A Silencer Promotes the Assembly of Silenced Chromatin Independently of Recruitment[▽]

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In *Saccharomyces cerevisiae*, silenced chromatin occurs at telomeres and the silent mating-type loci *HMR* and *HML*. At these sites, the Sir proteins are recruited to a silencer and then associate with adjacent chromatin. We used chromatin immunoprecipitation to compare the rates of Sir protein assembly at different genomic locations and discovered that establishment of silenced chromatin was much more rapid at *HMR* than at the telomere VI-R. Silenced chromatin also assembled more quickly on one side of *HMR-E* than on the other. Despite differences in spreading, the Sir proteins were recruited to *HMR-E* and telomeric silencers at equivalent rates. Additionally, insertion of *HMR-E* adjacent to the telomere VI-R increased the rate of Sir2p association with the telomere. These data suggest that *HMR-E* functions to both recruit Sir proteins and promote their assembly across several kilobases. Observations that association of Sir2p occurs simultaneously throughout *HMR* and that silencing at *HMR* is insensitive to coexpression of catalytically inactive Sir2p suggest that *HMR-E* acts by enabling assembly to occur in a nonlinear fashion. The ability of silencers to promote assembly of silenced chromatin over several kilobases is likely an important mechanism for maintaining what would otherwise be unstable chromatin at the correct genomic locations.

The packaging of DNA into transcriptionally repressed heterochromatin is an important process common to eukaryotic organisms. Just as important is the restriction of this heterochromatin to appropriate genomic loci. To understand why repressive chromatin forms in particular locations, we compared the rates of assembly of silenced chromatin at various loci in the budding yeast *Saccharomyces cerevisiae*.

In *S. cerevisiae*, domains of silenced chromatin are found at the silent mating-type cassettes *HMR* and *HML* and at most telomeres. The structural components of this chromatin are Sir2p, Sir3p, and Sir4p. The first step in establishment of silenced chromatin is the recruitment of Sir proteins to the chromosome, which is mediated by DNA sequences termed silencers. At the silent mating-type cassettes, the silencers *HMR-E* and *HMR-I* flank *HMR* and the silencers *HML-E* and *HML-I* flank *HML*. Each of these silencers consists of binding sites for the origin recognition complex (ORC) and for either Rap1p, Abf1p, or both. Sir proteins are assembled at the silencers through physical interactions with the DNA-binding proteins and each other. A fourth Sir protein, Sir1p, also participates in the initial assembly process through interactions with ORC and Sir4p (49). Telomeric silencers are distinct from the silencers at *HM* loci in that they are composed of an array of Rap1p binding sites embedded in the telomeric (TG_{1–3})_n repeats. Despite variation in composition, most silencers function to recruit the Sir complex and thus initiate silencing. The one exception is *HMR-I*, which does not recruit Sir proteins on its own and appears to play a supporting role in silencing *HMR* (35, 37).

Once recruited to a silencer, Sir2p, Sir3p, and Sir4p spread along the chromosome. A working model for spreading proposes that Sir proteins propagate in a stepwise manner facilitated by sequential deacetylation of histones (reviewed in reference 36). Sir2p, a histone deacetylase, generates hypoacetylated histone H3 and H4 tails that are preferentially bound by Sir3p and Sir4p, which in turn recruit additional Sir2p to deacetylate the next nucleosome (5, 14, 17, 37). Thus, the Sir proteins are dependent on each other for assembly. This model predicts that Sir-silenced chromatin should propagate linearly along a chromosome. Because silencers serve as the initiators of the spreading process, they determine where silenced chromatin will form. Although Sir proteins are recruited differently by silencers at *HM* loci and telomeres, spreading of the Sir complex occurs at all sites.

The ability of Sir proteins to propagate along a chromosome could potentially be toxic to the cell if silenced chromatin spreads beyond its appropriate domain or fortuitously assembles in the wrong locations. Consequently, mechanisms must exist to damp down the spreading of the Sir proteins. However, such damping mechanisms could prevent the Sir proteins from stably repressing promoters distant from a silencer, as required to maintain cell type identity. How the assembly of silenced chromatin is opposed throughout most of the genome and promoted in particular locations is incompletely understood. Two general models have been proposed: competition with euchromatin and discrete DNA sequences that generate barriers between active and silenced chromatin. A naturally occurring barrier is a tRNA^{Thr} gene located on the telomere-proximal side of *HMR* (8, 9, 32). However, barrier elements have not been identified at most junctions between silenced and active chromatin in *S. cerevisiae*, and competition is proposed to limit spreading at these sites. In this model, active chromatin is characterized by a set of histone modifications that reduce the affinity of the Sir proteins for nucleosomes.

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Thus, an equilibrium between active and silenced chromatin is reached, and silenced chromatin spreads only a short distance from a silencer. Evidence for this model comes from observations that in the absence of proteins that characterize active chromatin, Sir proteins spread farther at telomeres. These proteins include, but are not necessarily limited to, the histone acetyltransferase Sas2p, the bromodomain protein Bdf1p, the histone variant H2A.Z, and the histone methyltransferases Dot1p and Set1p (20, 22, 23, 27, 39, 47, 51). Although these "antisilencers" clearly play a role in restricting the spread of Sir proteins, their absence results in only modest extensions of silenced chromatin rather than global redistribution of Sir proteins.

The impact of antisilencers on silenced chromatin often varies at different genomic loci. Sas2p, for example, is arguably the most significant antagonist of telomeric silencing through its acetylation of histone H4 at the K16 residue (H4K16). In addition to competing with Sir2p for the state of H4K16, Sas2p also fortifies the function of other antisilencers by facilitating the deposition of H2A.Z and boosting the ability of Dot1p to compete with Sir3p for access to histones (1, 20, 43, 47). Despite exhibiting a clear antisilencing phenotype at telomeres, SAS2 has a much less severe impact at *HMR* (19). Furthermore, SAS2 differentially influences silencing at the two mating-type loci, *HMR* and *HML* (10, 54). Such discrepancies have been observed for other antisilencers (39, 51) and suggest that silenced chromatin may not assemble equivalently at all locations.

In this study, we characterized the rates of Sir complex assembly at several genomic locations. We discovered that spreading rates vary at different genomic loci and that much of this variation can be attributed to the silencer. Sir proteins assembled rapidly at *HMR* over a region of about 3 kb, and the association of Sir proteins occurred virtually simultaneously throughout the locus. In contrast, assembly at a telomere (VI-R) was significantly slower and proceeded in a linear fashion, such that the Sir proteins associated with regions closer to the telomere earlier than regions farther from the telomere. Remarkably, despite the differences in the rates of spreading, the Sir proteins were recruited to the silencers (*HMR-E* or the telomeric repeat) at equivalent rates and at similar levels. Furthermore, insertion of the *HMR-E* silencer into the telomere resulted in more-rapid spreading of Sir proteins, indicating that the slower spreading observed at the telomere was not simply due to the telomeric chromatin being restrictive to spreading. From these observations, we conclude that the *HMR-E* silencer does not simply recruit Sir proteins to the chromosome. It also has the capacity, which the telomeric repeat does not have, to promote the assembly of silenced chromatin over a distance of several kilobases. We propose that silencers permit a specialized chromatin structure to exist that would otherwise be unstable and that the silencer's ability to promote spreading is an important parameter for determining the size and stability of silenced chromatin domains. Furthermore, we hypothesize that the *HMR-E* silencer promotes the assembly of Sir proteins over a distance by creating a situation in which spreading is not strictly linear, as predicted by the stepwise model of sequential deacetylation.

