Persistence of an alternate chromatin structure at silenced loci in the absence of silencers

(Transcriptional repression and silencers/*S. cerevisiae*/mating-type locus/heterochromatin/DNA topology/recombination)

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ABSTRACT In *Saccharomyces cerevisiae*, genes placed near telomeres or the silent *HML* and *HMR* mating-type loci are transcriptionally repressed by a heterochromatin-like structure. We have generated nonreplicating DNA rings by recombination *in vivo* to examine the role of chromosomal context on transcriptional repression. Specifically, recombination at *HMR* was used to produce rings that lacked the E and I silencers. An altered level of DNA supercoiling was observed in these rings but not in comparable rings from derepressed loci. Our results indicate that a repressive chromatin structure persists in an extrachromosomal environment immediately following removal of the *cis*-acting control elements. Examination of both chromatin footprints and DNA sequence dependence revealed that changes in nucleosome number could account for the topology shifts. Upon continued cell growth, the differences in supercoiling were lost and transcriptional competence was restored. These results show that silencers are required for sustained persistence of repressive chromatin structure, even in the absence of DNA replication.

The chromosomes of higher eukaryotes consist of both euchromatic and heterochromatic domains, and these regional variations in DNA packaging profoundly influence the transcriptional activity of genes. In *Drosophila*, for example, relocation of an active euchromatic gene near a heterochromatic domain results in a variegated pattern of gene expression; the gene is on in a subset of cells and off in others (1). The expression states are maintained stably over many generations because of the clonal propagation of heterochromatin. Switching between states occurs rarely.

Position effects also play an essential role in determining cell type in the yeast *Saccharomyces cerevisiae*. In this organism, haploid mating type is governed by the expression of either **a**- or α-specific genes at the *MAT* locus. Additional copies of the mating-type genes are stored at *HML* and *HMR* where they are transcriptionally repressed because of the combined action of *cis*-acting regulatory sequences, termed silencers, and *trans*-acting components (2). Silencers consist of different combinations of binding sites for Rap1p, Abf1p, and the origin recognition complex (ORC). Together, the DNA binding proteins recruit critical silencing factors encoded by the *SIR* genes, *SIR1–4* (3), through a network of protein–protein contacts (4, 5).

Transcriptional silencing at *HML* and *HMR* is gene-independent and results from a heterochromatin-like structure that suppresses a number of physiological DNA transactions (2). The repressed chromatin is also refractory to probes of DNA accessibility (6), a property also observed at telomeres where the Sir2–4 proteins silence telomere-proximal genes (7).

In addition, the amino-terminal tails of histones H3 and H4 are hypoacetylated at the silent mating-type loci (8). The deacetylated tails bind Sir3p and Sir4p directly *in vitro* (9), and crosslinking experiments have shown that Sir2p, Sir3p, and Sir4p span repressed chromosomal domains *in vivo* (10). Despite this extensive characterization, little is known about the detailed structure of silent chromatin and the mechanism by which transcriptional machinery is blocked.

An epigenetic pattern of gene expression has been observed in a subset of silencing pathway mutants. In a *sir1* null strain, for example, repression of *HML* and *HMR* occurs in only a fraction of cells within a population and individual cells switch between states infrequently (11). Based on these and other data, Pillus and Rine (11) posited that transcriptional repression involves both establishment and maintenance processes. According to this view, Sir1p is required for efficient establishment of the silent state but not its maintenance. The establishment function of the protein is likely to be linked to its association with silencers, to which it is tethered (5), because mutations in silencers and in Rap1p illicit a similar response (12, 13). However, silencers also are involved in perpetuating the repressed state; the elimination of the elements results in reactivation of silent genes within a single generation (14).

In this report, we examine the role of chromosomal context in maintaining an altered chromatin structure at the silent *HMR* locus. We used site-specific recombination *in vivo* to produce nonreplicating DNA rings from different chromosomal positions. In *SIR* strains, rings from *HMR* initially maintained an altered DNA topology even though the excised DNA fragments lacked both E and I silencers. The altered supercoiling level was lost at later times, and transcriptional competence was eventually restored. Our results reveal new features of the chromatin structure at a repressed locus and show that silencers are required for long-term but not short-term persistence of repressive chromatin structure in DNA fragments removed from their normal chromosomal context.

