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An Auxiliary Silencer and a Boundary Element Maintain High Levels of Silencing Proteins at *HMR* in *Saccharomyces cerevisiae*

Patrick J. Lynch and Laura N. Rusche[1]

Institute for Genome Sciences and Policy and Department of Biochemistry, Duke University, Durham, North Carolina, 27708

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ABSTRACT

Heterochromatin is notable for its capacity to propagate along a chromosome. The prevailing model for this spreading process postulates that silencing proteins are first recruited to silencer sequences and then spread from these sites independently of the silencers. However, we found that in *Saccharomyces cerevisiae* silencers also influence the extent of silenced chromatin domains. We compared the abilities of two different silencers, *HMR-E* and a telomeric repeat, to promote silencing and found that the *HMR-E* silencer contributed to an increased steady-state association of Sir proteins over a region of several kilobase pairs compared to the telomeric repeat, even though both silencers recruited similar levels of Sir proteins. We also discovered that, although the *HMR-E* silencer alone was sufficient to block transcription of the *HMR* locus, a secondary silencer, *HMR-I*, boosted the level of Sir proteins at *HMR*, apparently beyond the level necessary to repress transcription. Finally, we discovered that a tRNA^{Thr} gene near *HMR-I* helped maintain silenced chromatin and transcriptional repression under conditions of reduced deacetylase activity. This study highlights the importance of auxiliary elements, such as *HMR-I* and the tRNA^{Thr} gene, in enhancing the association of Sir silencing proteins with appropriate genomic locations, thereby buffering the capacity of silenced chromatin to assemble under suboptimal conditions.

SILENCED chromatin and some other specialized chromatin states have the capacity to propagate along a chromosome. This ability is important for assembling extended chromatin domains but must be controlled to prevent one chromatin domain from encroaching into another. The prevailing model of the spreading process postulates that silencing proteins are first recruited to specific sequences, termed silencers, and then spread from these sites through interactions with nucleosomes (Hoppe *et al.* 2002; Rusche *et al.* 2002; Grewal and Elgin 2007). Thus, the primary role of silencers is thought to be the nucleation of silenced chromatin, and the spreading process is thought to occur independently of the silencer. However, we recently discovered that at least one silencer in *Saccharomyces cerevisiae* can promote the assembly of silenced chromatin at a step after recruitment (Lynch and Rusche 2009). We have now compared the abilities of various silencers and combinations of silencers to shape the steady-state distributions of silencing proteins in the yeast *S. cerevisiae* and find that silencers differ in their abilities to promote the steady-state association of silencing proteins with neighboring nucleosomes.

In *S. cerevisiae*, domains of silenced chromatin are found at the silent mating-type, loci (*HMR* and *HML*) and at most telomeres (reviewed in Rusche *et al.* 2003). At the silent mating-type loci, silenced chromatin maintains haploid cell identity by preventing the expression of extra copies of the mating-type genes. This silenced chromatin also protects *HMR* and *HML* from cutting by the HO endonuclease that triggers mating-type switching (reviewed in Haber 1998). The role of silenced chromatin at subtelomeric domains is less well understood, although it is speculated to contribute to the stability of the ends of the chromosomes.

The structural components of silenced chromatin are the silent information regulator (SIR) proteins, Sir2p, Sir3p, and Sir4p, which are recruited to the chromosome by silencers. At the *HMR* locus, the *HMR-E* and *HMR-I* silencers flank auxiliary copies of the **a** mating-type genes, and at the *HML* locus the *HML-E* and *HML-I* silencers flank copies of the α mating-type genes. Each of these four silencers is composed of binding sites for the origin recognition complex (ORC) and for Rap1p, Abf1p, or both. In contrast, multiple Rap1p binding sites embedded within the terminal telomeric repeats facilitate the recruitment of Sir proteins to the chromosome ends. In addition, binding sites for ORC and Abf1p occur in the "core X" subtelomeric element associated with all telomeres, and these binding sites contribute to silencing at some (Fourel *et al.* 1999; Pryde and Louis 1999) but not all telomeres (Mondoux and

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[1]*Corresponding author:* Institute for Genome Sciences and Policy, Duke University, 101 Science Dr., Box 3382, Durham, NC 27708.
E-mail: lrusche@biochem.duke.edu

Zakian 2007). Following recruitment to silencers, the Sir protein complex is thought to spread along the chromosome by repetitive cycles of histone deacetylation and binding in a process referred to as sequential deacetylation (Hoppe *et al.* 2002; Rusche *et al.* 2002). Sir2p, a histone deacetylase, generates hypoacetylated nucleosomes that are preferentially bound by Sir3p and Sir4p, which in turn recruit an additional molecule of Sir2p to the chromatin (Hoppe *et al.* 2002; Rusche *et al.* 2002). Thus, the Sir protein complex is predicted to spread away from the silencers in a linear step-wise manner.

Our discovery that the *HMR-E* silencer promotes the establishment of silenced chromatin over several kilobases more efficiently than does the telomeric repeat at chromosome VI-R (Lynch and Rusche 2009) suggests that spreading may not be strictly linear. We hypothesize that proteins associated with the *HMR-E* silencer promote a looped or compact arrangement of the chromatin fiber that brings the silencer-associated Sir complex into close proximity with multiple nucleosomes at once, such that multiple nucleosomes can be deacetylated independently of one another, enabling assembly to occur in a nonlinear fashion. Consistent with this model, chromosome conformation capture (3C) experiments have indicated that silenced chromatin adopts either a looped or compact chromatin structure at the *HMR* locus (Valenzuela *et al.* 2008; Miele *et al.* 2009).

To explore how silencers shape the steady-state distribution of Sir proteins at *HMR*, we have now examined the association of silencing proteins with *HMR* in the presence and absence of three known regulatory sequences—two silencers and a boundary element. The *HMR-E* silencer is composed of binding sites for ORC, Rap1p, and Abf1p and is required for silencing at *HMR*. A second silencer, *HMR-I*, is not required for the silencing of *HMR**a1***, although it does contribute to the silencing of a reporter gene located in the place of *HMR**a1*** (Rivier *et al.* 1999). Like *HMR-E*, *HMR-I* has binding sites for ORC and Abf1p. However, *HMR-I* does not have a binding site for Rap1p and cannot recruit Sir proteins to DNA on its own (Rusche *et al.* 2002). A third important element at *HMR* is a tRNA^{Thr} gene, designated tT(AGU)C in the *Saccharomyces* Genome Database, which is located ~1 kb beyond the *HMR-I* silencer. This gene acts as a boundary to the spread of silenced chromatin (Donze *et al.* 1999; Donze and Kamakaka 2001; Oki and Kamakaka 2005). Both the recruitment of RNA polymerase III and the depletion of histones in the vicinity of the tRNA^{Thr} gene contribute to barrier function (Donze and Kamakaka 2001; Dhillon *et al.* 2009). Additionally, the tRNA^{Thr} gene is important in establishing sister chromatid cohesion at *HMR* (Dubey and Gartenberg 2007), and cohesins have been implicated in the regulation of silenced chromatin (Lau *et al.* 2002; Suter *et al.* 2004). Thus, the *HMR-E* and *HMR-I* silencers together with the tRNA^{Thr} boundary are thought to shape the domain of silenced chromatin at *HMR*.

For comparison, we examined the steady-state distribution of Sir proteins at telomere VI-R, where spreading is more likely to occur in a linear fashion. Telomere VI-R is one of the most strongly silenced telomeres (Mondoux and Zakian 2007) and lacks subtelomeric elements known to antagonize silencing, such as the X-combinatorial repeats and Y′ elements. Telomere VI-R does have a core X element, containing ORC and Abf1p binding sites, located within 350 bp of the terminal repeat. It is not known whether ORC and Abf1p contribute to the recruitment of Sir proteins to telomere VI-R, but the deletion of the core X element only reduced silencing of a *URA3* reporter by twofold (Mondoux and Zakian 2007). A gene of unknown function, *YFR057W*, whose promoter is ~1 kb from the terminal repeat, is silenced by the Sir proteins (Vega-Palas *et al.* 2000). No other functional elements are known to exist within 5 kb of the terminal repeat at telomere VI-R. Therefore, once Sir proteins have been recruited to the end of the chromosome (through Rap1p associated with the terminal repeat, and perhaps ORC and Abf1p in the adjacent core X element), the propagation of silenced chromatin along the chromosome is not expected to be influenced by either positive or negative elements.

In this study, we analyzed the contributions of each of these regulatory elements to the steady-state distribution of Sir proteins and examined how they contribute to the known biological functions of silencing at *HMR*. Consistent with our previous report, we discovered that the *HMR-E* silencer by itself promotes silenced chromatin more efficiently over a region of several kilobases compared to a telomeric repeat. Additionally, we discovered that although the *HMR-E* silencer alone was sufficient to maintain high levels of Sir proteins and block transcription of the *HMR* locus, the *HMR-I* silencer boosts the level of Sir proteins at *HMR* and modestly extends the domain of silenced chromatin. Intriguingly, we also discovered a role for the tRNA^{Thr} gene in promoting Sir protein association with the *HMR* locus under conditions of weakened silencing.

MATERIALS AND METHODS

Yeast strains and plasmids: Yeast strains used in this study (Table 1) were derived from W303-1b. The following alleles were described previously: *sir2Δ::TRP1* and *sir4Δ::HIS3* (Rusche and Rine 2001), *LEU2::sir2-N345A* (Imai *et al.* 2000; Armstrong *et al.* 2002), *hmr-ΔE* (silencer deletion 358–223, YAB71; and 546–30, YAB65) (Brand *et al.* 1987), ΔtRNA^{Thr} (Donze *et al.* 1999), and *TELVI-R::Stuffer* and *TELVI-R::HMR-E* (Lynch and Rusche 2009).

To create the *HMR*-ΔtRNA^{Thr} allele used in this study, a previously described ΔtRNA^{Thr} deletion was amplified from ROY1681 (Donze *et al.* 1999) genomic DNA by PCR with *Pfu* Turbo DNA polymerase (Stratagene) using primer sequences

TABLE 1

Strains used in this study

Strain	Genotype	Source
LRY1007 (W303)	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
LRY0800	W303 *MAT*α *sir2*Δ::*TRP1 LEU2*::*sir2-N345A*	
LRY0804	W303 *MAT*α *LEU2*::*sir2-N345A*	
LRY1021	*MAT***a** *his4*	P. Schatz
LRY1068	W303 *MAT*α *sir2*Δ::*TRP1*	
LRY1815	W303 *MAT*α *hmr-*Δ*I LEU2*::*sir2-N345A*	
LRY2148	W303 *MAT*α *TELVIR*::*STUFFER*	
LRY2150	W303 *MAT*α *TELVIR*::*HMR-E*	
LRY2302	W303 *MAT*α *HMR-*ΔtRNA^{Thr}	
LRY2303	W303 *MAT*α *HMR-*ΔtRNA^{Thr} *LEU2*::*sir2-N345A*	
LRY2309	W303 *MAT*α *hmr-*Δ*I* ΔtRNA^{Thr}	
LRY2352	W303 *MAT*α *hmr-*Δ*I* ΔtRNA^{Thr} *LEU2*::*sir2-N345A*	
LRY2315	W303 *MAT*α *hmr-*Δ*I*	
LRY2316	W303 *MAT*α *hmr-*Δ*I LEU2*::*sir2-N345A*	
LRY2379	W303 *MAT*α [pJR831; P_{GAL1}-*HO*; *URA3*][a]	
LRY2384	W303 *MAT*α *hmr-*Δ*I* [pJR831; P_{GAL1}-*HO*; *URA3*][a]	
LRY2467	W303 *MAT***a** [pJR831; P_{GAL1}-*HO*; *URA3*][a]	
LRY2482	W303 *MAT*α *sir4*Δ::*HIS3* [pJR831; P_{GAL1}-*HO*; *URA3*][a]	
YAB65	W303 *MAT*α *hmr-*Δ*E* (546–30; 516-bp deletion)	A. Brand
YAB71	W303 *MAT*α *hmr-*Δ*E* (358–223; 135-bp deletion)	A. Brand

[a] Brackets denote transformation with the indicated plasmid. See MATERIALS AND METHODS for plasmid details.

