MOLECULAR AND CELLULAR BIOLOGY, Feb. 2006, p. 852–862 Vol. 26, No. 3
0270-7306/06/$08.00+0 doi:10.1128/MCB.26.3.852–862.2006

Cell Cycle Requirements in Assembling Silent Chromatin in *Saccharomyces cerevisiae*

Ann L. Kirchmaier[1]* and Jasper Rine[2]

Department of Biochemistry and Purdue Cancer Center, Purdue University, West Lafayette, Indiana 47907,[1] and Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, California 94720-3202[2]

Received 23 August 2005/Returned for modification 13 October 2005/Accepted 10 November 2005

The establishment of silencing at the silent mating-type locus, *HMR*, in *Saccharomyces cerevisiae* requires that yeast pass through S phase of the cell cycle, yet requires neither the initiation of DNA replication at the locus destined to become silenced nor the passage of a replication fork through that locus. We tested whether this S-phase requirement reflects a window within the cell cycle permissive for recruitment of Sir proteins to *HMR*. The S-phase-restricted event necessary for silencing occurred after recruitment of Sir proteins to *HMR*. Moreover, cells arrested in early S phase formed silent chromatin at *HMR*, provided *HMR* was on a nonreplicating template. Replicating templates required a later step for silencing. These results provide temporal resolution of discrete steps in the formation of silent chromatin and suggest that more than one cell cycle-regulated event may be necessary for the establishment of silencing.

Epigenetic processes play a critical role in creating stable patterns of gene expression during normal growth and differentiation. Epigenetic processes regulate gene expression through heritable chromatin structures, creating distinctly different states of gene expression in genetically identical cells. Formally, the process of creating epigenetic influences on gene expression has three requirements: (i) the assembly of a specialized chromatin structure at a locus or loci, (ii) the maintenance of that structure throughout the cell cycle, and (iii) the ability of that structure to template its own replication, akin to the ability of complementary strands of DNA to template their replication.

In *Saccharomyces cerevisiae*, the Sir proteins play key roles in epigenetic states of gene expression, including the silencing of mating-type genes at *HML* and *HMR*, silencing genes near telomeres and controlling telomere length, and regulating expression, replication, and recombination in the rRNA genes. Sir proteins are the principal structural components of *Saccharomyces* silent chromatin. The localized formation of silent chromatin at *HML* and *HMR* is governed by events initiated at regulatory sites known as silencers, which flank *HML* and *HMR*. Recent studies of silencing at telomeres and the *HM* loci have revealed key insights into how heterochromatin is assembled (19, 30, 39, 40).

The *HMR-E* silencer consists of binding sites for three proteins: ORC, Rap1p, and Abf1p. Interactions between the four Sir proteins and these three DNA binding proteins recruit the Sir proteins to the silencer. Affinities among the Sir proteins for each other also contribute to the recruitment and initial assembly of heterochromatin (5, 14, 19, 30, 40). The affinity of Sir1p for Orc1p facilitates the initial assembly of silenced chromatin (5, 14, 15, 40, 49, 52). Although the *HMR-E* silencer is an origin of replication, initiation of replication at *HMR E* is not required for silencing, as evidenced by the ability of Sir1p, when tethered to a silencer, to bypass ORC's role in silencing (9, 13, 15).

In the current model for the mechanism of silencing, the histone deacetylase activity of Sir2p, when recruited to the silencer, deacetylates key positions on the tails of histones H3 and H4 in the flanking nucleosomes. A complex of Sir proteins then binds the deacetylated tails, and these newly bound Sir complexes then deacetylate their adjoining nucleosomes, resulting in the recruitment of new Sir protein complexes. Additional cycles of deacetylation and recruitment lead to the spreading of the Sir proteins throughout the *HMR* locus, resulting in the deacetylation of the underlying chromatin (19, 40). The assumption has been that the deacetylation of the underlying chromatin leads to its repression, though how deacetylated chromatin actually blocks gene expression is not fully known. Steric occlusion of proteins from their recognition sequences seems to be important for the mechanism (16, 28, 43, 45, 48), but recent data suggest an additional effect on the recruitment of TFIIB in silenced chromatin (7).

Studies using a temperature-sensitive *SIR3* allele to determine when silencing can be abrogated or established revealed that silencing can be lost at any point in the cell cycle (32). In contrast, silencing cannot be established in G_1, and rather requires passage through the cell cycle, with silencing established by G_2/M (32). This requirement for passage through S phase was originally interpreted as a role for DNA replication in silencing. However, silencing on a nonreplicating *HMR* excised in G_1 from the chromosome still displays a cell cycle requirement for silencing, even though that *HMR* is never replicated (22, 25). Thus, there is a cell cycle requirement for silencing that is not DNA replication. To investigate what that cell cycle requirement might be, we used a regulated source of Sir1p that could be tethered to the silencer to determine whether the recruitment of Sir proteins, the spreading of Sir

* Corresponding author. Mailing address: Department of Biochemistry and Purdue Cancer Center, Purdue University, 175 S. University St., West Lafayette, IN 47907. Phone: (765) 494-0977. Fax: (765) 494-7897. E-mail: kirchmaier@purdue.edu.

TABLE 1. Histone deacetylation in G_1

Gal4-Sir1p[a]	Relative efficiency of coprecipitation[b]					
	AcH3		AcH4		H4AcK16	
	HMR-GalSS	**a***1*	*HMR-GalSS*	**a***1*	*HMR-GalSS*	**a***1*
−	1	1	1	1	1	1
+	0.33 ± 0.056	0.85 ± 0.40	0.24 ± 0.17	0.53 ± 0.40	0.20 ± 0.28	0.74 ± 0.40

[a] Cells grown in the absence of Gal4-Sir1p (−) were arrested in G_1 with α factor, transferred to medium with α factor and without methionine to express Gal4-Sir1p (+), and harvested for chromatin immunoprecipitation as described for Fig. 1. DNA was coprecipitated with the indicated antibodies, and DNA from the synthetic silencer (*HMR-GalSS*), the **a***1* open reading frame, and *SSC1* were analyzed by real-time PCR.

[b] The efficiency of coprecipitation of each locus relative to *SSC1,* at each time point was calculated as (locus IP)/(*SSC1* IP) = $2^{(SSC1\ C_T\ -\ locus\ C_T)}$ relative to locus sheared DNA/*SSC1* sheared DNA = $2^{(SSC1\ C_T\ -\ locus\ C_T)}$ and normalized to the G_1 arrest in the absence of Gal4-Sir1p, which was set to 1. Data reflect the average ± the standard deviation of three or four (anti-AcH3 and -AcH4 or -H4AcK16, respectively) independent experiments, one of which corresponds to the experiment in Fig. 1. Ac, acetyl.

proteins, or the deacetylation of the underlying chromatin might be a cell cycle-regulated event in silencing.

MATERIALS AND METHODS

Plasmids and strains. JRY7130 or JRY7131 {*mat*Δ::*ADE2* FRT-4xGal4-Rap1-Abf1 *HMR***a** Δ*I*-FRT *leu2-3,112*::*LEU2 FLP1* (pFV17 integrated at *leu2-3,112* [38] *ade2-1 his3-11,15 trp1-1 ura3-1 can1-100* [*cir*^{0}] plus pJR1811 (*GAL4DBD-SIR1* expressed from the *MET3* promoter in pRS313 [13]) (22), or AKY1686 or AKY1687 (JRY7130 or JRY7131, respectively, plus *bar1*Δ*::KanMX*) were used for experiments summarized below in Table 1 and Fig. 1, 5, 6, and 7A and B. AKY912 and AKY913 (*mat*Δ::*ADE2* 4xGal4-Rap1-Abf1 *HMR***a** Δ*I ade2-1 his3-11,15 LEU2::sir2-N345A trp1-1 ura3-1 sir2*Δ::*TRP1 can1-100 hml*Δ*::hisG* plus pJR1811 [13]) were used in experiments summarized in Fig. 2 and 3, below. AKY855 and AKY856 (*mat*Δ::*ADE2* 4xGal4-Rap1-Abf1 *HMR***a** Δ*I ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 mec1*Δ::*TRP1 sml1-1 can1-100* plus pJR1811 [13]) were used in experiments shown below in Fig. 7C and D. AKY912, AKY913, AKY855, and AKY856 were derived from the JRY4806 (13) using standard genetic techniques (2, 38). RY260 (*MAT***a** *ura3-52 rpb1-1*) (33) was used to determine the half-life of **a***1* mRNA in G_1-arrested cells (see Fig. 4, below).

