DOI: 10.1534/genetics.106.055491

New Alleles of *SIR2* Define Cell-Cycle-Specific Silencing Functions

Mirela Matecic,*[,1,2] Kristen Martins-Taylor,*[,1] Merrit Hickman,*[,3] Jason Tanny,[†,4] Danesh Moazed[†] and Scott G. Holmes*[,5]

**Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, Connnecticut 06459 and [†]Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115*

Manuscript received January 5, 2006
Accepted for publication June 1, 2006

ABSTRACT

The establishment of transcriptional silencing in yeast requires cell-cycle progression, but the nature of this requirement is unknown. Sir2 is a protein deacetylase that is required for gene silencing in yeast. We have used temperature-sensitive alleles of the *SIR2* gene to assess Sir2's contribution to silencing as a function of the cell cycle. When examined *in vivo*, these conditional alleles fall into two classes: one class exhibits a loss of silencing when raised to the nonpermissive temperature regardless of cell-cycle position, while the second class exhibits a mitosis-specific silencing defect. Alleles of the first class have a primary defect in protein deacetylase activity, while the alleles of the second class are specifically defective in Sir2–Sir4 interactions at nonpermissive temperatures. Using a *SIR2* temperature-sensitive allele, we show that silencing can be established at the *HML* locus during progression through the G_2/M–G_1 interval. These results suggest that yeast heterochromatin undergoes structural transitions as a function of the cell cycle and support the existence of a critical assembly step for silent chromatin in mitosis.

In *Saccharomyces cerevisiae*, transcriptional silencing is observed at the silent mating-type loci (*HML* and *HMR*), telomeres, and the ribosomal DNA repeats (for reviews see Huang 2002 and Rusche *et al.* 2002). At the *HM* loci and telomeres, silencing depends on the action of three Sir proteins, Sir2, Sir3, and Sir4. A Sir2–Sir4 complex is observed *in vivo* (Moazed *et al.* 1997), and this complex can also associate with Sir3 (Moazed *et al.* 1997; Hoppe *et al.* 2002; Luo *et al.* 2002; Rusche *et al.* 2003; Liou *et al.* 2005). The Sir protein complex can be recruited to telomeres and the *HM* loci via a specific interaction between Sir4 and the DNA-binding factor Rap1p (Moretti *et al.* 1994). Silencing at the rDNA locus does not require Sir3 or Sir4, but does depend on Sir2; here Sir2 is recruited as part of the RENT complex (Shou *et al.* 1999; Straight *et al.* 1999). Therefore, the Sir2 protein is central to all forms of transcriptional silencing in yeast. Sir2 is the founding member of a large family of NAD-dependent lysine deacetylases (Imai *et al.* 2000; Landry *et al.* 2000b; Smith *et al.* 2000) that includes members from bacteria to humans (Brachmann *et al.* 1995; Afshar 1999; Frye 2000; for Sir2 reviews see Moazed 2001; Smith *et al.* 2002). Yeast Sir2's likely substrate is histones. A relative lack of histone deacetylation is observed at all three silenced loci (Braunstein *et al.* 1993, 1996; Bryk *et al.* 2002; Buck *et al.* 2002), and Sir2's deacetylase activity is specifically required for silencing to be established (Hoppe *et al.* 2002; Luo *et al.* 2002; Rusche *et al.* 2002) and maintained (Bedalov *et al.* 2001). Deacetylation of the histone H4 N-terminal tail may increase interactions with the Sir3 protein, promoting the spread of the Sir protein complex (Hecht *et al.* 1995; Carmen *et al.* 2002; Liou *et al.* 2005).

The establishment of silencing requires progression through the cell cycle (Miller and Nasmyth 1984; Fox *et al.* 1997; Kirchmaier and Rine 2001, 2006; Li *et al.* 2001; Lau *et al.* 2002; Martins-Taylor *et al.* 2004), but the nature of this constraint is unknown. Determining the requirement for cell-cycle dependence is likely to provide key insights into the mechanism of silencing in yeast and uncover general insights into the maintenance and propagation of transcriptional states. In this study we examine Sir2's specific contribution to silencing as a function of the cell cycle using conditional alleles of the *SIR2* gene. Our data suggest that Sir2 makes distinct contributions to silencing and that a crucial step for the assembly of silent chromatin occurs in mitosis.

MATERIALS AND METHODS

Strains and plasmids: To generate random mutations in the *SIR2* gene, we used error-prone PCR followed by gap repair (Muhlrad *et al.* 1992). Error-prone PCR reactions included

[1]These authors contributed equally to this study.

[2]*Present address:* Department of Biochemistry and Molecular Genetics, University of Virginia Medical School, Charlottesville, VA 22908.

[3]*Present address:* Malaria Vaccine Development Branch, National Institutes of Health, Rockville, MD 20892.

[4]*Present address:* Laboratory of Chromatin Biology, Rockefeller University, New York, NY 10021.

[5]*Corresponding author:* Department of Molecular Biology and Biochemistry, Hall-Atwater Laboratories, Lawn Ave., Wesleyan University, Middletown, CT 06459-0175. E-mail: sholmes@wesleyan.edu

TABLE 1

Description of strains

Strain	Genotype	Source[a]
YSH189 (DMY1)	*MAT***a** *ura3 ade2 lys1 his5 leu2 can1*	Mahoney and Broach (1989)
YSH501	*MAT***a** *ura3 ade2 lys1 his5 leu2 can1* Δ*sir2::KAN*	
YSH502	YSH501; *leu2::sir2-604-LEU2*	
YSH498	YSH501; *leu2::sir2-614-LEU2*	
YSH499	YSH501; *leu2::sir2-620-LEU2*	
PJ69-4a	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200* Δ*gal4* Δ*gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James *et al.* (1996)
YSH563	PJ69-4α; Δ*sir3::NAT1MX* Δ*sir4::URA3*	
YSH625	PJ69-4α; Δ*sir2::HYG* Δ*sir3::NAT1MX* Δ*sir4::URA3*	

[a] Unless noted, strains were constructed during the course of this work.

5 units of Taq polymerase, 7 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dCTP and dTTP, 0.2 mM dATP and dGTP, as well as the *SIR2* template. Mutated *SIR2* DNA fragments were cotransformed with *SIR2* plasmid pAW2 that had been cut with *Sma*I into the *HML*α *MAT***a** *HMR*α Δ*sir2* strain YSH311. Ura+ transformants were then screened for those able to promote mating at 23°, but not at 37°. All constructs were sequenced to identify or verify the existence of mutations. Temperature-sensitive alleles of *SIR2* were cloned into pRS305 (Sikorski and Hieter 1989) to produce plasmids pMMi1 (bearing the *sir2-604* allele), pMMi2 (*sir2-614*), and pMMi3 (*sir2-620*). These plasmids were then integrated into the *leu2* locus of strain YSH501 by transforming plasmids cut with *Eco*RI. Yeast strains used in this study are listed in Table 1. *SIR2* plasmids used in silencing assays are based on pR415 (Sikorski and Hieter 1989) and include pKAM1 (*SIR2*), pMM80 (*sir2-604*), pMM81 (*sir2-614*), and pMM82 (*sir2-620*). All plasmid-borne *SIR2* alleles described in this work have identical flanking sequences and include 300 bp of wild-type sequences 5′ to the open reading frame and 320 bp 3′ to the open reading frame. A complete description and characterization of *SIR2* temperature-sensitive alleles will be presented elsewhere (M. Hickman and S. Holmes, unpublished results).

Silencing assays: To measure silencing at *HML* using a pheromone response assay, cultures were grown in YPD (1% bacto yeast extract, 2% bactopeptone, 2% dextrose) to early log phase (~2×10^6 cells/ml) when α-factor was added at 10 μg/ml. Cultures were further incubated with shaking at the indicated temperatures for 5 hr. Cell-cycle arrest was determined by microscopic examination of cell morphology. Unbudded cells were assumed to be in G_1 phase. Unbudded cells with obvious growth projections were further designated as "shmoos." Cells with buds composing <50% of the volume of the mother cell were designated as small-budded cells, while cells with buds composing >50% of the volume of the mother cells were designated as large-budded cells. A minimum of 100 cells was assayed for each determination. For pedigree analysis of α-factor sensitivity, cultures were applied directly to YPD media containing α-factor. Mating assays were performed as described (Dula and Holmes 2000). Reverse-transcriptase PCR (RT–PCR) measurements of *ACT1* and α*1* message were performed exactly as described (Martins-Taylor *et al.* 2004) except that PCR products were run on 8% acrylamide gels, stained using sybr gold dye (Invitrogen, San Diego), and the gels were converted to tif files using a Storm 840 PhosphorImager. Identical results were achieved in at least two independent experiments and in repeated determinations from RNA collected from individual experiments.

