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DNA in Transcriptionally Silent Chromatin Assumes a Distinct Topology That Is Sensitive to Cell Cycle Progression

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Transcriptionally silent regions of the *Saccharomyces cerevisiae* genome, the silent mating type loci and telomeres, represent the yeast equivalent of metazoan heterochromatin. To gain insight into the nature of silenced chromatin structure, we have examined the topology of DNA spanning the *HML* silent mating type locus by determining the superhelical density of mini-circles excised from *HML* (*HML* circles) by site-specific recombination. We observed that *HML* circles excised in a wild-type (*SIR*$^+$) strain were more negatively supercoiled upon deproteinization than were the same circles excised in a *sir*$^-$ strain, in which silencing was abolished, even when *HML* alleles in which neither circle was transcriptionally competent were used. *cis*-acting sites flanking *HML*, called silencers, are required in the chromosome for establishment and inheritance of silencing. *HML* circles excised without silencers from cells arrested at any point in the cell cycle retained *SIR*-dependent differences in superhelical density. However, progression through the cell cycle converted *SIR*$^+$ *HML* circles to a form resembling that of circles from *sir*$^-$ cells. This decay was not observed with circles carrying a silencer. These results establish that (i) DNA in transcriptionally silenced chromatin assumes a distinct topology reflecting a distinct organization of silenced versus active chromatin; (ii) the altered chromatin structure in silenced regions likely results from changes in packaging of individual nucleosomes, rather than changes in nucleosome density; and (iii) cell cycle progression disrupts the silenced chromatin structure, a process that is counteracted by silencers.

The eukaryotic genome is divided into domains of distinct regulatory potential, dictated in part by local differences in chromatin structure. Mating type determination of the yeast *Saccharomyces cerevisiae* presents a readily accessible example of such chromatin-mediated effects on gene expression. Sequences resident at the *MAT* locus dictate the mating type of *S. cerevisiae*. The same sequences also reside at the homothalic mating locus *HML* or *HMR*, but at these loci the sequences are transcriptionally inactive and do not contribute to mating type determination (reviewed in references 28, 29, and 38). Repression of the silent mating type loci represents a region-specific but non-gene-specific form of gene regulation: translocation of the mating type genes resident at the silent loci to a different site activates expression of the genes, and insertion of heterologous genes into the *HM* loci results in repression of the inserted genes. Insertion of genes into the telomeric regions of yeast chromosomes similarly results in their repression, through a process mechanistically similar to that underlying silencing of the mating type loci (3).

Several lines of evidence suggest that transcriptional silencing of the *HM* mating type loci and telomeres derives from a heterochromatin-like organization of these loci. First, the silent loci and telomeres are relatively inaccessible to DNA-modifying agents, such as DNA methyltransferase and DNA repair factors, in vivo and to endonucleases in chromatin preparations in vitro (22, 41, 59, 63). Second, similar to DNA in heterochromatin in higher cells, transcriptionally silenced regions in yeast replicate late in S phase (56). Third, the nucleosomes from silenced chromatin, as probed by chromatography on methyl mercury columns, exhibit a distinction from those from active chromatin similar to that of nucleosomes from heterochromatin versus euchromatin in metazoans (13). Fourth, nucleosomes from the transcriptionally silent *HM* loci and telomeres in yeast exhibit reduced acetylation of lysine residues in the N-terminal domains of histones H3 and H4 relative to that of nucleosomes from active regions of the genome (9, 10). Further, the particular pattern of acetylation of histone H4 lysines in nucleosomes from the silent loci is identical to that of nucleosomes in centric heterochromatin in *Drosophila melanogaster* (9), indicating that the chromatin from the silent regions in yeast bears a structural signature of metazoan heterochromatin.

Formation and maintenance of the heterochromatin-like structure of the silent mating type loci require both *cis*-acting sites and *trans*-acting factors (38). The *cis*-acting sites involved in transcriptional silencing, known as the *E* and *I* silencers, are small negative regulatory sequences flanking each of the *HM* loci (2, 16). Silencers are both necessary and sufficient to support silencing: removal of silencers leads to full expression of *HML* and *HMR* (8, 44), while introduction of silencers next to *MAT* represses it (57). Silencers consist of various combinations of binding sites for Rap1p, Abf1p, and the origin recognition complex (ORC) (reviewed in reference 38) and are required for establishment of silencing, inheritance of the repressed state, and maintenance of repression during progression through the cell cycle (30).

Various *trans*-acting factors are required for transcriptional silencing. A number of proteins that perform essential functions in the cell are also required for silencing activity. These include histones as well as the proteins that bind to silencer elements. Specific alleles of the genes encoding histone H3 or H4 can attenuate silencing and alleviate position effect repression at the telomeres (35, 53, 65; for a review, see reference 38). Similarly, specific mutations in *RAP1*, *ABF1*, *ORC2*, and *ORC5* (the genes encoding Rap1p, Abf1p, and two subunits of the ORC, respectively) can activate the silent loci (5, 18, 37, 39, 40). In addition to these essential proteins required for silenc-

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ing, four proteins are involved exclusively in transcriptional silencing: mutational inactivation of *SIR2*, *SIR3*, or *SIR4* yields complete derepression of the mating type genes at *HML* and *HMR* and complete loss of telomeric silencing, and mutational inactivation of *SIR1* leads to defects in the establishment or maintenance of silencing of the silent loci, although such inactivation has no effect on telomeric silencing (3, 54).

The interactions among the proteins required for silencing provide a model by which these proteins promote silencing. Genetic and biochemical evidence indicates that Sir3p and Sir4p can both homodimerize and heterodimerize (47), and both of these proteins can bind to the amino-terminal domains of histones H3 and H4 (25, 33). Similar experiments have shown that Sir3p also interacts with histones H2A and H2B (26); that Sir2p interacts with Sir3p and Sir4p (31, 62); that Sir1p and Sir3p bind to ORC and Rap1p, respectively; and that both these proteins bind Sir4p (47, 66). In addition, Sir2p may modify the acetylation state of nucleosomes with which it associates (10). These observations suggest that silencers initiate local alteration of the chromatin structure by recruiting Sir1p and/or Sir3p through their direct interactions with ORC and Rap1p, respectively. Sir1p and/or Sir3p in turn recruits Sir4p and Sir2p to the silencer, which with Sir3p polymerize outward along the chromatin. In this fashion, Sir2p, Sir3p, and Sir4p form an extended complex that serves as an integral part of the silenced chromatin (26, 66). The precise nature of this silenced chromatin, though, remains unresolved.

The topology of eukaryotic DNA reflects the chromatin structure in which it resides, making topology an attractive tool for exploring the relation between chromatin structure and gene expression. Abraham et al. (1) examined the topology of DNA at the silent *HM* loci by using plasmids bearing a restriction fragment of chromosome III containing *HMR*. These investigators showed that a plasmid carrying *HMR* in a SIR^+ strain was more negatively supercoiled than the same plasmid propagated in an isogenic sir^- strain. This indicated that a change in DNA topology accompanies a change in the silencing state of *HMR*, at least for *HMR* propagated on a plasmid. However, in contrast to *HM* loci on the chromosome, *HM* loci carried on plasmids are not fully repressed (1, 2, 16). Moreover, the ARS elements in *HML* silencers do not serve as replication origins on the chromosome, although they do when resident on plasmids (15). Finally, the presence of a telomere can influence the silencing capacity of *HMR* as well as the replication timing of nearby sequences (17, 64). Therefore, the chromosomal context and the plasmid context for the *HM* loci are significantly different, and the topological features of silenced DNA on a plasmid do not necessarily reflect the topology or chromatin structure of a silenced locus in its chromosome configuration.

