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Analyses of *SUM1-1*-Mediated Long-Range Repression

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ABSTRACT

In *Saccharomyces cerevisiae*, local repression is promoter specific and localized to a small region on the DNA, while silencing is promoter nonspecific, encompasses large domains of chromatin, and is stably inherited for multiple generations. Sum1p is a local repressor protein that mediates repression of meiosis-specific genes in mitotic cells while the Sir proteins are long-range repressors that stably silence genes at *HML*, *HMR*, and telomeres. The *SUM1-1* mutation is a dominant neomorphic mutation that enables the mutant protein to be recruited to the *HMR* locus and repress genes, even in the absence of the Sir proteins. In this study we show that the mutation in Sum1-1p enabled it to spread, and the native *HMR* barrier blocked it from spreading. Thus, like the Sir proteins, Sum1-1p was a long-range repressor, but unlike the Sir proteins, Sum1-1p-mediated repression was more promoter specific, repressing certain genes better than others. Furthermore, repression mediated by Sum1-1p was not stably maintained or inherited and we therefore propose that Sum1-1p-mediated long-range repression is related but distinct from silencing.

IN *Saccharomyces cerevisiae,* mating-type information is present at three locations on chromosome III. The transcriptionally active *MAT* locus determines the mating type of a cell and encodes either *MAT***a** or *MAT*α genes in **a** or α cells, respectively. Cells of opposite mating type mate to produce **a**/α diploids. Additional copies of the mating-type genes are present on the distal arms of chromosome III at the *HML* and *HMR* loci. In most yeast strains, *HML* contains an unexpressed but intact copy of the *MAT*α allele whereas *HMR* contains an unexpressed intact copy of the *MAT***a** allele. Expression of *HML* and *HMR* is stably repressed by a mechanism called silencing, which requires the Sir proteins and specific flanking DNA sequences called silencers (reviewed in Rusche *et al.* 2003). The silencers and the Sir proteins function together to inactivate most genes in a distance- and orientation-independent manner. The silencers contain binding sites for the origin replication complex (ORC), Rap1p, Abf1p, and Sum1p (Laurenson and Rine 1992; Loo and Rine 1995; Irlbacher *et al.* 2005) and these silencer-associated proteins initiate the assembly of the silenced chromatin by recruiting the Sir proteins Sir1p, Sir2p, Sir3p, and Sir4p (reviewed in Moazed 2001). Sir2p is an NAD-dependent protein deacetylase, which deacetylates the N-terminal tails of histones, and Sir3p and Sir4p interact with the hypoacetylated histone tails, resulting in silencing.

SUM1 encodes a sequence-specific DNA-binding repressor protein that binds to the operator (middle sporulation elements, or MSEs) of middle-sporulation genes (Xie *et al.* 1999) and to the *HML-E* silencer (Irlbacher *et al.* 2005) in mitotic cells. While Sum1p binding to the MSE element represses the genes (Pierce *et al.* 2003), binding to the silencer is necessary for Sir-mediated silencing at *HML* (Irlbacher *et al.* 2005). Sum1p is present in a protein complex with the Sir2p homolog, Hst1p, which is required to repress the middle sporulation genes (Xie *et al.* 1999; Rusche and Rine 2001; Sutton *et al.* 2001; McCord *et al.* 2003), and this Hst1p-mediated repression is gene specific and highly localized (Xie *et al.* 1999).

SUM1-1 (suppressor of *mar*) was identified in a screen of extragenic suppressors of the silencing defect in *sir2*Δ cells (Klar *et al.* 1985). *SUM1-1* is a dominant mutation that restores silencing at *HMR* in *sir1*Δ, *sir2*Δ, *sir3*Δ, or *sir4*Δ mutants as well as mutations in the *HMR-E* silencer (Livi *et al.* 1990; Laurenson and Rine 1991; Chi and Shore 1996). The mutation in *SUM1-1* is neomorphic since neither a null mutation in, nor overexpression of, *SUM1* gives rise to the *SUM1-1* phenotype (Chi and Shore 1996). Although Sum1p normally does not associate with *HMR*, it was likely that ORC recruited Sum1-1p to the silenced loci (Rusche and Rine 2001; Sutton *et al.* 2001). While Sum1-1p can repress in the absence of the Sir proteins, repression is dependent on Hst1p (Rusche and Rine 2001; Sutton *et al.* 2001; Bedalov *et al.* 2003). Following recruitment to the *HMR* silencers, Sum1-1p and Hst1p spread across the *HMR* domain, deacetylating the histones and thereby mediating repression (Lynch *et al.* 2005).

To gain further insight into Sum1-1p-mediated repression, we analyzed how this neomorph repressed genes at the *HML* and *HMR* loci. We determined if Sum1-1p was a long-range repressor that could silence a

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variety of genes and whether this repression was stable. We show that Sum1-1p is a long-range repressor and that the chromatin domain generated by Sum1-1p can repress genes independently of their position and orientation within this domain. However, Sum1-1p can repress certain genes better than others and this repression was not stably sustained. Together, these data suggest that the long-range repression mediated by Sum1-1p was distinct from Sir-mediated silencing.

MATERIALS AND METHODS

Yeast growth, transformations, and integrations: Standard yeast media and genetic methods were used as described (Sherman 1991). PCR-based integrations were performed with oligonucleotides whose sequences are available upon request. PCR-based integrations and transformation with plasmids followed the standard lithium acetate procedures (Ito *et al.* 1983). All fragments of DNA were PCR amplified with Expand High Fidelity DNA polymerase, and 5–10 μl of PCR reaction containing 0.5 μg of product were used for a single transformation. Integrations were PCR amplified and their sequences were confirmed.

Plasmids: *pGBD-SUM1* (pRO706) was obtained from the yeast *GBD* fusion protein collection (Ito *et al.* 2001). In this plasmid the *GAL4* DNA-binding domain (1–147 aa) was fused in frame to the N terminus of *SUM1*. The *ADH1* promoter drove transcription of the chimera in the pGBK-RC-*TRP1* plasmid. *pGBD-SUM1-1* (pRO707) was constructed by replacing the *Spe*I–*Pst*I C-terminal fragment of *SUM1* in *pGBD-SUM1* with that of the *SUM1-1* allele. This fragment was amplified by PCR using genomic DNA from the *SUM1-1* strain ROY1971 (derived from strain YMC89; Chi and Shore 1996) as template.

To overexpress *SUM1* and *SUM1-1*, the *Pst*I–*Bam*HI fragments encompassing the Sum1p coding region from p*GBD-SUM1* (pRO706) and p*GBD-SUM1-1* (pRO707) were cloned into the *Pst*I–*Bam*HI site of pRS425 (Sikorski and Hieter 1989). The *SUM1* promoter region (725 bp upstream of ATG) was PCR amplified from yeast genomic DNA and cloned into the *Sac*I-*Bam*HI sites of pRS425 to create the plasmids p*SUM1* (pRO709) and p*SUM1-1* (pRO711). p*HST1* in pRS423 (pRO713) was constructed by cloning a *Bam*HI–*Sal*I fragment from pRO575 (gift of Masaya Oki) and contains *HST1* with 300 bp of upstream and downstream regulatory sequences. *SIR2* was cloned with its promoter in pRS315 (pRO46).

Serial dilutions: A single colony of yeast cells was used to inoculate 5 ml of liquid YP or YM medium with glucose or galactose as the carbon source and the appropriate supplements to allow maintenance of the plasmids. The cells were grown overnight at 30°. All cells were diluted to an initial concentration of 1.0 A_{600}/ml in YM medium and serially diluted fivefold. Approximately 3 μl of each serial dilution was spotted onto appropriately supplemented plates using a cell spotter. For mating assays, supplemented YMD plates were spread with 1.0 A_{600} of mating lawn (strains JRY19a, *MAT***a** *his4*, or *MAT*α *his4*) diluted in 300 ml of YPD. Where necessary, selection for plasmids was maintained throughout this analysis. Cells were allowed to grow at 30° for 48 hr prior to photography.

Patch mating: Patches of the appropriate strains were grown on YMD plates for 1–2 days at 30°. The mating potential of the cells was monitored by replica plating the patches onto selective YMD plates previously spread with a mating lawn, maintaining the selection for the plasmids prior to and following mating.

α-Factor arrest: Cells grown in YPAD liquid media (Sherman 1991) were collected by centrifugation and diluted to 1 A_{600}/ml with fresh YPAD media. Five milliliters of the culture was transferred to a flask and Na-succinate, pH 3.5, was added to a final concentration of 25 mm. Cells were allowed to grow for 15 min (30°, 200 rpm) prior to addition of α-factor. α-Factor (stock concentration 1 mm in 0.1 n HCl; Sigma, St. Louis) was added to the cultures to a final concentration of 0.012 mm. After 3 hr (30°, 200 rpm), the cells were transferred onto a YPAD plate. Five microliters of 0.1 mm α-factor solution was spotted at different locations on the plate. Cells that had arrested in α-factor (Shmoo) were moved to the regions on the plate that lacked or contained α-factor, using a dissecting microscope. Plates were placed at 30° and allowed to grow. Every 3 hr, over a period of 15 hr, the plates were removed and photographed with a digital camera. Arrested and dividing cells from two separate experiments were quantified.

RNA isolation and RT-PCR: Strains (ROY4026, ROY4027, ROY 4028, JRY4563, JRY4013, and ROY1924) were grown overnight in YPD and were used to inoculate 80 ml of fresh YPAD. Cells were grown to an A_{600}~1.0, harvested, and washed with DEPC-treated water, and total RNA was isolated as described (Schmitt *et al.* 1990). cDNA was prepared using a RT-PCR kit from Invitrogen (San Diego) with random primers and Superscript III reverse transcriptase and the Platinum Taq DNA polymerase. Quantitative analyses of the expression of the genes at *HMR* were performed using multiplex PCR with primers specific to *URA3* or *MAT***a***1* and *ACT1*. The sequences of the PCR primers specific for *MAT***a***1*, *URA3*, and *ACT1* are available upon request. Reaction volumes were typically 50 μl and contained 2 μl of cDNA in 20 mm Tris-HCl, pH 8.4, 50 mm KCl, 1.5 mm $MgCl_2$, 0.2 mm of each dNTP, 0.2 μm of each primer and 0.034 μm of [α-^{32}P]dATP and [α-^{32}P]CTP, and 1 unit of platinum Taq DNA polymerase (Invitrogen). Templates were amplified in 25 cycles and 3 μl of the reaction was resolved on a 5%-polyacrylamide-TBE gel. The gel was dried, and the radioactive bands were analyzed on a Typhoon PhosphorImager using ImageQuant software (Amersham-Pharmacia).