MATERIALS AND METHODS

Plasmid Constructions. Cassettes for excision of mating-type sequences were constructed by inserting fragments of the *HMR***a** locus from pLSD12 (D. Shore, University of Geneva) between a pair of tandemly repeated 58-bp *RS* sites (15) in plasmid pABX22 (M.R.G., unpublished results). The fragments along with flanking *RS* sites were then transferred back to pLSD12. This procedure yielded hmr::rHMR, which contains *RS* sites at *Sna*BI and *Pvu*I sites of *HMR* (outside E and I), and hmr::rA(1.8), which contains *RS* sites at *Bsp*MI and *Bcl*I sites (within E and I). Δhmr::rAΔp(1.6) was constructed by similar manipulations with the exception that overlap PCR was used to generate an *hmr* fragment that abutted the start codons of the

Abbreviations: MNase, micrococcal nuclease; kb, kilobase.
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divergent **a**1 and **a**2 genes. 523- and 735-bp *LYS2* fragments were inserted downstream of the **a**2 gene in hmr::rA(1.8) and Δhmr::rAΔp(1.6), respectively, to generate hmr::rA1A2 and Δhmr::rA1A2Δp. To construct Δhmr::rKWD50N, a 2.75-kilobase (kb) rKWD50N excision cassette from plasmid pKWD50N (16) was used to replace the *Xba*I-*Bgl*II fragment between the E and I silencers in pLSD12. Δhmr::r50NY/Z was constructed by replacing a 275-bp *Nco*I-*Hpa*I *LYS2* fragment in Δhmr::rKWD50N with a Y/Z junction-containing fragment of equal length (starting from the *Bgl*II site of *HMR*). The Δhmr::r50NY/Z construction was then transferred to pRS414.

pUC18-Δlys2 was created by deleting the *Bgl*II-*Xho*I *LYS2* fragment from pUC18-LYS2. The 2.75-kb rKWD50N excision cassette described above was inserted into the deletion site of pUC18-Δlys2 to generate Δlys2::rKWD50N. A *Not*I site within this cassette was destroyed, introducing an additional 4 bp relative to Δhmr::rKWD50N. pΔmat::URA3 was constructed by replacing the *Bgl*II-*BstB*I region of the *MAT***a** locus in plasmid pDC283 (17) with the *URA3* gene.

Strain Constructions. The yeast strains used in this study were derived from either W303–1A or a W303–1A derivative, THC1, in which the mating-type genes of *HMR***a** were replaced by *URA3* (T.-H.C., unpublished results). All of the gene disruptions or replacements were performed by the one-step method (18) and confirmed by Southern hybridization or PCR. THC3 and THC13 were derived from THC1 and W303–1A, respectively, by disruption of *SIR3* with psir3::HIS3 (16). THC9, THC23, and THC24 were derived from THC3 by replacement of the chromosomal Δhmr::URA3 with plasmids Δhmr::rKWD50N, hmr::rA1A2, and Δhmr::rA1A2Δp, respectively. THC10, THC25, THC26, and YCL1 were derived from THC9, THC23, THC24, and YCL2, respectively, by regeneration of the chromosomal *SIR3* using plasmid pAR3 (S. Holmes, Wesleyan University). THC16 and THC17 were derived from THC13 and W303–1A, respectively, by disruption of *LYS2* with plasmid Δlys2::rKWD50N. THC27 and THC28 were derived from THC10 and THC9, respectively, by disruption of *LYS2* with plasmid pUC18-Δlys2. THC40 and

Table 1. Yeast strains

Strain	Genotype	Source
W303-1A	*MAT***a** *HMLα HMR***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein, Columbia University
THC1	W303-1A Δ*hmr::URA3*	This study
THC3	W303-1A Δ*hmr::URA3* Δ*sir3::HIS3*	" "
THC9	W303-1A Δ*hmr::rKWD50N* Δ*sir3::HIS3*	" "
THC10	W303-1A Δ*hmr::rKWD50N*	" "
THC13	W303-1A Δ*sir3::HIS3*	" "
THC16	W303-1A Δ*lys2::rKWD50N* Δ*sir3::HIS3*	" "
THC17	W303-1A Δ*lys2::rKWD50N*	" "
THC23	W303-1A hmr::rA1A2 Δ*sir3::HIS3*	" "
THC24	W303-1A Δ*hmr::rA1A2Δp* Δ*sir3::HIS3*	" "
THC25	W303-1A hmr::rA1A2	" "
THC26	W303-1A Δ*hmr::rA1A2Δp*	" "
THC27	W303-1A Δ*hmr::rKWD50N* Δ*lys2*	" "
THC28	W303-1A Δ*hmr::rKWD50N* Δ*lys2* Δ*sir3::HIS3*	" "
THC40	W303-1A hmr::rA1A2 Δ*mat::URA3* Δ*sir3::HIS3*	" "
THC41	W303-1A hmr::rA1A2 Δ*mat::URA3*	" "
YCL1	W303-1A hmr::rHMR	" "
YCL2	W303-1A hmr::rHMR Δ*sir3::HIS3*	" "

THC41 were generated from THC23 and THC25, respectively, by disruption of *MAT***a** with Δmat::URA3. YCL2 was derived from THC3 by replacement of the chromosomal Δhmr::URA3 with hmr::rHMR.

