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DNA Replication is not Required in Re-establishment of *HMRE* Silencer Function at the *HSP82* Yeast Heat Shock Locus

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We have examined the re-establishment of *HMRE* mediated silencing function on the transcriptional activity of yeast heat shock gene *HSP82*. To test whether the onset of *SIR* repression can occur in growing cells in the presence of a potent inhibitor of DNA replication, *HMRa/HSP82* strains with *sir4*$^-$ and *SIR4*$^+$ genetic backgrounds were arrested in S phase by incubation of a culture in 200 mM hydroxyurea for 120 min. It was clear that following a 20 minute heat shock, silencing of the *HMRa/HSP82* allele in cells pretreated with hydroxyurea does occur in a *SIR4*-dependent fashion, even though the kinetics of repression appears to be substantially delayed. We also have tested whether re-establishment of silencing at the *HMR/HSP82* locus can occur in G1-arrested cells. Cell-cycle arrest at G1 phase was achieved by treatment of early log a cell cultures with α-factor mating pheromone, which induces G1 arrest. The result suggests that passage through S phase (and therefore DNA replication) is not required for re-establishing silencer-mediated repression at the *HMRa/HSP82* locus. Finally, to test whether *de novo* protein synthesis is required for re-establishment of silencer-mediated repression, cells were pretreated with cycloheximide (500 μg/ml) for 120 min. It was apparent that inhibiting protein synthesis delays, but does not prevent, re-establishment of silencer-mediated repression. Altogether, these results indicate that re-establishment of silencer-mediated repression is not dependent on the DNA replication and has no requirement for protein synthesis.

Key words: DNA replication, saccharomyces cerevisiae, silencer

Eukaryotic chromosomes contain many replication origins from which DNA is duplicated faithfully during S phase of the cell cycle (12, 13, 17, 25). Control of the initiation of DNA synthesis is a key regulatory step and the origin of replication is the key *cis*-acting DNA element. Origins of replication have been best studied in the budding yeast *Saccharomyces cerevisiae* (7, 10). Autonomously replicating sequence (ARS) elements, which confer autonomous replication of plasmids, consist of only 100~200 bp including a conserved 11 bp core consensus sequence (7, 38).

It has been suggested that DNA replication and transcription share regulatory mechanisms. First, initiation of viral replication often depends on sequence elements and proteins that also activate transcription (11). Thus, eukaryotic DNA replication and transcription apparently can share common activation mechanisms. Second, activation of transcription of viral genes is dependent on replication of the phage or virus (23, 40). Thus, the process of replication itself can activate transcription. Likewise, several lines of evidence suggest that DNA replication may be involved in transcriptional repression, silencing, in yeast. The first clue came from the discovery that the silencers have ARS activity and that they have matches to the ARS consensus sequence (1, 18). A second clue that DNA replication may play a role in silencing came from a study of the cell cycle requirements for repression of the silent mating-type loci, *HMRa* and *HML α* (29). When *sir3*^{ts} cells are shifted to the nonpermissive temperature, the *HM* loci become transcriptionally derepressed. However, upon a shift from the nonpermissive to the permissive temperature, the recovery of transcriptional repression at the silent mating-type loci requires passage through the S phase of the cell cycle.

Table 1. Yeast strains

Strain	Genotype	Source
IV16-17A	*MATα can1 his4 leu2-3,112 trp1-1 ura3-52 sir4-351*	J.R. Broach
W303-1A	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R.J. Rothstein
W303-1B	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R.J. Rothstein
B-7056	*MATα leu2-3,112 ura3-52 cyc1::CYH2^s cyc7-67 cyh2^r*	F. Sherman
SLY101	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 cyh2^r*	this study
SLY102	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 hsp82::CYH2^s cyh2^r*	this study
SLY103	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 hsp82::CYH2^s sir4△-2::HIS3*	this study
SLY105	*MATα can1 his4 leu2-3,112 trp1-1 ura3-52 sir4-351 HMRE/HSP82*	this study
SLY106	*MATα can1 leu2-3,112 trp1-1 ura3 HMRE/HSP82*	this study
SLY107	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 cyh2^r HMRa/HSP82 sir4△-2::HIS3*	this study
SLY207A	*MATα can1 his3-11,15 leu2-3,112 trp1-1 ura3 ade2-1 sir4△-2::HIS3 HMRa/HSP82 bar1::LEU2*	this study