Chromatin immunoprecipitation. All chromatin immunoprecipitation experiments were performed using at least two independent yeast strains in two to four independent replicates of each experiment as described elsewhere (40, 41) using 10 optical density units (OD) of cells and 4 μl of either preimmune serum, unimmunized rabbit antiserum (100853; Roche), or rabbit polyclonal antisera that recognized the recombinant Sir fusion proteins LacZ-Sir2p (rabbit 2931) and LacZ-Sir3p (rabbit 2934) or the C-terminal 46% of Sir4p (rabbit 2913) (3), anti-acetyl-histone H3 (K9 and K14; 06-599; Upstate Biotechnology), or anti-acetyl-histone H4 (06-866; Upstate Biotechnology) or anti-acetyl H4 K16 (06-072; Upstate Biotechnology). Immunoprecipitated DNAs were analyzed as described previously (40) (see Fig. 1 and 2, below) or by real-time PCR (see Table 1 and Fig. 3 and 6, below). The weak signal at *HMR* in some Sir protein immunoprecipitates relative to *MAT* compared to preimmune sera or input DNA in experiments with a regulated source of Gal4-Sir1p likely reflects a small percentage of cells in which Gal4-Sir1 protein is still present after repressing transcription of the *GAL4-SIR1* fusion gene from the *MET3* promoter. In experiments using wild-type, *sir2*Δ, *sir3*Δ, or *sir4*Δ yeast harboring a vector instead of the Gal4-Sir1p plasmid, this weak signal was not detected (data not shown).

Real-time PCR. Dilutions (1/400) of coimmunoprecipitated DNA samples from chromatin immunoprecipitation experiments (shown below in Table 1 and Fig. 3 and 6) were analyzed by real-time PCR using Sybr Green PCR master mix (Applied Biosystems) according to the manufacturer's protocols. Real-time PCR was performed and analyzed on an ABI Prism 7000 using the following primers: for *MAT*, oALK283, 5′ GCC CCT GGA CTA CGA AAA CTT A 3′, and oALK284, 5′ ACA ATT CAT CAT TTG CGT TCG TT 3′; for *HMR-GalSS*, oALK265, 5′ GCC AAA CAA AAC CCA GAC ATC 3′, and oALK266, 5′ CCT GAC CCG CGG TGA TAT AT 3′; for **a***1*, oALK270, 5′ TTT AGA AGA AAG CAA AGC CTT AAT TCC 3′, and oALK271, 5′ CTT GTC TTC TCT GCT CGC TGA A 3′; for *HML-E*, oALK255, 5′ TTT TTC GCC TTT TAT ACA GAC TTC AA 3′, and oALK256, 5′ ACT TAA GAA ATT ACA TTC CAT TGC GAT AC 3′; for α*1*, oALK272, 5′ CTT GTC TTC TCT GCT CGC TGA A 3′, and oALK273, 5′ TCC CAT ATT CCG TGC TGC AT 3′; for *SSC1*, oALK467, 5′ CCT CCT CTG CCT GCT GTA CAT A 3′, and oALK468, 5′ AAT ACA AAA GTT GTC ACT CTG GCA AA 3′. Relative quantification of coprecipitated DNA was performed using the comparative C_T method according to the manufacturer's instructions (User bulletin 2, ABI Prism 7700 sequence detection system) (27). Briefly, the amount of DNA coprecipitated from each chromosomal locus at each time point during the experiments shown below in Fig. 2, 3, and 6 is expressed relative to that of the reference locus, *MAT* (see Fig. 3 and 6) or *SSC1* (see Table 1) from the same immunoprecipitation (IP) at that same time point and was calculated as follows: locus IP/reference IP = $2^{(REF\ C_T\ -\ locus\ C_T)}$. For the representative experiment shown below in Fig. 6, data represented the average of at least three PCRs for each locus for each chromatin immunoprecipitation reaction at each time point. The data shown below in Table 1 and Fig. 3 were calculated similarly and represent the averages of three or four or of two independent experiments, respectively.

RNA and DNA analyses. Total RNA was isolated, **a***1* mRNA levels relative to control *SCR1* mRNA levels were monitored by RNA blot assays, and data were quantified using a Typhoon 8600 imager (Molecular Dynamics) as described previously (22, 40, 42). *RNR4* mRNA levels were monitored similarly by RNA blot assays using a 1,040-bp probe generated by amplification of yeast genomic DNA by PCR using oALK92 (5′CA ATG GAA GCA CAT AAC CAA 3′) and oALK93 (5′CTT AGA AGT CAT CAT CAA AGT 3′) (SGD Chr7; 855257 to 856297). For monitoring the stability of **a***1* mRNA in G_1-arrested cells versus log-phase cultures, *MAT***a** yeast containing the temperature-sensitive mutant of RNA polymerase II, *rpb1-1* (33), were grown logarithmically in yeast extract-peptone-dextrose (YPD) at 23°C. The culture was adjusted to 0.5 OD/ml and split in two, and one culture was arrested in G_1 by adding α-factor to a final concentration of 10 μg/ml and incubating at 23°C for approximately 3 h. Both cultures were then shifted rapidly to 37°C by collecting cells by centrifugation and resuspending cells in YPD prewarmed to 37°C with or without α-factor. Aliquots were harvested for RNA analysis prior to the temperature shift (zero min) and at 5, 10, 15, 20, 25, 30, 40, 50, and 60 min after shifting to restrictive temperature. RNA blot assays were performed to monitor **a***1* mRNA and *SNR17A* (U3 snoRNA) levels over time (33, 34). *SNR17A* RNA levels were monitored using a 490-bp probe generated by amplification of yeast genomic DNA by PCR using oALK208 (5′ GTC GAC GTA CTT CAG TAT GT 3′) and oALK209 (5′ ACT TGT CAG ACT GCC ATT TG 3′).

Excision of *HMR* from the chromosome upon induction of FLP recombinase was monitored by DNA blot assays as described previously (22).

Flow cytometry. Aliquots of approximately 0.5 OD_{600} of cells were collected by centrifugation, resuspended in 70% ethanol, and stored at 4°C. Cells were later collected by centrifugation, washed and resuspended in phosphate-buffered saline, and prepared for flow cytometry as described previously (17), except cells were sonicated prior to staining with propidium iodide and stained cells were filtered through 35-μm strainer-capped tubes (catalog no. 2235; Falcon) by centrifugation to remove debris prior to analysis.

Cell cycle experiments. For cell cycle experiments, to regulate the expression of Gal4-Sir1p from the methionine-repressible promoter, *pMET3*, yeast were grown in synthetic complete media lacking histidine or lacking histidine and methionine at 23°C or 30°C. During G_1 arrests, the amount of methionine in the medium was reduced from 134 to 75 μM to facilitate derepression of the *MET3* promoter upon removal of methionine entirely during later steps of experiments. To induce expression of Flp recombinase from the galactose-inducible promoter, *GAL10*, powdered galactose was added to cultures grown in raffinose to a final concentration of 2% and cultures were incubated at 30°C for 1.5 h. To repress expression of Flp recombinase, yeast were grown in synthetic complete media lacking histidine or lacking histidine and methionine and containing 2% glucose or raffinose. To arrest cells in G_1, yeast were grown in media containing 10 μg/ml

