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Disturbance of Normal Cell Cycle Progression Enhances the Establishment of Transcriptional Silencing in *Saccharomyces cerevisiae*

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Previous studies have indicated that mutation of *RAP1* (*rap1^s*) or of the *HMR-E* silencer ARS consensus element leads to metastable repression of *HMR*. A number of extragenic suppressor mutations (*sds*, suppressors of defective silencing) that increase the fraction of repressed cells in *rap1^s hmrΔA* strains have been identified. Here we report the cloning of three *SDS* genes. *SDS11* is identical to *SWI6*, a transcriptional regulator of genes required for DNA replication and of cyclin genes. *SDS12* is identical to *RNR1*, which encodes a subunit of ribonucleotide reductase. *SDS15* is identical to *CIN8*, whose product is required for spindle formation. We propose that mutations in these genes improve the establishment of silencing by interfering with normal cell cycle progression. In support of this idea, we show that exposure to hydroxyurea, which increases the length of S phase, also restores silencing in *rap1^s hmrΔA* strains. Mutations in different cyclin genes (*CLN3*, *CLB5*, and *CLB2*) and two cell cycle transcriptional regulators (*SWI4* and *MBP1*) also suppress the silencing defect at *HMR*. The effect of these cell cycle regulators is not specific to the *rap1^s* or *hmrΔA* mutation, since *swi6*, *swi4*, and *clb5* mutations also suppress mutations in *SIR1*, another gene implicated in the establishment of silencing. Several mutations also improve the efficiency of telomeric silencing in wild-type strains, further demonstrating that disturbance of the cell cycle has a general effect on position effect repression in *Saccharomyces cerevisiae*. We suggest several possible models to explain this phenomenon.

Mating type genes in *Saccharomyces cerevisiae* are subject to a position effect (reviewed in reference 27). When located at the *MAT* locus, near the centromere of chromosome III, mating type genes are transcribed. However, identical copies of these genes, located near the telomeres of the same chromosome, are transcriptionally repressed. The silent (*HM*) mating type loci are repressed by the action of *cis* regulatory elements (silencers) and the products of the four *SIR* (silent information regulator) genes. Efficient silencing at *HM* loci also requires the highly conserved N terminus of histone H4 (24, 32, 36), suggesting the involvement of an altered chromatin structure. A similar Sir- and histone H4-dependent mechanism of silencing also operates at telomeres in *S. cerevisiae* (2, 19). Because telomeric silencing is variegated and spreads to varying extents away from the telomere in different cells, silencing in yeast cells has been compared to position effect variegation in *Drosophila melanogaster* (39).

A series of observations link transcriptional silencing in yeast cells to DNA replication. Initial characterization of the four silencer elements flanking the two *HM* loci indicated that they all have ARS (autonomously replicating sequence) activity (1, 16). Subsequent dissection of two of these elements (*HMR-E* and *HML-E*) showed that they contain ARS consensus sequences that are important silencer regulatory elements (8, 25, 29). More recent studies have shown that the ARS consensus sequence binding proteins (the origin recognition complex) play a direct role in repression (6, 18, 33). Finally, Miller and Nasmyth (34), using a *sir3^{ts}* mutation to turn silencing on and off by shifting temperature, showed that progression through the S phase of the cell cycle is required for the reestablishment of repression.

Studies by Pillus and Rine (38) suggested that the establishment of silencing might involve a special mechanism acting during DNA replication, separate from that required for the maintenance of repression throughout the rest of the cell cycle. They showed that the Sir1p is not required for the maintenance of silencing but instead seems to be essential for the efficient reestablishment of repression. This conclusion followed from the observation that *sir1* mutants exist as a mixed population of repressed and derepressed cells. In contrast, the other three *SIR* genes are absolutely required for the maintenance of repression, and cells mutated in any of these genes are uniformly derepressed at *HM* loci and telomeres. Pillus and Rine argued that when silencing is lost in *sir1* cells (a rare, stochastic event), it is inefficiently reestablished because Sir1p plays an important role in this process. The idea of a special establishment function in silencing received additional support when it was discovered that *cis*-acting silencer mutations (30, 49) and mutations in a silencer binding protein, Rap1 (*rap1^s* [46, 47]), lead to epigenetic switching of transcriptional states at either *HML* or *HMR*.

We have taken a genetic approach to understanding the establishment of repression at *HMR* by focusing on the silencing-defective *rap1^s* mutations. Four *rap1^s* alleles were isolated in an *hmrΔA* background and found to affect the silencing functions of Rap1p while leaving its essential activation functions intact (48). In this paper, we deal only with two alleles: *rap1-12*, which was the most strongly derepressing allele, and *rap1-13*. To gain further insight into the molecular defect of these mutants, a large number of extragenic suppressors that could restore repression in an *hmrΔA rap1^s* strain (49) were isolated. These suppressors were hypothesized to enhance the establishment of transcriptional silencing at *HMR*. Here we report the cloning and identification of three of these suppressor genes and present evidence in support of the idea that they

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act by causing a disruption of progression through the cell cycle that favors the reestablishment of silencing, both at *HMR* and at telomeres.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in this study and their genotypes are listed in Table 1. All strains are derived from W303-1B (51). Yeast strains were grown and manipulated by standard procedures (43). For the color assays, strains were plated on rich medium (YEPD) at 30°C for 2 to 3 days and then shifted to 4°C for at least 24 h before being photographed.

Complementation of *sds* mutations. *rap1^s sds hmrΔA::TRP1* strains (which are phenotypically Trp$^-$) were transformed with a *CEN*-based genomic yeast library via the lithium acetate method (21). Transformants were selected on plates lacking uracil and then screened for complementation of the *sds* mutation (which should render the strain phenotypically Trp$^+$) by replica plating to plates lacking both uracil and tryptophan. Plasmids were then isolated from Ura$^+$ Trp$^+$ transformants and retransformed into the above *rap1^s sds hmrΔA::TRP1* strain to confirm that complementation of the *sds* mutation was plasmid linked. The plasmid was also introduced into a *trp1-1* strain by transformation to determine whether or not the insert carried *TRP1*, which could also confer a Trp$^+$ phenotype. Once these two criteria were satisfied, the plasmids were further analyzed by restriction endonuclease digestion and sequence analysis.

Growth of cells in hydroxyurea. Hydroxyurea (Sigma) was diluted from a filter-sterilized 1 M stock solution into both liquid and solid media to concentrations from 1 to 200 mM. To determine what concentration of hydroxyurea affected S phase in strain W303-1B, YEPD cultures containing from 1 to 200 mM hydroxyurea were inoculated with an equal number of cells and allowed to incubate in a shaker at 30°C overnight. The optical density at 600 nm was then measured, and a budding index was also done for some cultures to observe the cell morphology and to note the proportion of cells having small and large buds compared with cells grown in the absence of hydroxyurea. Tenfold serial dilutions of *rap1^s hmrΔA::TRP1* strains were spotted onto plates of SC medium with 0, 10, 20, 25, 30, and 40 mM hydroxyurea and onto SC plates lacking tryptophan at the same concentrations of hydroxyurea. Plates were incubated at 30°C for 2 days before being photographed.