TABLE 1. Strains used in this study

Strain	Genotype ^a	Source
LRY1007 (W303)	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	R. Rothstein
LRY0750	W303 <i>MATα sir3Δ::LEU2</i> [pJR517]	
LRY0800	W303 <i>MATα sir2Δ::TRP1 LEU2::sir2-N345A</i>	
LRY0804	W303 <i>MATα LEU2::sir2-N345A</i>	
LRY1021	<i>MATα his4</i>	P. Schatz
LRY1068	W303 <i>MATα sir2Δ::TRP1</i>	
LRY1780	W303 <i>MATα sir3Δ::LEU2 RPB1::18myc::TRP1</i> [pJR517]	
LRY1781	W303 <i>MATα sir3Δ::LEU2 hmr-ΔI</i> [pJR517]	
LRY1815	W303 <i>MATα hmr-ΔI LEU2::sir2-N345A</i>	
LRY1861	W303 <i>MATα hmr-ss(5GAL-RAP1-ABF1)ΔI</i> [pLR529]	
LRY2019	W303 <i>MATα sir3Δ::LEU2 TELVIR::STUFFER</i> [pJR517]	
LRY2020	W303 <i>MATα sir3Δ::LEU2 TELVIR::HMRE</i> [pJR517]	
LRY2042	W303 <i>MATα sir3Δ::LEU2</i> [pLR577]	
LRY2043	W303 <i>MATα hmr::HMR LEU2::RecR SIR3-myc::klTRP1</i> [pLR577]	
LRY2165	W303 <i>MATα hmr-ΔI</i> [pLR577]	
LRY2287	W303 <i>MATα LEU2::HA-sir2-N345A</i>	
LRY2288	W303 <i>MATα LEU2::HA-sir2-N345A sir2Δ::TRP1</i>	
DRY0450	W303 <i>MATα hmr-ΔI</i>	D. Rivier

^a Brackets denote transformation of the indicated plasmid. See Materials and Methods for plasmid details.

MATERIALS AND METHODS

Yeast strains and plasmids. Strains used in this study were derived from W303-1b and are described in Table 1. The following alleles were described previously: *sir3Δ::LEU2* (38), *RPB1::18myc::TRP1* (33), *hmrΔI* (35), *LEU2::sir2-N345A* (3, 18), and *HMRss(5xGal4DBS-RAP1-ABF1)ΔI* (11). To create the *SIR3-myc* allele, 9×-Myc DNA was amplified by PCR from a plasmid containing 9× Myc and the *TRP1* marker from *Kluyveromyces lactis* in the pUC19 vector backbone (pWZV87; courtesy of Kim Nasmyth, Oxford University, Oxford, United Kingdom) using the primers 5'-GAGACTGCATGTGTACATAGGCATATCTATGGCGGAAGTGGGCCAGAAAGACTAAGAGGTG and 5'-CCTTTTCGATGGATGAAGAATCAAAAATATGGACTGCATTGGTTC TGCTGCTAGTGGT, with the underlined sequences annealing to the plasmid and the remaining sequences homologous to the 3' end of the *SIR3* gene. The resulting DNA was integrated at the *SIR3* locus by homologous recombination to generate *SIR3-myc*. To generate strains with a hemagglutinin (HA)-tagged *sir2-N345A* allele, a plasmid containing N-terminal HA-tagged *SIR2* plus approximately 1 kb of the *SIR2* promoter (pRO298; courtesy of Rohinton Kamakaka, University of California, Santa Cruz) was cut with SacII and BglII and the fragment containing the promoter and 5' end of the *SIR2* gene was ligated into the same sites of the plasmid pRS305-*sir2N345A* (3, 18), resulting in pLR0727. To integrate *HA-sir2-N345A* into the genome, pLR0727 was cut within the *LEU2* gene by AflII and integrated into yeast via homologous recombination, resulting in a *LEU2::HA-sir2-N345A* allele.

The *TELVIR::HMRE* and *TELVIR::STUFFER* alleles were constructed in four steps. (i) A *TELVIR::URA3* allele was created by homologous recombination in a *sir3Δ::LEU2* strain using DNA amplified from pRS406 (44) with primers 5'-TCATAAACATAAGCGTATCCAATTTTGACATATCCTTCACCTGTG CGGTATTTACACACCG and 5'-AACGAGTGGATGCACAGTTCAGAGTTA TCTAACAAATATTCGATTGTACTGAGAGTGCACC, resulting in LRY1862. (ii) *TELVIR::URA3* was amplified from LRY1862 genomic DNA by PCR with high-fidelity *Pfu* Turbo DNA polymerase (Stratagene) using primers 5'-CACG AGGTACCGACCAATAAGAAATGTGAGCATAC and 5'-GAGTCGGAG CTCGTGCTAAAGGAATCCCCAGAGAC, with the underlined sequences corresponding to engineered recognition sites for KpnI and SacI, respectively. The PCR products were digested with SacI and KpnI and cloned into the vector pRS412 (44) to generate pLR566. (iii) Four hundred thirty-one bases of DNA

containing either *HMR-E* or a fragment of the *TRP1* open reading frame were amplified from wild-type (W303) genomic DNA with high-fidelity polymerase using the primers 5'-AATATAAATGATATATCATAAACATAAGCGTATC CAATTTTGACATATCCTTCACCTAAATCGCATTTCTTTTCGTCCAC and 5'-TTCGAACGTGATCCTAACGAGTGGATGACACAGTTCAGAGTTA TCTAACAAATATTCTAACAACCAAGAGTACCTGCGC for *HMR-E* and 5'-AATATAAATGATATATCATAAACATAAGCGTATCCTAATTTTG ACATATCCTTCACGAATGTGCTCTAGATTCCGATGCTG and 5'-TTCG AACGTGATCCTAACGAGTGGATGACAGTTCAGAGTTA TCTAACAA TATCTCTCTCTGCTTCCAACCCAGTC for *STUFFER*, with the underlined sequences annealing to the template and the remaining sequences homologous to subtelomeric VI-R DNA. The resulting PCR products were recombined into *AccI*-gapped pLR566 by homology-driven *in vivo* gap repair to generate pLR568 (*TELVR::HMRE*) and pLR573 (*TELVR::STUFFER*). (iv) The *TELVR::HMRE* and *TELVR::STUFFER* alleles were integrated by gene conversion of *TELVR::URA3* in strain LRY1862 using high-fidelity PCR products amplified from pLR568 and pLR573 with primers 5'-CAGCAATAAGAAAATGTGAGCATAC and 5'-GTGCTAAAGGAATCCCCAGAGAC. The resulting integrations were positioned 60 bases adjacent to the core-X sequence.

The plasmid pJR517 contains *SIR3* under the control of the *GALI* promoter and was constructed in the laboratory of J. Rine (University of California, Berkeley). The plasmid pLR577 was constructed by ligating a PCR-generated *SIR3-myc* fragment, amplified from genomic DNA from LRY1827, into an *EagI* site located within the *SIR3* gene on pJR517. The primers used to amplify the *SIR3-myc* insert were 5'-CATCTGTGCTTTCAAGTAAAC and 5'-GAGTCGC GCGCCGAGTGAATGATCGTTCCAC, in which the underlined sequence corresponds to an *EagI* site. The plasmid pLR529 was a HA-tagged derivative of pJR1811, a *P_{MET3}-GAL4_{DBD}-SIR1* construct in the pRS313 vector previously described (11). To tag *SIR1*, the plasmid was gapped with *EcoNI* and *AflII* and then repaired by *in vivo* homologous recombination with chromosomal HA-tagged *SIR1* from CFY416 (13).

Cell growth conditions. All cultures were grown in selective, supplemented media (CSM; MP Biomedicals) and maintained in logarithmic growth throughout the time courses. For the induction of *P_{GAL}-SIR3*, cultures pregrown in 2% raffinose were brought to an optical density at 600 nm of approximately 1.0 (± 0.1) and then induced by the addition of galactose to a final concentration of 2%. For the induction of *P_{MET3}-GAL4_{DBD}-SIR1-HA*, cultures were grown under conditions lacking methionine. These strains were grown in 2% glucose.

