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Promoter Strength Influences the S Phase Requirement for Establishment of Silencing at the *Saccharomyces cerevisiae* Silent Mating Type Loci

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ABSTRACT

In *Saccharomyces cerevisiae*, the two cryptic mating type loci, *HML* and *HMR*, are transcriptionally silent. Previous studies on the establishment of silencing at *HMR* identified a requirement for passage through S phase. However, the underlying mechanism for this requirement is still unknown. In contrast to *HMR*, we found that substantial silencing of *HML* could be established without passage through S phase. To understand this difference, we analyzed several chimeric *HM* loci and found that promoter strength determined the S phase requirement. To silence a locus with a strong promoter such as the **a***1*/**a***2* promoter required passage through S phase while *HM* loci with weaker promoters such as the α*1*/α*2* or *TRP1* promoter did not show this requirement. Thus, transcriptional activity counteracts the establishment of silencing but can be overcome by passage through S phase.

EPIGENETIC silencing refers to a transcriptionally inactive state and its heritable transmission. It involves the formation, maintenance, and inheritance of a specialized, constitutively compact chromatin structure, termed heterochromatin. This kind of transcriptional silencing plays an important role in establishing and maintaining distinct patterns of gene expression in genetically identical cells during growth and differentiation. Examples of transcriptional silencing include the inactive mammalian X chromosome, position effect variegation in *Drosophila melanogaster*, and the cryptic mating-type loci in fission and budding yeasts (Rusche *et al.* 2003; Probst *et al.* 2009).

In the budding yeast *Saccharomyces cerevisiae*, the *MAT* locus encodes transcriptional regulatory proteins that are responsible for the differences between the two mating types. *HML* and *HMR* harbor cryptic copies of the mating type information genes, α and **a**, respectively. Transcriptional silencing at these loci relies on *cis*-regulatory DNA elements called silencers and on a number of *trans*-acting gene products. Previous studies revealed that establishment of silencing involves a series of protein–DNA and protein–protein interactions (reviewed in Gasser and Cockell 2001; Rusche *et al.* 2003; Fox and Mcconnell 2005). The silencer elements flanking the *HM* loci recruit the DNA binding proteins Rap1, Abf1, and ORC, which in turn recruit the silent information regulator (Sir) proteins, Sir1, Sir2, Sir3, and Sir4. A Sir2–Sir3–Sir4 complex spreads from the silencers into nearby nucleosomes (Hoppe *et al.* 2002; Rusche *et al.* 2002; Liou *et al.* 2005; Rudner *et al.* 2005). This spreading requires Sir2, which deacetylates histone H4 K16, thereby creating a binding site for Sir3 and Sir4 and hence the Sir2/3/4 complex (Carmen *et al.* 2002; Liou *et al.* 2005). Multiple rounds of deacetylation by Sir2 allow the Sir complex to spread to adjacent nucleosomes, thus creating a stretch of silent chromatin.

To investigate the establishment of silencing and its relationship to the cell cycle, previous studies utilized conditional or inducible alleles of the Sir proteins to create a transition from Sir^- to Sir^+ and then examined the establishment of silencing (Miller and Nasmyth 1984; Fox *et al.* 1997; Kirchmaier and Rine 2001; Li *et al.* 2001; Lau *et al.* 2002; Martins-Taylor *et al.* 2004; Kirchmaier and Rine 2006; Xu *et al.* 2006; Wang *et al.* 2008; Osborne *et al.* 2009). For example, in a classic study, Miller and Nasmyth (1984) used a *sir3* temperature-sensitive allele (*sir3-8*) and shifted cells from a nonpermissive temperature to a permissive temperature to follow the establishment of silencing as functional Sir3 protein became available. This strain contained **a**-information at both *HML* and *HMR* cassettes while *MAT* was deleted, so that it could be arrested in G1 phase by α-factor at either temperature. Therefore, the establishment of silencing at *HML* and *HMR* could not be distinguished in this strain. They tested the establishment of silencing under two conditions, arresting in G1 phase or released for cell-cycle progression. They found that silencing could not be

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TABLE 1

Yeast strains used in this study

Strain	Genotype[a]
W303-1a	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1*
RS547	*HML***a** *MAT***a** *HMR***a** *leu2-1 can1-100 met trp1-1 his3, his4, ade2-1*
RS1230	*MAT*α *sir3-8 ade2 trp1-1 ura3 leu2 his3 his4*
RS1231	*MAT***a** *sir3-8 ade2 trp1-1*
XRY19	*HML*α *mat*::*LEU2 hmr*::*TRP1 sir3*Δ::*kanMX6 trp1-1 leu2 ura3 his3 can 1-100 ade2-1*
JRY17	*HML***a** *mat*::*LEU2 hmr*::*TRP1 sir3*Δ::*kanMX6 bar1*Δ::*S.p.his5*$^+$ *trp1-1 leu2 ura3 his3 can 1-100 ade2-1*
JRY19	*HML***a** *mat*::*LEU2 hmr*::*TRP1 sir3-8 bar1*Δ::*S.p.his5*$^+$ *trp1-1 leu2 ura3 his3 can 1-100 ade2-1*
JRY25	*HML***a** *mat*::*LEU2 hmr*::*TRP1 sir3-8 bar1*Δ::*S.p.his5*$^+$ *trp1*Δ::*kanMX6 leu2 ura3 his3 can 1-100 ade2-1*
JRY27	*HML-P***a** *mat*::*LEU2 hmr*::*TRP1 sir3-8 bar1*Δ::*S.p.his5*$^+$ *trp1*Δ::*kanMX6 leu2 ura3 his3 can 1-100 ade2-1*
JRY30	*HML*α *mat*::*kanMX6 HMR***a** *sir3-8 ade2 trp1-1 ura3 leu2 his3 his4*
JRY32	*HML***a** *mat*::*LEU2 HMR*α *sir3-8 bar1*Δ::*S.p.his5*$^+$ *trp1*Δ::*kanMX6 leu2 ura3 his3 can 1-100 ade2-1*

[a] *S.p.his5*$^+$, *Schizosaccharomyces pombe his5*$^+$ gene.

established while the cells were held in G1 phase. Furthermore, they determined that passage through S phase was required for silencing because cells released from an α-factor block and then arrested in G2/M phase were able to silence the *HM* loci substantially.

It was generally assumed that this S phase requirement was DNA replication. However, a subsequent study in which ORC binding sites were deleted from the *HMR* silencers found that silencing of the locus still required passage through S phase, suggesting that the S phase requirement was not replication (Fox *et al.* 1997). This was later demonstrated convincingly by two groups who used modified extrachromosomal copies of an *HMR* locus whose origins of replication had been deleted and hence could not replicate (Kirchmaier and Rine 2001; Li *et al.* 2001). They showed that silencing on these plasmids could still occur and thus was independent of DNA replication, but, surprisingly, still required passage through S phase. In a later study, Lau *et al.* (2002) identified an additional cell-cycle requirement in M phase and suggested it to be the dissolution of sister-chromatid cohesion. They also showed that the two cell-cycle requirements were independent because loss of sister-chromatid cohesion could not bypass the requirement of passage through S phase. Interestingly, the underlying mechanism for the S phase requirement remains unknown.