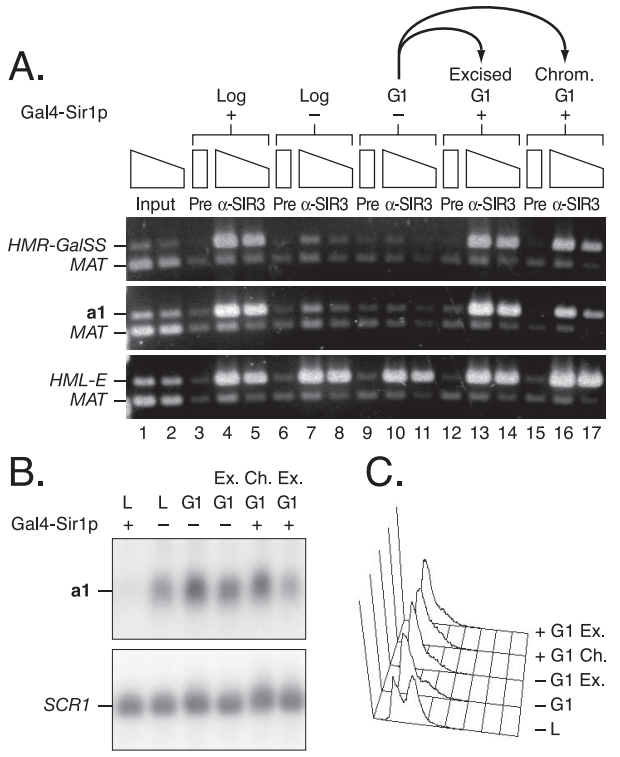

FIG. 1. Sir3 protein associates with *HMR* during G_1. Yeast containing *mat*Δ and a genomic *HMR* locus in which the *HMR-E* silencer was replaced with a synthetic silencer containing four Gal4p binding sites, a Rap1p binding site, and an Abf1p binding site and in which the *HMR-I* silencer was deleted were used. The entire *HMR* locus was flanked by two FRT sites in the same orientation to allow excision of *HMR* from the chromosome by FLP recombinase (22), which was expressed from the regulatable *GAL10* promoter (18). Silencing at *HMR* was controlled by regulating the expression of the chimeric Gal4-Sir1p via the methionine-repressible promoter *MET3* (13). A. Cells were grown logarithmically in the presence or absence of Gal4-Sir1p (lanes 3 to 5 and 6 to 9, respectively). Cells grown in the absence of Gal4-Sir1p were arrested in G_1 with α-factor (lanes 9 to 11). The culture was divided into two, and FLP was induced with 2% galactose, resulting in the excision of *HMR* in one culture. *HMR* was left in the chromosome in the other culture. Expression of Gal4-Sir1p was then induced in both G_1-arrested cultures, and FLP recombinase was repressed by incubating yeast in medium lacking methionine and containing 2% raffinose (lanes 12 to 14 and 15 to 17, respectively). Aliquots were harvested at each time point for chromatin immunoprecipitation (A), RNA blot assays (B), flow cytometry (C), DNA blots to confirm excision of *HMR* (not shown), and microscopy (not shown). DNA coprecipitating with either preimmune serum (pre) or antibodies against Sir3p (α-SIR3) was analyzed by simultaneous amplification of the synthetic silencer (*HMR-GalSS*), the *HMR***a***1* open reading frame, or the *HML-E* silencer and sequences adjacent to the *MAT* locus. Dilutions (1/15,000 and 1/30,000) of the input DNA and 1/200 and 1/400 of the immunoprecipitated DNA were analyzed. B. Total RNA was isolated from each time point, and **a***1* and *SCR1* mRNA were monitored via RNA blot assays. (No aliquot was analyzed by chromatin immunoprecipitation or by flow cytometry for G_1-arrested cells in which *HMR* had been excised from the chromosome but Gal4-Sir1p had not yet been induced.) **a***1* mRNA levels at each time point (*n*), relative to logarithmic cultures lacking Gal4-Sir1p (−L), were calculated as $[(\mathbf{a}1/\mathrm{SCR1})_n/(\mathbf{a}1/\mathrm{SCR1})_{-\mathrm{L}}]$ and were as follows: +L, 0.084; −L, 1.0; $-G_1$, 2.7; −Ex. G_1, 1.6; +Ch. G_1, 2.7; +Ex. G_1, 1.2. C. Flow cytometry of cells indicating DNA content of cells throughout the course of the experiment. + and −, presence or absence of Gal4-Sir1p; L, log-phase cells; G_1, α-factor-arrested cells; Excised or Ex., *HMR* excised from the chromosome; Chrom. or Ch., *HMR* within the chromosome.

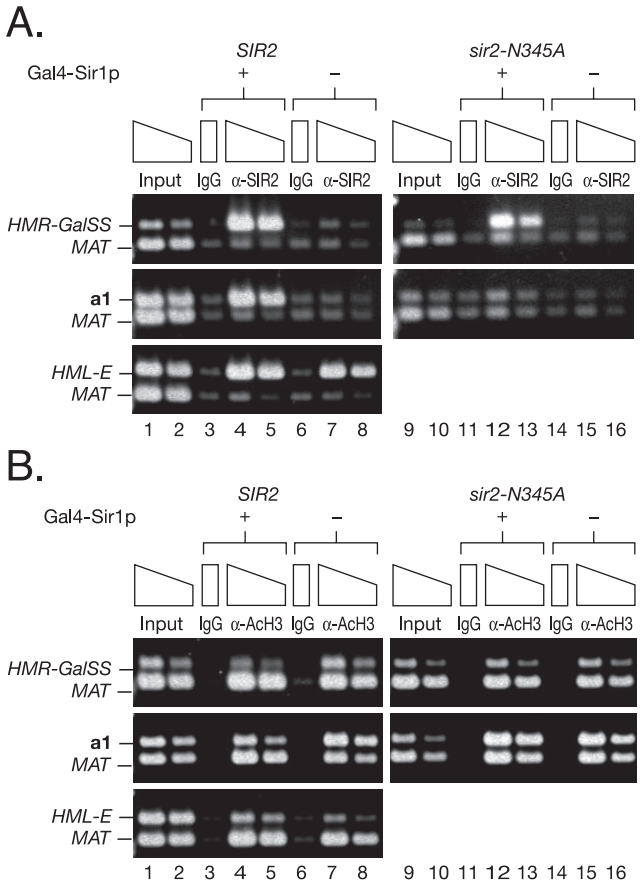

FIG. 2. Histone deacetylation upon Sir protein loading at *HMR*. A. Association of Sir2p or sir2-345p (20) with *HMR*. B. Histone acetylation at *HMR* upon expression of Gal4-Sir1p. Cells were grown logarithmically in the presence (+) or absence (−) of Gal4-Sir1p. DNA coprecipitating with either unimmunized rabbit serum (immunoglobulin G [IgG]) or antibodies against diacetylated H3 (α-AcH3) or Sir2p (α-SIR2) were analyzed by simultaneous amplification of the synthetic silencer (*HMR-GalSS*), the *HMR***a***1* open reading frame, or the *HML-E* silencer and sequences adjacent to the *MAT* locus. Dilutions (1/15,000 and 1/30,000) of the input DNA and 1/200 and 1/400 of the immunoprecipitated DNA were analyzed.

α-factor, or 0.5 μg/ml α-factor for *bar1* strains, for 3 to 3.5 h at 30°C until >90% of the cells exhibited a shmoo morphology. To release cells from the G_1 arrest, yeast were transferred to fresh medium containing 10 μg/ml pronase and incubated at 30°C. To rearrest cells in early S phase, powdered hydroxyurea was also added to the medium to a final concentration of 0.2 M and incubated at 30°C for 2 or 3 hours. To rearrest cells at G_2/M, yeast were released into medium containing 30 μg/ml benomyl and 10 μg/ml nocodazole and incubated at 30°C for 2 hours. Samples were harvested at each time point for microscopy, flow cytometry, chromatin immunoprecipitation, and analysis of RNA and DNA (2, 42).

The weak residual **a***1* message detected in silenced samples reflects the method used here to regulate silencing. As Gal4-Sir1p is expressed from a *HIS3*-marked plasmid, at any given time, a small fraction of the cells in the population grown under selection will have lost that plasmid and therefore will express **a***1* mRNA. The plating efficiency of cells containing the *HIS3*-marked plasmid encoding Gal4-Sir1p in the absence versus the presence of histidine was 91 ± 10% (n = 4).

RESULTS

Sir proteins associated with *HMR* in G_1. Previous experiments defined Sir protein interactions necessary for their re-