Micrococcal nuclease analysis: The *HMR* locus was analyzed by micrococcal nuclease digestion and indirect end labeling (Avendano *et al.* 2005). Briefly, strains were grown in 200 ml of YPD to 1.0 A_{600}/ml and treated with lyticase to produce spheroplasts. Chromatin in the nystatin-permeabilized spheroplasts was digested with different amounts of micrococcal nuclease (0, 1, 2, 3, and 4 units) for 20 min at 37°. Naked DNA was prepared by phenol/chloroform extraction of spheroplasts and this was digested with 0.03 and 0.06 units of MNase for 5 min at 37°. MNase reactions were stopped with 1% SDS and 5 mm EDTA (final concentration) and proteinase K treatment, and DNA was purified by three phenol/chloroform extractions and treated with RNAse. After digestion with *Bgl*II (100 units/sample), DNA samples were run on a 1.5% agarose-Tris-borate-EDTA gel, transferred to Hybond N+ nylon membrane (Amersham, Buckinghamshire, UK), and analyzed by end labeling using a 138-bp radioactive probe that recognizes the *Bgl*II region at the C terminus of *HMRa1*. A pair of oligos (forward: CCAAGGAAAAAGAAGAAGTTGC and reverse: AGA TCTCATACGTTTATTTATGAAC) was used to label this probe with [α-^{32}P]dCTP by PCR amplification. A GIBCO (Gaithersburg, MD) 50-bp ladder radioactively labeled was included in the gel as a molecular size reference. Chromatin blots were scanned and analyzed with the program ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA).

Strain construction: *SIR2*, *PPR1*, and *SUM1* genes were deleted from the start to the stop codon and replaced with *HIS3*, *TRP1*, or *kanMX* markers by homologous recombination to produce *sir2Δ::HIS3*, *sir2Δ::TRP1*, *ppr1Δ::kanMX*, and *sum1Δ::kanMX* strains. The *SUM1-1* allele used in this study is derived from strain YMC89 (Chi and Shore 1996). The genotypes of the yeast strains used in this study are presented in Table 1.

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
	MAT**a** his4	J. Rine
	MATα his4	J. Rine
JRY19a	MAT**a** ura3 leu2 trp1 his4	J. Rine
ROY3790	MATα HMR::TRP1 SIR2 SUM1	
ROY3792	MATα HMR::TRP1 sir2Δ::HIS3 SUM1	
ROY3839	MATα HMR::TRP1 sir2Δ::HIS3 SUM1-1	
ROY3774	MATα HMR::ADE2 SIR2 SUM1	
ROY3770	MATα HMR::ADE2 sir2Δ::HIS3 SUM1	
ROY3818	MAT**a** HMR::ADE2 sir2Δ::TRP1 SUM1-1	
ROY3787	MATα HMR::URA3 ppr1Δ::kanMX SUM1 SIR2	
ROY3797	MAT**a** HMR::URA3 ppr1Δ::kanMX SUM1 sir2Δ:: TRP1	
ROY2677	MATα HMR::URA3 ppr1Δ::kanMX SUM1-1 sir2Δ::TRP1	
ROY4027	MATα HMR::URA3 ppr1Δ::kanMX SUM1 SIR2 ura3Δ::HIS3	
ROY4028	MAT**a** HMR::URA3 ppr1Δ::kanMX SUM1 sir2Δ:: TRP1 ura3Δ::HIS3	
ROY4026	MATα HMR::URA3 ppr1Δ::kanMX SUM1-1 sir2Δ:: TRP1 ura3Δ::HIS3	
JRY4013	MATα HMR**a** SUM1 SIR2	J. Rine
JRY4563	MATα HMR**a** SUM1 sir2Δ:: TRP1	J. Rine
ROY1924	MATα HMR**a** SUM1-1 sir2Δ:: TRP1	
ROY3786	MAT**a** HMLα hmrΔ::URA3 SUM1-1 sir2Δ::HIS3	
ROY3753	MATα HML**a** hmrΔ::URA3 SUM1-1 sir2Δ::HIS3	
ROY3754	MATα HML**a** hmrΔ::URA3 SUM1 SIR2	
ROY3755	MATα HML**a** hmrΔ::URA3 SUM1 sir2Δ::HIS3	
ROY3785	MATα hmlΔ::TRP1 HMR**a** SUM1-1 sir2Δ::HIS3	
ROY3725	MAT**a** hmlΔ::TRP1 HMRα SUM1 sir2Δ::HIS3	
ROY3726	MAT**a** hmlΔ::TRP1 HMRα SUM1 SIR2	
ROY3743	MAT**a** hmlΔ::TRP1 HMRα SUM1-1 sir2Δ::HIS3	
ROY3861	MAT**a** hmlΔ::TRP1 hmrΔ::HMLα SUM1 SIR2	
ROY3859	MAT**a** hmlΔ::TRP1 hmrΔ::HMLα SUM1 sir2Δ::HIS3	
ROY3863	MAT**a** hmlΔ::TRP1 hmrΔ::HMLα SUM1-1 sir2Δ::HIS3	
ROY4030	MAT**a** hmlΔ::TRP1 hmrΔ::[HMR-E-α-HML-I] SUM1 sir2Δ::HIS3	
ROY4031	MAT**a** hmlΔ::TRP1 hmrΔ::[HMR-E-α-HML-I] SUM1 SIR2	
ROY4032	MAT**a** hmlΔ::TRP1 hmrΔ::[HMR-E-α-HML-I] SUM1-1 sir2Δ::HIS3	
ROY4053	MAT**a** hmlΔ::TRP1 hmrΔ::[HML-E-α-HMR-I] SIR2 SUM1	
ROY4045	MAT**a** hmlΔ::TRP1 hmrΔ::[HML-E-α-HMR-I] sir2Δ::HIS3 SUM1	
ROY4048	MAT**a** hmlΔ::TRP1 hmrΔ::[HML-E-α-HMR-I] sir2Δ::HIS3 SUM1-1	
ROY2863	MATα sum1Δ::kanMX sir2Δ::TRP1 HMR-[E+ a2::prma1-a1cds+ a1Δp+ I]	
ROY3306	MATα sum1Δ::kanMX sir2Δ::TRP1 HMR-[E+ a2::prma1-URA3cds+ a1Δp+ I]	
ROY3723	MATα sum1Δ::kanMX sir2Δ::TRP1 HMR-[E+ a2::prmURA3-a1cds+ a1Δp+ I] ppr1Δ::kanMX	
ROY3769	MATα sum1Δ::kanMX sir2Δ::TRP1 HMR-[E+ a2::prmURA3-URA3cds+ a1Δp+ I] ppr1Δ::kanMX	
ROY3322	MATα sir2Δ::TRP1 SUM1-1 HMR**a**1Δp::a1	
ROY3324	MATα sir2Δ::TRP1 SUM1-1 HMR**a**1Δp - a1- tRNA	
ROY3259	MATα sir2Δ::TRP1 SUM1-1 HMR**a**1Δp tRNA barrier - a1	
ROY4029	MATα sir2Δ::TRP1 SUM1-1 HMR**a**1Δp tRNA barrierΔ - a1	
ROY4043	MATα HMR-prma1+ URA3cds-hmraΔp SIR2 SUM1	
ROY4042	MATα HMR-prma1+ URA3cds-hmraΔp sir2Δ::TRP1 SUM1	
ROY3364	MATα HMR-prma1+ URA3cds-hmraΔp sir2Δ::TRP1 SUM1-1	
ROY2666	MATα HMR sir2Δ::HIS3 sum1Δ::kanMX	
ROY4038	MATα HMRss(1xGAL4-RAP1-ABF1)+I sir2Δ::HIS3 sum1Δ::kanMX	
ROY4039	MATα HMRss(1xGAL4-RAP1-ABF1) Δ I sir2Δ::HIS3 sum1Δ::kanMX	
ROY4040	MATα HMRss(3xGAL4-RAP1-ABF1) Δ I sir2Δ::HIS3 sum1Δ::kanMX	
ROY4041	MATα HMRss(5xGAL4-RAP1-ABF1) Δ I sir2Δ::HIS3 sum1Δ::kanMX	

SUM1-1 sir2Δ strains with *TRP1*, *ADE2*, and *URA3* reporter genes placed at *HMR* were obtained as follows. *HMR::TRP1* strains ROY3790, ROY3792, and ROY3839 were obtained by crossing YLS195 (*HMR::TRP1*; Buck and Shore 1995) with strains containing *sir2Δ sum1Δ* and *sir2Δ SUM1-1* alleles. *HMR::ADE2* strain YLS409 (Sussel *et al.* 1993) was crossed with *sum1Δ sir2Δ* and *sir2Δ SUM1-1* strains to generate ROY3770, ROY3774, and ROY3818. In strain ROY2584 (*MAT***a** *HMR::URA3 sir2Δ*), the *URA3* gene with the promoter of *URA3* proximal to *HMR-E* replaced sequences in the *HMRa2* coding region [Saccharomyces Genome Database (SGD) coordinates 293212–293410]. The *HMR::URA3* strains ROY3787, ROY3797, and ROY2677 are derivatives of ROY2584 crossed with *ppr1Δ* and *ppr1Δ SUM1-1* strains.

Strains containing *HMR*α or *HML***a** were derived from XW652 [*ho MAT***a** *HML*α *HMR*α*-Bura3 ade1 ade3::GAL-HO (His-) leu2 trp::hisG ura3-52 LYS2*] from J. Haber (Wu and Haber 1996) or from K1107 (*MAT***a** *HML***a** *HO::lacZ46 can1-100 ade2-1 leu2-3,112 trp1-1 his3 ura3*) from K. Nasmyth (Cvrckova and Nasmyth 1993). These strains were initially backcrossed into W-303 (JRY5078 and JRY3024 from J. Rine) four and three times, respectively. The strains were finally crossed with a *sir2*Δ*::HIS3*, *sum1*Δ*::kanMX*, or *SUM1-1* strain to obtain the requisite genotype.

Strains ROY3859, ROY3861, and ROY3863 were constructed by integrating *HML*α (sequences 415 bp upstream of the ARS301 to 511 bp downstream of the ARS302 element) at the *HMR* locus. *HML*α was amplified using genomic DNA from ROY175 (W-303 strain) as template and integrated by homologous recombination at the *Spe*I–*Pst*I sites that flank *hmr*Δ*::URA3* (SGD coordinates 292388–295324) in strain ROY3573 (*MAT***a** *hml*Δ*::TRP1 hmr*Δ*::URA sir2*Δ *sum1*Δ). Following transformation, 5-FOA-resistant colonies were selected to obtain ROY3825 (*MAT***a** *hml*Δ*::TRP1 hmr*Δ*::HML*α *sir2*Δ *sum1*Δ). This strain was crossed with strains carrying *SIR2 SUM1* and *sir2*Δ *SUM1-1* alleles.

Silencer swap strains: The *hmr*Δ*::HML*α strain ROY3825 described above was used to replace *HML-E* (from 415 bp upstream of the ARS301 element to the stop codon of *HML*α*2*) with the *URA3* cassette. The *URA3* gene was then replaced by the *HMR-E* silencer (SGD coordinate 292388 upstream of *HMR* to the stop codon of *HMRa2*) to generate ROY4036 (*MAT***a** *hml*Δ*::TRP1 hmr*Δ*::HMR-E-*α*HML-I sir2*Δ *sum1*Δ). ROY3825 was also used to replace *HML-I* (the region of *HML*α from the stop codon of *HML*α*1* to 511 bp downstream of the ARS302 element) with *URA3*, which was then replaced with the *HMR-I* silencer (from the stop codon of *HMRa1* to SGD coordinate 295324) to give ROY4037 (*MAT***a** *hml*Δ*::TRP1 hmr*Δ*::HML-E-*α*HMR-I sir2*Δ *sum1*Δ). Replacement of the *URA3* cassette was done by selection on 5-FOA, and ROY4036 and ROY4037 were crossed with *SIR2 SUM1* and *sir2*Δ *SUM1-1* strains to obtain ROY4030, ROY4031, ROY4032, ROY4045, ROY4048, and ROY4053.