All the studies described above focused on the *HMR* locus. A single report previously investigated the S phase requirement at the *HML* locus and concluded that passage through M phase, but not S phase, was required for establishment of silencing of this locus (Martins-Taylor *et al.* 2004). But their experimental protocol differed substantially from those used previously to study the S phase requirement at *HMR*; furthermore, they did not compare the two loci (see discussion). Therefore we decided to compare establishment of silencing of *HML* and *HMR* in the same strain under similar conditions. Consistent with the previous observations on *HMR*, we found that silencing was not established without passage through S phase. However, we found that substantial silencing could be established at the *HML* locus under the same conditions. To understand this difference, we analyzed the *HM* loci and attributed the difference to the transcription units of these loci. We then used modified *HM* loci containing transcription units with different promoter strength and found that transcription counteracted the establishment of silencing: the stronger the promoter, the more stringent the cell-cycle requirement. On the basis of these observations, we propose possible cell-cycle events that may determine the S phase requirement.

MATERIALS AND METHODS

Yeast strains and culture conditions: Strains used in this study are listed in Table 1. Gene replacements were performed as described (Scherer and Davis 1979; Longtine *et al.* 1998). JRY17 was derived from a cross of RS547 with XRY19, followed by deletion of *BAR1* with the *S.p.his5*$^+$ marker. JRY19 was constructed from JRY17 by gene replacement of *sir3*Δ::*kanMX6* with *sir3-8*. JRY25 is a *trp1*Δ::*kanMX6* version of JRY19. JRY27 was constructed by replacing the wild-type (WT) *HML* locus with a modified *HML* locus from plasmid pHML-P**a**. This plasmid contains a hybrid *HML* locus with the α*1* gene driven by the promoter from **a***1*/**a***2* transcription unit. It was generated by overlapping PCR, substituting *HML* sequences from the Yα segment (Chr III coordinates 12,944–13,244) with *HMR* sequences from the Y**a** segment (Chr III coordinates 293,734–293,819). JRY32 was constructed by replacing the *hmr*::*TRP1* locus in JRY25 (from *HMR-E* through the *TRP1* gene and Z1 segment) with a hybrid *HMR*α locus from plasmid pHMRα. This plasmid contains ligated genomic fragments of *HMR-E* (Chr III coordinates 292,385–293,029) fused with *HML* sequences containing the X, Yα, and Z1 segments (Chr III coordinates 12,239–13,909).

Strains with the *sir3-8* mutation (RS1230, RS1231, JRY19, JRY25, JRY27, and JRY32) were all grown to early log phase in yeast extract–peptone–dextrose medium (YPD) before synchronizing with 0.2 M hydroxyurea (HU) at the nonpermissive temperature. In the case of JRY27, an initial synchrony with 150 nM alpha factor (αF) was used for a better outcome before

synchronizing with HU as above. To restore silencing, strains were shifted back to permissive temperature either with HU to prevent passage through S phase or released into fresh YPD to allow for cell-cycle progression. Samples were taken at 1-hr intervals and subjected to DNA and RNA measurements.

Plasmids: The **a***1* promoter, α*1* promoter, and *TRP1* promoter present at *hmr::TRP1* were fused to a *yEmRFP* reporter gene in 2-μm plasmids pJR67, pJR68, and pJR69, respectively. W303-1a was transformed with each plasmid for measurements of expression of reporter gene from corresponding promoter.

Flow cytometry: Flow cytometry analysis of DNA content was performed as described (Haase and Reed 2002). Briefly, cells were harvested and fixed with 70% ethanol. After sonication, cells were treated with RNaseA (Sigma) and pepsin (Sigma). Samples were stained with CYTOX Green (Invitrogen) and analyzed on a FACScan using Cell Quest Pro software (BD).

RT–PCR: Total RNA was extracted with a RiboPure–Yeast kit (Applied Biosystems) followed by treatment with RNase-free DNase (Applied Biosystems). RT–PCR was performed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

Real-time PCR and quantification: Real-time PCR was performed with a LightCycler 480 SYBR Green I Master kit according to recommended conditions. Experiments were conducted and analyzed in Mastercycler ep realplex^2 (Eppendorf) according to manufacturer's instructions. Primer sequences for **a***1*, α*1*, *ACT1*, *18S*, and *yEmRFP* are listed in supporting information, Table S1. For each set of samples, the RNA level at the 0-hr time point, normalized to either an ACT1 or 18S internal control, was set to 1.0 and RNA levels from subsequent time points were normalized relative to the initial state. For comparison of the RNA level from derepressed *HMR***a***1* and *HMLα1*, the relative amount of HMR**a**1 and HMLα1 RNA was measured by normalizing to the corresponding locus in genomic DNA isolated from JRY30, which only contains one copy of each transcription unit. The relative amount of HMR**a**1 RNA was set to 1.0. For measurement of promoter strength, the RNA level of *yEmRFP* was normalized to the ACT1 internal control, and the relative amount of transcript from **a***1* promoter was set to 1.0.

RESULTS

Silencing at *HMR* requires passage through S phase; however, it could be partially established at *HML* during early S phase arrest: Previous work from our laboratory identified a point mutation in the *SIR2* gene, which caused mating defects in haploid strains of either mating type at 37° but not at 23° (Wang *et al.* 2008). RNA measurements demonstrated that the lack of mating at 37° was due to loss of silencing. We also noted that when cultures were shifted from 37° to 23°, it took >8 hr for silencing to be reestablished at the *HMR* locus (Wang *et al.* 2008). On the other hand, it took <4 hr to achieve a similar extent of silencing at *HML* (data not shown). These observations prompted us to consider the possibility that establishment of silencing at *HML* might not have the same cell-cycle requirement as had been described previously for *HMR*. To characterize the difference in cell-cycle requirement we monitored the establishment of silencing at the *HMR* locus in a *MAT*α strain and at the *HML* locus in a *MAT***a** strain with the same *sir3-8* temperature-sensitive allele that had been used in several previous studies on this topic. Cells were grown to log phase at 37° (the nonpermissive temperature that disrupts silencing), synchronized in early S phase with HU, then shifted to 23° (the permissive temperature) either in the presence of HU to prevent passage through S phase or released into fresh medium without HU to allow cell-cycle progression (Figure 1A). HU rather than αF was used because only the *MAT***a** strain is sensitive to αF, while HU allowed us to compare strains of either mating type under the same condition. Samples were withdrawn at the times indicated and their DNA content monitored by flow cytometry. The cells held in HU maintained a 1n peak of DNA content during the course of the experiment, demonstrating an early S phase arrest by HU (Figure 1B), whereas cells incubated without HU progressed through the cell cycle (data not shown). RNA was extracted from the above samples, subjected to RT–PCR, and quantified by real-time PCR. The amount of HMLα1 and HMR**a**1 RNA level was normalized to the ACT1 RNA control, respectively. At the 0-hr time point, just after cells have been shifted to 23°, when they were still fully derepressed, the ratio of HMRa1/ACT1 RNA (Figure 1C) or HMLα1/ACT1 RNA (Figure 1D) for each strain was set to 1.0. As shown in Figure 1C, the expression level of HMR**a**1 RNA remained high in cells held in HU, consistent with previous studies (Miller and Nasmyth 1984; Fox *et al.* 1997; Kirchmaier and Rine 2001; Li *et al.* 2001; Lau *et al.* 2002; Kirchmaier and Rine 2006; Osborne *et al.* 2009). Although there was a slight decrease during incubation at 23° in HU (to 0.85 at the 4-hr time point), this extent of silencing may have been due to a small portion of the cells that escaped the HU block and entered the cell cycle. In contrast, as shown in Figure 1D, the HMLα1 RNA level showed a significant decrease under the same condition (to 0.28 at the 4-hr time point). This demonstrated that substantial silencing of the *HML* locus could occur without passage through S phase and thus contrasted with the well-documented cell-cycle requirement for establishment of silencing at *HMR*. Cells released from the S phase block had an even greater drop in the HMLα1 RNA level, to 0.028 at the 4-hr time point. This is to be compared with cells grown at 23° for 11 hr in which the HMLα1 RNA level was even lower, 0.004 (Figure 1D). Thus, it took several generations for silencing to be fully established, consistent with previous observations and our results on the *HMR* locus, which also required several cell division cycles for the locus to be fully silenced (Katan-Khaykovich and Struhl 2005) (Figure 1C).