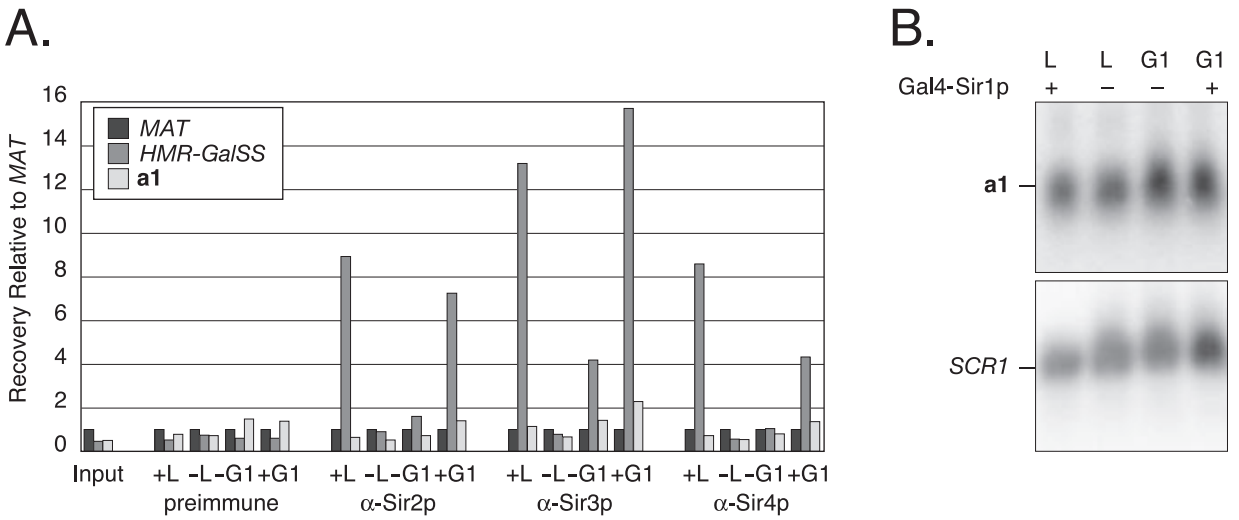

FIG. 3. Sir protein recruitment to *HMR* during G_1 does not require the catalytic activity of Sir2p. Yeast containing *mat*Δ, *hml*Δ *sir2-345*, and a genomic *HMR* locus containing *HMR-GalSS* at *E* and lacking the *I* silencer were used. The expression of the chimeric Gal4-Sir1p was regulated as described in the Fig. 1 legend. Cells were grown logarithmically in the presence or absence of Gal4-Sir1p. Cells grown in the absence of Gal4-Sir1p were arrested in G_1 with α-factor. Expression of Gal4-Sir1p was then induced in G_1-arrested cells in medium lacking methionine. Aliquots were harvested at each time point for chromatin immunoprecipitation (A), RNA blotting (B), and microscopy (not shown). A. DNA coprecipitating with either preimmune sera (pre) or antibodies against Sir2p, Sir3p, or Sir4p were analyzed using real-time PCR by amplification of DNA within the synthetic silencer (*HMR-GalSS*), the *HMR***a***1* open reading frame, and sequences adjacent to the *MAT* locus. A dilution (1/400) of the immunoprecipitated DNA was analyzed in each PCR. Data represent an average of at least three PCRs for each time point per experiment, and the efficiency of coprecipitation of each chromosomal locus at each time point was calculated as described in Materials and Methods, except the average of the relative efficiency of coprecipitation from two independent experiments is shown. *MAT* is the reference locus. B. Total RNA was isolated at each time point, and **a***1* and *SCR1* mRNA were monitored via RNA blot assays. + or −, presence or absence of Gal4-Sir1p; L, log-phase cells; G_1, α-factor-arrested cells.

cruitment to *HMR* and their subsequent spreading and deacetylation of histones (19, 30, 40). As these experiments were performed in logarithmically growing cells, they did not address whether the initial association of one or more Sir proteins with *HMR* was cell cycle regulated. To provide temporal resolution to steps in silencing, we determined whether Sir proteins can be recruited outside of S phase. We monitored Sir association at *HMR* by chromatin immunoprecipitation using yeast in which silencing could be regulated and in which the *HMR* locus could be evaluated either in the chromosome or excised from the chromosome (22). This *HMR* contains a synthetic version of the *E* silencer in which the ORC binding site is replaced with four Gal4 binding sites (*HMRGal-SS*). In these cells, silencing can be regulated by controlling expression of a chimeric protein consisting of the Gal4 DNA binding domain fused in frame to the amino terminus of Sir1p (13, 22) from the methionine-repressible promoter *MET3*. In the absence of Gal4-Sir1p, the Sir proteins do not associate with *HMR*, and **a***1* mRNA is expressed (13, 22, 40). Our previous experiments testing whether DNA replication was required for silencing used an extrachromosomal *HMR* locus which was created from an integrated *HMR* locus flanked by binding sites for FLP recombinase upon induction of Flp1p from a galactose-inducible promoter (22). Both the chromosomal and the extrachromosomal loci were used to test the cell cycle requirements for each step of silencing, as we expected that either the recruitment, or spreading of Sir proteins, or perhaps deacetylation of the underlying chromatin would be the cell cycle-regulated step.

In these experiments, yeast expressing **a***1* mRNA from *HMR* were arrested in G_1, and *HMR* was either maintained within the chromosome or excised from the chromosome while the cells remained in G_1. Upon induction of Gal4-Sir1p in both G_1-arrested cultures, Sir3p (Fig. 1) and Sir2p (not shown; see also Fig. 3 and 6, below) efficiently localized to the synthetic silencer at *HMR* (*HMR-GalSS*) relative to the *MAT* locus regardless of whether *HMR* was chromosomal or extrachromosomal (Fig. 1A, upper panel). The efficiency of loading Sir proteins at the regulated silencer in G_1 was similar to that observed in silenced, logarithmically growing cells (Fig. 1A, upper panel, +Log lane) as well as at the natural *HML* silencer (Fig. 1A, bottom panel). Because efficient loading of Sir2p, Sir3p, and Sir4p at the silencer is codependent (19, 40), the efficient loading of Sir3p also indicates that Sir2p and Sir4p all loaded at the silencer in G_1-arrested cells (data not shown; see also Fig. 3 and 6, below). Thus, the initial recruitment of Sir proteins to *HMR* could occur outside of S phase.

To test whether Sir proteins could spread across *HMR* in G_1-arrested cells, coprecipitation of the **a***1* open reading frame at *HMR* with Sir3p was also compared to that of *MAT* (Fig. 1A, middle panel). **a***1* from either the excised or the chromosomal locus efficiently coprecipitated relative to *MAT*, indicating that, like Sir protein loading, spreading of Sir proteins was temporally separable from silencing per se, and hence Sir protein spreading was not the S-phase-regulated step in silencing (Fig. 1 and data not shown; see also Fig. 6). RNA blot analysis indicated that **a***1* mRNA was still expressed during G_1 (Fig. 1B), even upon association of Sir proteins across *HMR* (Fig. 1A). Flow cytometry profiles (Fig. 1C) and microscopy (data

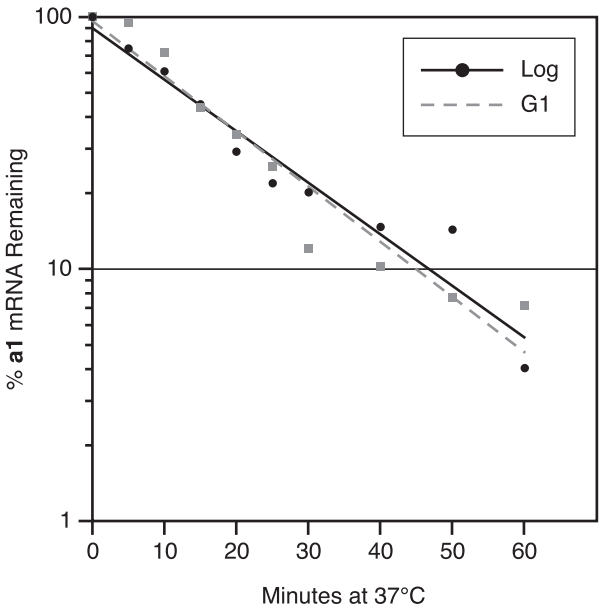

FIG. 4. Cell cycle effects on **a***1* mRNA half-life. *MAT***a** *rpb1-1* yeast (33) grown logarithmically at 23°C in YPD were divided into two cultures in fresh YPD and allowed to grow logarithmically or were arrested in G_1 with α-factor. Cells were pelleted and resuspended in YPD at 37°C with or without α-factor, and aliquots were harvested every 5 minutes for the first half hour and then every 10 min for an additional half hour for RNA blot assay or flow cytometry (not shown). Total RNA was isolated at each time point, and **a***1* mRNA and *SNR17A* (U3 snoRNA) were monitored via RNA blot assays. The log percent **a***1* mRNA remaining at each time point relative to time zero, $\log\{([\mathbf{a}1]_n/[\mathrm{U3}]_n)/([\mathbf{a}1]_o/[\mathrm{U3}]_o)\}$, where n = RNA harvested at n minutes and o = RNA harvested at zero minutes, was plotted with respect to time.

not shown) confirmed that the cells remained arrested in G_1 throughout this experiment.

Sir2p's catalytic activity was not required for Sir protein binding in G_1. The catalytic activity of Sir2p is required for the efficient spreading of Sir proteins from the silencer throughout *HMR* (19, 30, 40). Therefore, the spreading of Sir2p, Sir3p, and Sir4p to the **a***1* gene at *HMR* in G_1 (Fig. 1A; see Fig. 6A; data not shown; Fig. 2A) provided strong evidence that the deacetylase activity of Sir2p was not tightly cell cycle regulated. Nevertheless, to provide an independent test of whether the deacetylase activity of Sir2p might be cell cycle regulated, we monitored acetylation of histones H3 and H4 in G_1-arrested cells upon induction of Gal4-Sir1p and found the levels of acetylated H3 and H4 at *HMR-E* were reduced (Table 1). This reduction in acetylation could not be explained simply by displacement of histones from the silencer region when Sir proteins were present (Fig. 2). No difference in coprecipitation of the synthetic silencer at *HMR-E* was observed with antibodies against acetylated histones in the presence of Gal4-Sir1p and loading of Sir proteins (Fig. 2A and B, lanes 12 and 13) in catalytically inactive *sir2-N345A* mutants, versus the absence of Gal4-Sir1p with no loading of Sir proteins (Fig. 2A and B, lanes 15 and 16).

