MOLECULAR AND CELLULAR BIOLOGY, Nov. 1989, p. 4621–4630
0270-7306/89/114621-10$02.00/0

Vol. 9, No. 11

The *HML* Mating-Type Cassette of *Saccharomyces cerevisiae* Is Regulated by Two Separate but Functionally Equivalent Silencers

DEBORAH J. MAHONEY AND JAMES R. BROACH*

Department of Biology, Princeton University, Princeton, New Jersey 08544

Received 24 April 1989/Accepted 12 July 1989

Mating-type genes resident in the silent cassette *HML* at the left arm of chromosome III are repressed by the action of four *SIR* gene products, most likely mediated through two *cis*-acting sites located on opposite sides of the locus. We showed that deletion of either of these two *cis*-acting sites from the chromosome did not yield any detectable derepression of *HML*, while deletion of both sites yielded full expression of the locus. In addition, each of these sites was capable of exerting repression of heterologous genes inserted in their vicinity. Thus, *HML* expression is regulated by two independent silencers, each fully competent for maintaining repression. This situation was distinct from the organization of the other silent locus, *HMR*, at which a single silencer served as the predominant repressor of expression. Examination of identifiable domains and binding sites within the *HML* silencers suggested that silencing activity can be achieved by a variety of combinations of various functional domains.

In *Saccharomyces cerevisiae*, genes encoding regulatory proteins that determine the mating type of the cell are located at three different positions on chromosome III (2, 39, 57). Those genes resident at *MAT* are transcribed to establish and maintain the mating type of the cell (14, 17, 30, 38, 54). The same genes resident at either end of the chromosome, at loci designated *HML* and *HMR*, are not transcribed and do not contribute to the mating phenotype of the cell (29, 37, 40). Rather, these loci serve solely as repositories of mating-type information, which usually can be activated only by transposition to *MAT* (18, 25, 26, 28, 31).

Differential expression of the mating-type genes at *MAT* versus those at *HML* and *HMR* results from repression of transcription at these "silent" loci. The products of three genes, *SIR2*, *SIR3*, and *SIR4*, act to maintain *HML* and *HMR* in a transcriptionally inactive state (15, 27, 42, 43). Mutational inactivation of any one of these genes yields expression of the mating-type genes at *HML* and *HMR* at a level equivalent to that of the genes present at *MAT*. The product of a fourth gene, *SIR1*, is required for full repression of the silent loci. Inactivation of this gene yields derepression of the loci in only a subset of the cells in the population (22, 42). Repression of the silent loci also requires the integrity of two specific, *cis*-active sites, designated E and I, which flank each locus (1, 13). These sites are relatively small (<150 base pairs [bp]) and are located more than 1 kilobase away from the promoters of the genes they regulate. A reasonable working hypothesis is that these sites serve as the loci through which the *SIR* products act to establish and maintain repression of the silent cassettes.

The E site at *HMR* has been studied in detail by several groups. *HMR* E can act in an orientation-independent fashion over a range of distances to mediate *SIR* repression of the mating-type genes at *HMR* (5). In addition, the E site can fully repress transcription of the *TRP1* and *LEU2* genes, when these are inserted into *HMR*, and can partially repress expression from the polymerase III promoters of the tRNA genes *SUP3* and *SUP4* (5, 47). On the basis of the similarity of these properties of the E site to those of eucaryotic enhancer elements and acknowledging that the E site mediates repression rather than activation, this site had been termed a "silencer."

The structural features of the *HMR* E region salient to its silencer activity have been investigated. This particular silencer region encompasses three recognizable domains: an 11-bp consensus element associated with yeast autonomously replicating sequences (ARSs); a binding site for an abundant DNA-binding protein, termed variously RAP1, GRF1, TUF1, or TBF1; and a binding site for a protein, called ABF1, that also binds near, but not at, the consensus ARS element at a number of chromosomal origins of replication (1, 6–8, 13, 49, 51). Mutational analysis of the *HMR* E region has shown that inactivation of any one of these elements does not significantly diminish the silencing capacity of the locus, but that inactivation of any two of these elements yields essentially complete derepression of *HMR* (6, 51). Thus, any two of these three elements is sufficient for imparting silencer activity.

In this report, we describe our analysis of the silencer domains associated with the *HML* mating cassette. In contrast to earlier studies of the regulation of *HML* and distinct from the situation at *HMR*, the *cis*-acting regulatory loci on either side of *HML* appear to be functionally equivalent. Either site alone is sufficient to maintain repression of *HML*. In addition, both sites show silencer activity, as judged by their ability to repress heterologous genes placed in their vicinity. Since both of these sites have only a subset of the elements present in the *HMR* E silencer, these results help define the salient features of natural yeast silencers.