The difference in cell-cycle requirement for establishment of silencing at *HML vs. HMR* was due to the transcription units of these loci rather than the flanking silencers: Despite some similarities between the *HML* and *HMR* loci, they are composed of different tran-

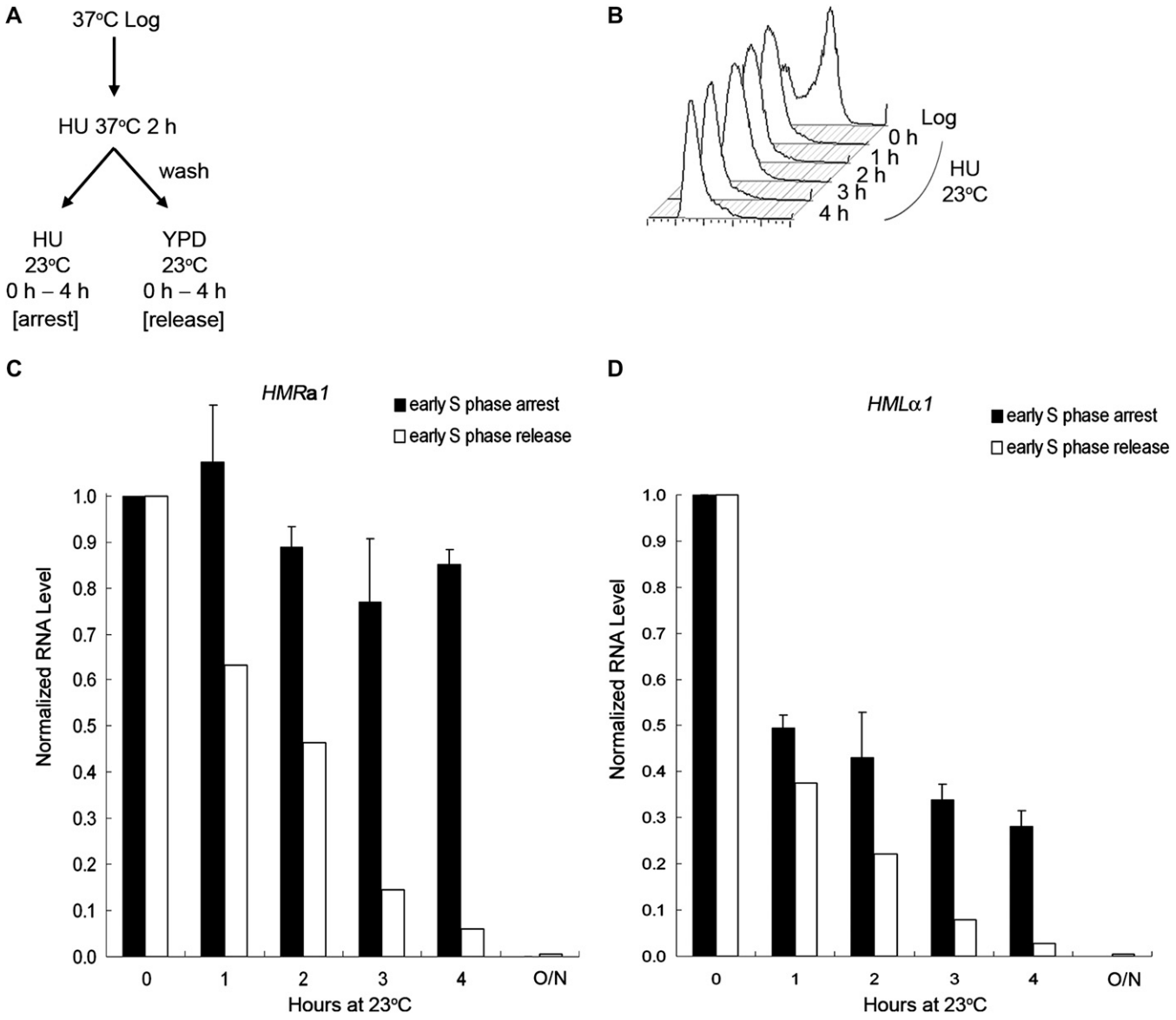

FIGURE 1.—Silencing can be partially established at *HML* without passage through S phase, while silencing at *HMR* cannot. (A) Experimental outline. *MAT*α *sir3-8* cells (RS1230) and *MAT***a** *sir3-8* cells (RS1231) were used to analyze silencing at *HMR* and *HML*, respectively. Cells were grown to log phase at 37°, synchronized in early S phase with HU, and then shifted to 23° either with HU to prevent passage through S phase or released into fresh YPD to allow for cell-cycle progression. (B) DNA content. Samples at 23° were withdrawn at the times indicated and their DNA content monitored by flow cytometry. A representative result of samples held in HU is shown. (C) *HMR***a***1* expression. RNA was extracted at the indicated time points from both S phase-arrested and -released samples, subjected to RT–PCR, and quantified by real-time PCR. The HMRa1 RNA level at the 0-hr time point, normalized to the *ACT1* control, was set to 1.0. The average of two independent experiments is shown for the S phase-arrested samples (shaded bars) and one representative experiment is shown for S phase-released samples (open bars). (D) *HML*α*1* expression. A similar procedure and analysis was done as described in C, except that the HMLα1 RNA level was measured. In C and D, the RNA level for fully silenced cells grown at 23° overnight is also shown (labeled O/N).

scription units and somewhat different flanking silencers. Therefore, there were two possible explanations for the difference in cell-cycle requirement, the transcription units or the flanking silencers. To distinguish these possibilities, we constructed a strain carrying an *HML***a** locus, which contained the **a***1*/**a***2* transcription unit from the *HMR* locus instead of the usual *HML* α*1*/α*2* transcription unit, but flanked by the usual *HML* silencers, as diagrammed in Figure 2A. Thus, if the silencers caused the different cell-cycle requirement of the *HML* locus, they should be able to convey the difference to the *HML***a** locus, allowing silencing to be partially reestablished without passage through S phase. On the other hand, if the difference was linked to the transcription units, this substitution should prevent the establishment of silencing before S phase.