Strain constructions. Each of the gene disruptions made or received as gifts from others was used to transform a *rap1^s hmrΔA::TRP1* diploid (YDV42-44) that is heterozygous for the *rap1-12* and *rap-1-13* alleles. The disruptions were selected by growth in the absence of the amino acid, and all disruptions were confirmed by Southern analysis. Diploids were then sporulated and dissected. Plasmid pMA1186 was a gift from A. Hoyt. It contains the *cin8::LEU2* construct described previously (20). The *clb6::LEU2* disruption construct was a gift from E. Schwob and K. Nasmyth. It has the *Mlu*I-*Nsi*I fragment of *CLB6* replaced with the *LEU2* gene cloned into pKS Bluescript. The *clb6::LEU2* construct was liberated by digestion with *Xba*I and *Bgl*II for transplacement. The *mbp1::URA3* disruption construct was a gift from C. Koch and K. Nasmyth. It has 2.7 kb between the *Eco*RI sites in *MBP1* replaced by the *URA3* gene. The plasmid (containing a pKS Bluescript backbone) was digested with *Xba*I in order to release the disruption fragment for transplacement. *cln3::URA3* was a gift from F. Cross. The YIp5-based plasmid contained the 3′ *Hin*dIII-*Xho*I fragment and the 5′ *Eco*RI-*Xho*I noncoding sequence of *CLN3* cloned into the *Hin*dIII and *Sal*I sites of YIp5. This construct was linearized with *Eco*RI, and the transplacement resulted in *URA3* replacing the *Xho*I-*Hin*dIII fragment of *CLN3*. Plasmid p101-1D, a gift of F. Cross, contains the dominant *CLN3-2* mutation cloned into a derivative of YCp50 that has a *Hpa*I-*Sma*I deletion which removes the centromere sequences, thus allowing the construct to be integrated. The plasmid was cut with *Sac*I for targeting to the *CLN3* locus. Plasmid pJ098, a gift from R. Tabtiang, has an *Xba*I-*Sma*I fragment of *HIS3* inserted into the *Xba*I and *Pvu*I sites of *SWI4*. The plasmid was cut with *Eco*RI and *Sal*I to release the disruption construct for transplacement.

The *clb5::URA3* construct was made as follows. The *CLB5* gene (a gift of F. Cross) was subcloned as a 3-kb *Xho*I-*Cla*I fragment into pRS316. A *Hin*dIII fragment containing the *URA3* gene was used to replace *CLB5* sequences from the 5′ *Hin*dIII site up to the *Hin*dIII site in the polylinker of pRS316. Sequences from the *CLB5* 3′ end were added in a three-way ligation with an *Eco*RI-*Xho*I fragment containing a *clb5::URA3* disruption, a 3′ *Cla*I-*Eco*RI fragment of *CLB5*, and the vector pIC19H, cut with *Cla*I and *Xho*I. Digestion with *Cla*I and *Xho*I liberated the disruption construct. *CLB2* was subcloned as a 2.8-kb *Eco*RI fragment into pIC20R. The internal 1.34-kb *Spe*I-*Xba*I fragment was replaced with the *HIS3* gene on an *Xba*I fragment. The *clb2::HIS3* disruption construct was released by digestion with *Eco*RI for transplacement. The *SDS11* clone, which contains a genomic library fragment containing the wild-type *SWI6* gene, was used to make a gene disruption. The plasmid was digested with *Xba*I, which removes 1.3 kb of 3′ coding sequence as well as additional downstream sequences, and an *Xba*I fragment containing the *HIS3* gene was used to replace these sequences. The disruption construct was released by digestion with *Cla*I and *Bam*HI.

Confirming the identity of putative *SDS* gene clones. To confirm that the *SDS15*-complementing clone actually contained the *SDS15* gene, a 1.1-kb *Eco*RI-*Bgl*II restriction fragment was cloned into pRS306, a *URA3*-containing plasmid, and integrated by homologous recombination of the insert DNA into the chromosome of a *rap1-13 SDS15 hmrΔA::TRP1 ura3-1* strain. The *SDS15* locus was then mapped in relation to the integrated *URA3* marker by mating this transformant to a *rap1-13 sds15-1 hmrΔA::TRP1* strain. Among the 21 tetrads analyzed, every Ura$^+$ segregant was also Trp$^+$, indicating that the complementing clone mapped at or very near to *SDS15*. In a similar manner, a 0.9-kb *Xba*I-*Bgl*II fragment was used to confirm that the *SDS11*-complementing clone contained the *SDS11* gene. Among the 22 tetrads dissected, every Ura$^+$ segregant was also Trp$^+$, indicating that the complementing clone mapped at or near *SDS11*.

Other techniques. Southern and Northern (RNA) blot analyses were performed essentially as described before (4). Assays for cell growth on selective (SC-Trp) medium (spot assays) were performed as described before (48). Briefly, strains were grown overnight in YEPD medium, and 10-fold serial dilutions of each strain were prepared in water. Aliquots (5 μl) of each dilution were then spotted directly onto SC plates, to measure the number of viable cells, and onto SC plates lacking tryptophan, which assayed the level of derepression of the *TRP1* reporter gene located at *HMR*.

RESULTS

***SDS11* is identical to *SWI6*, a transcriptional regulator of cyclins and other cell cycle-regulated genes.** Two alleles of *sds11* were isolated as strong suppressors of the *rap1^s* allele *rap1-13* (50). The *SDS11* gene was cloned from a yeast genomic library by complementation of an *sds11-1 rap1-13 hmrΔA::TRP1* strain, YRS83 (see Materials and Methods). Twelve complementing plasmids were isolated. All complemented the *sds11-1* mutation upon retransformation and appeared not to contain the *TRP1* gene. These 12 plasmids all contained common restriction fragments, and one was chosen for further analysis. An internal *Xba*I deletion of this clone (which contained three *Xba*I sites unique to the insert) abolished the ability of the plasmid to complement the *sds11-1* mutation. A 0.9-kb *Eco*RI-*Bgl*II fragment which overlapped some of the sequence between the *Xba*I sites was subcloned and sequenced. Comparison with the GenBank database showed that this subclone contained a portion of *SWI6*, a gene involved in regulating the cell cycle-specific transcription of a large number of genes (10, 13, 28). From the published sequence of *SWI6*, a 2.4-kb *Xho*I-*Bgl*II subclone predicted to contain just the *SWI6* open reading frame was constructed and shown to partially complement the *sds11-1* mutation (data not shown).

To determine whether a disruption in the *SWI6* open reading frame would confer an *sds* phenotype, we replaced sequences between the *Xba*I sites of the *SDS11* clone with the *HIS3* gene and transformed a *rap1^s hmrΔA::TRP1* diploid, YDV43, with this *sds11::HIS3* disruption (see Materials and Methods). After confirmation of the gene disruption by Southern blot analysis, the diploids were sporulated, and haploid *rap1^s sds11::HIS3 hmrΔA::TRP1* segregants were tested for the extent of repression at *HMR*. Serial dilutions of each strain were placed on synthetic complete medium or medium lacking tryptophan. Derepression of the *TRP1* reporter gene enables the strain to grow on plates without tryptophan, while repression of the locus results in an auxotrophic phenotype and thus an inability to grow on plates lacking tryptophan. As shown in Fig. 1, the *sds11::HIS3* allele is able to restore silencing in *rap1-12 hmrΔA* strains, so that repression is as efficient as in an isogenic *RAP1* strain, YLS59 (Fig. 1). We conclude from these data that *SDS11* is allelic to *SWI6*.

Mutations in both *SWI6* partners, *MBP1* and *SWI4*, restore repression in *rap1^s hmrΔA* strains. Swi6p is known to regulate the cell cycle-specific transcription of two different classes of genes, those having MCBs (*Mlu*I cell cycle boxes) and those having SCBs (SWI4/6 cell cycle boxes) in their upstream regulatory sequences. Since we found that mutations of *SWI6* could suppress a *rap1^s hmrΔA::TRP1* strain, we wanted to