Cells arrested in G1 fail to restore repression. The majority of cells restore repression within the second round of the cell cycle, and many can restore repression within the first round. Thus, an S phase event, probably DNA replication, is required to establish transcriptional repression at the silent mating-type loci (29). These findings led to the hypothesis that initiation of DNA replication from silencer elements is required to establish repression at the silent mating-type loci.

A direct test of the ARS activity of silencers has been made possible by a replicon mapping technique that uses two-dimensional agarose gel electrophoresis (6, 31). Using this technique, Rivier and Rine have demonstrated that *HMRE* does in fact act as a chromosomal replication origin *in vivo* (35). Interestingly, they showed that transcriptional repression of the *HMR* locus was not required for initiation of replication from *HMRE* and that the individual SIR proteins were not required components of the initiation machinery at the *HMRE* origin (35). Thus, initiation of replication from *HMRE* was not dependent on repression of *HMR*. Surprisingly, chromosomal DNA replication does not initiate at the *HMLE* silencer (15). This result is consistent with the finding of Mahoney *et al.* that some of the mutations that disrupt ARS activity at *HMLE* have little effect on repression (28) and suggests that repression at *HML* is not related to the initiation of DNA replication, at least at *HMLE* itself.

The results described in earlier studies appear to be at odds with current dogma, which says that silencing of the yeast mating-type locus *HMR* must be established during S-phase. Since complete repression at both *HMR/HSP82* alleles is re-established within 15 min, a time that is much shorter than one cell cycle, it is important to determine whether conditional silencing at these genes has a requirement for DNA replication. We have addressed this question in two complementary ways. First, we have tested whether silencing at *HMRa/HSP82* can occur in cells exposed to a chemical inhibitor of DNA synthesis (hydroxyurea). Second, we have tested if silencing at *HMRa/HSP82* can occur in G1-arrested cells using pheromones (27, 37). Finally, we have also tested if re-establishment of silencer function can occur when protein synthesis is inhibited (2).

Materials and Methods

Yeast strains

Yeast strains included in this study and their genotypes are listed in Table 1. All *Saccharomyces cerevisiae* haploid strains constructed for this study are derivatives of W303-1B (*MATα, ade2-1, can1-100, his3-11,15, leu2-3, 112, trp1-1, ura3-1*), IV16-17A (*MATα, can1-100, his4, leu 2-3,112, trp1-1, ura3-52, sir4-351*), and B-7056 (*MATα, leu2-3,112, ura3-52, cyc1::CYH2s, cyc7-67, cyh2^r*). These strains were obtained from R. Rothstein, A. Rose/J. R. Broach, and M. Hampsey, respectively.

Cell-cycle synchronization

Cell-cycle arrest at G1 phase was achieved by treatment of early log a cell cultures with α-factor mating pheromone, which induces G1 arrest. Biologically active α-factor, obtained from Sigma, was added to a final concentration of 500 ng/ml and cell cycle arrest was monitored under the microscope. Pictures were taken using a Olympus T041 microscope equipped with Nomarski optics before and after incubation with α-factor. After 120 min of incubation with α-factor, 95% of cells were unbudded which is the indication of cell cycle arrest.

Thermal up- and down-shift protocol

Sequential heat-shock and recovery experiments were performed in a Reciprocating Water Bath Shaker (New Brunswick) or a Shaker Bath (Forma Scientific). In a typical experiment, 50 ml of culture was subjected to a 30℃ to 39℃ heat-shock for varying times by rapid mixing with an equivalent volume of medium prewarmed to 51℃. Culture was then rapidly returned to non-stressful conditions (30℃) by pouring 50 ml of chilled

medium (4℃). 30 ml of cultures were poured into pre-frozen 50 ml Sarstedt tubes containing sodium azide (20 mM) at appropriate time intervals.

