Yeast heterochromatin is a dynamic structure that requires silencers continuously

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Transcriptional silencing of the *HM* loci in yeast requires *cis*-acting elements, termed silencers, that function during S-phase passage to establish the silent state. To study the role of the regulatory elements in maintenance of repression, site-specific recombination was used to uncouple preassembled silent chromatin fragments from silencers. DNA rings excised from *HMR* were initially silent but ultimately reactivated, even in G_1- or G_2/M-arrested cells. In contrast, DNA rings bearing *HML*-derived sequence were stably repressed due to the presence of a protosilencing element. These data show that silencers (or protosilencers) are required continuously for maintenance of silent chromatin. Reactivation of unstably repressed rings was blocked by overexpression of silencing proteins Sir3p and Sir4p, and chromatin immunoprecipitation studies showed that overexpressed Sir3p was incorporated into silent chromatin. Importantly, the protein was incorporated even when expressed outside of S phase, during G_1 arrest. That silencing factors can associate with and stabilize preassembled silent chromatin in non-S-phase cells demonstrates that heterochromatin in yeast is dynamic.

[*Key Words*: Transcriptional repression; silencers; *Saccharomyces cerevisiae*; mating-type locus; Sir proteins; heterochromatin]

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Large regions of eukaryotic chromosomes are transcriptionally quiescent due to the packaging of DNA in repressive chromatin structures that are heritably propagated from one generation to the next. Heterochromatin, a constitutively condensed form of inactive chromatin, represents a primary example (Elgin 1996). Although heterochromatic regions are generally gene poor, heritable inactivation of either one of the two X chromosomes in female mammals involves formation of heterochromatin over the entire chromosome (Lee and Jaenisch 1997). In addition, chromosomal translocations that reposition active euchromatic genes near heterochromatin frequently result in a metastable form of repression that persists in subsequent generations.

Heritable inactivation of chromosomal domains in yeast *Saccharomyces* is typified by the *HM* loci (*HMR* and *HML*); in which endogenous copies of the mating-type genes are normally stored in a transcriptionally repressed state (Herskowitz et al. 1991). Inactivation of either *HMR* or *HML* involves a pair of flanking *cis*-acting regulatory sequences, referred to as the *E* and *I* silencers, that bind Rap1p, Abf1p, and the replication origin recognition complex (ORC) in various combinations (Loo and Rine 1995). An additional factor, Sir1p, is localized to silencers via protein–protein interactions (Triolo and Sternglanz 1996). Together, silencer-bound proteins recruit other Sir factors, Sir2p, Sir3p, and Sir4p, which associate with one another and histones throughout the repressed domain (Moretti et al. 1994; Hecht et al. 1995, 1996; Moazed and Johnson 1996; Strahl-Bolsinger et al. 1997). The resulting chromatin form, termed silent chromatin, bears many structural similarities to heterochromatin of higher eukaryotes. Both are assembled with hypoacetylated histones (Braunstein et al. 1993), both involve ORC as a DNA-binding component (Bell et al. 1993; Huang et al. 1998), and both are refractory to an array of DNA modification enzymes (Singh and Klar 1992; Loo and Rine 1994; Wallrath and Elgin 1995). This generalized chromatin inaccessibility, termed silencing in yeast, accounts for the block to transcription of both native and heterologous genes (Brand et al. 1985; Schnell and Rine 1986), as well as the diminished capacity for DNA repair (Terleth et al. 1989). Although heterochromatic structures are commonly assumed to be more or less static and inert, recent evidence suggests that cell cycle-dependent fluctuations occur. During mitotic chromosome condensation, much of the mouse heterochromatin protein HP1 is displaced from chromosomes (Murzina et al. 1999) and the accessibility of silenced yeast telomeric regions is increased in G_2/M-arrested cells (Aparicio and Gottschling 1994).

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A prevailing model for silencing has emerged in which repression consists of both establishment and maintenance phases. Accordingly, the establishment phase is one in which silent chromatin is reformed on nascent daughter duplexes following DNA replication. The maintenance phase, on the other hand, is one that sustains the silent state between successive establishment events. These concepts were first introduced by Miller and Nasmyth (1984) who used a conditional *sir3* allele to show that de novo establishment of the silent state occurred during S-phase passage exclusively. Subsequent genetic studies isolated mutations in *SIR1*, *RAP1*, and silencers that impaired establishment but not maintenance of silencing (Pillus and Rine 1989; Mahoney et al. 1991; Sussel et al. 1993). In such mutants genotypically identical cells displayed variegated silencing phenotypes: In some cells the *HM* loci were "off", whereas the loci were "on" in others. Switching between expression states occurred, albeit infrequently, indicating that the conditions that specify a particular state were reversible. The interpretation of this epigenetic behavior was that silencing could be maintained once established but that establishment in the mutants was an inefficient process. That all of these mutations were linked to silencers supported the notion that the elements were critical for establishment. Sir3p, on the other hand, was shown to be required continuously to maintain silencing; inactivation of the protein during any stage of the cell cycle led to immediate derepression (Miller and Nasmyth 1984). Therefore, Sir3p and other structural components that span the repressed domain (Sir2p and Sir4p) have come to be viewed as maintenance factors.

An unexpected role for silencers in maintenance of the silent state was first suggested by mutations in the ORC complex. Inactivation of conditional ORC subunits led to partial derepression of *HMR***a** in G_2/M-arrested cells, indicating that the silencer binding complex was required at times other than S phase for silencing (Fox et al. 1995). Complimentary data were obtained from more recent experiments in which preassembled silent chromatin domains were uncoupled from silencers by an inducible site-specific recombinase. Using this strategy, Holmes and Broach (1996) showed that removal of silencers from the chromosomal *HML*α led to reactivation within a single cell cycle. In reciprocal studies by the Broach laboratory and our own, focus was placed on extrachromosomal rings that were excised from silent loci. Though initially silent, the nonreplicating rings were also not able to maintain silencing in the absence of silencers (Bi and Broach 1997; Cheng et al. 1998). In the case of *HML*-derived rings, cell cycle progression between G_1 and G_2 was required for reactivation (Bi and Broach 1997). A general view that emerges from this work is that derepression in the absence of silencers is triggered by a cell cycle specific event.

Here we capitalize on the DNA ring excision approach to investigate the relationship between silencers and silent chromatin stability. We show that the elements function continuously in *cis* to maintain silent chromatin, even in G_1- and G_2/M-arrested cells. Furthermore, we show that silencing proteins can be recruited to silent chromatin during G_1 arrest. We propose that silencers act continuously throughout the cell cycle for this purpose. The experiments provide a unique and informative glimpse at the unexpected dynamic nature of silent chromatin.