In this work, we explored the topology of silent chromatin at the *HM* loci more directly. Instead of examining DNA of plasmids containing both *HM* sequences and vector sequences, we analyzed *HM* sequences exclusively. This was achieved by excising the *HM* sequences from its chromosomal location as a circle in vivo by using site-specific recombination (see Fig. 1). This circle, bearing solely the in situ chromatin structure of the *HM* locus, can be readily examined. Using this strategy, we studied the supercoiling of DNA in circles excised from *HML* and obtained evidence demonstrating that DNA within the transcriptionally silenced *HML* locus is more negatively supercoiled than that in the active *HML* locus. We further show that this topological difference was due to the differential chromatin structures, but not the differential transcriptional states, of silenced and active *HML* loci. We also show that certain cell cycle-dependent events disrupted the silenced chromatin structure and that silencers can counteract this disruption. These results expand our understanding of the architecture of transcriptionally silenced chromatin and its establishment and maintenance in the cell cycle.

TABLE 1. Yeast strains

Strain	Genotype	Source or reference
Y2047b	*MAT***a** *HMR***a** *HML*α *E*Δ*79-113*::*SUP4-o I*Δ*242 LEU2-GAL10-FLP1 ura3-52 ade2-1 lys1-1 his5-1 can1-100* [cir^0]	30
YXB1	Y2047b *FRT-E-HML*α*-I-FRT*	This work
YXB1s	YXB1 *sir3*::*URA3*	This work
YXB2	Y2047b *FRT-E-hml*α*1*α*2*Δ*-I-FRT*	This work
YXB2s	YXB2 *sir3*::*URA3*	This work
YXB3	Y2047b *E-FRT-HML*α*-FRT-I*	This work
YXB3s	YXB3 *sir3*::*URA3*	This work
YXB4	Y2047b *E-FRT-hml*α*1*α*2*Δ*-FRT-I*	This work
YXB4s	YXB4 *sir3*::*URA3*	This work
YXB5	Y2047b *E-FRT-hml*::β*1-FRT-I*	This work
YXB5s	YXB5 *sir3*::*URA3*	This work
YXB6	Y2047b *E-FRT-hml*::β*2-FRT-I*	This work
YXB6s	YXB6 *sir3*::*URA3*	This work

MATERIALS AND METHODS

Plasmids and strains. Plasmids for yeast strain constructions were derived from pUC26, which consists of the *Bam*HI fragment of yeast chromosome III containing *HML* (see Fig. 2A) cloned into the *Bam*HI site of pUC19. Various modifications of the *HML* sequence carried on pUC26 are illustrated below (see Fig. 2A and 3A). Two *FLP1* recombination target sites (*FRT* [11]) were inserted at the *Bsu*36I and *Sna*BI sites that bracket *HML* to make pYXB1. Plasmid pXYB3 was similarly constructed except that *FRT* sites were inserted at the *Cla*I and *Pvu*II sites. A 294-bp fragment (coordinates 3319 to 3613 of the *Bam*HI fragment) containing the promoters for the α*1* and α*2* genes of *HML* was deleted from pYXB1 and pYXB3, generating pYXB2 and pYXB4, respectively. Plasmids pYXB5 and pYXB6 were derived from pYXB3 by replacing its *Bst*BI fragment with the 1.94-kb *Acc*I-*Cla*I fragment of the *lacZ* gene sequence and the 3.75-kb *Cla*I fragment of plasmid pBRβH (6), respectively. The latter consists of a 2.77-kb fragment of *lacZ* including the 1.94-kb *Acc*I-*Cla*I fragment and a 0.98-kb *tetA* sequence of pBR322 (coordinates 23 to 1002).

Yeast strains were derived from Y2047b (30) and are listed in Table 1. The *SUP4-o* allele, which can suppress the *can1-100* mutation, is present at *HML* in strain Y2047b, rendering it sensitive to canavanine. The *HML*-containing *Bam*HI fragment of pYXB1 was used to transform Y2047b to canavanine resistant (due to the loss of *SUP4-o*), resulting in strain YXB1. Strains YXB2 through YXB6 were similarly constructed by using the *Bam*HI fragments of plasmids pYXB2 through pYXB6, respectively. Strains YXB1s through YXB6s were derived from YXB1 through YXB6, respectively, by disrupting the *SIR3* gene with *URA3* as described previously (44). The relevant genotypes of each strain were confirmed by Southern blotting.

Growth of yeast cultures. Yeast cultures were grown at 30°C in YPR medium (1% yeast extract, 2% Bacto Peptone, and 2% raffinose). When needed, galactose was added to YPR cultures at a concentration of 2%; α-factor (Sigma), hydroxyurea (Sigma), and nocodazole (Sigma) were used at 10 μg/ml, 0.2 M, and 10 μg/ml, respectively. Cells were judged to be in stationary phase when there was no increase in the optical density of the culture during the previous 24-h period and >95% of cells were unbudded (4).

Analysis of DNA circles excised from the *HML* locus by *FLP1*. Yeast strains listed in Table 1 (except Y2047b) were grown in YPR medium to early log phase (optical density at 600 nm = 0.6). Galactose was then added to the culture to induce the expression of *FLP1*. After 2.5 h of incubation, cells were collected by centrifugation. Nucleic acid was then isolated by the glass bead method (34) and fractionated on agarose gels in 0.5× TPE (45 mM Tris, 45 mM phosphate, 1 mM EDTA [pH 8.0]) supplemented with chloroquine (Sigma). DNA circles were detected by Southern blotting. Following hybridization, the washed filters were exposed to phosphor screens and images were scanned on a Molecular Dynamics model 425 PhosphorImager. Gaussian centers of topoisomer distributions were determined as described previously (69). As in similar experiments reported previously (30), excision of the *FRT*-bracketed sequences was greater than 80% efficient during the induction period in all the experiments described.

FACS analysis. Cells for fluorescence-activated cell sorter (FACS) analysis were prepared as described previously (49). Briefly, cells were fixed in 70% ethanol and treated with 1 mg of RNase A per ml and 0.5% pepsin before their

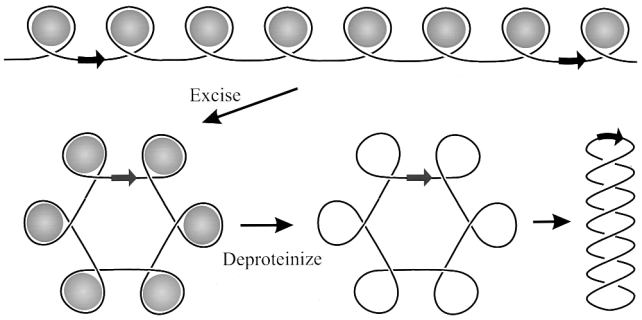

FIG. 1. Strategy for examining the topology of chromosomal DNA at its resident locus. Two sites for a site-specific recombinase are inserted in direct orientation to flank the chromosome region of interest. Recombination between these sites leads to the excision of a circular mini-chromosome. After deproteinization, the topology of DNA in the circle can be readily examined. Shaded circles, nucleosomes; thick arrows, target sites for the site-specific recombinase.

DNA was stained by propidium iodide. Cells were diluted to 10^6/ml and sonicated for 10 s before being analyzed on a Coulter Electronic Epics 753 cell sorter.

RESULTS

Transcriptional silencing increases negative supercoiling of DNA in vivo. To study the topology of DNA in transcriptionally silenced chromatin in its chromosomal configuration, we constructed strains in which a segment of the chromosome at *HML* could be excised as a mini-circle in a controlled manner. This was achieved by inserting two recombination target sites (*FRT* [67]) for the *FLP1* site-specific recombinase in direct orientation at positions flanking *HML* (Fig. 1 and 2A). Induction of *FLP1* from a *GAL10-FLP1* construct resident elsewhere in the genome (30, 67) yielded recombination between the two *FRT* sites and excision of *HML* as a closed circular species. DNA spanning *HML* in the chromosome passes over itself a number of times as it threads around the nucleosomes packaging the locus. Recombination between the two *FRT* sites captures the chromatin structure over the locus at the time of excision by closing the path of the DNA. As a consequence, the loops of DNA around the nucleosomes become topologically equivalent to negative supercoils of the DNA (Fig. 1), which can be visualized by deproteinization of the chromatin, fractionation on chloroquine agarose gels, and detection by Southern hybridization. This strategy has been successfully applied previously in studies of DNA circles excised from plasmids in yeast (19) and *Escherichia coli* (43).