Verification of inhibition of protein synthesis

To verify that cycloheximide was able to block protein synthesis in the experiment to test whether *de novo* protein synthesis is required for re-establishing silencer-mediated repression, the extent of protein synthesis rate determined. Cultures of early log phase cells (1×10^7 cells /ml) were split into two aliquots. Cycloheximide was added to one of them at a concentration of 500 μg/ml (The concentration employed in functional analysis) and two cultures were incubated for 15 min. Cells were collected by centrifugation and the same number of cells were resuspended in SDC^{-Leu} containing ^{3}H-leucine (1 mM, 2 μCi) and incubated for 15 min. Cells were metabolically arrested by addition of sodium azide (20 mM) and then applied to a membrane (Whatman GF/C glass microfibre filters) under vacumn. 5 ml of 10% trichloroacetic acid and 5 ml of 95% ethanol were sequentially added. The membrane was air-dried and radioactivity was determined by scintillation counting.

Probes used

The probe specific for *HSP82* mRNA was a 62-mer antisense oligonucleotide spanning +2226 to +2287 of the transcriptional unit. 5U/5L was an *in vitro* synthesized *HSP82* specific riboprobe spanning the −1300 (*Eco*RI) to −1050 (*Hin*dIII) site using the pGEM-3 based construct for Southern analysis. p1U was *in vitro* synthesized *HSP82* specific riboprobe spanning the −273 (*Hin*dIII) to −174 (*Xba*I) site using pSP65 based construct for MNase nucleosome-protected ladder assay. pSL901 was an *in vitro* synthesized a1 specific riboprobe using pGEM-3 based construct for Northern analysis. pN161 was an *in vitro* synthsized riboprobe specific to the ACT1 gene.

Results

Regaining position effect is delayed in cells pretreated with hyroxyurea

To test whether the onset of *SIR* repression can occur in growing cells in the presence of a potent inhibitor of DNA replication, we performed the following experiment. The basic strategy is depicted in the figure below (Fig. 1.A). Briefly, it involves arresting *sir4*$^-$ and *SIR4*$^+$ cells in S phase by incubation of a culture in 200 mM hydroxyurea for 120 min, upshifting the temperature of the medium from 30℃ to 39℃ for 20 min followed by

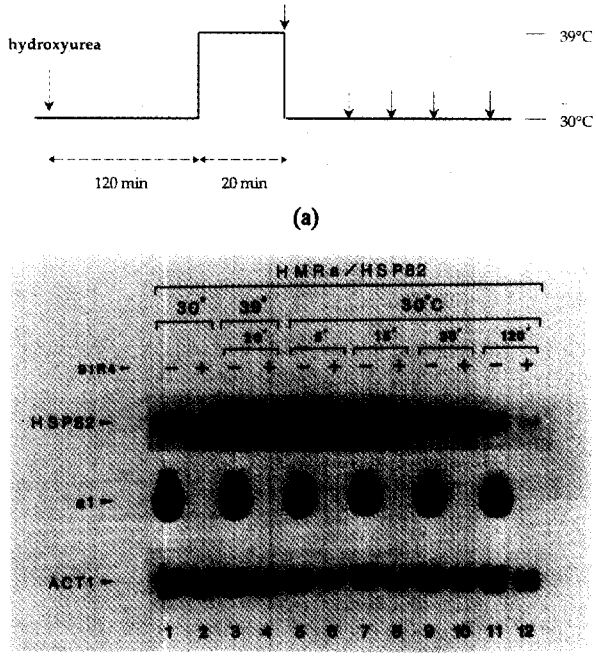

Fig. 1. Kinetics of re-establishment of *SIR4*-dependent repression at the *HMRa/HSP82* locus in cells pretreated with the DNA replication inhibitor, hydroxyurea. A. Experimental strategy for studying the kinetics of re-establishment of silencer funtion in cells pretreated with hydroxyurea. B. Northern analysis of total RNAs from early log cultures of SLY107 pretreated with 200 mM hydroxyurea for 120 min subjected to a sequential thermal upshift/downshift protocol as indicated (39℃ and 30℃). *SIR4*$^+$ and *sir4*$^-$ derivatives were generated by transformation with pJR368 (Fig. 2. top) and a control plasmid, YCP50, respectively.