TABLE 1. Yeast strains used in this study

Strain	Relevant genotype or description	Source or reference
YDS2	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
YDS3	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
YDS38	YDS3 *hmrΔ77-144::sup4-0*	7
YLS59	YDS38 *hmrΔA::TRP1*	48
YLS238	YLS59 *rap1-12*	48
YLS239	YLS59 *rap1-13*	48
YLS497	YDS59 *rap1-13::URA3*	L. Sussel
YLS506	YDS59 *rap1-12::URA3 MAT***a**	L. Sussel
YDV32	YDS59 *rap1-12::LEU2 MAT***a**	D. Vannier
YDV33	YDV32 *MAT*α	D. Vannier
YDV36	YDS59 *rap1-13::HIS3*	D. Vannier
YDV42	YLS506 × YDV36	D. Vannier
YDV43	YDV32 × YLS497	D. Vannier
YDV44	YDV32 × YDV36	D. Vannier
YRS83	YLS59 *rap1-13::LEU2 sds11-1*	50
YRS87	YLS59 *rap1-13::LEU2 sds12-1*	50
YRS111	YLS59 *rap1-13::LEU2 sds15-1*	50
HLY79	YDV33 *mbp1::URA3*	This study
HLY83	YLS506 *clb6::LEU2 MAT*α	This study
HLY87	YLS506 *cin8::LEU2 MAT*α	This study
HLY95	YDV33 *clb5::URA3*	This study
HLY99	YDV33 *clb2::HIS3*	This study
HLY103	YLS506 *clb5::URA3 clb6::LEU2 MAT*α	This study
HLY108	YDV33 *CLN3-2::URA3*	This study
HLY137	YDV33 *swi4::HIS3*	This study
HLY165	YDV33 *cln3::URA3*	This study
HLY248	YDV33 *swi6::HIS3*	This study
HLY151	YLS59 *sir1::LEU2*	This study
HLY113	HLY151 *clb5::URA3*	This study
HLY159	HLY151 *swi4::HIS3*	This study
HLY272	HLY151 *swi6::HIS3*	This study
YLS178	YDS3 *hmrΔA*	L. Sussel
HLY308	YLS178 *clb2::HIS3*	This study
HLY309	HLY308 *rap1-12::URA3*	This study
HLY310	YLS178 *clb5::URA3*	This study
HLY311	HLY310 *rap1-12::LEU2*	This study
HLY312	YLS178 *mbp1::URA3*	This study
HLY313	HLY312 *rap1-12::LEU2*	This study
HLY314	YLS178 *cln3::URA3*	This study
HLY315	HLY314 *rap1-12::LEU2*	This study
HLY316	YLS178 *swi4::HIS3*	This study
HLY317	HLY316 *rap1-12::LEU2*	This study
HLY318	YLS178 *swi6::HIS3*	This study
HLY319	HLY318 *rap1-12::LEU2*	This study
HLY320	YLS178 *clb6::LEU2*	This study
HLY321	HLY320 *rap1-12::URA3*	This study
HLY322	HLY320 *rap1-13::HIS3*	This study
HLY440	YDS2 *ADE2* at telomere VIIL	A. Lustig
HLY441	HLY440 *MAT*α	This study
HLY442	HLY440 *rap1-12::LEU2*	This study
HLY443	HLY442 *MAT*α	This study
HLY445	HLY441 *mbp1::URA3*	This study
HLY447	HLY443 *mbp1::URA3*	This study
HLY448	HLY440 *clb5::URA3*	This study
HLY449	HLY442 *clb5::URA3*	This study
HLY451	HLY441 *clb2::HIS3*	This study
HLY453	HLY443 *clb2::HIS3*	This study
HLY455	HLY441 *cln3::URA3*	This study
HLY456	HLY443 *cln3::URA3*	This study
HLY458	HLY441 *swi6::HIS3*	This study
HLY460	HLY443 *swi6::HIS3*	This study
HLY461	HLY441 *swi4::HIS3*	This study
HLY463	HLY443 *swi4::HIS3*	This study
HLY172	YDS2 *RAP1::URA3 hmrΔA::ADE2*	This study
HLY502	HLY172 *RAP1 sir4::LEU2 MAT*α	This study
HLY562	HLY172 × HLY502	This study
HLY348	YDS2 *RAP1::HIS3 cln3::URA3 hmrΔA::ADE2*	This study
HLY514	HLY348 *RAP1 sir4::LEU2 MAT*α	This study

Continued on following page

TABLE 1—*Continued*

Strain	Relevant genotype	Source or reference
HLY563	HLY348 × HLY514	This study
HLY184	YDS2 *RAP1*::*HIS3 mbp1*::*URA3 hmrΔA*::*ADE2*	This study
HLY516	HLY184 *RAP1 sir4*::*LEU2 MATα*	This study
HLY564	HLY184 × HLY516	This study
HLY180	YDS2 *RAP1*::*HIS3 clb5*::*URA3 hmrΔA*::*ADE2*	This study
HLY518	HLY180 *RAP1 sir4*::*LEU2 MATα*	This study
HLY565	HLY180 × HLY518	This study
HLY251	YDS2 *RAP1*::*URA3 swi4*::*HIS3 hmrΔA*::*ADE2*	This study
HLY536	HLY251 *RAP1 sir4*::*LEU2 MATα*	This study
HLY566	HLY251 × HLY536	This study
HLY275	YDS2 *RAP1*::*URA3 swi6*::*HIS3 hmrΔA*::*ADE2*	This study
HLY537	HLY275 *RAP1 sir4*::*LEU2 MATα*	This study
HLY567	HLY275 × HLY537	This study
HLY174	YDS2 *RAP1*::*URA3 clb2*::*HIS3 hmrΔA*::*ADE2*	This study
HLY538	HLY174 *RAP1 sir4*::*LEU2 MATα*	This study
HLY568	HLY174 × HLY538	This study

determine if a particular subset of genes, MCB controlled or SCB controlled, was involved in silencing. We therefore constructed disruptions of two genes whose products have been shown to interact with Swi6p to control either MCB- or SCB-regulated genes.

Mbp1p has been demonstrated to be a component of the complex that, along with Swi6p, binds upstream of MCB-regulated genes, including *RNR1*, *POL1*, and *CLB5* (26). A disruption of *MBP1* was integrated into the chromosome of a *rap1^s hmrΔA*::*TRP1* diploid. The transformed diploid was sporulated, and haploid *rap1-12 mbp1*::*URA3 hmrΔA*::*TRP1* segregants (HLY79) were assayed for the level of derepression at *HMR* by testing their ability to grow on plates lacking tryptophan. The *mbp1*::*URA3* mutation was able to suppress the *rap1-12 hmrΔA* mutations partially compared with an isogenic strain, YLS238 (Fig. 1).

Swi4p and Swi6p interact directly with each other to form a complex that binds SCBs (13, 45). These elements are located upstream of many genes, including those for *HO* (which encodes the endonuclease that initiates mating type switching) and the G_1 cyclins (*CLN1*, *CLN2*, and *CLN3*) (10, 35). A disruption of *SWI4* was integrated into YDV43. After sporulation and dissection of this diploid, the level of silencing of *rap1-12 swi4*::*HIS3 hmrΔA*::*TRP1* haploid segregants (HLY137) was assayed. The *swi4*::*HIS3* disruption was able to silence the expression of *TRP1* caused by the *rap1-12 hmrΔA* mutations strongly (Fig. 1). Thus, both the *mbp1* and *swi4* mutations were able to suppress these mutations. Since *MBP1* and *SWI4* appear to regulate different sets of genes, these results argue that the restoration of silencing is not caused by the failure to express a specific gene or set of genes. Instead, they suggest that a more general perturbation in the regulated expression of any one of several classes of genes needed for normal cell cycle progression might be capable of restoring repression.

Hydroxyurea restores silencing in *rap1^s hmrΔA*::*TRP1* strains. It has been reported previously that cells with a mutation in *swi6* have a prolonged S phase (28). Since *sds11* was an allele of *SWI6*, we decided to test whether the S-phase delay induced by the DNA synthesis inhibitor hydroxyurea would also result in an *sds* phenotype. *rap1^s hmrΔA*::*TRP1* strains YLS238 and YLS239 were grown with increasing concentrations of hydroxyurea from 1 to 200 mM. We then did a budding index to determine the concentration of hydroxyurea that affected the cell cycle without greatly affecting viability (data not shown). *rap1^s hmrΔA*::*TRP1* strains were assayed for derepression of the *TRP1* gene by checking their growth on plates containing 10 to 40 mM hydroxyurea and lacking tryptophan. As shown in Fig. 2, *rap1^s hmrΔA*::*TRP1* strains grown on 20 mM hydroxyurea were both significantly suppressed compared with an isogenic *RAP1* strain, YLS59. This suggests that a general lag in S phase caused by either genetic or chemical means is able to enhance silencing in *rap1^s hmrΔA*::*TRP1* strains.