In *sir2-N345A* mutants, Sir3p and Sir4p as well as Sir2-N345Ap loaded efficiently at the synthetic *HMR-E* silencer in G_1 following the induction of Gal4-Sir1p (Fig. 3A). However, none of the Sir proteins spread efficiently to the **a***1* gene at *HMR* in cells containing the catalytically inactive Sir2 protein, in contrast to *SIR2* cells (Fig. 1A and 2; see also 6A, below). Thus, the loading and spreading of Sir proteins in G_1-arrested cells and the requirement for Sir2's enzymatic activity for spreading in G_1 indicated that the cell cycle-regulated event in silencing occurred after spreading of Sir proteins over *HMR*.

This experiment revealed an interesting aspect of spreading and deacetylation. The antibodies against acetylated histones H3 and H4 revealed a greater reduction in the acetylation state of the chromatin at *HMR-E* than at *HMR***a***1* despite the spreading of Sir proteins to *HMR***a***1* chromatin and the requirement for the deacetylase activity of Sir2p for this spreading in G_1. Possible mechanisms by which this could occur are outlined in the Discussion, below.

a*1* **mRNA remained unstable in G_1-arrested cells.** Monitoring **a***1* mRNA steady-state levels to infer the silenced state of cells (13, 22, 24, 25, 32) has long proven useful in silencing studies due to **a***1* mRNA's very short half-life of 3 minutes (31). The utility of this measurement for this study is predicated upon the half-life of **a***1* mRNA being similar in all portions of the cell cycle. If **a***1* mRNA's half-life were longer in G_1 than in exponentially growing cells, it would provide misleading clues as to when silencing occurs in the cell cycle. To determine whether the stability of **a***1* mRNA increased significantly in G_1-arrested cells relative to logarithmically growing cells, the decay of **a***1* mRNA was compared under both conditions in *MAT***a** cells expressing *rpb1-1*. This temperature-sensitive mutant of RNA polymerase II (Pol II) blocks 90% of Pol II-dependent transcription within 2 minutes of shifting to restrictive temperature and does not affect the ability of α-factor to arrest cells in G_1 (33). RNA blot assays monitoring **a***1* mRNA relative to the stable *SNR17A* transcripts indicated that **a***1* mRNA from both cultures decayed at similar rates (Fig. 4). Measured in this way, **a***1* mRNA appeared to decay more slowly than previously reported (32). This altered half-life of **a***1* mRNA in *rpb1-1* cells has been observed previously and may, in part, reflect residual Pol II activity in these mutants (50). Regardless, this analysis indicated that the transcripts detected in G_1 upon Sir protein loading were not due to altered stability of **a***1* mRNA during G_1 relative to dividing cells. Together, these data indicate that Sir proteins can associate throughout *HMR* in G_1-arrested cells, yet transcription of **a***1* mRNA continues.

Silencing between G_1 and early S phase. To define more closely where the cell cycle regulation of silencing is temporally executed, we tested whether the silencing that is established somewhere between the G_1 arrest and the nocodazole arrest at G_2/M could be established at an intermediate step. Except for hydroxyurea, there are few robust cell cycle blocks to allow analysis of the intermediate positions of the cell cycle. The ability of hydroxyurea to arrest cells in early S phase was used to test an intermediate position in the cell cycle for the establishment of silencing. These experiments revealed the surprising conclusion that whether or not silencing is completed by early S phase depended upon whether *HMR* was chromosomal or extrachromosomal. In experiments in which silencing of an excised *HMR* locus was monitored following Gal4-Sir1p induction in G_1-arrested cells, the excised *HMR* locus was silenced at the hydroxyurea arrest point in early S phase (Fig. 5A, lane 6

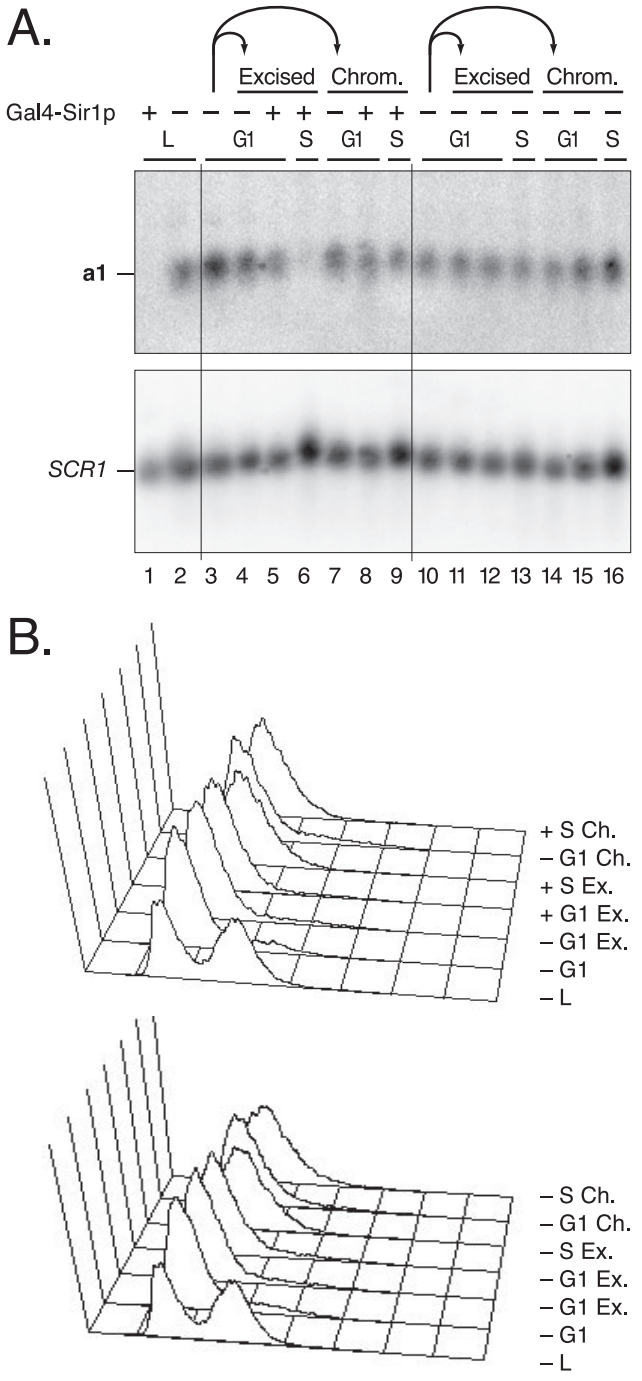

FIG. 5. Establishment of silencing between G_1 and an S-phase arrest. A. Yeast strains described in the Fig. 1 legend were grown in the absence of Gal4-Sir1p, resulting in the expression of **a***1* from *HMR* (lane 2). Yeast were arrested in G_1 with α-factor (lane 3), and then each culture was divided in two. Either FLP1 recombinase was induced with 2% galactose, leading to excision of the *HMR* locus from the chromosome in G_1 (lane 4), or *HMR* was left within the chromosome (lane 7). Expression of Gal4-Sir1p was induced (lanes 5 and 8) and FLP recombinase was repressed (lane 5) in the G_1-arrested cells by incubation in medium lacking methionine and containing 2% raffinose. Cells were then released from G_1 arrest into medium containing 0.2 M hydroxyurea to rearrest cells in S phase (lanes 6 and 9). Parallel experiments were performed in the absence of expression of Gal4-Sir1p (lanes 10 through 16). Total RNA was isolated from each time point, and **a***1* and *SCR1* mRNA were monitored via RNA blot assays. B. Flow cytometry analysis, indicating DNA content of cells at each time point of the experiment described for panel A. The upper panel includes time points corresponding to lanes 2 to 6, 8, and 9, and the lower panel includes time points corresponding to lanes 2, 10 to 13, 15, and 16 of RNA blots shown in panel A). + or −, presence or absence of Gal4-Sir1p; L, log-phase cells; G_1, α-factor-arrested cells; S, hydroxyurea-arrested cells; Excised or Ex., *HMR* excised from the chromosome; Chrom. or Ch., *HMR* within the chromosome.

versus lane 5; in S-phase-arrested cells, **a***1* mRNA levels were 9.8 ± 6.7% [$n = 3$] relative to G_1-arrested cells expressing Gal4-Sir1p). This result indicated that a crucial cell cycle requirement for silencing was executed after the α-factor arrest point and prior to the hydroxyurea arrest point, corresponding to a narrow cell cycle window between late G_1 and early S (Fig. 5A).