Chromatin immunoprecipitation. Chromatin immunoprecipitations were performed essentially as previously described (38) using 10 optical density equivalents of cells and 3 μ l rabbit polyclonal antiserum to recombinant LacZ-Sir2p or LacZ-Sir3p (rabbits 2932 and 2934, respectively; gifts from J. Rine, University of California, Berkeley) or 3 μ l of antibodies to histone H4 acetyl-K16, the C terminus of histone H3, Myc, or HA tag (07-329, 05-928, 06-549, or 05-902; Upstate Biotechnology). Cells were treated with 1% formaldehyde for 20 min to cross-link proteins to DNA, after which the cross-linking reaction was quenched by the addition of glycine to a final concentration of 0.125 mM. For Fig. 7B and C, cells were treated with formaldehyde for 35 min and the cross-linking reaction was not quenched. Quantitative real-time PCR was performed as previously described (26), except a different control locus was selected for normalization. For most reactions, *PHO5* was chosen as the internal control locus since this site is subject to neither silencing nor transcription under the specified growth conditions. For Fig. 2E, an uncharacterized open reading frame (YKL105C) on chromosome XI which lacks significant levels of RNA polymerase II (Pol II) (45) was selected as the internal control for Rpb1p-Myc chromatin immunoprecipitation (IP) analysis. Relative IP values represent the ratio of the query locus to the internal control locus. Where appropriate, the relative IP data were normalized to either the maximum ratio of the query to the control locus obtained in the particular experiment or the ratio observed under uninducing conditions. Unless otherwise indicated, reported values represent averages for at least two independent immunoprecipitations analyzed in at least three separate PCRs. Sequences of the oligonucleotides used are available upon request.

Mating assay. One optical density equivalent of logarithmically growing *MAT α* haploid cells was collected by centrifugation and resuspended in 100 μ l of minimal medium (YM). A 10-fold dilution series was prepared in YM, and 3 μ l from each dilution was spotted on yeast extract-peptone-dextrose to control for growth. To induce mating, an equivalent volume of *MAT α* tester cells (LRY1021), suspended in yeast extract-peptone-dextrose at a dilution of 10 optical density equivalents per ml, was added to the dilution series. Three microliters of the mating mixture was spotted on YM plates to select for diploids. Plates were imaged after 2 days growth at 30°C.

RNA blotting. Total RNA was isolated via the hot phenol method (40), separated on formaldehyde agarose gels, and transferred to Zeta Probe nylon

membranes (Bio-Rad) by capillary action. DNA probes were generated by PCR using total yeast genomic DNA as a template. The sequences of primers are available upon request. Probes were labeled with [α -³²P]dCTP using the RediPrime II DNA labeling kit (Amersham). The mRNA of interest was normalized to *ACT1* mRNA using a Storm PhosphorImager.

Protein blotting. Proteins were extracted from whole cells using a trichloroacetic acid precipitation technique described previously (16). Proteins from 0.2 optical density equivalents of cells were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membranes (Amersham), and probed with antibodies to Myc (06-549; Upstate Biotechnology) and 3-phosphoglycerate kinase (A-6457; Molecular Probes/Invitrogen).

RESULTS

Following induction of *SIR3*, *HMRa1* was largely silenced within one generation. To compare the rates at which Sir proteins spread at different genomic locations, an inducible *SIR3* gene was employed. First, the establishment of silencing was assessed by quantitative Northern blotting for *HMRa1* mRNA (Fig. 1A and B), which has a half-life of less than 3 min (28). After an initial delay of ~10 to 20 min, *HMRa1* levels dropped dramatically. By 60 min, the precipitous drop in *HMRa1* levels began to level out, and within 105 min, total mRNA was at 10% of the original value, approaching its steady-state level (Fig. 1B). The doubling time of the yeast during this experiment was approximately 120 min. Thus, the majority of silencing occurred within one generation. The amount of *HMRa1* mRNA was inversely related to that of *SIR3* mRNA, which steadily increased until maximum expression was reached around 105 min. To follow the accumulation of the Sir3 protein, a 9x-Myc tag was fused to the C terminus of Sir3p and protein samples were collected for immunoblotting at various times after induction (Fig. 1C). Sir3p-Myc was first detected 20 min after induction. The Myc tag did not alter the rates of *SIR3* expression or silencing of *HMRa1* (data not shown).

It should be noted that expression of *SIR3* driven by the *GALI* promoter results in overproduction of the protein compared to endogenous levels (Fig. 1D). Since silenced chromatin is sensitive to the dosage of *SIR3*, an alternative method to establish silencing was examined in which initiation is controlled by a synthetic silencer that acts through the recruitment of a Gal4_{DBD}-Sir1p-HA fusion protein, whose expression is dictated by the inducible *MET3* promoter (11). In this system, regulation was achieved without altering the endogenous levels of the Sir proteins that spread (Sir2/3/4p), guaranteeing that the rate of spreading is not affected by unusually high levels of Sir3p. Establishment rates were slightly slower in this system compared to those observed upon overexpression of *SIR3*, which could be explained by the absence of the *HMR-I* silencer (see Fig. 4). However, the majority of *HMRa1* mRNA was still repressed within the first generation (Fig. 1E). Thus, overproduction of Sir3p did not result in a significant change in the rate of establishment.

Association of Sir2p and deacetylation of H4K16 at the *HMRa1* promoter occurred in a similar time frame to that of repression of *HMRa1*. A prior study following establishment of silencing using a similar inducible *SIR3* gene reported that it takes several generations to reach a fully mature silenced state (19). However, in both the previous study and our work, around 90% of transcriptional repression occurred within the

FIG. 1. *HMRa1* was silenced rapidly following induction of *SIR3*. (A) RNA was isolated at the indicated times following the addition of galactose to a *sir3Δ* strain (LRY0750) with a plasmid expressing wild-type *SIR3* under the control of the inducible *GAL1* promoter (pJR517). The blot was probed for *HMRa1*, *SIR3*, and *ACT1*. (B) Quantification of *HMRa1* and *SIR3* mRNAs following induction of *SIR3*. Each number represents the average from two independent experiments, normalized first to a control mRNA, *ACT1*, and then to the experimental maximum. Rel. mRNA, relative mRNA level. (C) Immunoblot of protein from a *sir3Δ* strain (LRY2042) with a plasmid containing a 9×-Myc-tagged *SIR3* under the control of the *GAL1* promoter (pLR577). Protein samples were collected under similar conditions as for panel A and probed with antibodies against the Myc tag and 3-phosphoglycerate kinase (loading control). (D) Expression of *SIR3*-myc from a strain with chromosomal *SIR3*-myc expressed by its endogenous promoter (LRY2043) and transformed with plasmid pLR577 under noninducing (Raf.) or inducing (Gal.) conditions. The Sir3p-Myc observed under noninducing (Raf.) conditions represents the endogenous levels of the protein. (E) RNA was isolated from a strain with a modified *HMR* allele containing a synthetic silencer in the place of *HMR-E* and no *HMR-I* silencer. The strain was transformed with a plasmid containing *GAL4_{DBD}*-*SIR1*-HA (LRY1861). Expression

first generation. Therefore, this early phase of silencing is likely to be the time during which the Sir complex associates with *HMR*. To test this hypothesis, we repeated the experiment described above and assessed the association of Sir2p with the *HMRa1* promoter by chromatin IP assays at various times after induction of *SIR3* (Fig. 2A). Sir2p was chosen as the representative Sir protein based on the consistency of results with anti-Sir2p antibodies. After induction of *SIR3*, accumulation of Sir2p at the *HMRa1* promoter was relatively rapid. There was little change in the total levels of Sir2p after 90 min, and these levels were very close to those observed in the late time points of the experiment (Fig. 2B). This was roughly the same length of time required for the majority of *HMRa1* repression (Fig. 2B). Therefore, the initial phase of silencing at *HMR* represents the time during which Sir proteins assemble along the chromatin.