MATERIALS AND METHODS

DNA manipulations. Plasmid DNA was prepared from strain DH1 (16) or MC1066 (10) by the method of Birnboim and Doly (3), as described in Maniatis et al. (32). Yeast DNA was prepared as described by Hoffman and Winston (19) or Abraham et al. (1). Restriction enzymes were purchased from New England and BioLabs, Inc., Beverly, Mass., and were used according to the directions. For Southern blot analysis (53), digested yeast DNA was run on 0.8% agarose gels and transferred to activated nylon membranes, using the alkaline transfer method of Reed and Mann (41). Probes were prepared by nick translation or random primer tech-

* Corresponding author.

nique (32), using [α-^{32}P]dATP purchased from Dupont, NEN Research Products, Boston, Mass. Hybridization and washing of membranes were performed as described by Church and Gilbert (11).

Plasmid constructions. E and I deletion plasmids used in this study were derived from plasmids described previously (13). E deletion plasmids pDM22 and pDM23 were derived from plasmids 637 (E^+) and 504 (E^-) (13) by cloning the *Bam*HI to *Hin*dIII fragment containing *HMLα* and differing amounts of the telomere-proximal sequences into *Bgl*II-plus-*Hin*dIII-digested plasmid pDM17 DNA. Plasmid pDM17 carries the *Bam*HI to *Xba*I telomere-proximal sequences of *HMLα*, with the *Xba*I site converted to a *Bgl*II site (see Fig. 1A). A *Hin*dIII fragment spanning *URA3* was cloned into the *Hin*dIII site of these intermediate plasmids to generate plasmids pDM22 and pDM23. I deletion plasmids pDM18 and p242-3 were derived from R547 (I^+) and R242 (I^-), respectively. An *Eco*RI to *Hin*dIII fragment containing *HMLα* and differing amounts of centromere-proximal sequences was cloned into *Eco*RI-plus-*Hin*dIII-digested pSI20-4 plasmid DNA. Plasmid pSI20-4 carries the *Hin*dIII to *Bam*HI centromere-proximal fragment of *HMLα* (see Fig. 1A). Plasmid pDM68 was created by cloning the *URA3* *Hin*dIII fragment into the *Hin*dIII site of plasmid p242-3. Plasmid pDM60 was created by cloning a *Bam*HI fragment containing the 243-bp $SUP4_o$ tRNA gene from plasmid YC689 (48) into the *Bgl*II site of plasmid pDM11. Plasmid pDM11 carries the *Bam*HI to *Hin*dIII fragment spanning *HMLα*, in which a *Bgl*II octanucleotide linker has been substituted for nucleotides 79 to 113 within *HML* E (numbering system from reference 13), rendering the site E^- (Mahoney and Broach, unpublished data). Similarly, plasmid pDM30 carries the same *Bam*HI to *Hin*dIII fragment as pDM11 and has a *Bgl*II octanucleotide linker substituted for nucleotides 59 to 76. This substitution does not affect E activity (Mahoney and Broach, unpublished data). Insertion of the *URA3* *Hin*dIII fragment into the *Bgl*II site of pDM11 and pDM30 gave plasmids pDM41 and pDM40, respectively.

Plasmid pJH3A is an autonomously replicating plasmid carrying the 3′ one-fourth of the *SIR4* gene. This plasmid acts as a dominant SIR^- allele (22, 34).

Plasmid pSC4, used for creating $mat\alpha1^-\alpha2^-$ strains, was derived from the $mat\alpha1^-\alpha2^-$ C46 *Xho*I linker mutant described by Siliciano and Tatchell (52) by cloning a *Sal*I to *Xho*I fragment spanning *LEU2* into the *Xho*I linker insertion.

Plasmid pGAL-*HO*, which carries the yeast *HO* under partial control of the *GAL10* promoter, is described in Rose and Broach (44). Expression of *HO* carried on this plasmid is sufficient to induce mating-type interconversion even when strains harboring this plasmid are grown on raffinose, a noninducing, nonrepressing carbon source.

Plasmid pDM42 carries a *sir3*::*URA3* allele, in which 369 nucleotides between two *Bgl*II sites within the *SIR3* coding region are replaced by a *Hin*dIII fragment spanning the *URA3* gene (50).

Plasmid pSC3 contains the *Eco*RI to *Bam*HI fragment spanning *HMLα*, into the *Hin*dIII site of which is inserted a *Hin*dIII fragment encompassing *URA3* (see Fig. 1A).

Plasmid pGJ1 contains the *Eco*RI to *Bam*HI fragment spanning *HMLα*, in which the 1.1-kilobase *Hin*dIII fragment encompassing *URA3* has been substituted for the 500-bp *Xba*I fragment lying completely within the X region.