However, a different picture emerged in the analysis of the chromosomal *HMR* locus. Here, *HMR* was not silenced at the hydroxyurea arrest (Fig. 5, lane 9; see also Fig. 7, below). Indeed, there was no expectation that the chromosomal *HMR* would become silenced, as earlier work implied that the cell cycle-dependent step in silencing occurred after this block (13, 24, 25, 32). Thus, the two different contexts for *HMR*, chromosomal and extrachromosomal, provided greater temporal resolution of a cell cycle requirement than had been previously achieved and hinted at the possibility of a second cell cycle requirement in silencing (see below).

To understand the difference in silencing outcome between chromosomal and excised *HMR* loci in hydroxyurea-treated cells, we considered the possibility that Sir proteins bound and spread throughout chromosomal *HMR* might be less stably associated than those on an extrachromosomal *HMR* and that some difference between the excised and chromosomal *HMR*, perhaps topology, may have resulted in the dissociation of Sir proteins from the chromosomal locus but not the extrachromosomal one. However, chromatin immunoprecipitation analysis indicated that Sir2, Sir3, and Sir4 proteins that were recruited in G_1 remained associated with chromosomal *HMR* in hydroxyurea-treated cells (Fig. 6). Similarly, hydroxyurea treatment was unable to disrupt silencing in either G_1-arrested or hydroxyurea-arrested cells that had been silenced in previous cell cycles (Fig. 7A and B). Thus, hydroxyurea does not prevent Sir proteins from localizing to *HMR*, but rather blocks a step critical to the establishment of silencing in the chromosomal context that is not necessary for maintaining the silent state.

One difference between untreated and hydroxyurea-treated cells is the presence in the latter of stalled replication forks and activation of the intra-S-phase checkpoint. For a checkpoint activation to be responsible for the results we obtained, the consequences of checkpoint activation would have to be *cis*-limited to molecules capable of inducing the checkpoint. Since the excised *HMR* lacks a replication origin (22) and, thus, would lack any stalled replication fork, the excised *HMR*, under this scenario, would be spared the inhibitory effect of checkpoint activation on silencing that acted in *cis* to stalled forks on the chromosome. As S-phase checkpoint activation is largely dependent on the *MEC1*-encoded kinase (10, 29, 47), we tested whether Mec1p activation blocked chromosomal *HMR* silencing. We used a *mec1* null allele in a strain analo-

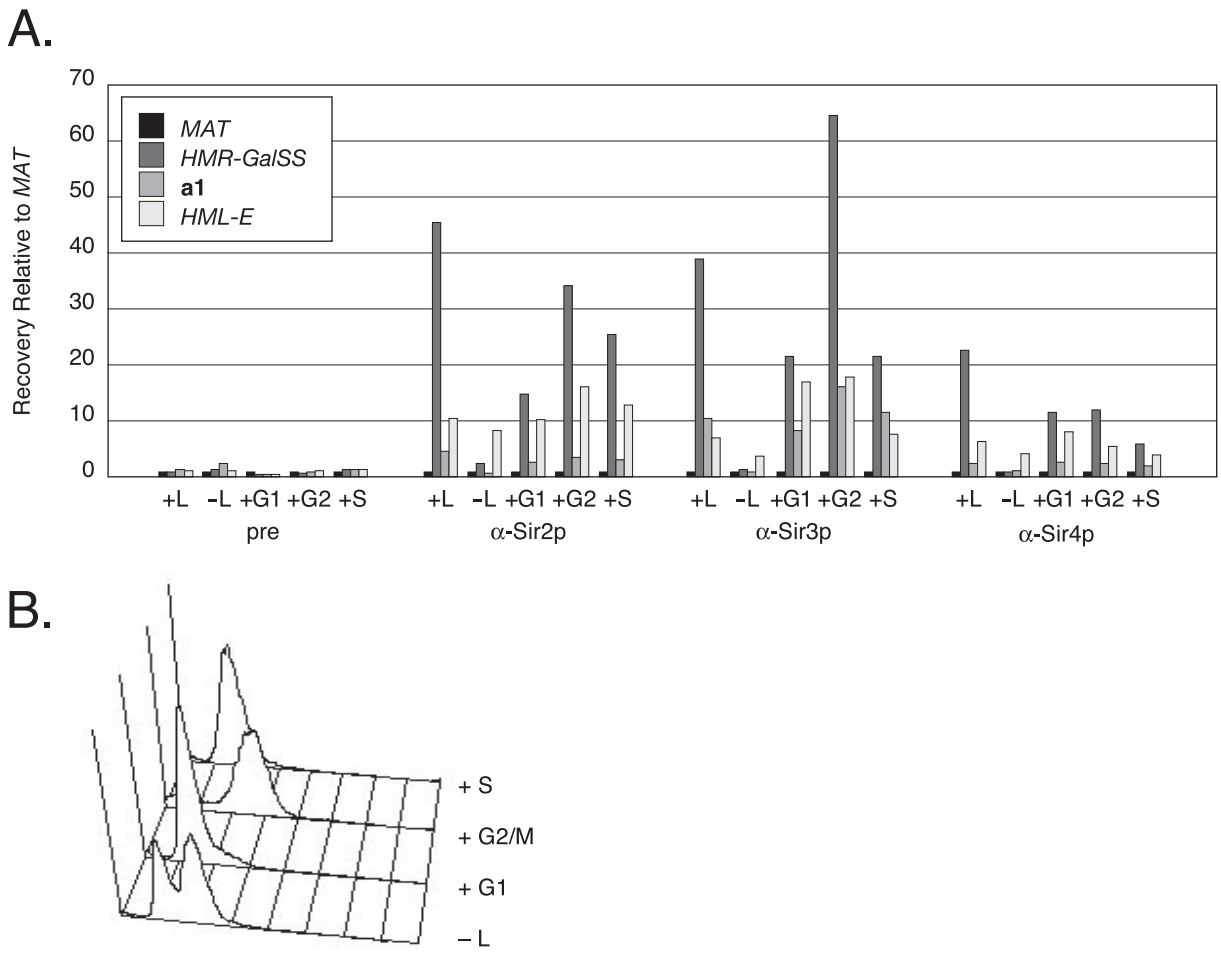

FIG. 6. Sir protein association. Yeast described in the legend for Fig. 1 were grown logarithmically in the presence or absence of Gal4-Sir1p. Cells grown in the absence of Gal4-Sir1p were arrested in G_1 with α-factor. Expression of Gal4-Sir1p in the G_1-arrested cells was then induced by incubating the yeast in medium lacking methionine. The culture was divided into two, and one culture was released into medium containing benomyl and nocodazole to rearrest cells at G_2/M and the other culture was released into medium containing hydroxyurea to rearrest cells in early S phase. Aliquots were harvested at each time point for chromatin immunoprecipitation (A) and flow cytometry (B). A. DNA coprecipitating with either preimmune serum (pre) and antibodies against Sir2p, Sir3p, or Sir4p were analyzed using real-time PCR by amplification of DNA within the synthetic silencer (*HMR-GalSS*), the *HMR***a***1* open reading frame, or the *HML-E* silencer and sequences adjacent to the *MAT* locus. A dilution (1/400) of the immunoprecipitated DNA was analyzed in each PCR. Data represent averages of at least three PCRs for each time point from one representative experiment. The efficiency of coprecipitation of each locus at each time point during the time course is expressed relative to that of the *MAT* locus at that same time point and was calculated as follows: locus IP/*MAT* IP $= 2^{(MAT\ C_T\ -\ locus\ C_T)}$. (See Materials and Methods.) B. Flow cytometry of cells indicating DNA content of cells throughout the course of the experiment. + or −, presence or absence of Gal4-Sir1p; L, log-phase cells; G_1, α-factor-arrested cells; G_2/M, benomyl- and nocodazole-arrested cells.

gous to that used in Fig. 1 to determine whether silencing of a chromosomal *HMR* would be restored upon release from a G_1 arrest into hydroxyurea in the absence of checkpoint function. However, silencing in such a strain was not restored in hydroxyurea (Fig. 7C and D). In contrast, silencing was established between release from G_1 and rearrest at G_2/M in this mutant. Therefore, *mec1*Δ cells were not inherently defective in establishment of silencing. As expected, *RNR4*, a checkpoint-responsive gene, was not induced in *mec1* cells compared to *MEC1* cells in hydroxyurea (Fig. 7C versus E), confirming that the S-phase checkpoint was indeed nonfunctional. An analogous experiment with *mec1*Δ *tel1*Δ double mutants to test for residual checkpoint function could not be conducted, as *mec1 tel1* cells exhibit rapid senescence (6, 36), precluding their efficient synchronization for cell cycle experiments (data not shown).

