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Persistence of an alternate chromatin structure at silenced loci *in vitro*

(transcriptional repression and silencers/*Saccharomyces cerevisiae*/mating-type locus/heterochromatin/recombination)

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ABSTRACT **In *Saccharomyces cerevisiae*, transcriptional repression at the *HM* mating-type loci and telomeres results from the formation of a heterochromatin-like structure. Silencing requires at least three Sir proteins (Sir2p–4p), which are recruited to chromatin by silencers at the *HM* loci and $TG_{1–3}$ tracts at telomeres. Sir proteins and telomeres colocalize at the nuclear periphery, suggesting that this subnuclear position may also contribute to transcriptional repression. To evaluate the contribution of nuclear context to silencing, we developed methodology to isolate silent chromatin for analysis *in vitro*. Site-specific recombination was used *in vivo* to produce DNA rings from the silent *HMR* locus, and differential centrifugation was used to isolate the rings from whole-cell lysate. The partially purified rings retained many of the intracellular hallmarks of transcriptionally repressed domains. Specifically, rings from repressed strains were resistant to restriction endonuclease digestion, bore an altered DNA topology, and were associated with Sir3p. The recombination approach also was used to form rings from *HMR* that lacked silencers. Despite the uncoupling of these cis-acting regulatory elements, similar but nonidentical results were obtained. We conclude that an alternate chromatin structure at silent loci can persist *in vitro* in the absence of silencers and nuclear compartmentalization.**

Genes within the chromosomes of higher eukaryotes are subject to position-dependent transcriptional regulation. In *Drosophila*, for example, translocation of an active euchromatic gene near a region of centromeric heterochromatin results in a variegated pattern of expression: the gene is on in some cells and off in others (1). Inactivation is attributed to the linear spread of a repressive heterochromatic structure into adjacent DNA, a packaging that is inherited through subsequent generations. In at least one case, heterochromatic repression of a nonadjacent gene occurs via the three-dimensional juxtaposition of the gene with centromeric heterochromatin (2, 3).

Position effects also occur in the yeast *Saccharomyces cerevisiae*, where genes near telomeres and the *HM* mating-type loci are repressed because of the formation of a heterochromatin-like structure (4). Gene silencing at these locations requires a number of trans-acting factors, including the Sir proteins (Sir2p–4p), which are associated with chromatin throughout the repressed domain under normal conditions (5, 6). The Sir proteins are recruited to telomeres by interactions with Rap1p, a protein that binds repeatedly to telomeric $TG_{1–3}$ tracts (7). At the *HM* loci, recruitment is attributed to interactions with Rap1p, Abf1p, and the origin recognition complex (ORC), factors that bind in various combinations to discrete cis-acting control sequences (termed silencers) (4). An additional Sir protein, Sir1p, is tethered by protein–protein interactions to silencers, where it contributes to the recruitment of the other Sir proteins (8, 9).

Saccharomyces telomeres are clustered in discrete foci that contain Sir2p and a significant fraction of the cellular Sir3p, Sir4p, and Rap1p (10–12). The clusters are localized at the nuclear periphery, as are the telomeres of fission yeast *Schizosaccharomyces pombe* (13), where repression of telomere-proximal genes also has been observed (14). In a number of other organisms, telomeres also are clustered and peripherally localized, although this arrangement is not observed in all cell types nor in all stages of the cell cycle (13, 15). At least some nontelomeric heterochromatin in *Drosophila* and the inactive X chromosome of female mammals also reside at the periphery of the nucleus (16, 17). Whether this localization is important for repressive chromatin structure and telomeric silencing or merely coincident with them is unclear. Recently, however, attachment of a defective yeast mating-type silencer to the nuclear membrane was shown to restore Sir-dependent silencing of a neighboring reporter gene (18). This observation suggests that perinuclear localization contributes to the formation of silent chromatin in yeast.

Factors involved in Sir-dependent silencing can be divided into two operational classes: those that participate in establishment of the silent state and those that participate in its maintenance. Silencers and the proteins that bind them traditionally have been viewed as establishment factors. A subset of mutations in these components produce cells that establish silencing rarely but maintain it faithfully when it occurs (19–21). Sir3p, on the other hand, is required continuously to maintain silencing (22). Structural components of the repressed domain, such as the Sir3 protein (and presumably Sir2p and Sir4p), are therefore considered maintenance factors. A role for silencers in maintenance of the repressed state recently was examined with site-specific recombination, which was used to uncouple the elements from preexisting silent DNA *in vivo*. Immediately following recombination, silencing was shown to persist, as indicated by phenotypic criteria, direct examination of transcriptional state, and an altered topology of recombined extrachromosomal DNA rings (23–25). Upon continued cell growth, however, the Sir-dependent structure was lost and transcriptional competence was restored. It is difficult to determine what role, if any, nuclear localization plays in the initial preservation and subsequent loss of silencing in the rings. Whereas the excised DNA is uncoupled from the chromosome, it is unclear whether it remains within the original nuclear domain and near silencers or diffuses away.

