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Yeast Silencers Can Act as Orientation-Dependent Gene Inactivation Centers That Respond to Environmental Signals

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The mating-type loci located at the ends of chromosome III in *Saccharomyces cerevisiae* are transcriptionally repressed by a region-specific but sequence-nonspecific silencing apparatus, mediated by *cis*-acting silencer sequences. Previous deletion analyses have defined the locations and organizations of the silencers in their normal context and have shown that they are composed of various combinations of replication origins and binding sites for specific DNA-binding proteins. We have evaluated what organization of silencer sequences is sufficient for establishing silencing at a novel location, by inserting individual silencers next to the *MAT* locus and then assessing expression of *MAT*. The results of this analysis indicate that efficient silencing can be achieved by inserting either a single copy of the E silencer from *HMR* or multiple, tandem copies of either the E or I silencer from *HML*. These results indicate that while all silencers are functionally equivalent, they have different efficiencies; *HMR* E is more active than *HML* E, which itself is more active than *HML* I. Both *HMR* E and *HML* E exhibit orientation-dependent silencing, and the particular organization of binding elements within the silencer domain is critical for function. In some situations, silencing of *MAT* is conditional: complete silencing is obtained when cells are grown on glucose, and complete derepression occurs when cells are shifted to a nonfermentable carbon source, a process mediated in part by the *RAS*/cyclic AMP signaling pathway. Finally, the E silencer from *HMR* is able to reestablish repression immediately upon a shift back to glucose, while the silencers from *HML* exhibit a long lag in reestablishing repression, thus indicating distinctions between the two silencers in their reestablishment capacities. These results demonstrate that silencers can serve as nonspecific gene inactivation centers and that the attendant silencing can be rendered responsive to potential developmental cues.

Two identical genomic sequences in a mammalian cell can sometimes be regulated in quite distinct fashions. For example, one of the two X chromosomes in every somatic cell of a female is inactivated, while the other remains fully active (reviewed in references 19 and 41). Similarly, several loci exhibit chromosomal imprinting, in which one allele of a locus is active and the other is completely inactive; the pattern of inactivation depends solely on the parental source of the allele (reviewed in references 21 and 63). In both situations, repression of the inactive allele persists through extended mitotic divisions, resulting in epigenetic inheritance of the transcription state; the inactive loci are generally reactivated only upon entry into meiosis. X-chromosome inactivation is achieved by condensation of the chromosome into a heterochromatic state, and a similar condensation may underlie chromosomal imprinting. In neither of these cases are the molecular events leading to chromatin condensation understood.

Silencing of mating-type loci in the budding yeast *Saccharomyces cerevisiae* is remarkably similar to these examples of long-term, epigenetic inactivation of specific genomic domains in mammalian cells. Mating-type sequences reside at three different locations on chromosome III in yeast cells (Fig. 1). Those sequences near the center of the chromosome, at a locus called *MAT*, are expressed to yield regulatory proteins that determine the mating type of the cell (reviewed in references 23 and 24). Identical sequences, including the promoters of the mating-type genes, are also located at opposite ends of the chromosome, at loci designated *HML* and *HMR*. However, the genes at these loci are transcriptionally inactive and do not contribute to the phenotype of the cell (32, 49; reviewed in references 24 and 37). Thus, as for X-chromosome inactivation and chromosomal imprinting, essentially identical sequences in yeast cells exhibit quite distinct expression states.

A variety of observations suggest that repression of the silent cassettes is accomplished through condensation of chromatin across the region. First, the silent cassettes are relatively inaccessible to DNA-modifying agents, both in vivo and in chromatin preparations in vitro (40, 48, 62). Second, nucleosomes spanning the silent cassettes and other silenced regions are uniquely hypoacetylated, like those in inactive chromatin from metazoan cells, and fractionate on methyl mercury columns in a pattern similar to that of inactive chromatin (6, 11). Third, several genetic observations implicate a role for histones and histone modification in transcriptional silencing (28, 30, 46, 52). Finally, silencing acts through a region-specific but sequence-nonspecific mechanism: removal of the mating-type genes from silent domain yields their activation, while insertion of other RNA polymerase II or RNA polymerase III genes into the silent loci inactivates the inserted gene (4, 25, 58). Thus, a reasonable hypothesis is that the silent loci are rare instances of heterochromatic domains in yeast cells.

The apparatus required for establishing and maintaining *HML* and *HMR* in a transcriptionally silent state has been defined genetically. The products of three loci, *SIR2* (*MAR1*), *SIR3* (*MAR2*), and *SIR4*, are required for maintenance of repression (20, 31, 55, 56), while the product of a fourth gene, *SIR1*, has been suggested to facilitate establishment of repression (27, 53). In addition, mutations in a gene encoding histone H4, *HHF2*, or the genes encoding heterodimeric N-terminal acetyltransferase, *NAT1* and *ARD1*, yield partial derepression of the silent cassettes (30, 38, 47, 52, 67). The product of the *SIR2* gene promotes deacetylation of lysine residues in the

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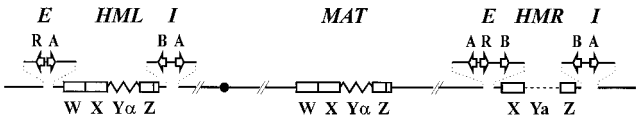

FIG. 1. Organization of the silent mating-type loci and their associated silencers. An abbreviated map of chromosome III is shown, on which are indicated the location of *MAT*, *HML*, and *HMR*. The regions of homology between the three loci are indicated as open boxes, as are the locations and structures of the silencer regions flanking the two storage cassettes. B, R, and A refer, respectively, to Abf1p binding sites, Rap1p binding sites, and ARS core consensus sequences. The orientations of these sites, reading in the 5′-to-3′ direction as designated by the arrows, are as follows: B, (A/G)TC(A/G)YN NNNNACG; R, (A/G)NACCCANNCAPuPu; and A, (A/T)TTTAT(A/G)TTT(A/T).

amino-terminal domains of three of the four core histones, a likely prelude to chromatin condensation (6). The function of the other *SIR* proteins is unknown, although a reasonable hypothesis is that they also assist in converting chromatin across the silent loci into a heterochromatic state.

Silencing of the mating-type loci is also dependent on small, *cis*-acting sites termed silencers (1, 16). The silencers appear to function in an orientation-independent manner over a reasonable distance to promote repression of the silent mating-type loci (4). Thus, these sites likely serve as foci for the silencer proteins, localizing the activity of these proteins to the region of the genome in the vicinity of the silencer. These sites may constitute the yeast homologs of the mammalian X-chromosome inactivation centers.

The locations and functional organizations of the silencers at *HML* and *HMR* are well defined. Both silent loci are bracketed by two silencers (1, 16). However, the roles that the two silencers play in silencing the adjacent mating-type genes differ at the two loci. At *HML*, both silencers appear independently capable of promoting full repression of the mating-type genes at the locus. Deletion of neither the centromere-distal silencer, termed E, nor the centromere proximal-silencer, termed I, alone affects repression of the resident mating-type genes; deletion of both E and I, though, results in full activation of *HML* (42). In contrast, deletion of the centromere-proximal silencer at *HMR* (the *HMR* E silencer) yields full derepression, even with the centromere distal silencer, I, intact. Deletion of I has only a marginal affect on expression of the genes at *HMR* (4).