DISCUSSION

The critical discoveries reported here include the ability of SIR proteins to bind to, spread over, and deacetylate the *HMR* chromatin in the G_1 phase of the cell cycle in a manner similar to that seen in logarithmically growing cells (Fig. 1, 2, and 6 and Table 1). These data provided important temporal resolution of steps involved in forming silenced chromatin. Moreover, these observations indicate the existence of at least one undescribed step in the formation of silenced chromatin. Analysis of *HMR* chromatin in G_1 cells lacking or expressing Gal4-

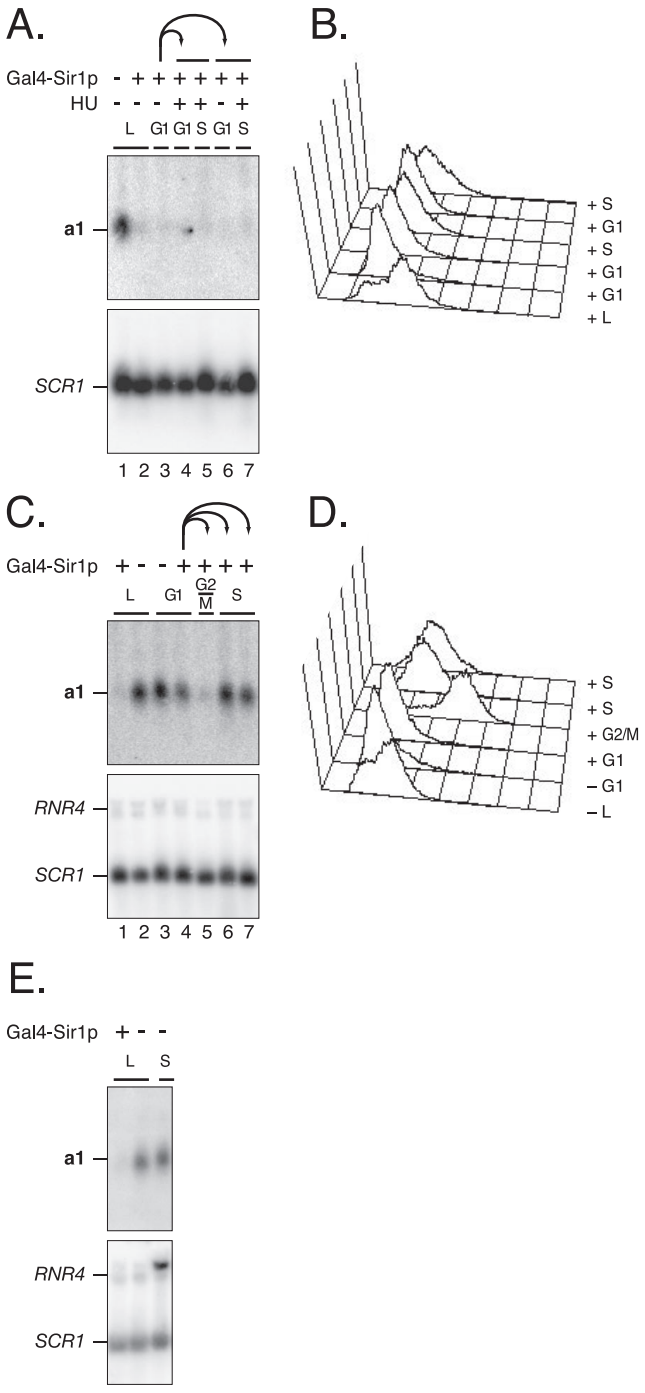

FIG. 7. Effects of hydroxyurea on silencing. A and B. Hydroxyurea does not disrupt silenced chromatin. A. Logarithmically growing cells expressing Gal4-Sir1p (lane 2) were arrested in G_1 with α-factor (lane 3). The G_1-arrested culture was split in two, hydroxyurea was then added to one culture during the G_1 arrest (lane 4), and both cultures were held in the G_1 arrest for one additional hour (lanes 4 and 6). Cells from both cultures were released from the G_1 arrest into medium containing 0.2 M hydroxyurea for 2 hours (lanes 5 and 7) to rearrest cells in S phase. Total RNA was isolated at each time point, and **a***1* and *SCR1* mRNA were monitored via RNA blot assays. B. Flow cytometry of the experiment shown in panel A, indicating DNA content of cells at each time point. C and D. Hydroxyurea blocks the establishment of silencing in *mec1*Δ cells. C. Logarithmically growing *mec1*Δ cells lacking Gal4-Sir1p (lane 2) were arrested in G_1 with α-factor (lane 3). Gal4-Sir1p expression was induced in G_1-arrested cells (lane 4), and cells were released from the G_1 arrest into benomyl plus nocodazole to rearrest cells at G_2/M (lane 5) or into 0.2 M hydroxyurea for 2 or 3 hours (lanes 6 and 7, respectively). Total RNA was isolated at each time point, and **a***1*, *RNR4*, and *SCR1* mRNA were monitored via RNA blot assays. D. Flow cytometry of the experiment shown in panel C, indicating DNA content of cells at each time point. E. Hydroxyurea-activated checkpoint induces *RNR4* in *MEC1* cells. Logarithmically growing cells in the absence of Gal4-Sir1p were arrested in S phase by incubating in 0.2 M hydroxyurea for 3 hours. + or −, presence or absence of Gal4-Sir1p; L, log-phase cells; G_1, α-factor-arrested cells; S, hydroxyurea-arrested cells; G_2/M, benomyl- and nocodazole-arrested cells.

Sir1p clearly showed that Sir2p could deacetylate chromatin in G_1 at *HMR* (Table 1). The residual histone acetylation observed at *HMR***a***1* in G_1 may be related to the inability of Sir proteins to silence transcription from the **a***1* promoter in G_1 due to competition between a histone acetyltransferase responsible for this acetylation linked to transcription and the Sir2 deacetylase, as the nature of silenced chromatin is dynamic (8). The essential paradox revealed by these results was that the known events involved in silenced chromatin had occurred by G_1, yet silencing was not established. Thus, the cell cycle requirement for silencing must identify some heretofore-unanticipated step. The quantitatively dramatic reduction in acetylation at the *HMR-E* silencer compared to that at *HMR***a***1* in G_1-arrested cells superficially seems to conflict with the requirement for the deacetylase activity of Sir2p for its efficient spreading to *HMR***a***1*. However, at least two models could explain how these apparently conflicted states could coexist. One view is that spreading of Sir proteins can be accomplished in G_1 even if only a subset of Sir2p's possible substrate sites are deacetylated in a region. In this view, the cell cycle requirement for silencing might be an event that allows Sir2p to finish deacetylating H3 and H4 tails after Sir2, 3, and 4 proteins have already spread. In a competing view, while spreading to *HMR***a***1* in G_1, Sir2p did deacetylate all of its substrates, but an acetylase(s), presumably including Sas2p, was able to reacetylate H3 and H4 tails during G_1 and lost this ability later during the cell cycle.

Significantly, we observed that robust silencing could be established by early S phase if silencing was measured on a nonreplicating extrachromosomal cassette containing *HMR*. A previous study also deduced the existence of a post-G1 cell cycle-regulated event in silencing; however, in that study the cell cycle-regulated event was post-hydroxyurea block and even post-G_2/M arrest (see below) (24). The results reported here were the first to reveal the existence of a cell cycle-regulated step in silencing that could be completed prior to the hydroxyurea arrest. Indeed, these data were compatible with that step occurring any time after release from an α-factor arrest and before early S phase, which indicates that the missing step in silencing may actually occur in late G_1 or at "start." In support of this notion, prearrest of cells lacking Sir1p in S phase with hydroxyurea and then expression of a Sir1 fusion protein does not permit silencing of an excised *HMR* arrested in S phase (25).