To determine whether deacetylation of histones occurred concomitantly with association of Sir2p, we examined the acetylation of K16 of histone H4, an *in vitro* substrate of Sir2p (18, 48). In the same samples used to follow association of Sir2p, acetylation of H4K16 declined during the same period of time that association of Sir2p increased (Fig. 2C). This decline was probably not due to loss of histones, since the total levels of H3 were relatively constant during the time that a decrease in acetyl-H4K16 was observed (Fig. 2D). Interestingly, the acetyl-H4K16 signal continued to decrease between 90 and 180 min after induction of *SIR3*, whereas the association of Sir2p didn't change significantly during this time. It is therefore possible that Sir2p becomes saturated at the *HMRa1* promoter prior to complete deacetylation of resident nucleosomes. In any event, the association of Sir2p with the *HMRa1* promoter was accompanied by a loss of acetylation of H4K16, a known substrate for Sir2p.

Pol II was displaced by Sir proteins. The mechanism by which Sir chromatin blocks transcription remains disputed, and two different models have been proposed. The first model argues that silenced chromatin blocks the transcription complex from assembling at promoters within the silenced domain (6, 24). The other model suggests that Pol II-containing preinitiation complexes do form at the promoter but are unable to clear the promoter and enter the elongation phase of transcription (12, 41, 45). In light of conflicting data on the presence of Pol II within silenced chromatin, we examined whether Pol II is displaced during the assembly of silenced chromatin. Pol II occupancy at the *HMRa1* promoter was assayed by chromatin IP of Myc-tagged Rpb1p, the largest subunit of the polymerase. Following induction of *SIR3*, Rpb1-Myc levels dropped precipitously (Fig. 2E), indicating that the Sir complex does displace RNA Pol II from chromatin. This drop occurred over the same time period as loss of H4K16 acetylation (compare Fig. 2C and E). Control experiments revealed that the rate of silencing of *HMRa1* mRNA in this Rpb1-Myc strain was comparable to the rate observed in the untagged strain (data not

of *GAL4_{DBD}*-*SIR1*-HA was driven by the inducible *MET3* promoter (pLR529), and RNA was collected at the indicated times following transfer to medium lacking methionine. *HMRa1* mRNAs were detected and quantified by Northern blotting as described above.

FIG. 2. The association of Sir2p and loss of acetylated H4K16 occurred rapidly at the *HMRA1* promoter. (A) Diagram of the *HMR* locus. The bar indicates the position of the amplicon used for PCR analysis. (B) Association of Sir2p with *HMRA1* following induction of *SIR3* in strain LRY0750. Cultures were maintained under logarithmic growth conditions. For the overnight time points, cultures were diluted following overnight growth and brought to logarithmic growth conditions before the sample was collected. DNA coprecipitated with Sir2p was analyzed by real-time PCR with primers specific for the promoter of *HMRA1* (black bar in panel A). Values represent averages from two independent experiments normalized first to an internal control (*PHO5*) and then to the experimental maximum (see Materials and Methods). RNA was also collected and analyzed for *HMRA1* mRNA as for Fig. 1B. (C) Acetylation of H4K16 at *HMRA1*. DNA coprecipitated with an anti-acetyl H4K16 antibody from the same samples collected for panel B was analyzed as described above, with the exception that relative IP values were normalized to those obtained from uninducing conditions at the 0-min time point. (D) Total histone H3 levels were assessed by chromatin IP from the same samples collected for panel B.

shown). These results are consistent with the first model, in which Sir chromatin blocks the access of Pol II to silenced promoters, although it remains possible that some preinitiation complexes do form and are prevented from elongating.

Association of Sir2p was rapid and uniform across *HMR*. To determine the rate at which the Sir complex propagates along the chromosome, we examined the association of Sir2p with DNA as a function of distance from the *HMR-E* silencer (Fig. 3A). Since the *HMR-I* silencer cannot independently silence chromatin and does not recruit the Sir complex (4, 37), *HMR-E* is the point at which Sir proteins are first recruited to *HMR*. The association of Sir2p with DNA immediately adjacent to *HMR-E* and at distances of approximately 1 and 2 kb on the telomere side of the silencer was assessed by chromatin IP (Fig. 3B). The accumulation of Sir2p was rapid at the *HMR-E* silencer, approaching saturation within 90 min after induction of *SIR3*. Remarkably, Sir2p levels were also approaching saturation within 90 min of *SIR3* induction at distances of 1 and 2 kb from the silencer, although the maximum chromatin IP signals for Sir2p at these sites were lower than at the silencer (Fig. 3B). In an attempt to resolve the times at which Sir2p arrived at these three positions, samples were collected at close intervals during the first 45 min after induction of *SIR3*. The association of Sir2p was first observed at *HMR-E* between 10 and 15 min following induction of *SIR3*, consistent with the *E* silencer being the initial point of contact between Sir proteins and *HMR* (Fig. 3C). However, Sir2p enrichment was observed simultaneously at 1- and 2-kb distances approximately 5 to 10 min after the protein was detected at the silencer.

The observation that the association of Sir2p reaches a maximum at about the same time at all three locations implies that the assembly of Sir chromatin is rapid throughout the locus. Therefore, the gradual buildup of Sir2p over a 90-min window can be attributed to heterogeneity within the population rather than gradual assembly of the complex within a single cell. The reciprocal relationship between the accumulation of *SIR3* mRNA and the loss of *HMRA1* mRNA (Fig. 1B) also suggests that silencing was established asynchronously in the population, with individual cells establishing silencing once a certain threshold of Sir3p was reached. It is interesting to note that no significant difference was observed in the association of Sir2p at sequences 1 and 2 kb from the silencer. Two explanations were considered for this result: either linear spreading is more rapid than can be resolved by chromatin IP or spreading is not strictly linear at *HMR*.

Rates of Sir2p spreading were different on the two sides of the *HMR-E* silencer. We next addressed whether the assembly of Sir proteins is equally rapid on the other side of the *HMR-E* silencer. The simple sequential deacetylation model would predict that spreading should be equivalent in both directions.

Samples were analyzed as for panel C. (E) Association of RNA polymerase II with the *HMRA1* promoter following induction of *SIR3* was determined by chromatin IP of Myc-tagged Rpb1p from a strain of the genotype *sir3Δ RPB1-myc* (LRY1780) transformed with *P_{GAL1}-SIR3* on a plasmid (pJR517). Relative chromatin IP values plotted on the y axis were analyzed as for panels C and D with the exception that data were first normalized to a different internal control (YKL105C) and then to the uninduced time point.

FIG. 3. Sir2p associated with chromatin more rapidly on the telomere-proximal side of *HMR-E*. (A) Diagram of *HMR* and flanking regions. Bars represent amplicons for PCR analysis. Distances are relative to *HMR-E* (± 100 bp) as measured from the endpoints of the consensus sequences for silencer binding proteins (ORC and Abf1p) to the midpoint of the amplicon. (B) Association of Sir2p with three regions of *HMR* following induction of *SIR3* in strain LRY0750. The y axis represents total Sir2p enrichment relative to an internal control locus (*PHO5*). (C) Association of Sir2p with three regions of *HMR* was assessed at short intervals following the induction of *SIR3*. Data were collected from one experiment and are independent of those represented in panel B. (D) Association of Sir2p with regions at equivalent distances on either side of *HMR-E* following induction of *SIR3* in strain LRY0750. (E) Relative level of acetylation of H4K16 at regions at equivalent distances on either side of *HMR-E*. (F) Relative levels of histone H3 at equivalent distances on either side of *HMR-E*. Relative enrichment values on the y axis were determined as for Fig. 2C and D. Samples for panels B, D, E, and F were collected from the same experiment. (G) Association of Sir2p with regions at equivalent distances on either side of *HMR-E* at steady state in a strain (LRY1007) expressing endogenous levels of Sir3p.