To test it, we used a similar experimental strategy (Figure 2B) as we did for WT *HM* loci. A strain with the

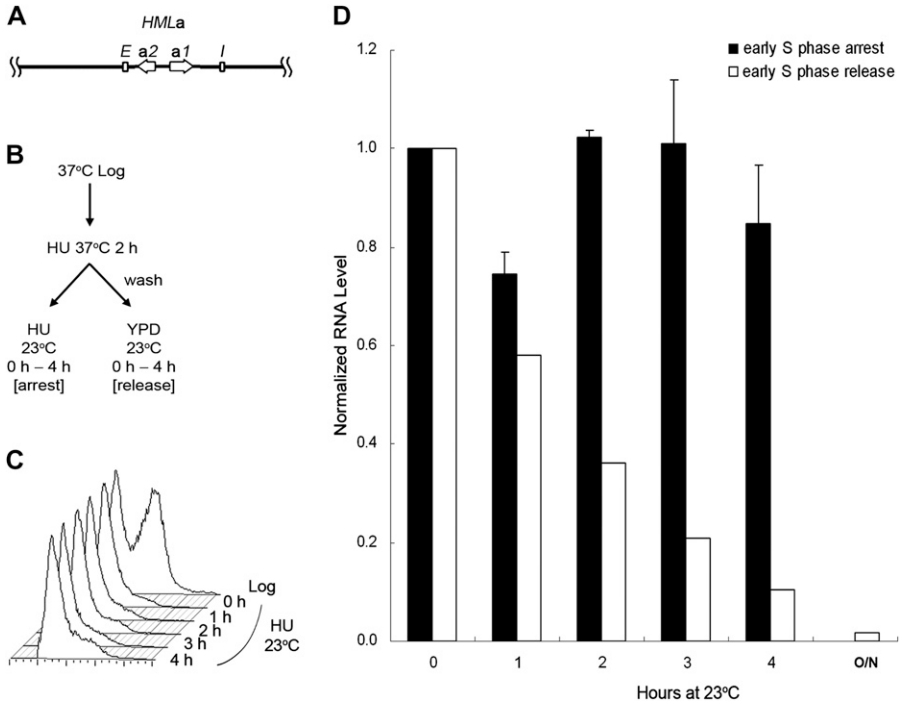

FIGURE 2.—Silencing is not established at an *HML***a** locus without passage through S phase. (A) A diagram of the modified *HML* locus, *HML***a**, is shown. It contains the **a***1*/**a***2* transcription unit from *HMR* instead of the usual *HML*α*1*/α*2* transcription unit, but flanked by the usual *HML* silencers. (B) Experimental outline. The scheme for this experiment is similar to that described in Figure 1, except that *HML***a** *sir3-8* cells (JRY19) were used. (C) DNA content. Samples for S phase arrest were withdrawn at the time points indicated and their DNA content monitored by flow cytometry. (D) *HML***a***1* expression. RNA was extracted at the indicated time points from both S phase-arrested and -released samples, subjected to RT–PCR, and quantified by real-time PCR. The HMLa1 RNA level at the 0-hr time point, normalized to either an 18S rRNA or the *ACT1* internal control, was set to 1.0. The average of two independent experiments is shown. Also shown is the RNA level for cells grown at 23° overnight (labeled O/N).

*HML***a** locus and mutations at *MAT* and *HMR* so that there was no other source of a1 mRNA was synchronized in early S phase with HU at the nonpermissive temperature, then shifted to the permissive temperature, either with HU for early S phase arrest or released into fresh medium to allow for cell-cycle progression (Figure 2, B and C). Cells arrested in early S phase kept expressing a1 RNA from the *HML***a** locus at a high level, while cells released from the block established silencing as cells progressed through the cell cycle (Figure 2D). The normalized HMLa1 RNA level was 0.85 after arresting in early S phase for 4 hr. In contrast, the HMLa1 level decreased to 0.10 at the 4-hr time point in the released samples (Figure 2D). To check that the cell-cycle requirement was not an artifact caused by an HU-induced checkpoint, the same strain was synchronized in G1 with α-factor in a similar experiment. Cells arrested in G1 phase still did not establish silencing at this hybrid locus (Figure S1), indicating that arresting with α-factor or HU gave the same result. Therefore, silencing was not established at the *HML***a** locus without passage through S phase. These results indicated that the difference in the cell-cycle requirement for establishment of silencing at *HML vs. HMR* was linked to the transcription units.

The difference in cell-cycle requirement for establishment of silencing between *HML* and *HMR* was due to transcription, rather than the gene product: To further delineate which part of the transcription units, *i.e.*, the promoter or the open reading frame (ORF) caused this difference, we constructed a strain (JRY27) with a hybrid *HML-P***a** locus by substituting the usual α*1*/α*2* divergent promoter with the **a***1*/**a***2* promoter. This construct expressed the α1 protein from the **a***1* promoter instead of the usual α*1* promoter (Figure 3A). HU was used to synchronize cells at 37° as in Figures 1 and 2. After the HU block cells were shifted back to 23°, either with HU for continued S phase arrest or released into fresh medium to allow cell-cycle progression. Similar to the result with an *HML***a** locus (Figure 2), the α1 RNA level expressed from the **a***1* promoter at the hybrid *HML-P***a** locus showed no significant decrease without passage through S phase (Figure 3D, 0.77 for the 4-hr time point). On the other hand, in cells allowed to pass through the cell cycle, silencing was reestablished and transcription dropped to 0.11 after 4 hr. Since silencing was not established at the hybrid *HML-P***a** locus without passage through S phase, the difference in cell-cycle requirement between *HML* and *HMR* was due to the promoter-based transcription activity, rather than to the gene product from the ORF.