To gain insight into the role of nuclear and chromosomal context on the maintenance of silencing in yeast, we developed methodology to examine the persistence of silent chromatin structure *in vitro*. DNA rings either containing or lacking

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silencers were excised from HMR by *in vivo* recombination and isolated from whole-cell lysate by using differential centrifugation. We show that several characteristics of intracellular repressed chromatin are preserved in excised rings after isolation from the nucleus and the removal of silencers.

MATERIALS AND METHODS

DNA and Strain Constructions. Plasmid pSIR3-HA was generated by in-frame insertion of three copies of the hemagglutinin (HA) epitope (preceded by a six-histidine linker) into the extreme carboxyl terminus of *SIR3*. The modified gene, including its own promoter, was inserted into pRS416 (26). Plasmid psir4::TRP1 was generated by replacing the *HIS3* gene that disrupts *SIR4* in plasmid pMM10.7 (27) with *TRP1*. Plasmid pRS415-RecR was generated by transferring the *GAL1*::*R* recombinase gene fusion from pHM153 (28) to pRS415 (26). All strains were derived from W303–1A (see Table 1). Isogenic *SIR3* and *sir3* derivatives were obtained by transforming *sir3* null mutants with a plasmid-borne, HA-tagged *SIR3* allele (pSIR3-HA) that complemented the *sir3* mating defect (A.A., unpublished results). Strain AA4 was derived from THC9 by one-step gene disruption of *SIR4* with plasmid psir4::TRP1. The modification was confirmed by using Southern hybridization.

Isolation of Chromatin Rings by Using Differential Centrifugation. Cell extracts were prepared according to published methods (29) with the following modifications. Strains were grown to OD_{600} 2.0–2.5 in synthetic dropout media supplemented with raffinose (2%). Excision was induced by the addition of galactose (final concentration = 2%) for exactly one hour. Cell pellets from 1 liter of culture were first washed with ice-cold water and then with 25 ml of ring isolation (RI) buffer, (10 mM Hepes·KOH, pH 7.9/1.5 mM $MgCl_2$/0.5 mM DTT/0.125 mM spermidine/0.05 mM spermine) containing either 100 or 150 mM KCl and either 10% or 20% (vol/vol) glycerol. The exact KCl and glycerol concentrations are specified in each experiment (see *Results*). The pellets were resuspended in 10 ml of the same buffer, frozen in liquid nitrogen, and stored at −70°C. Frozen aliquots were homogenized to a very fine powder by using a mortar and pestle, allowed to thaw, and subsequently stirred gently for 1 hr. (All steps were performed in a 4°C cold room.) The resulting whole-cell lysate was clarified by using centrifugation at 20,000 rpm for 45 min in a Sorvall SS-34 rotor (47,800 × *g*). Supernatant was spun again at 37,000 rpm for 1 hr in a Beckman type 70 Ti rotor (100,736 × *g*). The resulting pellet (P2) was washed with 2 ml of RI buffer containing 140 mM KCl/10% glycerol and resuspended in 200 μl of the same buffer.

Dot-Blot Analysis. Aliquots of resuspended P2 (10 μl) were deproteinized by adding 10 μl of proteinase K (10 mg/ml) and 2 vol of TSN detergent solution (2% Triton X-100/1% SDS/100 mM NaCl). After 30 min at 65°C, the samples were extracted twice with phenol/chloroform and precipitated. The processed samples were denatured with 0.2 N NaOH and spotted on Zetaprobe GT membrane (Bio-Rad).

Table 1. Yeast strains

Strain	Genotype	Reference
W303-1A	*MAT***a** *HML*α *HMR***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	45
THC9	W303-1A Δ*hmr*::*rKWD50N* Δ*sir3*::*HIS3*	25
THC16	W303-1A Δ*lys2*::*rKWD50N* Δ*sir3*::*HIS3*	25
THC40	W303-1A *hmr*::*rA1A2* Δ*mat*::*URA3* Δ*sir3*::*HIS3*	25
YCL2	W303-1A *hmr*::*rHMR* Δ*sir3*::*HIS3*	25
AA4	THC9 Δ*sir4*::*TRP1*	This study

Restriction Enzyme Accessibility Assays. Aliquots of resuspended P2 (10 μl) were incubated at 30°C for 1 hr in 100 μl of restriction digestion reactions containing 50–100 units of a specific endonuclease. Each reaction was buffered to the manufacturer's specifications, taking into account the contribution of P2 resuspension buffer. Reactions were deproteinized as described above and subjected to electrophoresis in 0.8% agarose gels. DNA was transferred to Zetaprobe GT membrane and hybridized with selected probes sequentially.