However, mechanisms may exist to promote the formation of silenced chromatin specifically over the mating-type genes, and hence there may be a directional bias in rates of assembly. Silenced chromatin emanates bidirectionally from the *HMR-E* silencer, as assayed by restriction endonuclease accessibility, transcriptional repression, and chromatin IP of Sir proteins (8, 25, 37). However, although the silencer can induce bidirectional silencing, it does demonstrate an orientation preference, silencing reporter genes more efficiently on the side that binds

Abf1p than on the side that binds ORC (55). This directionality favors silencing in the direction of the *HMRa1* gene, located on the telomere side of the *HMR-E* silencer (Fig. 3A). We compared the rates of Sir2p accumulation at 1-kb distances on both sides of *HMR-E* (Fig. 3D). Once saturated, both sides reached similar levels of Sir2p enrichment. However, in the first 90 min following induction of *SIR3*, the accumulation of Sir2p on the telomere side of *HMR-E* was clearly more rapid than that on the centromere side. By 180 min, both locations

appeared to reach maximum levels. Thus, it took two to three times longer to reach this maximum level at the centromere side of *HMR-E*.

The rates of deacetylation of H4K16 were also compared on the two sides of *HMR-E*. Interestingly, on the centromere side of *HMR-E*, deacetylation of H4K16 occurred concomitantly with association of Sir2p (Fig. 3E). At this location, H4K16 was largely deacetylated by the time Sir2p approached its maximum level at 180 min. In contrast, on the telomere side of *HMR-E*, acetylated H4K16 was still detected at 45 and 90 min, when Sir2p was close to its maximum level (compare Fig. 3E to D). To control for total histone occupancy, histone H3 levels were also assayed on the two sides of *HMR-E* (Fig. 3F). Although the total chromatin IP levels of H3 were slightly reduced at both loci at the later time points (Fig. 3F), this decline was less than the decline in acetylated H4K16. Thus, differences in acetylated histone H4K16 levels were unlikely to be due solely to a loss of total histone levels. Collectively, these data indicate that the silencer promotes the association of Sir proteins more on one side than on the other and may even have the ability to stabilize the assembly of Sir proteins on the telomere side prior to full deacetylation. Therefore, the rapid and stable accumulation of Sir2p throughout *HMR* (Fig. 3B) represents a special case rather than a general property of Sir proteins.

The overexpression of Sir3p leads to the extension of silenced chromatin domains (15). Therefore, at endogenous levels of Sir3p, silenced chromatin might form preferentially on the more stable, telomere side of *HMR-E*. To test this idea, Sir2p enrichment was assayed at the same sites at steady state with endogenous levels of Sir3p. Under these conditions, total Sir2p enrichment was three- to fourfold higher on the telomere side of *HMR* (Fig. 3G), suggesting that Sir proteins are stabilized on this side of *HMR-E*.

***HMR-I* accelerated accumulation of Sir2p at *HMR*.** Two features that could contribute to the difference in rates of assembly on the two sides of *HMR-E* are the asymmetry of the *HMR-E* silencer itself and the presence of the *HMR-I* silencer on the favored side of *HMR-E*. The *HMR-I* silencer does not recruit Sir proteins (37) or contribute to the steady-state silencing of *HMRa1* (35). *HMR-I* does, however, collaborate with *HMR-E* to silence a reporter gene (35) and diminishes the antisilencing activity of barrier elements inserted between the *HMR-E* silencer and reporter genes (8). To determine whether *HMR-I* influences the rate of spreading, we assayed the rates of transcriptional repression and Sir2p accumulation with and without the *HMR-I* silencer. In the absence of *HMR-I*, repression of *HMRa1* mRNA was slightly but consistently delayed, although mRNA levels were comparable to wild-type levels 24 h after induction (Fig. 4A). As in the presence of the *HMR-I* silencer, recruitment of Sir2p to *HMR* occurred more or less simultaneously throughout *HMR* (Fig. 4B), although it was slightly delayed compared to assembly in the presence of *HMR-I* (Fig. 4C). These data indicate that although *HMR-E* alone is sufficient to silence *HMR*, *HMR-I* accelerates the accumulation of the Sir proteins.

Spreading of Sir2p at telomere VI-R was significantly slower. Thus far, we have demonstrated that variation exists in the rates at which the Sir complex accumulates on the telomere and centromere sides of *HMR-E* and that these rates are in-

FIG. 4. In the absence of *HMR-I*, the accumulation of Sir2p at *HMR* was slightly delayed. (A) Quantification of *HMRa1* mRNA in strains containing wild-type *HMR* (LRY0750) or lacking the *HMR-I* silencer (LRY1781) at indicated times following induction of *SIR3*. The y axis represents *HMRa1* mRNA normalized first to *ACT1* and then to the levels prior to induction of *SIR3*. (B) Association of Sir2p with three regions of *HMR* following induction of *SIR3* in a strain in which the *HMR-I* silencer has been deleted (LRY1781). (C) Association of Sir2p with a region 2 kb from the *HMR-E* silencer in the presence or absence of the *HMR-I* silencer.

fluenced by neighboring silencers. Importantly, assembly occurred more rapidly on the side of *HMR-E* that is more strongly silenced. Silencing at *HMR* is known to be more resistant to disruption by genetic mutations than silencing at other loci. At the other end of the spectrum, telomeric silencing is easily perturbed by such mutations. To determine whether the rate of assembly of Sir proteins correlates with the strength of silencing, we examined the association of Sir2p with telomere VI-R. The most obvious difference between the mechanisms of silencing at telomeres and *HMR* is the recruitment step. The Sir complex is recruited to telomeres via an array of Rap1p binding sites as opposed to a compact cluster of ORC, Rap1p, and Abf1p binding sites. Additionally, what role, if any, Sir1p plays in recruiting the Sir complex to telomeres remains incompletely understood (2, 31, 34). In contrast, there are no known differences in the mechanisms of spreading at telomeres and *HMR*.

To directly compare rates of spreading at the two loci, the association of Sir2p was assessed at distances along telomere VI-R comparable to those examined at *HMR* using the

FIG. 5. Rates of spreading but not recruitment were slower at telomere VI-R than at *HMR*. (A) Sir2p association with telomere VI-R following induction of *SIR3* in strain LRY0750. Distances were measured from the end of the telomeric repeats (TG_{1-3}) and accurate to about 170 bases (see lower diagram). (B) Sir2p association at *HMR*, assessed by PCR using the same samples as in panel A. (C) Association of Sir2p and acetylation of histone H4K16 at a region 1 kb away from the end of telomere VI-R. Enrichment levels for acetylated H4K16 were determined as described for Fig. 2C. (D) Histone H3 levels assessed at 1 kb from the end of telomere VI-R. Relative IP values were determined as for Fig. 2C. Samples analyzed in panels A, B, C, and D were collected from the same experiment. (E) Association of Sir2p with regions at equivalent distances from the *HMR-E* and telomere VI-R silencers at steady state in a strain (LRY1007) expressing endogenous levels of Sir3p.

same chromatin IP samples analyzed previously (Fig. 5A and B). Only one open reading frame, YFR057, is located within 4 kb of telomere VI-R, and it is silenced (52). Remarkably, the accumulation of Sir2p was significantly slower at the telomere, taking more than 7.5 h to develop compared to less than 1.5 h at *HMR*. The lower rate of spreading at the telomere could not be attributed to differences in initial recruitment, since both silencers achieved maximum levels of Sir2p within 90 min of *SIR3* induction (Fig. 5, compare panels A and B). In fact, in terms of total Sir2p enrichment, recruitment was slightly greater at the telomere than at *HMR*.