As a first test of this approach, we constructed strains YXB1 and YXB1s, isogenic *SIR*$^+$ and *sir3*$^-$ strains in which two *FRT* sites flank the entire *HML* locus, including the *E* and *I* silencers (Fig. 2A). The two *FRT* sites lie 4.1 kb apart in these strains, dictating the size of the excised circle. To excise the *HML* circle from these two strains, we grew cells under noninducing conditions and then shifted them to galactose-containing media to induce the expression of *FLP1*. Two and one-half hours after induction, we isolated DNA from the two strains, fractionated total DNA by agarose gel electrophoresis in the presence of chloroquine, and identified *HML*-specific sequences by Southern hybridization. As shown in Fig. 2B, the *HML* circle excised from the *SIR*$^+$ strain YXB1 had a different superhelical density

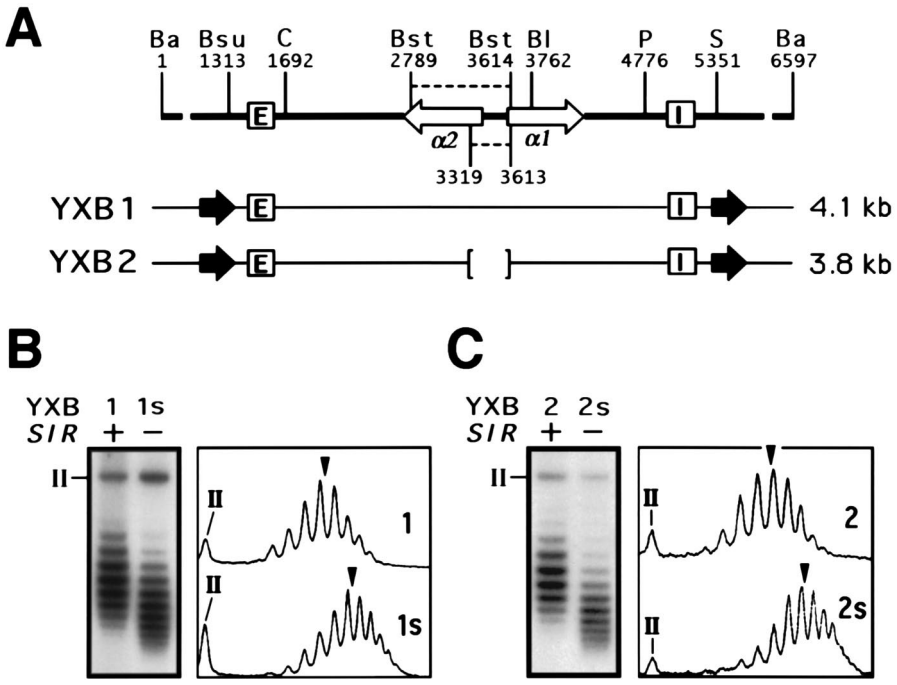

FIG. 2. DNA in transcriptionally silenced chromatin is more negatively supercoiled than in active chromatin. (A) At the top, a map of the genomic *Bam*HI fragment of yeast chromosome III containing *HML*, drawn to scale, is shown. Open arrows, α*1* and α*2* genes; open squares, *HML* silencers. Ba, Bl, Bst, Bsu, C, P, and S, sites for restriction endonucleases *Bam*HI, *Blp*I, *Bst*BI, *Bsu*36I, *Cla*I, *Pvu*II, and *Sna*BI, respectively. In strain YXB1, two *FRT* (*FLP1* recombination target) sites were inserted at Bsu and S in direct orientation. Strain YXB2 was derived from YXB1 by deleting the 3319-3613 fragment of *HML* from positions 3319 to 3613. The distance between the *FRT* sites in each strain is shown to the right of the construct. (B and C) Cells of the indicated strains were grown in YPR medium to early log phase, at which time galactose was added and the cultures were incubated for 2.5 h. DNA isolated from cells was fractionated by agarose gel electrophoresis in the presence of 30 μg of chloroquine per ml. Under this condition, the more negatively supercoiled circles migrate more slowly in the gel. *HML* circles were revealed by Southern blotting (left sides of panels in B and C). The position of form II (nicked) circles is indicated. Densitometer scans of the phosphorimages in the left panels are shown on the right. The Gaussian center of each distribution is indicated (arrowhead).

than the same circle excised from the isogenic *sir3*$^-$ strain YXB1s (for convenience, circles excised from a *SIR*$^+$ strain are referred to as *SIR*$^+$ circles hereafter and circles excised from a *sir3*$^-$ strain are referred to as *sir*$^-$ circles). The topological difference (distance between the Gaussian centers of the Boltzmann distributions of topoisomers) was a linking number difference (ΔLk) of ca. 2, and, since under these conditions the more negatively supercoiled species migrate more slowly (42), the *SIR*$^+$ circle was more negatively supercoiled. Additional studies using two-dimensional chloroquine gels (71) confirmed that the *SIR*$^+$ circle was more negatively supercoiled than the *sir*$^-$ circle (see Fig. 3D). Thus, we conclude that DNA passes over itself more often in chromatin from the silenced *HML* locus than in chromatin from the activated version of the same locus.

Transcription in yeast induces detectable topological changes in the DNA template, which can be attributed to unwinding of DNA by RNA polymerase binding (52). Two genes (α*1* and α*2*) at *HML* are transcriptionally repressed in a *SIR*$^+$ strain (e.g., YXB1) but are transcribed in strains defective in transcriptional silencing. Although the effect of transcription on topology of the *HML* circles would be predicted to be the opposite of that which we observed (52), we tested directly whether the difference in the topology of *HML* DNA between YXB1 and YXB1s was due to the differential transcriptional states of *HML* in these strains. To do so, we constructed strains YXB2 and YXB2s from YXB1 and YXB1s, respectively, by deleting a 294-bp sequence containing the divergent promoters for α*1* and α*2* (Fig. 2A). In strain YXB2, as in strain YXB1, *HML* is repressed. In strain YXB2s, the *HML* locus is transcriptionally inactive even though the locus is not silenced. As shown in Fig. 2C, the *SIR*$^+$ circle from strain YXB2 is more negatively supercoiled than the *sir*$^-$ circle from strain YXB2s, with a topological difference (ΔLk) of slightly greater than 2. This is comparable to the difference in superhelical density of *HML* circles from strains YXB1 and YXB1s. These results indicate that the greater negative supercoiling of transcriptionally silenced *HML* DNA is not the result of the absence of transcription but rather the result of the distinct chromatin structures at this locus in a silenced versus active state.

Silencers are not required for maintenance of the distinct topology of silenced DNA. *HML* is flanked by the *HML E* and *I* silencers, which serve as foci through which *trans*-acting components are recruited to promote transcriptional silencing of the intervening region (reviewed in reference 38). While silencers are essential for establishment and inheritance of silencing, it was recently determined that they are dispensable for the maintenance of silencing in the absence of cell cycle progression (30). To test whether silencers are required for maintaining the higher degree of negative supercoiling of DNA in transcriptionally repressed chromatin, we constructed strain YXB3 and its *sir*$^-$ derivative YXB3s, in which two *FRT* sites bracket the coding region of *HML* but exclude the silencers (Fig. 3A). Accordingly, transcriptionally silenced chromatin at *HML* could be excised as a circle bearing no silencer by recombination between the *FRT* sites. As shown in Fig. 3B, the silencer-free *HML* circle excised from the *SIR*$^+$ strain YXB3 is more negatively supercoiled than its counterpart excised from the *sir3*$^-$ strain YXB3s. As with silencer-bearing circles, deletion of the promoter for α*1* and α*2* had no effect on the topological difference between the silencer-free *SIR*$^+$ and *sir*$^-$ circles (Fig. 3B, lanes 4 and 4s). We conclude from these experiments that the presence of a silencer on an excised circle is not required for *SIR*-dependent differential supercoiling.