Results

Maintenance of silencing at HMR**a** *requires silencers*

Previously, we found that DNA rings excised from *HMR* did not maintain silencing in logarithmically growing cells if the rings lacked silencers. Specifically, a ring-borne copy of the **a***1* gene was initially not expressed, yet it became fully activated within one to two doublings in cell density following excision. To determine whether reactivation required cell cycle progression, we mea-

Table 1. *Yeast strains*

W303-1A	*MAT***a** *HML*α *HMR***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
THC23	W303-1A *hmr::rHMR***a** *Δsir3::HIS3*
THC37	W303-1A *Δmat::URA3 hmr::rHMR***a** *+ EI*
THC42	W303-1A *Δmat::TRP1 hmr::rHMR***a** *Δbar1::hisG Δlys2 Δsir3::HIS3*
THC43	W303-1A *Δmat::TRP1 hmr::rHMR***a** *Δbar1::hisG Δlys2*
THC51	W303-1A *Δmat::TRP1 Δhmr::rURA3 Δbar1::hisG Δlys2 Δsir3::HIS3*
THC57	W303-1A *Δmat::TRP1 hmr::rHMR***a** *ura3-1::GAL10P–SIR3::URA3 Δbar1::hisG Δlys2*
THC59	W303-1A *Δmat::TRP1 Δhmr::rHMR*α *Δbar1::hisG Δlys2 Δsir3::HIS3*
THC62	W303-1A *Δmat::TRP1 Δhmr::rHMR*α *Δbar1::hisG Δlys2*
THC67	W303-1A *Δmat::TRP1 hmr::rHMR***a** *ura3-1::GAL10P–SIR3HA::URA3 Δbar1::hisG Δlys2*
THC68	W303-1A *Δmat::TRP1 Δhmr::rHMR*α*(rap1pm) Δbar1::hisG Δlys2 Δsir3::HIS3*
THC69	W303-1A *Δmat::TRP1 Δhmr::rHMR*α*(rap1pm)* Δbar1::hisG Δlys2
THC70	W303-1A *Δmat::TRP1 hmr::rHMR***a** *Δhml::kanMX ura3-1::GAL10P–SIR3HA::URA3 Δbar1::hisG Δlys2 Δsir3::HIS3*
THC74	W303-1A *URA3 HML*α*::URA3P–ADE2*
THC75	W303-1A *Δmat::TRP1 Δhmr::rHMR***a**/α *Δbar1::hisG Δlys2 Δsir3::HIS3*
THC76	W303-1A *URA3 hml(rap1pm)::URA3P–ADE2*
THC77	W303-1A *Δmat::TRP1 Δhmr::rHMR***a**/α *Δbar1::hisG Δlys2*
THC78	W303-1A *Δmat::TRP1 hmr::rHMR***a** *Δhml::kanMX Δbar1::hisG Δlys2 Δsir3::HIS3*
THC79	W303-1A *Δmat::URA3 hmr::rHMR***a** *+ EI Δhml::kanMX Δsir3::HIS3*

sured the persistence of silencing in rings in non-cycling cells. To this end, chromosomal fragments containing the **a** mating-type genes, either with or without silencers (Fig. 1), were excised from *HMR* in cultures that had been treated with α-factor mating pheromone. Greater than 98% of cells arrested at G_1 and remained there for the duration of the experiment, as confirmed by flow cytometry and a persistent unbudded shmoo morphology (data not shown). In the silencing-competent *Δmat* strains used here, no **a***1* transcript was detected prior to recombination (Cheng et al. 1998). However, in a strain containing a conditional *sir3* allele, fully derepressed **a***1* levels were observed within 60 min of a shift to nonpermissive conditions (data not shown). These preliminary tests indicated that **a***1* gene expression could serve as a sensitive and rapid indicator of loss of silencing.

Ring formation was initiated by galactose-induced expression of the R recombinase, and **a***1* mRNA levels were measured by Northern analysis at timed intervals thereafter. In a ring that lacked silencers, no **a***1* transcript was detected at the 60-min time point, indicating that the ring was initially repressed (Fig. 2A, lane 2). After 210 min, however, **a***1* mRNA began to accumulate, and by 360 min, the expression level approached that seen in a *sir3* strain (lanes 1,3,4). In contrast, the **a***1* transcript was never observed if the ring contained both the *E* and *I* silencers (lanes 6–8). These data indicate that silencers are required for maintenance of repression in G_1-arrested cells and that absence of the *cis*-acting elements results in deterioration of the repressed state in a time-dependent fashion.

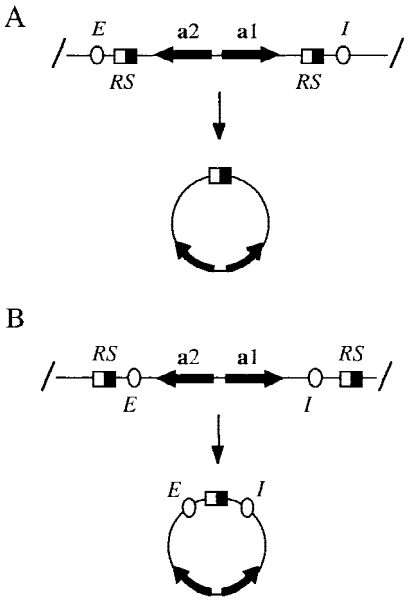

Figure 1. Excision of chromosomal fragments by site-specific recombination. *RS* target sites (half-filled boxes) for the *Zygosaccharomyces rouxii* R recombinase (Matsuzaki et al. 1990) were built into the *HMR* locus, either within the *E* and *I* silencers (*A*), or just beyond the *E* and *I* silencers (*B*) to produce rings that either lacked or contained the elements, respectively. In most laboratory strains, *HMR* contains the **a***1* and **a***2 mating-type genes (HMR***a**), whereas *HML* contains the α*1* and α*2* mating-type pair (*HML*α). A third mating-type locus, *MAT*, contains an active copy of either pair of mating-type genes. In the various experiments described herein, either **a** mating type sequences, α mating type sequences, or a composite of the two were positioned at *HMR* for subsequent excision. All excision cassettes are named according to the rings they produce (e.g., locus *hmr::rHMR***a** shown in *A* produces ring rHMR**a**).