All four silencers are composed of various combinations of clearly defined elements (Fig. 1). The *HMR* E silencer encompasses distinct recognition sites for the abundant DNA-binding proteins Rap1p and Abf1p as well as an origin of replication core consensus sequence, or autonomously replicating sequence (ARS) element (8, 9, 14, 60, 61). Deletion of any one of these elements has little effect on repression, while deletion of any two causes complete derepression (5). Reconstruction experiments suggest that these three elements alone are sufficient to manifest most of the properties of the *HMR* E silencer (45). Similarly, both *HML* E and *HML* I silencers each encompass two of these three elements: *HML* E contains a Rap1p binding site and an ARS element, and *HML* I contains an Abf1p binding site and an ARS element (8). Deletion analysis of *HML* E has shown that both of these elements are critical for silencer function (42). Thus, to a first approximation, yeast silencers can be defined as collections any two of three well-defined elements.

Despite these prior studies, though, a number of ambiguities and inconsistencies remain regarding the nature of yeast silencers. First, the activities of silencers appear to depend on their context. For instance, while the *HML* silencers appear to function as independent units in situ, both are required for efficient repression of *HML* on a plasmid (16, 42). Second, while deletion analyses suggests that silencers consist simply of collections of any two of three elements, other pairs of such elements are found at numerous other sites in the genome at which they do not evince silencing activity. For instance, ARS1 encompasses an ARS element and an Abf1p binding site, just as does *HML* I, and yet the adjacent *TRP1* locus is not silenced (8, 14). Similarly, the promoters of *ENO1*, *PGK1*, and *PEM1* contain both Rap1p and Abf1p binding sites, but at these locales, these elements promote transcription rather than silence it (7, 10, 12, 34, 51). Thus, structural or contextual features other than the elements themselves are critical for silencer function.

To address these conundrums and to explore more completely the contextual components of silencer activity, we have examined what is required to establish silencing at ectopic locations. That is, we have determined what organization and combination of silencers from *HML* or *HMR* are sufficient to induce silencing of a region of the genome that is not normally subject to silencing. The results of this analysis provide resolutions to these issues as well as demonstrate that silencers can serve as centers for inactivation. In addition, these results indicate that silencers can function in a conditional capacity, providing inactivation of a region of a genome that can be reversed under certain environmental cues. These results provide additional parallels between yeast silencing and mammalian chromosome inactivation and imprinting.

MATERIALS AND METHODS

Plasmids. Plasmid pDM33, used for integration of the *HML* E site immediately centromere proximal to *MAT*, was constructed by inserting a 1.0-kb *Xba*I fragment spanning *HML* E and a portion of the contiguous X-homology domain of *HML* into the *Xba*I site of vector YIp19. YIp19 consists of pUC19 carrying the 1.1-kb *Hin*dIII fragment spanning *URA3*.

Plasmid pGJ13, used for integration of the *HML* I site immediately centromere distal to *MAT*, was constructed as follows. The 2.3-kb *Hha*I fragment spanning *HML* I and a portion of the Z1Z2 homology region of *HML* was cloned into a Bluescript pKS+ such that the *Hpa*I site in Z1Z2 was positioned adjacent to the *Hin*dIII site in the polylinker, yielding plasmid pGJ6. The resulting 1.2-kb *Hin*dIII site was then cloned into plasmid pGJ12 to yield plasmid pGJ13. Plasmid pGJ12 consists of pUC18, from which the single *Nde*I site has been eliminated, carrying a 2.2-kb *Sal*I-*Xho*I fragment spanning *LEU2* in the *Sal*I site of the polylinker.

Plasmid pGJ27, used for insertion of the *HML* I site immediately centromere proximal to *MAT*, was constructed as follows. The 275-bp *Hpa*I-*Hin*dIII fragment spanning *HML* I was inserted into the *Bgl*II site in the *HML* E Δ80-216 plasmid (pDM30Δ80-216) described previously (42) to yield plasmid pGJ23. The *Hpa*I-*Sac*I fragment spanning this insertion was excised from plasmid pGJ23 and used to replace the corresponding fragment of pDM33, yielding plasmid pGJ27.

Plasmids used for insertion of *HML* E plus *HML* I centromere proximal to *MAT* were constructed by inserting the 275-bp *Hpa*I-to-*Hin*dIII fragment spanning *HML* I into the *Bgl*II site of plasmid pDM30Δ59-76 (42). The *Hpa*I-to-*Sac*I fragments from two resulting plasmids with the *HML* I fragment in opposite orientations were then used to replace the corresponding fragment from plasmid pDM33, yielding plasmid pGJ28 and plasmid pGJ41 (orientations are indicated in Fig. 2).

Plasmids used for insertion of *HMR* E centromere proximal to *MAT* were constructed by inserting the 328-bp *Xho*I-*Ssp*I fragment spanning *HMR* E into the *Bgl*II site of plasmid pDM30Δ80-216. The *Hpa*I-to-*Sac*I fragments from two resulting plasmids with the *HMR* E fragment in opposite orientations were then used to replace the corresponding fragment from plasmid pDM33, yielding plasmid pGJ29, in which the *HMR* E fragment is inserted in the same orientation relative to the *MAT* as it is at *HMR*, and plasmid pGJ32, in which the *HMR* E is in the opposite orientation (see Fig. 2).

Plasmids used for insertion of *HMR* E centromere distal to *MAT* were constructed by inserting the 328-bp *Xho*I-to-*Ssp*I fragment spanning *HMR* E into the *Bam*HI site of plasmid pGJ12. Two plasmids in which the fragment was inserted in opposite orientation were retained as plasmids pGJ44 and pGJ45, and the 1-kb *Ssp*I fragment spanning Z1Z2 from *MAT* was inserted into the *Sal*I site of each of the two plasmids to yield plasmids pGJ48 and pGJ49.

Strains. Except for those noted below, all strains used in this study (Table 1) were isogenic derivatives of strain Y1471, which was constructed by mating strains MS1554 and MS2290 obtained from M. Rose. Derivatives of strain Y1471 carrying *HML* or *HMR* silencers at *MAT* were constructed by circular integration

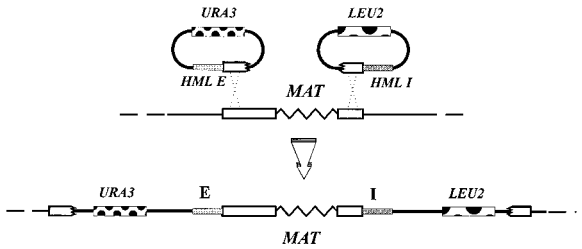

FIG. 2. Insertion of silencers at the *MAT* locus. Strains used in this study were constructed by circular integration of a *URA3*-based plasmid to the left (centromere-proximal side) of *MAT* or circular integration of a *LEU2*-based plasmid to the right (centromere-distal side) of *MAT*, or both. These plasmids consisted of pUC19 (heavy line) carrying the selectable marker (mottled region) and a fragment from *HML* spanning the *MAT*-homologous sequences (open box) and the sequences immediately flanking it (shaded boxes) encompassing the indicated *HML* silencer. The chromosomal structure surrounding *MAT* in a strain carrying both integrated plasmids is shown at the bottom of the panel.

of the appropriate plasmid described above. Those plasmids integrated centromere proximal to *MAT* carried sequences homologous to the W region of *MAT* and were digested prior to transformation with *Hpa*I, which cut uniquely within the W sequence. Those plasmids integrated centromere distal to *MAT* carried sequences homologous to the Z1Z2 region of *MAT* and were digested prior to transformation with *Nde*I, which cut uniquely within the Z1 region. The structures of the *MAT* loci in all transformants used in this study were determined by Southern analysis, using digestions that clearly distinguished between integration of one, two, or more plasmids at *MAT*.

Strains Y1854 and Y1855 are separate isolates of the same strain, constructed by mating appropriate progeny obtained from a cross between strain Y1359 and a *MAT***a** spore clone from strain Y1513. Y1856 was obtained by transforming Y1855 to uracil prototrophy with plasmid YCp-CYR1, which consists of the *CYR1* (*CDC35*) gene cloned into the vector YCp50.