To obtain strains in which transcription of the *URA3* and *MAT***a***1* genes is driven by the *URA3* or *MAT***a***1* promoter at the *HMR* locus, initially *URA3* was integrated at the *HMRa2* coding region (SGD coordinates 293212–293410) with transcription of *URA3* going toward the *HMR-E* silencer (ROY2795 *MAT*α *HMR::URA3 sir2*Δ *sum1*Δ). In this strain the *MAT***a***1* gene at *HMR* was made nonfunctional. For this, the *HMR* locus (*hmra*Δ*p*) was PCR amplified from plasmid pDR126 (gift of S. Loo). In this plasmid the promoter and the first 17 bp of the coding sequence of *HMR***a***1* were deleted (*hmra*Δ*p*). This PCR fragment was used to transform ROY2795. Transformants were selected on the basis of the recovery of the mating capacity of ROY2795. After transformation, the *MAT*α cells were allowed to grow for 4 hr in liquid YPAD (30°, 250 rpm), mixed with wild-type *MAT***a** W-303 cells, and plated onto YPAD plates and incubated at 30° overnight. Cells were then scrapped off the YPAD plate, washed with YM, and replated on YMD plates selecting for diploids. The *HMR/hmra*Δ*p* diploids were analyzed by PCR and dissected to give the strain ROY2800 (*MAT*α *HMR-URA3-hmra*Δ*p sir2*Δ *sum1*Δ).

The entire *URA3* cassette in ROY2800 was replaced with the *MAT***a***1* gene (SGD coordinates 293512–294505) to obtain ROY2863. In the same strain (ROY2800), the *URA3* coding sequence was replaced with the *MAT***a***1* coding sequence to generate strain ROY3469 (*MAT*α *HMR-prmURA3+a1cds-hmra*Δ*p sir2*Δ *sum1*Δ). ROY3469 was then crossed with a *ppr1*Δ strain to obtain ROY3723. The *MAT***a***1* coding region in strain ROY2863 was replaced with the *URA3* coding region to generate ROY3306. Finally, ROY3769 was obtained by crossing ROY2800 with a *ppr1*Δ strain. Transcription of all the reporter genes in these strains runs toward the *HMR-E* silencer.

Integration of *MAT***a***1* along the *HMR locus* was achieved as follows. First, *URA3* was integrated between *HMR-I* and the tRNA (SGD coordinates 295070–295281), or beyond the tRNA (SGD coordinates 296382–296482), and then the promoter of the resident *HMR***a***1* gene was deleted as described for ROY2800 to generate ROY2798 (*HMR-E-hmra*Δ*p-HMR-I-URA3*-tRNA *sir2*Δ *sum1*Δ) and ROY3141 (*HMR-E-hmra*Δ*p-HMR-I*-tRNA-*URA3 sir2*Δ *sum1*Δ). The barrier element present to the right of *HMR* (111–1301 bp downstream of *ARS318*) was replaced in strain ROY2798 with sequences derived from pUC18 (1200 bp), and *URA3* was reintegrated beyond the pUC18 sequences as in ROY3141 to make strain ROY4034 (*HMR-E-hmra*Δ*p-HMR-I*-barrierΔ*::pUC18-URA3 sir2*Δ *sum1*Δ). The *MAT***a***1* gene (SGD coordinates 293512–294505) was PCR amplified and used to replace the *URA3* cassettes in ROY2798, ROY3141, and ROY4034. These strains along with ROY2863 were crossed with a *sir2*Δ *SUM1-1* strain to obtain the final *sir2*Δ *SUM1-1* genotype in strains ROY3322, ROY3324, ROY3259, and ROY4029.

Strains ROY4038, ROY4039, ROY4040, and ROY4041 with a synthetic silencer in place of *HMR-E* were obtained as follows. Strains bearing a synthetic silencer at the *HMR* locus (Jasper Rine) in which the *ARS* element was substituted with one *GAL4*-binding site and the *HMR-I* silencer was left intact (JRY4529 *1xGEB+HMR-I*)—or in which the *ARS* element was substituted with one (JRY4531 *1xGEB+hmrI*Δ; Ehrenhofer-Murray *et al.* 1999), three (JRY4804 *3xGEB+hmrI*Δ), or five (JRY4806 *5xGEB+hmrI*Δ; Fox *et al.* 1997) *GAL4*-binding sites and the *HMR-I* silencer was deleted—were transformed with a plasmid expressing *GBD-SIR1* (pCF117) for mating competence and crossed with a *hmr*Δ*::URA3 sir2*Δ *sum1*Δ strain, followed by selection of diploids and tetrad analysis.

RESULTS

Promoter specificity in Sum1-1p-mediated repression: *SUM1-1*-mediated silencing at *HMR* shares several aspects of *SIR*-mediated silencing: *SUM1-1* is recruited to the *HMR* silencers, is found at several sites within the *HMR* locus, and also generates a hypoacetylated domain that is transcriptionally repressed (Rusche and Rine 2001; Sutton *et al.* 2001). However, it is not clear whether *SUM1-1*-generated transcriptional repression fulfills other silencing criteria that include gene nonspecificity and repression that is stably inherited.

To address whether Sum1-1p could repress different promoters in the absence of Sir2p, we examined the repression of *ADE2* (Sussel *et al.* 1993), *URA3* (Donze *et al.* 1999), and *TRP1* (Buck and Shore 1995) at the *HMR* locus in *SUM1-1 sir2*Δ strains (Figure 1). Our results showed that in the absence of Sir2p, the *TRP1* gene at *HMR* (Figure 1A) was not repressed in *SUM1-1* cells, since the *sir2*Δ *SUM1-1 HMR::TRP1* strain grew as robustly as the *sir2*Δ *SUM1 HMR::TRP1* strain on medium lacking tryptophan. The smaller colony size of the *SUM1-1* strain was most likely caused by a growth defect associated with *SUM1-1* since this phenotype was also observed in rich medium.

The *URA3* and *ADE2* genes are commonly used as reporters for Sir-mediated silencing (Gottschling *et al.*

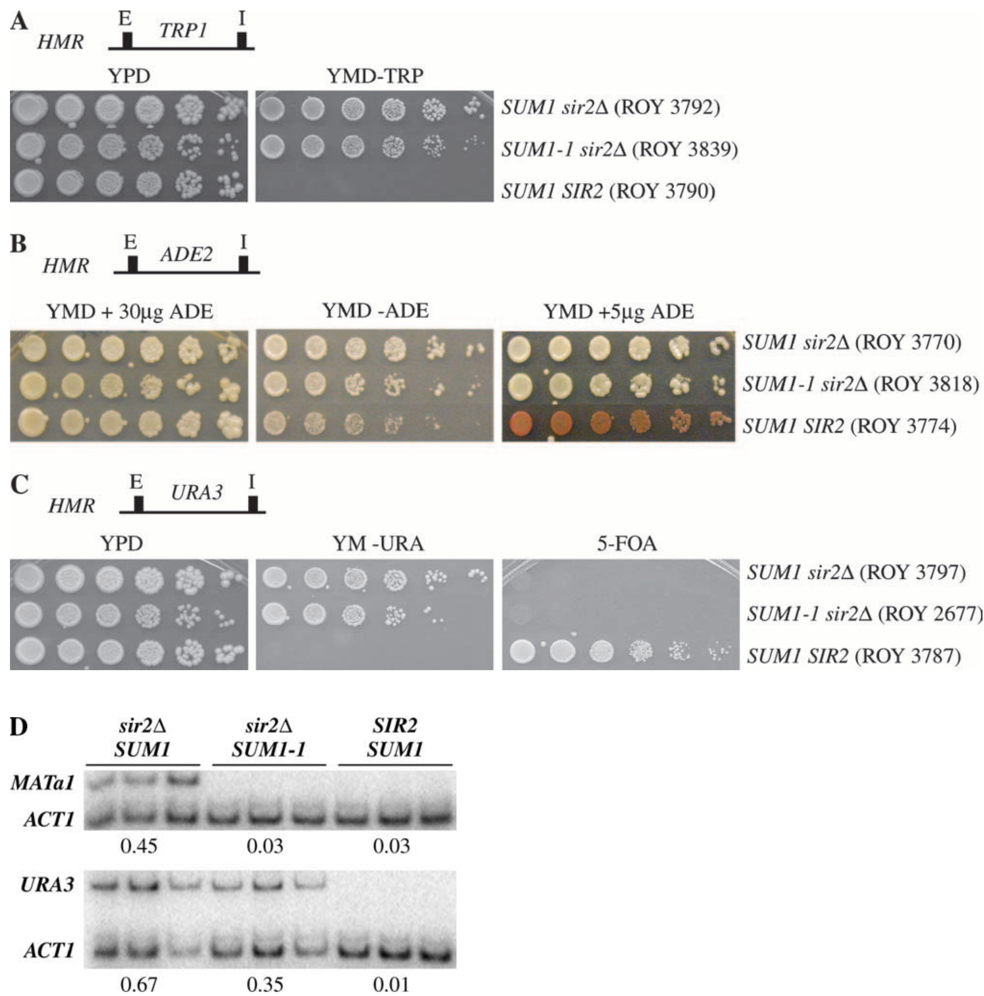

FIGURE 1.—Promoter-specific repression at *HMR* by *SUM1-1*. (A) The *TRP1* gene was integrated at the *HMR* locus, and expression of the gene was monitored by growth on YMD plates lacking tryptophan. (B) The *ADE2* gene was integrated at the *HMR* locus, and expression of the gene was monitored by growth on YMD plates lacking adenine or containing limiting amounts of adenine. (C) The *URA3* gene was integrated at the *HMR* locus, and expression of the gene was monitored by growth on YMD plates lacking uracil or containing 5-FOA. Approximately 3 μl of fivefold serial dilutions of overnight cultures was spotted on the different plates. Cells were allowed to grow at 30° for 2 days before the plates were photographed. The plates containing the *HMR::ADE2* strains were left at 4° for an additional day to allow for accumulation of the red pigment prior to photography. (D) mRNA levels of *MAT***a***1* and *URA3* present at *HMR*. *MAT***a***1* and *URA3* gene expression at *HMR* was quantitated by reverse transcribing total RNA from asynchronously growing cells followed by multiplex PCR. The levels of *ACT1* mRNA along with either *MAT***a***1* or *URA3* primers were quantitated. The average of the ratio of the intensities of the *MAT***a***1* or *URA3* signal to the *ACT1* signal was determined and is shown below.

1990). Silencing of *ADE2*, which is stable over several generations, gives rise to red colonies, while colonies where the gene is active, are white. Similarly, stably inherited repression of *URA3* over several generations allows cells to form colonies on medium containing 5-FOA and is a hallmark of the silenced state. Consistent with previously published data we find that the *TRP1*, *ADE2*, and *URA3* reporter genes were fully repressed by the Sir proteins.