***SDS12* and *SDS15* are genes required for cell cycle progression.** Among the collection of 21 *SDS* complementation groups, 5 shared the additional phenotype of being temperature sensitive for growth. These mutants have the additional phenotypes of slow growth (at 30°C) and clumpiness (50). We

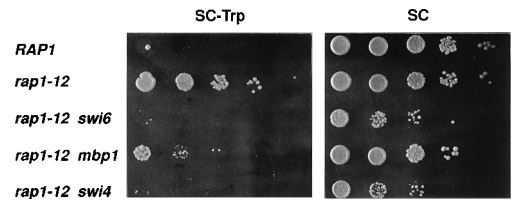

FIG. 1. Mutation of the cell cycle transcriptional regulators *SWI6*, *SWI4*, and *MBP1* restores silencing in *rap1-12 hmrΔA* strains. All of the strains tested have the *TRP1* gene placed at *HMR* as well as a mutation in the ARS consensus element at the *HMR-E* silencer (*hmrΔA*::*TRP1*). Cells were grown overnight in rich (YEPD) medium, and then 10-fold serial dilutions in water were placed onto complete synthetic medium (SC) and medium lacking tryptophan (SC-Trp) to assay for expression of the *hmrΔA*::*TRP1* reporter.

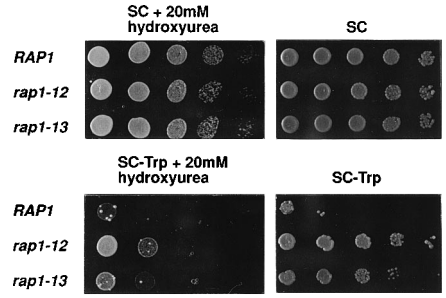

FIG. 2. Sublethal doses of hydroxyurea can restore silencing in *rap1^s hmrΔA* strains. All strains contain an *hmrΔA*::*TRP1* reporter and either a wild-type *RAP1* or *rap1^s* allele (*rap1-12* or *rap1-13*). Expression of the *hmrΔA*::*TRP1* reporter was assayed as described in the legend to Fig. 1 except for the addition of hydroxyurea (20 mM) to plates, as indicated.

cloned the *SDS12* gene by transforming a *rap1-13 sds12-1 hmrΔA*::*TRP1* strain (YRS87) with a yeast (*CEN URA3*) genomic library and selecting for growth at 37°C on medium lacking both uracil and tryptophan. Eight transformants were able to grow under these conditions, suggesting that they contained plasmids that could complement both the temperature-sensitive lethality and silencer suppression phenotypes of the *sds12-1* mutation. Plasmids were isolated from these eight strains and shown to contain inserts with common restriction fragments.

Partial DNA sequence analysis of one of these clones revealed a region of 78% homology to *RNR3* (*DIN1*), the damage-inducible regulatory subunit of ribonucleotide reductase. It had previously been reported that *RNR1* and *RNR3* are about 80% homologous (14, 52). Conversion of the putative *SDS12* sequence to the amino acid sequence revealed two cysteines that have been implicated in the catalytic reduction of ribonucleotides. Additionally, the locations of restriction endonuclease cleavage sites in a 5.9-kb *Kpn*I subclone that complements the *sds12-1* suppression phenotype are identical to those of *RNR1* (13a). Fluorescence-activated cell sorting (FACS) analysis of an *sds12-1* strain showed that it progressed slowly through S phase when grown at the permissive temperature. This delay was relieved when the complementing clone was introduced (data not shown). Thus, the clone is also able to restore normal progression through the cell cycle to an *sds12-1* strain. We conclude from these results that *sds12-1* is an allele of *RNR1*, the major regulatory subunit of ribonucleotide reductase. This finding was striking because ribonucleotide reductase is composed of a catalytic (*RNR2*) and a regulatory (*RNR1*) subunit. Hydroxyurea, which has also been shown to give an *sds* phenotype (see above), inhibits DNA synthesis by abolishing the ability of the catalytic subunit of this enzyme to function. Thus, interference with the activity of both subunits of ribonucleotide reductase can restore silencing in *rap1^s hmrΔA* strains.

A second temperature-sensitive mutation, *sds15-1*, was isolated as an extragenic suppressor of the *rap1-13* mutation (50). The wild-type *SDS15* gene was cloned by the same strategy described for *SDS11*. Comparison of a partial DNA sequence from the *SDS15* clone with the GenBank database revealed that it was identical to *CIN8*, which encodes a kinesin-related protein (20). It had previously been demonstrated that a *cin8* temperature-sensitive mutation resulted in a block in the G_2 phase of the cell cycle at the nonpermissive temperature. We confirmed by observing the cell morphology of YRS111 that the *sds15-1* mutation resulted in a G_2 block at 37°C and showed by FACS analysis that it had a G_2 delay at 30°C. Furthermore, this delay was relieved by the introduction of the complementing clone, returning the FACS profile to that of a wild-type strain (data not shown). Additionally, pMA1125, provided by A. Hoyt, which contains *CIN8* on a *CEN*-based plasmid, was able to complement the *sds15-1* mutation. To determine if a null mutation of *CIN8* would also have the ability to suppress the silencing defect of *rap1^s hmrΔA* strains, a *cin8*::*LEU2* disruption was transplaced into YDV42. After the correct transplacement was confirmed by Southern blot analysis, the diploid was sporulated, and *rap1-12 cin8*::*LEU2 hmrΔA*::*TRP1* segregants (HLY87) were tested for their ability to restore silencing by checking the level of growth on plates lacking tryptophan. The *cin8*::*LEU2* mutation was able to suppress the silencing defect of a *rap1-12 hmrΔA* strain (data not shown).

Effect of cyclin mutations on silencing. Because mutations in genes that resulted in elongation of the cell cycle (*cin8* causes a prolonged G_2, while *rnr1* and *swi6* cause a prolonged S phase) were able to restore repression in *rap1^s hmrΔA*::*TRP1* strains, we wanted to determine whether disruptions in any cyclin gene affecting progression through the cell cycle would also restore silencing in these strains. *CLB2*, a mitotic B cyclin, has been shown to be expressed in G_2 phase and is involved in the assembly and maintenance of the mitotic spindle (17). A mutation in this gene also results in an elongated G_2 phase (47). To test the ability of a *clb2* disruption to restore silencing in *rap1^s hmrΔA*::*TRP1* strains, we transplaced a *clb2*::*HIS3* mutation into YDV43 (see Materials and Methods). The resulting strain was induced to sporulate, and *rap1-12 clb2*::*HIS3 hmrΔA*::*TRP1* segregants (HLY99) were assayed for the level of derepression of the *TRP1* reporter gene. The *clb2* mutation could strongly suppress the *rap1-12 hmrΔA* mutations (Fig. 3), as judged from the failure of these strains to grow in the absence of tryptophan. We confirmed by FACS analysis that the *clb2* strain displayed delayed progression through the G_2 phase of the cell cycle relative to an isogenic parent strain (data not shown).

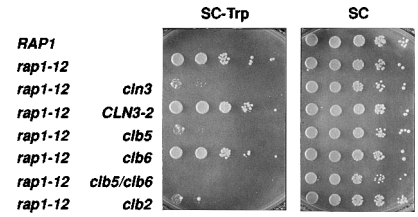

FIG. 3. Mutations in several cyclin genes can restore silencing in a *rap1-12 hmrΔA*::*TRP1* background. Expression of the *hmrΔA*::*TRP1* reporter was assayed as described in the legend to Fig. 1 in strains containing *RAP1* or *rap1-12* alleles together with mutations in different cyclin genes.

Two B-type cyclins, Clb5p and Clb6p, are present in late G_1/S phase and are involved in the timely progression through S phase (15, 44). A mutation in *CLB5* results in severely retarded progression through S phase, while mutations in *CLB6* have no apparent effect on the cell cycle (44). A *clb5*::*URA3* disruption was integrated into YDV44, a *rap1^s hmrΔA*::*TRP1* diploid, and used to generate HLY95. FACS analysis showed that the *clb5* strain had a prolonged S phase (data not shown). The *clb5*::*URA3* mutation was able to suppress the *rap1-12 hmrΔA* mutations fully (Fig. 3). A similar procedure was followed for the *clb6*::*LEU2* disruption. However, this mutation, which by itself does not cause a cell cycle delay, proved to be unable to suppress *hmrΔA*::*TRP1* strains with either the stronger *rap1-12* (HLY83) or the weaker *rap1-13* allele (Fig. 3 and data not shown). These results indicate that not all cyclin mutations suppress the *rap1^s hmrΔA* silencing defect and support the idea that suppression is a consequence of cell cycle delay.