downshifting to 30℃ for 5, 15, 30, and 120 min (indicated by small arrows). For this study, we employed the *HMRa/HSP82* strain, SLY107. Figure 1.B reveals that following a 20 minute heat shock, silencing of the *HMRa/HSP82* allele in cells pretreated with hydroxyurea does occur in a *SIR4*-dependent fashion, even though the kinetics of repression appears to be substantially delayed (120 min instead of 15 min). This delay is likely more apparent than real, since hydroxyurea appears to also stabilize *HSP82* transcripts (lanes 5, 7, 9, 11 of Figure 1.B). As before, a1 remains strongly repressed within the *HMR* locus at all times.

Re-establishment of silencer-mediated repression can occur in G1-arrested cells

To avoid such complications, we have tested whether re-establishment of silencing at the *HMR/HSP82* locus can occur in G1-arrested cells. Cell-cycle arrest at G1 phase was achieved by treatment of early log a cell cultures with α-factor mating pheromone, which induces

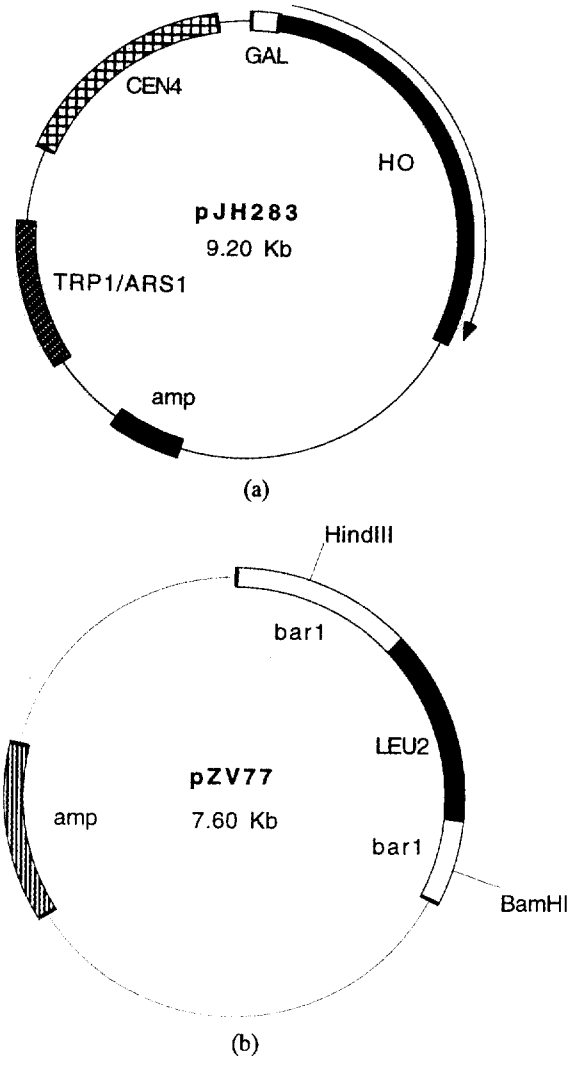

Fig. 2. Plasmid maps of pJH283 and pZV77.

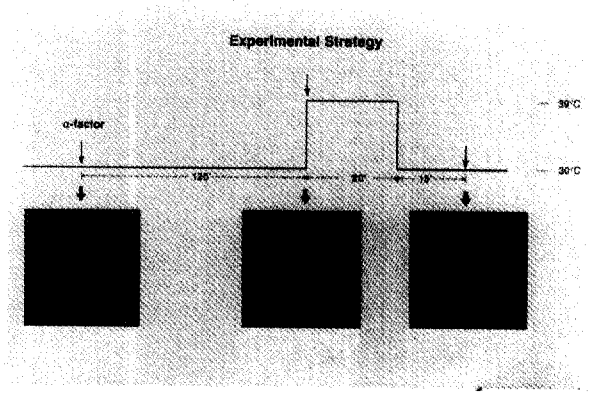

Fig. 3. Experimental strategy for testing whether re-establishment of *SIR4*-dependent repression at the *HMRa/HSP82* locus can occur in cells arrested in the G1 phase of the cell cycle. Top: Experimental protocol for Northern analysis of strain SLY207A (*MATa, HMRa/HSP82*) pretreated with 500 ng/ml α-factor for 120 min subjected to a sequential upshift/downshift protocol. Bottom: Nomarski micrographs of yeast cells before and after treatment with a-factor (cell samples removed at the times indicated).