Media. YEPD medium consisted of 1% yeast extract, 2% Bacto Peptone, and 2% glucose. YEPA medium was identical to YEPD except for the substitution of 2% potassium acetate for glucose. Sporulation medium consisted of 2% potassium acetate. Synthetic medium was as described previously (59).

RNase protection assays. RNase protection assays were performed as described previously (42).

Quantitative mating assays. Quantitative mating assays were performed by a modification of the procedure described in reference 42, using strains MS26 and MS30 as mating-type testers. Equal numbers of exponentially growing test and

TABLE 1. Strains used in this study

Strain	Genotype[a]	Source or reference
Y1359	*MAT*α *cdc35-1 pde2 ura1 his7 trp1 arg leu*	Laboratory stock
Y1471	*MAT***a**/*MAT*α *ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 ade2/ADE2 TRP1/trp1*	This study
Y1472	Y1471, *MAT***a**/*MAT*α*-HMLI-LEU2*	This study
Y1473	Y1471, *MAT***a**/*URA3-HMLE-MAT*α*-HMLI-LEU2*	This study
Y1474	Y1471, *MAT***a**/*MAT*α*-(HMLI-LEU2)*$_2$	This study
Y1476	Y1471, *MAT***a**-(*HMLI-LEU2*)$_{>4}$/*MAT*α	This study
Y1478	Y1471, *URA3-<HMRE-MAT***a**/*MAT*α	This study
Y1481	Y1471, *MAT***a**/*URA3-<HMRE-MAT*α	This study
Y1482	Y1471, *URA3-HMRE>-MAT***a**/*MAT*α	This study
Y1483	Y1471, *MAT***a**/*URA3-HMRE>-MAT*α	This study
Y1484	Y1471, *MAT***a**/(*URA3-HMLE*)$_2$*-MAT*α	This study
Y1485	Y1471, *MAT***a**/(*URA3-HMLE*)$_{>4}$*-MAT*α	This study
Y1486	Y1471, (*URA3-HMLE*)$_2$*-MAT***a**/*MAT*α	This study
Y1487	Y1471, (*URA3-HMLE*)$_{>4}$*-MAT***a**/*MAT*α	This study
Y1488	Y1471, *MAT***a**/*URA3-HMLE-MAT*α	This study
Y1489	Y1471, *URA3-HMLE-MAT***a**/*MAT*α	This study
Y1498	Y1471, *URA3-HMLI>-HMLE-MAT***a**/*MAT*α	This study
Y1503	Y1471, *MAT***a**/*URA3-HMLI>-HMLE-MAT*α	This study
Y1511	Y1471, *URA3-HMLE-MAT***a***-HMLI-LEU2/MAT*α	This study
Y1512	Y1471, *MAT***a***-HMLI-LEU2/MAT*α	This study
Y1513	Y1471, *MAT***a***-<HMRE-LEU2/MAT*α	This study
Y1514	Y1471, *MAT***a**/*MAT*α*-<HMRE-LEU2*	This study
Y1515	Y1471, *MAT***a**/*MAT*α*-HMRE>-LEU2*	This study
Y1768	Y1471, *MAT***a**/*URA3-<HMLI-HMLE-MAT*α	This study
Y1584	*HML*α *MAT*α *HMR*α *sir2::TRP1 ura3-52 leu2-3,112 trp1*	This study
Y1585	Y1584, *MAT*α*-HMLI-LEU2*	This study
Y1586	Y1584, *URA3-HMLE-MAT*α	This study
Y1588	Y1584, *URA3-HMLE-MAT*α*-HMLI-LEU2*	This study
Y1590	Y1584, (*URA3-HMRE>*)$_{>4}$*-MAT*α	This study
Y1762	*HML*α *MAT***a** *HMR*α *sir2::TRP1 ura3-52 leu2-3,112*	This study
Y1765	Y1762, *URA3-HMRE>-MAT***a**	This study
Y1766	Y1762, (*URA3-HMLE*)$_{>4}$*-MAT***a**	This study
Y1767	Y1762, *MAT***a**-(*HMLI-LEU2*)$_{>4}$	This study
Y1793	Y1471, *MAT***a**-(*HMRE>-LEU2*)/*MAT*α	This study
Y1854	*MAT***a**-(*<HMRE-LEU2*)/*MAT*α *cdc35-1/cdc35-1 pde2/pde2 ura3-52/ura3-52 leu2-3,112/leu2-3,112 ade2-1/ade2-1 his3Δ200/hisΔ200 TRP1/trp1-289*	This study
Y1855	*MAT***a**-(*<HMRE-LEU2*)/*MAT*α *cdc35-1/cdc35-1 pde2/pde2 ura3-52/ura3-52 leu2-3,112/leu2-3,112 ade2-1/ade2-1 his3Δ200/hisΔ200 TRP1/trp1-289*	This study
Y1855	Y1854[YCp-CYR1]	This study
DMY1	*MAT***a** *ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100*	42
MS26	*MAT*α *ura3-52 lys2 trp1-1*	M. Rose
MS30	*MAT***a** *ura3-52 lys2 his4*	M. Rose
MS1554	*MAT***a** *ura3-52 his3Δ200 leu2-3,112 ade2*	M. Rose
MS2290	*MAT*α *ura3-52 his3Δ200 leu2-3,112 trp1*	M. Rose

[a] > or < indicates the relative orientation of the silencer element.

tester strains were mixed and collected on a nitrocellulose filter. The filter was transferred to a YEPD plate and incubated for 5 h at 30°C, after which the cells were resuspended in water and plated on synthetic complete plates lacking lysine. After 2 days, colonies were replica plated to synthetic minimal medium (if the test strain was Ura^+) or synthetic minimal plus uracil medium (if the test strain was Ura^-). Percent mating was calculated as the number of colonies growing on the synthetic minimal or synthetic minimal plus uracil medium (diploids) divided by the number of Lys^+ colonies (total test cells). Mating efficiency was determined by dividing the percent mating observed for the test strain by the percent mating observed for control haploid strains, MS1554 and MS2290, examined at the same time.

Liquid sporulation assays. Overnight YEPD cultures of the test strains were inoculated into YEPA, and the cultures were grown at 30°C to a density of 10^7 cells per ml. Cells were harvested, washed, and resuspended at 5×10^7 cells per ml in 2% potassium acetate plus uracil, leucine, and histidine, each at a concentration of 20 μg/ml. Cells were incubated at 30°C for 48 h, at which point the percentage of sporulated cells was determined by microscopic examination in a hemocytometer. At least 500 cells were examined for each determination.

RESULTS

Individual silencers can repress expression from the *MAT* locus. We have explored the structural and organizational features of silencers by examining what sequences from *HML* or *HMR* are required to silence expression of some other gene. The target gene that we have examined in detail is the *MAT* locus itself. We chose *MAT* since the mating-type genes are clearly repressible by the silencing apparatus, at least when resident at *HML* and *HMR*, and since the various genetic and biochemical assays for *MAT* expression can both detect subtle changes in expression and provide clear quantitation of substantial changes. To examine the requirements for silencing, we inserted the *HML* or *HMR* silencers adjacent to the *MAT* locus and then assessed whether the resulting *MAT* allele was expressed or repressed. In all cases, both silent cassettes were left unaltered. The starting strain for these studies was a *MAT***a**/*MAT*α diploid strain, which, because of the coexpression of *MAT***a** and *MAT*α genes, does not mate but is capable of sporulation. Silencing one of the *MAT* alleles would unmask the mating type dictated by the other allele and suppress the sporulation potential of the strain.