Chloroquine Gel Electrophoresis. Aliquots of resuspended P2 (50 μl) were incubated with 1 μl of 100 mg/ml RNase A, 40 μl of TE (10 mM Tris·HCl, pH 8.0/1 mM EDTA) and 10 μl of buffer (100 mM NaCl/10 mM $MgCl_2$/50 mM Tris·HCl/1 mM DTT, pH 7.9) for 30 min at 30°C. Samples were deproteinized by adding 30 μl of prewarmed 250 mM EDTA/5% SDS (65°C), 30 μl of 10% Triton-X100, and 10 μl of proteinase K (10 mg/ml). After 30 min at 65°C, samples were phenol/chloroform-extracted three times and ethanol-precipitated. Topoisomers were resolved by electrophoresis in gels containing chloroquine (25).

Western Blots and Immunoprecipitations. For Western blot analysis, 50-μl aliquots of resuspended P2 were subjected to electrophoresis in SDS/PAGE gels alongside whole-cell lysate (30). Protein was transferred to Immobilon-P membrane (Millipore) according to the manufacturer's recommendations, incubated with 12CA5 mAbs against the HA epitope of the tagged Sir3p and visualized with enhanced chemiluminiscence (Renaissance, DuPont). For immunoprecipitations, 10-μl aliquots of resuspended P2 were incubated with 15 μg of 12CA5 mAb (protein A-Sepharose purified) in 500 μl of IIP150 buffer (10 mM Tris·HCl, pH 8.0/150 mM NaCl/0.1% Nonidet P-40) at 4°C for 1 hr. The reaction mixture was centrifuged to remove nonspecific precipitate, and the supernatant was mixed with 25 μl of protein A-Sepharose beads (1 g in 10 ml). After 1 hr at 4°C on a rocking platform, the beads were collected by a brief centrifugation and washed three times (5 min each) with 500 μl of IIP150 buffer. Finally, beads were resuspended in 200 μl of detergent solution and deproteinized as described above. Coprecipitating DNA was resuspended in 20 μl of TE buffer, and 25% of each sample was used for PCR analysis.

RESULTS

Biochemical Enrichment of Extrachromosomal DNA Rings. We developed a method to generate and purify small silent chromatin templates from intact cells (31). The approach uses the R site-specific recombinase of the yeast *Zygosaccharomyces rouxii* to promote excision and recircularization of chromosomal DNA fragments *in vivo* (25). Differential centrifugation is then used to isolate the rings from whole-cell lysate. Chromatin of the silent *HMR* mating-type locus was examined in this manner, using strains containing the excision cassettes shown in Fig. 1. Each cassette contains a pair of recombinase target sites, termed *RS* sites, that flank the sequence to be excised. Strain YCL2 yields ring rHMR that contains the entire locus, including both *E* and *I* silencers, whereas strain THC40 yields only the **a**1 and **a**2 genes. Elsewhere, we have shown that the R recombinase produces DNA rings rapidly and efficiently from *HMR* in both transcriptionally repressed and derepressed strains (25, 31).

Recombination was initiated by using galactose induction of a *GAL1*::*R* recombinase gene fusion. Cells were harvested and lysed, and the resulting extract was clarified by a low-speed centrifugation (see *Materials and Methods*). The supernatant was subjected to a second high-speed centrifugation, resulting in a thin glassy pellet, termed P2. Dot blots of deproteinized P2 showed that extrachromosomal DNA, including both plasmids and excised rings, was recovered from both *SIR4* and *sir4* strains (Fig. 2). Recovery of rings required galactose-induced recombination, whereas recovery of the endogenous 2-μm