Therefore, despite similar levels of recruitment, the Sir complex was unable to spread across subtelomeric chromatin with the same efficiency as observed at *HMR*. In addition, Sir2p accumulated at sites closer to the telomere earlier than at sites

more distant from the telomere. These results are consistent with linear propagation of the Sir complex along chromosomes, as predicted by the sequential deacetylation model, and further highlight the unique nature of Sir protein assembly at *HMR*. Additionally, the rate of H4K16 deacetylation mirrored that of Sir2p accumulation at the telomere (Fig. 5C), and no significant changes in total histone H3 levels were observed (Fig. 5D). Finally, we assessed the steady-state levels of Sir2p at 1-kb distances from *HMR-E* and telomere VI-R under endogenous levels of Sir3p (Fig. 5E). Similar to our observations at the centromere side of *HMR-E* (Fig. 3G), Sir2p levels were lower at the telomere than at *HMR* (Fig. 5E). From these data we infer that features of the *HMR* locus promote the assembly of Sir proteins in ways that do not occur at the telomere VI-R. Furthermore, the presence of a second silencer at *HMR* is not the sole contributor to the more rapid association of Sir pro-

teins with the *HMR* locus, because assembly of silenced chromatin is significantly faster at the *hmr-ΔI* locus than at telomere VI-R.

The *HMR-E* silencer promotes spreading of the Sir complex at telomere VI-R. Two models can explain the slower spreading of Sir proteins at the telomere than at *HMR*. The *HMR-E* silencer may be more effective at promoting the spreading process. Alternatively, subtelomeric chromatin may be less permissive to spreading. In support of the first model, *HM* silencers have been shown to favor the assembly of the Sir complex in a particular direction by positioning adjacent nucleosomes (55). In support of the second model, subtelomeric silencing is often more sensitive to the antisilencing activity of euchromatin-associated enzymes, such as the histone acetyltransferase Sas2p (19, 20, 42, 47). It is therefore plausible that these enzymes are more active at subtelomeric chromatin, making the telomere more restrictive to spreading. To distinguish between these two models, a 431-bp fragment containing the *HMR-E* silencer was integrated at the telomere VI-R adjacent to the core-X sequence. As a control, the same-size fragment containing a portion of the silencing neutral *TRP1* open reading frame was integrated at the same site. Parallel experiments were conducted to follow the rates of Sir2p accumulation in these two strains.

To verify that the transposed silencer was functional, the ability of telomeric *HMR-E* to recruit Sir proteins was tested. Maximum levels of Sir2p were reached within 90 min (Fig. 6A), and the level of enrichment of Sir2p at telomeric *HMR-E* was comparable to that observed at the native *HMR* locus (Fig. 3B). Therefore, the *HMR-E* silencer was functional in its new location. Next, the rates at which Sir2p accumulated at distances of 1 and 2 kb from the telomeric *HMR-E* silencer were assayed. If *HMR-E* promotes spreading, then Sir2p should accumulate rapidly, as at *HMR*. In contrast, if telomeric chromatin is more restrictive to the Sir complex, then spreading would occur at the same rate as that observed at the native telomere. Primers were selected at equivalent distances (1 and 2 kb) from the silencers in the two integrant constructs. Upon induction of *SIR3*, Sir2p accumulated more rapidly at positions 1 and 2 kb from telomeric *HMR-E* compared to the stuffer fragment (Fig. 6B and C), although the rate of accumulation was still lower than that observed at *HMR*. For example, at the modified telomere, Sir2p levels 2 kb away from the silencer begin to flatten out around 270 min, whereas at *HMR*, Sir2p levels reach a plateau within 90 min (compare Fig. 3B and 6C). It is likely that the exclusion of other *HMR*-specific components, such as the *HMR-I* silencer, accounts for the slightly lower rate of spreading. We cannot, however, rule out that the chromatin context also plays a role in restricting the spread of silenced chromatin at telomere VI-R. Nevertheless, these data clearly demonstrate that the *HMR-E* silencer does more than just recruit Sir proteins to the chromosome; it also participates in promoting their association with adjacent chromatin.

Sir protein turnover was similar at *HMR* and telomere VI-R.

To explore how *HMR-E* promotes the assembly of silenced chromatin independently of recruitment, we first considered whether the silencer influences the rate at which Sir proteins dissociate from chromatin. Silenced chromatin reverts to an active state upon removal of adjacent silencers, making it evident that silenced chromatin turns over with some frequency

FIG. 6. Integration of *HMR-E* at telomere VI-R accelerated the accumulation of Sir2p. Sir2p-associated DNA was isolated by chromatin IP following induction of *SIR3* in strains with modified telomeres. The genotypes were *sir3Δ TELVIR::STF* (LRY2019) and *sir3Δ TELVIR::HMR-E* (LRY2020), each with a 431-bp piece of DNA containing silencing neutral stuffer sequence or the *HMR-E* silencer, respectively. Both strains were transformed with a plasmid containing *P_{GALT}-SIR3* (pJR517). (A) Accumulation of Sir2p at the silencer as determined by quantification of coprecipitated DNA with primers located immediately adjacent to the telomeric repeats for *TELVIR::STF* (LRY2019) or next to the *HMR-E* silencer for *TELVIR::HMR-E* (LRY2020). (B and C) Accumulation of Sir2p at distances of approximately 1 kb (B) or 2 kb (C) from respective silencers. Represented distances were within 120 bp of actual distances.

(7). Therefore, the observed rate of Sir protein assembly on chromatin must be a function of the rates of association and dissociation. *HMR-E* may function to reduce the frequency of Sir protein turnover in a manner that is not shared by the telomere silencer. To test this hypothesis, we induced the expression of Myc-tagged *SIR3* in the presence of untagged endogenous *SIR3* and followed the incorporation of newly expressed Sir3p-Myc at *HMR* and telomere VI-R by chromatin IP. If *HMR-E* diminishes the rate of Sir protein displacement, then Sir3p-Myc should associate with *HMR* at a lower rate than at the telomere. However, incorporation of Sir3p-Myc occurred rapidly after induction, and no significant differences were observed at *HMR* compared to results at telomere VI-R (data not shown). These data suggest that Sir protein turnover is equally rapid at both locations. Thus, it is unlikely that the robust rate of Sir2p association near *HMR-E* is due to a significant difference in the rate of dissociation.

FIG. 7. Silencing at *HMR* was insensitive to coexpression of Sir2-N345Ap and wild-type Sir2p. (A) Mating ability was assessed by exposing 10-fold serial dilutions of *MATα* haploids containing only wild-type *SIR2* (LRY1007), *sir2Δ* (LRY1068), the catalytically dead *sir2-N345A* allele located at the *LEU2* locus (LRY0800), or both *SIR2* and *sir2-N345A* (LRY0804) to *MATa* tester haploids (LRY1021). The resulting diploids were selected on minimal medium. In the lower panel, mating was assessed in *MATα* haploids lacking the *HMR-I* silencer and containing either wild-type *SIR2* (LRY0450) or both *SIR2* and *sir2-N345A* (LRY1815). (B) Relative enrichment of total Sir2p was assayed at three regions of *HMR* by chromatin IP in the presence of *SIR2* alone, both *SIR2* and *sir2-N345A*, or *sir2Δ*. (C) Total Sir2p enrichment was assayed at three regions of telomere VI-R in the same chromatin IP samples as in panel B. (D) HA-tagged mutant Sir2-N345Ap levels were assayed at two regions of *HMR* in strains containing only untagged wild-type *SIR2* (LRY1007), both wild-type *SIR2* and the HA-tagged *SIR2-N345A* mutant (LRY2287), or the HA-tagged *SIR2-N345A* mutant alone (LRY2288). (E) Relative enrichment of HA-Sir2-N345Ap at two regions of telomere VI-R in the same chromatin IP samples as in panel D.

