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10. Analysis of the DNA sequence was performed by BLAST searches of the BDGP database. In addition, a second putative Trx gene (*dmtrx-2*, GenBank accession number AF236866) was identified. The 106–amino acid protein shows 37% sequence identity with DmTrx-1 and 49% with human Trx (JH0568).
11. The *dmtrxr-1* gene was identified after plasmid rescue using an Eco RI digestion of total genomic DNA of a *Drosophila* line carrying a transposon insertion in the X chromosome. The transposon insertion is located downstream of base 93 of a putative *Drosophila* disulfide reductase sequence. This *dmtrxr-1* sequence was found to be identical with the BDGP clot 1274. DNA sequencing was performed with the Pharmacia ALF express system. Based on the genomic sequence derived from the BDGP, the gene structure of *dmtrxr-1* allows two alternative transcripts that differ in the 5′ untranslated region and the first 15 bp. This corresponds to a difference in the NH_2-terminal amino acids: MAPVQ (BDGP EST GM02264; GenBank accession number AF301144) versus MSTKG (BDGP EST GM14215; GenBank accession number AF301145) (*29*). The present work exclusively contains data on the MAPVQ variant (Fig. 1). Furthermore, a putative DmTrxR-2 (GenBank accession number AAF64152) has been identified in the BDGP database, exhibiting 76% protein sequence identity with DmTrxR-1 and 50% with hTrxR. The deduced protein contains an NH_2-terminal peptide of 27 amino acids that is likely to represent a mitochondrial targeting sequence. Multiple TrxRs have been described in other organisms, including humans, mice, and *C. elegans* (*1–3*).
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14. The *dmtrxr* primers were designed according to the BDGP *trxr* sequence (BDGP clot 1274). For *dmtrxr-1*, the forward primer introduced a Sac I restriction site (5′-GCGCGAGCTCGCGCCCGTGCAAGGATCC-3′), and the reverse primer introduced a Hind III restriction site (5′-GCGCAAGCTTTCGACACGTCCCAGGCG-3′). The *dmtrx* primer design was based on the *dhd* sequence by Salz *et al.* (*12*). For *Dmtrx-1*, the forward primer (5′-GCGCGGATCCGCATCCGTACGCACCATGAA-3′) and the reverse primer (5′-GCGCAAGCTTTACGCCTTCACCAGCTTGG-3′) introduced the restriction sites Bam HI and Hind III, respectively. The primers were used in a hot start polymerase chain reaction (PCR) using a *D. melanogaster* head cDNA library as a template (at 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s, for 30 cycles). The resulting amplified PCR fragments were cloned into the *pQE-30* (Qiagen) expression vector, which placed a hexahistidyl tag at the NH_2-terminus of the proteins. The plasmid constructs were introduced into *E. coli* XL1-Blue cells (Stratagene). Gene expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C in an overnight culture. The recombinant proteins were purified over a Ni-NTA agarose column. As judged from silver-stained SDS–polyacrylamide gel electrophoresis, the resulting proteins were >98% pure. The yield was 20 mg of DmTrxR-1 and 10 mg of DmTrx-1 per 1 liter of cell culture.
15. DTNB reduction assays for TrxR activity were spectrophotometrically conducted at 25°C in 100 mM potassium phosphate of pH 7.4, containing 200 μM NADPH and 3 mM DTNB as a disulfide substrate (*6*). Trx reduction assays with the Trxs from other species as substrates were performed in the same buffer but in the presence of 100 μM NADPH (millimolar absorbance coefficient at 340 nm = 6.22 mM^{-1} cm^{-1}). GR activity was also assayed at 340 nm and 25°C, but in 47 mM potassium phosphate, 200 mM potassium chloride, and 1 mM EDTA (pH 6.9) in the presence of 100 μM NADPH and 1 mM GSSG.
16. hTrxR was isolated from placenta and hGR was recombinantly produced in *E. coli* (*6*). *E. coli* TrxR, *E. coli* Trx, and human Trx were kind gifts of C. H. Williams Jr. (University of Michigan). *P. falciparum* TrxR (PfTrx) was recombinantly produced as described (*22*). The expression vector *pQE30* was purchased from Qiagen. All chemicals were of the highest available purity.
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19. The DmTrxR-1 mutants C489S and C490S and the double mutant C489S/C490S were generated by site-directed mutagenesis using a plasmid DNA template. In all three cases, the same forward primer introducing a Sac I restriction site was used (5′-GCGCGAGCTCGCGCCCGTGCAAGGATCC-3′). A Hind III restriction site was introduced by the following mutagenesis reverse primers: for C489S, 5′-GCGCAAGCTTTAGCTGCAG**G**AGCTGGCCG-3′; for C490S, 5′-GCGCAAGCTTTAGCTG**G**AGCAGCTGGCCG-3′; for C489S/C490S, 5′-GCGCAAGCTTTAGCTG**G**AG**G**AGCTGGCCG-3′. Mutated bases are in bold; restriction sites are underlined. Gene expression and protein purification were conducted as described for the wild-type enzyme.
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23. Rate constants were determined using an enzymatic $TrxS_2$ reducing system and subsequently transforming it to a GSSG reducing system (*22*). In a cuvette containing 100 μM NADPH, 10 nM DmTrxR-1 subunit, and 10 μM $TrxS_2$, Trx reduction was measured. After the reaction had come to an end, GSSG was added. The system resumed NADPH consumption at a rate that now represents a Trx flux leading to GSSG reduction. On the basis of the employed concentrations, k_2, the rate constant for the reaction between reduced Trx and GSSG, was determined to be 170 M^{-1} s^{-1}, which is equal to 0.01 μM^{-1} min^{-1}. This value was confirmed by varying the total Trx concentration between 5 and 20 μM and the DmTrxR-1 subunit concentration between 1 nM and 10 nM.
24. *D. melanogaster* Schneider cells were grown in Schneider's medium (Gibco BRL). A cell pellet of 750 μl was resuspended in 3 ml of 100 mM potassium phosphate (pH 7.4) and lysed by freezing and thawing three times. The centrifuged lysate was assayed for enzyme activities and then applied to a 0.5-ml 2′,5′–adenosine diphosphate (ADP) Sepharose column. After the column was washed with 10 volumes of the phosphate buffer, disulfide reductases were eluted with 2 mM NADPH. To test the enzyme activity of whole flies, a fly homogenate was obtained by chilling 300 adult OREGON-R flies (~200 mg) and subjecting them to sonication in 5 ml of 100 mM potassium phosphate buffer (pH 7.4). Enzyme activity was determined in the extract or in eluate fractions of the 2′-5′-ADP Sepharose column.
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29. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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DNA Replication-Independent Silencing in *S. cerevisiae*