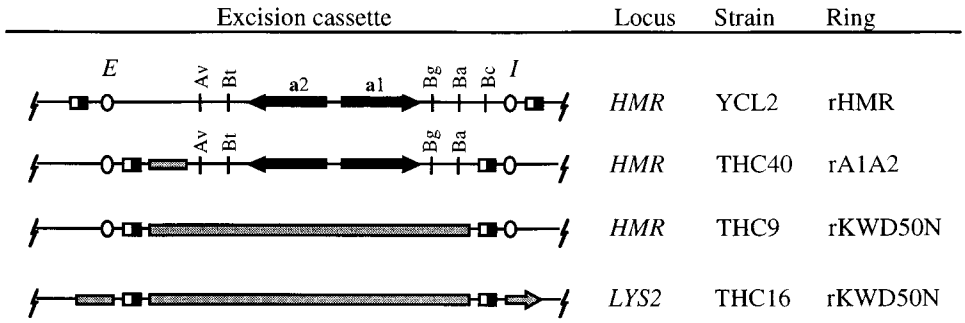

FIG. 1. Excision cassettes for the formation of extrachromosomal DNA rings. *RS* sites are shown as half-filled boxes. In strain YCL2, the entire *HMR* locus is flanked by *RS* sites, including the *E* and *I* silencers (shown as ovals). In strain THC40, only the **a**1 and **a**2 genes of the locus are flanked by *RS* sites. The excision cassette in this strain also contains a small piece of the *LYS2* gene (shaded box). THC9 differs from THC40 in that the region between the *RS* sites is replaced entirely by a fragment of the *LYS2* gene. The same fragment can be excised from the *LYS2* locus in strain THC16. Strain constructions are described in detail in ref. (25). Ba, *Bsa*AI; Bg, *Bgl*II; Bc, *Bcl*I; Bt, *Bst*BI; Av, *Ava*II.

plasmid was galactose-independent. This indicates that significant levels of chromosomal excision cassette were not isolated in the absence of galactose. Agarose gel electrophoresis showed that >95% of the rings were covalently closed and supercoiled (Fig. 3). These results demonstrate that intact DNA rings can be enriched relative to chromosomal sequences by using differential centrifugation of whole-cell lysate. We also have shown (and describe in greater detail) that enrichment of the rings exceeds 1,000-fold (31).

Extrachromosomal DNA Rings Bear Hallmarks of Silent Chromatin *in Vitro*. The differential-centrifugation procedure described above was employed to isolate DNA rings as chromatin templates. Indeed, rings isolated in this manner yielded micrococcal nuclease digestion patterns consistent with nucleosomal packaging (31). We were specifically interested in whether rings from silent loci retained an alternative chromatin structure when removed from the cell nucleus. Therefore, rings from wild-type and *sir3* strains were evaluated by two criteria: (*i*) accessibility to restriction endonucleases and (*ii*) the relative level of DNA supercoiling.

Repressive chromatin structure at the silent mating-type loci hinders most enzymatic probes of DNA accessibility including the yeast HO endonuclease that cleaves the active mating-type locus, *MAT*, to initiate mating-type switching (4, 32). In permeablized nuclei, common restriction endonucleases and HO have been used to demonstrate the refractory nature of the silent loci to DNA digestion (33). To test whether Sir-dependent differences in DNA accessibility are preserved during our enrichment procedure, preparations of pellet P2 were gently resuspended and incubated with a series of restriction enzymes. Fig. 3*a* shows that rHMR was more resistant to digestion by each of the enzymes tested when isolated from a *SIR3* strain than when isolated from a *sir3* mutant. In contrast, the 2-μm plasmid was cleaved equally well in both strains (Fig. 3*b*). These results indicate that the inaccessibility of the silent loci to endonucleases in nuclei is recapitulated with partially purified rings in an extranuclear context.

Closed circular plasmids isolated from eukaryotes are negatively supercoiled as a result of the wrapping of DNA around histone cores. Variations in the degree of supercoiling can arise from differences in the density of nucleosomes or subtle

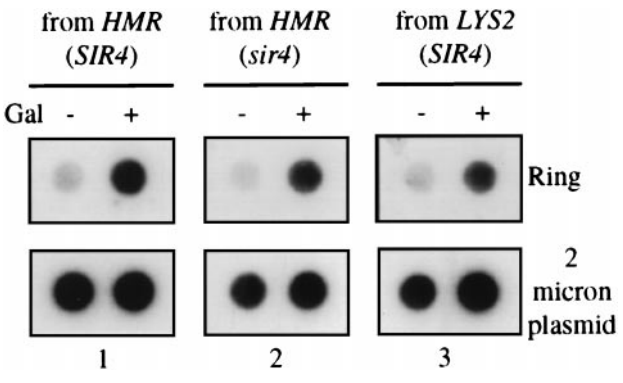

FIG. 2. DNA content in pellet P2. Chromatin rings were isolated in RI buffer containing 150 mM KCl and 10% glycerol. Samples were deproteinized and affixed to nylon membrane by dot blotting. Ring rKWD50N was excised from the *HMR* locus in strains THC9 (*Left*) and AA4 (*Center*) and from the *LYS2* locus in strain THC16 (*Right*). Each strain was transformed with pSIR3-HA and a *GAL1*::*R* recombinase expression vector, pRS415-RecR. Blots were hybridized with randomly primed probes specific for rKWD50N or the entire 2-μm plasmid.