Similar to the results obtained with the *TRP1* gene, the *ADE2* and *URA3* reporters were not susceptible to Sum1-1p-mediated repression when located at *HMR* (Figure 1, B and C). The *sir2*Δ *SUM1-1 HMR::ADE2* strain formed white colonies when grown under limiting amounts of adenine, suggesting that the *ADE2* gene was not repressed. Similarly, the *sir2*Δ *SUM1-1 HMR::URA3* strain did not grow on 5-FOA-containing plates, suggesting that this gene also was not repressed. Consistent with these observations, we find that *sir2*Δ *SUM1-1* cells were able to grow robustly on medium lacking uracil or adenine although there was a subtle difference between *sir2*Δ *SUM1* and *sir2*Δ *SUM1-1* strains. Whether this difference was due to partial repression of these promoters by *SUM1-1* or due to the slower growth of *SUM1-1* strains was difficult to determine by these assays.

To confirm the phenotypic analysis, we also measured transcript levels. We measured changes in transcription of two different reporter genes at *HMR-MAT***a***1* and *URA3* in wild-type, *sir2*Δ *SUM1*, and *sir2*Δ *SUM1-1* cells. The amount of specific transcript was determined by multiplex RT-PCR with primers specific for the *MAT***a***1* or *URA3* genes at *HMR* and the *ACT1* gene (Figure 1D). The level of the *URA3* and *MAT***a***1* transcript was normalized to *ACT1* to compensate for any differences in handling. Our data showed that the *MAT***a***1* gene at *HMR* was repressed in a *sir2*Δ *SUM1-1* strain nearly to the same extent as in the wild-type strain (*SIR2 SUM1*). The *URA3* gene at *HMR* was significantly active in a *sir2*Δ *SUM1-1* strain compared to the wild-type strain although we did observe a slight reduction in the expression levels in comparison to a *sir2*Δ *SUM1* strain. These data are consistent with our phenotypic results showing that *SUM1-1* repressed *MAT***a***1* more than *URA3* did.

Specificity in Sum1-1p-mediated repression at *HML* and *HMR*: Previous analysis had shown that Sum1-1p restored repression more efficiently at *HMR***a** than at *HML*α (Laurenson and Rine 1991; Chi and Shore 1996). It was possible that as in the case of *ADE2* and *URA3*, Sum1-1p was not able to repress *MAT*α genes at the silenced loci. We therefore reexamined the ability of Sum1-1p to repress *MAT***a** and *MAT*α genes at *HML* and *HMR* using a mating assay. A haploid yeast strain of a particular mating type will mate with cells of the opposite mating type to form diploids, which can then be selected for on appropriate selection plates. Derepression of the silent *HML* and *HMR* cassettes results in an inability of the haploids to mate and form diploid colonies.

Consistent with previous data (Laurenson and Rine 1991; Chi and Shore 1996), we found that the *MAT***a** genes at *HMR* were silenced by Sum1-1p in a *MAT*α *hml*Δ *HMR***a** *sir2*Δ *SUM1-1* strain (Figure 2A). This silencing was dependent on Sum1-1p since a *sir2*Δ strain expressing wild-type Sum1p was not able to silence *MAT***a***1* at *HMR* (data not shown). We also analyzed the ability of Sum1-1p to silence *MAT*α genes located at *HML* in a *MAT***a** *hmr*Δ *HML*α *sir2*Δ *SUM1-1* strain and, again consistent with previous data (Laurenson and Rine 1991), the *MAT*α genes at *HML* were not silenced by Sum1-1p (Figure 2B).

To determine whether the observed differences were due to the promoters or the silencers, we initially analyzed Sum1-1p-mediated repression of *MAT***a** genes located at the *HML* locus (Figure 2C). The *MAT***a** genes were efficiently repressed by Sum1-1p at *HML*. One possibility is that Sum1-1p was a promoter-specific repressor of the *MAT***a** genes or, alternatively, repression by Sum1-1p was effective only at weak promoters.

To distinguish between the two possibilities, we examined whether the *MAT*α genes, which were not repressed at *HML*, could be repressed when present at *HMR* (Figure 2D). Our analysis showed that *HMR*α was repressed in a *MAT***a** *hml*Δ *HMR*α *sir2*Δ *SUM1-1* strain. This repression was Sum1-1p dependent since a *MAT***a** *HMR*α *hml*Δ *sir2*Δ *SUM1* strain was a nonmater. These results would argue that *SUM1-1* could repress promoters of varying strengths and that the difference between *HML* and *HMR* may be due to differences in the silencers of the two loci or due to the chromosomal positions occupied by these two loci.

HML and *HMR* are located on opposite ends of chromosome III and it was possible that sequences adjacent to *HMR* cooperated with the silencers for Sum1-1p repression. We therefore replaced the *HMR***a** locus on the right arm of chromosome III with the *HML*α locus and monitored Sum1-1p-mediated repression at this locus (Figure 2E). The results showed that the *MAT*α genes were not repressed in the *MAT***a** *hmr*Δ::*HML*α *sir2*Δ *SUM1-1* strain. As a control, we monitored Sir-mediated silencing of this locus in a *SIR2 SUM1* background and found that the *MAT*α were fully repressed, indicating that the lack of repression by Sum1-1p was not an inherent property of having the *HML*α locus at *HMR*. The results indicate that the chromosomal location of *HML*α did not affect the extent of silencing by Sum1-1p and suggest that differences in the silencers may affect the outcome of Sum1-1p repression.

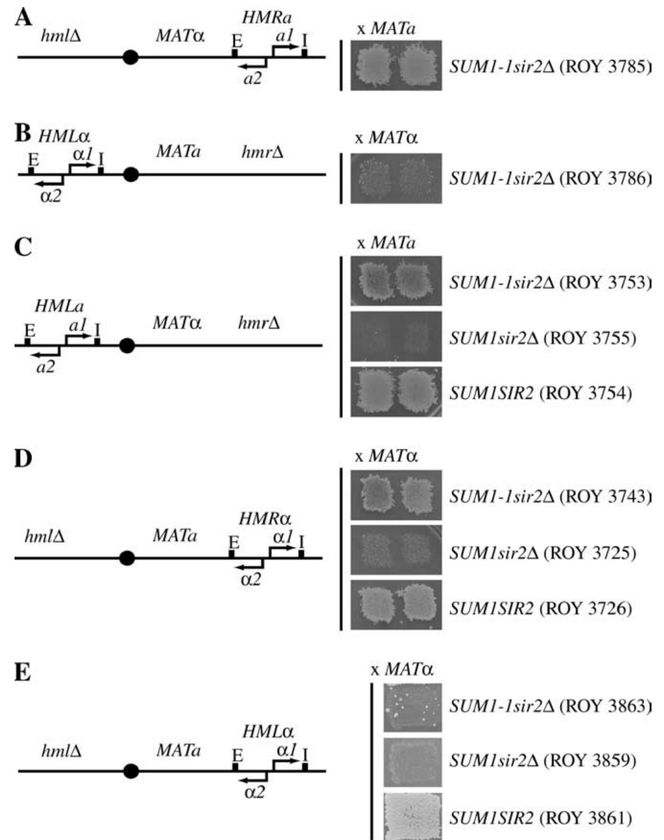

Figure 2.—*SUM1-1*-mediated repression of *MAT* genes at *HMR* and *HML*. (A–E) Strains containing the *MAT***a** genes at the *HMR* or *HML* loci (*HMR***a** and *HML***a**, respectively), the *MAT*α genes at the *HMR* or *HML* loci (*HML*α and *HMR*α, respectively), or *HML*α at *HMR* were generated and patched onto YPD plates. Mating assays with the appropriate mating-type tester strains were used to monitor expression of these genes and diploid colonies were allowed to grow on YMD plates for 2 days prior to documentation. The genotypes of the strains at *HML*, *MAT*, and *HMR* are shown schematically.

Silencer specificity in *SUM1-1*-mediated repression: To determine the role of the individual silencers in repressing *MAT*α genes, we generated four strains. All four *MAT***a** strains lacked *HML* and contained *MAT*α genes at *HMR*. In one strain, the *HMR-E* and *HMR-I* silencers flanked the *MAT*α genes, while in the second strain, the *MAT*α genes were flanked by *HML-E* and *HML-I*. In the third strain, these genes were flanked by *HMR-E* and *HML-I*, while in the fourth strain these genes were flanked by *HML-E* and *HMR-I*. We monitored repression of the *MAT*α genes in wild-type, *SUM1 sir2*Δ, and *SUM1-1 sir2*Δ backgrounds (Figure 3). Our data indicate that in a *sir2*Δ *SUM1-1* background the

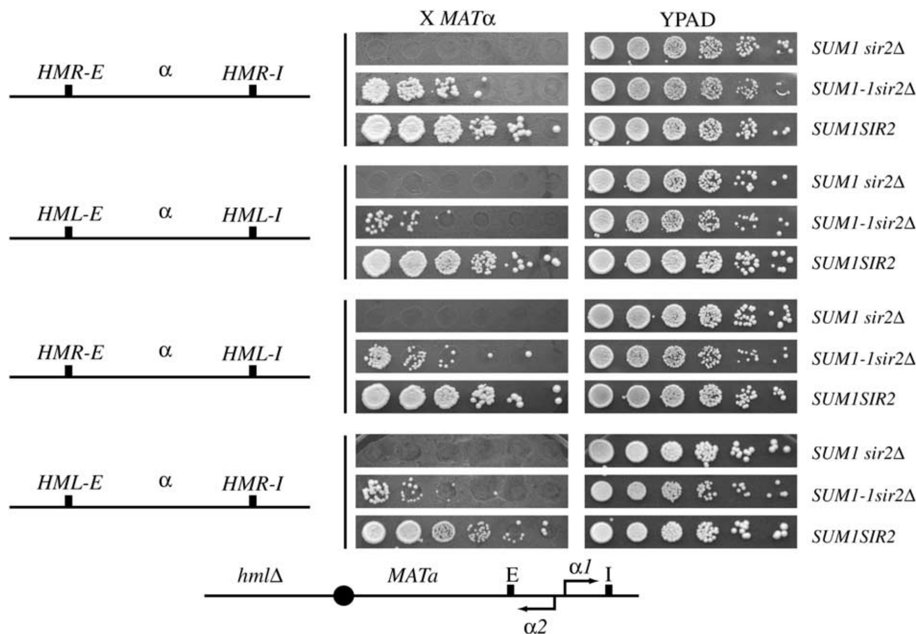

Figure 3.—Silencer specificity in Sum1-1p-mediated repression. *MAT***a** *hml*Δ strains with *MAT*α genes at *HMR* were constructed. In strains ROY3725 (*SUM1 sir2*Δ), ROY3743 (*SUM1-1 sir2*Δ) and ROY3726 (*SUM1 SIR2*), the *HMR-E* and *HMR-I* silencers flanked the *MAT*α genes. In strains ROY3859 (*SUM1 sir2*Δ), ROY3863 (*SUM1-1 sir2*Δ), and ROY3861 (*SUM1 SIR2*), the *HML-E* and *HML-I* silencers flanked the *MAT*α genes. In strains ROY4030 (*SUM1 sir2*Δ), ROY4032 (*SUM1-1 sir2*Δ), and ROY4031 (*SUM1 SIR2*), the *HMR-E* and *HML-I* silencers flanked the *MAT*α genes. In strains ROY4045 (*SUM1 sir2*Δ), ROY4048 (*SUM1-1 sir2*Δ), and ROY4053 (*SUM1 SIR2*), the *HML-E* and *HMR-I* silencers flanked the *MAT*α genes. Patch-mating assays using a *MAT*α tester lawn were performed to monitor silencing of the *MAT*α reporter gene.