The means by which we constructed the test strains is described in Materials and Methods and outlined in Fig. 2. In brief, a plasmid carrying a fragment from *HML* or *HMR* spanning the E or I silencer and a portion of the contiguous mating-type sequences was inserted at *MAT* by plasmid integration via the homologous mating-type sequences. This resulted in a circular integration of the plasmid, in which the *HML* or *HMR* flanking sequences (spanning the silencer) now reside at *MAT* in the same configuration as they do at their original sites. Lying immediately distal to the silencer are vector sequences, followed by *MAT* flanking sequences (Fig. 2). In some instances as noted below, this procedure resulted in multiple tandem copies of the plasmid integrated at *MAT*. In all cases, the location of the insertion and the number of copies of the plasmid integrated were determined by Southern analysis. The *HML* E silencer fragment consisted of the 658 bp of sequence immediately centromere distal to W at *HML*; the *HML* I silencer consisted of the 720 bp of sequence immediately centromere proximal to Z2 at *HML*. The *HMR* E silencer was extracted as a 328-bp *Xho*I-*Ssp*I fragment spanning the three silencer elements, which, for integration at *MAT*, was inserted into a fragment from the *HML* distal region in place of the *HML* E silencer (see Fig. 6).

In our initial experiment, we examined what sequences from *HML* or *HMR* were capable of silencing *MAT*. The results of this analysis are presented in Table 2. The starting strain for these studies, Y1471, does not mate but sporulates efficiently. Insertion of the *HML* E silencer to the left (centromere-proximal side) of either *MAT***a** or *MAT*α or insertion of the *HML* I silencer to the right of either allele does not alter the mating or sporulation phenotype of the strain. Thus, even though both E and I sites appear to have complete silencer activity at *HML*, neither alone at single copy is capable of silencing *MAT*. We obtained a different result with the E silencer from *HMR*. We inserted the *HMR* E silencer adjacent to *MAT***a** or *MAT*α in strain Y1471 in a manner identical to that used to place the *HML* E silencer at those sites (see Fig. 6). In these experiments, the orientation of the *HMR* E silencer relative to the mating-type genes at *MAT* was the same as that of the silencer at *HMR*. The presence of the *HMR* E silencer at either *MAT***a** or *MAT*α yielded significant repression of the adjacent mating-type locus, as judged by the mating assay. The sporulation results are addressed below. Thus, a single copy of *HMR* E, but not of *HML* E or *HML* I, placed adjacent to an otherwise active gene can silence that gene.

TABLE 2. Repression of *MAT* by individual silencers

Strain	*MAT* genotype[a]	% Mating as[b]:		Sporulation (%)[c]
		a	α	
Y1471	**a**/α	−	−	66
Y1488	**a**/E-α	−	−	59
Y1489	E-**a**/α	−	−	57
Y1472	**a**/α-I	−	−	68
Y1512	**a**-I/α	−	−	77
Y1483	**a**/RE-α	77	−	<0.1
Y1482	RE-**a**/α	−	50	54
Y1473	**a**/E-α-I	79	−	3
Y1511	E-**a**-I/α	−	49	47
Y1768	**a**/(E+I)-α	68	−	ND

[a] E, I, RE, and E+I refer to circular integration of a plasmid carrying *HML* E, *HML* I, *HMR* E, or a combination of E and I adjacent to the indicated *MAT* locus as diagrammed in Fig. 2. Constructs in which the silencer is inserted centromere proximal to *MAT* are denoted with the designation to the left of **a** or α, while those in which the silencer is inserted centromere distal have the silencer designation to the right of the locus designation.

[b] Mating efficiency with the haploid tester strains 14a and 17α relative to that of the haploid parents of strain Y1471. −, less than 0.1% of that of the haploid tester strains.

[c] Percentage of cells yielding asci after 48 h in sporulation medium, as described in Materials and Methods. ND, not determined.

To confirm that the phenotypic silencing of the *MAT* alleles by *HMR* E results from transcriptional silencing, total RNA was extracted from the first seven strains listed in Table 2 and the levels of *MAT***a**-specific and *MAT*α-specific transcripts were determined by RNase protection assays. As evident from the results of this analysis (Fig. 3), the presence of either *HML* silencer next to *MAT* does not significantly diminish the levels of *MAT*-specific transcripts. In contrast, no *MAT*α-specific transcripts are present in a strain in which the *HMR* E silencer is inserted adjacent to *MAT*α. Similarly, little *MAT***a**-specific transcripts are present in a strain in which the *HMR* E silencer is inserted adjacent to *MAT***a**. Thus, a single copy of a single yeast silencer is sufficient to repress an adjacent locus.

Different yeast silencers have similar activities but different potencies. While the difference in the abilities of *HMR* E and *HML* E or I to repress adjacent mating-type sequences could reflect a qualitative difference between these two silencers, an alternative hypothesis is that this difference is merely quantitative. Support for this latter interpretation emerged from analysis of strains in which multiple, tandem copies of the plasmid carrying the *HML* E or *HML* I silencer were inserted adjacent to the locus. As noted in Table 3, insertion of more than four tandem copies of the plasmid carrying the *HML* E

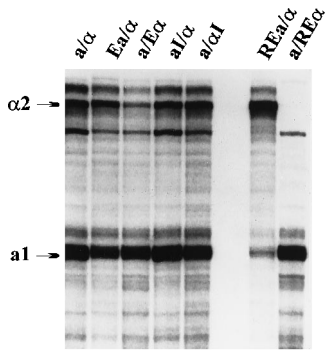

FIG. 3. *MAT*-specific transcript levels in strains carrying single silencers at *MAT*. Total RNA was extracted from the designated strains following growth in YEPD, and the level of *MAT***a***1*-specific and *MAT*α**2**-specific mRNA was determined by an RNase protection assay as described in Materials and Methods. The positions of migration of protected fragments corresponding to *MAT***a1** and *MAT*α*2* mRNAs are indicated. The fragment migrating slightly faster than that for *MAT*α*2* corresponds to unspliced *MAT***a***1* transcript. Strains: **a**/α, Y1471; E**a**/α, Y1488; **a**/Eα, Y1489; **a1**/α, Y1512; **a**/α**I**, Y1472; RE**a**/α, Y1482; **a**/REα, Y1483.

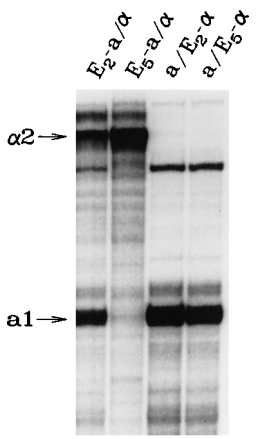

FIG. 4. Multiple copies of the *HML* E silencer represses the adjacent *MAT* locus. *MAT*-specific transcript levels were determined for the indicated strains as described in the legend to Fig. 3. Strains: E_2-**a**/α, Y1486; E_5-**a**/α, Y1487; **a**/E_2-α, Y1484; **a**/E_5-α, Y1485. E_2 designates two tandem integrated copies of the plasmid carrying *HML* E, and E_5 designates four or more tandem integrated copies of the same plasmid.

locus adjacent to either *MAT***a** or *MAT*α yielded full repression of the contiguous *MAT* locus. Insertion of even two copies of the plasmid carrying *HML* E was sufficient to repress *MAT*α, although it was not sufficient to repress *MAT***a**. We confirmed these results by an RNase protection assay as shown in Fig. 4. Similarly, multiple copies of the plasmid carrying the I silencer from *HML* were sufficient to promote some repression of the adjacent *MAT* locus, although two copies alone were less effective in repressing *MAT*α than were two copies of *HML* E. These results clearly demonstrate that the effects of silencers are cumulative, even when the individual silencers are separated by 3-kb intervals, as they are in the strains that we examined. In addition, these results suggest that all three silencers possess fundamentally similar properties, although with different potencies: all are able to repress an adjacent locus, but *HMR* E is more potent than *HML* E, which is itself more potent than *HML* I. Finally, these results indicate that all three silencers can serve as centers for gene inactivation, with repression emanating outward from the cluster of silencers to adjacent loci.