Plasmid pDM44, used to generate a probe for RNase protection studies, consists of plasmid pGEM3 (Promega Biotec, Madison, Wis.), into the *Hin*cII site of which is cloned a 690-bp *Eco*RV to *Fsp*I restriction fragment of *HMLα* containing the 5′ portions of the divergent α1 and α2 genes (see Fig. 1A). Similarly, plasmid pDM71 consists of an *Ssp*I to *Bgl*II fragment from MAT**a**, inserted into *Bam*HI-plus-*Hin*cII-digested pGEM3 plasmid DNA. This fragment covers most of exon 1 and a portion of exon 2 of MAT**a** (2).

TABLE 1. *S. cerevisiae* strains used in this study

Strain[a]	Genotype[b]
DMY1	*HMLα MAT***a** *ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100*
DMY2	DMY1; *sir3*::*LEU2*
DMY4	DMY1; *HMLα* E^-*Δ79-113*::$SUP4_o$
DMY5	DMY1; *HMLα* I^-*Δ242*::*URA3*
DMY7	DMY1; *HMLα* I^+::*URA3*
DMY8	DMY1; *HMLα* I^+::*URA3 sir3*::*LEU2*
DMY9	DMY1; *HMLα* E^-*Δ79-113*::$SUP4_o$ I^-*Δ242*::*URA3*
DMY10	DMY1; *HMLα* E^-*Δ79-113*::$SUP4_o$ I^+::*URA3*
DMY18	DMY1; *HMLα* I^-*Δ242*
DMY19	DMY1; *HMLα* E^-*Δ79-113*::$SUP4_o$ I^-*Δ242*
DMY20	DMY1; *HMLα* E^-*Δ79-113*::$SUP4_o$ I^+*Δ547*
DMY22	DMY1; *HMLα* E^+*Δ637* I^-*Δ242*
DMY23	DMY1; *HMLα* E^-*Δ504* I^-*242*
DMY30	DMY1; *HMLα* E^-*Δ504* I^+::*URA3*
DMY32	DMY1; *HMLα* E^-*Δ79-113* I^+::*URA3*
DMY53	DMY1; *MATα*
DMY55	DMY1; *mat*::*LEU2*
DMY56	DMY18; *mat*::*LEU2*
DMY57	DMY22; *mat*::*LEU2*
DMY62	DMY23; *mat*::*LEU2*
DMY81	DMY1; HMLα E^-*Δ504* I^+*Δ547*
DMY87	DMY1; HMLα E^-*Δ504*
DMY91	DMY1; HMLα E^+*Δ59-76*::*URA3*
DMY92	DMY1; *HMLα* E^-*Δ79-113*::*URA3*
DMY92	DMY18; *HMLα* E^+*Δ59-76*::*URA3*
DMY95	DMY18; *HMLα* E^-*Δ79-113*::*URA3*
DMY108	DMY18; *HML***a** *MATα*
DMY110	DMY87; *HML***a** *MATα*
DMY112	DMY108; *HML***a** I^+::*URA3*
DMY113	DMY110; *HML***a** I^-*Δ242*::*URA3*
DMY117	DMY81; *mat*::*LEU2*
DMY118	DMY87; *mat*::*LEU2*
GJY5	DMY1; *HMLα*::*URA3*
GJY6	DMY2; *HMLα*::*URA3*
YAB61	*MATα HMLα HMR***a** E^-I^+ *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*
DC14**a**	*MAT***a** *his1-1*
DC17α	*MATα his1-1*

[a] All strains except YAB61, DC14**a**, and DC17α were constructed as described in Materials and Methods. Strain YAB61 (described in reference 6) was kindly provided by Andrea Brand, and strains DC14**a** and DC17α were obtained from Cold Spring Harbor Laboratory stocks.

[b] E- and I-site deletion alleles with single numbers refer to deletion allele numbers described in reference 13. In this study, E-site deletions extend only to the *Xba*I site telomere proximal of *HML*, rather than to the *Bam*HI site used in reference 13. Hyphenated deletion allele numbers indicate the endpoints of sequences deleted and replaced by an octanucleotide *Bgl*II linker, using the numbering system adopted in reference 13.

Yeast strain construction. *Saccharomyces cerevisiae* strains used in this study are isogenic derivatives of the parental strain DMY1 and are listed in Table 1. These isogenic derivatives were constructed by using a one-step gene transplacement technique as described by Rothstein (45) or a two-step replacement technique essentially as described by Scherer and Davis (46) with the modifications of Boeke et al. (4). Yeast transformations were carried out by the lithium acetate procedure of Ito et al. (21).