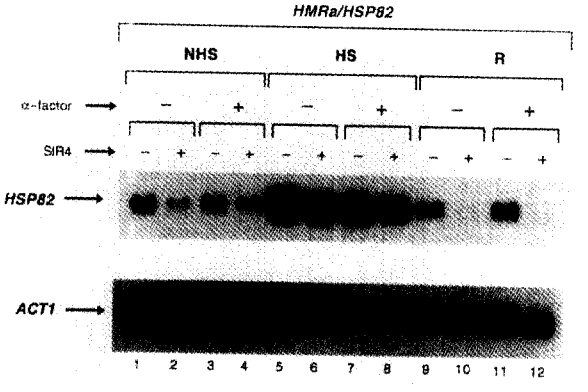

Fig. 4. Kinetics of re-establishment of *SIR4*-dependent repression at the *HMRa/HSP82* locus in cells arrested in G1 phase of the cell cycle. Northern analysis of total RNAs from early log cultures of SLY207A pretreated with a-factor for 120 min subjected to a sequential thermal upshift/downshift protocol as indicated (HS, 39°C for 20 min; R, 30°C for 15 min). *SIR4*$^+$ and *sir4*$^-$ genotypes were generated by transformation with pJR368 and a control plasmid, YCP50, respectively.

G1 arrest (37). To create an a, *HMRa/HSP82* strain that could be arrested with commercially available α-factor, HO endonuclease was introduced into the a strain, SLY 107, to stimulate mating-type switching (24). pJR368 (harboring *SIR4*) containing SLY107 (*MATα, HMRa/HSP82, sir4::HIS3*) was transformed with pJH283 (pGAL-HO; Fig 2.A) and selected on SDC$^{-Ura, -Trp}$. Transformants were streaked on SDC$^{-Ura, -Trp}$ plate. A clonally pure colony was incubated in SRG$^{-Ura, -Trp}$ (0.5% galactose) liquid medium for 4 hours to induce HO expression, then streaked on a SDC^{-Ura} plate (i.e., in the presence of tryptophan) to permit the loss of pJH283. Mating-types of individual colonies were identified. To render the cell supersensitive to α-factor, the *BAR1* gene, encoding a secreted protease that degrades α-factor, was knocked out by one step gene transplacement (36). Positive a strains were transformed with pZV77 (*bar1::LEU2*, digested with *Hi*-*nd*III and *Bam*HI; Fig. 2.B) and selected on SDC^{-Leu}. Transformants were screened by α-factor sensitivity. An a mating-type strain that is supersensitive to α-factor was identified and termed SLY207A (*MATα, HMRa/HSP 82, sir4::HIS3, bar1::LEU2*).

The basic strategy involved arresting *sir4*$^-$ and *SIR4*$^+$ cells in G1, upshifting the temperature of the medium from 30°C to 39°C for 20 min followed by downshifting to 30°C for 15 min (Figure 3). Biologically active α-factor, obtained from Sigma, was added to a final concentration of 500 ng/ml and cell cycle arrest was monitored under