Ann L. Kirchmaier and Jasper Rine*

In *Saccharomyces cerevisiae*, the silent mating loci are repressed by their assembly into heterochromatin. The formation of this heterochromatin requires a cell cycle event that occurs between early S phase and G_2/M phase, which has been widely assumed to be DNA replication. To determine whether DNA replication through a silent mating-type locus, *HMRa*, is required for silencing to be established, we monitored heterochromatin formation at *HMRa* on a chromosome and on a nonreplicating extrachromosomal cassette as cells passed through S phase. Cells that passed through S phase established silencing at both the chromosomal *HMRa* locus and the extrachromosomal *HMRa* locus with equal efficiency. Thus, in contrast to the prevailing view, the establishment of silencing occurred in the absence of passage of the DNA replication fork through or near the *HMR* locus, but retained a cell cycle dependence.

Heritable states of gene expression are central to the development of life. Gene repression and activation play pivotal roles in the differentiation of totipotent cells into different cell types, each of which selectively and stably expresses only a subset of the genes in the genome. DNA replication can play a role in changing patterns of gene expression (*1–3*) and thus is a possible mechanism for disrupting chromatin states before their reprogramming and for the de novo establishment of those states. There are also clear examples of changes in gene expression and differentiation that occur independently of DNA replication (*4*).

For years, one of the strongest suggestions of a role for DNA replication in establishing heritable transcriptional states came from studies of yeast mating types. In *S. cerevisiae*, mating competence requires

Division of Genetics and Development, Department of Molecular and Cell Biology, University of California, 401 Barker Hall, Berkeley, CA 94720, USA.

*To whom correspondence should be addressed. E-mail: jrine@uclink4.berkeley.edu

heritable repression at the silent mating-type loci, *HML* and *HMR*. The formation of heterochromatin at *HML* and *HMR* requires regulatory sites called silencers, which flank these silent loci. Silencers contain binding sites for the origin recognition complex (ORC), Rap1p and Abf1p [(*5*) and references therein]. In addition, the four Silent Information Regulator proteins, Sir1p, Sir2p, Sir3p, and Sir4p, are structural components of yeast heterochromatin (*3*, *5*, *6*). Sir2p is an enzyme with histone deacetylase activity (*7*). The principal role of Sir1p is the establishment rather than maintenance of silencing, presumably by enhancing a limiting step in heterochromatin formation (*5*). The recruitment of Sir1p to the silencer through interactions with ORC is thought to lead to silencing, resulting in the recruitment of Sir2p, Sir3p, and Sir4p (*8*, *9*).