Western blots: Protein extraction and detection by Western blotting was performed as described (Tanny *et al.* 1999). For cycling cells, protein was extracted from log-phase cultures (cell density was ~1.5×10^7 cells/ml) grown at either 23° or 37°. For cultures blocked in the cell cycle, cells were grown at 23° to an OD_{600} of ~0.2. Cell-cycle blocking agents were added and cells were allowed to block for ~4–5 hr until >90% of the cells were arrested. For G_1-S experiments, α-factor was added to a final concentration of 10 μg/ml. Once cells were >90% arrested in G_1, hydroxyurea was added to a final concentration of 30 mg/ml. Cultures were incubated at 23° for an additional 30 min when the culture was divided, half remaining at 23° and half shifted to 37°. Following an additional incubation of 3 hr, cells were pelleted and protein was extracted. For G_2/M-blocked cultures, nocodazole was added to a final concentration of 15 μg/ml. Once >90% of the cells were arrested, the cultures were divided; half of the culture remained at 23°, while the other half was shifted to 37° for 3 hr when cells were pelleted and protein was extracted. Bradford assays were performed on each sample to equalize protein loadings on the gel. Duplicate gels were run and stained with Coomassie to confirm consistent loading from lane to lane. Blots were probed with an antibody directed to the N terminus of Sir2 (Santa Cruz Biotechnology), used at a dilution of 1:2000. Secondary detection was performed using either the Renaissance enhanced chemiluminescence detection system (New England Nuclear, Boston) or a mouse anti-goat IgG–biotin secondary antibody from Santa Cruz used at a 1:500 dilution, followed by addition of streptavidin–HRP (Bio-Rad, Hercules, CA) at a dilution of 1:2500 and the Western Lightning chemiluminescence reagent (Perkin-Elmer, Norwalk, CT). Western blots run with varying dilutions of protein extracts indicated that the assay was sensitive to small differences in protein levels. Similar results were obtained in three independent experiments.

Cell-cycle blocks: Cell-cycle blocks and interval experiments were performed as described (Martins-Taylor *et al.* 2004). α-Factor (10 μg/ml), nocodazole (15 μg/ml), or hydroxyurea (20 mg/ml) was used to block cells in G_1, G_2/M, or early S phase, respectively. Unless noted, cells exhibited at least a 90% arrest in the cell cycle. Cell-cycle arrest was determined by microscopic examination of cell morphology. For all interval experiments, log-phase cells were incubated in the initial blocking agent until >90% of cells were arrested in the cell cycle. Media were then removed by filtration, and cells were washed with several volumes of water and resuspended in media containing the second blocking agent until at least 90% of cells exhibited cell-cycle arrest. Experiments were initiated when cultures were at early log phase (~$2–3 \times 10^6$ cells/ml).

Two-hybrid assays: Vectors and methods for performing two-hybrid screens have been described (James *et al.* 1996; Uetz *et al.* 2000). *SIR2* alleles were amplified using primers

SP280 (ACCCCACCAAACCCAAAAAAAGAGATCGAATTCCA GCTGACCACCATGACCATCC CACATATG) and SP281 (CTA CGATTCATAGATCTCTGCAGGTCGACGGATCCCCGGGAA TT GCCATGTTAGAGGGTTTTGGGATG). The *sir4Δ730N* allele was amplified using primers SP278 (ACCCCACCAAACC CAAAAAAAGAGATCGAATTCCAGCTGACCACCATGCC AA ATGACAATAAGAC) and SP279 (CTACGATTCATAGATCT CTGCAGGTCGACGGATCCC CGGGAATTGCCATGTCAATA CGGTTTTATCTCCT). These fragments were cotransformed with cut pOBD2 or pOAD; gap repair of the cut plasmids created plasmids expressing binding domain or activation domain fusions (Uetz *et al.* 2000). Deletions of *SIR2* and *SIR3* in strain PJ69-4α were made via PCR-mediated gene deletion using pAG25 and pAG32 as templates (Goldstein and McCusker 1999). *SIR4* was deleted by transforming PJ69α with pCTC77 cut with *Hin*dIII (Stone *et al.* 1991).

NAD hydrolysis assays: Sir2 enzymatic assays were performed on Sir2 protein complexes affinity purified from yeast (Tanny *et al.* 2004). *SIR2* alleles fused to an N-terminal tandem affinity purification (TAP) tag (Rigaut *et al.* 1999; Puig *et al.* 2001) were constructed by gap repair; this addition did not affect the mating behavior of these *SIR2* alleles (not shown). The TAP tag was amplified from plasmid pBS1761 (Rigaut *et al.* 1999) using PCR primers SP259 (CGCTAGTCTTTGAT ACGGCGTATTTCATATGTGGGATGGTCATCTTATCGTCA TCATCAAGTG) and SP269 (GTAGACACATTCAAACCATT TTTCCCTCATCGGCACATT AAAGCTGGATGGCAGGCCTT GCGCAACA). These primers amplify the TAP affinity tag with overhangs identical to sequences flanking the start of the *SIR2* open reading frame. This fragment was transformed into yeast along with plasmids containing alleles of the *SIR2* gene partially digested with *Nda*I, an enzyme that cuts near the start of the *SIR2* open reading frame. Successful gap repair creates plasmids expressing *SIR2* alleles from their own promoter, altered solely by the addition of the TAP tag to the N terminus. Plasmids were recovered from yeast and verified by sequencing. The TAP–*SIR2* plasmids that were created included pMM84 (*SIR2*), pMM87 (*sir2-604*), pMM85 (*sir2-614*), and pMM86 (*sir2-620*).

Purification and assay of Sir2 was essentially as described (Tanny *et al.* 2004). Cultures were grown in glucose medium lacking leucine. Fifty-milliliter cultures were grown to OD_{600} 1.5; cells were washed with ice-cold water and pelleted by centrifugation. Cell pellets were frozen using liquid nitrogen and stored at −70°. Each of the remaining steps was performed at 4°. The frozen cell pellets were resuspended in 400 μl of lysis buffer (50 mm HEPES KOH, pH 7.6, 10 mm MgOAc, 0.5 m KOAc, 5 mm EGTA, 0.1 mm EDTA, 0.25% NP-40, 5% glycerol, 1 mm DTT). Freshly made PMSF (1 mm) and the protease inhibitors leupeptin, bestatin, and pepstatin (2 μg/ml each) were added to lysis buffer immediately before use. Cells were disrupted by grinding with glass beads on a bead beater (2 × 30 sec with 5 min rest on ice in between). Eppendorf tubes were punctured at the bottom and lysates were collected in centrifuge tubes by centrifugation at 2 krpm for 1 min at 4°. The lysates were transferred to new tubes and microcentrifuged at 13 krpm for 10 min at 4°. After centrifugation, the amount of total protein in each lysate was determined using the Bradford assay. Equal amounts of the total protein from each lysate (10–15 mg) were added to 10 μl IgG sepharose beads (Pharmacia 17-0969-01) that had been washed with lysis buffer. After 2 hr of nutation at 4°, beads were collected by centrifugation at 2 krpm for 1 min at 4° and washed three times with wash buffer (50 mm HEPES KOH, pH 7.6, 150 mm NaOAc, 5 mm MgOAc, 5% glycerol, 1 mm DTT, 1 mm PMSF). For Western detection, the washed beads were resuspended in 1.5× sample buffer (75 m Tris–HCl, pH 6.8, 3% SDS, 15% glycerol, 0.15% bromophenol blue, 7.5% β-mercaptoethanol, 0.1 mm PMSF, and 1 mm DTT), boiled at 65° for 10 min, and run on an 8% PAGE gel. The proteins were transferred to nitrocellulose and incubated with Sir2-specific antibodies (used at 1:5000 dilution); protein bands were visualized using the New England Nuclear–Renaissance system.

For the enzymatic activity assay, TAP–Sir2 was affinity purified as described above. After the second wash, the beads were split. One-third of the beads were washed with wash buffer one more time and stored at −20° in 1.5× sample buffer to be used in the Western blot assay. The other two-thirds of the beads were washed with reaction buffer (50 mm Tris 7.5, 100 mm NaCl, 1 mm DTT), divided, and used in the assay for Sir2 activity by nicotinamide release. Sir2-bound beads were incubated in 8.5 μl of reaction buffer, 0.05 μCi [*carbonyl*-^{14}C]-NAD (Amersham Pharmacia; 51 mCi/mmol; label on the nicotinamide ring), and 10 μg of synthetic N-terminal H4 peptides with either acetylated or deacetylated lysines. Samples were incubated for 1 hr at either 23° or 37°. The reactions ware stopped with 0.5 m sodium borate, pH 8.0, and the released nicotinamide was extracted by ethyl acetate. After addition of ethyl acetate, the samples were vortexed for 5 sec and centrifuged for 1 min at 13 krpm in a microcentrifuge. The ethyl acetate phase was removed and counted in 2 ml of scintillation fluid. The NAD breakdown activity for each mutant protein was expressed as percentage of activity measured for the wild type in a concurrent experiment. The amount of Sir2 protein in individual experiments was normalized to the amount of wild-type Sir2 used in the same experiment. We performed quantitative Western blotting to ensure that equal amounts of Sir2 protein were added to each reaction. Samples from individual experiments were analyzed by Western detection, the autoradiography film was scanned, and the Sir2 bands were digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT). Serial dilutions of wild-type Sir2 samples were included to generate a standard curve. The data shown in Table 5 are the results of four independent experiments.