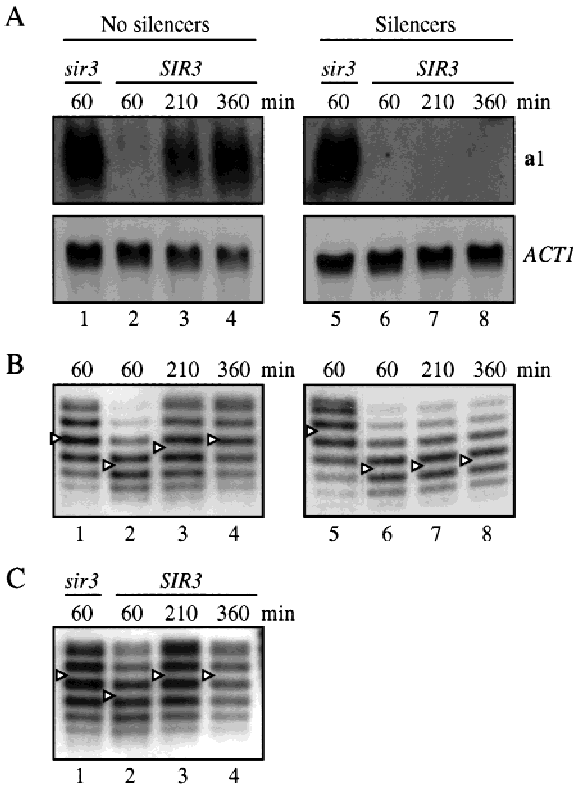

Figure 2. Maintenance of silencing requires silencers. DNA rings were excised from *HMR* by induction of recombination in cell cycle-arrested cultures. Strains THC78 *hmr::rHMR***a** *Δhml::kanMX Δsir3::HIS3* (lane *1*) and THC43 *hmr::rHMR***a** (lanes *2–4*) produced rings that lacked silencers. Strains THC79 *hmr::rHMR***a***+EI Δhml::kanMX Δsir3::HIS3* (lane *5*) and THC37 *hmr::rHMR***a***+EI* (lanes *6–8*) produced rings that contained silencers. *HML*α was deleted in the *sir3* strains; otherwise, expression of the locus would have prevented arrest by α-factor. *MAT***a** was deleted from all of the strains so that *HMR***a**-derived rings provided the sole source of **a***1* mRNA. In cells arrested with α-factor or nocodazole, nucleic acids were harvested at timed intervals following galactose addition. (*A*) Northern analysis of **a***1* gene silencing in cells arrested by α factor in G_1. Blots were hybridized sequentially with probes to **a***1* and the *ACT1* gene. When normalized to *ACT1*, the levels of **a***1* mRNA in lanes *1* and *4* are comparable. (*B*) Chloroquine gel electrophoresis of DNA rings from α-factor-arrested cells. Centers of the topoisomer distributions were determined by the Gaussian method and marked with an arrowhead for clarity. Changes in DNA supercoiling were attributable to changes in chromatin structure and not due to the mechanics of transcription because rings that lacked promoters also bore *SIR*-dependent DNA topology changes (Cheng et al. 1998). (*C*) Analysis of a ring lacking silencers from cells arrested at G_2/M with nocodazole.

The appearance of a ring-encoded **a***1* transcript suggested that changes in chromatin structure occur to relieve transcriptional repression following the removal of silencers. To test this idea more directly, we measured the level of supercoiling in excised DNA rings by electrophoresis in gels containing chloroquine. Previously, we showed that changes in the supercoiling of *HMR***a**-derived rings correlated closely with transitions between silent and nonsilent states (Cheng et al. 1998). This is recapitulated in Figure 2B with rings from α-factor-arrested cells. At the earliest time point following excision (60 min), a ring lacking silencers was more negatively supercoiled by one to two turns when isolated from a *SIR3* strain then when isolated from a *sir3* strain (lanes 1,2). However, by 210 min the supercoiling shift of the ring from the *SIR3* strain was slightly diminished, and by 360 min the supercoil density of the ring matched that of the ring from the *sir3* strain (lanes 1,3,4). In contrast, the *SIR*-dependent supercoiling shift of the ring containing silencers did not change during the course of the experiment (lanes 6–8). These results indicate that silencers are required continuously in G_1-arrested cells to maintain the alternate chromatin structure that is associated with transcriptional repression.

Cell cycle arrest in response to mating pheromones is mediated by a mitogen-activated protein (MAP) kinase pathway that triggers numerous physiological changes, including the hyperphosphorylation of Sir3 (Stone and Pillus 1996). Although activation of the pathway has been shown to strengthen telomeric silencing, repression of the extrachromosomal rings lacking silencers might be adversely affected. To test whether the persistence of silent chromatin structure was influenced by either α-factor treatment or G_1 arrest, we examined the level of supercoiling of DNA rings in cells that had been arrested at G_2/M with the microtubule destabilization agent nocodazole (Jacobs et al. 1988). Figure 2C shows that the supercoiling shift of a ring lacking silencers in nocodazole-arrested cells was similar to that in α-factor-arrested cells (cf. Fig. 2B). Sixty minutes after the induction of the recombinase, the ring was more negatively supercoiled when isolated from a *SIR3* strain than when isolated from a *sir3* mutant (lanes 1,2). At later time points, however, the altered supercoiling level of the ring reverted to that of the *sir3* strain (lanes 3,4). Changes in the Sir-dependent supercoiling shift of rings that possessed silencers were not observed under these conditions (data not shown). Therefore, the role of silencers in maintaining silent chromatin is not restricted to cells arrested in G_1 by α-factor. Rather, the results suggest that the *cis*-acting elements are required in a continuous manner throughout the cell cycle.

Maintenance of silencing at HMLα *in the absence of* E *and* I *silencers*

The observations described above are at apparent odds with those made by Holmes and Broach (1996), who showed that elimination of silencers from *HML*α in G_1-arrested cells did not disrupt silencing. The difference in persistence of transcriptional repression between *HML*α and *HMR***a** could be due to a host of factors, including long-range effects associated with either of the corresponding chromosomal domains or localized effects due to the specific sequences excised. To distinguish between these possibilities, we constructed a series of excision cassettes at *HMR* that contained sequences normally found at *HML*α. The *HML*-based fragments were excised in G_1-arrested cells, and persistence of the silent state was evaluated by measuring supercoil density of the resulting DNA rings. Figure 3 shows that the topoisomer distribution of a ring containing the α mating-type genes, but lacking silencers, remained constant during the 360-min experiment (lanes 2–4). At all times examined, the ring bore approximately one to two additional negative supercoils when isolated from a *SIR3* strain than it did when isolated from a *sir3* mutant. This result indicates that α genes, unlike **a** genes in Figure 2, maintain a silent chromatin structure upon excision and uncoupling from silencers at an *HM* locus.

Phenotypic evidence of persistent α gene repression was obtained to support the structural data. In *MAT***a** or *Δmat* haploid strains, α-factor treatment causes cells to arrest with an elongated shmoo morphology. If, however, the *HML*α locus is derepressed (e.g., by inactivation of a conditional *sir3* gene), cells adopt the **a**/α or α mating profile and rapidly escape α-factor-imposed arrest (Holmes and Broach 1996). Therefore, appearance of new buds in arrested cells can serve as a morphological marker for loss of silencing. A *Δmat* strain, THC62, was

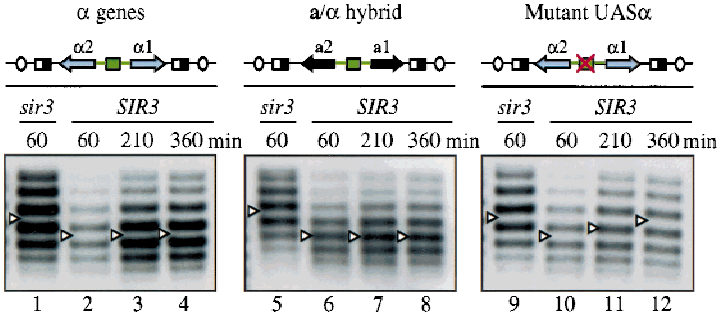

Figure 3. UASα maintains silencing in the absence of silencers. Following treatment with α-factor, DNA rings were formed in the following isogenic strain pairs: THC59 *Δhmr::rHMR*α *Δsir3::HIS3* (lane *1*), THC62 *Δhmr::rHMR*α (lanes *2–4*); THC75 *Δhmr::rHMR***a**/α *Δsir3::HIS3* (lane *5*), THC77 *Δhmr::rHMR***a**/α (lanes *6–8*); THC68 *Δhmr::rHMR*α(*rap1pm*) *Δsir3::HIS3* (lane *9*), THC69 *Δhmr::rHMR*α(*rap1pm*) (lanes *10–12*). Diagrams of the excision cassettes are shown above each panel with UASα depicted as a green box. DNA rings were examined as in Fig. 2. Asynchronous cultures of THC59, THC75, and THC68 were used as nonsilent control because these *sir3* strains contain *HML*α and do not respond to α-factor. However, the supercoiling of rings in *sir3* strains was influenced negligibly by α-factor arrest (data not shown).