Formation of Intracellular DNA Rings and Analysis of Topoisomer Distributions. Strains were transformed with the recombinase expression vector pHM153 (15) and pregrown at 30°C to mid-log phase in 50 ml of synthetic complete media lacking leucine and containing raffinose (2%). Recombinase expression was induced by the addition of galactose ($C_f = 2\%$) for 60 min, unless specified otherwise. DNA rings were isolated by a spheroplasting procedure (16). Then, 25% of each DNA sample was electrophoresed for 18 hr at 1.5 V/cm in 0.8% agarose gels containing 2.0 μg/ml chloroquine diphosphate (Sigma). After transfer to Zeta Probe GT membrane (Bio-Rad), blots were hybridized with a randomly primed 735-bp *LYS2* probe (beginning at *Stu*I and spanning upstream sequences), unless specified otherwise. Topoisomer bands were quantified by phosphorimaging (Bio-Rad), and the centers of the topoisomer distributions were determined by the Gaussian method (19).

Analysis of Chromatin Structure by Micrococcal Nuclease (MNase) Digestion and Indirect End Labeling. Footprinting by MNase was performed in NP-40 (Sigma) permeabilized spheroplasts essentially as described by Kent *et al.* (20) with the following exceptions. Ten times as many cells were used, and all of the buffer volumes were thus raised fivefold. Spheroplasting was accomplished by treatment with 1 ml of spheroplasting solution [20% Sorbitol, 0.3–0.5 mg/ml Zymolyase T100 (Seikagaku, Japan), and 0.5 mM β-mercaptoethanol] for 5 min at 30°C. MNase digests (5 min at 30°C) were terminated by the sequential addition of 125 μl of prewarmed 250 mM EDTA/5% SDS (65°C), 125 μl of 10% Triton X-100, and 25 μl RNase A (10 mg/ml). After a 30 min incubation at 37°C, 60 μl of Proteinase K (10 mg/ml) was added and the samples were incubated at 65°C overnight, extracted with phenol/chloroform twice, and precipitated. Sample were linearized with *Stu*I, electrophoresed in 1% agarose, and transferred to nylon membrane. Blots were hybridized with the *LYS2* probe described above. For naked DNA controls, DNA was isolated from spheroplasts, as described above, before MNase digestion.

Northern Blots. Aliquots of culture were harvested and preserved on dry ice until additional processing. Total RNA was extracted by the hot acid phenol procedure and electrophoresed in 1% agarose/formaldehyde gels (18). Nucleic acids were transferred to Zeta Probe membrane. Blots were hybridized sequentially with a randomly primed **a**1-specific probe (spanning the entire ORF as well as 3′-untranslated sequences) and a *SNR17a(U3 snRNA)*-specific probe (21).

RESULTS

Methodology. The R site-specific recombinase of yeast *Zygosaccharomyces rouxii* can excise and recircularize DNA fragments from appropriately designed constructs (15, 22). Inducible expression of the enzyme in living cells provides a means to separate DNA fragments from their original chromosomal positions (23). Furthermore, circularization of the excised fragments permits examination of the DNA topology of sequences that were formerly linear. Our initial experiments used an excision cassette consisting of a 2.4-kb *LYS2* fragment flanked by recombinase target sites, termed *RS* sites (16). The cassette was integrated either at the *HMR* locus on chromosome III, replacing the transcriptionally repressed **a**1 and **a**2 genes, or within the nonrepressed *LYS2* gene on chromosome II (Fig. 1). Excision from either location produced a nonreplicating ring of chromosomal DNA, designated rKWD50N, which lacked known promoters of transcription. Importantly, excision cassettes at *HMR* were positioned between the E and
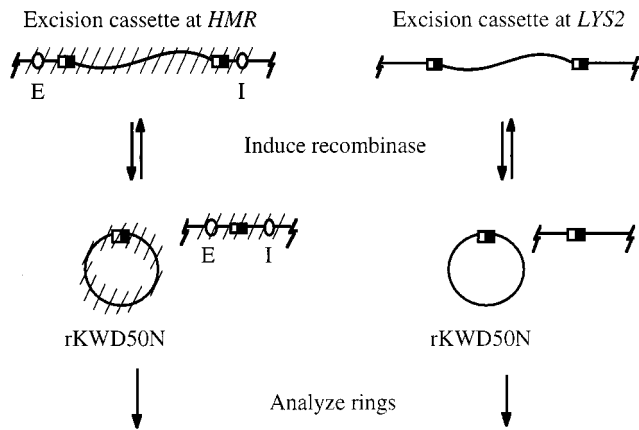
FIG. 1. Methodology for production of extrachromosomal DNA rings. A fragment of internal coding sequence of the *LYS2* gene was flanked by tandemly oriented *RS* sites (half-filled rectangles) and integrated at *HMR* or *LYS2*. Cross-hatches in the figure indicate the potential for Sir-mediated repression. Induction of the R recombinase gene creates a 2,465-bp extrachromosomal ring, rKWD50N.