RESULTS

A mitosis-specific function of Sir2? We assessed the contributions of the Sir2 protein to silencing as a function of the cell cycle using conditional alleles of *SIR2*. For these studies, we focused on three temperature-sensitive alleles (Table 2; also see materials and methods). Full characterization of these and other temperature-sensitive alleles of *SIR2* will be described elsewhere (M. Hickman and S. Holmes, unpublished results).

We created strains with integrated copies of temperature-sensitive *SIR2* alleles in a *MAT***a** strain lacking the wild-type *SIR2* gene. Silencing was initially examined using an α-factor sensitivity assay (Table 3). Cultures were grown to steady state (at least 10 cell doublings) at the permissive or nonpermissive temperature and then challenged with α-factor. Cells that lose silencing at *HML*α in these *MAT***a** strains will lose sensitivity to α-factor and progress through the cell cycle, while cells that retain silencing will block in G_1 and adopt an altered ("shmoo") morphology. Strains with these alleles were found to retain near wild-type levels of silencing at the permissive

TABLE 2

***SIR2* alleles**

SIR2 allele	Amino acid changes
sir2-604	D515Y Δ*30C*
sir2-614	E174G *N299I L438H* K523E K542R
sir2-620	S204P *K414R K475E V500A* Δ*24C*

A shorthand description for mutations present in each allele is used. For amino acid substitutions, the wild-type amino acid (in its one-letter code) and position are listed, followed by the amino acid replacing it in the mutated allele. For deletions caused by premature stop codons, the number of amino acids removed is listed after the "Δ," followed by a "C" for carboxy-terminal deletion. Amino acid changes known to be sufficient to confer the temperature-sensitive mating phenotype are in italics (M. Hickman and S. Holmes, unpublished results). Both N299I and L438H mutations of *sir2-614* are necessary to observe the temperature-sensitive phenotype. *SIR2* alleles containing only the Δ24C, K475E, or K414R/K475E mutations found in *sir2-620* exhibit no mating phenotypes.

temperature and exhibited essentially no silencing at the nonpermissive temperature by this assay (Table 3).

As an independent measure of silencing we used RT–PCR to measure the α*1* message transcribed from the *HML*α locus (see Figure 1). Loss of silencing in *MAT***a** strains allows expression of the α*1* and α*2* genes from *HML*. α*1* and, to a lesser extent, α*2* are subsequently subject to repression by the action of the **a**1/α2 heterodimer (Klar *et al.* 1981; Nasmyth *et al.* 1981; Siliciano and Tatchell 1984). However, α*1* message remains detectable in *MAT***a** strains lacking Sir2 (Chi and Shore 1996; Wyrick *et al.* 1999). In our experiments we find that assaying α*1* expression provides the most consistent and quantitative measure of *HML* expression. Similar to the prior experiment, cultures were grown to steady state at the permissive or nonpermissive temperature, RNA was collected, and the levels of α*1* message were determined. As a control for these experiments message from the *ACT1* gene was also measured. Consistent with the α-factor sensitivity assay, each of the strains bearing temperature-sensitive *SIR2* alleles exhibits wild-type silencing at the permissive temperature and is defective for silencing at the nonpermissive temperature (Figure 1A).

TABLE 3

Temperature-sensitive silencing mediated by conditional *SIR2* alleles

	% α-factor arrested	
SIR2 allele	23°	37°
SIR2	91	96
sir3-8	96	1
sir2-604	96	2
sir2-614	98	2
sir2-620	96	0

Yeast strains bearing different *SIR2* alleles were grown to log phase at 23° or 37° and then challenged with 10 μg/ml α-factor. The percentage of unbudded (G_1 blocked) cells after 5 hr incubation in α-factor is listed. A strain bearing the temperature-sensitive *sir3-8* allele was assayed as a control. A minimum of 200 cells was assayed for each determination.

The absence of silencing at nonpermissive temperatures could be due to a loss of Sir2 function or to a temperature-dependent defect in the synthesis or stability of Sir2. To examine the steady-state levels of Sir2 protein coded for by these alleles, we performed Western blotting on strains grown at permissive and nonpermissive temperatures. As shown in Figure 1B, we find that strains bearing mutated alleles of Sir2 have lower steady-state levels of Sir2 protein than a strain expressing wild-type Sir2, but in each of the strains Sir2 protein levels either do not change, or appear to increase (see sir2-614p in cycling cells), at the nonpermissive temperature. When strains bearing specific *SIR2* alleles are transformed with low-copy plasmids bearing the same *SIR2* allele, the mating behavior of the strain does not change (Figure 5). Thus, the Sir2 proteins coded for by these alleles are defective for function at elevated temperatures, and these defects are not likely due to limiting Sir2 protein levels.

To test for the requirement for Sir2 in G_1 phase, we grew cultures bearing conditional *SIR2* alleles to log phase at permissive temperature, blocked them with α-factor, and then shifted the culture to the nonpermissive temperature. This design allowed us to observe the immediate effects of Sir2 inactivation *vs.* the cumulative effects of the steady-state experiments. Cultures were monitored at several time points following the temperature shift using the α-factor sensitivity assay. As a control we conducted the same experiment with a strain bearing the temperature-sensitive *sir3-8* allele, known to lose silencing under these conditions (Miller and Nasmyth 1984; Holmes and Broach 1996). Results from these experiments are shown in Figure 2. We find that at the permissive temperature all alleles promote efficient silencing and remain arrested in G_1 phase (Figure 2A). However, when raised to the nonpermissive temperature, our strains bearing temperature-sensitive alleles of *SIR2* exhibit distinct phenotypes in this assay: the *sir-604* and *sir-620* alleles promote silencing at 37°, while a strain with the *sir2-614* allele rapidly loses silencing (Figure 2B). Therefore, the *sir2-604* and *sir2-620* alleles may be defective for a function of Sir2 that is dispensable in G_1 phase. Alternatively, the *sir2-604* and *sir2-620* alleles may code for Sir2 proteins that are slower than the sir2-614 protein to lose function at the nonpermissive temperature.

We conducted a similar experiment using RT–PCR to assay silencing of *HML*. Cultures were blocked in G_1 at the permissive temperature and then shifted to the nonpermissive temperature. In this experiment, cells