*MAT*α genes were silenced to a greater extent in constructs with *HMR-E* than with *HML-E*. As controls we also monitored repression of the genes in *SIR2 SUM1* or *sir2*Δ *SUM1* strains. In the former case the genes were silenced in all four silencer combinations while in the latter case we did not observe silencing in any of the constructs.

***SUM1-1*-mediated repression was not stably inherited:** Thus far, we found that only the mating-type genes were efficiently repressed by Sum1-1p in the absence of Sir2p. This may be due to the inherent difference in the assays used to measure repression of the *MAT* genes *vs.* the *ADE2* and *URA3* reporters (van Leeuwen and Gottschling 2002). The mating assay used to measure repression of the *MAT* genes was akin to taking a snapshot since it measured repression only during the G_1 phase of the cell cycle, and not repression through the cell cycle or inheritance of repression through multiple cell cycles. On the other hand, repression of *ADE2*, *URA3*, and *TRP1* measured by colony formation on medium lacking these nutrients or containing 5-FOA was akin to a movie in that repression was measured throughout the cell cycle and over multiple generations. We do not believe that the differences in repression between the *MAT* genes and *URA3* were due to different turnover rates in their mRNAs since transcripts of these genes have a similar half-life of ~3 min (Herrick *et al.* 1990). This raised the possibility that Sum1-1p was capable of repressing *URA3* and the other reporters but this repression was transient and not sustained.

To address these issues, we initially generated two *HMR* alleles in *sir2*Δ *sum1*Δ strains: one allele contained the *MAT***a***1* coding region under control of the *URA3* promoter while the second allele was a control that contained the *MAT***a***1* coding region under control of its own promoter (Figure 4A). If the *URA3* promoter was sensitive to Sum1-1p-mediated repression, at least during the G_1 phase of the cell cycle, then these cells would be able to mate. This assay also allows us to monitor solely the two promoters without having to worry about differences in the stability of the RNA or protein. Strains were transformed with plasmids expressing Sum1-1p, Sum1p, or Sir2p and cells were grown in selective medium. Mating assays were performed in minimal medium (selecting for the plasmids) to monitor repression of the promoters and our results showed that both the *MAT***a***1* and *URA3* promoters were repressed by Sum1-1p, although the *MAT***a***1* promoter was repressed to a much greater extent than the *URA3* promoter. While the repression of the *URA3* promoter is slight, it is reproducible, and an integrated version of *SUM1-1* at its chromosomal location was able to better repress the *URA3* promoter (data not shown). These results are consistent with our earlier results that Sum1-1p was better at repressing the *MAT***a***1* promoter compared to the *URA3* promoter (see Figure 1D). Wild-type Sum1p did not repress these hybrid genes, while Sir2p completely restored silencing.

We next addressed the question of whether Sum1-1p-mediated repression was stably inherited over many generations. We generated two *HMR* alleles in *sir2*Δ *sum1*Δ strains: in one allele, the *URA3* coding region was placed under the control of the *URA3* promoter, and in the second allele, the *URA3* coding region was placed under the control of the *MAT***a***1* promoter (Figure 4B). Once again this allows us to monitor solely the two promoters without having to worry about differences in the stability of the RNA or protein. These strains were transformed with plasmids that expressed Sum1-1p,

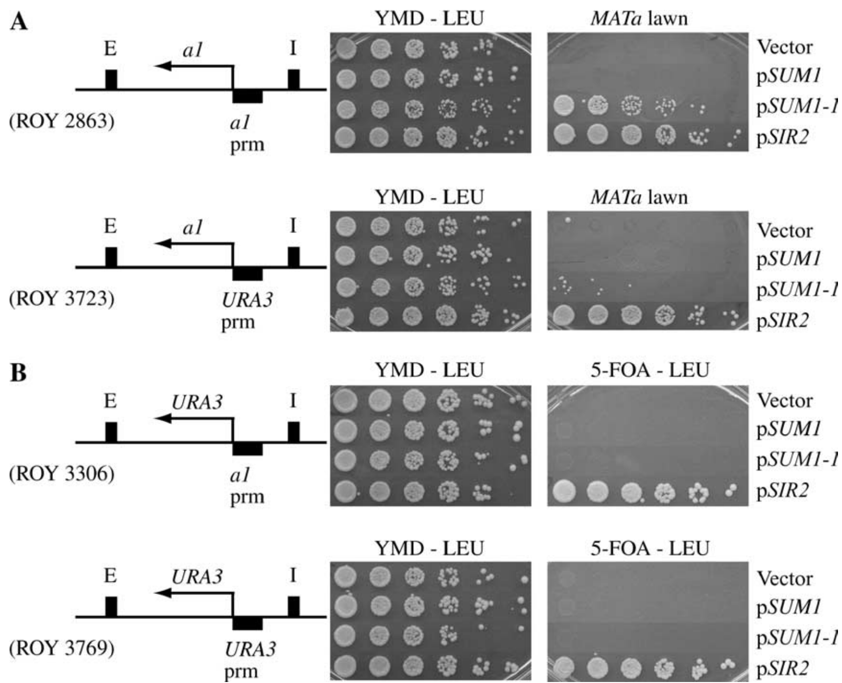

FIGURE 4.—*SUM1-1* repression was promoter specific and unstable. (A) *MAT*α *sir2*Δ *sum1*Δ strains in which transcription of the *MAT***a***1* coding region was driven by the *MAT***a***1* promoter (ROY2863) or by the *URA3* promoter (ROY3723) at the *HMR* locus were transformed with vector alone (pRS425), p*SUM1* (pRO709), p*SUM1-1* (pRO711), or p*SIR2* (pRO46). Transformants were grown selectively and fivefold serial dilutions were spotted onto YMD plates lacking leucine to monitor growth or onto YMD plates with a *MAT***a** tester lawn (*MAT***a** *his4*) to monitor for expression of the *MAT***a***1* gene using a mating assay. (B) *MAT*α *sir2*Δ *sum1*Δ strains with the *URA3* coding region under the control of the *MAT***a***1* promoter (ROY3306) or the *URA3* promoter (ROY3769) were transformed with vector alone (pRS425), p*SUM1* (pRO709), p*SUM1-1* (pRO711), or p*SIR2* (pRO46). The transformants were grown selectively and fivefold serial dilutions were spotted onto YMD plates without leucine as controls for growth or onto YMD plates containing 5-FOA (5-FOA–LEU) to monitor for stable repression of *URA3*.

Sum1p, or Sir2p and repression was monitored by growth on 5-FOA-containing plates. Neither strain could grow on 5-FOA media, indicating that the repression mediated by Sum1-1p was transient. Cells expressing wild-type Sum1p were also not able to repress the reporter genes, whereas Sir2p stably silenced both promoters. These results clearly demonstrated that Sum1-1p-mediated repression of *URA3* and even the *MAT***a***1* promoter was not stably inherited for many generations and may reflect a general characteristic of the repressive state mediated by Sum1-1p.

While Sum1-1p-mediated repression was not inherited for many generations, it was possible that Sum1-1p-mediated repression was stably inherited for a few generations but was not sufficient to form a visible colony on 5-FOA. To determine the number of generations in which the repressed state was inherited, we monitored the division of single cells over a period of 24 hr. We used three different strains: *SIR2 SUM1*, *sir2*Δ *SUM1*, and *sir2*Δ *SUM1-1*. The *HMR* locus in these strains was modified such that the *URA3* coding region was under the control of the *MAT***a***1* promoter. These strains were initially grown in YPD and >200 individual cells were manipulated for each strain and placed on plates lacking or containing 5-FOA. In the absence of FOA, the vast majority of cells divided and formed colonies (data not shown). Also as expected, the vast majority of wild-type *SIR2 SUM1* cells divided and formed colonies within 24 hr on plates containing 5-FOA (Figure 5). While most of the *sir2*Δ *SUM1* cells did not divide more than once and nearly 60% of the microcolonies had only two cells, 15–20% had three or four cells each. It should also be pointed out that the microcolonies scored with three and four cells could actually be two cells with large buds.

The *sir2*Δ *SUM1-1* cells also did not divide to form colonies on 5-FOA. The manipulated cells divided once or twice since there was an even distribution from one to four cells in the microcolonies. Since *SUM1-1*-mediated repression results in only 40% of cells in a population being repressed (Chi and Shore 1996), and since we were unable to determine which micromanipulated cells were repressed and which were derepressed at the start of this experiment, our data suggest that repression mediated by *SUM1-1* was at the most stably inherited for two generations and more likely no more than one generation.

SUM1-1*-mediated repression was not stably maintained:** Our data showed that *SUM1-1*-mediated repression was not stably inherited. We next asked if *SUM1-1*-mediated repression was stably maintained in the G_1 phase of the cell cycle using a modified shmoo-bud assay (Enomoto and Berman 1998). To perform this analysis, we used three *MATa** *hml*Δ *HMR*α strains. One of these was *SIR2 SUM1*, one was *sir2*Δ *SUM1*, and the third was *sir2*Δ *SUM1-1*. As shown in Figure 2, the *MAT*α genes at *HMR* were repressed by *SUM1-1*.