Constructions were designed to obtain strains that contained deletions of differing amounts of the chromosomal E or I sites of *HML* and that were free of exogenous DNA. The

basic strategy was to construct recipient strains with a *SUP4* ochre suppressor replacing *HML* E or a *URA3* gene replacing *HML* I. Alleles of I could be transplaced directly into this recipient strain by using DNA fragments carrying the alleles to transform the strain to resistance to 5-fluoroorotic acid (FOA), which selects for loss of the *URA3* gene. Alleles of E were introduced by a two-step procedure. For each case, a plasmid carrying the appropriate E allele and a *URA3* gene was transformed into the recipient strain to yield a circular integration of the plasmid into the *HML* locus. Excisions of the integrated plasmid were selected as FOA-resistant clones of the transformant. Those clones that had excised the integrated plasmid and left the new E allele in the chromosome were identified by the concurrent acquisition of FOA resistance and loss of *SUP4* suppressor activity. Recipient strains were constructed by introducing a *SUP4* ochre tRNA suppressor gene at the E site by transforming strain DMY1 with *Xba*I-digested pDM60 DNA and selecting for Lys^+ transformants. The resulting strain, DMY4, and the parental strain DMY1 were then transformed with *Eco*RI- and *Bam*HI-digested pDM68 to replace the wild-type I site with I^-::*URA3*. This yielded strains DMY9 and DMY5, respectively. Specific *HML* I alleles were introduced into these strains by transforming them to FOA resistance with *Eco*RI-plus-*Bam*HI-digested p242-3 or pDM18 DNA, generating strains DMY19 and DMY20. Strains containing deletions of the E site were made by a two-step transplacement technique. Strain DMY19 was transformed to Ura^+ with plasmid pDM22 or pDM23 DNA that had been linearized by digestion with *Xho*I. From individual transformants, clones in which the integrated plasmid had excised were selected by plating on FOA-containing media. These were subsequently screened for loss of suppressor activity. This yielded strains DMY22 and DMY23. Strains DMY81, DMY86, and DMY87 were created in a similar fashion, except that strains DMY4 and DMY20 served as the initial recipients and, prior to transformation with plasmid pDM22 or pDM23 DNA, the strains were rendered temporarily Sir^- by transforming them with plasmid pJH3A DNA (22, 34).

mat$\alpha 1^-\alpha 2^-$ derivatives of various of the above strains were obtained by transforming each of them with *Sca*I-digested plasmid pSC4 DNA. This yielded strains DMY55, DMY56, DMY57, DMY62, DMY114, and DMY115.

*HML***a** *MAT*α derivatives of some of the above strains were constructed by first rendering strains DMY18 (*HML*α E^+I^-) and DMY87 (*HML*α E^-I^+) *sir3*$^-$ by transforming them to Ura^+ with *Eco*RI-digested plasmid pDM42. These sterile strains were transformed with plasmid pGAL-*HO*, and individual transformants were grown on raffinose-containing media, plated to yeast extract-peptone-glucose agar (YEPD), and tested for mating type. *HML***a** *MAT***a** strains were picked as those that mated as **a** cells with the tester strain DC17α. We presumed that these cells had undergone an illegitimate switching event, using *MAT***a** as the donor and *HML*α as the recipient. The expected configuration of the mating-type loci in these strains was confirmed by Southern analysis of their genomic DNA. These strains were then crossed to strain DMY53, sporulated, and dissected. SIR^+ (Ura^-) spore clones were checked for mating ability, and the genotypes of their silent loci were scored by Southern analysis. Appropriate *HML***a** *MAT*α segregants were retained and designated DMY108 (*HML***a** E^+I^-) and DMY110 (*HML***a** E^-I^+). Strain DMY108 was transformed to Ura^+ with *Hpa*I- plus-*Xho*I-digested plasmid pSC3 DNA to yield strain DMY112 (*HML***a** E^+I^+). Strain DMY110 was transformed to Ura^+ with *Hha*I-digested plasmid pDM68 DNA to yield strain DMY113 (*HML***a** E^-I^-).

Strains used for heterologous gene expression study were either described above or created by integrating the *URA3* gene next to *HML*α by transforming the appropriate strain with *Eco*RI-plus-*Bam*HI-digested pSC3 DNA or *Xba*I-digested pDM40 or pDM41 DNA. In addition, strains GJY5 and GJY6 were constructed by transforming strains DMY1 and DMY2 to Ura^+ with *Eco*RI-plus-*Hin*dIII-digested pGJ1 DNA.

The configuration of the mating-type loci of every yeast strain used was confirmed by Southern blot analysis.

Quantitative matings. Quantitative matings were performed essentially as described by Dutcher and Hartwell (12). Strains were grown to a density of 5×10^6 to 1.5×10^7 cells per ml. Some 4×10^6 cells were mixed with an equal number of tester cells (DC14**a** or DC17α), filtered onto a membrane filter (Millipore Corp., Bedford, Mass.), and allowed to mate for 4.5 h before plating for single colonies on YEPD. After 2 days, colonies were replica plated to synthetic minimal and synthetic complete-minus-adenine media. Percent mating was calculated as the number of colonies growing on the synthetic minimal medium (diploids) divided by the number of diploids plus the number of Ade^- colonies.