The a*1* promoter was significantly stronger than the α*1* promoter: To understand the linkage between the cell-cycle requirement and the corresponding promoter, we measured the relative strength of the **a***1* and α*1* promoters. First, the RNA level from the derepressed *HML*α*1* and *HMR***a***1* loci was measured as an indicator of their promoter strength. We found that the HMLα1 RNA level was 0.13, relative to 1.0 for HMRa1 (Figure 4A). To confirm that the measurement of these RNA levels reflected the promoter strength rather than half-life of the RNAs, the **a***1* promoter and α*1* promoter were fused to a *yEmRFP* reporter gene (KEPPLER-ROSS *et al.* 2008) and the amount of this transcript from each promoter was measured. When the normalized yEmRFP RNA level from the **a***1* promoter was set to

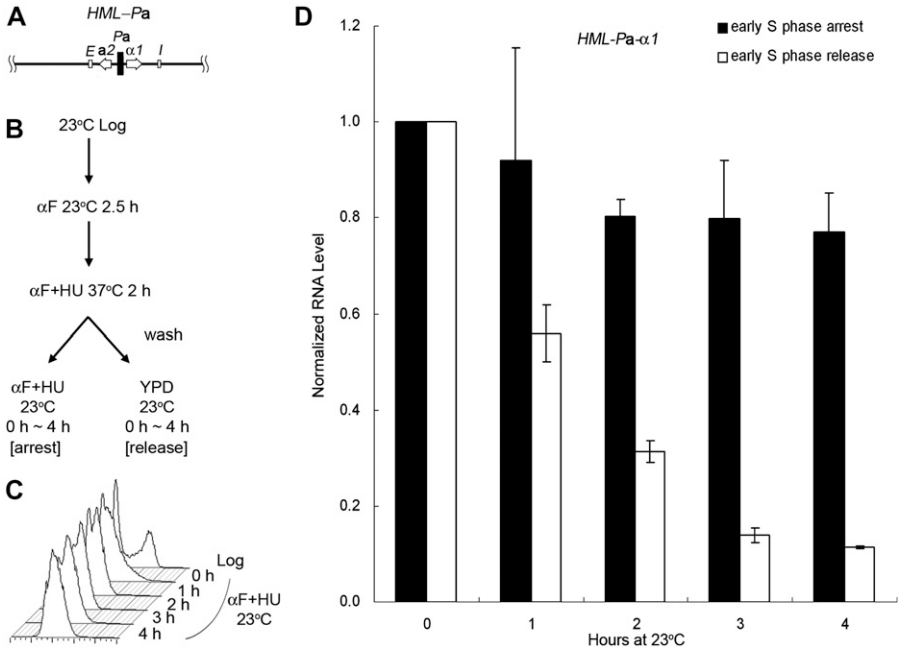

FIGURE 3.—Silencing is not established at a hybrid *HML-P***a** locus without passage through S phase. (A) A diagram of the hybrid *HML-P***a** locus is shown. It expresses the α1 protein from the **a**1 promoter instead of the usual α*1* promoter. (B) Experimental outline. An *HML-P***a** *sir3-8* strain (JRY27) was treated with αF at 23° for 2.5 hr, then shifted to 37° in the presence of αF and HU for 2 hr to synchronize cells in early S phase. The culture was then shifted back to 23°, either with HU for S phase arrest or released into fresh YPD to allow for cell-cycle progression. (C) DNA content. Samples for S phase arrest were withdrawn at the times indicated and their DNA content monitored by flow cytometry. (D) *HML*α*1* expression at the *HML-P***a** locus. RNA was extracted at the indicated time points from both S phase-arrested and -released samples, subjected to RT–PCR, and quantified by real-time PCR. For either cell-cycle condition, the HMLα1 RNA level at the 0-hr time point, normalized to 18S rRNA, was set to 1.0. The average of two independent experiments is shown.

1.0, the level from the α*1* promoter was 0.18 (Figure 4B). Therefore, using two different methods, we found that the **a***1* promoter was significantly stronger than the α*1* promoter.

Silencing was partially reestablished without passage through S phase at a chimeric *HMR* locus containing a weaker promoter, but not at the wild-type *HMR* locus: The results presented above indicated that the strength of the promoter and hence the amount of transcription through the locus determined the cell-cycle requirement or lack thereof. To test this in another way, a strain (JRY27) with an *hmr::TRP1* locus harboring a weakened *TRP1* promoter, flanked by the usual *HMR* silencers, was used (Figure 5A). Measurement of promoter strength with the *yEmRFP* reporter gene showed that this *TRP1* promoter was much weaker than the **a***1* promoter (Figure 4B). This strain also contained the hybrid *HML-P***a** locus. As we showed in Figure 3, silencing was not established at that locus without passage through S phase. In contrast, the *TRP1* transcript from the *hmr::TRP1* locus measured from the same samples decreased significantly during S phase arrest (Figure 5B). When the TRP1 RNA level at the 0-hr time point was set to 1.0, after 4 hr of arrest in early S phase, the RNA level from *hmr::TRP1* dropped to 0.31, a much greater drop than that seen from the *HML-P***a** promoter driving the α1 transcript in the same strain (compare Figures 5B and 3D). Therefore, in contrast to the WT *HMR* locus, silencing could be partially established at the hybrid *HMR* locus containing a weaker promoter.

We also tested this conclusion in a strain that had the α*1*/α*2* transcription unit from *HML* flanked by the *HMR* silencers (Figure 6A). In this case, we observed an intermediate phenotype (Figure 6D), presumably because the α*1* promoter strength is much weaker than the **a***1* promoter, but still stronger than the *TRP1* promoter. Silencing could be established to a certain extent, as the α*1* RNA level dropped to 0.48 after a 4-hr arrest in early S phase when the initial RNA level was set to 1.0. The α1 RNA level was somewhat higher than the TRP1 RNA level measured at the same time point (comparing Figures 6D and 5D, 0.48 *vs.* 0.31), showing less silencing with the stronger promoter. Although the *HMR* α strain showed significant silencing before passage through S phase, it was less than was seen at *HML*α (Figure 1). Therefore, it is possible that the silencers may have some effect on the kinetics of silencing.

DISCUSSION

Our results demonstrate a difference in the S phase requirement for establishment of silencing at *HML* and *HMR*. While silencing cannot occur at the *HMR* locus without passage through S phase (MILLER and NASMYTH 1984; FOX *et al.* 1997; KIRCHMAIER and RINE 2001; LI *et al.* 2001; LAU *et al.* 2002; KIRCHMAIER and RINE 2006; OSBORNE *et al.* 2009) (Figure 1C), it can be established to a significant extent at the *HML* locus under the same conditions (Figure 1D). This difference explains our previous result that silencing was established at *HML* much more rapidly than at *HMR* after shifting a *sir2* temperature-sensitive strain from a nonpermissive to a permissive temperature (WANG *et al.* 2008).

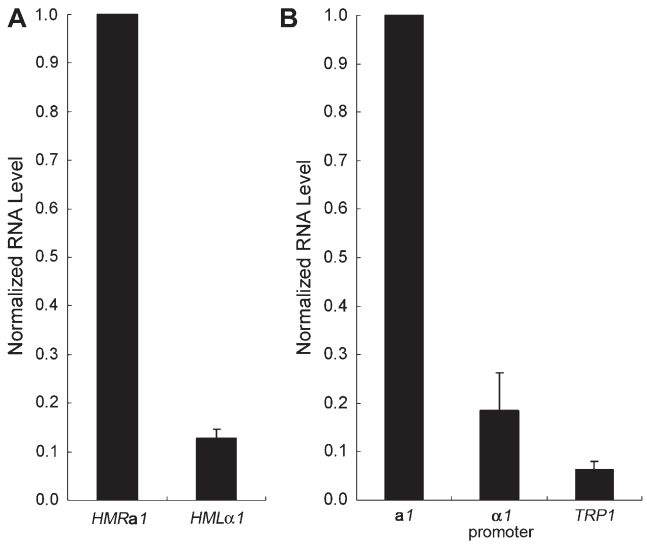

FIGURE 4.—The **a***1* promoter is significantly stronger than the α*1* promoter and a weakened *TRP1* promoter. (A) RNA levels from derepressed *HML*α*1* and *HMR***a***1*. An *HML*α *mat*Δ*::kanMX6 HMR***a** *sir3-8* strain (JRY30) was grown at the nonpermissive temperature and used to extract RNA for RT–PCR. RNA was quantified as described in MATERIALS AND METHODS. (B) Measurement of promoter strength. The **a***1* promoter, α*1* promoter, and *TRP1* promoter present at *hmr::TRP1* were fused to a *yEmRFP* reporter gene and expressed from 2μ plasmids. RNA was extracted, subjected to RT–PCR, and quantified by real-time PCR. The yEmRFP RNA level from the **a***1* promoter, normalized to the *ACT1* internal control, was set to 1.0. The average of two independent experiments is shown.