treated with α-factor, and a DNA ring containing a copy of the repressed α genes was excised from *HMR*. Greater than 98% of the cells adopted the shmoo morphology. Strikingly, no new buds emerged during the 6 hr incubation period following galactose-induced ring formation, indicating that expression of ring-borne α genes did not occur (data not shown). Together, these results show that silencing of the α genes, unlike the **a** genes, persists in G_1-arrested cells in the absence of silencers.

A Rap1-binding site in the α gene UAS contributes to silencing at HML

The **a** and α mating-type loci are remarkably similar in primary sequence organization (Astell et al. 1981). The most notable difference is the Y region that spans the divergent **a***1*/**a***2* promoters and the **a***1* gene at *HMR***a** (Y**a**) versus the α*1*/α*2* promoters and the α*1* gene at *HML*α (Yα). To identify the sequence determinants that facilitate maintenance of silencing at *HML*, an HMR/HML hybrid excision cassette was constructed in which the divergent **a** gene promoters were replaced with a fragment containing the α gene promoters (Fig. 3, middle). When this hybrid ring was excised from *HMR* in G_1-arrested cells, the ring bore a *SIR*-dependent alteration in DNA supercoiling that did not change throughout the 360-min time course (lanes 6–8). This result indicates that a site within the α gene promoter region is sufficient for maintenance of preassembled silent chromatin.

We hypothesized that the α gene promoters recruited a protein that favored the persistence of silencing. A well-characterized binding site for the silencer binding protein Rap1p within the α gene UAS (UASα) represented a likely candidate. When the α genes are located at *MAT*, binding of the dual function protein to the site is required for expression (Giesman et al. 1991; Kurtz and Shore 1991). Footprinting studies have shown that a region encompassing the 15-bp UASα is not occluded by nucleosomes at *HML*, indicating that the site might be available to Rap1p, even within the silent chromatin (Weiss and Simpson 1998). To test whether the UASα contributes to silencing of the genes when they are located at an *HM* locus, we examined the supercoiling of a ring bearing a nonfunctional Rap1p-binding site. The central cytosine of the conserved CCC triplet of the Rap1 site was converted to an adenosine, a mutation that blocks transactivation of *MAT*α in vivo and prevents the binding of Rap1p in vitro (Vignais and Sentenac 1989; Giesman et al. 1991). Sixty minutes after excision the mutated and nonmutated rings produced similar *SIR*-dependent supercoiling shifts (Fig. 3, lanes 2,10). At later time points, however, the ring with the mutant Rap1p-binding site reverted back to the nonsilent state (cf. lanes 11 and 12 to lanes 3 and 4). Together, these results indicate that the Rap1p-binding site in UASα is both necessary and sufficient for maintenance of the repressed state in rings that lack silencers.

A sensitive genetic assay was used to test whether UASα contributes to silencing at the chromosomal *HML* locus. A chimeric reporter gene in which the *URA3* promoter was fused to the *ADE2* ORF was integrated between α2 and the *HMLE* silencer (Fig. 4). Cells that express *ADE2* give rise to white colonies on media containing low adenine, whereas cells that do not express the gene give rise to red colonies (Roman 1957). Cells containing *URA3P–ADE2* at *HML* produced uniformly red colonies (Fig. 4, left), indicating that the reporter gene was silenced. When the gene was integrated at a derivatized *HML* locus that contained a point mutation in the UASα Rap1 site, a mixture of derepressed white colonies, partially repressed pink colonies, and fully repressed red colonies was observed (Fig. 4, right). Some colonies were either predominantly red or white but contained small sectors of the opposite color, indicating that a stable switch between expression states occurred during colony formation. The data reveal that the Rap1p-binding site in the α gene promoters is a significant contributor to repression of the genes when they are located at *HML*.

Elevated levels of Sir3p and Sir4p stabilize rings of silent chromatin

Silencing of the *HM* loci is compromised when they are ectopically positioned far from telomeres where the Sir proteins are normally concentrated (Thompson et al. 1994; Maillet et al. 1996). The defect can be suppressed, however, by overexpressing a subset of the *SIR* genes. To test whether elevated *SIR* gene dosage would improve silencing in a ring that lacked silencers, a strain carrying the excision cassette shown in Figure 1A was transformed with *CEN* -based plasmids carrying *SIR1*, *SIR2*, *SIR3*, or *SIR4*. Maintenance of silencing was then measured in G_1-arrested cells. Figure 5A shows that *SIR-*

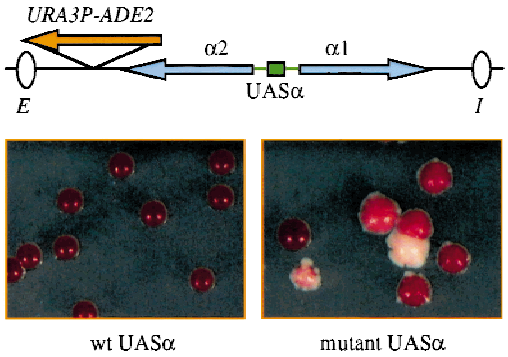

Figure 4. UASα contributes to silencing of the chromosomal *HML*α locus. Strains THC74 *hml::URA3P–ADE2* and THC76 *hml(rap1pm)::URA3P–ADE2* were plated on SC media containing minimal adenine after overnight growth on nonselective media. Red or pink pigmentation corresponds to full or partial repression of *ADE2*, respectively. Unpigmented (white) colonies correspond to complete *ADE2* derepression. Previously, the *URA3P–ADE2* construct was used to monitor transcriptional repression at *HMR***a** (Rivier et al. 1999). The *URA3* promoter is up-regulated when uracil is omitted from the growth media. On SC plates lacking uracil, more frequent derepression was observed in the UASα mutant strain (data not shown).