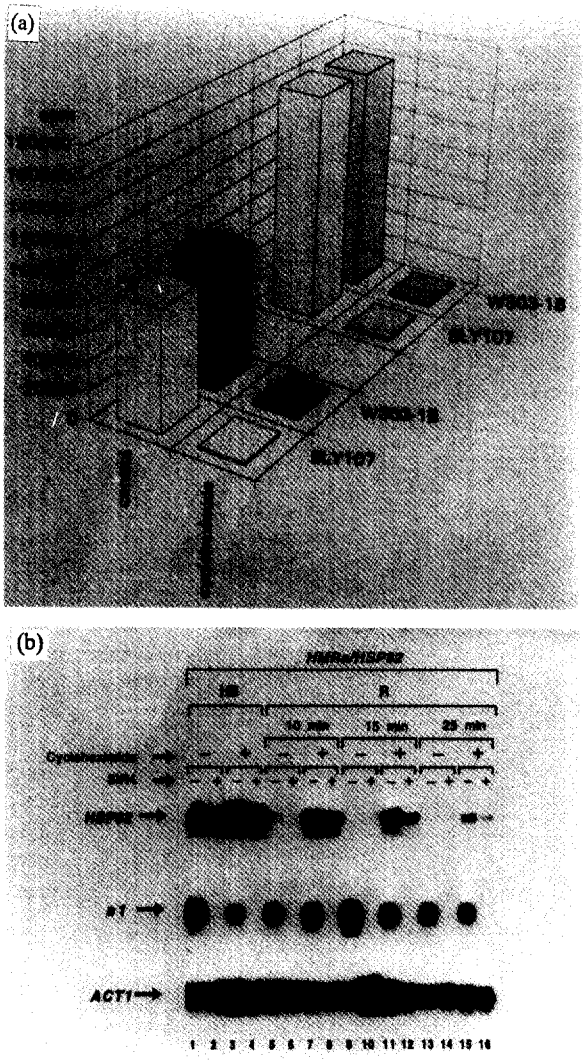

Fig. 5. Kinetics of re-establishment of *SIR4*-dependent repression at the *HMRa/HSP82* locus in cells pretreated with the protein synthesis inhibitor, cycloheximide. A. 3-D graph of the protein synthesis rate of cells ^{3}H-leucine incorporation (cpm) of SLY107 (*HMRa/HSP 82*) and W303-1B (*HSP82*$^{+}$) in the presence or absence of protein synthesis inhibitor, cycloheximide (500 μg/ml). Shown are two sets of experimental data. B. Northern analysis of SLY107 subjected to thermal downshift protocol Northern blot analysis of total RNA isolated from SLY107 pretreated with cycloheximide for 120 min followed by upshift/downshift protocol as before.

the microscope. Light micrographs were taken before and after incubation with α-factor (Figure 3). After 120 min of incubation with α-factor, 95% of cells were unbudded which is the indication of cell cycle arrest. This G1-arrest lasted at least 5 hr, more than enough of a window to perform up- and down-shift experiments. *HSP 82* transcript levels were measured in cells pretreated with α-factor, maintained at 30℃, upshifted to 39℃ for 20 min (HS), and then downshifted to 30℃ for 15 min (R). Figure 4 reveals that the silencer-mediated repression of *HSP82* was re-established even when cell cycle was arrested at G1 (compare lanes 9, 10 vs. 11, 12). This result suggests that passage through S phase (and therefore DNA replication) is not required for re-establishing silencer-mediated repression at the *HMRa/HSP82* locus.

Inhibiting protein synthesis delays re-establishment of silencer-mediated repression

Finally, to test whether *de novo* protein synthesis is required for re-establishment of silencer-mediated repression, *HMRa/HSP82* cells with *SIR4*$^{+}$ and *sir4*$^{-}$ genetic backgrounds were pretreated with cycloheximide (500 μg/ml) for 120 min, heat-shocked at 39℃ for 20 min and then downshifted to 30℃ for 15 min. To verify that cellular protein synthesis was blocked by the addition of 500 μg/ml cycloheximide, the *de novo* incorporation of ^{3}H-leucine was determined. The scintillation counts of both SLY107 and W303-1B cells revealed that *de novo* protein synthesis is blocked (>90%) in the cells pretreated with this concentration of cycloheximide (see Fig. 5.A). As illustrated in the Northern analysis in Fig. 5. B, inhibiting protein synthesis delays, but does not prevent, re-establishment of silencer-mediated repression (see lanes 15 and 16). This delay most likely reflects the requirement for *de novo* protein synthesis for rapid *HSP82* mRNA turnover, upon which the assay is dependent. We conclude that re-establishment of silencer-mediated repression most likely has no requirement for protein synthesis.