5′-gcagcttactcccaaga gtgc and 5′-gcaaggattgataatgtggtag. The PCR product was digested with *Xho*I and *Nde*I and cloned into a plasmid (pJR1270) bearing the *Eco*RI–*Hin*dIII fragment of *HMR*, lacking both the *HMR-I* silencer and a Ty1 LTR, in a pUC18 vector backbone to generate pLR0575. To replace the missing *HMR-I* silencer and Ty1 LTR with wild-type sequences, wild-type genomic DNA (LRY1007) was amplified by PCR using primer sequences 5′-gatgtgtttgtacatttggc and 5′-tcgacgtcggat ttgcg. The PCR product was digested with *Mfe*I and *Pst*I and cloned into pLR0575 to create pLR0667.

To create the *hmr-*Δ*I* ΔtRNA^{Thr} construct, mutagenesis was performed on pLR0667 using primers 5′-ctttctactgcgataaagtta ttatttagattaca**gctagc**gaaaatttgtcaacgaagttagagaaag and 5′-ctttc tctaacttcgttgacaaattttc**gctagc**tgtaatctaaataataactttatcgcagtagaaag, inserting a *Nhe*I site (boldface letters) in place of the *HMR-I* silencer, to generate pLR0683. The 305-bp deletion of *HMR-I* was filled with the same size fragment from the *TRP1* open reading frame DNA by amplifying wild-type genomic DNA (LRY1007) using primer sequences 5′-cacgat**gctagc**actccg aaa tacttggttggc and 5′-gagtcg**gctagc**cttccaacccagtcagaaatc, which contain *Nhe*I sites (boldface letters). The PCR product was digested with *Nhe*I and cloned into plasmid pLR0683 to generate pLR0690.

Finally, to create the *hmr-*Δ*I* construct, pLR0690 was digested with *Mfe*I and *Pst*I and the resulting fragment containing the *HMR-I* silencer deletion as well as the Ty1 LTR was cloned into pLR0689, which contains the *Eco*RI–*Nde*I fragment of *HMR* including the tRNA^{Thr} gene in a pUC18 vector backbone, to generate pLR0691.

To integrate the mutant *HMR* alleles into their native locus in the yeast genome, plasmids pLR0667, pLR0690, and pLR0691 were digested with *Eco*RI and *Nde*I and used to transform a yeast strain in which *HMR* was replaced by *URA3* (LRY2177). Approximately 10 OD equivalents of transformed cells were resuspended in 50 mL of rich medium (YPD) and allowed to recover overnight at 30°. To select for integrants in which the *URA3* marker was lost, 2 OD equivalents of cells were plated directly onto medium containing 5-FOA. Correct integration of *HMR* mutant alleles was confirmed by PCR and Southern blotting.

The plasmid pJR831 contains the HO endonuclease gene under the control of the *GAL1* promoter in a YCp50 vector backbone and was a gift from Jasper Rine (University of California Berkeley).

Chromatin immunoprecipitation: Chromatin immunoprecipitations were performed as previously described (RUSCHE and RINE 2001) using 10 OD equivalents of cells and 3 μl of rabbit polyclonal antiserum to recombinant LacZ-Sir2p or LacZ-Sir3p (rabbits 2931 and 2934, respectively; gifts from J. Rine, University of California Berkeley). Cells were grown in rich medium (YPD). Samples were collected in logarithmically growing cultures at an OD_{600} of ∼1.0 (±0.2). 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 M. Quantitative real-time PCR was performed as previously described (LYNCH *et al.* 2005), except *PHO5* was used as a control locus. First, the amount of product generated using each primer set was determined compared to a standard curve of input DNA. Then, relative IP values were determined by taking the ratio of the query locus to *PHO5* or a silencer, as indicated. Reported values represent averages of two to six independent immunoprecipitations derived from independent cultures. Sequences of the oligonucleotides are given in Table 2.

Reverse transcriptase PCR: RNA was isolated from logarithmically growing cells via the hot phenol method (SCHMITT *et al.* 1990). To remove contaminating DNA, RNA was treated with rDNase I using a DNA-free kit (Ambion). RNA was converted to cDNA using M-MLV reverse transcriptase (Invitrogen) as previously described (HICKMAN and RUSCHE 2007), with the exception that 400 ng of DNase-treated RNA was used in each reaction. The resulting cDNA was quantified by real-time PCR using genomic DNA isolated from wild-type yeast (LRY1007) for the standard curve. Transcript levels of query

TABLE 2

Oligos used in this study

Region	Sequence 1	Sequence 2
	RT–PCR	
NTG1	caaggttcctcgatttagtg	gactccagatcagacaagaac
ACT1	cagcgcttgcaccatccc	gagcttcatcaccaacgtaggag
YFR057w	caatagcctttcaaagcatac	gctttgttacgcttgcacttg
*HMR**a1***	atggaaagtaatttgactaaagtag	ccaaactcttacttgaagtggag
GIT1	gttgctgacgcttcactac	gaagactgctactacagaagtc
	Chromatin IP	
PHO5	cttgaacgatgattacgag	caagaagtcacgagcatg
HMR (−)1 kb	gcaatgactagagaactatcg	gatctgaaggttcagtaactc
HMR-E	gcaatagatcatgtactaaac	ctgcgcttattctcaaacg
HMR 1 kb	caatacatctccttatatcaaag	caatctcagtacctagaatg
HMR 2 kb	gttgatcataagtctcttc	ctatgtgtttatacaattgc
HMR 3 kb	ctacaatgcaaccccac	tcgacgtcggatttgcg
HMR 4 kb	gcgcagctatttcaattttgg	caattctaacataggatggcag
HMR 5 kb	cattcgacgcctactacagaac	gtaatgctggaccaggtgatatg
HMR 6 kb	cattgcgtccggttttgctc	gttggaaactctagtcgacac
TEL6R 0 kb	ctgagttcggatcactacacac	gatcattgaggatctataatcaac
TEL6R 1 kb	gtaggaatgcgaaaggatctgtc	gtgctaaaggaatccccagagac
TEL6R 2 kb	gacggaaagagggcagaaag	cagcgcacgtttgtttgatg
TEL6R 3 kb	gagttttgtagtagcgatccgac	gtagtgtaaccataagaaatccag
TEL6R 4 kb	cgtacttagagtaaccatagc	cagcaaaataaccactggtgtttaag
TEL::HMR-E 0 kb	gcaatagatcatgtactaaac	gtggatgcacagttcagag
TEL::HMR-E 1 kb	gaccttcataggatgtaagtag	catatcactaacttctctcagatc
TEL::HMR-E 2 kb	gacggaaagagggcagaaag	cagcgcacgtttgtttgatg
TEL::HMR-E 3 kb	gagttttgtagtagcgatccgac	gtagtgtaaccataagaaatccag
TEL::STF 1 kb	caatagcctttcaaagcatac	gctttgttacgcttgcacttg
TEL::STF 2 kb	caaattgcaggcaaataaacac	gcatgatgatccccaataac
TEL::STF 3 kb	gacatgaatctcctatcgttc	gataaatggacctgtccttc

genes were normalized to the transcript levels of the control gene *NTG1*. Reported values represent the averages of four independent RNA samples, each analyzed in duplicate PCR reactions.

Mating assay: One optical density equivalent of logarithmically growing *MAT*α haploid cells was collected by centrifugation and resuspended in 100 μl of minimal medium (YM). A 10-fold dilution series was prepared in YM, and 3 μl from each dilution was spotted onto rich medium (YPD) to verify that all samples were diluted equivalently. To assess mating proficiency, an equivalent volume of *MAT***a** tester cells (LRY1021), suspended in rich medium (YPD) at a dilution of 10 OD equivalents per mL, was added to the dilution series. A total of 3 μl of the resulting mixture was spotted onto minimal medium (YM) to select for prototrophic diploids. To assay mating in the presence of nicotinamide, *MAT*α haploid cells were grown to late log phase in medium supplemented with nicotinamide (NAM) (Sigma) and plated with *MAT***a** tester cells on minimal medium containing NAM. Plates were imaged after 2 days at 30°.

HO endonuclease cleavage assay: For experiments testing DNA cutting by the HO endonuclease, cells were grown in selective, supplemented medium lacking uracil (CSM) (MP Biomedicals) in 2% raffinose. Cells were brought to an OD_{600} of ∼0.8 (±0.1), and then arrested in S phase by the addition of hydroxyurea (HU) (US Biological) directly to the medium at a final concentration of 200 mm for 4 hr. For the induction of P_{GAL1}-*HO* endonuclease, galactose was added to the medium at a final concentration of 2%. Cells were collected at various times after the addition of galactose.

To detect cleavage by HO endonuclease, genomic DNA was isolated from cells by phenol extraction. Approximately 40 to 45 μg of genomic DNA was digested overnight at 37° with *Hin*dIII at a concentration of 2 units per μg DNA. DNA fragments were separated on 0.7% agarose gels, depurinated in 0.25 m HCl for 8 min, denatured in 0.5 m NaOH and 1 m NaCl for 30 min, and neutralized in 0.5 m Tris (pH 7.4) and 3 m NaCl for 30 min. DNA was transferred to Zeta Probe nylon membranes (Bio-Rad) by capillary action. For sequence specific hybridization, DNA probes were generated by PCR using total yeast genomic DNA as a template. Probes were labeled with [α-^{32}P] dCTP using the RediPrime II DNA labeling kit (Amersham).