The topological difference between the YXB3 and YXB3s circles (a ΔLk of ca. 1 [Fig. 3B]) is smaller than that between the YXB1 and YXB1s circles (a ΔLk of ca. 2 [Fig. 2B]). One potential factor contributing to this distinction between the two sets of circles is their relative sizes. To determine whether the size of the excised circle affects the magnitude of the *SIR*-dependent superhelical density shift, we constructed strains YXB5 and YXB6, in which both *FRT* sites reside between the silencers but flank sequences of 4.3 and 6.1 kb, respectively (Fig. 3A). Strain YXB5 was constructed by replacing a portion of the *HML* locus with DNA internal to the coding region of *lacZ*, a gene that can be repressed by the silencing apparatus when inserted into *HML* (7). Strain YXB6 was constructed by replacing a portion of *HML* with *lacZ* sequences plus *tetA* sequences from pBR322. As shown in Fig. 3C, the difference in the superhelical density of the *SIR*$^+$ circle versus the *sir*$^-$ circle excised from strains YXB5 and YXB5s (a ΔLk of ca. 5) was significantly greater than that of the corresponding circles excised from strains YXB3 and YXB3s. An even larger supercoiling difference (a ΔLk of 10) was observed between the *SIR*$^+$ and *sir*$^-$ circles of YXB6 and YXB6s. Thus, within these sets of strains, there exists a correlation between the size of the excised circle and the magnitude of the topological difference of a circle in a silenced versus an active state.

Cell cycle progression alters the topological state of silenced DNA lacking silencers. We previously demonstrated that relief of *HML* silencing following deletion of its associated silencer required progression through the cell cycle (30). To examine whether cell cycle progression was similarly required to convert a silencer-free *HML* circle from the highly supercoiled state associated with silencing to the more relaxed state associated with active chromatin, we monitored the supercoil state of an excised circle during growth of the culture after excision. A culture of strain YXB6 was grown in noninducing medium, shifted to galactose-containing medium for 2.5 h, and then shifted to glucose medium without galactose. The short half-life of Flp1p in the cell and the stringent repression by glucose of *FLP1* expression from *GAL10-FLP1* ensured that circles were excised during, and only during, the 2.5 h of galactose induction (30, 67). Thus, we could follow the fate during subsequent growth of the culture of only those circles that had been excised during the period of galactose induction. As shown in Fig. 4A and C, silencer-free circles excised from the *SIR*$^+$ strain YXB6 initially exhibited the high degree of negative supercoiling associated with silenced chromatin. However, within 2 h of growth (more than one generation), approximately half of the circles exhibited a topological form identical to that of a circle excised from a *sir*$^-$ strain, and after 4 h of growth (almost three generations), the topological profile of the excised circles was essentially indistinguishable from that of the *sir*$^-$ circles. In contrast, silencer-bearing *HML* circles excised in strain YXB2 showed no decay in supercoiling when subjected to an identical regimen (Fig. 4B and D). These data establish that cell growth triggers the relaxation of DNA in circles excised from silenced *HML* loci, suggesting that in the absence of silencers cell growth irreversibly disrupts the structure of silenced chromatin.

Three aspects regarding the decay of the silenced chromatin structure of silencer-free circles during growth are noteworthy. First, the decay from inactive to active chromatin is quite cooperative. Rather than a gradual transition from a high level of supercoiling to reduced supercoiling through a series of intermediate forms occurring, the population of inactive chromatin circles disappears and is replaced with a population of active-state circles. This is consistent with genetic experiments indicating a two-state model of active versus silenced chromatin (45, 54) and provides the first physical evidence of such a

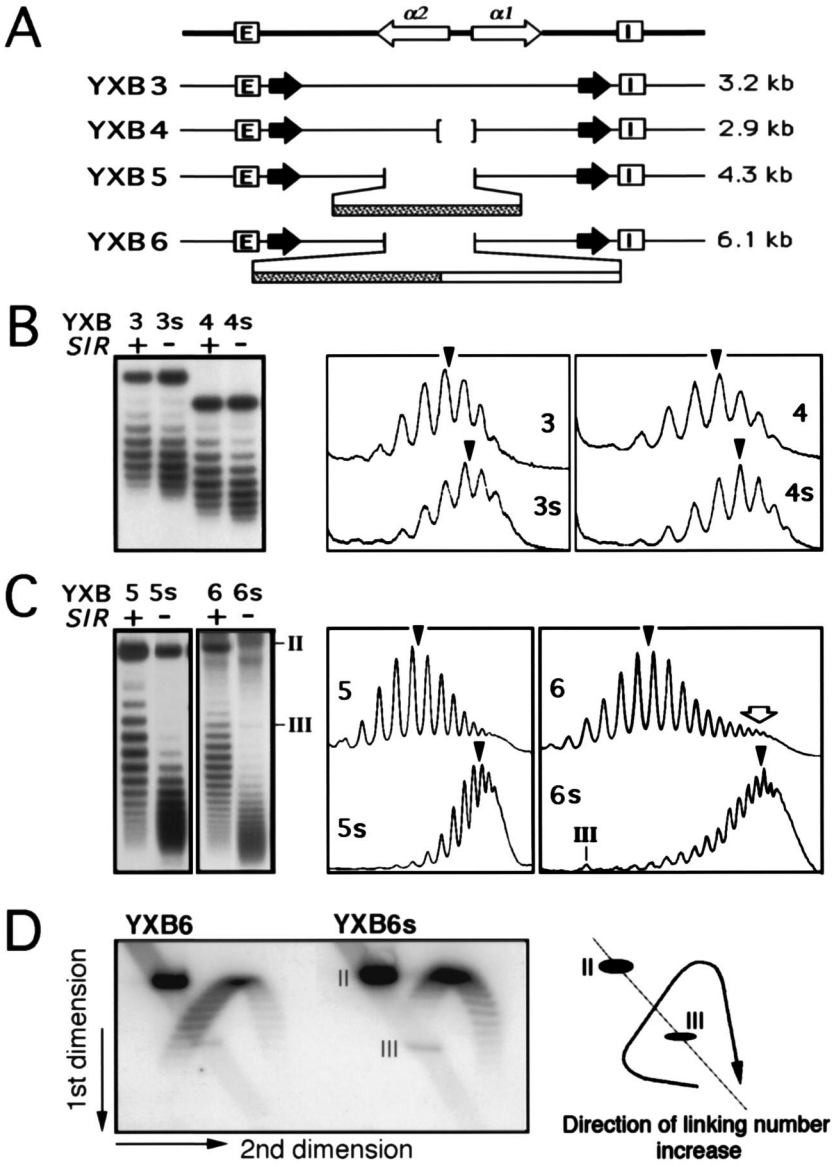

FIG. 3. Silencers are dispensable for maintenance of the distinct topology of silenced DNA. (A) *HML* alleles in strains constructed for excising silencer-free circles from *HML*. In strain YXB3, two *FRT* sites were inserted at C and P (Fig. 2A) in direct orientation. YXB4 was derived from YXB3 by deleting the fragment of *HML* from positions 3319 to 3613. YXB5 and YXB6 were derived from YXB3 by replacing the *Bst*BI fragment with the *Cla*I-*Acc*I fragment of the *lacZ* coding sequence, and the *Cla*I fragment of the plasmid pBRβH (6), respectively. The distance between the *FRT* sites in each strain is shown to the right of the construct. (B and C) *HML* circles were prepared and analyzed as described for Fig. 2). Results are shown for *HML* circles excised from strains YXB3, YXB3s, YXB4, and YXB4s (B) and YXB5, YXB5s, YXB6, and YXB6s (C). Chloroquine was present at 30 and 20 μg/ml in gels in panels B and C, respectively. (D) Left: *HML* circles from strains YXB6 and YXB6s were fractionated on two-dimensional chloroquine gels as described previously (71) and visualized by Southern blotting. Chloroquine was used at 3 and 20 μg/ml in the first and second dimensions, respectively. The locations of form II (nicked circles) and form III (linear molecules) species are indicated. Right: diagram of two-dimensional chloroquine gel patterns, indicating the direction of migration (dotted line) and the positions of migration of forms II and III and supercoiled species (arrow, pointing from the positions of circles with lower [more negative supercoiling] to those of higher [less negative supercoiling] linking number).