Passage of yeast cells through S phase is required to establish silencing at *HMR* (*10*), and DNA replication has been the leading candidate for the requisite S phase event. The hypothesized link between DNA replication and silencing is supported by indirect evidence. For example, two of the four silencers are chromosomal origins of replication (*11*). Similarly, ORC has roles in both replication and silencing (*12*–*14*). In addition, mutations in genes encoding an essential DNA helicase, *DNA2*, or the proliferating cell nuclear antigen (PCNA) loading factor, *RFC1*, disrupt silencing at the telomeres and ribosomal DNA (rDNA), respectively (*15*, *16*). Alterations of two replication-coupled chromatin assembly factors, CAF-1 and ASF1, also disrupt silencing (*15*–*18*). Finally, mutant forms of PCNA defective in CAF-1 interaction are defective in establishing silencing (*19*, *20*). These data suggest that CAF1 and PCNA link DNA replication to chromatin assembly and silencing.

However, some data raise doubt about this model. For example, DNA replication initiation at silencers is not required for silencing (*21*). Also, the roles of ORC in replication and silencing are genetically separable (*22*–*24*). Tethering Sir1p to a silencer bypasses a need for ORC in silencing that locus (*9*), but does not bypass the requirement for S phase passage (*21*). All observations could be explained if the link between replication and silencing were the passage of a replication fork though *HMR*, rather than a requirement for replication initiation at a silencer. Here we present a critical test of whether DNA replication is required to establish the silenced state.

To test directly whether DNA replication through *HMR* was the S phase event requirement for silencing, we excised a replication-defective *HMR* cassette from its chromosomal locus during G_1. Cells with the excised *HMR* cassette were induced to express Gal4-Sir1p, which is required to establish silencing, then were allowed to pass through S phase and subsequently rearrested at the G_2/M boundary. The establishment of chromosomal and extrachromosomal silencing was monitored by RNA blots (Fig. 1).

Both the *HMR-E* and *HMR-I* silencers contain origins of replication (*11*), which were incompatible with this experimental design that evaluated silencing in the absence of *HMR* replication. Thus, we used a strain with a replication-defective silencer containing a Rap1p binding site, an Abf1p binding site, and four Gal4p binding sites in place of the ORC binding site (4xGal4-Rap1-Abf1) flanking *HMR*. The *HMR-I* silencer was deleted and the entire locus was flanked by FLP1 recombination target (FRT) sites at which Flip recombinase (FLP) catalyzes site-specific recombination (*25*). The orientation of the sites allowed excision of *HMR* from the chromosome. The FLP enzyme, an Int-like recombinase, leaves no free broken DNA ends as intermediates or products of recombination (*26*). In these cells, silencing at *HMR* is dependent on the chimeric protein, Gal4-Sir1p, which binds Gal4 sites in the synthetic silencer, bypassing the requirement for ORC (*8*, *21*). In addition, silencing at *HMR* in this strain was dependent on *SIR2*, *SIR3*, and *SIR4*, indicating that silencing mediated by tethered Sir1p or a wild-type silencer was mechanistically similar (*27*). Moreover, the tethered Sir1p form of silencing causes an altered superhelical density and enrichment for deacetylated histones expected of ORC-dependent silencing (*28*).

Cells were arrested in G_1 by the pheromone α factor, and the culture was split in two. *HMRa* in these cells was de-repressed, expressing **a1** mRNA due to the absence of Gal4-Sir1p. In one culture, the *HMR* cassette was excised from the chromosome by inducing FLP recombinase from the *GAL10* promoter (*29*). The other culture was held in G_1 without inducing *FLP1*, leaving *HMR* in the chromosome. In G_1, *FLP1* expression was subsequently repressed in the first culture, and Gal4-Sir1p was then expressed for 1 hour via the *MET3* promoter in both cultures. Both cultures were then released from G_1, allowed to pass through S phase, and rearrested two hours later at the G_2/M boundary using microtubule inhibitors.