RESULTS

***HMR-E* increases the association of Sir proteins over a region of several kilobases:** Our previous work demonstrated that, although Sir proteins are recruited to the *HMR-E* silencer and the terminal repeats of telomere VI-R at similar rates, they assemble more rapidly across the entire *HMR* locus than they do across a similarly sized domain adjacent to telomere VI-R (Lynch and Rusche 2009). Moreover, this faster rate of assembly at *HMR* does not depend on the secondary silencer *HMR-I* (Lynch and Rusche 2009). We hypothesized that this increased rate of assembly reflects the ability of

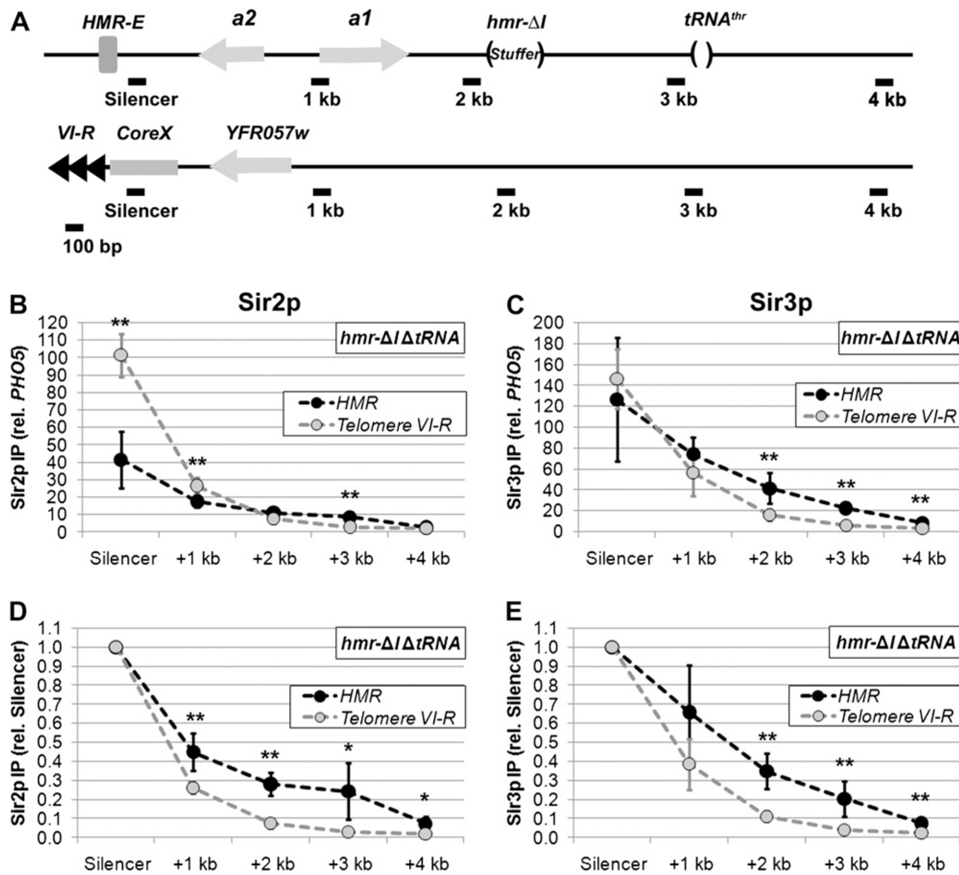

FIGURE 1.—*HMR-E* increases the enrichment of Sir proteins over a region of several kilobases. (A) Diagrams of *HMR* and subtelomere VI-R. Black bars indicate locations of amplicons used to quantify DNA isolated by chromatin IP. The approximate distances (in kilobase pairs) from the adjacent silencers are given. The *HMR* locus contains a previously described 85-bp deletion of the tRNA^{Thr} gene (DONZE *et al.* 1999) and a 305-bp deletion of the *HMR-I* silencer, which is filled in with an equivalent length DNA from the *TRP1* open reading frame (stuffer). In addition, a 448-bp Ty1 long-terminal repeat sequence located between the *HMR-I* silencer and the tRNA^{Thr} gene, which is missing in most modified *HMR* loci (RUSCHE *et al.* 2002), was restored. (B) Association of Sir2p with telomere VI-R and an *HMR* locus lacking the *HMR-I* silencer and tRNA^{Thr} gene. DNA coprecipitated with Sir2p from strain LRY2309 was analyzed by quantitative real-time PCR using the amplicons shown in A. Values represent the average of five independent immunoprecipitations analyzed in duplicate PCR reactions and normalized to a control locus (*PHO5*). An unpaired *t*-test was used to determine whether the enrichments were significantly different at equivalent distances from the telomere repeat and *HMR-E*. One asterisk indicates a *P-value $<$ 0.05; **P-value $<$ 0.01. (C) Association of Sir3p with telomere VI-R and an *HMR* locus lacking the *HMR-I* silencer and tRNA^{Thr} gene. DNA associated with Sir3p was isolated and analyzed in the same experiments as in B. (D and E) The relative enrichments of Sir2p and Sir3p with telomere VI-R and the modified *HMR* locus were normalized to the adjacent silencers rather than *PHO5*.

the *HMR-E* silencer to promote the association of Sir proteins with nucleosomes over a region of a few kilobases. A prediction of this model is that at steady state, the enrichment of Sir proteins would be elevated at sites within a few kilobases of *HMR-E* compared to sites at similar distances from telomere VI-R. To test this prediction, we compared Sir protein levels at equivalent distances from the *HMR-E* silencer and telomere repeat by chromatin immunoprecipitation. To eliminate the potential effects of *HMR-I* and the tRNA^{Thr} gene, these elements were deleted (Figure 1A). Similar levels of Sir3p associated with the two silencer sequences (Figure 1C), yet consistently higher levels of Sir3p, were associated with sites 1, 2, or 3 kb from *HMR-E* compared to sites at similar distances from the telomeric repeats. In the case of Sir2p, a significantly higher enrichment was detected at the telomeric repeat compared to the *HMR-E* silencer (Figure 1B). Nevertheless, Sir2p levels were comparable 2 kb from the sites of recruitment, and there was actually more Sir2p 3 kb from the *HMR-E* silencer than 3 kb from the telomere repeat. To facilitate the comparison of the two silencers' abilities to promote the assembly of silenced chromatin, Sir protein levels were normalized to the levels at the respective silencers (Figure 1, D and E). For both Sir2p and Sir3p, the decline in Sir protein association over a given distance was less steep when silenced chromatin was initiated by *HMR-E* compared to telomere VI-R. Therefore, although the telomeric repeat is as effective as *HMR-E* at recruiting Sir proteins (if not more so), the telomeric repeat is less efficient at promoting the association of Sir proteins with neighboring nucleosomes.

One explanation for the difference between the two silencers is genomic context. To determine whether *HMR-E* also promotes the association of Sir proteins with neighboring nucleosomes in the context of telomere VI-R, we examined a strain in which a 430-bp fragment containing the *HMR-E* silencer was integrated adjacent to telomere VI-R (Figure 2A). In this strain, higher enrichments of Sir proteins were observed over a region of several kilobases compared to a control strain, which had an equivalent-sized piece of silencing-neutral stuffer DNA (Figure 2, B and C). At this modified telomere, the terminal repeats and core X element likely cooperated with *HMR-E* to recruit Sir proteins to the chromosome. Nonetheless, the levels of Sir protein

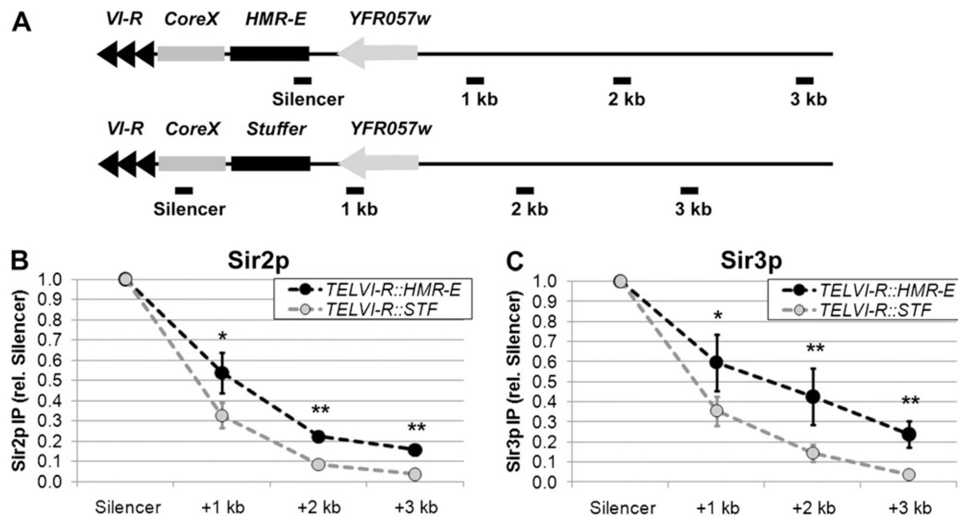

Figure 2.—A transposed *HMR-E* silencer increases the association of Sir proteins at telomere VI-R. (A) Diagrams of modified telomere VI-R loci. Either the entire *HMR-E* silencer (431 bp) or an equivalent-sized fragment of the *TRP1* ORF was integrated into telomere VI-R. (B) Relative association of Sir2p with modified telomere VI-R loci. DNA associated with Sir2p was isolated from strains LRY2150 and LRY2148, (*TELVI-R::HMR-E* and *TELVI-R::STF,* respectively). Sir2p-associated DNA was quantified by real-time PCR using the indicated amplicons. Data were analyzed as in Figure 1, D and E and represent the averages of two independent immunoprecipitation experiments and at least four PCR reactions. (C) Relative association of Sir3p with modified telomere VI-R loci. Sir3p-associated DNA was isolated in the same experiments and analyzed as in B.

enrichment observed at the telomere-localized *HMR-E* silencer were similar to the levels at the telomeric repeat sequences in the control strain (supporting information, Figure S1), suggesting that the efficiency of Sir protein recruitment was comparable in the two strains. Therefore, the *HMR-E* silencer promoted the association of Sir proteins with neighboring nucleosomes over a region of several kilobases to a greater extent than did the terminal repeat at telomere VI-R.

It is well established that silencing is less easily disrupted at *HMR* than at telomeres, and it has been assumed that this difference results from *HMR-E* having a greater ability to recruit Sir proteins. However, our results indicate that, if anything Sir proteins are recruited at higher levels immediately adjacent to telomere VI-R compared to *HMR-E*. Therefore, the critical difference between *HMR-E* and the telomere repeat must be in the ability of *HMR-E* to promote the association of Sir proteins with neighboring nucleosomes.

The *HMR-I* silencer increases the association of Sir proteins within *HMR*: The differences in the abilities of *HMR-E* and the terminal repeat at telomere VI-R to promote the distribution of Sir proteins over several kilobases may reflect the different roles of these two loci in the biology of *S. cerevisiae*. The repression of *HMR**a1*** is thought to be critical for an individual cell to mate. In contrast, no deleterious consequence is known to arise from the expression of *YFR057w*, a gene subject to Sir-mediated repression at the native telomere VI-R (Vega-Palas *et al.* 2000). Thus, it may be more important to have high levels of Sir proteins distributed across *HMR* to maintain repression. Consistent with the importance of repressing *HMR**a1***, two additional elements, *HMR-I* and a tRNA^{Thr} gene, are present at *HMR* and act in conjunction with *HMR-E*. To determine how these elements contribute to the silenced domain at *HMR*, we compared the distributions of Sir2p and Sir3p at wild-type and modified *HMR* alleles in which the *HMR-I* silencer and tRNA^{Thr} gene were deleted individually and in combination (Figure 1A).