Preparation of yeast RNA. Total yeast RNA was prepared essentially as described by Carlson and Botstein (9), except on a smaller scale. A total of 2×10^8 log-phase yeast cells, suspended in 0.2 ml of breaking buffer (0.5 M NaCl, 0.2 M Tris hydrochloride, pH 7.6, 0.01 M EDTA, 1% sodium dodecyl sulfate and an equal volume of water-saturated phenol-chloroform (1:1), were vortexed with 0.4 g of acid-washed glass beads (0.45 to 0.55 mm) for 2.5 min. The aqueous phase was removed, and the cell suspension was extracted with another 0.2 ml of breaking buffer. The two aqueous-phase samples were pooled and extracted twice with phenol-chloroform. RNA was precipitated from the final aqueous phase by addition of 2 volumes of ethanol. The samples were suspended in 0.5 ml of 6 mM $MgCl_2$ and incubated with RNase-free DNase (Promega Biotec) according to the directions. RNA was phenol extracted, ethanol precipitated, and suspended in 0.5 ml of water. RNA concentration was determined by A_{260}.

RNase protection assays. Labeled probes used for protection assays were prepared and hybridized to 25 or 50 μg of total yeast RNA by the procedure of Melton et al. (35). Probe specific for the $\alpha 2$ transcript was prepared by using *Nde*I-plus-*Hin*dIII-digested pDM44 DNA in a transcription reaction from the T7 promoter. This produces a 450-base RNA which is homologous to 310 bases of the $\alpha 2$ message as well as 159 bases of the **a***2* message. Probe specific for **a***1* was prepared from *Hin*dIII-digested pDM71 DNA in a transcription reaction with T7 polymerase. This yields a 265-base probe with 193-base homology to exon 2 of **a***1* and 18-base homology to exon 1.

OMP-DCase assays. Orotodine 5′-monophosphate decarboxylase (OMP-DCase) assays were performed essentially as described by Wolcott and Ross (60). YEPD cultures (10 ml) were grown to 10^7 cells per ml, harvested, washed once with extraction buffer (100 mM Tris hydrochloride, pH 8.0, 1 mM dithiothreitol, 20% glycerol), suspended in 0.3 ml of extraction buffer, and disrupted by vortexing with an equal volume of glass beads. Samples were diluted with 0.3 ml of extraction buffer and clarified by centrifugation, and supernatants were decanted and retained. Individual reactions (0.2 ml) consisted of 100 mM Tris hydrochloride, pH 8.0, 20 μg of pyridoxal phosphate per ml, 10 mM $MgCl_2$, 0.01 mM