I silencers; recombination uncouples the regulatory elements from the ring to be studied.

Excision from HMR Yields DNA Rings with Altered DNA Topology. In eukaryotes, DNA supercoiling is determined largely by the wrapping of the DNA double helix around histone cores. Each nucleosome constrains approximately one negative supercoil (24). Topoisomer distributions of intracellular DNA provide a sensitive probe of chromatin structure because nucleosome density and subtle structural changes in individual nucleosomes can affect DNA supercoiling (see ref. 25 for example). To test whether Sir-mediated silencing is accompanied by a change in supercoiling, we examined topoisomer distributions of ring rKWD50N from repressed and nonrepressed loci. Using a galactose-inducible recombinase expression plasmid (15), ≈80% of the excision cassette at *HMR* or *LYS2* was circularized within 60 min in both *SIR* and *sir3* strains (data not shown). The high rate and efficiency of excision indicates that recombination is not limited to a subpopulation of unrepresentative chromatin templates.

DNA topoisomers were resolved by electrophoresis in buffer containing chloroquine diphosphate such that more negatively supercoiled topoisomers migrated more rapidly. When exci-
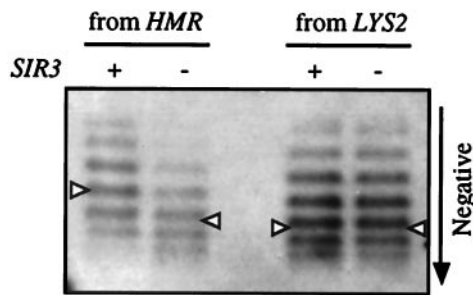

FIG. 2. DNA supercoiling of extrachromosomal rings. rKWD50N was produced by excision from either *HMR* in strains THC10 (*SIR*) and THC9 (*sir3*) or from *LYS2* in strains THC17 (*SIR*) and THC16 (*sir3*). Topoisomers were resolved by electrophoresis in buffer containing chloroquine diphosphate. Autoradiograms were cropped to show DNA rings exclusively. Gaussian centers of the topoisomer distributions are marked with a triangle. The *SIR3*-dependent shift in linking number was 1.1 ± 0.1 for the ring from *HMR* but negligible for the ring from *LYS2* (based on four independent measurements). The linking number difference between rings from *LYS2* and from the derepressed *HMR* locus was also negligible. A slight increase in mobility in topoisomers from *LYS2* arose from four additional bases in the excision cassette (see *Materials and Methods*).

sion occurred at the repressed *HMR* locus, the distribution of rKWD50N topoisomers was less negatively supercoiled, by approximately one turn, relative to the distribution in a *sir3* strain (Fig. 2). Similar results were obtained in *sir2* and *sir4* mutants (data not shown). In contrast, when rKWD50N was excised from the *LYS2* locus, the topology of the ring was not influenced by a *sir3* mutation (Fig. 2). Furthermore, the superhelical density of the ring from *LYS2* was comparable to the ring from the derepressed *HMR* locus (see Fig. 2 legend).

The supercoiling shift was not caused by restricted access of topoisomerases to silent chromatin. We found that the supercoil densities of silent and nonsilent rings increased rapidly upon an upshift in cell culture temperature, consistent with topoisomerase action in response to thermal unwinding of DNA (ref. 26 and T.-H.C., unpublished results). The relative difference in the topology of rings in *SIR* and *sir3* strains, however, was unaltered by the temperature jump (23°C to 38°C).

Three conclusions can be drawn from these observations. First, Sir-mediated effects on DNA topology occur in the vicinity of a silenced locus. Second, the altered DNA supercoiling of silent chromatin is not caused by a deficiency in topoisomerase action. Third, the structural differences detected by this approach persist in DNA rings despite uncoupling from the E and I silencers. Thus, identical DNA sequences are packaged differently at repressed and nonrepressed loci, and this difference persists on their removal from a chromosomal context.