One of the possible effects of lengthening the S or G_2 phase is a shortening of the G_1 phase. To test the possibility that a shorter G_1 was responsible for suppression of the *rap1^s hmrΔA* mutations, a *CLN3-2* allele was integrated into the chromosome of YDV44, and this strain was then dissected to generate HLY108. The *CLN3-2* mutation results in a hyperstable form of Cln3p because of a lack of the cyclin destruction sequences at the C terminus of the protein, and thus the strain progresses quickly through G_1 (11). Southern blotting confirmed that the correct allele was integrated, and FACS analysis showed that the strain had a shorter G_1 phase (data not shown). *rap1^s CLN3-2 hmrΔA*::*TRP1* strains were able to grow on medium lacking tryptophan, indicating that the *TRP1* reporter was fully derepressed (Fig. 3 and data not shown). Thus, the *CLN3-2* mutation was unable to restore silencing in *hmrΔA*::*TRP1*

strains with either the *rap1-12* or the *rap1-13* allele, indicating that shortening of the G_1 phase was not able to effect silencing.

The *clb5* mutation results in a delayed progression through S phase, while the *clb6* mutation had no apparent effect on the cell cycle. A strain containing both the *clb5* and *clb6* mutations shows normal-length S phase but delayed entry into S phase. This is apparent on a FACS plot as a greater number of cells in G_1 phase being present (44). We wanted to test whether the restoration of a normal progression through S phase in a *clb5 clb6* mutant would be unable to restore silencing (i.e., be derepressed at *HMR*) in *rap1^s hmrΔA*::*TRP1* strains. A *rap1-12 clb5*::*URA3 clb6*::*LEU2 hmrΔA*::*TRP1* strain, HLY103, was made by mating and dissecting strains with the single disruptions. The level of silencing was determined by growth on plates lacking tryptophan. Despite having a normal-length S phase, a *clb5 clb6* strain was able to suppress the *rap1-12 hmrΔA* mutations strongly, giving wild-type levels of silencing (Fig. 3). This result suggests that a longer G_1 phase could also restore repression in *rap1-12 hmrΔA*::*TRP1* strains.

To test directly the possibility that a longer G_1 phase could also suppress the *rap1^s hmrΔA* mutations, we lengthened this phase by integrating a *cln3*::*URA3* disruption into YDV44. This mutation has been previously reported to result in an elongated G_1 phase (11). The *cln3*::*URA3* disruption was able to suppress the *rap1-12 hmrΔA* mutations strongly but not completely compared with an isogenic parent strain (Fig. 3). Thus, mutations that lengthen the G_1, S, or G_2 phase of the cell cycle are able to suppress the silencing defect of an *hmrΔA rap1^s* strain, as judged from the *TRP1* reporter assay.

Because delays which suppressed the *rap1^s hmrΔA* mutations were not restricted to one phase of the cell cycle, we were concerned that this lack of specificity might indicate that simply slowing the growth rate of these strains could achieve the same effect. We tested this idea by growing *rap1^s hmrΔA* strains on media with alternative carbon sources (galactose or acetate plus glycerol) or at a lower temperature (23°C). Under all of these conditions, the strains grew more slowly but remained derepressed (data not shown), indicating that the suppression by the cell cycle mutants was not due solely to the slower growth of these cells allowing the reestablishment of repression.

Cell cycle mutations restore silencing at the transcriptional level. We wanted to verify that the restoration of silencing in the *rap1^s hmrΔA*::*TRP1* strains by each of the various cell cycle mutations described above was occurring at the level of transcription. Strains HLY308 through HLY322, in which the endogenous **a***1* and **a***2* genes were present at *hmrΔA* rather than the *TRP1* gene, were constructed. Northern blot analysis was performed on each of the strains, probing for the level of **a***1* mRNA (Fig. 4). None of the mutations tested (*clb2*, *clb5*, *cln3*, *mbp1*, *swi4*, *swi6*, and *clb6*) had an effect on transcription in cells with only the ARS consensus deletion. Comparison of the level of derepression in *rap1^s hmrΔA* mutants with that in isogenic strains containing the various cell cycle mutations revealed that the *swi4*, *swi6*, and *clb2* mutations have no **a***1* transcript present; the *clb5*, *cln3*, and *mbp1* strains have **a***1* transcript present at reduced levels compared with the parent strain, while the *clb6* strain has fairly high levels of **a***1* message, consistent with its inability to suppress the *TRP1* reporter. Thus, the cell cycle mutations that are able to suppress the derepression of the *TRP1* reporter are also able to repress the endogenous **a***1* gene, showing that the repression observed at *HMR* is at the level of transcription and, furthermore, is not limited to the *TRP1* gene.

Cell cycle mutations improve telomeric silencing. We wanted to determine whether the effect of enhanced repression at *HMR* was specific to mating type locus silencing or if telomeric silencing would also be strengthened by mutations that affect cell cycle progression. Thus, strains containing the various mutations described above were crossed to a strain containing the *ADE2* reporter gene located at the telomere of chromosome VIIL (19). After sporulation and dissection to generate strains with both the cell cycle mutation and the *ADE2* reporter gene (HLY442 through HLY463), the colony color phenotype was assessed. We observed that the *mbp1*, *clb5*, *clb2*, *cln3*, and *swi6* mutations showed a detectable increase in repression in an otherwise wild-type background, as evidenced by increased red sectoring in the colonies. When these strains also contained a *rap1-12* mutation in addition to the cell cycle mutations, they showed increased repression rel-

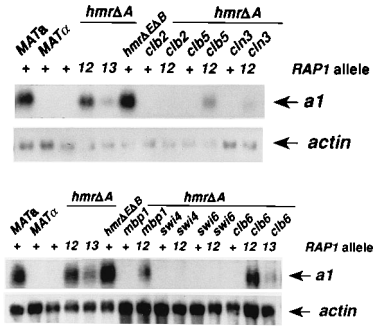

FIG. 4. Mutations in many cell cycle genes restore transcriptional repression of the endogenous **a***1* gene at *HMR*. RNA blot hybridization analysis of strains with the endogenous **a***1* and **a***2* genes present at *HMR* is shown. With the exception of the *MAT***a**, *MATα*, and *hmrΔEΔB* controls, all of the strains have an ARS consensus deletion at *HMR* (*hmrΔA*). Strains have either a wild-type *RAP1* (+), *rap1-12*, or *rap1-13* allele and additional mutations as indicated. Total RNA was isolated from cells grown in rich medium, electrophoresed, blotted to nylon, and sequentially hybridized with ^{32}P-labeled **a***1* and actin DNA probes.

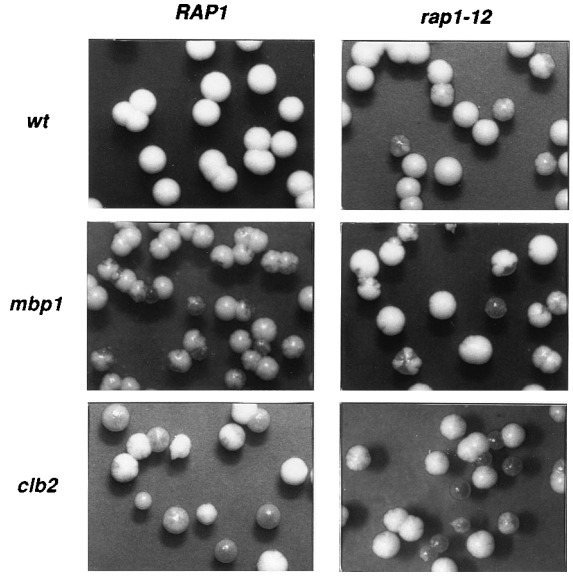

FIG. 5. Mutations in genes that control orderly progression through the cell cycle cause increased telomeric silencing. All strains have the *ADE2* gene at telomere VIIL. Strains with either *RAP1* or *rap1-12* in combination with other mutations in various cell cycle regulatory genes (as indicated) were grown overnight in rich medium and then plated onto rich (YEPD) plates. After incubation for 2 to 3 days, the plates were placed at 4°C for at least 24 h before being photographed.