Heterologous pairs of silencers are also effective in repressing expression from *MAT*. We inserted the *HML* E silencer to the left of *MAT* and the *HML* I silencer to the right of *MAT* to reconstruct at *MAT* the configuration of silencers found at *HML*. As noted in Table 2, this configuration of silencers yields complete repression of both *MAT***a** and *MAT*α, even though neither silencer alone is capable of silencing either allele. Thus, two silencers bracketing a locus can effect silencing of the region between them. However, the effect is likely achieved not because the silencers bracket the locus but rather because silencers function is additive. This conclusion is suggested by the behavior of strain Y1768, in which the *HML* E and *HML* I silencer are inserted adjacent to one another to the left of the *MAT* locus (see Fig. 6). As noted in Table 2, this configuration of silencers is just as effective in silencing the adjacent locus as is found with the two silencers bracketing the locus. Thus, we conclude that efficient silencing can be attained simply by increasing the number of homologous or heterologous silencers near a locus.

TABLE 3. Silencers exhibit a hierarchy of activity[a]

No. of copies	Relative mating efficiency					
	Silencer inserted at *MAT***a**			Silencer inserted at *MAT*α		
	HMR E	*HML* E	*HML* I	*HMR* E	*HML* E	*HML* I
1	0.25	<0.001	<0.001	0.77	<0.001	<0.001
2	0.90	0.005	—	—	0.89	0.007
>4	—	0.80	0.008	—	1.05	—

[a] Strains with the indicated number of plasmids tandemly integrated adjacent to *MAT***a** or *MAT*α were tested for mating ability by the quantitative mating assay described in Materials and Methods. Results are presented as the fraction of cells mating with the appropriate tester strain relative to that of the parental strains. The strain designations for the test strains are listed in Table 1. —, strain not constructed.

Ectopic silencing uses the same machinery as other forms of transcriptional silencing. To confirm that repression of *MAT* mediated by the E and I sites from *HML* and *HMR* is mechanistically identical to other examples of silencing in *S. cerevisiae*, we examined whether this repression required the same gene products as are required for establishing and maintaining repression of the silent cassettes. To test this hypothesis, we examined the effect of mutations in the *SIR* genes on repression of *MAT* by E or I. We inserted a single copy of *HMR* E or multiple copies of *HML* E or I adjacent to *MAT***a** in an *HML*α *MAT***a** *HMR*α strain. (As in the strains above, these configurations of silencers adjacent to *MAT***a** rendered the locus inactive: diploids formed by mating these strains to a *MAT*α strain mate as α.) We then made the strains *sir* and tested their mating types. If *HML*, *HMR*, and *MAT* are all repressed by the same *SIR*-dependent mechanism in these strains, then inactivating a *SIR* gene would derepress all three mating-type loci and the strains would be sterile. On the other hand, if repression of *MAT***a** were independent of the *SIR* genes, then inactivating a *SIR* gene would derepress only *HML*α and *HMR*α and the strains would mate as α. We found that *sir2* derivatives of these strains were sterile (data not shown). Thus, the same apparatus responsible for repressing the silent cassettes promotes ectopic silencing of *MAT* mediated by the repositioned E and I silencers.

We also performed a control experiment to address the fact that construction of the silencer-containing strains used in this study required, during the process of transformation, functional expression of the selectable marker carried on the plas-

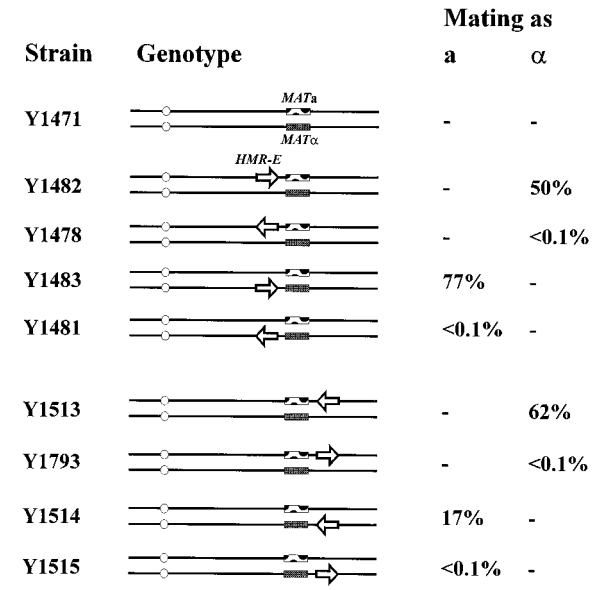

FIG. 5. Orientation dependence of a yeast silencer. The diagrams show the locations and orientations of the single *HMR* E silencer inserted adjacent to the *MAT* locus in the strains designated on the left. The construction of each strain is described in Materials and Methods. The orientation of the inserted *HMR* E silencer in strain Y1482 relative to the adjacent mating-type locus and to the chromosome as a whole is identical to that of the silencer in its normal position at *HMR*. The results of quantitative mating assays for the indicated strains are indicated on the right. −, less than 0.1%.

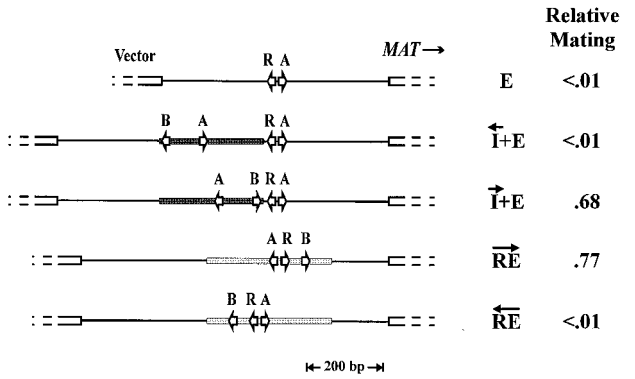

FIG. 6. Silencing depends on the particular organization of silencer elements. Scale diagrams show the structures of various hybrid silencers, indicating the locations and orientations of Abf1p binding sites (B), Rap1p binding sites (R), and consensus ARS sites (A). E, the normal *HML* E silencer (thin line), positioned with respect to mating-type genes as is found at *HML* (plasmid pDM33); I+E, the *HML* E silencer into which at the position shown is inserted a 275-bp fragment spanning the *HML* I silencer (hatched box) in either orientation (as found in plasmid pGJ28 [top] or plasmid pGJ41 [bottom]); RE, deletion of the *HML* E silencer domain and insertion of a 328-bp sequence spanning the *HMR* E silencer, in either orientation (as found in plasmid pGJ29 [top] or plasmid pGJ32 [bottom]).

mid. Since the selectable marker might have been subject to silencing elicited by the silencer also present on the plasmid, we assured ourselves that the selection process did not bias the behavior of the silencers in the resulting transformants. We integrated selected silencer-containing plasmids into the *sir2 HML*α *MAT*α *HMR*α strain Y1584. Since silencing is abrogated in this strain, no bias exists in selecting transformants. By this procedure, we obtained strains Y1585, Y1586, Y1588, and Y1590, carrying *MAT*α-*HMLI*, *HMLE-MAT*α, *HMLE-MAT*α-*HMLI*, and $(HMRE)_{>4}$-*MAT*α, respectively (Table 1). These strains were then mated with strain DMY1, and the mating behaviors of the resulting Sir^+ diploids were compared with those of strains that had the same genetic organization of the *MAT* loci as the corresponding Y1584 derivatives but were constructed in a Sir^+ background (Materials and Methods and Table 1). The results of this analysis demonstrated that Y1585/DMY1 and Y1586/DMY1 were sterile, just as were the corresponding Y1471 derivatives Y1472 and Y1488, while Y1588 and Y1590 exhibited efficient **a** mating, just as did Y1473 and Y1483 (data not shown). Thus, the activity of the silencer that we inserted at *MAT* was independent of the method used to construct the strains.