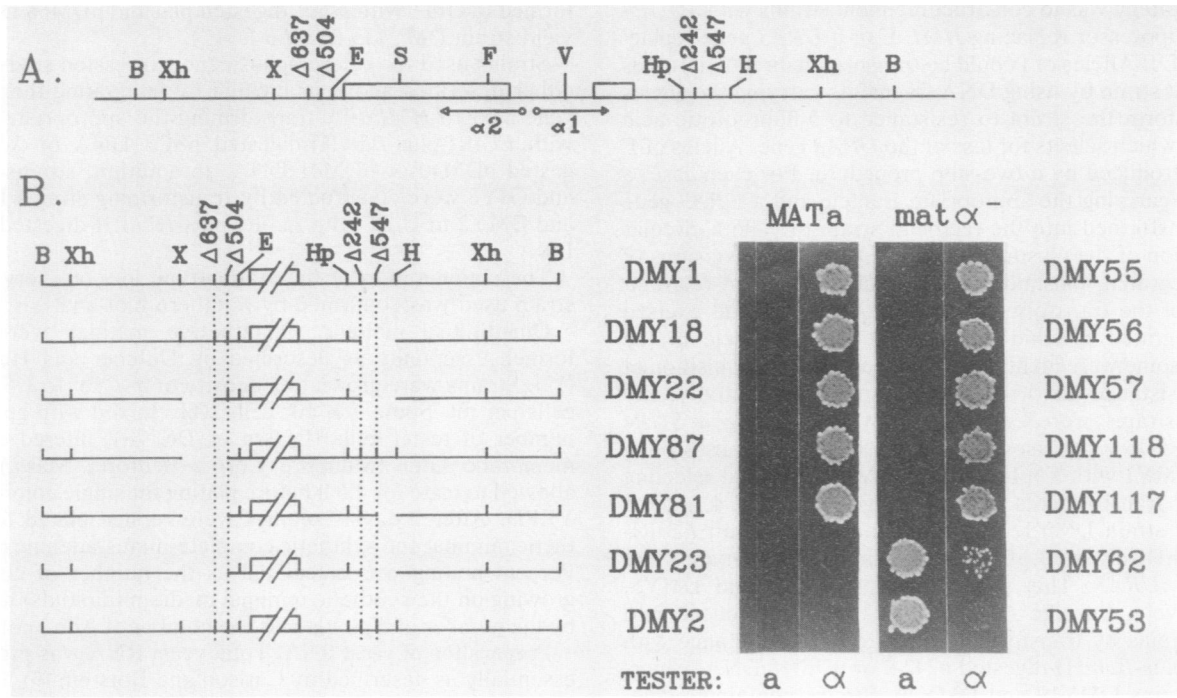

FIG. 1. Mating pattern of strains containing deletions of regulatory regions flanking *HML*. (A) Schematic diagram of the 6.6-kilobase *Bam*HI chromosomal fragment spanning *HML*α showing the positions of various restriction sites and indicating the regions of the locus conserved between *HML* and *MAT* (boxed segments). The location and direction of the two transcription units, α*1* and α*2*, within the locus are also shown. The E silencer was mapped previously to the region lying between the right-hand boundaries of deletions Δ*637* and Δ*504* (shown on the diagram), and the I silencer was mapped to the region lying between the left-hand boundaries of deletions Δ*242* and Δ*547* (also shown). The telomere lies approximately 10 kilobases to the left of the region shown, and the centromere lies approximately 150 kilobases to the right. Restriction site abbreviations: B, *Bam*HI; E, *Eco*RI; F, *Fsp*I; H, *Hin*dIII; Hp, *Hpa*I; V, *Eco*RV; X, *Xba*I; Xh, *Xho*I. (B) Structure of the regulatory regions flanking *HML* in the designated strains indicating the regions deleted (for simplicity, the central portion of the *HML* locus is not drawn). The previously delimited boundaries of the E and I regulatory regions are indicated by the vertical dotted lines. For strains listed to the left of the mating patches, the diagrammed *HML*α locus is carried in a *MAT***a** background. The abilities of these strains to mate with an **a**-mating tester strain and an α-mating tester strain are shown in the two columns under the designation "MATa." Growth of the patch indicates positive mating. For strains listed on the far right, the diagrammed *HML*α locus in carried in a *matα1*$^-$*α2*$^-$ background. The ability of these strains to mate with the same tester strains is shown in the two columns under the designation "matα$^-$." Strain DMY2 carries the wild-type *HML*α locus in a *MAT***a** *sir3*$^-$ background, and strain DMY53 carries the wild-type *HML*α locus in a *MAT*α background.

[*carboxyl*-^{14}C]-OMP (37.5 mCi/mmol; Dupont, NEN), and 10 to 200 μg of protein lysate. Units are given as picomoles of OMP hydrolyzed per minute per milligram of protein. Protein determinations were done by the method of Markwell et al. (33).

RESULTS

The E and I sites function independently to repress *HML*. Previous analysis of the *cis*-acting domains mediating repression of *HML* identified two regions, one on each side of the locus, that were required for repression (13). This delineation was obtained by measuring phenotypic expression of *HML* from a number of deletion derivatives of a plasmid-borne copy of the *HML* locus. Besides establishing the boundaries of these regulatory sites, the study concluded that both sites were required for efficient repression of the locus. Deletion of either site yielded significant expression of the mating-type information present in the test plasmid. In addition, the study concluded that the two sites were not equivalent: deletion of the site centromere distal to *HML* yielded full expression when the plasmid was present in the cell at single copy, whereas deletion of the centromere-proximal site yielded only limited expression under the same conditions. Accordingly, the centromere-distal site was denoted the E, or essential, site and the centromere-proximal site was designated the I, or important, site. Similar studies with the *HMR* locus revealed a strikingly similar organization (1).

To extend this investigation of the regulatory domains of *HML*, we initiated a mutational analysis of the normal chromosomal copy of *HML*. As described below, these studies confirm the boundary definitions for the silencer regions obtained in previous work. However, our current studies have yielded a story completely different from that reported previously regarding the relative contributions of the two silencers to repression of the locus.

To evaluate the regulatory role of specific sequences around *HML*, we have transplaced into the chromosome various in vitro constructed deletions of the regions flanking *HML*. As described in Materials and Methods, the transplacement procedure is performed in a manner that leaves no exogenous DNA at the locus. Thus, in all mutants we examined, the mutant strain differs from the wild type only in deletion of the specific sequences of interest. This approach ensures that any regulatory changes seen with a particular deletion mutation can be attributed to the deletion itself and not to the influence of exogenous DNA.