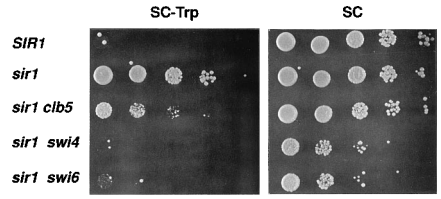

FIG. 6. Mutations in *CLB5*, *SWI4*, and *SWI6* can bypass the requirement for *SIR1*. All strains contain the *hmrΔA*::*TRP1* reporter, expression of which is assayed as described in the legend to Fig. 1. The relevant genotypes of strains are indicated.

ative to a strain with just the *rap1-12* mutation (Fig. 5). The *swi4* disruption did not have any effect on telomeric repression in either background (data not shown). These results suggest that altering normal cell cycle progression can also enhance telomeric repression and support the idea that transcriptional silencing, in general, is sensitive to the amount of time spent in certain phases of the cell cycle. We note that the *mbp1*, *clb5*, *clb2*, and *swi4* mutations did not result in any appreciable alteration in telomere length (data not shown).

Cell cycle mutations can suppress *sir1* but not *sir4* mutations. To determine if the restoration of silencing caused by the mutations in the cyclins and Swi6p-interacting proteins was due to the specific suppression of the *rap1^s* mutation and/or the ARS deletion and not to a general bypass of the silencing machinery, each of the strains containing the individual cell cycle mutations was mated to a *RAP1 sir1 hmrΔA*::*TRP1* or a *RAP1 sir4 hmrΔA*::*TRP1* strain. After sporulation and dissection, haploids containing each mutation were then assayed for their ability to suppress the derepression of the *hmrΔA*::*TRP1* reporter caused by either the *sir1* or the *sir4* mutation. None of the mutations tested (*clb2*, *clb5*, *mbp1*, *swi4*, and *swi6*) was able to suppress a *sir4* disruption (data not shown). However, as shown in Fig. 6, the *clb5*, *swi4*, and *swi6* mutations were able to suppress the *sir1 hmrΔA* mutations by at least 500- to 1,000-fold (despite the slower growth of *swi4* and *swi6* strains) compared with an isogenic *SIR1* strain. This effect was dependent on *RAP1*, as it was not evident in *rap1^s* strains. The *cln3*, *mbp1*, and *clb2* mutations were unable to establish repression in a *sir1 hmrΔA* background (data not shown). These data suggest that cell cycle disruption results in silencing that is still dependent on the normal machinery (Sir4p) to effect repression. However, some mutations can bypass the requirement for the establishment function of Sir1p.

Cell cycle delay is unable to suppress a decrease in *SIR4* gene dosage. In considering possible mechanisms by which cell cycle delay might improve silencing at *HMR* in a *rap1^s hmrΔA* strain, it is important to point out that this strain is extremely sensitive to the dosage of *SIR4*. In fact, one extra chromosomal copy of *SIR4* is sufficient to restore silencing in these strains (49). In addition to its ability to suppress the *rap1^s hmrΔA* mutations, *SIR4* on a *CEN*-based plasmid is also able to suppress *sir1 hmrΔA*::*TRP1* mutations and improve telomeric silencing (data not shown), which are among the phenotypes of some of the cell cycle mutations described here. Thus, one explanation for the suppression by these various mutations is that the disturbance in the cell cycle results in a rise in the concentration of silencing factors in the nucleus, such as Sir4p, to levels sufficient to promote silencing at *HMR*. To address this possibility, we tested the ability of each mutation to increase repression in a diploid containing only one functional copy of *SIR4* and two copies of the *hmrΔA*::*ADE2* reporter. The 0.5-fold reduction in *SIR4* dosage causes derepression of the *ADE2* genes at *HMR*, so that the strain (HLY562) produces white colonies. The introduction of *SIR4* on a *CEN*-based plasmid resulted in a uniformly pink appearance. Isogenic diploids (HLY563 through HLY568) homozygous for the various cell cycle mutations (*cln3*, *mbp1*, *clb5*, *swi4*, *swi6*, and *clb2*) remained white, indicating that Sir4p level or activity was not increasing to an extent sufficient to complement even the 0.5-fold reduction in *SIR4* gene dosage (data not shown). This result argues against the simple model that Sir4p accumulation in the nucleus due to slowed progression through the cell cycle is responsible for suppression of the *rap1^s hmrΔA* silencing defect.

DISCUSSION

Is a specific cell cycle event linked to the enhancement of silencing? Several previous studies had revealed an important role for DNA replication in the establishment of silencing at *HMR* (5, 34, 41). Our initial characterization of *sds* mutants provided further support for this idea but, in addition, suggested a more general connection between cell cycle progression and transcriptional silencing. Thus, although both *SDS11* (*SWI6*) and *SDS12* (*RNR1*) are required for proper progression through S phase, the *SDS15* (*CIN8*) gene functions in G_2/M, where it is required for mitotic spindle formation. Since the only connection between these three suppressor mutations that we could discern was a delay in the cell cycle, we tested the effect of mutations in other genes which were either known or suspected to interfere with normal cell cycle progression. The observation that mutations in several cyclin genes (*CLN3*, *CLB5*, and *CLB2*) and additional cell cycle transcriptional regulators (*SWI4* and *MBP1*) all lead to suppression further supports the idea that a general feature of cell cycle perturbation underlies this effect. Furthermore, these results indicate that this general effect can be exerted during a broad window of the cell cycle, from late G_1 (when *CLN3* acts) to G_2/M [when the *SDS15* (*CIN8*) gene product is required].

Is the cell cycle effect specific for the establishment of silencing? Mutations in *SIR1*, *RAP1*, or *cis* silencer elements can lead to metastable repression at *HM* loci (30, 38, 49). Pillus and Rine (38) proposed that the epigenetic effect observed in *sir1* mutants, in which genetically identical cells can exist in two distinct, heritable, transcriptional states (repressed or derepressed), reflects a failure to reestablish repression efficiently in the rare event that it is lost. In this view, Rap1p, Sir1p, and the silencers play a special role in assembling repressed chromatin, but (unlike Sir2p, Sir3p, and Sir4p) are not required for its maintenance throughout the cell cycle. It should be pointed out, though, that the efficiency of establishment of repression has not been measured directly in wild-type strains. Therefore, an alternative view is that Sir1p and Rap1p act to stabilize the repressed state, so that in their absence, repressed chromatin is slightly weakened and thus prone to disassembly.

Our finding that several mutations (*swi4*, *swi6*, and *clb5*) act as suppressors of both *rap1^s hmrΔA* and *sir1 hmrΔA* mutations but not other silencing mutations (e.g., *sir4* mutations) reinforces the idea that Rap1p, Sir1p, and the silencers play a common role in silencing which may be related to the establishment or assembly of repressed chromatin. In this regard, we think it is particularly significant that none of the cell cycle mutations that we have tested can suppress the effect of reduced *SIR4* gene dosage, a weak silencing defect caused by the decreased concentration of a protein clearly required for the maintenance of repression. This result indicates that cell cycle mutations do not act as general suppressors of weakened silencing and thus supports the idea that they act to improve the

establishment of repression. It should also be noted that the suppression by cell cycle mutations is not limited to partial silencing defects, since many of the mutations described here also suppress the double-mutant *hmrΔEΔB* silencer, which is completely defective in repression (see below).