Clearly, the situation in the chromosome was more complex in that the extrachromosomal *HMR* was silenced under conditions insufficient to complete the silencing of the chromosomal

locus. The question at hand is whether a second cell cycle requirement for silencing exists after the hydroxyurea arrest or whether the exposure of cells in G_1 to hydroxyurea which causes their subsequent arrest in S phase induces some process that precludes the establishment of silencing. Although these data do not definitively adjudicate the issue, they put limits on the possible interpretations. For example, if the block to silencing chromosomal loci resulted from checkpoint activation, then the effects of that activation must be limited to chromosomes but not to nonreplicating extrachromosomal loci in the same cell. Stalled replication forks are the likely candidates for a signal, but the effects of that signal must be restricted to the molecules that bear them. Mechanistically, this restriction invites comparisons with the ability of double-stranded DNA breaks to induce H2A phosphorylation at the site of damage and over tens of kilobases or more away from the site of the break, but only in *cis* (12, 37, 44). If this block to silencing reflects an activity of the S-phase checkpoint, then a *mec1* mutation would be expected to relieve this block to silencing, allowing silencing of chromosomal *HMR* in hydroxyurea-arrested cells. However, *mec1* mutant cells did not silence *HMR* under these conditions. Therefore, either an S-phase checkpoint is not responsible for the block to silencing chromosomal *HMR* loci or a protein that is not uniquely a target of the Mec1 kinase must be involved.

It is formally possible that only one cell cycle-dependent step in silencing exists but that this step can be executed earlier in S phase, prior to the hydroxyurea block, on nonreplicating templates than on replicating ones, after the hydroxyurea block. However, as a mechanism for this temporal separation awaits further studies, a two-step model could also accommodate our observations.

An alternative line of explanations for our observations invokes a post-hydroxyurea arrest event that is needed on replicating molecules but not on nonreplicating molecules. Although DNA replication itself seems to have no requisite role in silencing per se (13, 22, 25), other replication-dependent processes may, in turn, be required for silencing. Sister chromatid cohesion is established in S phase and dissolved in M phase and would be present on all replicated DNA molecules but not on the unreplicated extrachromosomal *HMR*. Hence, sister chromatid cohesion could be a candidate for a post-hydroxyurea block event that might impact silencing. Indeed, a recent study concluded that the dissolution of sister chromatid cohesion is an M-phase step required for the establishment of efficient silencing (24).

At face value, that earlier study would seem to conflict with some, but not all, of the conclusions presented here. In that study, a temperature-sensitive mutation in *SIR3*, *sir3-8*, was used to control the onset of silencing by shifting from the restrictive temperature in G_1-arrested cells to the permissive temperature and monitoring silencing of *HMR***a***1*. In the *sir3-8* experiments, by G_2/M, expression of **a***1* mRNA was reduced by only one-half. In contrast, using the protocol described here, expression was reduced by 89 or 86% by G_2/M (22) or 90% for an extrachromosomal *HMR* by S phase (Fig. 5). Importantly, once taking into account the subpopulation of cells that would have lacked the Gal4-Sir1p plasmid in our experiments (see Materials and Methods), nearly all cells that had the potential to be silenced were, in fact, silenced by G_2/M. All residual **a***1* mRNA detected at G_2/M could be explained by the fraction of cells that had lost the Gal4-Sir1p-encoded plasmid. And, since sister chromatid cohesion is not dissolved by G_2/M, the dissolution of sister chromatid cohesion would not be required to achieve quantitatively robust silencing in experiments using the *sir3-8* mutant and this protocol.

In the earlier study using *sir3-8*, complete silencing was not recovered until telophase unless the Scc1 cohesin was inactivated, in which case silencing was essentially complete by G_2/M (24). These and supporting data led to the conclusion that sister chromatid cohesion needs to be removed before silencing can be established. Of course, a nonreplicating excised *HMR* cassette would have no sister chromatid and, therefore, might be free of inhibitory effects on silencing caused by cohesion loading. However, if this were the explanation for the ability of the excised *HMR* to be silenced at the hydroxyurea arrest, then silencing of a chromosomal *HMR* would be established at the hydroxyurea arrest in *sir3-8* cells at the permissive temperature in cells lacking Scc1p, which it is not (24).

It is worth considering what role cohesins normally play in silencing or whether their apparent involvement is a function of the experimental protocol. All experimental manipulations of silencing use conditional gene expression or conditional protein function as an artificial switch, allowing the steps in silencing to be studied with a degree of synchrony. In our study, the switch was provided by Gal4-Sir1p induction in G_1, which allowed us to discover that all Sir proteins could bind the silencer, spread through *HMR*, and deacetylate histones, all in G_1. Under these conditions, Sir proteins have covered *HMR* before the time that the cohesins are laid down. In the previous study, with the temperature downshift of the temperature-sensitive *sir3-8* mutant, efficient loading or spreading of the Sir proteins was not observed until G_2/M, after the time at which cohesins are loaded. It seems entirely possible that cohesins have a chance to interfere with silencing only under experimental conditions in which their deposition can precede the spreading of Sir proteins, which is delayed by the nature of the *sir3-8* mutant. Nevertheless, both this study and the earlier study agree that Sir proteins can bind to and spread over the *HMR* locus without resulting in silencing of that locus, highlighting the existence of yet-to-be-discovered steps in silencing. The difference between these studies is when those events occur.

Our data clearly showed that silencing can be robustly established by G_2/M, although they leave open the possibility that silencing can be made more robust by later events in the cell cycle. Moreover, we revealed the existence of an event that occurred after the α-factor block and before the hydroxyurea block that can silence an unreplicated *HMR* locus. This event cannot be the dissolution of sister chromatid cohesion, which occurs later during the cell cycle. And, this event may not be the only event that is required for silencing a replicated *HMR* by G_2/M. Whether sister chromatid cohesion plays a role in regulating silencing in wild-type cells or interferes with establishment only during certain experimental designs cannot be resolved as yet. The pair-wise establishment of silencing that occurs in rare cell divisions in *sir1* mother-daughter pairs supports the notion of an early event critical to silencing occurring at or before the time that the *HM* loci are replicated (35). It is difficult to imagine how dissolution of sister chromatid cohe-

sion, which must happen on all chromosomes in all divisions, could accommodate rare but symmetric changes in chromatin state.

It is possible that a topological change to the *HMR* chromatin reflects a cell cycle and, in particular, the S-phase requirement for silencing. Recently, Xu et al. monitored the topology of an excised *HML* locus in *sir3-8* cells between a G_1 arrest at restrictive temperature and arrest in hydroxyurea at permissive temperature and observed similar topologies at both time points (51). Unfortunately, the unresolved kinetic issues with respect to how soon the sir3-8 protein returns to functional levels following such a temperature shift preclude a simple interpretation of this topological stasis.

We do not favor the model in which Sir protein concentrations are limiting in all phases of the cell cycle except S phase. *SIR* gene expression is not cell cycle regulated (46), cells harboring six copies of *HMR* on plasmids can stably silence all copies of *HMR* (1), Sir proteins were recruited to *HMR* in G_1 (Fig. 1, 3, and 6), and silent chromatin does not become derepressed at specific points in the cell cycle (Fig. 7) (22).

In summary, Sir protein recruitment to *HMR* in G_1 indicates that simply the presence of all known components of silent chromatin at *HMR* was insufficient to mediate silencing and suggests that histone modifications sufficient for spreading may be insufficient for silencing. Furthermore, our findings as well as others' observations that an inhibitor of Sir2p can disrupt silencing in G_1-arrested cells (4) indicate that Sir2p's catalytic activity is not limited to S phase. Thus, the cell cycle-regulated step(s) in building silent chromatin reflects a late event(s) that can be blocked by either hydroxyurea or sister chromatid cohesion. Such an event(s) may be the modification of a silencing component at *HMR* or a conformational or stoichiometric change in Sir proteins at *HMR* that must occur to silence transcription. The discovery of structural isomerization of a Sir protein complex in vitro (26) and the multiple changes to other histone modifications that occur upon silencing (21) offer an attractive range of possibilities. Further loss of chromatin modifications beyond deacetylation at *HMR* clearly correlate with decreased transcription from *HMR*, and these additional changes likely enhance the stability of the silenced state within any cell in a given population (21). Our data cannot exclude the existence of a regulatory role for sister chromatid cohesion in restricting the formation of silenced chromatin, but the data do highlight the need for further investigation of this possibility. Cohesins can interfere with Sir spreading, as Scc1p has been localized to boundaries of silent chromatin at *HMR* (23) and mutations in *SMC1* result in spreading of silenced chromatin to chromosomal regions flanking *HMR* (11). Understanding changes to components of silent chromatin between G_1 and S phase will provide insight into how silent chromatin is first formed.

ACKNOWLEDGMENTS

This work was supported by American Cancer Society postdoctoral fellowship PF-01-126-01-MBC (A.L.K.), USDA Hatch grant IND053072 (A.L.K.), Kimmel Scholar Award SKF-03-010 (A.L.K.), and National Institutes of Health grant GM31105 (J.R.). This project was also supported by an American Cancer Society Institutional Research Grant to the Purdue Cancer Center.

This is paper number 2005-17808 from the Purdue University Agricultural Experiment Station.

We thank Joe Ogas and Scott Briggs for helpful discussions and Marcia Kremer for technical assistance.

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