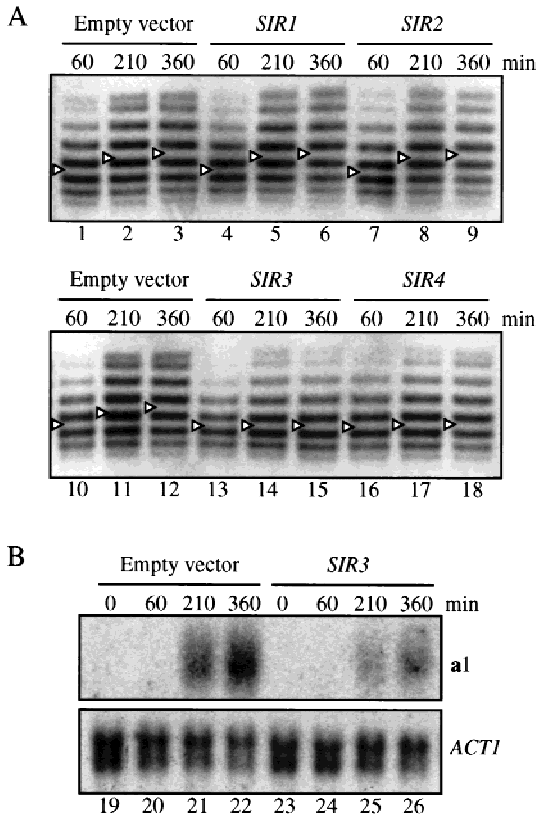

Figure 5. Stabilization of silent chromatin rings by elevated *SIR* gene dosage. Strain THC43 *hmr::rHMR***a** was transformed with single copy, *CEN*-based vectors pJR910 (*SIR1*, lanes *4–6*), pJR69 (*SIR2*, lanes *7–9*), pJR273 (*SIR3*, lanes *13–15*, *23–26*), pJR368 (*SIR4*, lanes *16–18*), or pRS416 (empty vector, lanes *1–3*, *10–12*, *19–22*). In each case, the gene of interest was transcribed from its own promoter. Supercoiling of DNA rings (*A*) and RNA levels (*B*) were examined as in Fig. 2.

dependent supercoiling shift of the ring did not persist in cells containing empty vector or an extra copy of *SIR1* or *SIR2* (lanes 1–9). In all three cases, the rings reverted to the level of supercoiling associated with the derepressed state. This result indicates that neither Sir1p nor Sir2p is limiting for maintenance of silencing under these conditions. In contrast, the silent state was stabilized by an extra copy of either *SIR3* or *SIR4* (lanes 13–18). Particularly in the case of elevated *SIR3* expression, supercoiling levels of the ring remained roughly constant throughout the duration of the experiment. Minor changes in the distribution of ring topoisomers probably reflect the presence of cells in the population that have lost the *SIR* expression vectors.

Northern analysis of the **a***1* transcript confirmed the structural data. Appearance of **a***1* mRNA following excision in strains containing an extra copy of *SIR3* was greatly reduced relative to strains that contained empty vector (Fig. 5B). These results indicate that elevated expression of the Sir3p and Sir4p structural components of the silent chromatin increases the persistence of the repressive structure, even in the absence of silencers.

Preassembled silent chromatin is stabilized by Sir3p overexpression in G_1

Stabilization of repressed rings by elevated Sir3p or Sir4p levels could occur during the establishment or maintenance phases of silencing. Conceptually, extra Sir proteins could facilitate the establishment of a more stable repressive structure. Alternatively, extra Sir proteins could add to and stabilize a pre-existing repressive structure. To determine whether elevated Sir3p increases the stability of preassembled silent chromatin, we induced expression of the protein in α-factor-treated cultures. Arrest with the pheromone in G_1 prevents cells from transiting through S phase, the period during which establishment of silencing is known to occur.

Induction of Sir3p was achieved with a chromosomally integrated *SIR3* gene fused to the *GAL10* promoter (*GAL10P–SIR3*). This tightly regulated construct provided functional Sir3p at nontoxic levels when induced (see Materials and Methods). Following uniform G_1 arrest, Sir3p and recombinase production were induced simultaneously by the addition of galactose. Recombination yielded the ring shown in Figure 1A. In a *SIR3* strain, production of additional Sir3p prevented loss of silencing of the ring (Fig. 6). The initial *SIR*-dependent DNA supercoiling level of the ring was maintained throughout the duration of the experiment (lanes 4–6). This result indicates that elevation of Sir3p levels can stabilize preassembled silent chromatin, even without passage through S phase.

To test whether non-S-phase expression of Sir3p could silence a locus that was initially derepressed, we induced *GAL10P–SIR3* in a *sir3* mutant strain. As before, cells were uniformly arrested in G_1 with α-factor and coproduction of recombinase and Sir3p was induced. Upon excision, the ring bore a nonsilent DNA topology (Fig. 6; cf. lanes 3 and 7) This condition was not changed by >6 hr of Sir3p expression (lanes 8,9). Therefore, Sir3p cannot impart silencing de novo to a derepressed locus in noncycling cells. Apparently, stabilization of silent chroma-

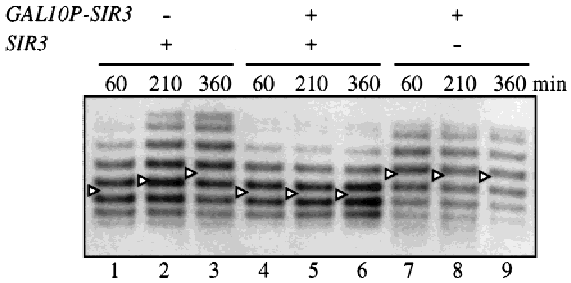

Figure 6. Stabilization of silent chromatin rings by elevated *SIR3* expression in G_1-arrested cells. Strains THC43 *hmr::rHMR***a** (lanes *1–3*), THC57 *hmr::rHMR***a** *ura3-1::GAL10P–SIR3::URA3* (lanes *4–6*), and THC70 *hmr::rHMR***a** *Δsir3::HIS3 ura3-1::GAL10P–SIR3HA::URA3* (lanes *7–9*) containing recombinase expression vector pHM153 were arrested with α-factor. Subsequently, expression of the *R* recombinase gene and *SIR3* were induced simultaneously with galactose. Supercoiling of DNA rings was examined as in Fig. 2.

tin by Sir3p in non-S-phase cells requires a pre-existing silent chromatin to be in place (lanes 4–6). The result is consistent with a requirement for passage through S phase to establish silencing (Miller and Nasmyth 1984; Fox et al. 1997). It seems likely that some specialized feature of chromatin assembly, such as histone deposition or modification, represents the critical S-phase event in the establishment of silent chromatin.

Association of Sir3p with silent chromatin does not require S-phase passage

Stabilization of the silent chromatin by Sir3p expression in non-S-phase cells could occur directly by incorporation of the protein into chromatin or by less direct means. To determine whether the protein was incorporated into preassembled silent chromatin structure, chromatin immunoprecipitation assays (ChImp assays) were performed with an induced, HA-tagged Sir3 derivative, Sir3HAp. Following uniform G_1 arrest, the epitope-tagged protein was expressed from a *GAL10P–SIR3HA* gene fusion. Immunoprecipitations were performed with anti-HA antibody and sonicated extract from formaldehyde cross-linked cells. Coimmunoprecipitation of representative genomic loci, including the **a***1* gene at *HMR***a**, as well as *GAL1*, *ACT1*, and *PHO5*, were evaluated by PCR using corresponding primer pairs. Figure 7 shows that **a***1* was selectively precipitated upon induction of *SIR3HA* in a *SIR3* strain (lane 3). Precipitation of the gene did not occur if Sir3HAp was not induced or if the epitope was removed from the expression vector (lanes 1,2). Finally, Sir3HAp was not incorporated into chromatin at **a***1* in a *sir3* strain (lane 4), in agreement with the DNA supercoiling data in Figure 6 (lanes 7–9). These data show that incorporation of Sir3p into the silent chromatin is not restricted to S phase. The results suggest that yeast heterochromatin is a dynamic structure that possess the ability to exchange chromatin-bound Sir3p for soluble unbound protein throughout the cell cycle.