Chromatin Footprints of rKWD50N. One simple explanation for the observed shift in DNA topology is that repressed chromatin contains fewer nucleosomes. To analyze the influence of *SIR*-mediated repression on nucleosome placement, we generated MNase footprints of the chromosomal excision cassette at *HMR* in spheroplasted cells. Cleavage sites were mapped by indirect end-labeling (43). Fig. 3 shows that the unexcised rKWD50N template yields similar although nonidentical digestion patterns in repressed and derepressed strains. Comparable results were obtained from digestion of the excised rings (data not shown). Regions of protection spanning ≈165 bp are indicative of positioned nucleosomes and are marked with ovals. Internucleosomal bands exist at
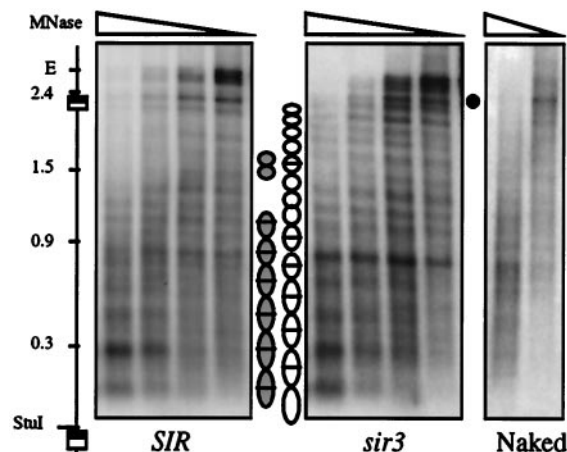
FIG. 3. MNase footprinting of chromatin templates. Spheroplasts were made from strains THC27 (*SIR*) and THC28 (*sir3*), and DNA was digested *in situ* with MNase (see *Materials and Methods*). The indirect end-labeling probe, a *LYS2* fragment, hybridizes within the rKWD50N excision cassette adjacent to the *Stu*I site (these strains lack the *LYS2* gene). The positions of *HMR* E, *RS* sites, and size markers (in kb) are denoted. (●) identifies a single hypersensitive site outside the excision cassette that appears upon derepression. Digestion of purified chromosomal DNA is also shown (marked Naked). Units of MNase/ml used in each lane: (*Left* and *Center*) 160, 80, 40, and 20; (*Right*) 10 and 5.

some locations and these are marked with bars. It seems
unlikely that the bands correspond to disrupted or "split"
nucleosomes because they were not observed in footprints of
the native *HMR* locus (ref. 27 and T.-H.C., unpublished
results). Rather, the pattern of bands suggests that there are
two possible settings for nucleosomes in the *SIR* strain (either
shaded ovals or open ovals) whereas only one setting is used
in the *sir3* strain (open ovals). Gain of a supercoil upon
derepression, according to one interpretation, is caused by the
presence of an additional nucleosome, on average, in the
derepressed setting. An alternative explanation holds that the
Sir-mediated topology shift is caused by a change nucleosome
conformation, not a change in nucleosome number (see *Dis-
cussion*). The data presently available cannot differentiate
between these two possibilities.

**The Role of DNA Sequence in Sir-Mediated Shifts in DNA
Topology.** Next, we asked whether the Sir-mediated supercoil-
ing shift at *HMR* was dependent on the sequence of the excised
fragment. Two additional excision cassettes were constructed
from fragments of the native *HMR* locus. The first yields ring
rA1A2, containing both the **a**1 and **a**2 mating-type genes and
the second yields ring rA1A2Δp, bearing the same genes with
their promoters deleted (Fig. 4*A*). When excised from *HMR*,
each ring possessed a different level of supercoiling in a *SIR*
strain than in a *sir3* strain (Fig. 4*B*). Upon repression, rA1A2
and rA1A2Δp gained a supercoil whereas rKWD50N lost a
supercoil. Nevertheless, rKWD50N and the two new rings bore
comparable DNA topoisomer distributions in the repressed
case. These results indicated that Sir-mediated changes in the
rings yielded similar repressed chromatin structures.

To sustain comparable levels of supercoiling in the re-
pressed state, each ring must also contain the same number of
nucleosomes. In isogenic *sir3* mutants, however, rKWD50N
was more negatively supercoiled than both rA1A2 and
rA1A2Δp by approximately two turns (Fig. 4*B*). The difference
cannot be attributed to transcription of ring-borne genes

because rA1A2Δp lacks the **a**1 and **a**2 promoters. Rather, the
difference is most easily explained by rA1A2 and rA1A2Δp
containing fewer nucleosomes in the derepressed state. Pre-
viously, chromatin footprints of *HMR* showed that nuclease
hypersensitive sites, indicative of disrupted or displaced nu-
cleosomes, appeared at a small number of positions upon
derepression (27). Indeed, incorporating one of these sites, the
Y/Z junction, into rKWD50N changed its DNA topology. The
new ring r50NY/Z contained 1–2 fewer supercoils than
rKWD50N in a *sir3* mutant (Fig. 4).