The *HMR-I* silencer contains binding sites for ORC and Abf1p, both of which interact with Sir proteins and are predicted to stabilize the association of the Sir complex with chromatin. However, *HMR-I* cannot recruit the Sir complex on its own and is not required for silencing of *HMR**a1*** (Rivier *et al.* 1999; Rusche *et al.* 2002). To determine how *HMR-I* contributes to the distribution of Sir proteins at *HMR*, the relative enrichments of Sir2p and Sir3p were examined in the presence and absence of *HMR-I*. The loss of *HMR-I* resulted in a considerable reduction of Sir2p and Sir3p in the immediate vicinity of the *HMR-I* silencer (2 kb) and more modest decreases at the other sites (Figure 3, B and C, squares). Therefore, the *HMR-I* silencer increases the levels of Sir proteins at *HMR*.

The tRNA^{Thr} gene acts as a boundary to the spread of silenced chromatin (Donze *et al.* 1999; Donze and Kamakaka 2001; Oki and Kamakaka 2005) and could shape the distribution of Sir proteins in two ways. First, the tRNA^{Thr} gene could prevent the Sir proteins from spreading into the telomere-proximal side of the locus. However, there is little spreading in this direction in its absence (Oki and Kamakaka 2005). In addition, the tRNA^{Thr} gene could maintain high levels of Sir proteins within the *HMR* cassette by preventing euchromatin from encroaching into the locus. For example, targeted histone acetyltransferases have been shown to modify histones across several kilobases (Vignali *et al.* 2000; Yu *et al.* 2006) and have been proposed to engage in a spreading reaction analogous to that of Sir proteins (Bulger 2005; Yu *et al.* 2006). To determine how the tRNA^{Thr} gene shapes the distribution of Sir proteins

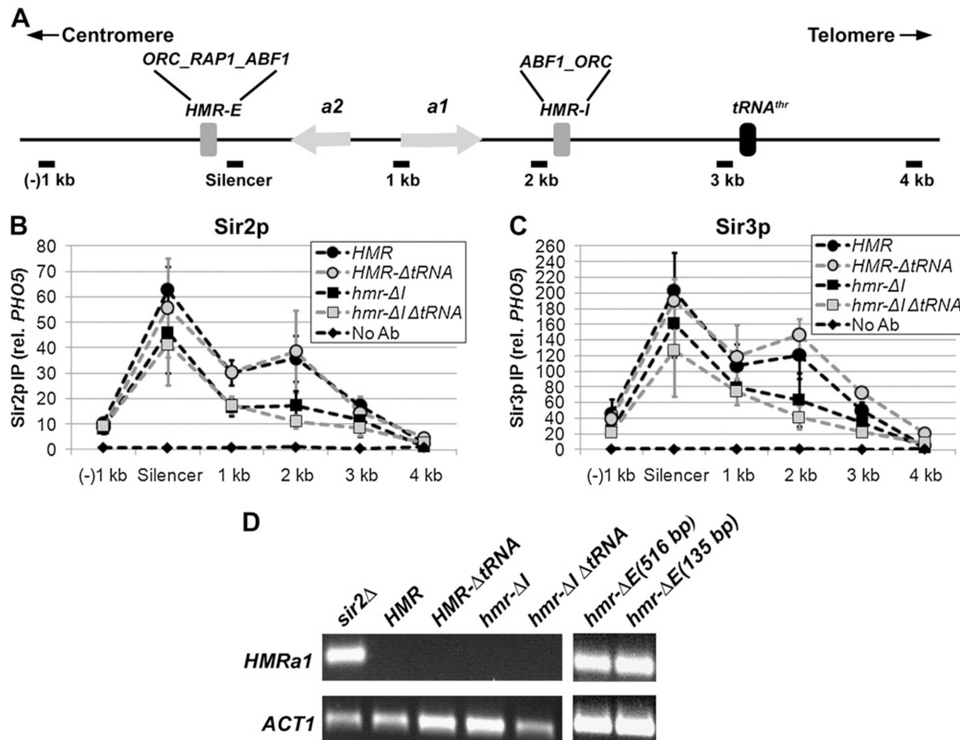

Figure 3.—The *HMR-I* silencer, but not the tRNA^{Thr} gene, boosts Sir protein levels within *HMR*. (A) Diagram of the wild-type *HMR* locus. (B) Association of Sir2p with *HMR* in the presence and absence of regulatory elements. Sir2p-associated DNA was isolated by chromatin IP from strains with the following genotypes: wild-type *HMR* (LRY1007), *HMR*-ΔtRNA^{Thr} (LRY2302), *hmr*-Δ*I* (LRY2315), and *hmr*-Δ*I* ΔtRNA^{Thr} (LRY2309). Data were analyzed as in Figure 1B and represent the averages of at least three independent immunoprecipitation experiments, each analyzed in duplicate quantitative PCR reactions. Primer sets and relative distances were the same for each mutant, with the exception that the +4-kb location is 85 bases closer to the silencer in strains lacking the tRNA^{Thr} gene. (C) Association of Sir3p with *HMR* in the presence and absence of regulatory elements. Sir3p-associated DNA was isolated and analyzed as in B. (D) Transcription of *HMR**a1*** in the presence and absence of regulatory elements. RNA was isolated from the same strains described above, a *sir2*Δ strain with wild-type *HMR* (LRY1068) and two different strains with large (YAB65, 516 bp) and small (YAB71, 135 bp) deletions of the *HMR-E* silencer. The mRNA transcripts were converted to cDNA and the resulting cDNA was amplified by conventional PCR using primer sequences specific to the coding regions of *HMR**a1*** and *ACT1*. The PCR products were run on 1% agarose gels and visualized by ethidium bromide staining.

at *HMR*, the relative enrichments of Sir2p and Sir3p were examined in the absence of the tRNA^{Thr} gene. Sir2p and Sir3p levels were comparable to wild type within the *HMR* cassette (Figure 3, B and C, shaded circles). As expected, slightly higher levels of Sir proteins were observed at a site on the telomere-proximal side of the tRNA^{Thr} gene (4 kb from *HMR-E*), consistent with the reported boundary activity of the tRNA^{Thr} gene (Figure 3, B and C; also see ahead to Figure 4). These results indicate that the tRNA^{Thr} gene is not critical for maintaining the association of Sir proteins within *HMR*.

It remained possible that the potential ability of the tRNA^{Thr} gene to block the encroachment of euchromatin was more important in the absence of the *HMR-I* silencer, which increases the association of Sir proteins. Therefore, chromatin IP was performed in strains lacking both elements. Under these conditions, there was no significant reduction in Sir protein levels compared to the single deletion of *HMR-I* (Figure 3, B and C, shaded squares). Therefore, even without the enhancing effect of *HMR-I*, the tRNA^{Thr} gene is not needed to maintain high levels of Sir protein within *HMR*. We conclude from these results that the *HMR-I* silencer, but not the tRNA^{Thr} gene, is important for maintaining high levels of Sir proteins at *HMR*.

The *HMR-E* silencer has been proposed to act in a directional manner (Zou *et al.* 2006a,b). Consistent with this proposal, even in the absence of both the *HMR-I* silencer and the tRNA^{Thr} gene, Sir proteins were distributed asymmetrically, being higher on the telomere-proximal side of *HMR-E* (Figure 3, B and C, compare (−)1-kb and 1-kb locations).

The elevated levels of Sir proteins due to the *HMR-I* silencer are not required for silencing *HMRa1*: The decrease in the association of Sir proteins observed in the absence of the *HMR-I* silencer suggests that transcriptional silencing may be compromised in these strains. However, quantitative mating assays, which indirectly reflect transcription of the *HMR* locus, revealed no obvious silencing defect in the absence of *HMR-I* (Rivier *et al.* 1999; see ahead to Figure 7A). To detect potential rare transcripts from *HMR*, we performed reverse transcriptase PCR on RNA isolated from strains with and without the *HMR-I* silencer. Controls revealed that *HMR**a1*** was transcribed as expected in the absence of silencing in a *sir2*Δ strain but was undetectable by conventional or real-time PCR in the presence of *SIR2* (Figure 3D). In the absence of the *HMR-I* silencer, no *HMR**a1*** cDNA was detected either by conventional PCR (Figure 3D) or real-time PCR. Two control genes, *ACT1* and *NTG1*, could be amplified (Figure 3D), indicating that cDNA synthesis was successful. Thus, *HMR**a1*** remained silenced in the absence of *HMR-I* despite the reduced association of Sir proteins with the promoter.