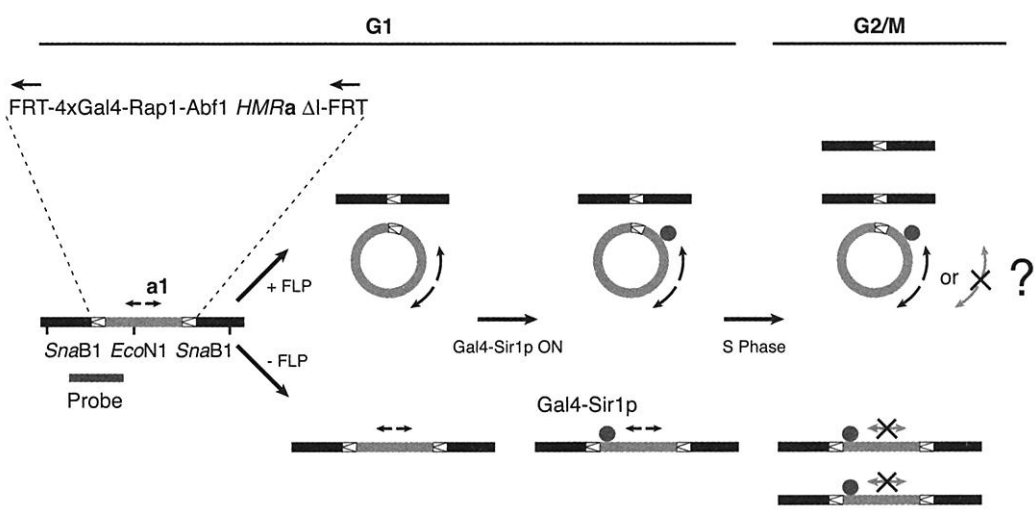

Fig. 1. Experimental Design. A genomic *HMR* locus consisting of a synthetic silencer containing four Gal4p binding sites, a Rap1p binding site and an Abf1p binding site integrated at the *HMR-E* silencer and the genes encoding **a1** and **a2** (black or gray double-headed arrow indicating expression or repression, respectively) was flanked by two FRT sites (white arrowhead) oriented to allow excision by FLP recombinase. The excised *HMR* locus lacked any origin of replication (*11*, *21*). The establishment of silencing at *HMRa* was regulated by controlling expression of the chimeric Gal4-Sir1p (gray circle) via the *MET3* promoter (*21*). FLP recombinase expression was regulated with the *GAL10* promoter (*29*). Cells grown with Gal4-Sir1p off, resulting in *HMRa* being on, were arrested in G_1 with α factor. Induction of FLP with 2% galactose led to excision of the *HMR* locus (light gray area) from the chromosome (black area) in G_1. Expression of Gal4-Sir1p was then induced, and FLP recombinase was repressed in medium lacking methionine and containing 2% raffinose. Cells expressing Gal4-Sir1p were then released from G_1, were allowed to proceed to G_2/M, and were rearrested with benomyl plus nocodazole. The probe (medium gray area) and restriction sites used (Figs. 2B and 3A and Table 1) are noted.

Thus, in one culture, *HMRa* was replicated in its chromosomal context, and, in the second culture, the excised *HMR* locus passed through S phase without being replicated. Parallel control experiments were performed without expression of Gal4-Sir1p, and silencing was not established in these cells. Cell cycle progression and arrest were monitored by microscopy.

After passage through S phase, the level of **a1** mRNA from *HMR* was monitored. The half-life of **a1** mRNA is less than 3 min, so changes in transcription initiation are rapidly reflected in the levels of **a1** mRNA. In cells at G_2/M, **a1** mRNA from the chromosomal locus was reduced to 11% of the level in G_1 cells. Thus, silencing was efficiently established at the chromosomal *HMR* locus ($P = 0.018$, $n = 3$) [Fig. 2A, lanes 8 and 9, and Web table 1 in (*30*)]. Similarly, after passage through S phase, the level of **a1** mRNA from the excised *HMR* cassette in cells at G_2/M was reduced to 14% of the level in G_1 cells ($P = 0.018$, $n = 3$) (Fig. 2A, lanes 5 and 6, and Web table 1). The efficiency of silencing of *HMR* in both contexts was quantitatively similar ($P = 0.51$, $n = 3$). Silencing was not established at *HMR* in either context without Gal4-Sir1p (Fig. 2A, lanes 15 and 16, or lanes 12 and 13, respectively, and Web table 1). In addition, the level of **a1** mRNA from the chromosomal *HMR* locus or from the excised *HMR* cassette in G_1-arrested cells was similar, with or without Gal4-Sir1p (Fig. 2A, lanes 3 and 5, and lanes 10 and 12; or lanes 5 and 8, and lanes 12 and 15). Thus, excision of *HMR* did not promote or inhibit transcription of **a1** from *HMR*. Also, **a1** mRNA levels in G_1-arrested cells from either the chromosomal *HMR* locus or excised *HMR* cassette was unaffected by expression of Gal4p-Sir1p for at least 1 hour (Fig. 2A, lanes 4 and 5, and lanes 7 and 8). Thus, silencing was established as efficiently on the excised *HMR* cassette as on the chromosomal *HMR* locus, and silencing in either context required both the expression of Gal4-Sir1p and passage through the cell cycle. These results implied that passage from G_1 to G_2/M was required for silencing, in support of earlier findings (*10*, *21*). However, the cell cycle requirement was not passage of the DNA replication fork through *HMR*.