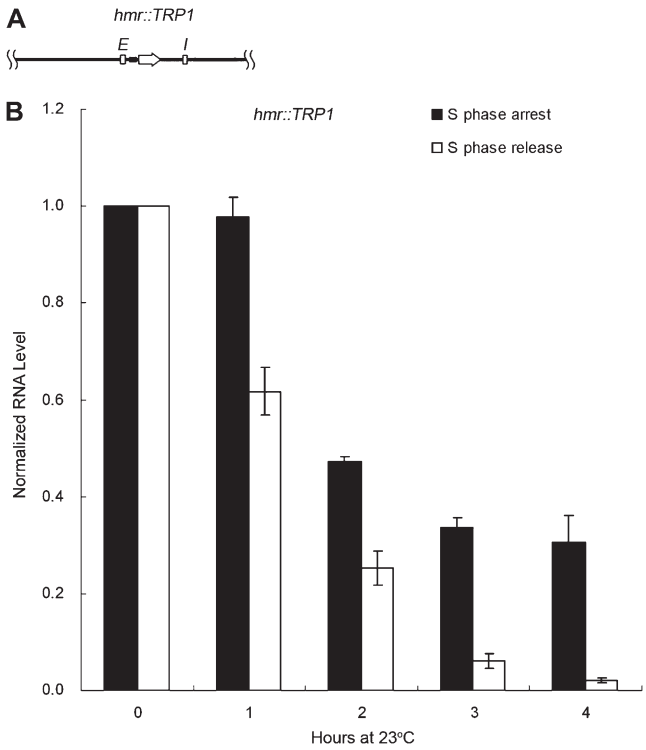

FIGURE 5.—Substantial silencing can occur at an *hmr::TRP1* locus without passage through S phase. (A) A diagram of the *hmr::TRP1* locus (JRY27), containing the *TRP1* transcription unit driven by a weakened *TRP1* promoter, flanked by the usual *HMR* silencers. (B) *TRP1* expression at the *hmr::TRP1* locus. The strain and the samples are the same ones used for the experiment shown in Figure 3, although TRP1 RNA quantification is shown here. For both S phase arrest and release, the TRP1 RNA level at time point 0 hr, normalized to an 18S rRNA internal control, was set to 1.0. The average of two independent experiments is shown.

Using various chimeric constructs we determined that the different S phase requirement for silencing *HML* and *HMR* was due primarily to the transcription units of these loci rather than to the flanking silencers. For example, an *HML* locus with the **a***1*/**a***2* transcription unit instead of the usual α*1*/α*2* transcription unit, but flanked by the usual *HML* silencer elements, could not be silenced without passage through S phase (Figure 2). We narrowed down this difference by showing that a substitution of the α*1*/α*2* promoter at *HML* with the **a***1*/**a***2* promoter also prevented the establishment of silencing before passage through S phase (Figure 3). Therefore, the different S phase requirement for silencing *HML*α and *HMR***a** was due to the different promoters present at those loci.

To test whether the two promoters had different strengths we measured transcription activity from each promoter and found that the **a***1* promoter was significantly stronger than the α*1* promoter (Figure 4). We did this in two ways. First we compared the amount of RNA from derepressed *HMR***a***1* with the amount from *HML*α*1* (Figure 4A). To correct for the possibility that a1 mRNA might have a greater half-life than α1 mRNA, we also fused each of these promoters to a reporter gene and measured the amount of RNA from this gene (Figure 4B). Both experiments showed that the **a***1* promoter was significantly stronger than the α*1* promoter. Furthermore, by substituting the **a***1*/**a***2* promoter and gene at *HMR* with the much weaker *TRP1* promoter and its gene, we observed that silencing could be established at the *HMR* locus without passage through S phase (Figure 5B). On the other hand, the silencers may also influence the S phase requirement. When we tested an *HMR*α construct, which had the *HMR* silencers but the α1/α2 transcription unit, less silencing was observed when holding cells in early S phase than when the α1/α2 transcription unit was at its natural locus, *HML* (Figure 6B). Nevertheless, the data from the various constructs tested support our conclusion that the amount of transcription through a gene counteracts establishment of silencing, and that influences the cell-cycle requirement. That is, the stronger the promoter, the more resistance there is to establishment of silencing and the more stringent is the S phase requirement. It seems reasonable that the frequent passage of RNA polymerase II from a relatively strong promoter inhibits the spreading of the Sir complex from the silencers. The euchromatin marks that result