**Altered DNA Topology and Transcriptional Silencing do
not Persist Indefinitely.** In our initial experiments, few if any
cells would have traversed a single cell cycle during the short
60-min excision period (Fig. 2). To test whether the DNA
supercoiling shifts of silent DNA persist indefinitely, we
examined the topoisomer distributions of rings from *HMR* at
later times. Cells were harvested at 210 and 360 min after the
addition of galactose, time points which correspond to one and
two doublings in cell number after an initial 60-min excision
interval. Upon extended growth, the supercoiling shift of
rA1A2 was lost; topoisomer distributions of the ring were
similar in both *SIR* and *sir3* strains by 210 min (Fig. 5*A*). Similar
results were obtained for rKWD50N, which became more
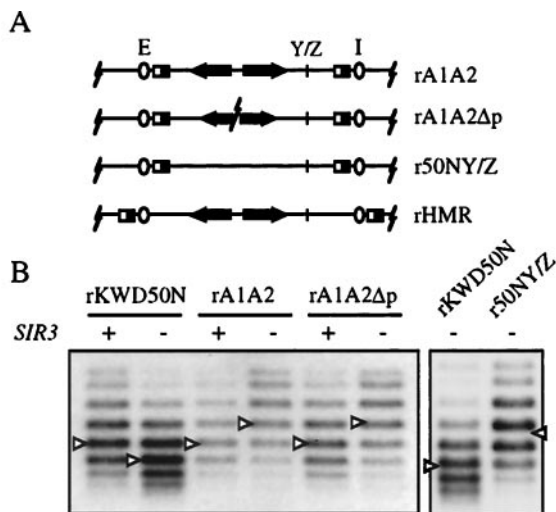negatively supercoiled at later times (data not shown). These

FIG. 4.   Sequence dependence of DNA supercoiling for rings
excised from *HMR*. (*A*) Excision cassettes based on *HMR* sequences.
rA1A2 contains the **a**1 and **a**2 genes. rA1A2Δp contains the same
genes but lacks the promoters. r50NY/Z is a derivative of rKWD50N
that contains the Y/Z junction. rHMR contains the entire *HMR* locus.
All rings, with the exception of rHMR, are 2,465 bp and contain a
fragment of *LYS2* (*Materials and Methods*). (*B*) Topoisomer analysis.
rA1A2 from strains THC25 (*SIR*) and THC23 (*sir3*), rA1A2Δp from
strains THC26 (*SIR*) and THC24 (*sir3*), and rKWD50N from THC10
(*SIR*) and THC9 (*sir3*) were examined as described in Fig. 2. r50NY/Z
was excised from a modified *hmr* locus on a CEN vector (pRS414) in
strain THC13 (*sir3*). The linking number shift was 1.0 for rKWD50N,
−1.2 for rA1A2, and −1.0 for rA1A2Δp. Additional trials of the
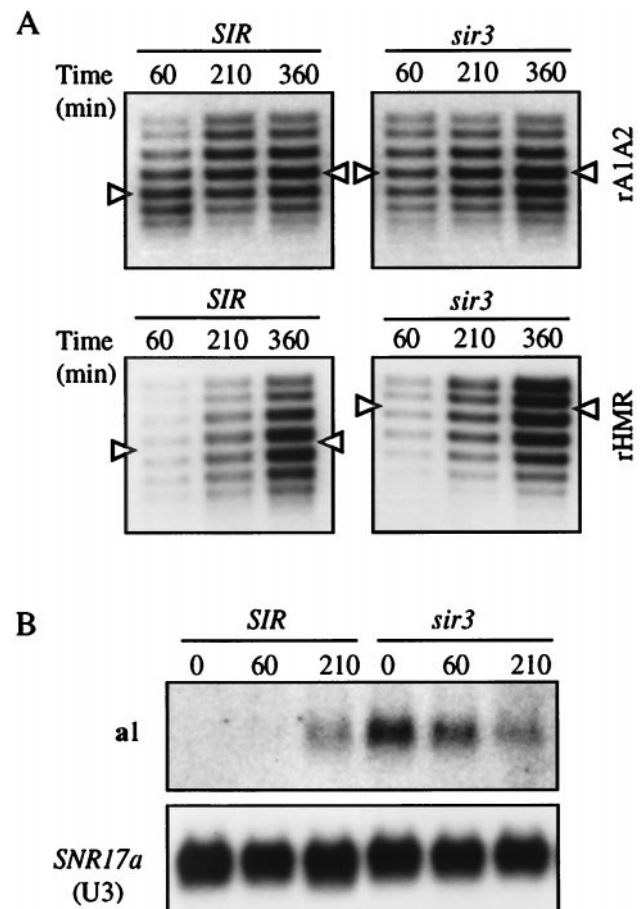experiment gave similar results.

FIG. 5.   Structural and functional fate of DNA rings from *HMR*. (*A*)
Topoisomer distributions of rings at extended intervals after galactose
induction (Time 0). rA1A2 (*Top*) was from strain THC25 (*SIR*) and
THC23 (*sir3*). rHMR (*Bottom*) was from YCL1 (*SIR*) and YCL2 (*sir3*).
rHMR was visualized by an *HMR* probe. (*B*) **a**1 mRNA determined by
Northern blot analysis. Experiment was performed in strains THC41
(*SIR*) and THC40 (*sir3*). Total RNA was isolated from cells at timed
intervals after induction of the R recombinase. Twenty micrograms of
nucleic acid were loaded in each lane. **a**1 mRNA and *SNR17a* (U3)
were visualized sequentially with gene-specific probes. *SNR17a* serves
as a loading control.

results demonstrated that Sir-mediated alterations in chromatin structure persisted for only a limited period after uncoupling from silencers and displacement from the chromosome. In contrast, a ring that contains silencers, rHMR (Fig. 4*A*), maintained its Sir-mediated supercoiling shift throughout the experiment (Fig. 5*A*). This suggests that silencers and not chromosomal continuity are required for long-term persistence of an altered DNA topology. In agreement, centromeric plasmids that contain the entire *HMR* locus also maintain a Sir-dependent supercoiling shift and support transcriptional repression (28).