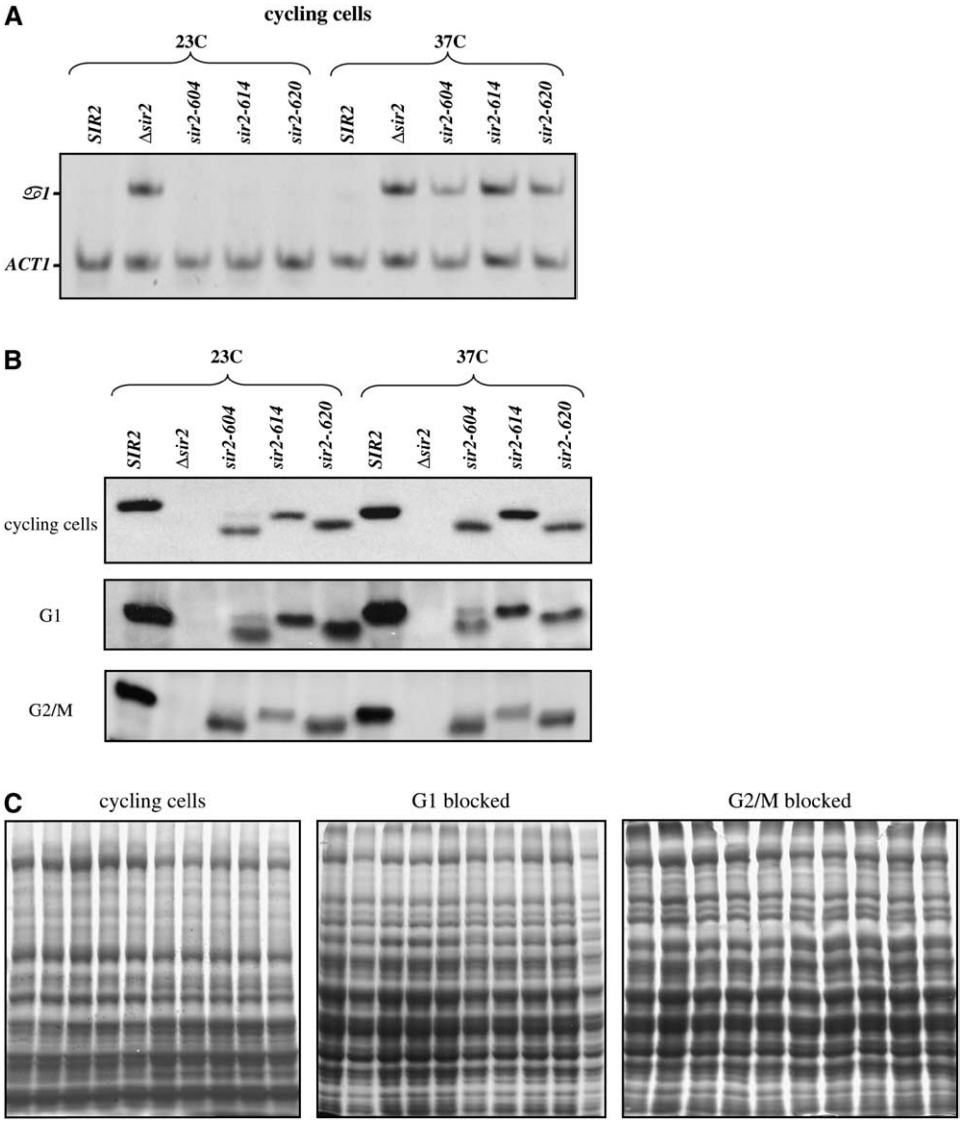

FIGURE 1.—Conditional alleles of *SIR2*. (A) Levels of α*1* and *ACT1* mRNA were measured by RT–PCR in strains bearing different alleles of *SIR2*. Cultures were maintained in log phase for >10 generations at the listed temperatures, and then RNA was collected and analyzed as described in MATERIALS AND METHODS. α*1* indicates steady-state levels of message transcribed from the *HML* locus; *ACT1* message is shown as a control. RNA was analyzed from a wild-type strain, a strain lacking the *SIR2* gene, and strains bearing the indicated alleles of *SIR2*. (B) Protein extracts made from cells bearing the indicated *SIR2* alleles were subjected to a Western blot using an antibody specific for the Sir2 protein, as described in MATERIALS AND METHODS. For the cycling cells, parallel cultures were grown to log phase at either 23° or 37°. G_1/early S-blocked cells were grown to early log phase at 23° and then blocked with α-factor. Half the culture was maintained at 23°, while the other half was shifted to 37° for 3 hr. G_2/M-blocked cells were grown to early log phase at 23° and then blocked with nocodazole. Half the culture was maintained at 23°, while the other half was shifted to 37° for 3 hr. (C) For each of the experiments shown in B, duplicate gels were run and stained with Coomassie to confirm consistent loading of samples from lane to lane.

that lose silencing escape the G_1 arrest and progress through the cell cycle. To restrict their progress, hydroxyurea was added to each culture prior to the temperature shift. Hydroxyurea blocks cells early in S phase, soon after the initiation of DNA replication; thus, cells escaping from G_1 were assayed in this short interval. Following 4 hr at the nonpermissive temperature, RNA was collected and α*1* message levels were determined. The RT–PCR experiment produced results identical to the α-factor sensitivity assay; no message was detected from strains bearing the *sir2-604* or *sir2-620* alleles, while message was readily detected from the strain bearing the *sir2-614* allele (see Figure 2C).

We next used the same set of strains to examine the requirement for Sir2 function in mitosis. For our first experiment, we used α-factor sensitivity to examine the role of Sir2 during the G_2/M–G_1 interval. Cells were blocked in G_2/M with nocodazole at the permissive temperature, where they arrest as large-budded cells. Cultures were then shifted to the nonpermissive temperature, held at the nonpermissive temperature for 1 hr, and then released from the nocodazole block into medium containing α-factor. In this medium, cells will complete mitosis and form two unbudded cells. If cells maintain silencing at *HML*, these cells will block in G_1 and remain unbudded. However, cells that lose silencing will be insensitive to α-factor and continue through G_1 into S phase, resulting in cells with small buds. Results from a block-and-release experiment are shown in Table 4. When performed entirely at the permissive temperature, we find that nearly all cells that progress out of the nocodazole block are sensitive to α-factor and are therefore silenced (note that in this experiment cultures with *SIR2* mutations fail to escape from the G_2/M arrest as efficiently as wild-type cells, more frequently persisting as large-budded cells). In contrast, when cells are shifted to the nonpermissive temperature, released from the nocodazole block, and allowed to progress through mitosis, we find that all strains bearing temperature-sensitive alleles of *SIR2* have lost silencing. The total

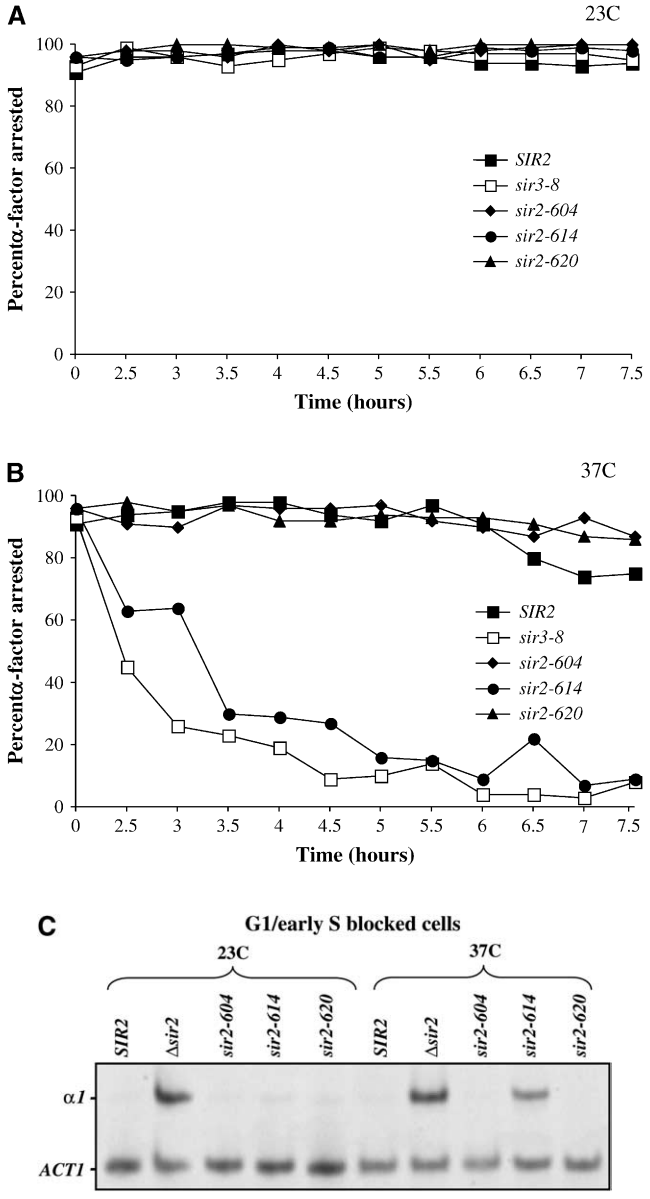

FIGURE 2.—Sir2 function in G_1 phase. (A and B) *SIR2* alleles with cell-cycle-specific silencing defects. Cells grown to log phase at 23° were arrested with α-factor (10 μg/ml) in G_1 phase. When at least 95% of cells exhibited an unbudded morphology, each culture was divided. One half was maintained at 23°, while the other was shifted to 37°. Silencing at *HML* was assessed by the bud morphology of the culture; unsilenced cells failed to arrest and form buds. The percentage of unbudded cells (α-factor-arrested cells) is plotted *vs.* time measured from the division of the culture. *HML* is efficiently silenced in all cultures maintained at 23° (Figure 2A). Silencing is lost in G_1 at the nonpermissive temperature (37°) only in strains bearing the *sir2-614* allele or the *sir3-8* allele (Figure 2B). (C) *α1* mRNA levels in G_1-arrested strains. Cells grown to log phase at 23° were arrested with α-factor (10 μg/ml) in G_1 phase. When at least 95% of cells exhibited an unbudded morphology, hydroxyurea (20 mg/ml) was added and each culture was divided. One-half was maintained at 23°, while the other was shifted to 37°. Following 4 hr of incubation, RNA was collected and RT–PCR was used to assay *α1* and *ACT1* message levels. RNA was analyzed from a wild-type strain, a strain lacking the *SIR2* gene, and strains bearing the indicated alleles of *SIR2*.

amount of time at the nonpermissive temperature is similar in the G_1 block and mitosis interval experiments, indicating that cells bearing the *sir2-604* and *sir2-620* alleles are more sensitive to a shift to the nonpermissive temperature in mitosis than in G_1 phase. Thus, the differences in the alleles cannot be attributed solely to a difference in the time that it takes to manifest their temperature-sensitive phenotypes.

We next used RT–PCR measurements to more narrowly define the point at which silencing is lost in the mitotic interval. First, we blocked cells in G_2/M with nocodazole at the permissive temperature and then shifted the culture to the nonpermissive temperature for 5 hr. RNA was collected and analyzed for expression of *α1* message. Figure 3A shows that the strain bearing the *sir2-604* allele remains substantially repressed despite the temperature shift, while strains bearing the *sir2-614* and *sir2-620* alleles are derepressed at *HML*. Finally, we measured *α1* message in cells progressing from the G_2/M boundary to early S phase. Cells were blocked with nocodazole, shifted to the nonpermissive temperature, and then released from the nocodazole block into media containing hydroxyurea. Figure 3B shows that all strains bearing conditional *SIR2* alleles lose repression of *HML* when traversing this interval at the nonpermissive temperature.

