT14)	*YIL127C*	167	++	uncharacterized; nucleolar; downregulated by rapamycin/during stationary phase	27 / 621	D
YLR162W-A (RRT15)	*YLR162W-A*	171	+++	uncharacterized	+162	D
YNL105W (RRT16)	*YNL105W*	150	+++	uncharacterized	394 / 429	R

[a]All mutants from the screen that passed the backcrossing test are shown, including multiple hits in specific genes.
[b]Reference number corresponds to strain numbers. For example, 75 is equivalent to PS175 and 212 to PS1212.
[c]Mutants chosen 1 day after replica-plating (+++), 2 days (++), 3 days (+).
[d]Nucleotide locations of the mTn3 transposon insertions. +1 corresponds to the first nucleotide of the open reading frame for each gene.
[e]Mutations were tested in heterozygous diploids to determine whether they were dominant (D), semi-dominant (SD), or recessive (R).

TABLE S4

Effects of mutations on *mURA3* silencing in the context of NTS1

mutant	nts1Δ phenotype	NTS1+ phenotype
bre1-Tn	FOA^R	normal
cmp2-Tn	FOA^R	normal
dpb11-Tn	FOA^R	Ura^+
gcn5-Tn	FOA^R	FOA^R
hos2-Tn	FOA^R	FOA^R
pre9-Tn	FOA^R	normal
rpn8-Tn	FOA^R	Ura^+
rrt1-Tn	FOA^R	normal
rrt8-Tn	FOA^R	normal
sap30-Tn	FOA^R	Ura^+
atg2-Tn	Ura^+	normal
gap1-Tn	Ura^+	normal
hms1-Tn	Ura^+	normal
mnn1-Tn	Ura^+	normal
rpn7-Tn	Ura^+	normal
rrt11-Tn	Ura^+	normal
rrt12-Tn	Ura^+	normal
rrt14-Tn	Ura^+	normal
sey1-Tn	Ura^+	FOA^R
uaf30-Tn	Ura^+	Ura^+

TABLE S5

Genes deletions tested in the Pol I transcription reporter strain (YRH4)

Gene	ORF	Summary of Function[a]	Phenotype
AHC1	*YOR023C*	ADA HAT specific subunit	wild type
ARD1	*YHR013C*	subunit of NatA N-terminal protein acetyltransferase complex	FOA^R
ASF1	*YJL115W*	nucleosome remodeling factor; associates with Bdf1p	Ura^+
CAC2	*YML102W*	subunit of the chromatin assembly complex (CAC)	FOA^R
FOB1	*YDR110W*	nucleolar replication fork block protein; required for rDNA recombination	wild type
HAT2	*YEL056W*	subunit of the Hat1p-Hat2p histone acetyltransferase complex	wild type
HOS1	*YPR068C*	class I HDAC; interacts with Tup1p-Ssn6p corepressor complex	wild type
HOS3	*YPL116W*	trichostatin A-insensitive HDAC	wild type
HTZ1	*YOL012C*	histone variant H2A.Z	Ura^+
IRA1	*YBR140C*	RAS GTPase activating protein	FOA^R
MER1	*YNL210W*	protein involved in chromosome pairing and meiotic recombination	wild type
PAF1	*YBR279W*	associates with pol II and Cdc73p; elongation factor for pol II	FOA^R
PIF1	*YML061C*	DNA helicase; telomerase formation and elongation	wild type
RAD6	*YGL058W*	E2 ubiquitin conjugating enzyme	FOA^R
RCO1	*YMR075W*	unique subunit of the Rpd3S HDAC complex	FOA^R
RED1	*YLR263W*	component of the synaptonemal complex	wild type
RPA14	*YDR165W*	Pol I non-essential subunit	Ura^+
RPD3	*YNL330C*	catalytic subunit of the Rpd3L and Rpd3S HDAC complexes	FOA^R
RSC1	*YGR056W*	subunit of RSC chromatin remodeling complex	FOA^R
RTG2	*YGL252C*	SLIK HAT specific subunit	wild type
SET1	*YHR119W*	histone H3 K4 methyltransferase; subunit of COMPASS	wild type
SET2	*YJL168C*	histone H3 K36 methyltransferase; involved in pol II elongation	FOA^R
SIR2	*YDL042C*	NAD-dependent histone deacetylase; involved in rDNA, MAT, and telomeric silencing in yeast	wild type
SIR3	*YLR442C*	silencing protein; interacts with Sir2p and Sir4p	FOA^R
SPT4	*YGR063C*	elongation factor for Pol I and Pol II	Ura^+
SPT7	*YBR081C*	SAGA HAT specific subunit; truncated version in SLIK	FOA^R
SPT8	*YLR055C*	SAGA HAT specific subunit	FOA^R
SWC5	*YBR231C*	subunit of SWR1 complex; function unknown	FOA^R
SWR1	*YDR334W*	structural component of SWR1 complex; Swi2/Snf2-related ATPase	wild type
TOP1	*YOL006C*	Topoisomerase I	Ura^+
UAF30	*YOR295W*	Pol I transcription factor; part of UAF	Ura^+
UME6	*YDR207C*	binds URS1 regulatory sequence; recruits Rpd3L complex to gene promoters	Ura^+
ZIP2	*YGL249W*	meiotic protein, involved in synaptonemal complex formation	wild type

[a]from the *Saccharomyces* Genome Database (www.yeastgenome.org)