Discussion

Intertwining of DNA replication and transcription has been well documented (11). A variety of transcriptional elements have been identified as components of replication origins (5, 14, 26, 39). Likewise, a correlation between transcriptional repression and DNA replication also has been implicated. As discussed in the introduction, there exists strong evidence to support the involvement of DNA replication in yeast mating-type silencing (1, 8, 9, 18, 34). Two simple models can be predicted: either repression at *HMR* is required for replication initiation from the silencer, or that replication initiation from the silencer or another replication origin is required for repression at *HMR*. However, analysis of the replication properties of the *HMRE* silencer in mutant strains defective in *SIR* genes disproves one of the models. Specifically, mutations in any of the four *SIR* genes, which result in derepression of *HMR*, have no detectable effect on replication initiation from *HMRE* (35). Thus, silencing at *HMR* appears to depend on initiation of DNA replication rather than visa versa. That both processes require

the ARS consensus sequence and the protein(s) bound to it has been demonstrated recently (3). A multiprotein complex, the origin recognition complex (ORC), binds specifically to the ARS. ORC is composed of at least 6 different proteins (4), and two of the genes encoding ORC have been genetically identified as regulators of transcriptional silencing, hinting at a role for this complex in both DNA replication and silencing. It is not, however, clear whether silencing is mechanistically dependent on replication. Also unclear is the situation at *HML*. In contrast to *HMR*, some of the mutations that disrupt ARS activity at *HMLE* have little effect on repression (28). One possible interpretation of these results, in combination with experiments that fail to detect the initiation of replication from *HML* (15), is that repression at *HML* has little to do with initiation of replication. Alternatively, *HML* silencing derives from DNA replication initiating at an origin other than *HMLE*. Also, it should be noted that the original studies of yeast telomeric position-effects involved artificially constructed telomeres lacking any known ARS element (21). Our data presented here clearly have demonstrated that DNA replication is not required for re-establishment of *HMRE*-mediated conditional silencing. Re-establishment of repression at the *HMRa /HSP82* locus can occur in cultures blocked at either S or G1 phase of their cell cycles and this event does not require *de novo* protein synthesis.

Different regions of eukaryotic chromosomes are replicated at specific times during S-phase (17). It has been known that active genes are replicated earlier in the S phase and inactive genes are replicated later in the S phase. Sequences affected by position-effect variegation, X chromosome inactivation, and yeast telomeric repression are all replicated late in S-phase (16, 20, 21, 22, 30). Likewise, a distinctive feature of *HMR* and *HML* is that they replicate late in S phase, whereas *MAT* replicates relatively early in the S phase (33). Thus, it is possible that when the silencer was integrated into the ectopic site in chromosome XVI, it might have lost the timing zone. Considering its high transcriptional activity, it is likely that the *HSP82* locus replicates early in S phase. This idea is supported by experiments demonstrating that the timing of initiation of a yeast origin can be controlled by a position effect (19). Proximity to a telomere results in late initiation whereas the same origin located elsewhere in the genome initiates replication early in S phase. It is unlikely that the telomere of chromosome XVI short arm has an effect on the timing of initiation or transcription of *HSP82*, considering the high level of *HSP82* basal expression. The exact role of replication in repression at silent mating-type loci will be understood better when we learn more about the functions of the proteins working at the silencers.

Earlier experiments have indicated that protein synthesis is not required for the induction of chromosomal puffs or for HSF binding to HSE (41). Fig. 5.B shows that when over 90% of ^{3}H-leucine incorporation is suppressed through addition of cycloheximide, the kinetics of re-establishment of silencer-mediated repression are delayed. This delay most likely reflects the requirement for *de novo* protein synthesis for rapid *HSP82* mRNA turnover, the basis of for this assay. Indeed, a previous study has shown that cycloheximide inhibits RNA turnover in *S. cerevisiae* (32). Thus, re-establishment of silencer-mediated repression has no requirement for protein synthesis.

Taken together, we conclude that re-establishment of conditional silencing at the *HMRa/HSP82* locus on chromosome XVI does not require DNA replication, passage through S phase, or *de novo* protein synthesis.

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