two-state model. Second, the excised, silencer-free circles are passive bystanders of the cell cycle events occurring in the cell, rather than active participants. In particular, these circles do not undergo DNA replication (the circles do not contain an origin of replication, and, as expected, the absolute number of silencerless circles does not increase during growth of the culture following excision of the circles [data not shown]) or active DNA segregation (the circles do not contain a centromere or a silencer and do not enhance the segregation potential of plasmids carrying them [reference 36 and data not shown]). Accordingly, decay of the silenced state is triggered by signals associated with the cell cycle rather than by participation in the events of the cell cycle. Third, since the circles do not undergo DNA replication and since the circles are closed entities not covalently linked to chromosomal DNA, the nucleosome density of excised circles cannot change by segregation to daughter molecules, by replication-associated nucleosome deposition, or by lateral diffusion along the DNA. Thus, decay in the superhelical density occurs in the absence of the usual means of altering the number of nucleosomes over the region. Accordingly, the difference in the topological state of the silenced versus active chromatin likely reflects a difference in the way

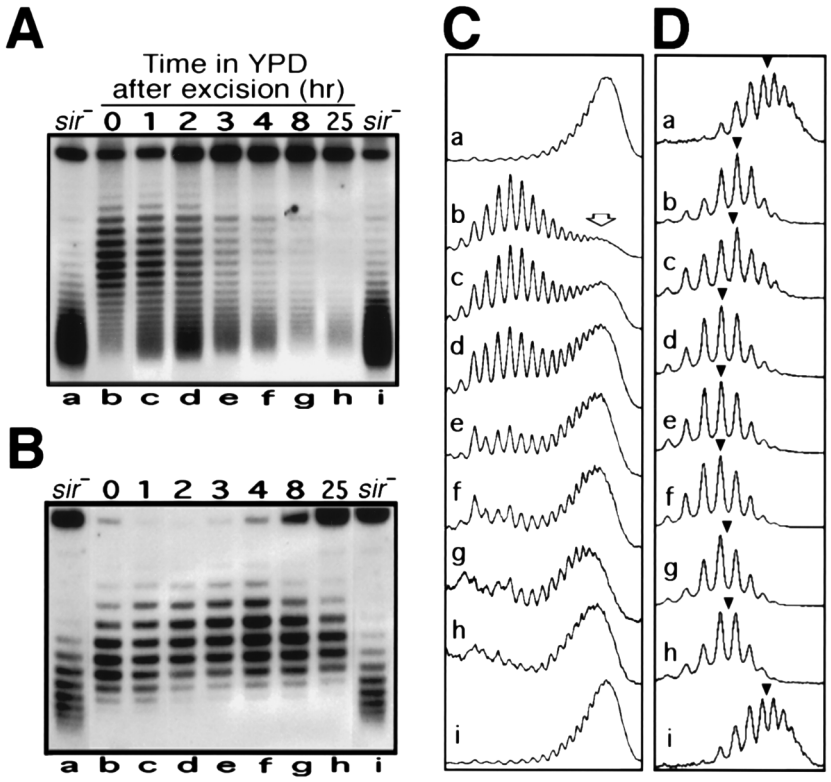

FIG. 4. The topological state of silenced DNA lacking silencers is sensitive to cell cycle progression. Cells of strains YXB6 and YXB2 were grown in YPR medium to early log phase and then grown for 2.5 h in the presence of galactose. Cells were then harvested, washed with YPD medium, and resuspended in YPD medium for further growth. Aliquots of cells were taken for DNA isolation after the indicated times of growth in YPD medium. DNAs from strains YXB6s and YXB2s cells were prepared as described for Fig. 2. (A) Phosphorimage showing *HML* circles in DNA samples from strains YXB6 and YXB6s. The agarose gels contained 20 μg of chloroquine per ml. Lanes b to h, DNA samples from strain YXB6; lanes a and i, DNA samples from strain YXB6s. (B) PhosphorImage showing *HML* circles in DNA samples from strains YXB2 and YXB2s. The agarose gels contained 30 μg of chloroquine per ml. Lanes are arranged as in panel A. (C and D) Densitometer tracings of the lanes from the phosphorimages in panels A and B, respectively.

the DNA is wrapped around nucleosomes or other chromatin proteins rather than a difference in the density of nucleosomes over the region.

Specific cell cycle events affect the topology of silenced *HML* circles. Since progression through the cell cycle disrupts the topology associated with silenced DNA, we were interested in determining whether such disruption could be ascribed to specific events within the cell cycle. As a preliminary step, we probed the topology of *HML* circles excised from cells arrested at different stages of the cell cycle. We grew YXB6 cells to early log phase in YPR medium, split the culture, and arrested cells at either the G_1, early S, or G_2/M phase of the cell cycle by treatment with α-factor (12, 27), hydroxyurea (60), or nocodazole (24, 32), respectively. Circles were excised by addition of galactose, and cells were harvested 2.5 h after the addition of galactose. As indicated by FACS analysis, most of the cells were arrested at the appropriate stage of the cell cycle prior to induction and remained arrested throughout the induction period (Fig. 5A). In a parallel experiment, cells in stationary phase were induced by addition of galactose. The topology of the circles obtained under these conditions was compared with that of circles obtained from asynchronous cultures of *SIR*$^+$ and *sir3*$^-$ strains.

The results of this analysis are presented in Fig. 5B and C. The silencer-free *HML* circles excised from cells in stationary phase, G_1, or early S phase all exhibited equivalent levels of negative superhelical density. Cells arrested by nocodazole treatment yielded *HML* circles with a superhelical density lower than that of circles from cells arrested at the other stages of the cell cycle, with a ΔLk of about 2. Nocodazole affected the superhelical density only of silenced DNA: no difference in supercoil density was observed in circles excised from nocodazole-arrested versus stationary-phase or hydroxyurea-arrested *sir*$^-$ strain YXB6s (data not shown). This observation suggests that the silenced chromatin over the *HML* locus in cells arrested in G_2/M by nocodazole is somewhat more relaxed than it is at other stages of the cell cycle. However, the silenced *HML* chromatin in nocodazole-arrested cells is not as relaxed as it is when the locus is active (a ΔLk of 2 versus 10).

As might be anticipated, circles excised from an asynchronous population contained a subpopulation with a topoisomer distribution equivalent to that of circles excised from a *sir*$^-$ strain (Fig. 5C). This likely reflects the decay of silencing of excised silencer-free circles that occurs in the ongoing growth during the 2.5-h induction period.