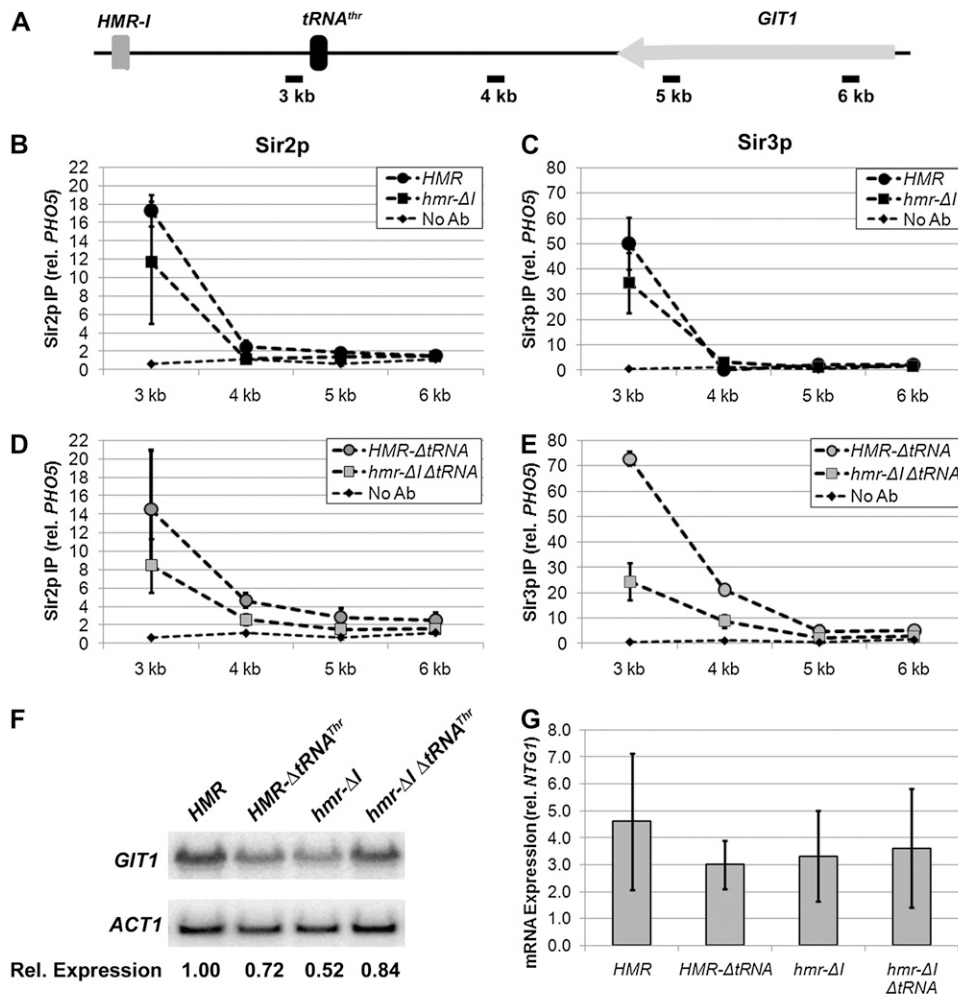

FIGURE 4.—*HMR-I* modestly extends the range of Sir proteins at *HMR*. (A) Diagram of the features on the telomere-proximal side of the *HMR* locus. (B) Relative association of Sir2p with the telomere-proximal side of *HMR* in the presence and absence of *HMR-I*. Quantitative PCR was performed on DNA isolated in the same chromatin IP experiments as in Figure 3. Note that the scale of the *y*-axis differs from Figure 3 to facilitate the examination of these data. (C) Relative association of Sir3p with the telomere-proximal side of *HMR*, as described for B. (D and E) Relative associations of Sir2p and Sir3p with the telomere-proximal side of the *HMR* locus in the absence of the tRNA^Thr gene and in the presence or absence of the *HMR-I* silencer. (F) Levels of *GIT1* mRNA in the presence and absence of regulatory elements, as analyzed by RNA blotting. RNA was isolated from the same strains used above with the indicated modifications at *HMR*, separated on an agarose gel, and transferred to a nylon membrane. The blot was probed for *GIT1* and *ACT1* mRNA. The values below each lane reflect the relative levels of *GIT1* expression, normalized first to *ACT1* and then to the value in wild-type conditions (*HMR*). (G) Levels of *GIT1* cDNA in the presence and absence of regulatory elements, as analyzed by RT–PCR. RNA was isolated from the indicated strains and the mRNA transcripts were converted to cDNA and quantified by real-time PCR using primers specific for *GIT1*. Transcript levels are shown relative to the control gene (*NTG1*). Values represent the average of four independent RNA preparations.

Similarly, no *HMR**a1*** cDNA was observed in strains lacking the tRNA^Thr gene alone or in combination with the *HMR-I* silencer (Figure 3D). In contrast, *HMR**a1*** mRNA was detected in the absence of the *HMR-E* silencer (Figure 3D and BRAND *et al.* 1987). Therefore, *HMR-E*, but not *HMR-I* or the tRNA^Thr gene, is necessary to silence *HMR**a1*** and maintain haploid cell identity, which is considered to be the critical function of silenced chromatin at the mating type cassettes. Additionally, these data, along with the chromatin IP results, reveal the surprising fact that more Sir proteins get recruited to *HMR* than are required for silencing.

***HMR-I* modestly extends the range of Sir proteins on the telomere-proximal side of *HMR*:** The observation that the *HMR-I* silencer increases the association of Sir proteins in its vicinity (Figure 3, B and C) raises the possibility that the *HMR-I* silencer enables the Sir proteins to propagate significantly farther along the chromosome. In this case, a boundary element may be important to block the extension of silenced chromatin. To determine whether the *HMR-I* silencer promotes the assembly of Sir proteins on its telomere-proximal side, we measured the levels of Sir proteins on the telomere-proximal side of *HMR* in the presence and absence of the silencer. When the *HMR-I* silencer was present, a modest enrichment of Sir proteins was observed at the 3-kb site (Figure 4, B and C; the scale of the *y*-axis is different than in Figure 3), suggesting that the *HMR-I* silencer has some ability to extend the range of silenced chromatin. A slight enrichment of Sir proteins was also observed at the 4-kb site beyond the boundary, in the presence but not the absence of *HMR-I* (Figure 4, B and C).

To observe the potential extension of Sir chromatin over a greater distance, the chromatin IP was repeated in the absence of the tRNA^Thr gene. Consistent with previous observations (OKI and KAMAKAKA 2005; DHILLON *et al.* 2009), in the absence of the tRNA^Thr gene, Sir2p and Sir3p levels were slightly elevated at the 4-kb site (Figure 4, compare D and E to B and C). However, Sir proteins were not as elevated in the absence of the

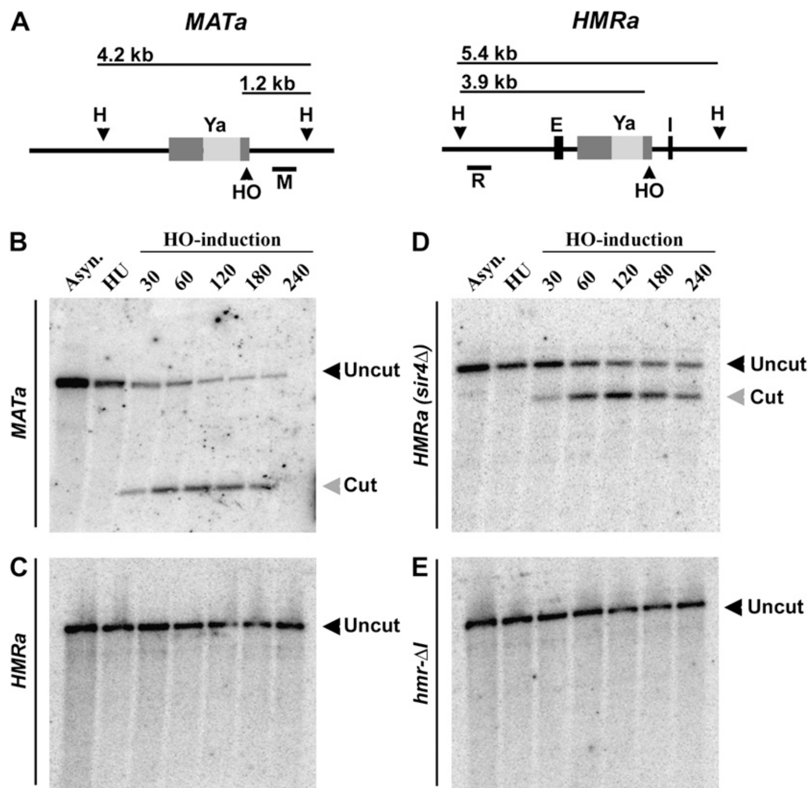

Figure 5.—*HMR* is resistant to HO-endonuclease digestion in the absence of *HMR-I*. (A) Diagram of the expected DNA fragment sizes generated by HO endonuclease (HO) and *Hin*dIII (H) digestion at *MAT***a** and *HMR****a***. Bars below the schematics represent the locations of DNA probes used for hybridizations to *MAT* (M) and *HMR* (R). Light shaded bands indicate **a**-gene specific sequence. Dark shaded bars represent sequences found at *MAT*, *HMR*, and *HML*, which include the recognition sequence for the HO endonuclease. (B) Time course of cutting by HO at the *MAT***a** locus. Samples were collected from a wild-type *MAT***a** strain (LRY2467) in asynchronously growing conditions (Asyn.), after S-phase arrest in HU, and at various times following induction of HO. Genomic DNA was isolated, digested with *Hin*dIII, and analyzed by Southern blotting. (C–E) Time courses of cutting by HO at the *HMR****a*** locus in strains of (C) wild-type *HMR* (LRY2379), (D) *sir4*Δ (LRY2482), and (E) *hmr*-Δ*I* (LRY2384).

HMR-I silencer, again indicating that the *HMR-I* silencer promotes a modest extension of the Sir proteins on its telomere-proximal side (Figure 4, D and E).

In the strains lacking the tRNA^{Thr} gene but retaining *HMR-I*, Sir protein levels were also slightly elevated over background at the 5- and 6-kb locations, which reside in the open reading frame of the *GIT1* gene (Figure 4A, see also Oki and Kamakaka 2005; Dhillon *et al.* 2009). This observation suggests that the expression of the *GIT1* gene, which is reported to be repressed in the absence of the tRNA^{Thr} gene (Donze and Kamakaka 2001; Oki and Kamakaka 2005), may be less affected in the absence of the *HMR-I* silencer. To test this possibility, we examined the level of *GIT1* mRNA in the presence and absence of *HMR-I* by RNA blotting and quantitative RT–PCR. Slight fluctuations in *GIT1* expression were observed in each of the mutants tested (Figure 4, F and G). However, *GIT1* levels in these strains were all within twofold of wild-type levels, and no statistically significant differences were observed. Therefore, we conclude that the occasional spread of Sir proteins into the *GIT1* open reading frame, as occurs in the absence of the tRNA^{Thr} gene, has little impact on the expression of *GIT1*.

HMR* is resistant to HO endonuclease in the absence of *HMR-I*:** *HMR-I* clearly enhances the association of Sir proteins with *HMR* but is not required for the transcriptional repression of the *HMR*a1*** gene. In addition to silencing of mating-type genes, which is critical to maintaining haploid cell identity, a second function of silenced chromatin at the *HM* loci is to protect DNA from being cut by the HO endonuclease during mating-type switching (reviewed in Haber 1998). *S. cerevisiae* cells change their mating type by site-directed recombination. The expression of the HO endonuclease during G1 of the cell cycle results in the formation of a double strand break at *MAT*, which is subsequently repaired via recombination with the *HM* locus of opposite mating type (reviewed in Haber 1998). Recombination is facilitated by conserved sequences found at all three locations (*MAT*, *HML*, and *HMR*) that include the recognition site for the HO endonuclease (Figure 5A). It is thought that silenced chromatin protects the *HM* loci from being cut by the HO endonuclease, ensuring that recombination only occurs at *MAT* (Klar *et al.* 1981; Strathern *et al.* 1982; Loo and Rine 1994).

The HO recognition site at *HMR* is $<$190 bases from the *HMR-I* silencer (Nickoloff *et al.* 1986). To address whether the increase in Sir protein association mediated by the *HMR-I* silencer helps protect *HMR* from HO-endonuclease digestion, we examined the ability of HO to cleave this site in a strain lacking *HMR-I*. We used a previously described assay in which cut intermediates are stabilized by arresting the cells with hydroxyurea (HU) (Connolly *et al.* 1988). Under these conditions, repair of double-strand breaks by homologous recombination is inhibited by the DNA replication checkpoint (Alabert *et al.* 2009). After arrest in HU, HO endonuclease was induced by the addition of galactose to the medium. Samples were collected at different times following induction, and genomic DNA was isolated, digested with *Hin*dIII (H), and analyzed by Southern