We used three different methods to measure expression of

TABLE 2. Quantitative mating assays of strains containing deletions of E or I

Strain(s)[a]	HML locus	Mating efficiency (%)[b]	
		MATa	matα1⁻α2⁻
1, 55	E⁺ I⁺	1.0	2×10^{-5}
18, 56	E⁺ I⁻ (Δ242)	0.91	3×10^{-5}
22, 57	E⁺(Δ637) I⁻(Δ242)	0.93	2×10^{-5}
87, 118	E⁻(Δ504) I⁺	0.96	5×10^{-5}
81, 117	E⁻(Δ504) I⁺(Δ547)	0.94	4×10^{-5}
23, 62	E⁻(Δ504) I⁻(Δ242)	0.002	1.05
2	E⁺ I⁺ (Sir⁻)	10^{-5}	
53	E⁺ I⁺ (MATα)		1.0

[a] Strains used were the same as those diagrammed in Fig. 1 and listed in Table 1. The number listed first refers to the *MAT***a** strain with the indicated *HML*α allele and the number listed second refers to the *matα1α2* strain with the same *HML*α allele.

[b] Quantitative mating assays were performed with tester strain DC17α for *MAT***a** strains and with DC14**a** for *matα1α2* strains. Assays were performed as described in Materials and Methods and are expressed as percent mating-competent cells relative to DMY1 (*HML*αE⁺I⁺) for *MAT***a** strains and relative to DMY53 (*HML*αE⁺I⁺ *MAT*α) for *matα1α2* strains.

HML in strains containing transplaced deletions of sequences flanking the locus. As a first approach, we determined the mating competence of a *MAT***a** strain carrying each of the mutant *HML* alleles. The *HML* locus we examined carries α mating-type information. If the *HML* locus is repressed, then only **a**-mating information from the *MAT***a** locus is expressed and the cell mates as an **a** (that is, it will form a diploid with an α cell, but not with another **a** cell). If repression of *HML* is relieved, then both **a**-mating information and α-mating information are expressed and the cell assumes a sterile **a**/α phenotype (that is, it will mate with neither an **a** cell nor an α cell) (17, 55). Thus, the degree to which the **a**-mating competence of the test strain is diminished reflects the extent to which the *HML* locus is derepressed.

The results of this analysis are presented in Fig. 1 and Table 2. The locations of the E and I sites of *HML* as determined previously (13) can be identified on the diagram of the locus at the top of Fig. 1. The E site lies to the left of the *HML* locus as it is drawn in the figure, between the endpoints of deletions 637 and 504. The I site lies to the right of the locus between deletion endpoints 242 and 547. As is evident, deletion of neither the E site nor the I site alone yields a reduction in **a** mating of the test strain, as measured by either qualitative patch tests (Fig. 1) or quantitative mating assays (Table 2). However, deletion of both sites results in a 500-fold reduction in the mating competence of the strain. Thus, as judged by this assay, *HML* is maintained in a repressed state even if E or I is deleted, while deletion of both sites yields nearly complete derepression of the locus.

Since a small level of derepression of *HML* could not be detected by the above assay, we also examined the mating behavior of a *matα1⁻α2⁻* strain carrying the *HML* deletion alleles. The null *matα1⁻α2⁻* allele does not contribute mating-type information to the cell. Since strains lacking mating-type information behave as **a** cells, the test strain usually mates as an **a**. However, if the silent α mating-type genes of *HML* are expressed, then the strain mates as an α cell, since no **a** mating-type information is present to cause a sterile **a**/α phenotype. Accordingly, the test strain mates as an **a** cell when *HML* is repressed and as an α cell when it is expressed (55). Thus, even a low level of derepression of *HML* can be detected as an increase in α mating. The results