Cells were grown in YPAD and then transferred to YPAD liquid medium containing α-factor for 3 hr to arrest the cells in the G_1 phase of the cell cycle. The *sir2*Δ *SUM1* strains never arrested in α-factor. For the other two strains, single cells that had formed shmoo projections were then micromanipulated on regions of YPAD plates containing or lacking α-factor (Figure 6). Cells were monitored for the maintenance of their shmoo projections or the appearance of buds. When shmooed

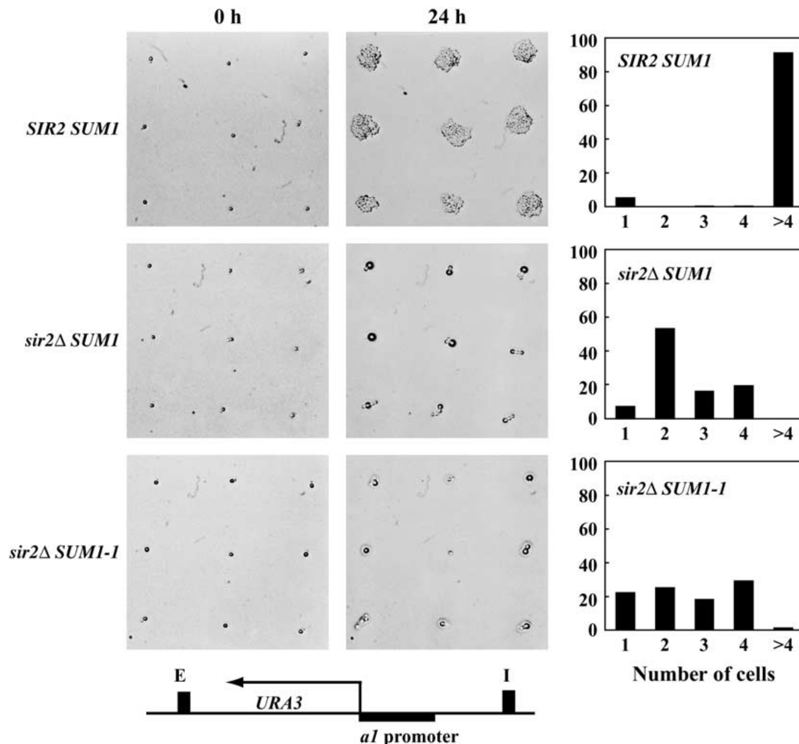

FIGURE 5.—Sum1-1p-mediated repression was not stably inherited. Three different *MAT*α strains with the *URA3* coding region under the control of the *MAT***a***1* promoter were generated. ROY4043 was *SIR2 SUM1* while ROY4042 was *sir2*Δ *SUM1* and ROY3364 was *sir2*Δ *SUM1-1*. The strains were grown in liquid YPD medium, and then placed onto YMD plates with 5-FOA. Single cells were micromanipulated on the plates and the plates were photographed to monitor growth and division of the cells. At 24 hr postmicromanipulation, the number of cells in each microcolony were counted. Approximately 200 microcolonies were counted for each strain and the data were plotted as a percentage of the total.

cells were placed on medium lacking α-factor, wild-type and *sir2*Δ *SUM1-1* strains were able to exit from the arrest, bud, and form microcolonies within a few hours.

On α-factor-containing plates, wild-type cells maintained their shmoo projections and remained arrested for close to 15 hr, often forming multiple shmoo projections. While a considerable number of *SUM1-1 sir2*Δ cells arrested in α-factor in liquid medium, when these arrested cells were manipulated onto plates containing α-factor and monitored over a period of time, a significant number of the arrested, shmooed cells escaped the arrest and began forming buds and microcolonies. Analyses of the data indicate that cells remained arrested for 6–9 hr before they escaped the arrest and began forming microcolonies, suggesting that Sum1-1p-mediated repression was not stably maintained (Figure 6 and Table 2). We do not believe that Sum1-1p-containing cells were exiting the arrest due to overexpression of Bar1p since it has been shown that *SUM1-1*-containing cells have significantly lower levels of *BAR1* RNA (Lynch *et al.* 2005).

The *SUM1-1*-repressed state spreads: One of the hallmarks of *SIR*-mediated silencing is that the Sir proteins spread between and beyond the *HMR-E* and *HMR-I* silencers up to the flanking barrier elements, and the genes present in this region are repressed independently of their orientation or location (Donze *et al.* 1999). While *SUM1-1* is present at different locations across the *HMR* domain (Rusche and Rine 2001), it is not known whether these regions are transcriptionally repressed.

To test whether genes located at any position or orientation within the *HMR* domain were repressed by *SUM1-1*, the native *MAT***a***1* gene was mutated and rendered nonfunctional, and *MAT***a***1* reporter genes were placed at three different locations along the *HMR* domain. *MAT***a***1* was placed either between the two silencers, to the right of the *HMR-I* silencer but between the silencer and the barrier element, or outside the barrier element as shown schematically in Figure 7. *MAT***a***1* was chosen as the reporter gene because it was most efficiently repressed by *SUM1-1*. As expected, the *MAT***a***1* gene located between the two silencers was repressed. It was also repressed when present to the right of the *HMR-I* silencer between the silencer and the barrier element, but not when it was located beyond the barrier element. Furthermore, overexpression of Sum1-1p alone or with Hst1p did not extend the repressed domain beyond the barrier element (data not shown). These results suggest that Sum1-1p-mediated repression encompassed the entire *HMR* domain and spread beyond the silencer and up to the barrier element.

The repressive domain generated by the Sir proteins blocks transcription of genes independently of their orientation. Similarly, the repressive domain generated by Sum1-1p also repressed genes independently of their orientation since transcription of the *MAT***a***1* gene present between the two silencers was repressed in either orientation (data not shown).

The spread of Sir-protein-mediated silencing is blocked by barrier elements (Donze and Kamakaka

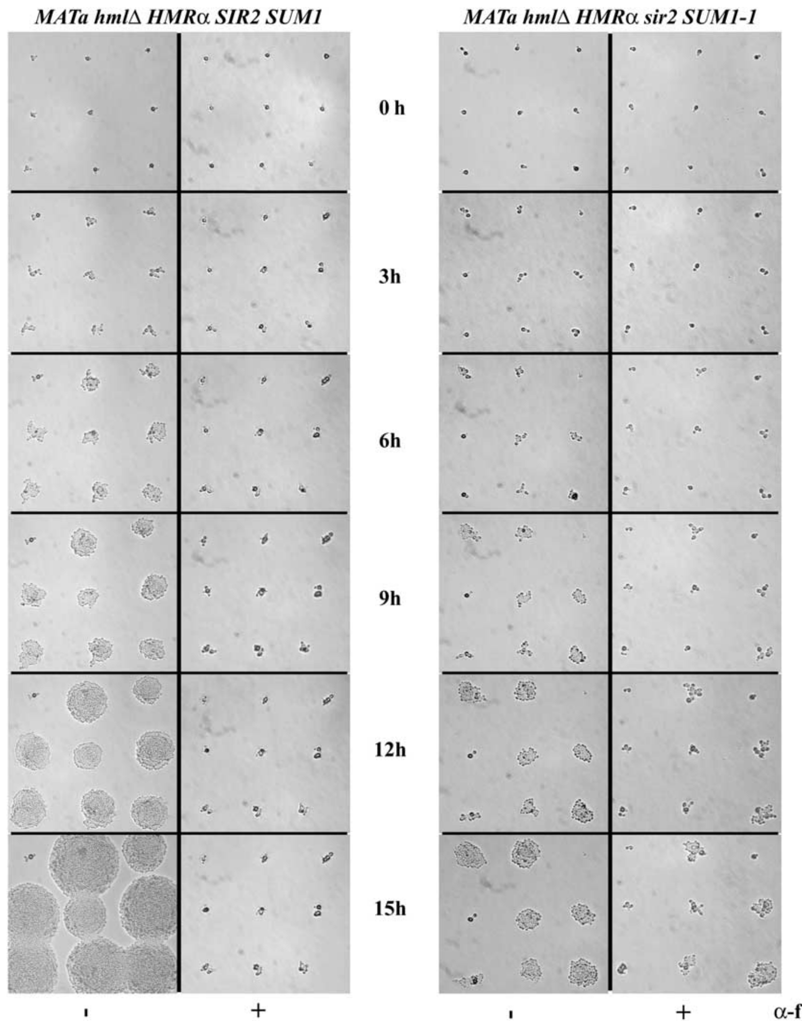

FIGURE 6.—Sum1-1p-mediated repression was not stably maintained. *MAT***a** *hml*Δ *HMR*α strains with *SIR2 SUM1* or *sir2*Δ *SUM1-1* alleles were initially grown in liquid YPAD medium. The cells were arrested with α-factor for 3 hr in liquid medium and then streaked onto YPAD plates. Single cells were micromanipulated on the regions of the plate containing or lacking α-factor and the micromanipulated cells were photographed every 3 hr for growth and cell division.

2001; OKI *et al.* 2004). We therefore inquired if the spread of Sum1-1p-mediated repression was also blocked by the barrier element. We replaced the entire *HMR* right barrier with pUC DNA and monitored expression of the *MAT***a***1* gene that was present outside the barrier. While Sum1-1p was unable to repress *MAT***a***1* when the barrier was intact, in the absence of the barrier, Sum1-1p was able to spread and partially repress the reporter gene. The analysis of the semiquantitative spot-mating assay suggests that in ~5–10% of the cells, the *MAT***a***1* gene located beyond the barrier became repressed when the barrier was deleted. While we were able to map Sum1-1p by chromatin immunoprecipitation at the *HMR* silencers, we were not able to see an increase in the spread of this protein when the barrier was deleted (data not shown). We believe that this is due to the fact that repression by Sum1-1p, beyond the barrier, was present in only a small percentage of cells (Figure 7).

Tethered Sum1-1p repressed *HMR*ΔI: Sum1-1p is recruited to *HMR* via interactions with ORC (RUSCHE and RINE 2001; SUTTON *et al.* 2001; LYNCH *et al.* 2005). This model implies that if Sum1-1p were recruited to the silencer, it would be able to silence in the absence of the ORC-binding site. To test this model, we generated *sir2*Δ *sum1*Δ strains with a synthetic *HMR-E* silencer containing Gal4-binding sites in place of the ORC-binding site (CHIEN *et al.* 1993; FOX *et al.* 1997). We also generated fusion proteins with the Gal4 DNA-binding domain fused in frame to *SUM1-1*. The Gbd-Sum1-1p chimera was active since it was able to repress wild-type *HMR* in the absence of Sir2p almost to the same extent as

TABLE 2

Single cell analyses for repression of *HMR*α

	*MAT***a** *hml*Δ *HMR*α			
	SIR2 SUM1		*sir2*Δ *SUM1-1*	
α-Factor	+	−	+	−
Budding	0	88	55	78
Shmoo	100	12	45	22

At 1 hr after micromanipulation, the cells in each microcolony were monitored for the presence of shmooed undivided single cells *vs.* cells that had divided multiple times to form microcolonies with greater than two cells per colony. The strains monitored were *MAT***a** *hml*Δ *HMR*α with *SIR2 SUM1* or *sir2*Δ *SUM1-1* alleles. Numbers are percentages.

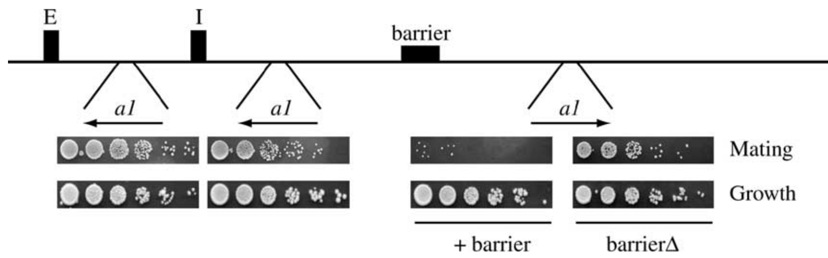

FIGURE 7.—*SUM1-1*-repressed genes within a large domain. *MAT*α *SUM1-1 sir2*Δ strains (ROY 3322, ROY 3324, ROY 3259, and ROY4029) containing the *MAT***a***1* reporter gene integrated at different locations were grown in YPD liquid medium and 3 μl of five-fold serial dilutions were spotted onto mating lawns to assay for the expression of the *MAT***a***1* genes. A schematic shows the sites of insertion and orientation of the *MAT***a***1* reporter gene integrated within the *HMR* locus. "BarrierΔ" refers to a replacement of the *HMR* barrier sequences with pUC DNA.

untagged Sum1-1p (Figure 8A). We then determined whether the fusion protein could silence *MAT***a***1* at the synthetic silencer containing a single Gal4p-binding site in place of the ORC-binding site at the *HMR-E* silencer. As Figure 8A shows, direct recruitment of Gbd-Sum1-1p was able to repress the reporter gene in the absence of the ORC-binding sites at *HMR-E*, while Sum1-1p was unable to silence this allele of *HMR*, presumably because it was not recruited to this locus.