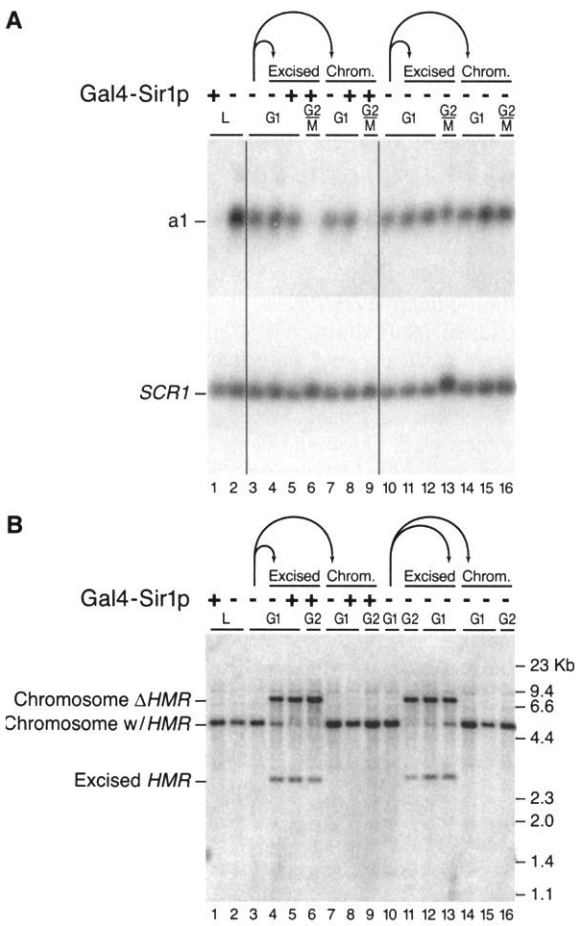

Fig. 2. Establishment of silencing in the absence of DNA replication. Cells were treated as described in Fig. 1 and text. (**A**) RNA analysis of the establishment of silencing. The blot was probed for **a1** mRNA (upper band) and subsequently stripped and reprobed for the *SCR1* loading control (lower band) (*30*). (**B**) Analysis of the excision efficiency of *HMR* and the nonreplication of the excised *HMR*. Cells were treated as described in text and in Fig. 1. Total genomic DNA was harvested, digested with Sna BI and Eco NI, and separated on a 0.7% agarose gel before analysis by DNA blots using a 1697–base pair (bp) probe that hybridized to both the *HMR* cassette and the flanking chromosomal DNA (*30*). This probe detected a 4.6-Kbp fragment from chromosomal *HMR* or a 2.6-Kbp fragment from the excised *HMR* cassette, and a 6.7-Kbp fragment, which harbored the chromosomal locus flanking the excised *HMR* (Fig. 1). Data were quantified using a PhosphorImager (Molecular Dynamics). Expression of Gal4-Sir1p is indicated by + or –. L, log phase cells; G_1, α-factor arrested cells; G_2/M, benomyl and nocodazole arrested cells; Excised, the excised *HMR* cassette; Chrom., *HMR* at the chromosomal locus.

Table 1. The excised *HMR* cassette was not efficiently replicated. Cells were treated as described in Fig. 1. Noted are the final arrests in α factor (10 μg/ml) (G_1) and the subsequent release and rearrest in benomyl (30 μg/ml) and nocodazole (10 μg/ml) (G_2/M). The relative level of excised *HMR* was calculated as the ratio of the PhosphorImager units as follows: [(excised *HMR*/chromosome Δ*HMR* in either G_1 or G_2/M arrest)/(excised *HMR*/chromosome Δ*HMR* in G1 arrest)] × 100 ± σ ; $n = 3$. The ratios during the G_1 arrest and the ratios during the G_2/M arrest either in the presence of absence of Gal4-Sir1p were compared to determine whether they were similar, and data were analyzed by using the one-sided Wilcoxon rank sum test ($P = 0.018$). The ratio during the G_2/M arrest in the presence of Gal4-Sir1p was compared to that in the absence of Gal4-Sir1p to determine whether they were similar, and data were analyzed by using the two-sided Wilcoxon rank sum test ($P = 0.51$). The ratio during the G_1 arrest has been set to 100% to normalize the data between independent experiments, one of which is shown in Fig. 2B.