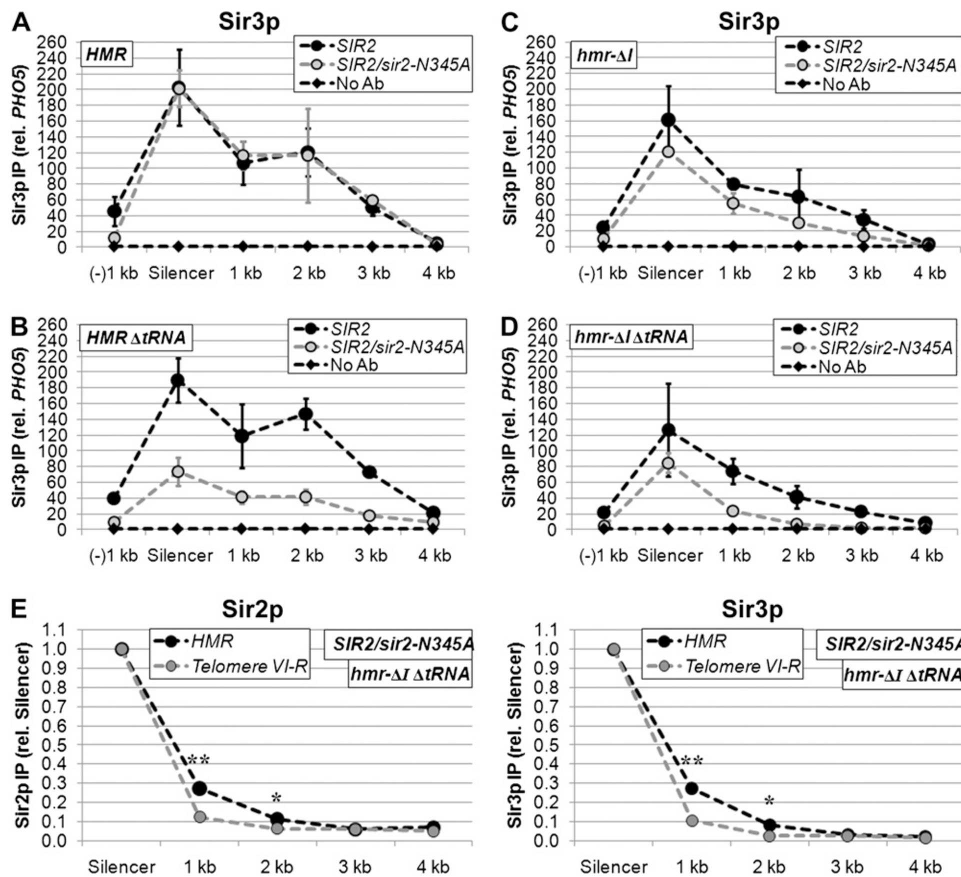

Figure 6.—The *HMR-I* silencer and tRNA^{Thr} gene help maintain silenced chromatin at *HMR* when deacetylase activity is reduced. (A) Sir3p association with *HMR* in the presence of either wild-type Sir2p alone (LRY1007) or Sir2p and catalytically inactive Sir2-N345Ap (LRY0804). Values in A–D represent the averages of at least three independent experiments analyzed as in Figure 1B. (B) Sir3p association with *HMR*-ΔtRNA^{Thr} in the presence of Sir2p alone (LRY2302) or Sir2p and Sir2-N345Ap (LRY2303). (C) Sir3p association with *HMR*-Δ*I* in the presence of Sir2p alone (LRY2315) or Sir2p and Sir2-N345Ap (LRY2316). (D) Sir3p association with *HMR*-Δ*I* ΔtRNA^{Thr} in the presence of Sir2p alone (LRY2309) or Sir2p and Sir2-N345Ap (LRY2352). (E) The relative enrichments of Sir2p and Sir3p with telomere VI-R and the modified *HMR* locus in the presence of Sir2-N345Ap (LRY2352). Data were analyzed as in Figure 1, D and E.

blotting using probes specific to *MAT* (M) or *HMR* (R) (Figure 5A). As a control, we examined the ability of HO to cut at *MAT***a** and wild-type *HMR**a***. Cleavage of the *MAT***a** locus was detected within 30 min of induction of HO (Figure 5B). In contrast, no cutting was detected at wild-type *HMR**a***, even after 4 hr of HO induction (Figure 5C). In the absence of Sir proteins, *HMR**a*** was cut with similar kinetics to those observed at the unprotected *MAT***a** locus, as expected (Figure 5D). However, cleavage was not observed in the absence of *HMR-I* (Figure 5E). Therefore, the *HMR-I*-mediated increase in Sir protein levels at *HMR* was not necessary to protect the adjacent HO recognition sequence from being cut.

The *HMR-I* silencer and tRNA^{Thr} gene cooperate to maintain Sir proteins at *HMR* when deacetylation is reduced: The *HMR-I* silencer and tRNA^{Thr} gene are not required to maintain transcriptional repression of *HMR**a1***, but their presence at the *HMR* locus suggests they have a biological function. One situation in which the increased association of Sir proteins at *HMR-I* might be important is when the Sir2p deacetylase has reduced activity. The deacetylase activity of Sir2p requires NAD+ (Imai *et al.* 2000; Landry *et al.* 2000; Smith *et al.* 2000) and is inhibited by nicotinamide (Bitterman *et al.* 2002). Sir2p activity may be reduced by fluctuations in the intracellular levels of these metabolites. To mimic reduced deacetylase activity, we coexpressed enzymatically inactive and wild-type alleles of *SIR2*, which should result in enzymatic inactivity for approximately half of Sir2p molecules. We previously found that the *HMR* locus is unaffected by this condition, but that silenced chromatin at telomere VI-R is disrupted (Lynch and Rusche 2009), as is repression of a reporter gene at telomere VII-L (Armstrong *et al.* 2002). This phenotype is not simply due to doubling the amount of Sir2p because two copies of the wild-type gene do not affect silencing at telomere VII-L (Armstrong *et al.* 2002).

To determine whether *HMR-I* or the tRNA^{Thr} gene contributed to the resistance of *HMR* to reduced deacetylase activity, Sir protein levels at *HMR* were assessed by chromatin IP in the presence of only wild-type *SIR2* or both *SIR2* and *sir2-N345A* (Figure 6 for Sir3p and Figure S2 for Sir2p). Remarkably, expression of *sir2-N345A* resulted in a profound decrease in Sir protein enrichment in the absence of the tRNA^{Thr} gene (Figure 6B). In contrast, a relatively modest, albeit reproducible, decrease in Sir protein association was observed in the absence of *HMR-I* (Figure 6C). In the absence of both the *HMR-I* silencer and the boundary, a more severe decrease in Sir3p distribution was observed than in either single mutant, with total Sir3p levels approaching background within 2–3 kb of the *HMR-E* silencer. As previously reported (Lynch and Rusche 2009), no loss of Sir3p was observed when the *sir2-N345A* mutant was expressed in a wild-type *HMR* strain. Therefore, the

tRNA^{Thr} gene and the *HMR-I* silencer together collaborate with the *HMR-E* silencer to prevent the loss of Sir proteins when deacetylation is reduced.

To determine whether *HMR-E* on its own retained an ability to promote the assembly of silenced chromatin over a distance under conditions of reduced deacetylase activity, we compared the distributions of Sir proteins adjacent to *HMR-E* or the telomeric repeat in the *hmr*-Δ*I* ΔtRNA^{Thr} strain. Indeed, although the association of Sir2p and Sir3p was reduced at both telomere VI-R and *HMR* in the presence of Sir2-N345Ap (Figure 6E), the level of Sir proteins 1 or 2 kb from the silencer represented a greater fraction of the level observed at the silencer at *HMR* compared to the telomere, suggesting that the *HMR-E* silencer provides some additional resistance to compromised deacetylation.

The *HMR-I* silencer and tRNA^{Thr} gene cooperate to maintain transcriptional silencing when deacetylation is reduced: To determine whether the reduction in Sir proteins associated with *hmr*-Δ*I* ΔtRNA^{Thr} under conditions of reduced deacetylase activity (Figure 6D) impacts silencing of *HMR**a1***, transcription was assessed in two ways. First, a mating assay was conducted. Only when *HMR**a1*** is silenced will *MAT*α strains mate. *MAT*α haploids containing different *HMR* alleles in combination with different *SIR2* alleles were mixed with haploids of the opposite mating type and plated on medium selective for diploids. Neither the deletion of the *HMR-I* silencer nor the tRNA^{Thr} gene alone resulted in a detectable defect in mating (Figure 7A). However, in the absence of both *HMR-I* and the tRNA^{Thr} gene, an ~10-fold defect in mating was observed in the presence of Sir2-N345Ap (Figure 7A, bottom row). To detect *HMR**a1*** transcripts, cDNA was prepared from each of the strains and quantified by real-time PCR. Consistent with the mating assay, *HMR**a1*** transcripts were close to the limit of detection in strains with the wild-type *HMR* or *hmr*-ΔtRNA^{Thr} alleles (Figure 7B and data not shown). In the absence of *HMR-I*, a very slight derepression of *HMR**a1*** occurred in the presence of Sir2-N345Ap, although the levels of *HMR**a1*** were ~1% of those in a *sir2*Δ strain (data not shown). However, in the absence of both *HMR-I* and tRNA^{Thr}, the coexpression of Sir2p and Sir2-N345Ap resulted in derepression of *HMR**a1*** levels at ~7% of a *sir2*Δ strain (Figure 7B). Therefore, the *HMR-I* silencer and tRNA^{Thr} gene together maintain repression of *HMR**a1*** when deacetylation is compromised.

To determine whether the *HMR-I* silencer and tRNA^{Thr} gene help maintain silencing of *HMR**a1*** in environmental conditions that reduce deacetylation, mating was assessed in the presence of nicotinamide, which inhibits Sir2p (Bitterman *et al.* 2002). Indeed, in the presence of 5 mm nicotinamide, mating was significantly reduced in each of the strains tested (Figure 7D). However, in the absence of *HMR-I*, the defect in mating was 10- to 100-fold more severe compared to wild-type *HMR*. In contrast, in the absence of the tRNA^{Thr} gene, mating occurred at levels similar to those observed in strains containing wild-type *HMR*. Additionally, no further mating defect was observed in the absence of both *HMR-I* and the tRNA^{Thr} gene than in the absence of *HMR-I* alone. Therefore, the *HMR-I* silencer helps maintain efficient silencing of *HMR* in conditions of reduced Sir2p activity.

Silencing at telomeres is known to be inhibited by the coexpression of Sir2-N345Ap and Sir2p (Armstrong *et al.* 2002; Lynch and Rusche 2009). To assess the ability of telomere VI-R to maintain transcriptional silencing in the presence of Sir2-N345Ap, we measured the level of *YFR057w* mRNA in the presence and absence of Sir2-N345Ap (Figure7C). In the presence of Sir2-N345Ap, *YFR057w* expression was partially derepressed to ~35% of the level in a *sir2* mutant (Figure 7C). This level of induction is greater than was observed for *HMR**a1*** in an *hmr*-Δ*I* ΔtRNA^{Thr} strain (7% of the level in a *sir2* mutant; Figure 7B), consistent with silencing being more easily disrupted at telomere VI-R than *HMR*, although there are several caveats to this interpretation, given that different genes with different promoters are being compared.

DISCUSSION

***HMR-E* increases the association of Sir proteins over several kilobases:** Historically, it has been thought that silencers act by recruiting silencing proteins to a particular site in the genome and that the spreading of silencing proteins along the chromosome occurs independently of the silencer. However, we found that the *HMR-E* silencer acts by a process distinct from recruitment to enable the rapid establishment of silenced chromatin over several kilobases (Lynch and Rusche 2009). These initial studies focused on the establishment of silencing following the induction of high levels of Sir3p, and it was important to investigate how *HMR-E* and other silencers shape the steady-state distribution of Sir proteins expressed at endogenous levels. We now report that *HMR-E* maintains Sir proteins over several kilobases at higher levels relative to the silencer than does the telomeric repeat at chromosome VI-R. This increased level of Sir proteins is observed both when *HMR-E* is in its native location at *HMR* (Figure 1) and when it is translocated to telomere VI-R (Figure 2). Moreover, the enhanced association of Sir proteins is observed in the absence of the auxiliary silencer *HMR-I* (Figures 1 and 2), indicating that *HMR-E* achieves this increase on its own. In addition, the association of Sir proteins is enhanced on one side of *HMR-E* compared to the other (Figure 3). Therefore, in addition to accelerating the rate of Sir protein assembly (Lynch and Rusche 2009), the *HMR-E* silencer increases the steady-state association of Sir proteins over several kilobases in a directional manner.