The *HMR-I* silencer is necessary for Sum1-1p-mediated repression (SUTTON *et al.* 2001) although its role in repression is not clear. We tested the requirement for

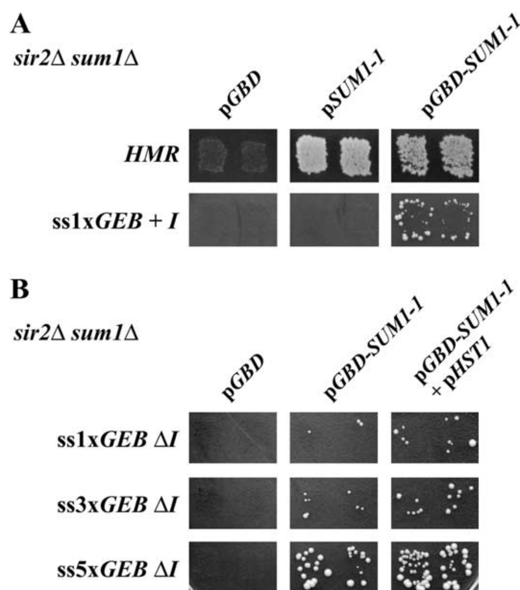

FIGURE 8.—*SUM1-1*-repressed genes in the absence of the *HMR-I* silencer. (A) *MAT*α *sir2*Δ *sum1*Δ strains bearing a wild-type or synthetic *HMR-E* silencer containing one Gal4p-binding site (GEB) were generated. Strains were transformed with vector or plasmids expressing *SUM1-1* (pRO711) or *GBD-SUM1-1* (pRO707) protein chimeras. Patch-mating assays using a *MAT***a** tester lawn were performed to monitor silencing of the *MAT***a***1* reporter gene. (B) *MAT*α *sir2*Δ *sum1*Δ strains bearing a synthetic *HMR-E* silencer without *HMR-I* were generated. One, three, or five *GAL4*-binding sites replaced the ARS element at *HMR-E* (ROY4039 *HMRss1xGEB*Δ*I*, ROY4040 *HMRss3xGEB*Δ*I*, and ROY4041 *HMRss5xGEB*Δ*I*). Strains were transformed with vector or plasmids expressing *GBD-SUM1-1* (pRO707) and *HST1* (pRO713) and patch-mating assays using a *MAT***a** tester lawn were performed to monitor silencing of the *MAT***a***1* reporter gene.

HMR-I by using strains with a synthetic silencer that contained one, three, or five binding sites for Gal4p in place of the ORC-binding sites at the *HMR-E* silencer but lacked the *HMR-I* silencer (Figure 8B). Under these conditions, Gbd-Sum1-1p was able to repress the *MAT***a***1* gene but only when there were multiple binding sites for Gal4p. These results demonstrated that the requirement for the *HMR-I* silencer could be bypassed by increased or more efficient recruitment of Sum1-1p to the silenced domain.

Sum1-1p-generated changes in the nucleosomal organization at *HMR*: The presence of the Sir proteins at *HMR* generates a characteristic organization of the nucleosomes (RAVINDRA *et al.* 1999), and it has been suggested that this special organization might be involved in the mechanism of Sir-mediated silencing. We asked if this characteristic pattern of nucleosome organization is recreated in the presence of Sum1-1p. We mapped the locations of the nucleosomes at the *HMR* locus by micrococcal nuclease digestion and indirect end labeling and the analysis of the pattern in a *sir2*Δ *SUM1-1* revealed characteristics of both wild-type and *sir2*Δ *SUM1* strains (Figure 9). Since *SUM1-1*-mediated repression occurs in only a fraction of cells in the population, one possibility is that the nucleosomal pattern observed was a composite of the patterns present in wild-type and *sir2*Δ *SUM1* cells. We are currently unable to sort silenced cells from nonsilenced cells to distinguish between this and other possible scenarios.

DISCUSSION

Transcriptional repression is either gene specific or general (reviewed in GRAY and LEVINE 1996; COUREY and JIA 2001): gene-specific repressors are usually short-range local repressors that affect transcription by blocking the function of specific activators in the immediate vicinity of the operator without affecting distal activators. General repression is usually long range, where the repressor encompasses an entire chromatin domain and is often referred to as silencing. Silencing is not gene specific and most genes packaged in the silenced chromatin are rendered inactive. Furthermore, and most importantly, the repressed state is stably inherited over many generations.

Figure 9.—Indirect end-labeling analyses of the *HMR* domain. Permeabilized spheroplasts from wild type (JRY4013), *sir2*Δ *SUM1* (JRY4563), and *sir2*Δ *SUM1-1* (ROY1924) strains were digested with micrococcal nuclease. After deproteinization and restriction enzyme digestion, the DNA was resolved on an agarose gel, blotted, and hybridized with a probe specific to *HMR*.

A key difference between local and long-range repressors is that the latter encompass large domains of chromatin into a repressed state that can repress most genes placed anywhere within this domain. Sir-mediated silencing at the *HMR* and *HML* loci are classic examples of the latter and most genes placed in these silenced domains are repressed (reviewed in Moazed 2001; Rusche *et al.* 2003). Sum1p is an example of a sequence-specific local repressor (Xie *et al.* 1999) while *SUM1-1* is a neomorphic allele of *SUM1* that transforms the wild-type protein from a promoter-specific repressor to a long-range repressor capable of repressing genes at *HMR* (Rusche and Rine 2001; Sutton *et al.* 2001). We have investigated Sum1-1p-mediated repression at *HMR* and our data suggest that this repressor is not as pleiotropic in repressing different promoters as the Sir proteins. We did not observe robust repression of the *TRP1*, *URA3*, or *ADE2* reporter genes (Figure 1), but as previously reported we did find that the *MAT***a** genes were repressed at *HMR* and, interestingly, even at *HML* (Figure 2).

It is interesting that while the *MAT*α genes were not repressed at *HML*, these genes were repressed by Sum1-1p when placed at the *HMR* locus. We showed that this specificity of repression was due to the presence of specific silencers, with the *HMR-E* silencer being more efficient for repression compared to the *HML* silencers. These data are consistent with observations that the *HMR* silencers are more proficient than the *HML* silencers in Sir-mediated repression (Shei and Broach 1995). However, a recent report has shown that wild-type Sum1p was bound to the D-element of the *HML-E* silencer and was necessary for Sir-mediated silencing at *HML* (Irlbacher *et al.* 2005). It is therefore paradoxical that *HMR-E*, rather than *HML-E*, is a better silencer for Sum1-1p-mediated repression. Since Sum1-1p is recruited via interactions with ORC (Rusche and Rine 2001; Sutton *et al.* 2001; Lynch *et al.* 2005), one possibility is that the *HMR-E* silencer is better at recruiting the mutant protein, given the large number of ORC-binding sites present around this silencer (Palacios DeBeer and Fox 1999). An alternative possibility is that in the absence of wild-type Sum1p, the *HML-E* silencer is weakened and is therefore unable to efficiently recruit Sum1-1p.

In addition to silencer specificity, promoter specificity may also be involved in influencing Sum1-1p-mediated repression since it is believed that *MAT*α genes are more active than *MAT***a** genes, which have no known transcriptional activators (Siliciano and Tatchell 1984, 1986). Our data also suggest that the *MAT*α promoter might be stronger than the *MAT***a** promoter since the former could overcome *SUM1-1* repression at *HML*. Thus, the observed differences in Sum1-1p-mediated repression of different genes may be a composite of promoter specificity or strength and silencer specificity or strength. The ability of Sum1-1p to silence some promoters and not others and the role of promoter strength is reminiscent of silencing at telomeres where Sir-mediated telomeric silencing is also promoter specific and depends on promoter strength (reviewed in van Leeuwen and Gottschling 2002).

Repression mediated by Sum1-1p is partial and only a fraction of the cells in a population are repressed at any one time (Laurenson and Rine 1991). In this aspect, too, Sum1-1p repression is more akin to telomeric silencing where repression is observed only in a fraction of the cells in a population (Gottschling *et al.* 1990). However, the partial repression mediated by Sum1-1p is different from the partial repression mediated by the Sir proteins at telomeres. Silencing at telomeres is epigenetically stable in that, in the fraction where the promoter is silenced, that state of the promoter is propagated for many generations while the cells where the promoter is active remain active for many generations (Gottschling *et al.* 1990; Iida and Araki 2004). Like telomeric silencing, *SUM1-1*-mediated repression is partial but our data with the *URA3* and *MAT***a***1* promoters (using the same transcript reporters) showed that neither promoter was stably repressed and that the repressed state was not stably inherited over many generations. We showed that Sum1-1p-directed repression either could be disrupted in the same mitotic cycle

(*i.e.*, a maintenance defect) as the silencing defects observed in *cac1*Δ mutants (Enomoto and Berman 1998) or could be lost following cell division. Thus the partial repression observed in *SUM1-1* cells was different from the partial repression observed for Sir-mediated silencing (Pillus and Rine 1989; Sussel *et al.* 1993; Gottschling *et al.* 1990; Mahoney *et al.* 1991).

One key difference between local gene-specific repressors and long-range repressors is that the latter usually spread along the chromatin to encompass a large chromatin domain into a repressed state. Silencing mediated by the Sir proteins initiates at specific elements (silencers) and then spreads along the chromatin (Luo *et al.* 2002; Rusche *et al.* 2002). This encroaching silenced chromatin is actively restricted from spreading by barrier elements (Donze *et al.* 1999). Localization studies with Sum1-1p have shown that, as with Sir proteins, this protein is also present across the entire *HMR* domain and it is believed that Sum1-1p recruited to the silencers by ORC spreads along the *HMR* locus with the aid of Hst1p (Rusche and Rine 2001; Sutton *et al.* 2001). While Sum1-1p is present across the entire *HMR* domain, the functional consequences of the physical presence across the domain were not known. We have now shown that while *URA3* was not robustly and stably repressed when placed at different sites within this domain (Figure 1 and data not shown), the *MAT***a***1* gene was repressed in an orientation- and location-independent manner across the entire domain (Figure 4), suggesting that, as with the Sir proteins, Sum1-1p could also spread and form a repressive chromatin domain. These results are consistent with recent data showing that Sum1-1p-mediated recruitment at ORC-binding sites and the resultant spreading from these sites does lead to repression of some neighboring genes (Lynch *et al.* 2005).