Silencers are orientation dependent. Prior studies of the *HMR* E silencer indicated that at its normal locus, it could exert repression in an orientation-independent fashion (5). We have found that when resident at *MAT*, this silencer exhibits a notable orientation dependence. We placed the *HMR* E silencer centromere proximal to *MAT* both in the same relative orientation to the mating-type genes as is found at *HMR* and in the opposite orientation. As evident from the analysis presented in Fig. 5, the normal orientation of *HMR* E yielded full repression of both *MAT***a** and *MAT*α. In contrast, *HMR* E positioned at the same site but in the opposite orientation failed to repress either *MAT***a** or *MAT*α.

To test whether this orientation dependence was due to the orientation of the silencer relative to *MAT* or to its absolute orientation on the chromosome, we also placed the silencer in both orientations adjacent, but centromere distal, to *MAT*. As shown in Fig. 5, the reciprocal results were obtained with these constructs as were found with strains carrying the silencer centromere proximal to *MAT*: the *HMR* E locus in the same absolute orientation, relative to the chromosome, as is found at *HMR* was less efficient in repressing *MAT* than it was in the opposite orientation. Similar results were obtained with *HML* E inserted centromere distal to *MAT* (data not shown). These results suggest that the strength or extent of *HMR* E's repressing activity is greater to one side of the silencer than to the other. When that side of the silencer is oriented toward the test locus, then repression occurs; when the opposite side faces the test locus, repression is not established as efficiently.

Given these results, we would anticipate that the organization of the elements within the silencer would affect its strength of repression, since this organization to some extent defines the orientation of the silencer. Evidence to support this hypothesis comes from our analysis of fused *HML* E-plus-*HML* I silencers. As shown in Fig. 6, we created two hybrid *HML* E-plus-I silencers that differed only in the relative orientation of the I silencer inserted next to E. In one case, the resulting hybrid was completely inactive as a unifocal silencer, while in the other case, the silencer was completely active. This is true in spite of the fact that the numbers of ARS elements and of Rap1p and Abf1p binding sites were the same in both constructs. Thus, the relative order of the elements that comprise the silencer is critical for silencer function. Not surprisingly, the hybrid *HML* E-plus-I silencer that was active consisted of an organization of elements that more closely resembled that found at *HMR*.

Ectopic silencers can respond to external stimuli. We found that several strains carrying a silenced *MAT* locus both mated well and sporulated well; that is, they exhibited the phenotypes both of a haploid strain and of a diploid strain. One possible explanation for this mixed phenotype was that cultures of such strains contained two populations of cells: one population would consist of cells in which the *MAT* locus was silenced, and the other would consist of cells in which the *MAT* locus was

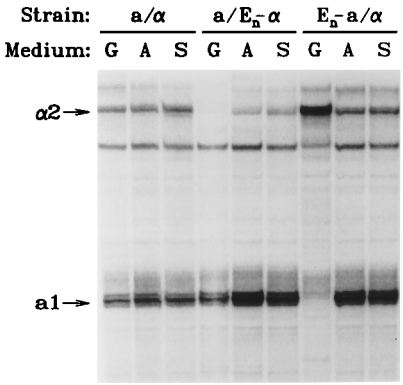

FIG. 7. Silencing in some strains is dependent on growth on glucose. RNA was isolated from the indicated strains after growth in YEPD (lanes G), YEPA (lanes A), or sporulation medium (lanes S), and *MAT*-specific transcript levels were determined as described in the legend to Fig. 3. Strains: **a**/α, Y1471; **a**/E$_n$-α, Y1485; E$_n$-**a**/α, Y1487. E$_n$ designates four or more tandem integrated copies of the plasmid carrying *HML* E.

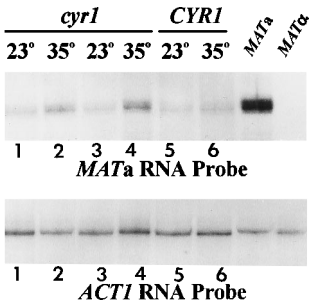

FIG. 8. Conditional silencing is mediated by the RAS/cAMP pathway. Strains Y1854 (lanes 1 and 2), Y1855 (lanes 3 and 4), and Y1856 (lanes 5 and 6) were grown at 23°C to 10^7 cells per ml. Each of the cultures was split in half; and one half was maintained at 23°C, and the other was shifted to 35°C. After 3 h, when greater than 80% of the Y1854 and Y1855 cells grown at 35°C were unbudded, cells were harvested. RNA was extracted, and the levels of *MAT***a***1* and *ACT1* mRNAs were determined by RNase protection assays as described in Materials and Methods. Specific RNA determinations were also performed with total RNA isolated from an equivalent number of cells from the *MAT***a** and *MAT*α strains, MS1554 and MS2290.

expressed. This epigenetic effect has been observed for *sir1* strains and for strains carrying certain mutations of *HML* E (43, 53). An alternative explanation for the dual phenotype was that the silencing in these strains was conditional. During mitotic growth, when mating capacity was measured, the *MAT* locus would be repressed, while under starvation conditions required for sporulation, the locus would be expressed. Such conditional silencing accounts for the same dual phenotype resulting from the *SAD1* allele of *MAT*, which arose as a spontaneous fusion of *HMR* E to *MAT*α (26, 29, 44). Results presented below demonstrate that conditional silencing accounts for the dual phenotypes of our strains as well.

Strain Y1487, in which *MAT***a** was silenced by multiple integrated copies of the plasmid carrying the *HML* E silencer, and strain Y1485, in which *MAT*α was silenced by two copies of the same plasmid, both mated well and sporulated efficiently. To test whether the dual phenotype of these two strains was due to conditional silencing, we isolated RNA from cultures of these strains after growth in a rich medium and after a shift to sporulation conditions. The levels of *MAT*-specific transcripts in these samples were determined by RNase protection assays, the results of which are presented in Fig. 7. As is evident for both strains, the silenced *MAT* locus was completely repressed when the strain was grown on a rich medium but was completely derepressed when the strain was shifted to sporulation medium. This result fully accounts for the dual phenotype of these strains. When grown on a rich medium, only one *MAT* locus was expressed and the strains could mate. When shifted to sporulation conditions, both *MAT* loci were expressed and the strains behaved as diploids and sporulated. Similar instances of conditional silencing were observed with strains Y1482 and Y1513, in which *MAT***a** is silenced by a single copy of *HMR* E, and strain Y1511, in which *MAT***a** is bracketed by single copies of *HML* E and *HML* I (Table 2 and data not shown). However, in strains such as Y1483 that do not show a dual phenotype, the silenced *MAT* locus is repressed under all growth conditions (data not shown). This observation provides additional confirmation that the dual phenotype seen with some of the strains results from the conditional expression of the silenced *MAT* locus.