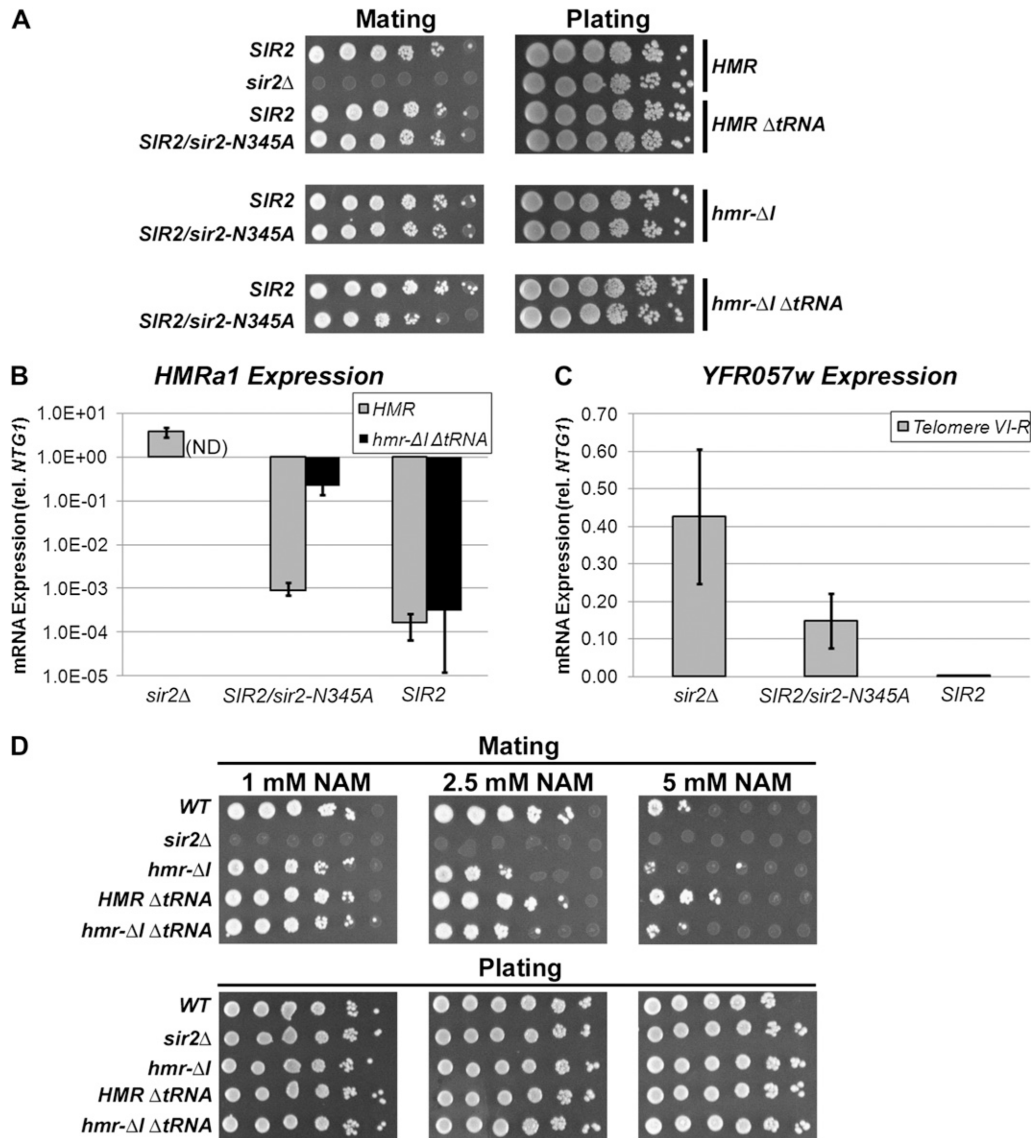

FIGURE 7.—Both the *HMR-I* silencer and the tRNA^{Thr} gene are required to maintain complete silencing of *HMR***a1** when deacetylation is reduced. (A) Mating ability was assessed by exposing 10-fold serial dilutions of *MAT*α haploids to *MAT***a** tester haploids (LRY1021). The resulting diploids were selected on minimal medium. The same strains described in Figure 6 were used, as well as a *MAT*α *sir2*Δ strain (LRY1068). (B) Levels of *HMR***a1** in a strain containing wild-type *HMR* and *sir2*Δ (LRY1068), and strains containing either wild-type *HMR* or *hmr-*Δ*I* Δ*tRNA^{Thr}* in the presence of both Sir2p and Sir2-N345Ap (LRY0804 and LRY2352) or only Sir2p (LRY1007 and LRY2309). *HMR***a1** levels were not assayed in a strain containing *hmr-*Δ*I* Δ*tRNA^{Thr}* and *sir2*Δ (ND). RNA was isolated from the indicated strains and the mRNA transcripts were converted to cDNA and quantified by real-time PCR using primers specific for the *HMR***a1**. Transcript levels are shown relative to the control gene (*NTG1*). Values represent the average of four independent RNA preparations and are plotted on a logarithmic scale. (C) Relative levels of *YFR057w* mRNAs were quantified in strains with *sir2*Δ (LRY1068), *SIR2* and *sir2-N345A* (LRY0804), and wild-type *SIR2* (LRY1007). Data from four independent RNA isolations are plotted as in B. (D) Mating ability was assayed upon exposure to the given concentrations of nicotinamide (NAM) in the same strains as in A.

The mechanism by which *HMR-E* enhances the association of Sir proteins remains to be determined. One possibility is that proteins associated with *HMR-E* favor the formation of a higher-order arrangement of the chromatin fiber, such as a looped or compact structure, and that this arrangement enables assembly to occur in a nonlinear, and hence more efficient, fashion. We observed a gradual decrease in Sir protein association as a function of distance from the *HMR-E* silencer (Figures 1, 2, and 3), a result inconsistent with the *HMR-E* silencer facilitating the formation of a single, defined higher-order structure. Instead, transient interactions between silencer-associated proteins and nearby nucleosomes may result in the formation of a set of related structures that enhance the assembly of silenced chromatin in a distance-dependent fashion. Moreover, the asymmetrical distribution of Sir proteins around *HMR-E* (Figure 3; *hmr-*Δ*I* Δ*tRNA* strain) could reflect a tendency of proteins at the *HMR-E* silencer to interact more frequently with sequences on the Abf1-binding side of the silencer, preferentially generating higher-order chromatin structures within the *HMR* cassette.

***HMR-I* impacts Sir protein levels at *HMR* but does not affect transcriptional silencing:** The Abf1p and ORC-binding sequences of *HMR-I* are conserved in a related species of yeast, *S. paradoxus*, although the surrounding sequences are highly diverged (Teytelman *et al.* 2008). Therefore, *HMR-I* probably has a biological function that positively impacts the fitness of a yeast cell. In keeping with this hypothesis, we found that the *HMR-I* silencer does elevate the levels of Sir proteins within the *HMR* cassette (Figure 3) and helps maintain the Sir

proteins at *HMR* when deacetylase activity is reduced (Figure 6C). However, despite the reduced association of Sir proteins in the absence of *HMR-I*, transcriptional silencing remained effective (Figure 3D; Brand *et al.* 1987). Similarly, we found that *HMR* remained protected from cleavage by the HO endonuclease in the absence of *HMR-I* (Figure 5E). Thus, it remains unclear how the increased association of Sir proteins with *HMR* due to *HMR-I* contributes to the biological function of this silencer. One possibility is that *HMR-I* protects the locus against loss of silencing under suboptimal conditions, such as a reduction in deacetylase activity, as discussed below.

It is striking that the *HMR* cassette appears to recruit more Sir proteins than are necessary to maintain silencing. In fact, transcriptional repression remains strong when the association of Sir proteins with the ***a1*** promoter is reduced to one-quarter of the maximum observed at the *HMR-E* silencer (Figure 6). Although it is not clear whether every cell is affected similarly by the loss of *HMR-I* given that the enrichments of Sir proteins observed by chromatin IP represent an average over the population, a fair number of cells must be depleted of Sir proteins at the promoter. Therefore, Sir proteins may not need to be present continuously throughout *HMR* to block transcription and thus may not act by hindering access of RNA polymerase II to the promoter, as was originally proposed. Instead, Sir proteins may generate modifications of histones that persist even when Sir proteins dissociate from the promoter. Alternatively, Sir proteins associated with the *HMR-E* silencer may act from a distance to prevent RNA polymerase II from initiating transcription. A final possibility is that Sir proteins, which have the capacity to multimerize, are normally present in "super-stoichiometric" amounts and thus, even with the decrease observed in the absence of *HMR-I*, every promoter remains associated with Sir proteins.

The tRNA^{Thr} gene plays both positive and negative roles in the regulation of silencing: The tRNA^{Thr} gene at *HMR* is notable as one of the few characterized boundary elements in *S. cerevisiae*. tRNA genes have also been shown to separate heterochromatin from other chromatin domains at centromeres and mating-type loci in *Schizosaccharomyces pombe* (Noma *et al.* 2006; Scott *et al.* 2006, 2007) and to block upstream activator sequences from acting on promoters in *S. cerevisiae* (Simms *et al.* 2008), indicating that these genes may have conserved functions in partitioning domains of chromatin. However, our analysis reveals that, although the tRNA^{Thr} gene does block the spread of Sir proteins, it probably is not the major mechanism controlling the extent of silenced chromatin. In the absence of the tRNA^{Thr} gene, Sir2p and Sir3p were only marginally enriched on the telomere-proximal side of *HMR* (Figure 4). Therefore, it appears that silenced chromatin naturally decays over a distance of 1 or 2 kb even without encountering a specific boundary element. The tRNA^{Thr} gene thus serves as a backup mechanism to check the propagation of silenced chromatin when it extends beyond its usual limit.

We also found that the tRNA^{Thr} gene helped maintain Sir proteins within the *HMR* cassette when Sir2p activity was compromised (Figure 6, B and D), although it had no effect on the distributions of Sir2p or Sir3p in the presence of wild-type *SIR2* (Figure 3, B and C). At least two mechanisms could account for this role of the tRNA^{Thr} gene in promoting the association of Sir proteins with *HMR*. One possibility is that the tRNA^{Thr} gene blocks the spread of euchromatin, and in particular acetyltransferases, into the silenced locus. It has been suggested that acetyltransferases participate in a spreading reaction similar to that of Sir proteins (Bulger 2005). For example, acetyltransferases targeted to a particular sequence can acetylate histones over several kilobases (Vignali *et al.* 2000; Yu *et al.* 2006), and this long-range acetylation is disrupted by nucleosome excluding sequences (Yu *et al.* 2006). Thus, the tRNA^{Thr} gene may block the spread of euchromatin into *HMR*. This effect may be particularly pronounced in the absence of *HMR-I*, which helps to maintain Sir proteins at *HMR* (Figure 6D).