Expression of Gal4-Sir1p	Stage of arrest	Relative level of excised *HMR* (x 100 ± σ)
Gal4-Sir1p	G_1	100
Gal4-Sir1p	G_2/M	62 ± 4.7
None	G_1	100
None	G_2/M	50 ± 14

The interpretation of the previous experiment hinged critically on knowing whether *HMR* was efficiently excised by FLP recombinase and whether the excised *HMR* had some unanticipated capacity to replicate. Analysis of DNA blots hybridized for *HMR* and for flanking chromosomal DNA resolved both issues. To determine the efficiency of the excision, we hybridized a probe homologous to both the *HMR* locus and chromosomal sequences flanking the site of excision to DNA from cells either expressing FLP recombinase or not, and either expressing Gal4-Sir1p or not (Fig. 2B, lanes 5 and 12) (*27*). The efficiency of excision in G_1 was 91 ± 7.7% ($n = 3$) for cells expressing Gal4-Sir1p and 93 ± 4.7 % ($n = 3$) for cells lacking Gal4-Sir1p (*30*). Thus, most cells expressing FLP recombinase had excised *HMR* from the chromosome before S phase.

To monitor whether the excised chromosomal locus was replicated, we compared the intensity of the hybridization signals from the excised *HMR* in both G_1 and G_2/M arrested cells to those from sequences flanking the site of excision. The flanking chromosomal sequences double each S phase. Whether the excised *HMR* could replicate was resolved by determining if the relative hybridization intensities changed between G_1 and G_2/M (*31*). The ratio from G_1-arrested cells was calculated, and the amount of the excised *HMR* cassette relative to the chromosomal DNA flanking the site of excision was set to 100%

to normalize between experiments (Table 1). If the excised *HMR* cassette did not replicate, this hybridization ratio should have been reduced to 50% at G_2/M. In contrast, if the excised *HMR* replicated, this hybridization ratio should have remained constant (100%). Indeed, this ratio was reduced to an average of 50% in cells lacking Gal4-Sir1p and to an average of 62% in cells containing Gal4-Sir1p (Fig. 2B, Table 1). These two ratios were not significantly different ($P = 0.51$, $n = 3$). Thus, no evidence of replication of either the expressed or silenced excised *HMR* locus was observed. Further analysis establishing that differential degradation of the excised *HMR* relative to the chromosomal locus was not responsible for the differences observed is provided (*30*).

To measure more sensitively whether the excised *HMR* could replicate, we monitored its loss in dividing cells (Fig. 3A). In this experiment, the initial G_1-arrested culture was split in two and the *HMR* cassette was excised by inducing Flp1p in both cultures. In one culture, Gal4-Sir1p was expressed while maintaining the G_1 arrest. However, upon release from G_1, the cells were grown logarithmically and samples were harvested hourly for 8 hours. In this experiment, hybridization to the extrachromosomal *HMR* relative to the flanking chromosomal sequences was reduced by approximately 50% per generation, reflecting a lack of replication of this locus (Fig. 3A). Together, these results confirmed that *HMR* was efficiently excised from the chromosome in G_1 and that the extrachromosomal *HMR* was not efficiently replicated, even after multiple cell divisions.

As an independent measure of whether the excised cassette could replicate, we monitored the passage of the replication fork through both a chromosomal and an excised nonsilenced *HMR* locus (Fig. 3B). In this experiment, *Escherichia coli dam* methylase, which methylates GATC sites, was expressed from the constitutive integrated *URA3* promoter. Passage of the replication fork though a locus during either origin-based or repair-based replication causes transient hemimethylation. Fully methylated sites are sensitive to cleavage by Dpn I, whereas hemimethylated (or nonmethylated) sites are resistant. Thus, passage of a replication fork leads to transient resistance of sites to cleavage by Dpn I. Upon release from G_1, transient resistance to digestion by Dpn I was observed for chromosomal *HMR* but not for excised *HMR*. Thus, a replication fork did not replicate Dpn I sites near **a1** in the excised *HMR*, providing direct physical evidence for lack of replication.

This study provided the first mechanistic test of whether DNA replication was required to form heterochromatin. The *HMR* locus of yeast was silenced efficiently regardless of whether the locus was in the chromosome or excised as a covalently closed circular DNA molecule. This excised *HMR* was not detectably replicated. Nevertheless, passage of the cells from G_1 to G_2/M through S phase was required for silencing *HMR* in either context. It is unlikely that the passage of time itself, rather than cell cycle passage, is required for Gal4-Sir1p to mediate silencing. Expression of Gal4-Sir1p for 4 hours between an arrest in G_2/M and rearrest in early S phase of the subsequent cell cycle did not allow establishment of silencing (*21*), whereas in this study expression for a total of 3 hours between G_1 and G_2/M did. Conceivably, passage of a replication fork through *HMR* might enhance the efficiency of silencing, even though these data demonstrated that it was not required to silence all, or virtually all, *HMR* loci in the population. Moreover, cells containing six *HMR*-bearing plasmids have all *HMR* loci silenced (*32*), indicating that all necessary silencing components are also likely in excess in this study. Therefore, at this level, silencing components do not seem to be limiting for *HMR* silencing. It is unclear what, if any, step would be enhanced by passage of the replication fork.