DNA replication of the rings represents a likely step during which repressive chromatin structure could be remodeled. However, two lines of evidence suggest that the extrachromosomal rings do not replicate. First, the rA1A2 excision cassette was constructed from sequences that possess no *ARS* activity (29). Second, and more importantly, the copy number of the excised ring remained constant over a 5-hr period of growth whereas chromosomal DNA content quadrupled (data not shown). Thus, we conclude that loss of the Sir-mediated shift in DNA topology does not require DNA replication-coupled remodeling of the rings.

Lastly, we examined whether the altered DNA supercoiling of rings from *HMR* was correlated with transcriptional repression. For this purpose, we used northern blot analysis to measure the steady–state levels of **a**1 mRNA from rA1A2. Before recombination, **a**1 mRNA was absent in the *SIR* strain and present in the isogenic *sir3* strain (Fig. 5*B*, lanes 1 and 4). This result indicates that incorporation of *RS* sites at *HMR* does not disrupt normal gene regulation. After a 60-min recombinase induction, **a**1 mRNA was barely detectable in the *SIR* strain. By the 210-min time point, however, the level of **a**1 transcript was comparable in *SIR* and *sir3* strains. The time-dependent diminution of transcript in the *sir3* strain can be explained, in part, by the dilution of the nonreplicating template in growing culture. These results demonstrated that transcriptional repression of *HMR*, along with altered DNA supercoiling, does not persist indefinitely in extrachromosomal rings that lack silencers. Loss of silent chromatin could be governed by either a time-dependent decay process or a cell–cycle-mediated event.

DISCUSSION

Silencing is Associated with an Altered DNA Path in Chromatin. *Saccharomyces* lacks the cytological features of constitutively condensed heterochromatin found in higher eukaryotes. Heterochromatin-like domains thus have been identified by their ability to repress transcription and to limit access to DNA modifying enzymes (2). Here, we show that DNA supercoiling is also a sensitive probe of silenced chromatin in yeast. Importantly, the altered superhelical density of rings excised from *HMR* indicates that the packaging of repressed DNA is different; silencing does not arise from simply encasing standard chromatin in an impenetrable sheath of silencing factors.

The simplest model to account for Sir-mediated changes in DNA supercoiling is that silencing alters the number of nucleosomes on a template. According to this model, the preferred settings and stabilities of nucleosomes may differ in repressed and derepressed states. For example, rings rA1A2 and rA1A2Δp contain the Y/Z junction, a site which is cut by nucleases at all mating-type loci in derepressed strains but only at *MAT* in repressed strains (27). Regulated cleavage of the site at *MAT* by the endogenous Ho endonuclease initiates mating-type interconversion (17). A *Y/Z* junction binding protein, YZbp, was recently purified from yeast (30), and it was proposed that the factor facilitates cleavage by maintaining an open chromatin conformation (27, 30). The loss of one super-coil at *HMR* upon derepression is consistent with YZbp, or
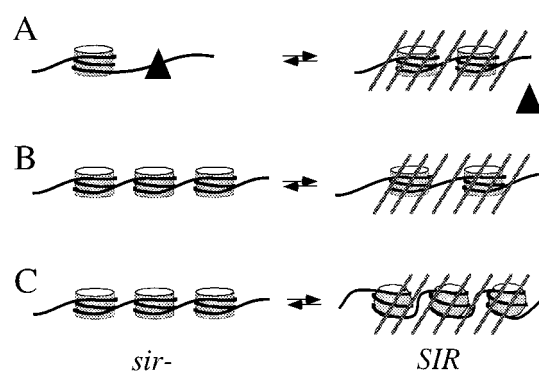

FIG. 6. Models for Sir-mediated transitions that can alter DNA topology. (*A*) Silencing favors nucleosome formation at the expense of binding sequence-specific binding factors (triangle). Negative supercoils are gained upon repression. (*B*) Silencing favors an altered nucleosome distribution, which can dictate a different total number of nucleosomes. Negative supercoils are either lost (as depicted here) or gained upon repression. (*C*) Silencing promotes a conformational change in all nucleosomes within a repressed region, which leads to a loss of negative supercoils upon repression (based on rKWD50N). Cross-hatched lines denote repressed chromatin.

some other factor, occluding or unwrapping nucleosomes (Fig. 6*A*).