Induction of sporulation requires both eliminating a nitrogen source and shifting cells from glucose to a nonfermentable carbon source, such as acetate. To determine whether both of these conditions were required to elicit derepression of the conditionally silenced *MAT* loci, we also measured *MAT*-specific transcripts in strains after simply shifting the cells from glucose to acetate as a carbon source. As documented in Fig. 7, shifting from glucose to acetate as carbon source gave the equivalent derepression of the silenced *MAT* locus as did shifting to complete sporulation conditions. Similar results were obtained in shifting from glucose to a number of other nonfermentable carbon sources, including ethanol, glycerol, and lactate. Thus, the presence of glucose is required for efficient silencing by all the conditional silencers; the absence of glucose yields full derepression.

Glucose-induced silencing is mediated in part by the Ras/cAMP pathway. Recent results have indicated that RAP1-mediated transcriptional activation of a number of genes is influenced by cyclic AMP (cAMP) levels in the cell (33, 50). Since cAMP levels vary in response to glucose and since RAP1 is a component of all of the silencers noted above that exhibit conditional silencing in response to glucose levels, we examined whether conditional silencing in response to glucose was mediated by the RAS/cAMP pathway. We constructed a diploid strain homozygous for *cdc35-1*, a temperature conditional mutation in the gene for adenylyl cyclase, and carrying the *HMR* E silencer adjacent to *MAT***a**, which in this configuration exhibits conditional silencing. We then compared the *MAT***a***1* transcript level in this strain with that of an isogenic derivative carrying *CDC35* on a *CEN* plasmid. The results of this analysis are presented in Fig. 8. When grown at 23°C, two independent *cdc35-1* homozygous strains as well as an isogenic *CDC35* derivative strain (designated *cyr1* and *CYR1*, respectively, in Fig. 8) show low levels of *MAT***a***1* transcription, indicating efficient silencing of the MAT locus by the *HMR* E silencer in these strains. Following a shift to 35°C, though, the *MAT***a***1* transcript levels increase in the *cdc35-1* strains but not in the *CDC35* strain. Thus, loss of adenylyl cyclase function leads to diminished silencing efficiency by the *HMR* E silencer adjacent to *MAT***a**, even in the presence of glucose. Thus, we can conclude that at least part of the glucose effect on silencing is mediated by the RAS/cAMP pathway.

HML* and *HMR* silencers establish silencing at different rates.** Since the presence of glucose is required for silencing by a variety of conditional silencers, we were able to use glucose addition as a means of examining the time course for establishing silencing over an initially derepressed locus. As noted above, *MATa** is conditionally silenced either by a contiguous

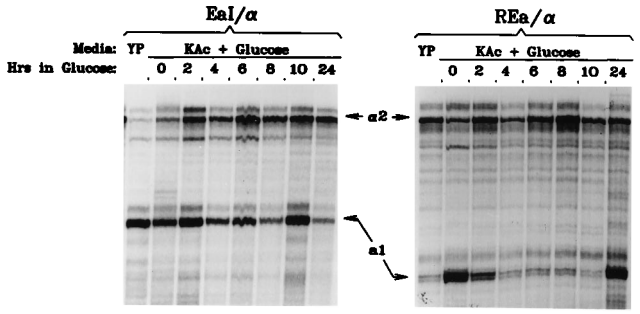

FIG. 9. The *HML* and *HMR* silencers differ in the ability to establish repression. The indicated strains were grown either in YEPD (YP) or in YEPA (KAc) to ca. 2×10^7 cells per ml. Glucose was added to the YEPA culture, and samples were removed immediately (time zero), at 2-h intervals for 10 h, and at 24 h. RNA was extracted from the samples, and *MAT*-specific transcript levels were determined as described in the legend to Fig. 3. Strains: E**a**I/α, Y1511; RE**a**/α, Y1482.

copy of *HMR* E or by bracketing the locus with single copies of *HML* E and I. Strains Y1482 and Y1511 carrying these constructions were grown in YEPA medium, resulting in derepression of the *MAT***a** locus. Glucose was then added to these cultures, samples were removed at 2-h intervals, and *MAT*-specific transcripts were measured in total RNA extracted from these culture samples. As evident from the results of this experiment (Fig. 9), addition of glucose to the culture of strain Y1513 led to a rapid repression of the *MAT***a** locus. Within 2 h the locus was more than 80% repressed, and by 4 h the locus was completely repressed. The *MAT***a** locus was again derepressed at 24 h, consistent with the fact that the culture was depleted for glucose by this time. In contrast, strain Y1511 failed to exhibit any repression of the *MAT***a** locus during the 10 h for which the culture was monitored. Repression could eventually be established in this strain: single cells isolated from the test culture and grown continuously in YEPD yielded cultures exhibiting full repression of *MAT***a**. Once silencing was established, though, both sets of silencers were equally active in maintaining the silenced state, as judged both by quantitative mating assays and by RNA transcript levels (Fig. 9).

DISCUSSION

A model for silencer function. The results presented in this report can best be appreciated in the context of our current model of the nature of *SIR*-mediated silencing. First, silencing is a state switch; it simply renders an active locus inactive. That is, the silencing apparatus does not modulate the level of expression of a locus but merely determines whether the locus is on or off (43). When the silencing apparatus is active, then the silenced locus is inactive; when the silencing apparatus is inactive, then the locus responds to the normal regulatory circuits mediated by its associated enhancer elements. Second, for the reasons outlined in the introduction, the physical nature of this state switch is likely to be chromatin structure. The properties of silenced chromatin is similar in many respects to that of heterochromatin in metazoan cells. While heterochromatin has not been identified in *S. cerevisiae* by cytological techniques nor directly correlated with silenced loci, silencing can most readily be conceptualized as a yeast version of heterochromatin.

Our analysis of the properties of silencer elements presented in this report clarifies their role in establishing and maintaining silencing. First, our results demonstrate that silencers serve as organization sites for heterochromatin formation and are consistent with a model in which heterochromatin emanates outward from the site of the silencer, although not necessarily symmetrically. This is consistent with recent results of Renauld et al. (54) indicating that silencing from the telomere extends in a continuous fashion into adjacent loci. Second, our results, in conjunction with those of Aparicio and Gottschling (3), can best be explained by assuming that the probability that a contiguous site is encompassed in heterochromatin and/or the extent to which the heterochromatin extends outward is the result of a competition between the silencer promoting a condensation of the chromatin and an enhancer or activator at the contiguous site promoting an open chromatin configuration. Thus, as we noted in this report, increasing the strength of a silencing center, either by using a stronger silencer or by adding more copies of the silencer, increases the likelihood that the adjacent locus will be silenced. Reciprocally, increasing the strength or activity of the enhancer associated with an adjacent locus would decrease the likelihood that the locus would be silenced (3). This would account for the fact that the basal, but not the induced, expression of *URA3* is silenced when it is positioned at telomeric sites (18) and that overexpression of *PPR1*, the *URA3* transcriptional activator, relieves silencing of *URA3* inserted at a telomere (3). It would also account for the fact that positioning *HMR* E adjacent to a yeast heat shock locus results in silencing of the basal, but not heat shock-induced, expression of the gene (39). Finally, as noted in this report, the strength of a silencer can be modified by external stimuli. Thus, the presence and extent of heterochromatin in *S. cerevisiae* may well be a function of the growth conditions of the cell.