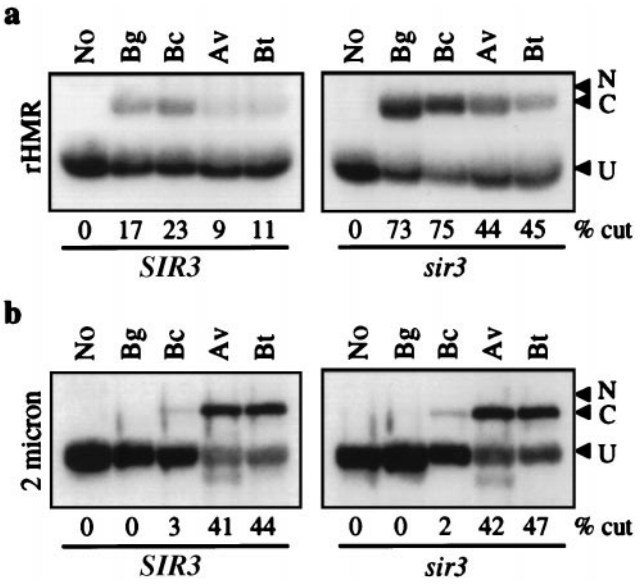

FIG. 3. Restriction endonuclease accessibility of partially purified chromatin rings. Samples were prepared from strain YCL2, which was transformed with pRS415-RecR and either pSIR3-HA or pRS416 to yield *SIR3* and *sir3* strains, respectively. Chromatin rings were isolated in RI buffer containing 150 mM KCl and 10% glycerol. After digestion and electrophoresis, blots were hybridized with either a probe to detect the rHMR ring (*a*) or a probe to detect the entire 2-μm plasmid (*b*). Linearized, closed-circular, and nicked species are denoted as **C** (cut), **U** (uncut), and **N** (nicked), respectively. Enzymes corresponding to unique sites in rHMR were used as indicated above each lane (see Fig. 1 for map and legend). In lane 1, no enzyme (No) was used. The extent of enzymatic digestion is reported as the percentage of band **C** relative to the sum of bands **C** and **U** in each lane. The 2-μm plasmid contains no *Bgl*II sites and one *Bcl*I site. There are multiple sites for *Ava*II and *Bst*BI yet singly cut species are observed because of partial cleavage of the chromatin templates.

structural changes in individual nucleosomes. Previously, it was shown that Sir-mediated silencing at *HMR* is accompanied by changes in DNA topology (24, 25, 34). To examine the supercoiling of rings in pellet P2, deproteinized samples containing ring rHMR were subjected to electrophoresis in gels containing varying concentrations of chloroquine. Fig. 4 shows that the ring was present as a distribution of negatively supercoiled topoisomers, corroborating that DNA rings can be recovered from whole-cell lysate as chromatin (Fig. 4, lanes 3 and 4). Moreover, the *HMR*-derived ring was more negatively supercoiled by two to three turns when derived from a silencing-competent *SIR3* strain than from a *sir3* strain. A similar topological shift was observed for rHMR when DNA was obtained from spheroplasts by using conventional nucleic acid isolation procedures (ref. 25; Fig. 4, lanes 1 and 2). These results indicate that the Sir-mediated differences in chromatin are preserved during the isolation procedure.

The data in Fig. 4 are particularly striking because the P2 pellet also contained a significant plasmid relaxation activity: exogenous supercoiled plasmids were completely relaxed when mixed with resuspended P2 (data not shown). The inability of this activity to eliminate the supercoiling differences between rings from *SIR3* and *sir3* strains indicates that the alternate chromatin structure is stable *in vitro*.

Structural Features of Repressed Chromatin Persist *in Vitro* in the Absence of Silencers. To study the role of silencers in maintaining the distinct features of silent chromatin described above, we used endonuclease digestion and topoisomer analysis to examine a ring from *HMR* that lacked silencers, ring rA1A2 (see Fig. 1). Chromatin rings were isolated in RI buffer containing 100 mM KCl and 20% glycerol. When using this buffer, rA1A2 was more resistant to cleavage when isolated from a *SIR3* strain than when isolated from a *sir3* strain (Fig. 5*a*). Correspondingly, the topoisomer distribution of rA1A2 was more negatively supercoiled in P2 pellets prepared from the *SIR3* strain (Fig. 5*c*). These results show that features of silent chromatin persist *in vitro* even when repressed DNA is uncoupled from silencers. The data are in agreement with earlier studies performed in permeabilized nuclei, where the inaccessibility of silent chromatin was preserved following the removal of silencers (33).