Progression through specific intervals of the cell cycle reduces the negative supercoiling of *HML* circles bearing no silencer. The above results suggested that arrest at none of the three stages tested yields significant decay of silenced chromatin separated from a silencer. To test whether passage through the intervals between these stages induces decay of silencing, we excised circles in cells arrested at one stage of the cell cycle and then examined the topology of the circles after cells had progressed specifically to a subsequent stage in the cell cycle. In the first experiment, cells of strain YXB6 were first arrested in the G_1 phase by α-factor and then treated with galactose for

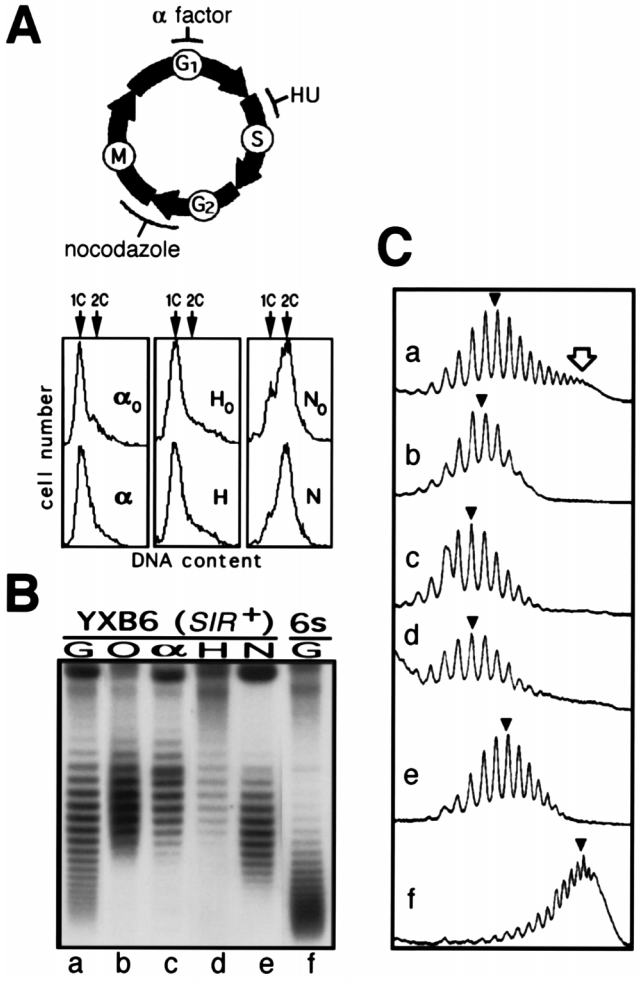

FIG. 5. Specific cell cycle events affect the topology of silenced DNA. (A) Experimental design. Early-log-phase cells growing in YPR medium were arrested in G_1, early S phase, and G_2/M of the cell cycle by a 2.5-h exposure to α-factor, hydroxyurea (HU), and nocodazole, respectively. Cells thus treated are designated α_0, H_0, and N_0, respectively. The arrested α_0, H_0, and N_0 cells were then incubated with galactose for 2.5 h while still in the presence of the growth-arresting agent. Cells are designated α, H, and N after incubation with galactose. Samples of the cultures cells were analyzed by FACS analysis, and the results are shown as histograms. (B) DNAs from exponentially growing cells of strain YXB6 incubated with galactose for 2.5 h (lane a), from stationary-phase cells incubated with galactose for 2.5 h (lane b), and from cells treated as described for panel A (lanes c, d, and e) were fractionated on agarose gels containing 20 μg of chloroquine per ml. DNA from an exponential-phase culture of strain YXB6s incubated with galactose for 2.5 h was also used (lane f). Shown is the phosphorimage of the fractionated DNA after probing for *HML* circles. (C) Densitometer tracings of the lanes from the phosphorimage in panel B. The Gaussian center of each distribution is indicated (filled arrowheads). The Gaussian center of the topoisomer distribution of *sir*$^-$ *HML* circles, as determined from lane f, is also shown (open arrow).

2.5 h to induce the excision of silencer-free *HML* circles. An aliquot of cells was shifted into yeast extract-peptone-dextrose (YPD) medium lacking α-factor but supplemented with hydroxyurea and was incubated for another 3 h. During this period, cells exited G_1 phase but progressed only as far as early S phase, as confirmed by FACS analysis (Fig. 6A). This transit induced the appearance of a small population of circles topologically equivalent to those excised from a *sir*$^-$ strain (referred to as a *sir*$^-$ configuration), although the majority of the *SIR*$^+$ circles maintained their topological profile (Fig. 6B and C).

A second aliquot of cells was shifted into YPD medium lacking α-factor but containing nocodazole. After incubation for 3 h, most cells had exited G_1 and accumulated with a G_2 content of DNA. In contrast to results observed with S-phase-arrested cells, the topology of *HML* circles in cells that traversed the G_1 to G_2/M interval of the cell cycle decayed almost entirely from the condensed *SIR*$^+$ configuration to the relaxed *sir*$^-$ configuration (Fig. 6B and C).

In a final experiment, we arrested cells with nocodazole, excised the silencer-free *HML* circle, and then transferred cells to nocodazole-free medium in the presence of α-factor. After 3 h, during which time most cells had progressed from the G_2/M block to G_1 (Fig. 6A), the topology of the circles remained largely condensed, although with a subpopulation of circles in a *sir*$^-$ configuration (Fig. 6B and C, lane h). In all these experiments, the *HML* circles lacked an origin of replication or a centromere and thus did not participate in DNA replication or mitosis. We conclude from these observations that in the absence of an attached silencer, the condensed chromatin is sensitive to signals in the cell that orchestrate the events of the cell cycle and in particular to those signals that occur between early S phase and mitosis.

Nocodazole induces a rapid decrease in negative supercoiling of DNA in *HML* circles bearing no silencer. To explore whether the decay in topology observed in the previous experiment resulted from transition of specific segments of the cell cycle or from accumulation at a particular point in the cell cycle, we examined the decay of silenced chromatin in synchronized cells. Cells of strain YXB6 were arrested with α-factor at G_1, and the silencer-free *HML* circle was excised. For one half of the culture, cells were transferred to fresh YPD medium in the absence of α-factor and allowed to grow without any further cell cycle arrest. Samples were taken from this synchronized culture at various times to assess the topological state of the circle. In a parallel culture, cells were transferred to YPD medium lacking α-factor and containing nocodazole. After 3 h, most of the cells had accumulated at G_2/M (Fig. 7B) and were harvested for analysis of the topological state of the circle.

The results of this analysis are shown in Fig. 7. As we had observed with the asynchronous population (Fig. 4), growth of the synchronized culture led to decay of the excised, silencer-free *HML* circle from a *SIR*$^+$ configuration to a *sir*$^-$ configuration. However, the decay of the *SIR*$^+$ configuration in the growing culture was not as rapid as that seen in cells released into nocodazole medium. For instance, in 2 h, by which time a significant proportion of the cells had completed mitosis (Fig. 7B, lane f), only about 30% of the circles had decayed to the *sir*$^-$ configuration. Even by 3 h, by which time most cells had entered a second cell cycle, only about 50% of the circles had decayed. In contrast, and as noted above, most of the circles in cells arrested by nocodazole after release from α-factor had decayed to the *sir*$^-$ state (Fig. 7A, lane j). These results confirm that cell cycle progression causes decay of silenced chromatin in the absence of silencers but indicate that nocodazole-induced arrest accelerates the decay process. Whether this accelerated decay results from an enhanced activity of the signal(s) responsible for decay during normal cell cycle progression or whether it represents a novel activity induced by nocodazole treatment is discussed below.

DISCUSSION

Transcriptional silencing in yeast shares many features with a variety of stable but nongenetic forms of gene regulation, including position effect variegation in *Drosophila* (70) and X-chromosome inactivation (20, 23) and chromosomal imprinting (46, 55, 61) in mammals. Position effect variegation results from metastable changes in the euchromatic versus