Our data also showed that the repressed domain was not constrained between the silencers but extended beyond them and that genes located in this flanking region were also repressed by Sum1-1p. Furthermore, our result also showed that genes repressed by Sum1-1p did not necessarily have to be flanked by silencers. Previous data have suggested a requirement for both *HMR-E* and *HMR-I* silencers to be present, flanking a gene for efficient repression by this protein (Sutton *et al.* 2001). We have now shown that the requirement for *HMR-I* can be partially bypassed by increasing the recruitment of Sum1-1p to the synthetic *HMR-E* silencer. While the exact function of *HMR-I* in Sum1-1p-mediated repression is not clear, it is likely that the role of *HMR-I* is to recruit Sum1-1p with the aid of ORC present at this silencer. However, we cannot exclude the possibility that *HMR-I* functions to stabilize the Sum1-1p complex recruited at *HMR-E* in a manner ascribed to proto-silencers (Boscheron *et al.* 1996).

Also, similar to Sir-mediated silencing (Oki *et al.* 2004), the spread of the *SUM1-1* repressed state could be disrupted by barrier elements, suggesting commonalities in the mechanisms that underlie the spread of these different repressor proteins and that spreading is important for this repression. Current models for Sum1-1p-mediated repression at *HMR* suggest that the interaction between ORC and Sum1-1p enables its own recruitment as well as that of Hst1p (through its interaction with Sum1-1p), leading to deacetylation of the histones and the propagation of the repressed state along the chromatin fiber (Lynch *et al.* 2005).

In conclusion, we have shown that Sum1-1p is a long-range repressor since it encompasses an entire chromatin domain to repress genes independently of their position or orientation within the domain. However, unlike Sir-mediated silencing, which is promoter nonspecific and stably inherited, repression mediated by Sum1-1p is more robust for certain promoters and is not stably inherited over many generations.

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LITERATURE CITED

Avendano, A., L. Riego, A. DeLuna, C. Aranda, G. Romero *et al.*, 2005 Swi/SNF-*GCN5*-dependent chromatin remodelling determines induced expression of *GDH3*, one of the paralogous genes responsible for ammonium assimilation and glutamate biosynthesis in *Saccharomyces cerevisiae*. Mol. Microbiol. **57:** 291–305.

Bedalov, A., M. Hirao, J. Posakony, M. Nelson and J. A. Simon, 2003 NAD+-dependent deacetylase Hst1p controls biosynthesis and cellular NAD+ levels in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **23:** 7044–7054.

Boscheron, C., L. Maillet, S. Marcand, M. Tsai-Pflugfelder, S. M. Gasser *et al.*, 1996 Cooperation at a distance between silencers and proto-silencers at the yeast *HML* locus. EMBO J. **15:** 2184–2195.

Buck, S. W., and D. Shore, 1995 Action of a *RAP1* carboxy-terminal silencing domain reveals an underlying competition between *HMR* and telomeres in yeast. Genes Dev. **9:** 370–384.

Chi, M. H., and D. Shore, 1996 *SUM1–1*, a dominant suppressor of *SIR* mutations in *Saccharomyces cerevisiae*, increases transcriptional silencing at telomeres and *HM* mating-type loci and decreases chromosome stability. Mol. Cell. Biol. **16:** 4281–4294.

Chien, C. T., S. Buck, R. Sternglanz and D. Shore, 1993 Targeting of *SIR1* protein establishes transcriptional silencing at *HM* loci and telomeres in yeast. Cell **75:** 531–541.

Courey, A. J., and S. Jia, 2001 Transcriptional repression: the long and the short of it. Genes Dev. **15:** 2786–2796.

Cvrckova, F., and K. Nasmyth, 1993 Yeast G1 cyclins *CLN1* and *CLN2* and a GAP-like protein have a role in bud formation. EMBO J. **12:** 5277–5286.

Donze, D., and R. T. Kamakaka, 2001 RNA polymerase III and RNA polymerase II promoter complexes are heterochromatin barriers in *Saccharomyces cerevisiae*. EMBO J. **20:** 520–531.

Donze, D., C. R. Adams, J. Rine and R. T. Kamakaka, 1999 The boundaries of the silenced *HMR* domain in *Saccharomyces cerevisiae*. Genes Dev. **13:** 698–708.

Ehrenhofer-Murray, A. E., R. T. Kamakaka and J. Rine, 1999 A role for the replication proteins PCNA, RF-C, polymerase epsilon and Cdc45 in transcriptional silencing in *Saccharomyces cerevisiae*. Genetics **153:** 1171–1182.

Enomoto, S., and J. Berman, 1998 Chromatin assembly factor I contributes to the maintenance, but not the re-establishment, of silencing at the yeast silent mating loci. Genes Dev. **12:** 219–232.

Fox, C. A., A. E. Ehrenhofer-Murray, S. Loo and J. Rine, 1997 The origin recognition complex, *SIR1*, and the S phase requirement for silencing. Science **276:** 1547–1551.

Gottschling, D. E., O. M. Aparicio, B. L. Billington and V. A. Zakian, 1990 Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. Cell **63:** 751–762.

Gray, S., and M. Levine, 1996 Transcriptional repression in development. Curr. Opin. Cell Biol. **8:** 358–364.

Herrick, D., R. Parker and A. Jacobson, 1990 Identification and comparison of stable and unstable mRNAs in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:** 2269–2284.

Iida, T., and H. Araki, 2004 Noncompetitive counteractions of DNA polymerase epsilon and ISW2/yCHRAC for epigenetic inheritance of telomere position effect in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **24:** 217–227.

Irlbacher, H., J. Franke, T. Manke, M. Vingron and A. E. Ehrenhofer-Murray, 2005 Control of replication initiation and heterochromatin formation in *Saccharomyces cerevisiae* by a regulator of meiotic gene expression. Genes Dev. **19:** 1811–1822.

Ito, H., Y. Fukuda, K. Murata and A. Kimura, 1983 Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:** 163–168.

Ito, T., T. Chiba, R. Ozawa, M. Yoshida, M. Hattori *et al.*, 2001 A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc. Natl. Acad. Sci. USA **98:** 4569–4574.

Klar, A. J., S. N. Kakar, J. M. Ivy, J. B. Hicks, G. P. Livi *et al.*, 1985 *SUM1*, an apparent positive regulator of the cryptic mating-type loci in *Saccharomyces cerevisiae*. Genetics **111:** 745–758.

Laurenson, P., and J. Rine, 1991 *SUM1-1*: a suppressor of silencing defects in *Saccharomyces cerevisiae*. Genetics **129:** 685–696.

Laurenson, P., and J. Rine, 1992 Silencers, silencing, and heritable transcriptional states. Microbiol. Rev. **56:** 543–560.

Livi, G. P., J. B. Hicks and A. J. Klar, 1990 The *sum1–1* mutation affects silent mating-type gene transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:** 409–412.

Loo, S., and J. Rine, 1995 Silencing and heritable domains of gene expression. Annu. Rev. Cell Dev. Biol. **11:** 519–548.

Luo, K., M. A. Vega-Palas and M. Grunstein, 2002 Rap1-Sir4 binding independent of other Sir, yKu, or histone interactions initiates the assembly of telomeric heterochromatin in yeast. Genes Dev. **16:** 1528–1539.

Lynch, P. J., H. B. Fraser, E. Sevastopoulos, J. Rine and L. N. Rusche, 2005 Sum1p, the origin recognition complex, and the spreading of a promoter-specific repressor in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **25:** 5920–5932.

Mahoney, D. J., R. Marquardt, G. J. Shei, A. B. Rose and J. R. Broach, 1991 Mutations in the *HML E* silencer of *Saccharomyces cerevisiae* yield metastable inheritance of transcriptional repression. Genes Dev. **5:** 605–615.

McCord, R., M. Pierce, J. Xie, S. Wonkatal, C. Mickel *et al.*, 2003 Rfm1, a novel tethering factor required to recruit the Hst1 histone deacetylase for repression of middle sporulation genes. Mol. Cell. Biol. **23:** 2009–2016.

Moazed, D., 2001 Common themes in mechanisms of gene silencing. Mol. Cell **8:** 489–498.

Oki, M., L. Valenzuela, T. Chiba, T. Ito and R. T. Kamakaka, 2004 Barrier proteins remodel and modify chromatin to restrict silenced domains. Mol. Cell. Biol. **24:** 1956–1967.

Palacios DeBeer, M. A., and C. A. Fox, 1999 A role for a replicator dominance mechanism in silencing. EMBO J. **18:** 3808–3819.

Pierce, M., K. R. Benjamin, S. P. Montano, M. M. Georgiadis, E. Winter *et al.*, 2003 Sum1 and Ndt80 proteins compete for binding to middle sporulation element sequences that control meiotic gene expression. Mol. Cell. Biol. **23:** 4814–4825.

Pillus, L., and J. Rine, 1989 Epigenetic inheritance of transcriptional states in *S. cerevisiae*. Cell **59:** 637–647.

Ravindra, A., K. Weiss and R. T. Simpson, 1999 High-resolution structural analysis of chromatin at specific loci: *Saccharomyces cerevisiae* silent mating-type locus *HMRa*. Mol. Cell. Biol. **19:** 7944–7950.

Rusche, L. N., and J. Rine, 2001 Conversion of a gene-specific repressor to a regional silencer. Genes Dev. **15:** 955–967.

Rusche, L. N., A. L. Kirchmaier and J. Rine, 2002 Ordered nucleation and spreading of silenced chromatin in *Saccharomyces cerevisiae*. Mol. Biol. Cell **13:** 2207–2222.

Rusche, L. N., A. L. Kirchmaier and J. Rine, 2003 The establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. Annu. Rev. Biochem. **72:** 481–516.

Schmitt, M. E., T. A. Brown and B. L. Trumpower, 1990 A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res. **18:** 3091–3092.

Shei, G. J., and J. R. Broach, 1995 Yeast silencers can act as orientation-dependent gene inactivation centers that respond to environmental signals. Mol. Cell. Biol. **15:** 3496–3506.

Sherman, F., 1991 Getting started with yeast. Methods Enzymol. **194:** 3–21.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Siliciano, P. G., and K. Tatchell, 1984 Transcription and regulatory signals at the mating type locus in yeast. Cell **37:** 969–978.

Siliciano, P. G., and K. Tatchell, 1986 Identification of the DNA sequences controlling the expression of the *MAT* alpha locus of yeast. Proc. Natl. Acad. Sci. USA **83:** 2320–2324.

Sussel, L., D. Vannier and D. Shore, 1993 Epigenetic switching of transcriptional states: cis- and trans-acting factors affecting establishment of silencing at the *HMR* locus in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:** 3919–3928.

Sutton, A., R. C. Heller, J. Landry, J. S. Choy, A. Sirko *et al.*, 2001 A novel form of transcriptional silencing by Sum1–1 requires Hst1 and the origin recognition complex. Mol. Cell. Biol. **21:** 3514–3522.

van Leeuwen, F., and D. E. Gottschling, 2002 Assays for gene silencing in yeast. Methods Enzymol. **350:** 165–186.

Wu, X., and J. E. Haber, 1996 A 700 bp cis-acting region controls mating-type dependent recombination along the entire left arm of yeast chromosome III. Cell **87:** 277–285.

Xie, J., M. Pierce, V. Gailus-Durner, M. Wagner, E. Winter *et al.*, 1999 Sum1 and Hst1 repress middle sporulation-specific gene expression during mitosis in *Saccharomyces cerevisiae*. EMBO J. **18:** 6448–6454.

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