Interestingly, we found that stability of an alternate chromatin structure in rings lacking silencers depended on the composition of the isolation buffer. For example, when RI buffer containing 150 mM KCl and 10% glycerol was used, only a minor Sir-dependent difference in cutting was observed (Fig. 5*b*). In contrast, the alternate chromatin structure of rings

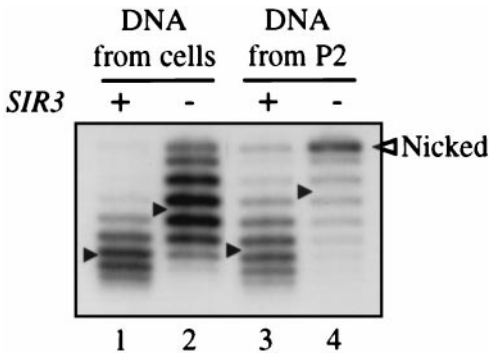

FIG. 4. Topoisomer analysis of partially purified chromatin rings. Electrophoresis was performed in buffer containing 1.5 μg/ml chloroquine. Samples were obtained from strains described in Fig. 3. (Lanes 1 and 2) DNA isolated directly from intact cells. (Lanes 3 and 4) DNA isolated via P2 pellets that were obtained in RI buffer containing 150 mM KCl and 10% glycerol. Topoisomer bands were quantified by phosphorimaging (Bio-Rad), and the centers of the topoisomer distributions (marked with filled arrows) were determined by the Gaussian method (44).

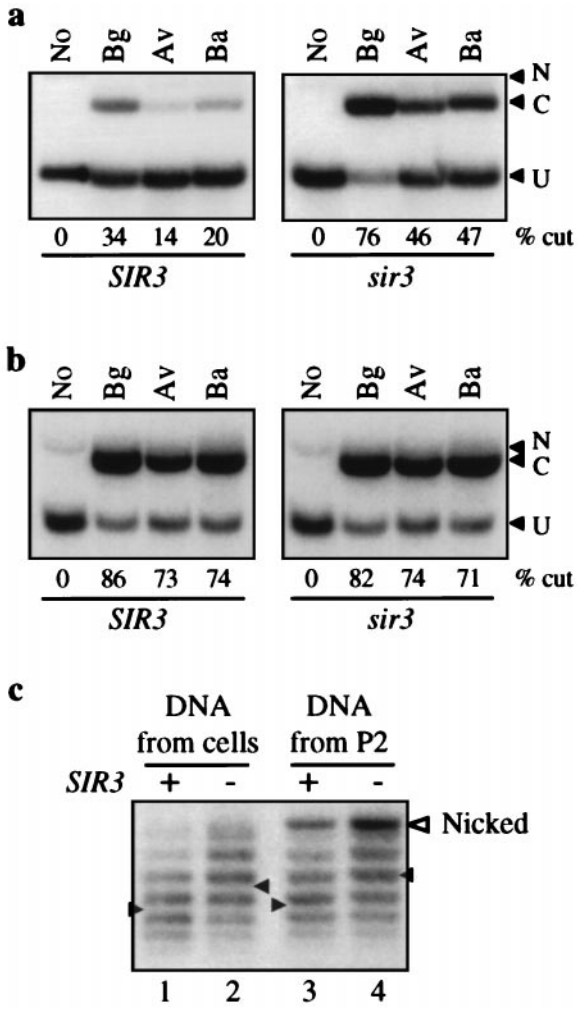

FIG. 5. Analysis of rings lacking silencers. Samples were prepared from strain THC40, which was transformed with pRS415-RecR and either pSIR3-HA or pRS416. P2 pellets were obtained in RI buffer containing either 100 mM KCl and 20% glycerol (*a*) or 150 mM KCl and 10% glycerol (*b*). Enzymes corresponding to unique sites in rA1A2 were used (see Fig. 1 for map and legend). (*c*) Topoisomer distributions of uncut samples were determined by using electrophoresis in buffer containing 2.0 μg/ml chloroquine.

containing silencers persisted over the range of buffer conditions employed (100 mM KCl/20% glycerol to 150 mM KCl/10% glycerol). These results suggest that silencers may participate in maintaining silent chromatin stability.

Sir3p Remains Associated with Rings from Silent Loci. The restriction digestion and DNA topology results reported above suggested that DNA rings from repressed loci may retain protein components of the silencing apparatus. Therefore, Western blot analysis was used to test for the presence of Sir3p in chromatin preparations of extrachromosomal DNA rings.

In all experiments described herein, the wild-type *SIR3* gene was replaced with a functional HA epitope-tagged allele. Fig. 6*a* shows that Sir3p (Sir3-HA) copurified with ring rKWD50N if the ring originated from a repressed *HMR* locus. rKWD50N lacks mating-type sequences and silencers. Therefore, the association of Sir3p with the ring was determined by the chromosomal locus from which the ring was excised. When samples were prepared from a derepressed *sir4* strain or with rings from the nonrepressed *LYS2* locus, Sir3p was not detected. In addition, Sir3p did not sediment in P2 in the absence of recombination. These experiments show that Sir3p remains associated with rings from silent loci after uncoupling from chromosomal silencers and removal from the nucleus.