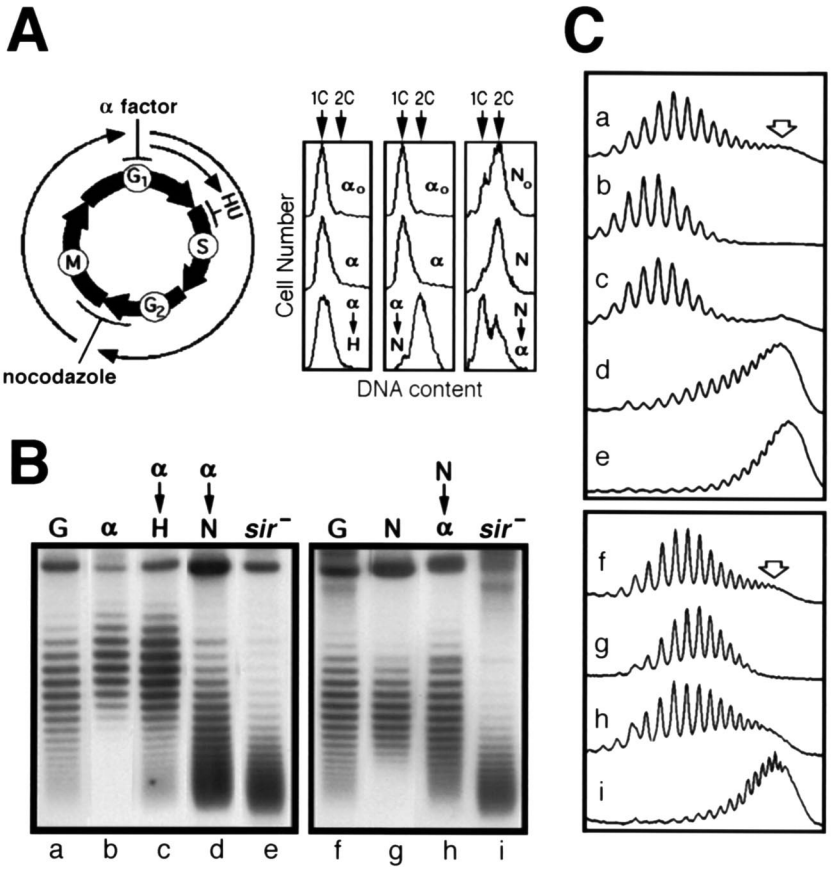

FIG. 6. Transit in certain intervals of the cell cycle reduces the negative supercoiling of *HML* circles bearing no silencer. (A) Experimental design. YXB6 cells grown to early log phase in YPR medium were incubated with α-factor (α_0 cells) or nocodazole (N_0 cells) for 2.5 h and then induced with galactose for 2.5 h (to yield α cells and N cells). α cells were washed, resuspended in YPD medium containing hydroxyurea (HU) or nocodazole, and incubated for 3 h (α→H cells or α→N cells, respectively). N cells were washed, resuspended in YPD medium containing α-factor, and incubated for 3 h (N→α cells). FACS analysis profiles of the indicated cell populations are shown. (B) Phosphorimage showing topoisomer distribution of *HML* circles isolated from the indicated cell populations. Lanes a and f, asynchronous culture of strain YXB6; lanes e and i, asynchronous culture of strain YXB6s. (C) Densitometer tracings of the lanes from the phosphorimage in panel B. The gaussian center of the topoisomer distribution of *sir*⁻ *HML* circles, as determined from lanes e and i, is indicated (arrows).

heterochromatic state of genes inserted near heterochromatic regions of the genome. X-chromosome inactivation is established by heterochromatization emanating from a *cis*-acting X-chromosome inactivation center, and a similar mechanism may underlie chromosomal imprinting. In none of these cases is the structural difference between heterochromatin and euchromatin known. We have explored that difference using yeast silent mating type loci as a model system.

DNA in transcriptionally silenced chromatin is more negatively supercoiled. The topology of eukaryotic DNA reflects the density of nucleosomes along the DNA, the configuration of the nucleosomes, and the overall organization of the chromatin domain. We have analyzed the topology of DNA within *HML* by excising *HML* from its resident locus as a mini-circle (*HML* circle) in vivo and examining the supercoiling of DNA in the circle. We found that an *HML* circle in a SIR^+ strain is more negatively supercoiled than that in an isogenic sir^- strain. This validates an earlier, less direct observation that a plasmid harboring *HMR* is more negatively supercoiled in a SIR^+ strain than in a sir^- strain (1) and establishes that DNA within transcriptionally silenced chromatin assumes a distinct topology in which the DNA crosses over itself more often than it does in the active locus (Fig. 1).

The major contribution to the observed negative supercoiling of DNA in eukaryotic chromatin is wrapping of DNA into nucleosomes in vivo (21). Wrapping of DNA on a nucleosomal core particle introduces an average ΔLk of about −1 per nucleosome in vitro (58). This change is dependent on the acetylation level of the core histones. Nucleosomes assembled with highly acetylated histones exhibit a smaller ΔLk (about −0.8 per nucleosome), suggesting that histone acetylation could release negative supercoils previously constrained by nucleosomes (50). The acetylation level of histones H3 and H4, but not H2A or H2B, is responsible for reducing the DNA linking number change during nucleosome assembly (51). If the above in vitro results are also true in vivo, the negative supercoils released by acetylating histones would be relaxed by DNA topoisomerases (68), and thus the local DNA would be less negatively supercoiled. Therefore, DNA in chromatin with less histone acetylation would be more negatively supercoiled in vivo than would the same DNA in chromatin with greater histone acetylation.

The fact that hypoacetylation of histones H3 and H4 is strictly associated with transcriptionally silenced chromatin (9, 10) may explain our observation that circles excised from silenced *HML* loci were more negatively supercoiled than those from active *HML* loci. For example, the 6.1-kb segment of silenced *HML* bracketed by *FRT* sites in strain YXB6 could accommodate 37 nucleosomes (165 bp per nucleosome). The total linking number deficit due to chromosomal assembly with

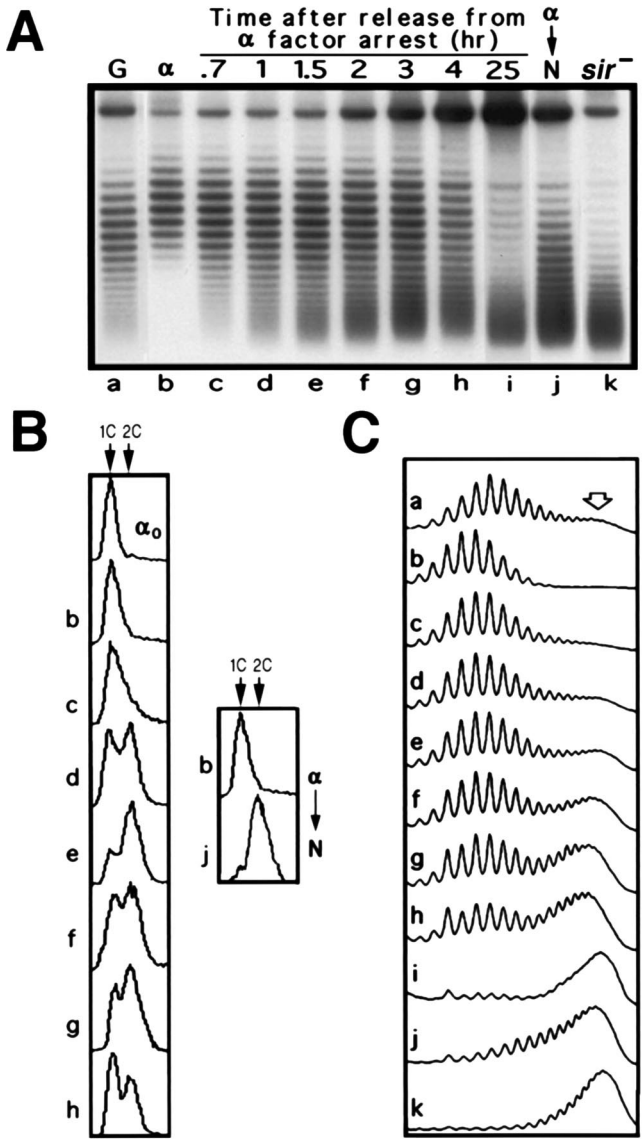

FIG. 7. Nocodazole induces a rapid decrease in the level of negative supercoiling of DNA in *HML* circles bearing no silencer. (A) YXB6 cells were grown in YPR medium to early log phase. An aliquot of the culture was incubated with galactose for 2.5 h before being collected for DNA isolation (designated G). The remainder of the culture was incubated with α-factor for 2.5 h (designated α_0) and then with galactose for an additional 2.5 h. An aliquot of the α cells was removed for DNA isolation (designated α), and the remainder was transferred to YPD medium without α-factor or galactose. Nocodazole was added to an aliquot of the YPD culture, and incubation continued for 3 h before DNA isolation (designated α→N). The remainder of the YPD culture was incubated without further treatment, and aliquots of cells were taken at the indicated times following transfer to YPD medium for DNA isolation. DNAs isolated from the above aliquots of YXB6 cells were analyzed by gel electrophoresis in the presence of 20 μg of chloroquine per ml. The sample in lane k was isolated from YXB6s cells treated with galactose for 2.5 h. (B) FACS analysis of the indicated cell samples. (C) Densitometer scan of the topoisomer distributions of the *HML* circles shown in panel A. The Gaussian center of the topoisomer distribution of *sir*⁻ *HML* circles, as determined from lane k, is indicated (arrow).