A second possibility is that the ability of the tRNA^{Thr} gene to recruit cohesins may help maintain silenced chromatin when deacetylation is reduced. The tRNA^{Thr} gene adjacent to *HMR* promotes the association of cohesin proteins with the silenced *HMR* locus (Dubey and Gartenberg 2007). Although the loss of cohesins in the absence of the tRNA^{Thr} gene has no impact on silencing of *HMR**a1*** (Chang *et al.* 2005), it remains possible that it reduces the ability of Sir proteins to remain associated with *HMR* under conditions of reduced deacetylation.

A final possibility we do not favor is that the increased spreading of a finite pool of Sir proteins reduces the availability of these proteins to associate with normally silenced domains. This model has been suggested in other contexts (Kimura *et al.* 2002; Suka *et al.* 2002; van Leeuwen *et al.* 2002), but seems less likely in the case of the *hmr* ΔI $\Delta tRNA$ strain, given that Sir proteins do not associate significantly with sequences beyond the position of the deleted tRNA gene (Figure 6D).

The biological functions of *HMR-I* and the tRNA^{Thr} gene: The contributions of *HMR-I* and the tRNA^{Thr} gene to silencing are only observed in the context of reduced Sir2p activity. Under normal laboratory conditions, *HMR-E* alone is strong enough to silence *HMR**a1***, maintain haploid cell identity, and protect against HO endonuclease cleavage. Why then, is it necessary to have an auxiliary silencer to boost Sir protein association and a boundary to block the subsequent spreading? Our experiments with reduced Sir2p deacetylase activity offer some clues. In contrast to laboratory growth conditions, in nature yeast are subjected to variations in

available nutrients. The direct link between Sir2p activity and NAD+ metabolism suggests that under some conditions deacetylation may be compromised and consequently yeast may have evolved insulating mechanisms for maintaining silenced chromatin at *HMR* in such circumstances. Indeed, in oxidative stress conditions, silencing at *HMR* is improved upon overexpression of Sir2p (Oberdoerffer *et al.* 2008). Similarly, the natural boost in Sir protein levels conveyed by *HMR-I* (and in some cases the $tRNA^{Thr}$ gene) may have a similar effect. Furthermore, the apparent overabundance of Sir proteins at *HMR* may mitigate the loss of Sir protein enrichment at silenced loci that occurs during aging (Lin *et al.* 2009), thus delaying the onset of sterility in older cells.

Perspective: These studies extend our previous work by demonstrating that, in addition to accelerating the rate of assembly of silenced chromatin, the *HMR-E* silencer increases the steady-state level of Sir proteins within several kilobase pairs of the silencer. Moreover, this work reinforces our previous conclusion that in the absence of such a silencer the ability of Sir proteins to spread is limited (Lynch and Rusche 2009; Rusche and Lynch 2009). For example, even when the $tRNA^{Thr}$ boundary element was deleted, robust levels of Sir proteins were detected only within a few kilobase pairs of the *HMR-E* and *HMR-I* silencers (Figures 3 and 4). This limited capacity to spread probably mitigates the potentially toxic effects of fortuitous assembly and spreading of Sir proteins at inappropriate genomic locations. At the same time, these limitations increase the importance of silencers, such as *HMR-E* and *HMR-I*, in stabilizing the associations of Sir proteins in appropriate locations, particularly when deacetylation is compromised. Thus, these elements probably serve to buffer the capacity of silenced chromatin to assemble under suboptimal conditions.

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An Auxiliary Silencer and a Boundary Element Maintain High Levels of Silencing Proteins at *HMR* in *Saccharomyces cerevisiae*

Patrick J. Lynch and Laura N. Rusche

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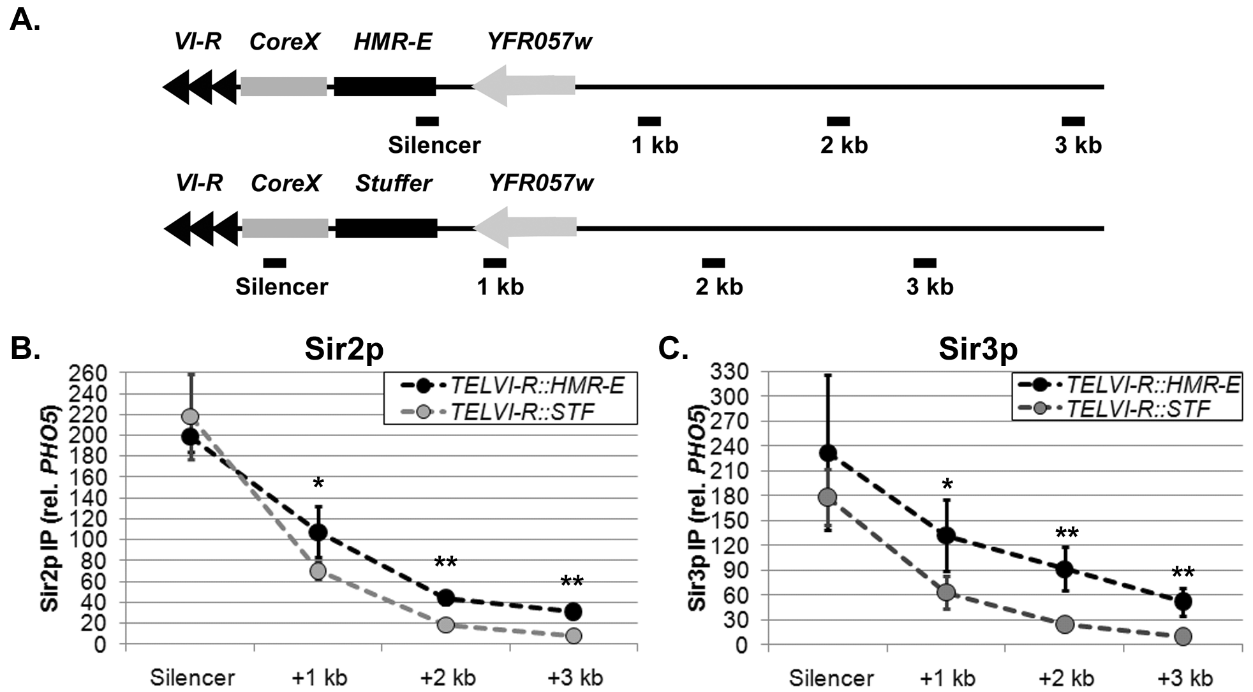

FIGURE S1.—A transposed *HMR-E* increases the association of Sir proteins at telomere VI-R. (A) Diagrams of modified telomere VI-R loci. (B) Relative association of Sir2p with modified telomere VI-R loci. The same chromatin IP samples analyzed in Figure 2B were normalized to *PHO5* rather than the silencer. Data were analyzed as in Figure 1B and C and represent the averages of 2 independent immunoprecipitation experiments and at least 4 PCR reactions. (C) Relative association of Sir3p with modified telomere VI-R loci. The same chromatin IP samples analyzed in Figure 2C were normalized to *PHO5*.

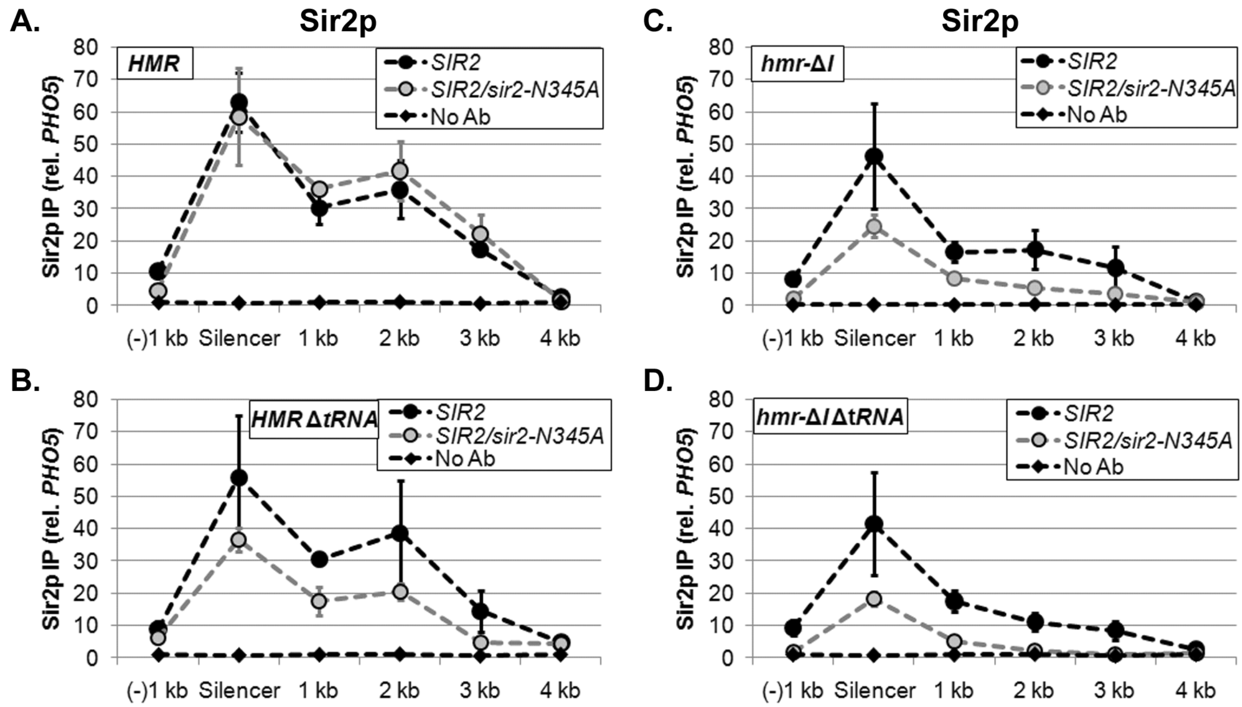

FIGURE S2.—The *HMR-I* silencer and tRNA[Thr] gene help maintain Sir2p at *HMR* when deacetylase activity is reduced. (A) Sir2p association with *HMR* in the presence of either wild-type Sir2p alone (LRY1007) or Sir2p and catalytically inactive Sir2-N345Ap (LRY0804). Values in A-D represent the averages of at least 3 independent experiments. It is expected that the polyclonal antibody against Sir2p recognizes both wild-type Sir2p and mutant Sir2-N345Ap, which differs from the wild-type at only one amino acid. (B) Sir2p association with *HMR*-ΔtRNA[Thr] in the presence of Sir2p alone (LRY2302) or Sir2p and Sir2-N345Ap (LRY2303). (C) Sir2p association with *HMR*-Δ*I* in the presence of Sir2p alone (LRY2315) or Sir2p and Sir2-N345Ap (LRY2316). (D) Sir2p association with *HMR*-Δ*I* ΔtRNA[Thr] in the presence of Sir2p alone (LRY2309) or Sir2p and Sir2-N345Ap (LRY2352).