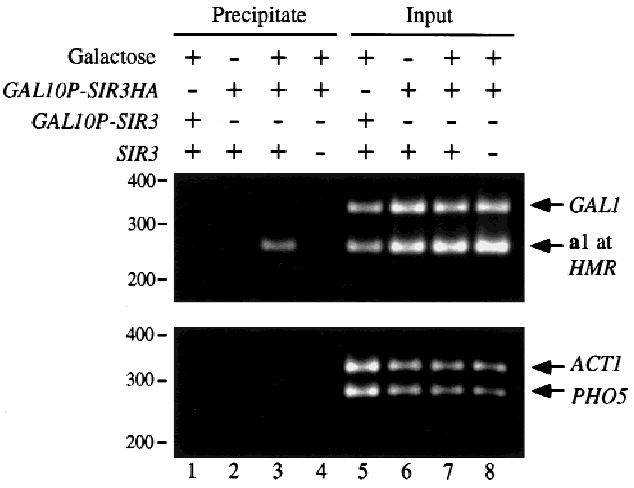

Figure 7. Incorporation of Sir3p into silent chromatin in G_1-arrested cells. Strains THC57 *ura3-1::GAL10P–SIR3::URA3* (lanes *1,5*), THC67 *ura3-1::GAL10P–SIR3HA::URA3* (lanes *2,3,6,7*), and THC70 Δ*sir3::HIS3 ura3-1::GAL10P–SIR3HA::URA3* (lanes *4,8*) were arrested with α-factor. Following induction of *SIR3* expression with galactose, cells were treated with formaldehyde. Processed samples were immunoprecipitated with anti-HA antibody, and the precipitated material was probed for the presence of DNA with PCR using primer pairs specific for the **a***1*, *GAL1*, *ACT1*, and *PHO5* genes. The *MAT***a** locus was deleted from all of these strains so that *HMR***a** provided the sole copy of **a***1* DNA. The positions of DNA mobility standards are marked at *left*.

Discussion

Silencers are required continuously to maintain silent chromatin

Heritable propagation of the stably repressed state at *HM* loci requires that (1) a heterochromatin-like structure be re-established following each round of DNA replication, and (2) that the structure be maintained throughout the duration of the cell cycle. A role for silencers in establishment of silencing is already well appreciated. Relocation of silencers to some ectopic loci can result in the silencing of adjacent genes (Lee and Gross 1993; Shei and Broach 1995; Maillet et al. 1996). In this report we have examined the role of silencers in the maintenance of the silent state and found that the elements contribute a critical function at this stage too. By unlinking fragments of preassembled silent chromatin from silencers in vivo, we have shown that silencers are required in *cis* to preserve silent chromatin; in G_1-arrested cells, removal of silencers resulted in reactivation of silenced genes and loss of *SIR*-dependent alterations in DNA supercoiling (Fig. 2). Similar observations were made in cells arrested at G_2/M using nocodazole. These findings demonstrate that silencers act in a continuous manner to maintain the structure and function of silent chromatin.

A protosilencer maintains silencing in the absence of silencers

The conclusion that silencers maintain silent chromatin is supported by our parallel studies of the **a** and α mating-type genes positioned at *HMR*. Unlike the situation with the **a** genes, uncoupling of the α gene pair from flanking silencers resulted in silent DNA rings that were stably repressed in arrested cells (Fig. 3). Persistent silencing in this case relied on a *cis*-acting silencing element located within the α gene promoter. By point mutation and add-back experiments we showed that the Rap1-binding site constituting UASα was both necessary and sufficient for repression in the absence of silencers. On the basis of this observation, we conclude that silencers, as well as individual silencer binding site sequences, are sufficient to maintain the silent state. Isolated binding sites for silencer binding proteins, termed protosilencers, although lacking intrinsic silencing function, have been shown to interact cooperatively with intact silencers to

strengthen silencing (Boscheron et al. 1996). Specifically, repression of a sensitive reporter gene by a sole silencer was aided by tethering either Rap1p, Abf1p, or ORC to a DNA site 4 kb away. By inference, it was posited that the Rap1p-binding site at UASα might contribute similarly to silencing at *HML*. That UASα performs this function in a near native chromosomal context was demonstrated by comparing wild-type and mutant UASα sequences in a colony color assay for derepression (Fig. 4). The Rap1 protein (repressor/activator protein) derived its name from the discovery that it contributes to either transcriptional repression when bound to silencers or transcriptional activation when bound at the promoters of numerous genes, including *MAT*α (Shore 1994). UASα provides the first example of a specific Rap1p site that possesses dual function, either activating or repressing the associated gene/genes, depending on the chromosomal context of the gene/genes.

Holmes and Broach (1996) have argued that maintenance of repression does not require silencers because derepression of the α genes did not occur when they removed the *E* and *I* silencers from *HML* in G_1-arrested cells. This conclusion must now be reconsidered in light of the discovery that UASα functions as a protosilencer. Nonetheless, silencers were shown to be required for inheritance of the silent state; following removal of the elements, reactivation of *HML* occurred within a single cell cycle, particularly during passage between the G_1 and G_2 stages of the cell cycle (Holmes and Broach 1996; Bi and Broach 1997). Remarkably, reactivation occurred even in the absence of DNA replication of the silent template. We too have found that nonreplicating DNA rings containing the UASα protosilencer reactivated during this interval (T.-H. Cheng, unpubl.). Although critical constructs of Bi and Broach (1997) lacked UASα, it should be noted that they did contain regions of *HML* and bacterial DNA fragments not present in our clones. It is possible that a sequence with protosilencer activity resided within this additional DNA. In either case, the results indicate that protosilencers on their own are not capable of propagating the silent state. Whereas the elements can prevent loss of silencing in G_1, they do not suffice during subsequent cell cycle progression. In this regard, bona fide silencers appear either to be more efficient or to provide additional functions.

Silent chromatin is dynamic

How do silencers function in the preservation of silent chromatin? The answer may lie in the relationships between Sir protein availability, recruitment, and silent chromatin stability. Sir proteins have been shown previously to be limiting for repression at *HM* loci due to competition for the factors by telomeres where Sir-mediated repression also occurs (Aparicio et al. 1991; Buck and Shore 1995; Marcand et al. 1996). In a number of studies, alteration of the level of free Sir2p, Sir3p, or Sir4p influenced either the efficiency of the silencing of reporter constructs or the span of the silenced domain (Renauld et al. 1993; Sussel et al. 1993; Maillet et al. 1996; Enomoto and Berman 1998). Here, persistence of silencing in DNA rings was shown to be extended by increasing the levels of Sir3p and Sir4p (Figs. 5 and 6). The striking feature was that stabilization by elevated Sir3p did not require passage through S phase. Moreover, stabilization appeared to be the result of direct incorporation of the protein into the repressive structure (Fig. 7). These findings demonstrate that recruitment of Sir factors can occur during maintenance of the silent state, as well as during its establishment. Together with the facile loss of silent chromatin upon removal of silencers, the observations indicate that silent chromatin is dynamic with critical components, such as Sir3p, equilibrating on and off the structure. Therefore, a reasonable role for silencers during maintenance may be to continually recruit new Sir proteins. For example, if silencing components turn over naturally, due either to dissociation or degradation, *cis*-acting elements would promote replenishment of the depleted components. Recruitment could be facilitated by direct protein-protein interactions with silencer-bound proteins, as described above, as well as by silencers targeting silent chromatin to regions of the nucleus that are enriched in Sir proteins (Andrulis et al. 1998).