Sir2–Sir4 interactions: These results suggest that the mutations conferring the temperature-sensitive phenotype define distinct functions for Sir2. Silencing is sensitive to disruptions in the function defined by the *sir2-614* allele at all points in the cell cycle that we have tested, while silencing is preferentially sensitive to disruptions in the function or functions defined by the *sir2-604* and *sir2-620* alleles during metaphase and mitosis. To determine the specific functions compromised in the Sir2 proteins produced from these alleles, we examined two known functions of Sir2 likely to be essential for silencing at *HML*: the ability to interact with Sir4 (MOAZED *et al.* 1997; COCKELL *et al.* 2000) and the ability to deacetylate histone substrates (IMAI *et al.* 2000; LANDRY *et al.* 2000b; SMITH *et al.* 2000).

To examine Sir2's ability to interact with Sir4, we performed two-hybrid assays (TOBY and GOLEMIS 2001). For bait we used the C-terminal 629 amino acids of Sir4 fused to the DNA-binding domain of the Gal4 protein. Prior studies found that truncations of Sir4's N terminus increased the ability to detect interactions with Sir2 (MOAZED *et al.* 1997; CHANG *et al.* 2003). The two-hybrid reporter strain that we used includes a *HIS3* reporter gene with binding sites for the Gal4 DNA-binding domain in its promoter (JAMES *et al.* 1996). Interaction of the Sir4-binding domain fusion protein with a Sir2–Gal4 activation domain fusion protein allows expression of the *HIS3* gene, permitting growth on media lacking histidine. To determine whether the Sir2–Gal4AD fusion proteins used in this study retained their silencing function, we performed the control experiment shown

TABLE 4

Sir2 function is required in mitosis

	23° → 23°				23° → 37°			
SIR2 allele	Small budded	Large budded	Unbudded	Shmoo	Small budded	Large budded	Unbudded	Shmoo
SIR2	0	2	98	92	1	5	94	80
sir2-604	10	14	76	66	52	11	37	8
sir2-614	16	25	59	50	59	24	17	0
sir2-620	3	9	88	80	60	29	11	1

Cultures bearing the indicated *SIR2* alleles were grown to log phase at 23° and then blocked in G_2/M phase with nocodazole (15 μg/ml). When >90% of cells exhibited large buds, the cultures were divided. Half was maintained at 23° for 1 hr and then released from the nocodazole block into media containing α-factor and incubated at 23° (23° → 23° columns). The other half was shifted to 37° for 1 hr and then released into media containing α-factor and incubated at 37° (23° → 37° columns). Budding morphology was monitored at various times and is reported here 4 hr after release from nocodazole. Values shown are the percentage of cells in each morphological class; shmoos are a subset of unbudded cells.

in Figure 4A. Plasmids expressing fusion proteins were introduced into a strain lacking the wild-type *SIR2* gene and a mating assay was performed. In each case, the activation domain fusions recapitulated the temperature-sensitive silencing phenotypes seen with the unfused *SIR2* alleles.

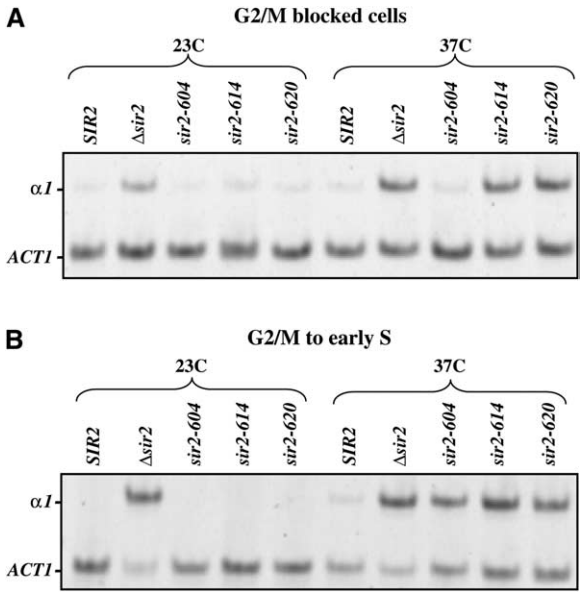

FIGURE 3.—Sir2 function during mitosis. (A) α*1* mRNA levels in nocodazole-arrested strains. Log-phase cultures grown at 23° were blocked at the G_2/M boundary with nocodazole. Each culture was then divided; half was maintained at 23°, and the other half was shifted to 37°. After maintaining cultures at their respective temperatures for 5 hr, RNA was collected and RT–PCR was used to assay α*1* and *ACT1* message, as described in MATERIALS AND METHODS. (B) α*1* mRNA levels in strains progressing from G_2/M to early S phase. Log-phase cultures grown at 23° were blocked in G_2/M with nocodazole. Each culture was then divided; half was maintained at 23°, and the other half was shifted to 37°. Cultures were retained at the nocodazole block for 4 hr and then released at their respective temperatures into media containing hydroxyurea. Eight hours following release from the nocodazole block, RNA was collected and RT–PCR was used to assay α*1* and *ACT1* message.

To decrease the possibility of observing signals dependent on indirect interactions between the fusion proteins and endogenous Sir proteins, we initially performed our assay in a strain deleted for the endogenous *SIR2*, *SIR3*, and *SIR4* genes. Assays were performed at 23°, 30°, and 37° to detect temperature-dependent effects. As shown in Figure 4B, at 23° and 30° all Sir2 proteins tested were able to interact with Sir4, while at 37° the sir2-604 protein has a significantly reduced interaction with Sir4. We also performed this assay in a strain containing the wild-type *SIR2* gene. In this experiment, we observed that both the sir2-604 and the sir2-620 protein are unable to interact with the Sir4–BD fusion protein at the nonpermissive temperature, while the sir2-614 protein shows a milder defect (Figure 4C). Competition between the wild-type and Sir2–AD fusion proteins in the *SIR2*$^+$ strain may have helped to reveal defects in the ability of sir2-620 and sir2-614 to interact with Sir4. Differences in the two strains could also be explained by more complex models invoking interactions between wild-type and mutant Sir2 proteins (CUBIZOLLES *et al.* 2006).

Deacetylase activity of Sir2 mutants: Silencing likely requires a Sir2-mediated deacetylation of histones at silenced locations (IMAI *et al.* 2000; HOPPE *et al.* 2002; LUO *et al.* 2002; MATECIC *et al.* 2002; RUSCHE *et al.* 2002). Removal of acetyl groups from lysines by Sir2 is coupled to the hydrolysis of NAD (LANDRY *et al.* 2000a; TANNER *et al.* 2000; TANNY and MOAZED 2001). To examine whether Sir2 enzymatic activity was altered by these *SIR2* alleles, we measured its ability to hydrolyze NAD in the presence of a peptide mimicking an acetylated histone tail. Sir2-containing protein complexes were purified from yeast and then assayed for NADase activity (TANNY *et al.* 2004 and MATERIALS AND METHODS). Even at the permissive temperature, each of the alleles has reduced activity compared to wild-type Sir2 (Table 5). At the

nonpermissive temperature, the activity of each allele is further reduced; this decrease is most pronounced for the *sir2-614* allele. Thus, while these assays do not allow an unambiguous assignment of the specific defect causing the loss of silencing in strains bearing these *SIR2* alleles, they do distinguish the alleles in a way that correlates with their phenotypes: one class of alleles, represented by the *sir2-614* allele, is particularly defective in enzymatic activity, and silencing is lost at all cell-cycle positions tested. A second class, composed of the *sir2-604* and *sir2-620* alleles, has primary defects in Sir2–Sir4 interactions and exhibits a cell-cycle-specific defect in silencing.

Complementation of *SIR2* alleles: In some cases, distinct alleles of the same gene are able to complement each other. For instance, coexpression of two distinct, nonfunctional alleles of *SIR4* restores silencing to cells lacking the *SIR4* gene (Marshall *et al.* 1987). The observation of transcomplementation can suggest distinct functional domains in the protein. To test the ability of our *SIR2* alleles to complement each other, plasmids bearing *SIR2* alleles were introduced into strains containing integrated alleles of *SIR2* (Figure 5). We used a mating assay to assess silencing at *HML*. As shown in Figure 5, these plasmid-borne alleles support mating at 23°, but not at 37°, in a strain lacking a chromosomal copy of *SIR2*. The remaining experiments show that (1) each of the temperature-sensitive alleles is recessive to

TABLE 5

NADase activity of Sir2 proteins

Sir2 protein	23°	37°
sir2-604	55 ± 29	29 ± 13
sir2-614	60 ± 22	6 ± 3
sir2-620	60 ± 39	23 ± 6
sir2-H364Y	3 ± 2	1.6 ± 1.5

The ability of the indicated Sir2 proteins to hydrolyze NAD in the presence of a substrate bearing an acetylated lysine was measured. Values are expressed as a percentage of a wild-type control purified and tested at the same time. The sir2-H364Y protein has been previously shown to have minimal activity in *in vitro* deacetylase assays (Tanny *et al.* 1999; Imai *et al.* 2000). In these assays, wild-type Sir2 had ~5% higher activity at 37° *vs.* 23°.