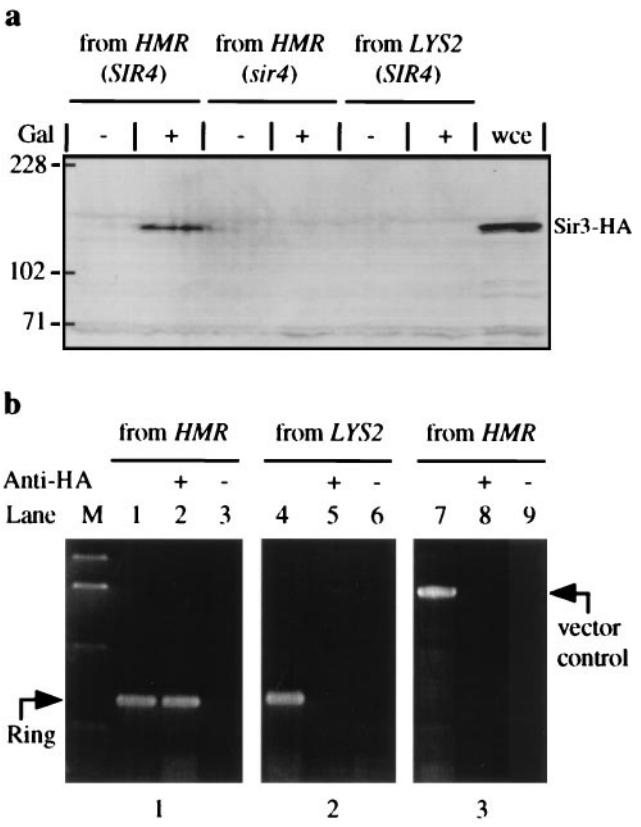

FIG. 6. Association of Sir3p with rings lacking silencers. Samples were obtained from strains described in Fig. 2. An anti-HA antibody was used to detect epitope-tagged Sir3p. (*a*) Resuspended P2 preparations were subjected to Western blot analysis. Mobility of size markers is shown at left, in kDa. Approximately equal amounts of protein were loaded in each lane, as judged by comparing the levels of a 71-kDa crosshybridizing species. wce, whole-cell extract of strain in lane 1. (*b*) Resuspended P2 preparations were subjected to immunoprecipitation by anti-HA antibody and PCR analysis of precipitates. Strain THC9 transformed with pSIR3-HA was used for panels 1 and 3. Strain THC16 transformed with pSIR3-HA was used for panel 2. PCR primers were specific for the circular form of the rKWD50N excision cassette (panels 1 and 2) or pSIR3-HA (panel 3). Lanes 1, 4, and 7 were PCR products of resuspended P2. Lanes 2, 5, and 8 and 3, 6, and 9 were PCR products of immunoprecipitations performed with or without anti-HA antibody, respectively.

Coimmunoprecipitation techniques were employed to demonstrate that Sir3p and ring rKWD50N were physically linked. Precipitations were performed with resolubilized P2 pellets and anti-HA antibody. The DNA content of the immunoprecipitates was evaluated with PCR by using primers specific for either rKWD50N or the SIR3-HA expression vector. Fig. 6*b* shows that rKWD50N coprecipitated with Sir3p if the ring originated from *HMR*. The observation depended strictly on the addition of anti-HA, discounting the possibility that rings were precipitated by nonspecific interactions with protein A-Sepharose. Furthermore, the inability to pull-down the SIR3-HA expression vector, which also sedimented in P2, showed that Sir3p does not transfer between DNA templates during isolation. Thus, Sir3p in the P2 pellet is associated specifically with rings from silent loci. This last experiment strengthens our contention that chromatin obtained in this manner is intact and physiologically relevant.

DISCUSSION

Features of Silent Chromatin Persist *in Vitro*. To study the role of silencers in maintenance and inheritance of heterochromatin-like structure in yeast, we and others have used site-specific recombination to uncouple transcriptionally repressed chromatin fragments from the silent mating-type loci *in vivo* (23–25). A limitation of the approach is that DNA rearrangements can eliminate the actions of silencers in *cis* but not in *trans*. It is impossible to determine whether a silencer on an unlinked yet nearby DNA segment influences a property of an excised ring. Similarly, the unlinked ring may or may not diffuse from a nuclear compartment critical to the phenomenon under study. Concerns regarding nuclear context are particularly relevant to silencing because of the body of evidence that suggests repressed domains occupy and require specific nuclear locations. In this report, we have addressed these issues by analyzing properties of silent chromatin in extrachromosomal DNA rings fractionated from whole-cell extracts. We have shown that features of silent chromatin structure persist *in vitro* despite the loss of a defined nuclear context and the removal of silencers, telomeres, and all other chromosomal DNA. On the basis of restriction enzyme accessibility, DNA supercoiling, and Sir3p association, we infer that DNA rings excised from the silent mating-type loci are transcriptionally repressed. This work illustrates that intact heterochromatin-like fragments can be isolated from yeast.