Silencers are not likely to act alone in recruitment of the Sir proteins. A large network of protein–protein interactions could favor binding of free Sir proteins by those already bound. Sir3p and Sir4p form homomeric and heteromeric complexes (Chien et al. 1991; Moretti et al. 1994), and Sir2p and Sir4p also form a complex (Moazed and Johnson 1996; Strahl-Bolsinger et al. 1997). In addition, Sir3p and Sir4p bind preferentially to hypoacetylated amino-terminal tails of histones H3 and H4 (Hecht et al. 1995), which are enriched at the silent loci (Braunstein et al. 1993). Finally, tethering Sir3p and Sir4p to DNA directly leads to repression of adjacent genes (Lustig et al. 1996; Marcand et al. 1996). This last example demonstrates that silencers can be bypassed if a high local concentration of Sir protein is maintained. Conceivably, pre-existing silent chromatin could be propagated by self-recruitment if the intracellular concentration of Sir proteins was elevated. In flies and humans, such a mechanism has been proposed to explain the epigenetic behavior of kinetochores, the specialized chromatin-based structures that segregate chromosomes (Murphy and Karpen 1998; Wiens and Sorger 1998, and references therein). Functional kinetochores sometimes assemble on noncentromeric locations, where they are propagated in a heritable fashion despite the lack of discernible centromeric DNA sequences. Perpetuation of this class of kinetochores may rely entirely on self-templating by the pre-existing chromatin structure. In yeast, Sir2p and Sir3p must be maintained at low levels because they are toxic, potentially due to the promiscuous silencing of critical genes (Holmes et al. 1997). Therefore, silencers may have evolved to recruit Sir proteins efficiently and specifically to the *HM* loci in an environment where telomeres and other sites, such as rDNA (Smith et al. 1998), compete for limited pools of Sir proteins.

Enomoto and Berman (1998) showed that maintenance of silent chromatin was influenced by mutations in a replication-coupled chromatin assembly factor encoded by the *CAC* genes. In G_1-arrested *cac* mutants, derepression of the mating-type loci in individual cells was recorded with a sensitive time-lapse microscopy assay. These investigators argued that a defect during silent chromatin assembly was manifest in a metastable repressive structure that could not be maintained appropriately. Given our results, an alternative testable hypothesis is that the chromatin assembly factor acts directly on preassembled silent chromatin in G_1-arrested cells to maintain the silent state. In support of this notion, the mouse Cac1p homolog CAF-1 p150 was shown recently to associate with heterochromatin in non-S-phase cells (Murzina et al. 1999).

Maintenance of repressed chromosomal domains in other organisms

Variegated repression of genes adjacent to heterochromatin is thought to occur by the stochastic but stable spread of heterochromatic structure into adjacent DNA. It was shown recently that reporter genes subject to this form of repression in *Drosophila* reactivated upon excision from the genome, even in mitotically quiescent cells (Ahmad and Golic 1996). This observation indicates that maintenance of heterochromatic repression in flies, like yeast, requires preservation of proper genomic context. It is not clear whether loss of continuity with chromosomal heterochromatin or nuclear compartmentalization accounts for reactivation in the *Drosophila* studies. However, recombination-based studies with engineered excision cassettes hold promise of identifying *cis*-acting sequences sufficient for maintenance of heterochromatic repression.

Maintenance of the inactive X chromosome (Xi) in female mammals is notably different from examples in yeast and flies. Like yeast, establishment of the repressed state requires a *cis*-acting regulatory element, the X inactivation center (*XIC*). However, inactivation of Xi is heritably propagated following removal of *XIC*, indicating that the element is not required for maintenance of the repressed state (Brown and Willard 1994). Xi is structurally distinct in numerous ways, including differential DNA methylation, histone acetylation, and the presence of an RNA chromatin component (Lee and Jaenisch 1997, and references therein). Any one of these features could participate in a self-templating mechanism to propagate heterochromatic repression. It also seems possible that the X chromosome contains stabilization elements, like protosilencers of yeast, that promote maintenance of repression but that lack the ability to establish repression on their own. Based on Xi-autosome translocation data, Gartler and Riggs (1983) postulated early on that the X chromosome contains stabilization elements that serve as "booster" sites to help spread heterochromatic structure throughout Xi. It was posited recently that long interspersed nuclear elements (LINEs), which are enriched in the X chromosome (Boyle et al. 1990), function in this way (Lyon 1998). Precedent for booster sites is found in yeast where native protosilencers within subtelomeric repeat sequences propagate silencing away from chromosomal termini (Fourel et al. 1999; Pryde and Louis 1999). Although the mechanisms of booster site action may differ widely between humans and yeast, the underlying requirement for amplification of a silencing signal along the chromosome appears to be conserved.

Materials and methods

Strain and plasmid constructions

pΔhmra::rHMRα was constructed by replacing the entire **a** gene fragment within the excision cassette of phmr::rA1A2 (Cheng et al. 1998) with a PCR fragment containing the α genes from *HML* (chromosome III coordinates 11695–14018). pΔhmra::rHMRα(rap1pm) was derived from pΔhmra::rHMRα by using overlap PCR to introduce a C-to-A transversion in the Rap1p-binding site of UASα (see Results). pΔhmr::rHMR**a**/α was obtained by inserting a PCR fragment containing the α gene divergent promoters (coordinates 12909–13332) into phmr::rA1A2Δp at a *Bam*HI site that replaced the **a** gene promoters (Cheng et al. 1998). pRS406GAL10P–SIR3 was obtained by ligating a *Eco*RV–*Bam*HI fragment that contained a *GAL10P–SIR3* chimeric gene from pAR42 (S. Holmes, Wesleyan University, Middletown, CT) into the multiple cloning site of pRS406. pRS406GAL10P–SIR3HA was generated by replacing the carboxyl terminus of *SIR3* in pRS406GAL10P–SIR3 (*Xba*I–*Xho*I) with an HA-tagged version from pRS416–SIR3HA (Ansari and Gartenberg 1999). phmlα::URA3P–ADE2 was derived from plasmid puc19–HML (D. Shore, University of Geneva, Switzerland) by replacing the *Eag*I–*Cla*I fragment of *HML* with a 2.0-kb, PCR-amplified fragment that contained the *URA3P–ADE2* chimeric gene from plasmid pDR859 (D. Rivier, University of Illinois, Urbana-Champagne). phmlα-(rap1pm)::URA3P–ADE2 was constructed by replacing the *Eag*I–*Blp*I fragment of phmlα::URA3P–ADE2 with the mutagenized version from pΔhmr::rHMRα(rap1pm). pΔmata::TRP1 was created by replacing the *Apa*I–*Sma*I fragment of pΔmat::URA3 with a 1.6-kb fragment (*Apa*I–*Sna*BI) containing the *TRP1* gene from pRS414 (Cheng et al. 1998). Plasmids pJR910, pJR69, pJR273, and pJR368 were constructed in the laboratory of J. Rine (UC, Berkeley) and provided by J. Berman (University of Minnesota, St. Paul).