hypoacetylated histones would be −37 (assuming a ΔLk for assembling each nucleosome of −1 [58]). On the other hand, if this region is derepressed and histones are highly acetylated, the linking number deficit would be reduced to −30 (assuming a ΔLk for each nucleosome of −0.8 [50]). Therefore, for a 6.1-kb segment of chromatin, the maximum ΔLk between silenced and derepressed states attributable to acetylation would be −7. The actual topological difference between the 6.1-kb YXB6 circle and its *sir*⁻ counterpart is a ΔLk of about −10. Therefore, hypoacetylation of nucleosomes could account for a majority, although not all, of the superhelical difference between the YXB6 *SIR*⁺ and *sir*⁻ circles. On the other hand, histone acetylation differences can fully accommodate the topological difference between pairs of *SIR*⁺ and *sir*⁻ circles of smaller sizes (calculations not shown).

The higher negative supercoiling of silent *HML* DNA could also be the result of a higher nucleosomal density at *HML*, since the more nucleosomes present, the greater the change in the linking number. However, Nasmyth (48) found that silencing at the *HM* loci did not cause a significant change in either the number or the positions of the nucleosomes. In addition, our finding that the silenced chromatin can decay to an active conformation in inert, excised circles suggests that the higher supercoil density state can likely convert to the lower supercoil density state in the cell without a change in nucleosome density. Nevertheless, further studies are needed to establish the extent of contribution by nucleosomal density to DNA topology at silent loci.

Numerous genetic and biochemical studies have indicated that Sir2p, Sir3p, and Sir4p are integral components of transcriptionally silenced chromatin (25, 26, 33, 47, 62). These *SIR* proteins could play a direct role in influencing the topology of DNA within the silent chromatin. For instance, these proteins could function in silenced chromatin in a manner analogous to the role of linker histones (H1 and H5) in metazoan chromatin. These linker histones promote chromatin condensation by increasing the extent to which DNA wraps around individual nucleosomes. Such increased wrapping would result in a higher superhelical density of silenced versus active chromatin. An alternative possibility is that the SIR proteins could form scaffolds around which DNA could wrap or, at a higher-order organization, around which nucleosome-packaged DNA could wrap. In either case, the association of the SIR proteins with the locus would yield increased superhelical density. In this context, Strahl-Bolsinger et al. (62) have suggested that chromosome folding occurs at telomeric heterochromatin. Finally, the *SIR* proteins could also help maintain the lower level of acetylation of histones by either excluding histone acetyltransferases or recruiting deacetylases. In summary, the higher degree of negative superhelicity of DNA in transcriptionally silent chromatin may be the result of hypoacetylation of histones, higher nucleosome density, and certain SIR-mediated nucleoprotein structures. To what extent each factor contributes remains to be determined.

The effect of cell cycle progression on transcriptionally silent chromatin. We have demonstrated that *SIR*⁺ *HML* circles bearing no silencer maintained their higher level of negative supercoiling in cells in G_0 or arrested at any of three different stages of the cell cycle. However, the higher negative supercoiling decayed to the lower supercoiling state as cells progressed through the cell cycle. In contrast, the superhelical density of silencer-bearing circles was not affected by cell cycle progression. These results are consistent with and extend the previous observation that silencing at *HML* is maintained in G_1-arrested cells after in vivo deletion of silencers but abolished within one generation of growth following deletion of the silencer (30).

What event(s) might disrupt the silent chromatin structure during transition of the cell cycle? Our results suggest that decay occurs primarily, although not exclusively, in the interval between early S phase and mitosis. However, since the excised plasmid lacks either an origin of replication or a centromere,

neither replication nor segregation of the plasmid is responsible for decay. Accordingly, we conclude that the silenced chromatin structure is sensitive to the signals in the cell that orchestrate the events of the cell cycle.

We note that decay of silencing of a silencerless *HML* locus in the chromosome is more rapid than that of a silencerless locus on an excised circle. Greater than 95% of cells carrying a silencerless chromosomal locus exhibited loss of silencing in one generation (30), while only 50% of excised HML circle exhibited a decay of supercoiling in one generation. This suggests that silenced chromatin, while sensitive to cell cycle signals, may also be sensitive to processes in the cell cycle, such as DNA replication.

We observed that *SIR*$^+$ *HML* circles excised from cells arrested in G_2/M by nocodazole are less negatively supercoiled than circles in cells arrested in G_0, G_1, or S phase. While the topological different is small ($\Delta Lk = 1$ to 2 for YXB6 circles) relative to the difference in *SIR*$^+$ versus *SIR*$^-$ cells (ΔLk of 10 for the same circle), the circles comprise a uniform population and the effect is quite reproducible. These data suggest that silenced chromatin in nocodazole arrested cells is somewhat more relaxed than in cells at other stages of the cell cycle. Aparicio and Gottschling (4) previously reported that telomere silencing of *URA3* could be disrupted by overexpression of Ppr1p, a transcriptional activator of *URA3*, in nocodazole-arrested cells but not in G_0 cells or in cells arrested in G_1 or S. They proposed that transcription factors overcome silencing by competing with silencing factors during a hypothetical reestablishment phase of silencing following DNA replication. Our observations provide an alternative explanation for these results. We propose that nocodazole induces a relaxation of silent chromatin, which enables the transcription factor to access its target. This relaxation of silenced chromatin could be a process that occurs prior to mitosis during normal cell cycle progression. Alternatively, the relaxation could be a response to nocodazole arrest, perhaps as a result of increased cyclin-dependent kinase (CDK) activity or of activation of the mitotic checkpoint pathway.

The role of silencer in maintaining transcriptionally silenced chromatin structure in the cell cycle. The results presented in this report indicate that the covalent association of a silencer was necessary in order to maintain the integrity of silenced chromatin during transition of various stages of the cell cycle. A previous study of the role of silencers in maintenance of silencing demonstrated that in the absence of a silencer, silenced loci would decay into an active state within one generation (30). Since in that previous study the state of the silencer-free locus at intermediate stages of the cell cycle was not probed, a model in which the silencer served as a maintenance element versus one in which it served as a reestablishment element could not be distinguished. The results presented in this report provide strong evidence that silencers serve to maintain the integrity of the silenced chromatin throughout the cell cycle. That is, in contrast to silencerless *HML* circles, silencer-containing circles maintain their higher negative supercoiling at every stage of the cell cycle. These indicate that transcriptionally silenced chromatin structures associated with silencers are stably maintained and inherited. This could be achieved in two ways. Cell cycle progression may disrupt silent chromatin linked to silencers, as it does to that not associated with silencers. Silencers may simply initiate the reestablishment of silenced chromatin whenever it is disrupted. This is consistent with the fact that the ORC binds to silencers throughout the cell cycle in vivo (14) and may be always available to trigger establishment of silencing by recruiting Sir1p (66). Alternatively, silencers may safeguard the integrity of silent chromatin by counteracting events that may disrupt it. When the silent chromatin is not challenged, as in G_0 cells or cell cycle-arrested cells, silencers are dispensable. However, when it is challenged by events involved in, or triggered by, cell cycle progression, silencers may be able to prevent the array of *SIR* complexes from dissociating from the nucleosomes by serving as a strong anchoring point for the SIR complex. Along this line, we propose that certain cell cycle-dependent factors may modify either histones or *SIR* proteins in the silent chromatin, rendering their interactions weaker. Further analysis of the structural nature of the silenced heterochromatin and its relationship with the silencer complex should distinguish between these models.

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