A Role for Silencers in the Maintenance of the Repressed State. A Sir-dependent alternate chromatin structure was demonstrated in rings lacking silencers, although the structure appeared to be unstable (because its persistence depended on isolation conditions). In contrast, rings containing silencers retained an alternate structure reproducibly. These results support the belief that silencers play a role in maintenance of transcriptional repression. Previous observations also have indicated that silencer function is not strictly limited to establishment of the silent state. For example, inactivation of subunits of the ORC silencer-binding complex resulted in derepression in M phase-arrested cells (35). Likewise, DNA rings from silent loci that lacked silencers lost repression during normal cell growth (24, 25), particularly during progression between the G_1 and G_2 phases of the cell cycle (24). In the experiments presented here, instability of silent chromatin in such rings might have arisen from a derepression process that began *in vivo*. We hypothesize that biochemical fractionation preempts steps in the process that would otherwise eliminate silencing of rings altogether.

A Role for Nuclear Localization in Silencing? Sir proteins appear to be limiting in the nucleoplasm but are enriched at telomeres, which reside in clusters near the nuclear periphery (see ref. 36 and references therein). A number of recent findings have shown that silencing of the mating-type loci relies, in part, on their proximity to telomeres and the pools of Sir proteins associated with them. For example, repositioning silent loci to various internal chromosomal sites reduced silencing (37, 38). The impairment was suppressed by creating a new telomere in adjacent DNA or by overexpressing the Sir proteins (38). Similarly, artificially targeting Sir3p or Sir4p to an attenuated silent locus restored repression (39, 40). Furthermore, targeting a defective *HMR* silencer to the nuclear membrane also restored repression, presumably because this led to colocalization with telomeres (18). Our experiments show that features of silent chromatin structure persist *in vitro* in the absence of telomeric pools of Sir proteins. One explanation for this seemingly contradictory result is that nuclear compartmentalization is required for establishment but not maintenance of the silent state. For instance, formation of a repressive chromatin structure may require a threshold level of Sir proteins only available near telomeric clusters. Once the factors are incorporated into chromatin, however, the repressed structure can persist despite displacement from cell.

An alternative explanation holds that nuclear compartmentalization is important for both establishment and maintenance of repression. According to this view, loss of repression in DNA rings lacking silencers in cycling cells results from their

escape from a compartment critical for silencing. If this is the case, persistence of an alternate chromatin structure after purification may reflect the removal of nuclear activities that disassemble misplaced chromatin.

Retention of Sir3p by Silent Chromatin in the Absence of Silencers. A critical role for Sir3p in maintenance of silencing was first implicated from experiments that used a temperature-sensitive *sir3* allele, where inactivation of the gene led to immediate loss of repression (22). The direct association of Sir3p with silent chromatin was demonstrated only recently by an *in situ* formaldehyde crosslinking technique (5). We have shown here that Sir3p remains associated with DNA rings that lack silencers. Thus, the cis-acting regulatory elements are not explicitly required to maintain the association of Sir3p with chromatin. The eventual reactivation of rings lacking silencers *in vivo*, however, most likely correlates with loss of the protein (24, 25). Our current experiments do not yield information regarding the stoichiometry of Sir3p with the extrachromosomal DNA rings, and it is likely that the time required for full excision affects this parameter when silencers are not included.

A New Tool for Chromosome Structure Analysis. Isolation of site-specific recombination products with differential centrifugation represents a powerful approach for studying chromatin structure. Any chromosomal region of interest can be obtained by virtue of designing appropriate recombination substrates. Inducible control of recombination permits uncoupling of the fragments from chromosomes according to specific criteria such as growth conditions, position within the cell cycle, or stages of development. We showed that the technique procures intact chromatin fragments in the form of closed circular rings with minimal chromosomal contamination. Moreover, our work was accomplished without the need for crosslinking agents to retain associations between components. Additional chromatographic steps are currently being developed to eliminate unrelated cosedimenting species, such as ribosomes and associated mRNA (41). Isolation of intact chromatin fragments from a genetically tractable system such as yeast should be useful in further structural and functional studies of heterochromatin. The approach should also be adaptable to higher eukaryotes, where inducible recombination is being used increasingly for programmed genomic rearrangements (42, 43).

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