Unlike rA1A2 and rA1A2Δp, rKWD50N gains a supercoil upon derepression, as do other rings composed of non-*HMR* yeast DNA (Y.-C.L., unpublished results). Ascribing the shift to a change in nucleosome number is difficult because chromatin footprints of rKWD50N do not provide clear evidence of such an event. However, the footprints are consistent with the presence of two preferred settings for nucleosomes with one strongly favored in the derepressed state and either one used in the repressed state. The supercoiling of rKWD50N can be explained if the derepressed setting permits binding of additional histone octamers (Fig. 6*B*).

If silencing does not change the number of nucleosomes in rKWD50N then the supercoiling shift must reflect a conformational change in those nucleosomes present, as depicted in Fig. 6*C*. At least two types of transitions can be envisioned. Silencing could alter the three-dimensional path or helical repeat of DNA within nucleosomes, and small differences in each nucleosome could contribute additively to a change in supercoiling. Alternatively, silencing could alter interactions between nucleosomes (31). This second type of transition might not perturb nucleosome structure directly, but it would impact the DNA topology of internucleosomal linker DNA. Either of the transitions could be facilitated by interactions of the Sir proteins with histones (9) or by the altered acetylation pattern of histone tails in repressed regions (32). The direct influence of histone acetylation on DNA supercoiling, however, is still controversial (33, 34).

Short-Term vs. Long-Term Persistence of Repressive Chromatin Structure in the Absence of Silencers. An altered chromatin structure persisted in silenced DNA immediately after excision from the chromosome and unlinkage from silencers. At extended times, however, the repressive DNA packaging was lost and the silenced genes were transcribed (Fig. 5). Thus, long-term preservation of silent chromatin requires silencers in *cis*. Our results confirm and extend earlier work of others. In DNA modification experiments with isolated nuclei, removal of silencers by endonucleolytic cleavage did not increase the accessibility of restriction endonuclease sites within *HMR* (6). Similarly, when a sole silencer was deleted from a chromosome by *in vivo* recombination, the adjacent mating-type genes were not derepressed, as long as cells were held in G$_1$ (14). Upon release from growth arrest, however, expression occurred within a single generation, in-

dicating that repression was not permanent. Taken together, these observations indicate that cell growth is required for reactivation of gene expression and topological reorganization. According to this view, the role of silencers may be to reinforce or reestablish silencing in the face of a specific physiological challenge during the cell cycle.

Global changes in chromosome structure during cell cycle progression could promote disassembly or decay of repressive chromatin. In this regard, DNA replication during S phase represents the most logical remodeling process. However, we show that nonreplicating DNA rings lose conformational distinctions and undergo changes in expression state. Therefore, replication of the extrachromosomal templates *per se* is not required for derepression. Inactivation of Sir3p, a constituent of silent chromatin, also leads to derepression without DNA replication (35).

Mitotic compaction of DNA represents another cell cycle-mediated event that could lead to dissociation of factors necessary for sustained repression. In human cells, for instance, nearly all sequence-specific transcription factors are displaced from highly condensed mitotic chromosomes every cell cycle (36). Two experimental observations suggest that maintenance of repression is compromised at or near this point in yeast. First, *HMR* is partially derepressed at the G_2/M border when silencer binding proteins, Orc2p and Orc5p, are inactivated (37). Second, silencing of a telomere-proximal *URA3* gene can be overcome exclusively at the G_2/M border by overexpression of its cognate transcriptional regulator (38). Programmed degradation of a critical silencing components at this stage in the cell cycle could also account for these results.

Finally, excision of sequences from *HMR* may permit relocalization of DNA fragments from a nuclear compartment that supports transcriptional repression to those that do not. There is evidence in yeast that Sir proteins are sequestered at telomeres and that efficient transcriptional repression afforded by the silent mating-type loci relies, in part, on their proximity to chromosome ends (39). Potentially, extrachromosomal rings without silencers are reactivated because they escape from localized pools of silencing factors.

Persistence of Silencing in Other Organisms. In female mammals, a *cis*-acting silencer-like element termed the X-inactivation center is required for establishment of repression of one of the two X chromosomes. Surprisingly, repression persists through successive generations after deletion of the element, indicating that the X-inactivation center is not required for inheritance of the silent state (40). A reasonable explanation is that structural features of the inactive chromosome are self-templating, even if the X-inactivation center is missing. The persistence of repressed chromatin also has been examined with *in vivo* recombination in *Drosophila*. In this case, transposed chromosomal elements that were subject to heterochromatic repression were excised (41). Similar to our results with yeast, the elements became transcriptionally active upon excision. Strikingly, reactivation occurred in cells that were mitotically quiescent and, therefore, did not require active growth as in *Saccharomyces* (14). Structural changes were shown to occur during cell-cycle progression, primarily during the G_1 to G_2/M interval.

Note. After this work was completed, a similar study of the structural fate of silent chromatin at *HML* was published (42). Structural changes were shown to occur during cell-cycle progression, primarily during the G_1 to G_2/M interval.

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