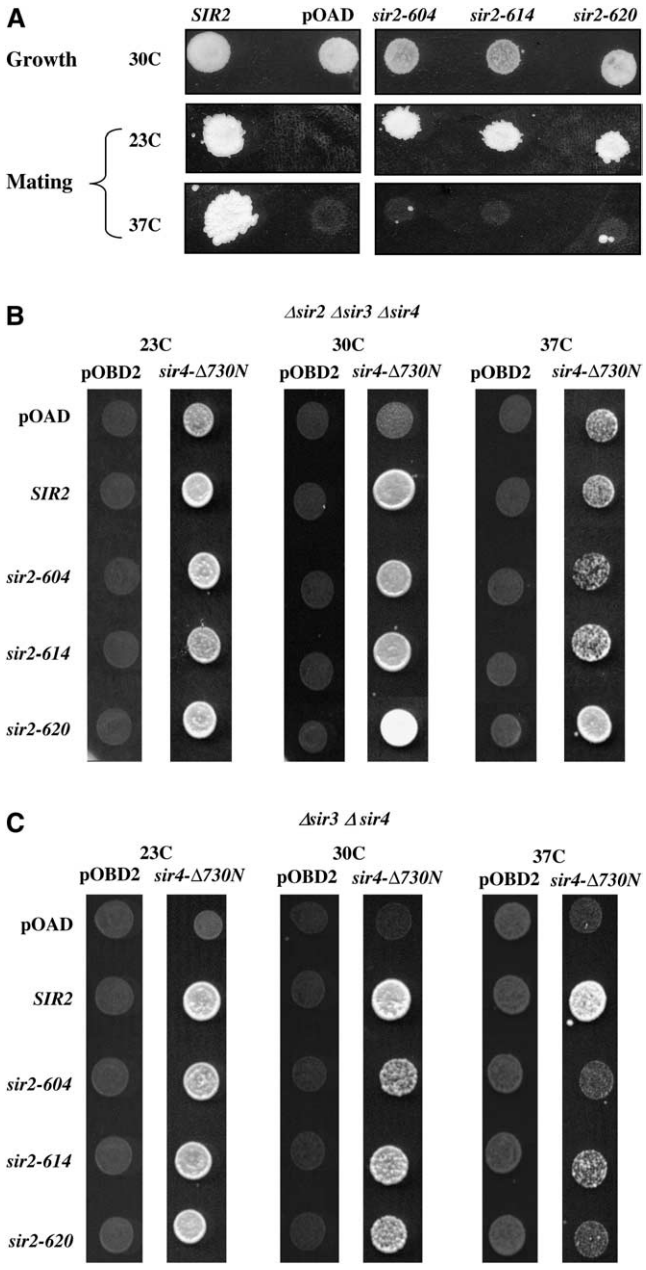

Figure 4.—Two-hybrid assays of Sir2–Sir4 interactions. (A) Sir2 fusion proteins maintain function. A mating assay was performed on strains expressing Sir2 proteins fused at their N terminus to the Gal4 activation domain. An equal number of cells from each culture were spotted onto plates spread with a *MAT*α haploid strain. These plates were incubated overnight at 23° or 37° to allow mating, replica plated to media selecting for diploids, and then incubated an additional night at their respective temperature, when the photos shown were taken. "Growth" shows the same haploid cultures spotted onto nonselective plates lacking a mating partner. Equivalent growth controls were done at 23° and 37° with identical results. pOAD is the base vector expressing only the Gal4-activation domain. (B) Sir2–Sir4 interactions. Each row is labeled with the activation domain fusion used; pOAD is the vector control expressing only the Gal4-activation domain. Each column lists the binding domain fusion used; pOBD2 is the vector control expressing only the Gal4-binding domain. Cultures were grown and plated at the temperatures indicated. This experiment was conducted in a strain lacking the endogenous *SIR2*, *SIR3*, and *SIR4* genes. Equal numbers of cells were applied to media selecting for expression of a *HIS3* reporter gene containing Gal4-binding sites in its promoter. Duplicate platings on media selecting only for the plasmids bearing the binding domain and activation domain fusions ensured that platings were equal (not shown). The Sir4–Δ730N binding domain fusion lacks the N-terminal 730 amino acids of Sir4. (C) An experiment was conducted exactly as described in B in a strain lacking the *SIR3* and *SIR4* genes.

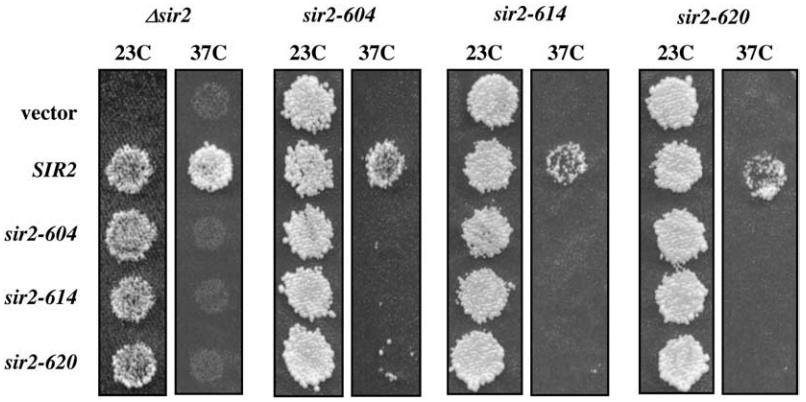

FIGURE 5.—Complementation of *SIR2* alleles. Plasmids bearing the *SIR2* allele listed at the left were introduced into strains bearing integrated alleles of the *SIR2* alleles listed at the top. Mating assays were performed at 23° and 37° as described in the Figure 4 legend.

wild type, (2) increasing the dosage of individual alleles does not significantly affect silencing by this assay, and (3) these sir2 temperature-sensitive alleles do not complement each other. This could mean that the mutations do not affect distinct functions of the protein or that independent functions must reside in a single molecule of Sir2 to promote silencing.

Strains bearing these *SIR2* alleles exhibit cell-cycle silencing defects. To examine the possibility that Sir2 levels exhibit cell-cycle dependent changes in stability, we performed Western blots on extracts from cells blocked at G_1 or G_2/M, at permissive and nonpermissive temperatures (Figure 1B). We found that the levels of each temperature-sensitive protein decreases relative to the wild-type Sir2 protein. However, the level of individual proteins is not affected by temperature, and the ability to promote silencing does not correlate with Sir2 protein levels within the range that we observed. Coupled with our observation that increases in gene dosage do not affect the temperature-sensitive phenotype, we consider it unlikely that alterations in protein levels are responsible for the phenotypes that we see.

Sir2 and the establishment of silencing: In all contexts reported thus far, the establishment of silencing requires progression through the cell cycle. Our experiments using conditional *SIR2* alleles suggest that silencing is particularly sensitive to decreases in the ability of the Sir2 and Sir4 proteins to interact during mitosis, possibly indicating a crucial assembly step for silent chromatin at this point of the cell cycle. To determine if silencing can be established in this interval, we performed a pedigree assay on a yeast strain bearing the *sir2-614* allele (Figure 6). Cells grown at the permissive temperature were blocked at the G_2/M boundary with nocodazole, subjected to a temperature shift to inactivate Sir2, and then applied to solid media containing α-factor. G_2/M-blocked cells were then monitored as they resumed progress through the cell cycle. If cells restore silencing in this interval, they will be sensitive to α-factor, blocked in the subsequent G_1 phase of the cell cycle, and eventually adopt a shmoo morphology. Unsilenced cells will be insensitive to α-factor and enter a new cell cycle. Control cells maintained at the permissive temperature throughout the experiment efficiently blocked in α-factor following release from the G_2/M block (87% shmoo; see Figure 6), while cells that were maintained at the nonpermissive temperature following release from G_2/M showed little response to α-factor (7% shmoo). When cells blocked at G_2/M were shifted to the nonpermissive temperature for 3 hr, shifted back to the permissive temperature for 2 hr, and then applied to media containing α-factor, a significant fraction were sensitive to α-factor (66% shmoo). We can compare this number to cells that were shifted to the nonpermissive temperature for 3 hr and then directly transferred out of the nocodazole-containing media to a plate containing α-factor (40% shmoo). Although the pedigree experiment does not allow us to

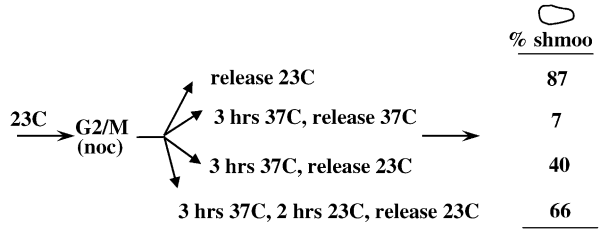

FIGURE 6.—Establishment of silencing in M phase. A strain bearing the *sir2-614* allele was grown at 23° and blocked at G_2/M with nocodazole. After >90% of the cells in the culture exhibited a large-bud morphology, the culture was divided and subjected to the indicated temperature shifts. At the release point, cells were applied to solid media containing α-factor. Released from the nocodazole-induced block, large-budded cells continued through the cell cycle and were either sensitive to α-factor, forming shmoos, or not sensitive to α-factor, forming buds (cells that neither budded nor formed shmoos, always <10% of the total, were not counted). The percentage of large-budded cells in which at least one of the cell–cell pair exhibited sensitivity to α-factor by forming a shmoo is indicated. Data shown are the cumulative results of two independent experiments. At least 90 large-budded cells were assayed for each condition. The difference between 40 and 66% is significant (χ^2, $P < 0.001$), given the number of events assayed for each condition.

measure the fraction of cells that were unsilenced when released from the nocodazole block, comparison of the last two conditions indicates that significant repression (χ^2, $P < 0.001$) is established when cells are permitted more time at the permissive temperature in this interval.