Two different assays for replication of the excised *HMR* locus detected no replication of this DNA molecule. However, we could not exclude the possibility that a low, undetectable level of DNA replication occurred. It is possible that a small subset of excised molecules could have replicated or that a very small amount of replication, such as repair-coupled DNA synthesis, could have occurred on a larger fraction of the population. The first possibility would not affect our conclusions because we observed complete repression of *HMRa* expression. Thus, even if a few molecules did replicate, the nonreplicated majority of the molecules were silenced.

A low level of repair-coupled synthesis could conceivably deliver replication-coupled proteins like PCNA or CAF1 to the excised *HMR* molecules. Repair synthesis can occur at any time during the cell cycle, even in nondividing cells, and hence would not explain the cell cycle dependence of silencing. Moreover, for repair-coupled synthesis to explain silencing, essentially all of the excised *HMR* molecules must have suffered some damage that requires repair. The only event experienced by all excised *HMR* mol-

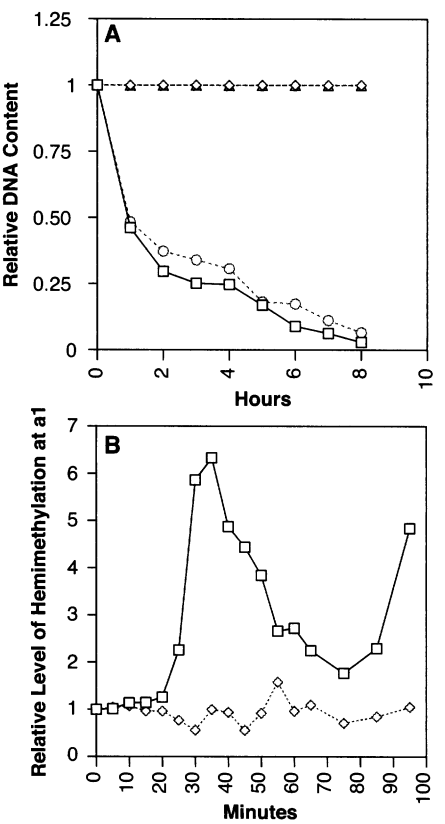

Fig. 3. The excised *HMR* cassette was not replicated. Cells were treated similarly to the description in Fig. 1, except that upon release from the G_1 arrest [0 hours in (A) or 0 min in (B) and Web fig. 1], cells were grown logarithmically and samples were harvested hourly for 8 hours (A) or every 5 min for 60 min and then every 10 min for an additional 30 min (B). Samples were analyzed by DNA blots and quantified using a PhosphorImager. Data represent one experiment from two independent experiments with comparable results. (A) The excised *HMR* cassette was rapidly lost from cycling cells in either the presence or absence of silencing. Samples were analyzed as described in Fig. 2B. The relative DNA content for each time point was calculated as follows: [(excised *HMR*/chromosomeΔ*HMR* at time indicated)/(excised *HMR*/chromosomeΔ*HMR* during G_1)] or [(chromosomeΔ*HMR*/chromosomeΔ*HMR* at time indicated)/(chromosomeΔ*HMR*/chromosomeΔ*HMR* during G_1)]. The doubling time for all cells was about 90 min. (□), excised *HMR* in the absence of Gal4-Sir1p; (◇), chromosomeΔ*HMR* in the absence of Gal4-Sir1p; (○), excised *HMR* in the presence of Gal4-Sir1p; and (△), chromosomeΔ*HMR* in the presence of Gal4-Sir1p. (**B**) Monitoring *HMR* for hemimethylation as a mark for passage of the replication fork using cells that constitutively expressed *dam* methylase (JRY7144). Total genomic DNA was harvested, and analyzed as described in (*30*). The relative level of hemimethylation at **a1** was calculated as follows: [(full-length restriction fragment at time indicated/fragment generated by sensitivity of Dpn I site closest to **a1** at time indicated)/(full-length restriction fragment during G_1/fragment generated by sensitivity of Dpn I site closest to **a1**during G_1)]. The signal from the full- length restriction fragment relative to the fragment generated by sensitivity of Dpn I site closest to **a1** at 0 min was 13 and 14% for the chromosomal locus and excised *HMR* cassette, respectively. (□), chromosome containing *HMR* in the absence of Gal4-Sir1p, (◇), excised *HMR* in the absence of Gal4-Sir1p. FACS analysis of cells from (**B**), indicating that both cultures entered and passed through S phase at the same rate, is available in Web fig. 1 (*30*).