Unless specified otherwise, all strains were constructed by the one-step gene disruption method and confirmed by Southern hybridization. THC42 was derived from THC23 in three steps: (1) *LYS2* was disrupted with plasmid pUC18–Δlys2 (Cheng et al. 1998); (2) *BAR1* was disrupted with plasmid pTM47 (Menees and Sandmeyer 1994); and (3) *MAT***a** was disrupted with pΔmata::TRP1. THC78 was derived from THC42 by replacing the α genes at *HML*α with a PCR-amplified *kanMX* gene from plasmid pUG6 (Wach et al. 1994). THC51 was derived from THC42 by replacing the **a** genes at *HMR***a** with *URA3*, as described previously (Cheng et al. 1998). THC59, THC68, and THC75 were derived from THC51 by replacing the *Δhmr::rURA3* locus with modified *hmr* loci from plasmids pΔhmra::rHMRα, pΔhmra::rHMRα(rap1pm), and pΔhmra::rHMR**a**/α, respectively. THC43, THC62, THC69, and THC77 were derived from THC42, THC59, THC68, and THC75, respectively, by regeneration of chromosomal *SIR3* using pAR3. THC57 and THC67 were derived from THC43 by targeted integration of one copy of pRS406GAL10P–SIR3 and

pRS406GAL10P–SIR3HA, respectively, into *ura3-1*. THC70 was derived from THC78 by targeted integration of one copy of pRS406GAL10P–SIR3HA into *ura3-1*. THC37 was derived from YCL1 (Cheng et al. 1998) by disrupting *MAT***a** with pΔmat::URA3. THC79 was derived from YCL2 (Cheng et al. 1998) by disrupting *MAT***a** with pΔmat::URA3 and replacing the α genes at *HML*α with *kanMX*. Strains THC74 and THC76 were derived from PJ1 (W303-1A *URA3*) by replacing *HML*α with modified loci in plasmids phmlα::URA3P–ADE2 and phmlα(rap1pm)::URA3P–ADE2, respectively. Selection of PJ1 derivatives was aided by prior transformation with an "anti-sir" plasmid, pCTC23 (Chien et al. 1998), which was subsequently evicted. Note that chromosomal excision cassettes *hmr::rHMR***a** and *hmr::rHMR***a**+*EI* previously were named *hmr::rA1A2* and *hmr::rHMR*, respectively (Cheng et al. 1998).

Cell growth and analysis of nucleic acids

Strains were transformed with a recombinase expression vector (pHM153) and grown at 30°C in synthetic dropout media containing 2% raffinose. At mid-log phase, cells were treated for 3 hr with 2 μg/ml α-factor (Sigma) for *Δbar1* strains and 10 μg/ml pheromone for *BAR1* strains. Persistent α-factor-mediated arrest in G_1 was confirmed by flow cytometry and/or visual inspection of cell morphology. Nocodazole-mediated arrest at G_2/M was achieved by treatment with 10 μg/ml nocodazole (Sigma) for 3 hr. Approximately 90% of the cells arrested with large buds (dumbbell shaped), indicative of G_2/M block, and remained in this configuration for the duration of the experiment.

DNA ring formation was induced with galactose (C_f = 2%), and parallel aliquots of culture were used for both Northern blots and DNA supercoiling analyses. Isolation, electrophoresis, and detection of nucleic acids were described previously (Cheng et al. 1998). Topoisomer distributions were evaluated by the Gaussian method following electrophoresis in gels containing 2 μg/ml chloroquine, such that more negatively supercoiled rings migrated more rapidly (Depew and Wang 1975).

Colony color assays

Uniformly red colonies of strains THC74 and THC76 were grown overnight in nonselective liquid media (YPDA) and plated on SC plates that contained limiting adenine (6 μg/ml). Following 2 days of incubation at 30°C, plates were stored for 3 days at 4°C for enhanced color development.

Characterization of integrated GAL10P–SIR3 *fusions*

Overexpression of *SIR2* and *SIR3* from high copy vectors is cytotoxic (Holmes et al. 1997). To verify that expression of a single integrated *GAL10P–SIR3* fusion gene was not overtly deleterious, *SIR3* strains containing or lacking the chimera were grown side by side on plates containing galactose. In both cases, equivalent numbers of colonies appeared and grew at equivalent rates. Galactose-induced expression of either the *GAL10P–SIR3* or *GAL10P–SIR3HA* was sufficient to suppress the mating defect in a *sir3* strain. When the strains were grown in raffinose, however, no silencing could be detected with a quantitative mating assay and Sir3HAp could not be visualized by Western blot analysis. Together, these data show that the integrated *GAL10P–SIR3* chimeras are functional, tightly regulated, yet not harmful.

ChImp assays and PCR analysis

ChImp assays were performed essentially as described in Aparicio et al. (1997), with the following exceptions. Following cell cycle arrest with α-factor, *GAL10P–SIR3HA* expression was induced by galactose addition. After a 45-min incubation, cells were treated with formaldehyde and chromatin-containing extracts were prepared. Epitope-tagged Sir3p was immunoprecipitated with anti-HA monoclonal antibody HA.11 (BAbCO, Richmond, CA) bound to protein A–Sepharose CL-4B beads (Pharmacia, Piscataway, NJ). PCR reactions were performed with either 1/50 of the precipitated DNA or 1/500 of the input material. Two pairs of gene-specific primers were used simultaneously in each reaction (A1-1/A1-2 for the **a***1* gene and GAL1-1/GAL1-2 for the *GAL1* gene, or ACT-1/ACT1-2 for the *ACT1* gene and PHO5-1/PHO5-2 for the *PHO5* gene). Primer sequences are as follows: A1-1 (5′-ATGGATGATATTTGTAGTATGGCG-3′); A1-2 (5′-GGTGGTATATTTCTAACCTATTGTTAG-3′); GAL1-1 (5′- CTGCAAGTCTTCTGTGAGG-3′); GAL1-2 (5′-GATACAACAAGGGTGTTCGC-3′); ACT1-1 (5′-AGACCAAGACACCAAGGTATC-3′); ACT1-2 (5′-GAGTACAAGGACAAAACGGCT-3′); PHO5-1 (5′-ACTTGACCTCAACTGACGC-3′); and PHO5-2 (5′-AGGATATCGGTATCGTGGG-3′). Twenty-five cycles of PCR were performed with an annealing temperature of 51°C. PCR products were separated by agarose gel electrophoresis, stained with EtBr, and photographed using Polaroid 665 film.

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