HMR-E may promote nonlinear spreading of Sir proteins.

Another explanation for the ability of *HMR-E* to enhance the assembly of silenced chromatin is that the spreading of Sir proteins is not strictly linear at *HMR*. According to the working model of stepwise spreading by sequential deacetylation, Sir proteins are first recruited to a silencer and then deacetylate an adjacent nucleosome, occupy that nucleosome, and repeat the process. Based on this model, sequences close to the silencer should become occupied by Sir proteins before sequences more distal to the silencer. Our data for silenced chromatin assembly at telomere VI-R were consistent with this model. In contrast, on the telomere-proximal side of *HMR-E*, the association of Sir2p occurred simultaneously across more than 2 kb.

To test the hypothesis that *HMR-E* promotes nonlinear spreading of Sir proteins, we utilized a catalytically inactive allele of *SIR2*. The mutant Sir2-N345Ap (18) is predicted to be structurally intact (30) and is incorporated into the Sir protein complex (3). Once recruited to the silencer, the protein cannot deacetylate histones and the Sir complex should not spread (3, 17, 37). Based on the model of stepwise spreading, incorporation of Sir2-N345Ap into silenced chromatin during assembly

would act as a chain terminator and prevent further spreading. In support of this prediction, *sir2-N345A* has been shown to exert a dominant-negative effect on silencing at the truncated telomere VII-L when expressed alongside wild-type *SIR2* (3). Therefore, if silenced chromatin assembly is linear at *HMR*, then *sir2-N345A* would also be expected to have a dominant-negative effect. However, if spreading at this locus is not strictly linear, then silencing might be insensitive to *sir2-N345A* coexpressed with wild-type *SIR2*. To determine the silencing status of *HMR*, mating assays were performed in which *MATα* haploids containing the mutant alleles were mixed with haploids of the opposite mating type and grown on medium selective for diploids. Only when *HMRa1* is silent will *MATα* cells mate and form diploids. As expected, haploids expressing only wild-type *SIR2* were proficient for mating, whereas those expressing the mutant were defective (Fig. 7A). Interestingly, when both alleles of *SIR2* were coexpressed, mating occurred at levels indistinguishable from those of wild-type Sir2p (Fig. 7A). Importantly, resistance to *sir2-N345A* was also observed in the absence of *HMR-I*, indicating that the *HMR-E* silencer alone is capable of insulating the *HMR* locus from disruption by *sir2-N345A* (Fig. 7A, bottom panel). Thus, in contrast to reported

results at telomere VII-L (3), *sir2-N345A* does not have a dominant-negative phenotype at *HMR*. These data argue that assembly of silenced chromatin is not strictly linear at *HMR*. Additionally, although *HMR-I* plays a supporting role, the *HMR-E* silencer alone is apparently sufficient to promote the nonlinear assembly of Sir proteins at *HMR*.

To determine the effect of the *sir2-N345A* allele on the assembly of Sir proteins at *HMR* and telomere VI-R, the steady-state distributions of total Sir2p and HA-Sir2-N345Ap were assessed at both loci by chromatin IP. Consistent with results of the mating assays (Fig. 7A), coexpression of *sir2-N345A* and wild-type *SIR2* had no effect on the distribution of total Sir2p at *HMR* (Fig. 7B). Furthermore, in the presence of wild-type Sir2p, HA-Sir2-N345Ap associated robustly with both the *HMR-E* silencer and a site 1 kb from the silencer (Fig. 7D), indicating that the enzymatically inactive Sir2p could be incorporated into the silenced chromatin structure without impeding assembly of silenced chromatin. As previously reported (17, 37), the spreading of silenced chromatin does require some deacetylation, since the association of HA-Sir2-N345Ap was limited to the silencer in the absence of wild-type Sir2p (Fig. 7D). In fact, under these conditions, the enrichment of Sir2-N345Ap with the silencer was reduced (Fig. 7D). We speculate that under these circumstances, the association of the Sir complex with the silencer is somewhat less stable and there are fewer epitopes available. In contrast to results at *HMR*, at telomere VI-R, total Sir2p enrichment was dramatically reduced in the presence of both *sir2-N345A* and wild-type *SIR2* compared to results with wild-type *SIR2* alone (Fig. 7C). This reduction was presumably due to an inhibitory effect of the mutant Sir2p, which associated with the telomeric silencer (Fig. 7E). Thus, the incorporation of enzymatically inactive Sir2p into silenced chromatin is more disruptive to the stability of the structure at telomere VI-R than it is at *HMR*, consistent with the model that assembly does not proceed in a strictly linear fashion at *HMR*.

DISCUSSION

Silencers can promote the assembly of Sir proteins independently of recruitment. The current model for establishment of silenced chromatin in *S. cerevisiae* suggests that the primary role of a silencer is to recruit Sir proteins. Once recruited to the chromosome, the Sir complex spreads autonomously. In its simplest form, this model predicts that any silencer proficient at recruiting Sir proteins would instigate identical spreading reactions into any DNA environment, assuming no barrier element is present. In our analysis of silenced chromatin assembly at *HMR*, we found that following recruitment, Sir2p associated nearly simultaneously with sequences throughout the locus, even in the absence of the *HMR-I* silencer. A difference in the accumulation rate was observed only at 4 kb from the silencer, which is beyond the tRNA^{Thr} barrier (Fig. 5B). The rate of Sir2p association observed at *HMR* raised the question of whether this rapid spreading is in fact a general property of the Sir complex, since most silent domains lack a barrier to block such efficient spreading. To address this question, we compared the rates of spreading at three different locations: the centromere and telomere facing sides of the *HMR-E* silencer and the subtelomeric region on the right arm

of chromosome VI. Three distinct spreading rates were observed at these locations, despite the absence of known barrier elements in the regions examined. One model to explain this observation is that one silencer's ability to outcompete another silencer for limited pools of Sir proteins might generate different rates of spreading. However, this model does not explain our data, since different spreading rates were observed on either side of the same silencer. Furthermore, the two silencers analyzed, *HMR-E* and telomere VI-R, had similar recruitment rates and levels of Sir2p association. We also considered the possibility that certain genomic locations may be less permissive to spreading. However, integration of the *HMR-E* silencer into the subtelomere, where spreading was initially slow, accelerated spreading, arguing that the silencer itself was a critical determinant for the rate of Sir protein assembly.

The ability of the *HMR-E* silencer, assisted by the *HMR-I* silencer, to promote the assembly of silenced chromatin may be crucial for maintaining Sir proteins at *HMR* when Sir3p is expressed at its normal levels. In this case, there is less Sir2p 1 kb outside of the *HMR* domain and at telomere VI-R than within *HMR* (Fig. 3G and 5E). Consistent with this interpretation, Cheng and Gartenberg (7) demonstrated that silenced chromatin could not be stably maintained in the absence of silencers. This role in maintenance was not restricted to reestablishment on newly synthesized templates, since the silenced state remained unstable in noncycling cells (7). What was not clear from the previous study but is demonstrated in this report is that the silencer contributes to the maintenance of silenced chromatin in a manner beyond recruitment.

Association of Sir proteins is not restricted by the cell cycle.

An early study of the establishment of silencing in *S. cerevisiae* using a temperature-sensitive *sir3* allele concluded that establishment required passage through S phase (29). Twenty-four years later, the nature of this cell cycle requirement remains elusive. To date, evidence in support of a cell cycle requirement for silencing has been based on gene expression data, and no such requirement has been described for spreading of Sir proteins. In fact, Sir proteins are still recruited to *HMR* in arrested cultures, suggesting that the cell cycle requirement occurred after association of Sir proteins (21).