Possible mechanisms to explain the relationship between cell cycle events and establishment of silencing. One model to explain how cell cycle delay might enhance repression concerns the correlation between DNA replication initiation and silencing (27, 42). All four *HM* silencers are ARSs (1, 16). Furthermore, a detailed mutagenesis study of the *HMR-E* silencer has revealed a strong correlation between the firing of this origin in the chromosome and silencer function, leading to the hypothesis that initiation of DNA replication from *HMR-E* is required for repression (41). Even though all of the experiments described above use strains that have a deletion of the ARS consensus element at *HMR*, it has been demonstrated that removal of this element does not completely abolish ARS activity of the remaining sequences. The B element together with neighboring sequences has ARS activity in the absence of the A element (8), so it is conceivable that chromosomal deletion of the A element results in the activation of secondary or cryptic elements nearby. In any event, it seems possible that the derepression caused by the *rap1^s hmrΔA* double mutation results from a decrease in the efficiency of initiation at the *HMR-E* origin. Thus, one possible mechanism through which various cell cycle mutations could be improving repression in these strains is by enhancing the firing of alternative ARS elements at *HMR*, including that at the *HMR-I* silencer (1). It is unclear whether this model could apply to silencing at the artificial chromosome VIIL telomere, for which the location of nearby ARS elements and their relationship to silencing at the telomere have not been studied. A more general problem with this model is that it does not explain how a gene acting during G_2/M, such as *SDS15* (*CIN8*), could affect origin firing during S phase. However, the recent observation that the protein complex bound at ARS elements in vivo undergoes changes during late M phase (12) indicates that such a possibility should be seriously considered.

An alternative explanation related to the initiation of DNA replication at *HMR-E* concerns the time during S phase when the silencer origin fires. It has been observed that the *HM* loci and telomeres, like other transcriptionally inactive regions of chromatin in multicellular eukaryotes, are replicated late in S phase (40). At *HMR*, this is presumably due to late initiation from the *HMR-E* silencer origin. This correlation may reflect a requirement for a specific time during S phase when origin firing optimally enhances repression. Axelrod and Rine (5) isolated a temperature-sensitive allele of *cdc7* as a suppressor of the mating defect caused by mutation of both the Rap1p binding site and the Abf1p binding site at *HMR*. They proposed that the *cdc7* mutation could be suppressing this defect by causing *HMR*, whose origin normally fires late in S phase, to fire even later. With the exception of the *swi4* mutation, all of the cell cycle mutations described here can restore silencing in strains with an *hmrΔEΔB* silencer (data not shown). These mutations could also be acting through a similar mechanism, allowing the later firing of alternative or cryptic ARS elements at *HMR* to promote silencing.

Another related possibility is that late replication per se, independent of (or in addition to) origin firing from the silencer, promotes silencing. It may therefore be possible that the timing of replication of the *HM* loci and telomeres during S phase could determine whether or not transcriptional repression is established by affecting the kind of chromatin structure assembled at that locus. One scenario could involve the depletion of positively acting factors during the assembly of active chromatin in early S phase, allowing more efficient assembly of repressed chromatin (heterochromatin) late in S phase. This model is consistent with recent results which indicate that the establishment of repressed chromatin at telomeres in yeast cells is in direct competition with the assembly of active transcription complexes during a window of the cell cycle between late G_1 and the G_2-M transition (3). Alternatively, specific silencing factors (e.g., the Sir proteins) may be more prevalent or active, or the environment of the nucleus may change in a more general way to promote silencing at the end of S phase. For example, histone deacetylation may be favored late in S phase, thereby promoting the assembly of repressed chromatin (9). Thus, the relative timing of replication of the silencers with respect to the onset of S phase may play an important role in the establishment of silencing. In this way, elongation of the cell cycle could enable a crippled silencer to establish silencing by changing the relative time of replication or of origin initiation, or by allowing additional time for the assembly of silenced chromatin at the end of S phase and into G_2 phase.

Finally, we suggest a completely different model to explain our results. One general consequence of cell cycle delay that may be common to all of the mutations described here is an increase in cell volume. This effect may lead to an alteration in the relative concentrations of regulatory proteins within the nucleus if the synthesis and import of nuclear proteins are not tightly regulated to compensate for possible increases in overall biosynthesis. We propose that an increase in cell size caused by cell cycle delay may lead to a rise in the nuclear concentration of silencing factors so that the establishment of silencing is favored. However, slower growth due to a poor carbon source, which results in a decrease in overall biosynthetic rates and smaller cell size (23), would not be expected to increase the relative nuclear concentrations of silencing factors. This would explain why, in contrast to our observations with cell cycle mutants, *rap1^s hmrΔA* strains remain derepressed under conditions that nonspecifically slow the growth of these cells. This model fits well with a number of observations which indicate that transcriptional silencing is extremely sensitive to the dosage of several *SIR* genes (22, 31, 39, 46, 48, 49) and to the concentration of activators of silenced genes (3, 39). In addition, this model has the virtue of explaining how such a diverse set of cell cycle-related mutations can have the same effect on gene regulation. We have tested this model for the limited case of *SIR4* dosage, which clearly has a profound effect on silencing at *HMR*. Our results suggest that the effects of the cell cycle mutations tested cannot be due to an increase in the concentration or activity of Sir4p alone. We are currently attempting to design a more general experimental test of this model.

Regardless of which, if any, of the models proposed above is the correct molecular explanation of our results, they clearly indicate a surprising sensitivity of transcriptional silencing to modest perturbations in the cell cycle. For example, mutations in *CLN3* and *MBP1* have minor effects on cell cycle progression and cell size, yet both dramatically increase the fraction of cells in which the *HMR* locus is repressed. The case of *MBP1* mutation is particularly curious, since the deregulation of DNA replication genes directly controlled by this factor has little or no phenotypic consequence with respect to either cell growth or viability (26). As discussed above, we imagine that the effect on silencing that we observe may be a consequence of two important characteristics: (i) the cell cycle-dependent nature of the establishment of silencing and (ii) the sensitivity of establishment to the gene dosage of silencing factors and to the concentration of activators with which these factors compete. At a more fundamental level, our results could reflect the

unusual nature of silent chromatin in *S. cerevisiae*, which is presumably assembled from silencers and telomeres in a highly cooperative process. Furthermore, once it is assembled, silent chromatin appears to be stably inherited through multiple cell divisions by an independent mechanism (discussed in reference 27). Both of these factors imply that even subtle effects acting at the level of establishment of silencing could have profound consequences on the actual fraction of repressed cells within a culture. Since transcriptional silencing in yeast cells has many features in common with position effect variegation in *D. melanogaster*, it might be interesting to determine whether this latter phenomenon, and related events such as the establishment of stable states of homeotic gene expression (37), is regulated by changes in the cell cycle that occur normally during development.

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REFERENCES

1. **Abraham, J., K. A. Nasmyth, J. N. Strathern, A. J. S. Klar, and J. B. Hicks.** 1984. Regulation of mating-type information in yeast. J. Mol. Biol. **176:**307–331.
2. **Aparicio, O. M., B. L. Billington, and D. E. Gottschling.** 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell **66:**1279–1287.
3. **Aparicio, O. M., and D. E. Gottschling.** 1994. Overcoming telomeric silencing: a *trans*-activator competes to establish gene expression in a cell cycle-dependent way. Genes Dev. **8:**1133–1146.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, New York.
5. **Axelrod, A., and J. Rine.** 1991. A role for *CDC7* in repression of transcription at the silent mating type locus *HMR* in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**1080–1091.
6. **Bell, S. P., R. Kobayashi, and B. Stillman.** 1993. Yeast origin recognition complex functions in transcription silencing and DNA replication. Science **262:**1844–1849.
7. **Brand, A. H., L. Breeden, J. Abraham, R. Sternglanz, and K. Nasmyth.** 1985. Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. Cell **41:**41–48.
8. **Brand, A. H., G. Micklem, and K. Nasmyth.** 1987. A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell **51:**709–719.
9. **Braunstein, M., A. B. Rose, and S. G. Holmes.** 1993. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev. **7:**592–604.
10. **Breeden, L., and K. Nasmyth.** 1987. Cell cycle control of the yeast HO gene: cis- and trans-acting regulators. Cell **48:**389–397.
11. **Cross, F. R.** 1988. *DAF1*, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8:**4675–4684.
12. **Diffley, J. F. X., J. H. Cocker, S. J. Dowell, and A. Rowley.** 1994. Two steps in the assembly of complexes at yeast replication origins in vivo. Cell **78:**303–316.
13. **Dirick, L., T. Moll, H. Auer, and K. Nasmyth.** 1992. A central role for *SWI6* in modulating cell cycle Start-specific transcription in yeast. Nature (London) **357:**508–513.