ecules was FLP-mediated recombination. FLP recombinase catalyzes the entire recombination reaction, requiring no other proteins (*26*) and leaving no substrates for repair synthesis. Also, no repair-coupled synthesis of the excised *HMR* molecules was detected by transient resistance to Dpn I cleavage. Thus, it was unlikely that replication-coupled process contributed to the quantitative silencing observed here. We are unaware of any evidence that mutations or damage occurs more readily on circular plasmids in yeast than on the chromosome.

In addition to offering a new mechanistic insight on silencing, these data place renewed importance on the role of proteins involved in DNA replication, such as PCNA, Rfc1p, Asf1p, Dna2p, and CAF-1, in silencing. An important challenge is to learn how those proteins affect silencing when silencing can be mechanistically divorced from both replication initiation and from the passage of a replication fork. PCNA left behind on a previously replicated template can mark that template as "competent" for CAF-1–dependent chromatin assembly (*33*). If PCNA from the previous cell cycle remains associated with *HMR* upon entering the subsequent G_1 phase, it may be excised with *HMR* and therefore available to establish heterochromatin. The efficiency of silencing observed here would require that some feature of *HMR* causes retention of PCNA. Alternatively, these proteins may have a role in silencing other than in its establishment (*18*). For example, once heterochromatin is established at a locus, it must be maintained throughout that cell cycle and duplicated in each subsequent cell cycle. Indeed, recent data underscore the dynamic nature of heterochromatin composition in vivo, even on nonreplicating DNA molecules (*3*). Perhaps proteins like CAF1 and PCNA have a replication-coupled role in the inheritance of heterochromatin at *HMR*, or possibly in its maintenance.

The results of this study have reframed the essential outstanding issues in establishing heterochromatin. One goal now is to learn what replication-independent event happens in this cell cycle window that is essential for silencing. The second goal is to uncover how replication proteins play a role in silencing when replication itself is not required. Both questions should provide fundamental insights into how cells assemble specific structures of chromatin in a spatially and temporally organized manner.

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Establishment of Transcriptional Silencing in the Absence of DNA Replication

Yao-Cheng Li, Tzu-Hao Cheng,* Marc R. Gartenberg†‡

Transcriptional repression of the silent mating-type loci in *Saccharomyces cerevisiae* requires a cell cycle–dependent establishment step that is commonly assumed to involve DNA replication. Using site-specific recombination, we created a nonreplicating DNA ring in vivo to test directly the role of replication in establishment of silencing. Sir1 was tethered to the ring following excision from the chromosome to activate a dormant silencer. We show here that silencing can be established in DNA that does not replicate. The silenced ring adopted structural features characteristic of bona fide silent chromatin, including an altered level of DNA supercoiling and reduced histone acetylation. In addition, the process required silencing factors Sir2, Sir3, and Sir4 and progression between early S and M phases of the cell cycle. The results indicate that passage of a replication fork is not the cell-cycle event required for establishment of silencing in yeast.

Silencing of large chromosomal domains involves specialized, heritable chromatin structures that repress transcription in a gene-independent fashion. The silent *HM* mating-type loci of budding yeast (*HMR* and *HML*) represent well-studied examples of this type of transcriptional control (*1*). Silencing of *HMR* and *HML* is governed by cis-acting sequences, known as the *E* and *I* silencers, which flank both loci and consist of various combinations of sites for Rap1p, Abf1p, and the multisubunit origin recognition complex (ORC). Despite an essential role for ORC in initiation of DNA replication, substantial evidence indicates that this is not its function at silencers. Only a subset of silencers act as chromosomal replication origins and *orc* mutants have been isolated that are defective in replication initiation but not silencing (*2*, *3*). Instead, the function of ORC at silencers appears to be recruitment of Sir1 (*4*–*6*), which, along with the other silencer binding proteins, facilitates incorporation of Sir2, Sir3, and Sir4 into a heterochromatin-like structure, termed silent chromatin. Indeed, artificially

Department of Pharmacology, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA.

*Present address: Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA.

†Member, The Cancer Institute of New Jersey, New Brunswick, NJ 08901, USA.

‡To whom correspondence should be addressed. E-mail: gartenbe@umdnj.edu