DISCUSSION

Sir2 and mitosis: Analysis of our conditional alleles of *SIR2* suggests a cell-cycle-dependent requirement for Sir2 function. When shifted to the nonpermissive temperature, cells bearing the *sir2-614* allele lose silencing in G_1 phase and during mitosis. We observed a distinct phenotype when analyzing the *sir2-604* and *sir2-620* alleles. Strains bearing these alleles did not lose silencing when raised to the nonpermissive temperature in G_1 phase, but did during progression from the G_2/M boundary to G_1. There are two general possibilities for the different phenotypes exhibited by these alleles. First, mutations in *SIR2* could affect a single function of the protein, with the *sir-614* allele having a more pronounced effect at high temperature than the *sir-604* or *sir-620* mutations. In this model, cells in mitosis are more sensitive to this deficit, and sir2-614p is more deficient in this function than sir2-604p or sir2-620p. However, if the copy number of the *SIR2* temperature-sensitive alleles is increased in haploid strains, there is no change in mating behavior, suggesting that the silencing defects are not simply due to dosage effects. Alternatively, the mutations could affect independent functions of Sir2, with the *sir2-604* and *sir2-620* alleles defining a cell-cycle-specific function.

In our conditional alleles of *SIR2*, the mutations that are sufficient to confer the temperature-sensitive mating defect cause alterations of the conserved region of Sir2 known to be sufficient for enzymatic function, within the carboxy-terminal region required for interacting with Sir4, or both (see Table 2). The *sir2-614* and *sir2-620* alleles introduce amino acid substitutions within the enzymatic core, while the mutations in *sir2-604* and *sir2-620* introduce a premature stop codon resulting in the deletion of 30 or 24 amino acids, respectively, from the carboxy terminus. None of these mutations alter amino acids highly conserved in the Sir2 family. However, this C-terminal region has been shown to be important for Sir2's interaction with Sir4 (Cockell *et al.* 2000). Our experiments suggest that the Sir2-614 protein has a temperature-dependent defect in enzymatic activity. This result is consistent with the prior observation that a small molecule inhibitor of Sir2's catalytic activity causes loss of silencing in log-phase cells or in cells arrested in G_1 phase (Bedalov *et al.* 2001). While we cannot rule out the possibility that the Sir2-614 protein has defects in addition to impaired enzymatic function, these results are consistent with a requirement for Sir2's enzymatic activity throughout the cell cycle. In cycling cells, the levels of the sir2-614 protein appear to increase at the nonpermissive temperature (Figure 1B). A recent report indicates that Sir2 levels are subject to autoregulation (Michel *et al.* 2005); our results suggest that Sir2's enzymatic activity may be required for feedback inhibition.

The Sir2-604 and Sir2-620 proteins exhibit a decreased ability to interact with Sir4 and also a more minor impairment in enzymatic activity. Again, while these alleles could have additional defects not yet revealed by our assays, these results may reflect a greater sensitivity to the Sir2–Sir4 interaction at specific points of the cell cycle, including mitosis. Interaction with Sir4 increases the catalytic activity of Sir2 (Tanny *et al.* 2004); thus, this decrease in Sir4 interaction could contribute to the decrease in catalytic activity that we measured in these proteins.

There is no evidence that silencing fails at any point in the cell cycle. However, our results suggest that the structure maintaining transcriptional repression is not static; decreasing the function of Sir2 has differential effects depending on cell-cycle position. A conclusion that silent chromatin is dynamic is supported by prior observations. First, expression of a transactivator is able to promote expression of a telomere-linked *URA3* gene when cells are blocked at the G_2/M boundary, but is unable to do so in cells blocked in G_1 phase (Aparicio and Gottschling 1994), suggesting that a transition in silencing occurs in mitosis. A silenced *HML* locus uncoupled from the *cis*-acting silencer sequences is derepressed specifically by passage through mitosis (Martins-Taylor *et al.* 2004), supporting the view that silent chromatin is particularly vulnerable to disruption at this point. Several studies have demonstrated that the establishment of silencing requires cell-cycle progression. The cell-cycle interval necessary for the establishment of silencing may depend upon the experimental system. In specific instances, S-phase passage has been shown to be necessary and sufficient to establish silencing (Miller and Nasmyth 1984; Fox *et al.* 1997; Kirchmaier and Rine 2001; Li *et al.* 2001; Kirchmaier and Rine 2006). However, establishment of silencing following inactivation and reactivation of a conditional *SIR3* allele occurs primarily in M phase (Lau *et al.* 2002; Martins-Taylor *et al.* 2004) while M-phase passage is necessary and sufficient to silence a telomere-linked gene following induced elevation of Sir3p levels (Martins-Taylor *et al.* 2004). Here we show that passage through the G_2/M–G_1 interval is sufficient to restore silencing in a strain bearing the *sir2-614* allele. Our identification of *SIR2* alleles with a mitosis-specific defect is consistent with a critical assembly step coinciding with mitosis.

In wild-type cells, silencing rarely fails and thus does not require frequent reestablishment. The significance of establishment experiments is in identifying intervals of dynamic reassembly or alteration of silent chromatin.

In particular, these experiments suggest that a transition from a tentatively silenced state to a more stable state occurs during mitosis. What is the crucial event in mitosis promoting the assembly of silent chromatin? The identification of the Scc1 cohesin as a modifier of silencing (Lau *et al.* 2002; Papacs *et al.* 2004; Suter *et al.* 2004) is consistent with a hypothesis that silencing is tied to the dynamic changes in chromosome structure that accompany cell-cycle progression.

While several proteins necessary for silencing have been identified and characterized, the specific mechanism of silencing and the physical state of chromatin sufficient to repress gene transcription are not clear. Detailed characterization of these dynamic transitions will likely continue to provide valuable insights into the silencing mechanism.

We thank members of the Holmes Lab for valuable discussions, Tania Rozario for assistance in data analysis, Katrina Catron for comments on the manuscript, and the University of Washington Yeast Resource Center for strains and plasmids. M.H. gratefully acknowledges support from a Howard Hughes Foundation Undergraduate Research Grant to Wesleyan University. This work was supported by grants from the American Cancer Society (RPG-98-351-01-MGO) and the National Science Foundation (MCB-0096561) to S.G.H.

LITERATURE CITED

Afshar, G., and J. P. Murnane, 1999 Characterization of a human gene with sequence homology to *Saccharomyces cerevisiae SIR2*. Gene **234:** 161–168.

Aparicio, O. M., and D. E. Gottschling, 1994 Overcoming telomeric silencing: a trans-activator competes to establish gene expression in a cell cycle-dependent way. Genes Dev. **8:** 1133–1146.

Bedalov, A., T. Gatbonton, W. P. Irvine, D. E. Gottschling and J. A. Simon, 2001 Identification of a small molecule inhibitor of Sir2p. Proc. Natl. Acad. Sci. USA **98:** 15113–15118.

Brachmann, C. B., J. M. Sherman, S. E. Devine, E. E. Cameron, L. Pillus *et al.*, 1995 The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. Genes Dev. **9:** 2888–2902.

Braunstein, M., A. B. Rose, S. G. Holmes, C. D. Allis and J. R. Broach, 1993 Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev. **7:** 592–604.

Braunstein, M., R. E. Sobel, C. D. Allis, B. M. Turner and J. R. Broach, 1996 Efficient transcriptional silencing in Saccharomyces cerevisiae requires a heterochromatin histone acetylation pattern. Mol. Cell. Biol. **16:** 4349–4356.

Bryk, M., S. D. Briggs, B. D. Strahl, M. J. Curcio, C. D. Allis *et al.*, 2002 Evidence that Set1, a factor required for methylation of histone H3, regulates rDNA silencing in S. cerevisiae by a Sir2-independent mechanism. Curr. Biol. **12:** 165–170.

Buck, S. W., J. J. Sandmeier and J. S. Smith, 2002 RNA polymerase I propagates unidirectional spreading of rDNA silent chromatin. Cell **111:** 1003–1014.

Carmen, A. A., L. Milne and M. Grunstein, 2002 Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. J. Biol. Chem. **277:** 4778–4781.

Chang, J. F., B. E. Hall, J. C. Tanny, D. Moazed, D. Filman *et al.*, 2003 Structure of the coiled-coil dimerization motif of Sir4 and its interaction with Sir3. Structure **11:** 637–649.