We assayed the accumulation of Sir2p at the *HMRa1* promoter in asynchronous cultures following induction of *SIR3* and discovered that the protein achieved maximum levels within 60 to 90 min. The experimental population doubling time was nearly 120 min, and therefore, spreading of Sir2p at *HMR* occurred well within one generation. If passage through a specific point in the cell cycle were required for association of Sir2p, then accumulation of Sir2p on the DNA would be expected to occur throughout the first generation, reflecting the distribution of cells at different stages of the cell cycle. These results argue that whatever the cell cycle requirement for silencing may be, it has a minimal effect on the association of Sir proteins.

A previous study using a similar inducible *SIR3* gene reported that features of silenced chromatin, including association of Sir3p and repression of *HMRa1*, required several generations to mature fully (19). In contrast, we did not observe significant changes in the amount of Sir2p associated with *HMR* after the first cell doubling. It is not clear whether Sir2p behaves differently than Sir3p or whether other differences

account for this discrepancy. A second difference between the two studies is that the silencing of *HMRa1* occurred more rapidly in our hands. This difference could result from the different genetic contexts of the two experiments. The previous study utilized an inducible tagged *SIR3* construct, which differs in sequence at 15 amino acids from the "standard" yeast strain (M. Gartenberg, personal communication), expressed in yeast cells that have several genetic differences with our strain, including a recombinant *HMR* with RecR recognition sequences next to the silencers and deletions of the *MAT*, *HML*, and *BARI* loci (7, 19).

Accumulation of Sir2p at *HMR* results in reduction of RNA polymerase II. Although the properties of silenced chromatin are relatively well understood, the mechanism by which it represses transcription has been surprisingly controversial. Evidence for two different models of repression has been presented in the literature. The first model suggests that silenced chromatin inhibits recruitment of RNA polymerases to silenced promoters. In support of this model, previous studies demonstrated that Pol II levels at the promoters of both a *URA3* reporter gene located at the *HMR* locus (6) and native *HMRa1* were significantly higher in the absence than in the presence of Sir proteins (6, 24). However, an alternative model suggests that transcriptional repression occurs downstream of Pol II recruitment. Consistent with this second model, another study provided evidence that promoters within silenced chromatin associate with proteins of PIC (the preinitiation complex), including Pol II, but not proteins involved in elongation and mRNA capping (12). Additionally, genome-wide analysis of Pol II levels revealed that some polymerases are present at the silent mating-type cassettes (45). In this study, we observed a precipitous drop in total Rpb1p-Myc, the large subunit of Pol II, accompanying the onset of silencing. Thus, our results are more consistent with a model in which silenced chromatin displaces Pol II.

Mechanisms by which silencers promote Sir protein-chromatin interactions. The discovery that the *HMR-E* silencer can promote spreading of Sir proteins in a manner beyond the recruitment of Sir proteins suggests that it has the ability to promote the association of Sir proteins with distant nucleosomes, either directly or indirectly. The mechanism by which the silencer achieves this effect is unknown. One possibility is that silencers may prime the chromatin for spreading. A prior study found that the organization of protein binding sites at the silencer was important for positioning the adjacent nucleosomes to promote spreading in one direction over the other (55). At *HMR-E*, for example, positioning of the ORC and Abf1p binding sites results in a nucleosome arrangement that favors silencing on the Abf1p side of the silencer (55). Our data clearly demonstrate a bias for assembly of Sir2p on the Abf1p side of *HMR-E*, consistent with this model. On the other hand, while a localized arrangement of nucleosomes may improve the probability that spreading will engage in a particular direction, it is unclear how such an arrangement promotes the assembly of silenced chromatin at more than a kilobase distance.

Another model is that *HMR-E* promotes a higher-order structure that favors assembly of silenced chromatin. For example, recent evidence indicates an interaction between the ends of the *HMR* cassette (50) that could represent a loop or

FIG. 8. Some silencers can promote the association of Sir proteins with chromatin independently of recruitment. (A) The *HMR-E* silencer may promote the assembly of silenced chromatin in a nonlinear fashion. Higher-order chromatin structures, such as loops or clustering of nucleosomes, could enable silencer-bound Sir proteins to associate simultaneously with multiple nucleosomes along the chromosome. (B) At telomere VI-R, the assembly of Sir proteins is consistent with the sequential deacetylation model, in which the silencer recruits Sir proteins, which then spread in a linear fashion.

otherwise condensed structure. Such a higher-order structure could bring silencer-bound Sir2p into the proximity of multiple nucleosomes, allowing it to deacetylate these nucleosomes without first spreading (Fig. 8A). In this case, Sir proteins would likely saturate the entire locus uniformly, since the occupancy status of one nucleosome would no longer be dependent on the occupancy status of nucleosomes closer to the silencer. Furthermore, a higher-order chromatin structure at *HMR* might stabilize the Sir proteins by enabling them to make more interactions with each other and the silencer binding proteins than is possible on an extended linear template. In contrast, the lack of such a structure at the telomere might explain the slower, directional assembly of Sir proteins at this locus (Fig. 8B). This model is consistent with the association of Sir2p occurring simultaneously throughout the *HMR* locus and the insensitivity of *HMR* to the coexpression of catalytically inactive Sir2-N345Ap and wild-type Sir2p. Interestingly, *HMR-E* alone was sufficient to promote Sir protein associations both at the telomere and at *HMR* in the absence of the *HMR-I* silencer, suggesting that *HMR-E* by itself can facilitate higher-order chromatin structures. However, the *HMR-I* silencer is likely to enhance the formation of such structures.

Role for silencers in determining silenced chromatin domains. To date, much of the research on how the spreading of silenced chromatin is restricted to the appropriate locations has focused on elucidating mechanisms by which euchromatic factors oppose encroachment of silenced chromatin. Nevertheless, eliminating these euchromatic factors or increasing the amount of Sir proteins primarily extends normally silenced loci rather than causing promiscuous occupation genome-wide (20, 42, 46, 47). The sequestration of Sir proteins to a small fraction of the genome is particularly curious in light of the fact that

each of the silencer binding proteins, ORC, Rap1p, and Abf1p, is found throughout the genome and yet functions as a silencer only at *HM* and telomeric sites. One explanation for continued restriction of silenced chromatin in the absence of antisilencers is that redundant mechanisms are at work. A prior study demonstrated that deleting both the methyltransferase *SET1* gene and the histone variant *HTZ1* gene results in the *SIR2*-dependent repression of genes more than 100 kb away from normally silent domains (53). However, Sir3p enrichment at these newly repressed genes was undetectable by chromatin IP, suggesting that association was ephemeral, a characteristic inconsistent with uninhibited spreading. Thus, even in a permissive environment, the Sir complex appears unable to assemble stable, long-range structures at new locations.

Here we have presented evidence suggesting that a significant impediment to the spreading of silenced chromatin is its own inherent instability. The Sir complex can physically interact with nucleosomes, but in the absence of a reinforcing silencer, this interaction is limited. We have also demonstrated that the composition of a silencer matters; simply bringing Sir protein to the DNA, as may happen at various ORC, Rap1p, or Abf1p sites in the genome, is insufficient to instigate consequential spreading reactions. These observations magnify the role that silencers play in restricting silenced chromatin to discrete loci. An efficient way to prevent the accidental assembly of silenced chromatin in the wrong location is for such chromatin to be unstable and unable to persist unless stabilized by a silencer. Thus, rather than restricting silenced chromatin by actively excluding it from most of the genome, the main mechanism of regulation may be the promotion of its assembly at a few appropriate loci. It is intriguing to speculate on the biological ramifications of using silencers to differentially regulate silenced chromatin domains. For example, perhaps the employment of spreading deficient silencers at the telomeres spares the organism from having to strategically position barrier elements. In contrast, utilization of a strong silencer, assisting silencer, and barrier combination would be beneficial at *HMR*, where complete silencing of *HMRa1* is critical to haploid cell identity.

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