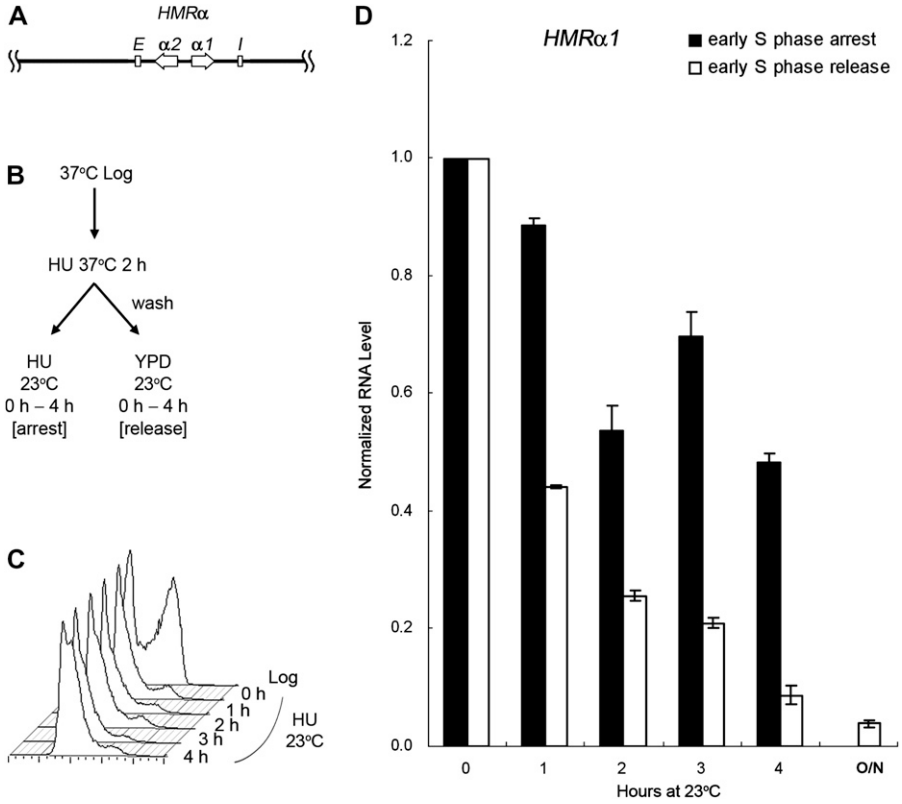

FIGURE 6.—Substantial silencing can occur at an *HMR*α locus without passage through S phase. (A) A diagram of the hybrid *HMR*α locus is shown. (B) Experimental outline. The scheme for this experiment is similar to that described in Figure 1, except that an *HMR*α *sir3-8* strain (JRY32) was used. (C) DNA content. Samples for S phase arrest were withdrawn at the times indicated and their DNA content monitored by flow cytometry. (D) α*1* expression at the *HMR*α locus. RNA was extracted at the indicated time points from both S phase-arrested and -released samples, subjected to RT–PCR, and quantified by real-time PCR. For either cell-cycle condition, the HMRα1 RNA level at the 0-hr time point, normalized to 18S rRNA, was set to 1.0. The average of two RNA measurements is shown. Also shown is the RNA level for cells grown at 23° overnight (labeled O/N).

from active transcription may also hinder the establishment of heterochromatin.

Previous studies have also observed a competition between transcription and silencing. For instance, a *URA3* reporter gene could be silenced at a greater distance from the telomere when *PPR1*, the *trans*-activator of *URA3*, was deleted (RENAULD *et al.* 1993). In addition, it was found that a silent telomeric *URA3* gene could become expressed if cells were arrested in G2/M and that depended on the *PPR1* activator (APARICIO and GOTTSCHLING 1994).

A recent study using galactose induction of Sir3 to study the kinetics of spreading of the Sir complex during reestablishment of silencing found that the Sir complex spread more rapidly at *HMR* than at a telomere, and evidence was presented that the *HMR-E* silencer was responsible for this effect (LYNCH and RUSCHE 2009). However, that study did not use cells blocked in the cell cycle and thus probably does not apply to the results presented here.

Additional support for the competition between transcription and silencing came from studying silencing in mutants lacking the chromatin-modifying enzymes Dot1 or Set1, responsible for euchromatic methyl marks on histone H3K79 and H3K4, respectively. In *dot1*Δ and *set1*Δ mutants, establishment of silencing was more rapid than in wild-type cells, probably because active transcription was compromised by the hypomethylated chromatin and hence was less resistant to silencing (OSBORNE *et al.* 2009). However, it may also have been caused by the better binding of Sir proteins to hypomethylated histones (ONISHI *et al.* 2007; SAMPATH *et al.* 2009).

Interestingly, the reason why S phase passage is necessary for establishing silencing at *HMR* is still not understood. Studies with nonreplicating *HMR* circles provided strong evidence that it is not DNA replication itself that is needed for establishing silent chromatin (KIRCHMAIER and RINE 2001; LI *et al.* 2001). On the basis of our findings that promoter strength influences the S phase requirement, we propose two different S phase events that may facilitate the spreading of the Sir complex and allow it to overcome the competition from transcription. One is an S phase-dependent post-transcriptional modification of a Sir protein or a histone that would strengthen the association between the Sir complex and nucleosomes. A recent study by HOLT *et al.* (2009) identified Sir2, Sir3, and Sir4 among 308 substrates of the cyclin-dependent kinase Cdc28/Cdk1 in cells synchronized at M phase. Conceivably, similar modifications of Sir proteins or histones could explain the S phase requirement.

Another explanation could be that histone synthesis and deposition occur during S phase and that facilitates silencing. It is well established that transcription tends to reduce histone occupancy on chromosomal DNA. For example, the histone occupancy on the *GAL10* coding region is inversely correlated with transcription activity (SCHWABISH and STRUHL 2004). Using antihistone H3 chromatin immunoprecipitation (ChIP), we

obtained a similar result. We observed a bigger decrease in histone occupancy at the *HMR***a***1* transcription unit than at the *HMLα1* transcription unit when shifting an exponentially growing *sir3-8* ts strain from 23° to 37° (data not shown), agreeing with our result that the **a***1* promoter is stronger than the α*1* promoter. The frequent passage of RNA polymerase II from the relatively strong **a**1 promoter may cause reduced nucleosome occupancy, which in turn, provides less binding surface for the Sir complex, thus counteracting silencing. During passage through S phase, when histone synthesis and deposition are robust, more nucleosomes may be incorporated into the silent regions, providing a better binding surface for the Sir complex. This process is not necessarily coupled to DNA replication since it can take place on a nonreplicating *HMR* circle (Kirchmaier and Rine 2001; Li *et al.* 2001).

Martins-Taylor *et al.* (2004) previously observed that establishment of silencing at *HML* did not require passage through S phase, but did require passage through G2/M. However, their protocol was very different than ours and did not compare *HML* and *HMR*. They synchronized *sir3-8* ts cells in G2/M at 23° and then released them into αF at 37°. They measured the fraction of cells blocked in G1 by αF as a measure of silencing at *HML*. Our results agree with their conclusion and extend it by showing that it is the strength of the promoter that influences the S phase requirement.

One interesting question not answered by our results is how the amount of silencing observed for the population relates to that of the individual cell. For example, in the experiment shown in Figure 1D, when the amount of HMLα1 RNA during S phase arrest decreased to 30% of its original level after 4 hr at a permissive temperature, was that because 70% of the cells were fully silenced or because the entire population was partially silenced? The two possibilities correspond to two different views for the establishment of silencing. One is that intermediate states of silencing exist and complete silencing is achieved gradually as cells continue to divide. The other assumes an all-or-none model, that a locus is either completely silenced or derepressed (Gottschling *et al.* 1990). Two recent studies showed that complete silencing required several generations and thus favor the former model (Katan-Khaykovich and Struhl 2005; Osborne *et al.* 2009). Therefore, the decrease in RNA level we detected at *HML* in the first few hours at the permissive temperature (Figure 1) is likely to reflect a reduced RNA level in the population of cells, few or none of which are completely silenced.

Even though substantial silencing was established without passage through S phase at the *HM* loci with a weak promoter, *e.g.*, *HMLα* and *hmr::TRP1*, it didn't reach the same extent as that seen for cells allowed to pass through the cell cycle. For example, as shown in Figure 1D, the HMLα1 RNA level decreased substantially to 0.28 after 4 hr in early S phase arrest, while it showed an even greater drop to 0.028 at the corresponding time point when released from the S phase block. A similar difference was observed at the *hmr::TRP1* locus (Figure 5B). One possible cause is the previously described G2/M phase requirement, which is independent of the S phase requirement (Lau *et al.* 2002). That study concluded that it was the dissolution of sister-chromatid cohesion at anaphase that accounted for the G2/M-phase requirement (Lau *et al.* 2002).