Chi, M.-H., and D. Shore, 1996 SUM1–1, a dominant suppressor of SIR mutations in Saccharomyces cerevisiae, increases transcriptional silencing at telomeres and HM mating-type loci and decreases chromosome stability. Mol. Cell. Biol. **16:** 4281–4294.

Cockell, M. M., S. Perrod and S. M. Gasser, 2000 Analysis of Sir2p domains required for rDNA and telomeric silencing in *Saccharomyces cerevisiae*. Genetics **154:** 1069–1083.

Cubizolles, F., F. Martino, S. Perrod and S. M. Gasser, 2006 A homotrimer-heterotrimer switch in Sir2 structure differentiates rDNA and telomeric silencing. Mol. Cell **21:** 825–836.

Dula, M. L., and S. G. Holmes, 2000 MGA2 and SPT23 are modifiers of transcriptional silencing in yeast. Genetics **156:** 933–941.

Fox, C., A. Ehrenhofer-Murray, S. Loo and J. Rine, 1997 The origin recognition complex, SIR1, and the S phase requirement for silencing. Science **276:** 1547–1551.

Frye, R. A., 2000 Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem. Biophys. Res. Commun. **273:** 793–798.

Goldstein, A. L., and J. H. McCusker, 1999 Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast **15:** 1541–1553.

Hecht, A., T. Laroche, S. Strahl-Bolsinger, S. M. Gasser and M. Grunstein, 1995 Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell **80:** 583–592.

Holmes, S. G., and J. R. Broach, 1996 Silencers are required for inheritance of the repressed state in yeast. Genes Dev. **10:** 1021–1032.

Hoppe, G. J., J. C. Tanny, A. D. Rudner, S. A. Gerber, S. Danaie *et al.*, 2002 Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. Mol. Cell. Biol. **22:** 4167–4180.

Huang, Y., 2002 Transcriptional silencing in Saccharomyces cerevisiae and Schizosaccharomyces pombe. Nucleic Acids Res. **30:** 1465–1482.

Imai, S., C. M. Armstrong, M. Kaeberlein and L. Guarente, 2000 Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature **403:** 795–800.

James, P., J. Halladay and E. A. Craig, 1996 Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144:** 1425–1436.

Kirchmaier, A. L., and J. Rine, 2001 DNA replication-independent silencing in S. cerevisiae. Science **291:** 646–650.

Kirchmaier, A. L., and J. Rine, 2006 Cell cycle requirements in assembling silent chromatin in Saccharomyces cerevisiae. Mol. Cell. Biol. **26:** 852–862.

Klar, A. J., J. N. Strathern, J. R. Broach and J. B. Hicks, 1981 Regulation of transcription in expressed and unexpressed mating type cassettes of yeast. Nature **289:** 239–244.

Landry, J., J. T. Slama and R. Sternglanz, 2000a Role of NAD(+) in the deacetylase activity of the SIR2-like proteins. Biochem. Biophys. Res. Commun. **278:** 685–690.

Landry, J., A. Sutton, S. T. Tafrov, R. C. Heller, J. Stebbins *et al.*, 2000b The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc. Natl. Acad. Sci. USA **97:** 5807–5811.

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

Li, Y. C., T. H. Cheng and M. R. Gartenberg, 2001 Establishment of transcriptional silencing in the absence of DNA replication. Science **291:** 650–653.

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

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

Mahoney, D. J., and J. R. Broach, 1989 The *HML* mating-type cassette of *Saccharomyces cerevisiae* is regulated by two separate but functionally equivalent silencers. Mol. Cell. Biol. **9:** 4621–4630.

Marshall, M., D. Mahoney, A. Rose, J. B. Hicks and J. R. Broach, 1987 Functional domains of SIR4, a gene required for position effect regulation in Saccharomyces cerevisiae. Mol. Cell. Biol. **7:** 4441–4452.

Martins-Taylor, K., M. L. Dula and S. G. Holmes, 2004 Heterochromatin spreading at yeast telomeres occurs in M phase. Genetics **168:** 65–75.

Matecic, M., S. Stuart and S. G. Holmes, 2002 SIR2-induced inviability is suppressed by histone H4 overexpression. Genetics **162:** 973–976.

Michel, A. H., B. Kornmann, K. Dubrana and D. Shore, 2005 Spontaneous rDNA copy number variation modulates Sir2 levels and epigenetic gene silencing. Genes Dev. **19:** 1199–1210.

Miller, A., and K. Nasmyth, 1984 Role of DNA replication in the repression of silent mating type loci in yeast. Nature **312:** 247–251.

Moazed, D., 2001 Enzymatic activities of Sir2 and chromatin silencing. Curr. Opin. Cell Biol. **13:** 232–238.

Moazed, D., A. Kistler, A. Axelrod, J. Rine and A. Johnson, 1997 Silent information regulator protein complexes in Saccharomyces cerevisiae: a SIR2/SIR4 complex and evidence for a regulatory domain in SIR4 that inhibits its interaction with SIR3. Proc. Natl. Acad. Sci. USA **94:** 2186–2191.

Moretti, P., K. Freeman, L. Coodly and D. Shore, 1994 Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. Genes Dev. **8:** 2257–2269.

Muhlrad, D., R. Hunter and R. Parker, 1992 A rapid method for localized mutagenesis of yeast genes. Yeast **8:** 79–82.

Nasmyth, K. A., K. Tatchell, B. D. Hall, C. Astell and M. Smith, 1981 A position effect in the control of transcription at yeast mating type loci. Nature **289:** 244–250.

Papacs, L. A., Y. Sun, E. L. Anderson, J. Sun and S. G. Holmes, 2004 REP3-mediated silencing in *Saccharomyces cerevisiae*. Genetics **166:** 79–87.

Puig, O., F. Caspary, G. Rigaut, B. Rutz, E. Bouveret *et al.*, 2001 The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods **24:** 218–229.

Rigaut, G., A. Shevchenko, B. Rutz, M. Wilm, M. Mann *et al.*, 1999 A generic protein purification method for protein complex characterization and proteome exploration. Nat. Biotechnol. **17:** 1030–1032.

Rusche, L. N., A. L. Kirchmaier and J. Rine, 2002 Ordered nucleation and spreading of silenced chromatin in Saccharomyces cerevisiae. Mol. Biol. Cell **13:** 2207–2222.

Rusche, L. N., A. L. Kirchmaier and J. Rine, 2003 The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu. Rev. Biochem. **27:** 481–516.

Shou, W., J. H. Seol, A. Shevchenko, C. Baskerville, D. Moazed *et al.*, 1999 Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell **97:** 233–244.

Sikorski, R., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Siliciano, P. G., and K. Tatchell, 1984 Transcription and regulatory signals at the mating type locus in yeast. Cell **37:** 969–978.

Smith, J. S., C. B. Brachmann, I. Celic, M. A. Kenna, S. Muhammad *et al.*, 2000 A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc. Natl. Acad. Sci. USA **97:** 6658–6663.

Smith, J. S., J. Avalos, I. Celic, S. Muhammad, C. Wolberger *et al.*, 2002 SIR2 family of NAD(+)-dependent protein deacetylases. Methods Enzymol. **353:** 282–300.

Stone, E. M., M. J. Swanson, A. M. Romeo, J. B. Hicks and R. Sternglanz, 1991 The *SIR1* gene of *Saccharomyces cerevisiae* and its role as an extragenic suppressor of several mating-defective mutants. Mol. Cell. Biol. **11:** 2253–2262.

Straight, A. F., W. Shou, G. J. Dowd, C. W. Turck, R. J. Deshaies *et al.*, 1999 Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. Cell **97:** 245–256.

Suter, B., A. Tong, M. Chang, L. Yu, G. W. Brown *et al.*, 2004 The origin recognition complex links replication, sister chromatid cohesion and transcriptional silencing in *Saccharomyces cerevisiae*. Genetics **167:** 579–591.

Tanner, K. G., J. Landry, R. Sternglanz and J. M. Denu, 2000 Silent information regulator 2 family of NAD-dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. Proc. Natl. Acad. Sci. USA **97:** 14178–14182.

Tanny, J. C., and D. Moazed, 2001 Coupling of histone deacetylation to NAD breakdown by the yeast silencing protein Sir2: evidence for acetyl transfer from substrate to an NAD breakdown product. Proc. Natl. Acad. Sci. USA **98:** 415–420.

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

Tanny, J. C., D. S. Kirkpatrick, S. A. Gerber, S. P. Gygi and D. Moazed, 2004 Budding yeast silencing complexes and regulation of Sir2 activity by protein-protein interactions. Mol. Cell. Biol. **24:** 6931–6946.

Toby, G. G., and E. A. Golemis, 2001 Using the yeast interaction trap and other two-hybrid-based approaches to study protein-protein interactions. Methods **24:** 201–217.

Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson *et al.*, 2000 A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature **403:** 623–627.

Wyrick, J. J., F. C. P. Holstege, E. G. Jennings, H. C. Causton, D. Shore *et al.*, 1999 Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast. Nature **402:** 418–421.

Communicating editor: M. Johnston