What constitutes a silencer? The results in this study allow us to clarify the critical anatomy of a silencer and resolve several of the conundrums of silencer activity noted in the introduction. First, by using an assay that defines what is sufficient for silencing, rather than previous assays that defined only what was necessary for silencing, we have resolved the minimum requirements for silencer function. By this criteria, both *HML* E and I are required for silencing, a result consistent with initial plasmid-based studies of *HML* but inconsistent with chromosomal deletion analysis (16, 42). Similarly, *HMR* E alone is sufficient for silencing in rich media but not in starvation media. This finding is also consistent with the initial plasmid-based studies but inconsistent with chromosomal deletion analysis (1). Thus, other elements in addition to E and I that contribute to the efficiency of silencing at the loci likely exist at both *HML* and *HMR*. On the basis of the phenotypes of certain specific chromosome rearrangements, these sites likely lie centromere distal of both loci (26, 65). Second, our results indicate that the organizations of specific elements within the silencer are critical for proper silencer function. Thus, the mere presence in close proximity of two of the three elements normally found in a silencer is not sufficient to constitute silencer function. What the organization constraints are have yet to be determined. Nonetheless, these observations account for the absence of silencer function of the various ARSs and enhancers composed of combinations of elements found at silencers.

Establishment of silencing in *S. cerevisiae*. The results in this report highlight the differences between establishment and maintenance of silencing in *S. cerevisiae*. Distinction between establishment of silencing and maintenance of silencing was previously invoked by Pillus and Rine (53) and Mahoney et al. (43) to explain the epigenetic characteristics of repression of *HML* in *sir1* strains or in strains with mutations in *HML* E. The results presented in this report allow us to observe establishment of repression directly. By using glucose-mediated formation of repression, we obtained concerted conversion of the

test locus in the entire population of cells from fully expressed to fully repressed. This has allowed us to document that different types and organizations of silencers have different potencies in establishing repression of an adjacent locus.

The *HML* and *HMR* silencers exhibit different rates for establishing repression. That is, the *HMR* E silencer converts the locus in all the cells to the repressed state within essentially one generation. In contrast, the *HML* E silencer requires a number of generations before a significant number of cells have established repression across the *MAT* locus. The differences in the efficiencies in establishing repression by the *HML* E-plus-I silencer as opposed to the *HMR* E silencer may explain a variety of distinguishing features of the two silent loci. For instance, *HML* is significantly more sensitive to derepression resulting from perturbation of the silencing apparatus than is *HMR*. That is, *ard1* or *nat1* strains exhibit derepression of *HML* but not of *HMR* (47, 67). Similarly, mutations of *HHF2* that cause derepression of *HML* do not affect repression of *HMR* to nearly the same extent (30, 52). These mutations may adversely affect equally the ability of *HML* and *HMR* silencers to maintain repression of the silenced state. However, in any cell in which the *HMR* locus became derepressed, the locus would be rapidly rerepressed within one generation. On the other hand, once derepressed, the *HML* locus would remain derepressed, leading to accumulation of the derepressed cells in a population.

What could account for the differences in efficiencies of establishment by different silencers? Replication initiation at the E silencer of *HMR* is more efficient than that at the E silencer of *HML* (15, 57). However, since initiation at the ARS adjacent to *HMR* occurs in only a relatively small percentage of cells in any one generation and since repression can be established in essentially one generation, replication initiation at the locus does not appear to be a prerequisite for establishing repression. Another potentially relevant distinction between the two loci is the actual organization of protein binding sites at the *HMR* and *HML* silencers. Assuming that the A, E, and B elements at a silencer provide binding sites for the Orc, Rap1p, and Abf1p proteins and that at least two of these sites must be occupied for the silencing apparatus to initiate chromatin condensation, then *HMR* E, which carries more sites, might be expected to bind at least one protein more readily that would either *HML* E or *HML* I, each of which carries only two such sites. If binding of these proteins exhibits cooperativity, then binding of one protein would nucleate formation of the complete structure. In addition, differences in the specific affinities or cooperativity of the particular elements at *HML* and *HMR* for their corresponding DNA proteins, either intrinsically or due to context effects, could enhance the differences in the initial binding kinetics. This may explain why a synthetic *HMR* E silencer is not quite as efficient as is the wild-type allele (45) and why the different organizations of the E-plus-I silencer at *MAT* have different effects on the adjacent locus.

Glucose-dependent silencing. The results presented in this report clearly document that in certain organizations of silencers in *S. cerevisiae*, silencing is dependent on nutrient availability. In particular, silencing by either the *HMR* E silencer alone or the *HML* E-plus-I silencer in concert is fully effective when strains carrying such constructs are grown on glucose, but repression by these silencers is completely abrogated, and the formerly repressed locus is fully activated, when the strain is grown on a nonfermentable carbon source. We expect that glucose affects the silencing apparatus itself and not the *MAT* genes that are being repressed, since none of the genes at *MAT* are subject to modulation by glucose (23). In this respect, our results differ from those of Lee and Gross (39), who noted that heat shock could abrogate ectopic silencing of a heat shock gene. In this case, the environmental stimulus affected the competition between silencing and expression through effects on the gene being silenced. In our case, we have revealed a sensitivity of the silencing apparatus itself to the growth conditions of the cell.

This observation could be relevant to naturally occurring cases of silencing in yeast cells. In standard laboratory strains, the silent mating-type cassettes are normally not subject to conditional silencing: both silent mating-type cassettes remain fully repressed even under sporulation conditions (**a**/**a** and α/α strains fail to sporulate, even though they carry *HML*α and *HMR***a** loci). However, certain strains of *S. cerevisiae* when subjected to starvation conditions undergo a transition from symmetrical budding to an asymmetrical growth pattern that results in a pseudohyphal morphology (17). This transition generally only occurs in diploids, since filamentous growth requires the polar budding pattern exhibited only by diploids. Recently, though, certain haploid strains that also exhibit pseudohyphal growth following starvation have been described (36). One explanation for this behavior could be that the silent cassettes in these strains exhibit conditional silencing. In this fashion, starvation would activate the silent cassettes to render the cells phenotypically diploid and thus allow them to access pseudohyphal growth, a potentially advantageous starvation response. Similarly, telomeric regions, which are naturally less efficiently silenced than are the mating-type storage loci, may well exhibit conditional silencing even in standard laboratory strains (2, 18). One consequence of such a conditional silencing would be that the telomere structure could be altered during meiosis, allowing, for example, a resetting of the length of the telomere upon passage through meiosis. An analogous mechanism in mammalian cells could explain the different behaviors of telomere lengths in meiotic and somatic cells.

The mechanism by which glucose could affect the activity of the various silencers is unresolved. A likely candidate for mediating this effect is Rap1p. A number of genes—those encoding ribosomal protein or glycolytic enzymes, for example—exhibit altered transcription as a function of the presence or absence of a fermentable carbon sources. That portion of the upstream activator region in these genes required for this transcriptional modulation in response to different carbon sources corresponds to Rap1p binding sites (7, 12, 22). A reasonable hypothesis is that the activity of Rap1p is altered in response to the carbon source, which translates into altered transcriptional activation by the protein (33, 50). Since Rap1p plays a critical role in silencer function (66), the altered activity of Rap1p in response to glucose could well account for the altered silencer function. Our results indicating that mutations in *CDC35* mimic the starvation signal suggest that the RAS/cAMP system comprises at least part of the pathway for glucose repression of silencing.

Conditional silencing has numerous correlates in mammalian cells. X-chromosome inactivation occurs in primordial germ cells, but the inactivated X chromosome is reactivated during meiosis such that expression from both X chromosomes occurs in oocytes (19, 35). Imprinted genes are similarly reset during meiosis: an autosome inherited by an individual from the parent of the opposite sex is reset during meiosis to yield the imprinting pattern dictated by the sex of that individual (13, 21, 63). Finally, imprinted genes can be specifically reactivated in specific cell types within an individual, suggesting that imprinting can be regulated by the particular cellular environment in which it resides (13). Thus, as in *S. cerevisiae*, cases of mammalian silencing are subject to specific reactivation, particularly during the process of meiosis.

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