13a. **Elledge, S.** Personal communication.

14. **Elledge, S. J., and R. W. Davis.** 1990. Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase. Genes Dev. **4:**740–751.
15. **Epstein, C. B., and F. R. Cross.** 1992. CLB5: a novel B cyclin from budding yeast with a role in S phase. Genes Dev. **6:**1695–1706.
16. **Feldman, J. B., J. B. Hicks, and J. R. Broach.** 1984. Identification of sites required for repression of a silent mating-type locus in yeast. J. Mol. Biol. **178:**815–834.
17. **Fitch, I., C. Dahmann, U. Surana, A. Amon, K. Nasmyth, L. Goetsch, B. Byers, and B. Futcher.** 1992. Characterization of four B-type cyclin genes of the budding yeast Saccharomyces cerevisiae. Mol. Biol. Cell **3:**805–818.
18. **Foss, M., F. J. McNally, P. Laurenson, and J. Rine.** 1993. Origin recognition complex (ORC) in transcriptional silencing and DNA replication in *S. cerevisiae*. Science **262:**1838–1844.
19. **Gottschling, D. E., O. M. Aparicio, B. L. Billington, and V. A. Zakian.** 1990. Position effect at *S. cerevisiae* telomeres: reversible repression of pol II transcription. Cell **63:**751–762.
20. **Hoyt, M. A., L. He, K. K. Loo, and W. S. Saunders.** 1992. Two *Saccharomyces cerevisiae* kinesin-related gene products required for mitotic spindle assembly. J. Cell Biol. **118:**109–120.
21. **Ito, H., V. Fukuda, D. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
22. **Ivy, J. M., A. J. S. Klar, and J. B. Hicks.** 1986. Cloning and characterization of four *SIR* genes from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**688–702.
23. **Johnston, G. C., C. W. Ehrhardt, A. Lorincz, and B. L. A. Carter.** 1979. Regulation of cell size in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. **137:**1–5.
24. **Kayne, P. S., U. J. Kim, M. Han, J. R. Mullen, F. Yoshizaki, and M. Grunstein.** 1988. Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell **55:**27–39.
25. **Kimmerly, W., A. Buchman, R. Kornberg, and J. Rine.** 1988. Roles of two DNA-binding factors in replication, segregation and transcriptional repression mediated by a yeast silencer. EMBO J. **7:**2241–2253.
26. **Koch, C., T. Moll, M. Neuberg, H. Ahern, and K. Nasmyth.** 1993. A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase. Science **261:**1551–1557.
27. **Laurenson, P., and J. Rine.** 1992. Silencers, silencing, and heritable transcriptional states. Microbiol. Rev. **56:**543–560.
28. **Lowndes, N. F., A. L. Johnson, L. Breeden, and L. H. Johnston.** 1992. SWI6 protein is required for transcription of the periodically expressed DNA synthesis genes in budding yeast. Nature (London) **357:**505–508.
29. **Mahoney, D. J., and J. R. Broach.** 1989. The *HML* mating type cassette of *Saccharomyces cerevisiae* is regulated by two separate but functionally equivalent silencers. Mol. Cell. Biol. **9:**4621–4630.
30. **Mahoney, D. J., R. Marquardt, G.-J. Shei, A. B. Rose, and J. R. Broach.** 1991. Mutations in the *HML* E silencer of *Saccharomyces cerevisiae* yield metastable inheritance of transcriptional repression. Genes Dev. **5:**605–615.
31. **Marshall, M., D. Mahoney, A. Rose, J. B. Hicks, and J. R. Broach.** 1987. Functional domains of *SIR4*, a gene required for position effect regulation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**4441–4452.
32. **Megee, P. C., B. A. Morgan, B. A. Mittman, and M. M. Smith.** 1990. Genetic analysis of histone H4: essential role of lysines subject to reversible acetylation. Science **247:**841–845.
33. **Micklem, G., A. Rowley, J. Harwood, K. Nasmyth, and J. F. X. Diffley.** 1993. Yeast origin recognition complex is involved in DNA replication and transcriptional silencing. Nature (London) **366:**87–89.
34. **Miller, A. M., and K. A. Nasmyth.** 1984. Role of DNA replication in the repression of silent mating-type loci in yeast. Nature (London) **312:**247–251.
35. **Nasmyth, K., and L. Dirick.** 1991. The role of SWI4 and SWI6 in the activity of G1 cyclins in yeast. Cell **66:**995–1013.
36. **Park, E.-C., and J. W. Szostak.** 1990. Point mutations in the yeast histone H4 gene prevent silencing of the silent mating type locus *HML*. Mol. Cell. Biol. **10:**4932–4934.
37. **Paro, R.** 1990. Imprinting a determined state into the chromatin of Drosophila. Trends Genet. **6:**416–421.
38. **Pillus, L., and J. Rine.** 1989. Epigenetic inheritance of transcriptional states in *S. cerevisiae*. Cell **59:**637–647.
39. **Renauld, H., O. M. Aparicio, P. D. Zierath, B. L. Billington, S. K. Chhablani, and D. E. Gottschling.** 1993. Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by *SIR3* dosage. Genes Dev. **7:**1133–1145.
40. **Reynolds, A. E., R. M. McCarroll, C. S. Newlon, and W. L. Fangman.** 1989. Time of replication of ARS elements along yeast chromosome III. Mol. Cell. Biol. **9:**4488–4494.
41. **Rivier, D. H., and J. Rine.** 1992. An origin of DNA replication and a transcriptional silencer require a common element. Science **256:**659–663.
42. **Rivier, D. H., and J. Rine.** 1992. Silencing: the establishment and inheritance of stable, repressed transcription states. Curr. Opin. Genet. Dev. **2:**286–292.
43. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Press, Plainview, N.Y.
44. **Schwob, E., and K. Nasmyth.** 1993. *CLB5* and *CLB6*, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. Genes Dev. **7:**1160–1175.

45. **Sidorova, J., and L. Breeden.** 1993. Analysis of the SWI4/SWI6 protein complex, which directs G_1/S-specific transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**1069–1077.
46. **Stone, E. M., M. J. Swanson, A. M. Romeo, J. B. Hicks, and R. Sternglanz.** 1991. The *SIR1* gene of *Saccharomyces cerevisiae* and its role as an extragenic suppressor of several mating-defective mutants. Mol. Cell. Biol. **11:**2253–2262.
47. **Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A. B. Futcher, and K. Nasmyth.** 1991. The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. Cell **65:**145–161.
48. **Sussel, L., and D. Shore.** 1991. Separation of transcriptional activation and silencing functions of the *RAP1*-encoded repressor/activator protein 1: isolation of viable mutants affecting both silencing and telomere length. Proc. Natl. Acad. Sci. USA **88:**7749–7753.
49. **Sussel, L., D. Vannier, and D. Shore.** 1993. Epigenetic switching of transcriptional states: *cis*- and *trans*-acting factors affecting establishment of silencing at the *HMR* locus in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**3919–3928.
50. **Sussel, L., D. Vannier, and D. Shore.** Silencing-defect suppressors in yeast: differential effects on transcriptional silencing, telomere structure, and cell growth. Submitted for publication.
51. **Thomas, B. J., and R. Rothstein.** 1989. Elevated recombination rates in transcriptionally active DNA. Cell **56:**619–630.
52. **Yagle, K., and K. McEntee.** 1990. The DNA damage-inducible gene *DIN1* of *Saccharomyces cerevisiae* encodes a regulatory subunit of ribonucleotide reductase and is identical to *RNR3*. Mol. Cell. Biol. **10:**5553–5557.