In summary, the results presented have clarified the different cell-cycle requirement for establishment of silencing at *HML* and *HMR*. That is, silencing can be partially established at *HML* without passage through S phase, but not at *HMR*. We have analyzed the difference and attributed it to the transcriptional activity of these loci. We found that the greater the transcriptional activity, the more resistance there is to silencing and the more stringent the S phase requirement. The competition between transcription and silencing may allow for a certain amount of plasticity for switching to the opposite phenotype, and this may be particularly important in metazoans.

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GENETICS

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Promoter Strength Influences the S Phase Requirement for Establishment of Silencing at the *Saccharomyces cerevisiae* Silent Mating Type Loci

Jie Ren, Chia-Lin Wang and Rolf Sternglanz

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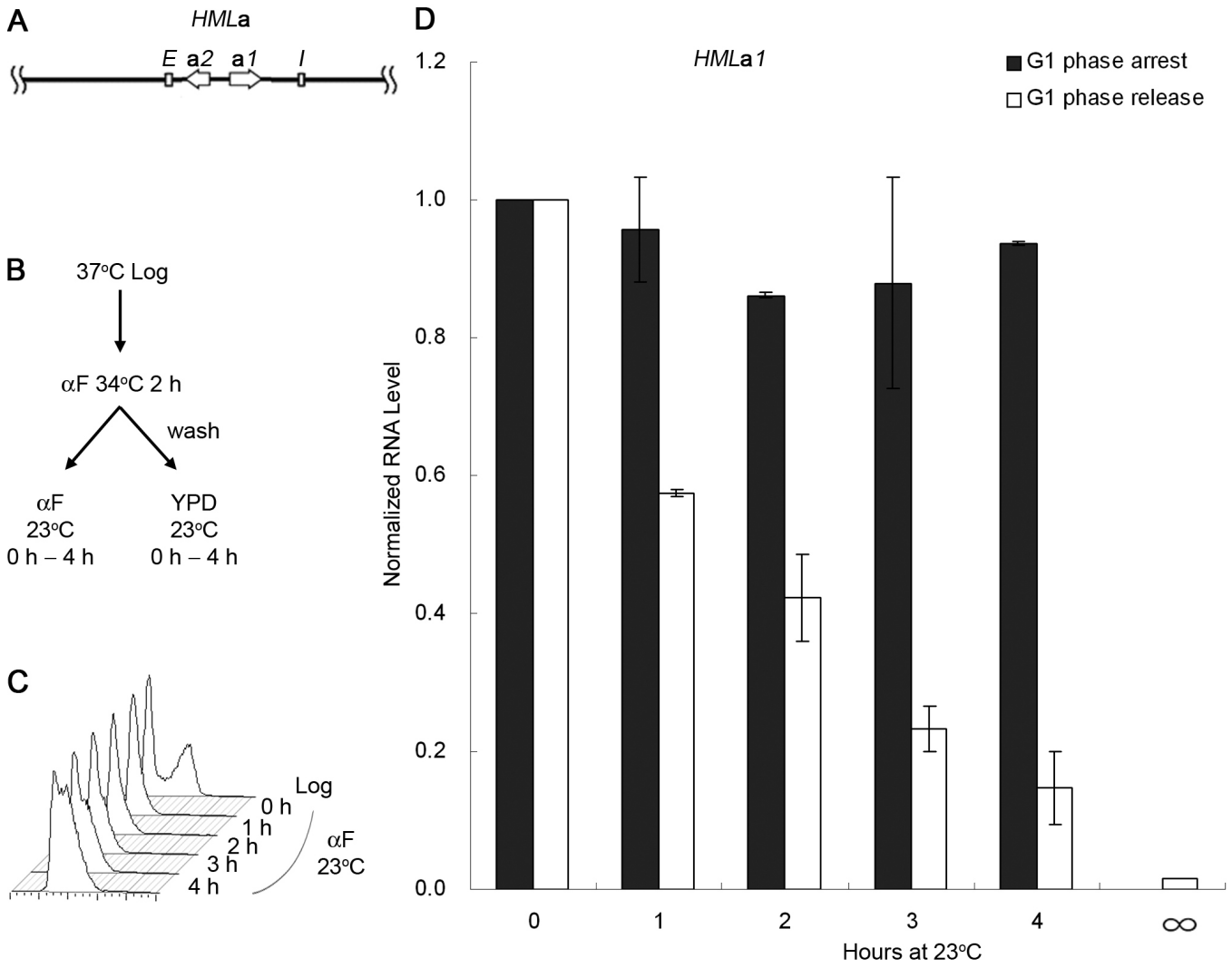

FIGURE S1.—Silencing is not established at an *HML***a** locus without passage through S phase. (A) Experimental outline. The scheme for this experiment is similar to that described in FIGURE 1, except that *HML***a** *sir3-8* strains (JRY19 or JRY25) were synchronized in G1 phase by aF at 34°C, then shifted to 23°C, either with αF for G1 phase arrest, or released into fresh YPD to allow for cell-cycle progression. (B) DNA content. Samples for G1 phase arrest were withdrawn at the time points indicated, and their DNA content monitored by flow cytometry. (C) *HML***a***1* expression. RNA was extracted at the indicated time points from both G1 phase arrested and released samples, subjected to RT-PCR, and quantified by real-time PCR. The HMLa1 RNA level at the 0 h time point, normalized to either an 18S rRNA or the *ACT1* internal control, was set to 1.0. The average of two independent experiments is shown. Also shown is the RNA level for cells grown at 23°C for 11 h after release from aF (labeled O/N).

TABLE S1

Primers used in this study

Name	Locus[a]	Coordinates	Sequence
JRP53	α*1* F	Chr III 13313-13336	AGAACAAAGCATCCAAATCATACA
JRP54	α*1* R	Chr III 13422-13399	GAGTGGTCGAATAATATTGAAGCA
JRP102	**a***1* exon 2 F	Chr III 294112-294129	CAATATCACCCCAAGCAC
JRP103	**a***1* exon 2-3[b] R	Chr III294302-294287 + 294235-294226	CGTTTATTTATGAAC CAAACTCTTA
JRP94	*TRP1* F	Chr IV 461934-461951	ATGCTGACTTGCTGGGTA
JRP95	*TRP1* R	Chr IV 462038-462055	GTATTTCGGAGTGCCTGA
JRP93	*ACT1* exon1 F	Chr VI 54707-54686	ACTGAATTAACAATGGATTCTG
JCP122	*ACT1* exon2 R	Chr VI 54256-54275	CATGATACCTTGGTGTCTTG
YY43F	*RDN18-1* F	Chr XII 456332-456315	GCCGATGGAAGTTTGAGG
YY43R	*RDN18-1* R	Chr XII 456083-456106	TACTAGCGACGGGCGGTGT
JRP132	*yEmRFP* F		AACTATGGGTTGGGAAGC
JRP133	*yEmRFP R*		CACCTGGTAATTGAACTG

[a] "F" stands for forward primer, "R" stands for reverse primer

[b] JRP103 was designed to span the **a**1 exon2 and exon3.