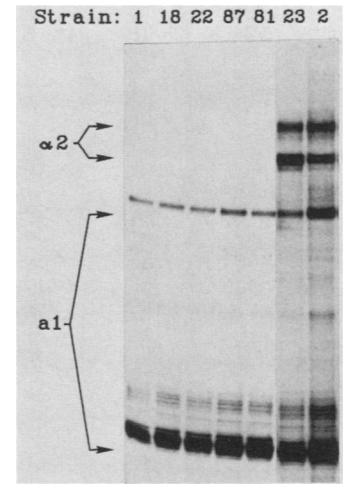

FIG. 2. Levels of *HML*-specific transcripts in strains containing various deletions of the regulatory sites flanking *HML*. Total RNA was isolated from the designated yeast strains, whose *HML* loci are diagrammed in Fig. 1, and 50-μg samples were hybridized with a mixture of 5×10^5 cpm of α*2*-specific probe and 5×10^5 cpm of **a***1*-specific probe (10^7 cpm/μg). After hybridization and then digestion with RNases A and T_1, samples were fractionated on a 6% polyacrylamide-8 M urea gel. A section of the autoradiograph of the gel is shown, on which is indicated the position of migration of RNA fragments protected by α*2* mRNA and those protected by **a***1* mRNA. The upper **a***1* fragment most likely corresponds to unspliced **a***1* mRNA, and the lower **a***1* fragment corresponds to mature **a***1* mRNA. Relevant strain genotypes: DMY1, *HML* E⁺I⁺; DMY18, *HML* E⁺I⁺Δ*547*; DMY22, *HML* E⁺Δ*637* I⁻Δ*242*; DMY87, *HML* E⁻Δ*504* I⁺; DMY81, *HML* E⁻Δ*504* I⁺Δ537; DMY23, *HML* E⁻Δ*504* I⁻Δ*242*; DMY2, *HML* E⁺I⁺ *sir3*.

of this assay (Fig. 1; Table 2) show that deletion of either the E or the I site does not yield even a minimal level of *HML* expression.

To confirm the results of the mating assays, we measured transcript levels for the α*2* gene resident at *HML* in the various deletion alleles. RNA was isolated from *MAT***a** strains containing the *HML* deletion alleles depicted in Fig. 1. The amount of α*2*-specific mRNA in the samples, relative to that of **a***1* mRNA derived from the *MAT***a** locus, was determined by RNase protection as described in Materials and Methods. The results of this analysis (Fig. 2) mirror those of the mating assays described above. Namely, in RNA samples from *HML*E⁺I⁺ (DMY1 and DMY18), *HML* E⁺I⁻ (DMY22), and *HML*E⁻I⁺ (DMY87 and DMY81) strains, no α*2*-specific mRNA is detectable. However, in an *HML*E⁻I⁻ strain (DMY23), the relative level of α*2*-specific mRNA is equivalent to that in an *HML*α Sir⁻ strain (DMY2). Given the intensity of the α*2* mRNA-specific band and examining longer exposures of the gel shown in Fig. 2, we can conclude that the level of expression from the E⁻I⁺ or E⁺I⁻ allele is <1% that of the E⁻I⁻ allele.

The information presented above delimits the boundaries of the E and I sites. The left-hand boundary of the E site lies between the right-hand endpoints of the centromere-distal deletions in strains DMY22 and DMY23 (Fig. 1). This is the same position to which the left-hand E site boundary had been mapped previously. Similarly, the right-hand boundary of the I site lies between the left-hand endpoints of the centromere-proximal deletions in strains DMY81 and DMY23. This is also the boundary site identified previously. Thus, despite the differences observed in this study in the

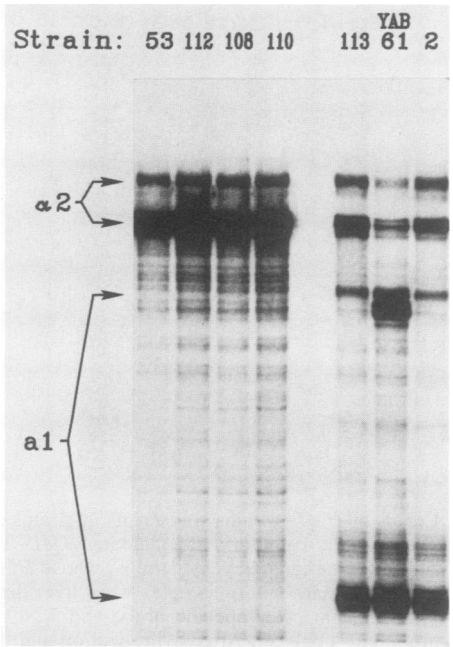

FIG. 3. Levels of *HML*-specific transcripts in strains containing various deletions of the regulatory sites flanking *HML* carrying **a**-mating information. RNA samples from *MAT*α strains containing the *HML* or *HMR* allele indicated (Table 1; Fig. 1) were prepared and analyzed as described in the legend to Fig. 2. For strain YAB61, which is not isogenic with the other strains, the major protected fragment corresponding to the α2 transcript migrates just ahead of the that corresponding to the **a***1* unspliced transcript. Relevant strain genotypes: DMY53, *MAT*α *HML*αE$^+$I$^+$; DMY112, *MAT*α *HML***a** E$^+$I$^+$; DMY108, *MAT*α *HML***a**E$^+$I$^-$; DMY110, *MAT*α *HML***a**E$^-$I$^+$; DMY113, *MAT*α *HML***a**E$^-$I$^-$; YAB61, *MAT*α *HMR***a**E$^-$I$^+$; DMY2, *MAT***a** *HML*αE$^+$I$^+$ *sir3*.

relative contribution to *HML* repression by the two silencer regions, definition of the location of the silencer regions remains unaltered.

***HML* has a different regulatory organization than *HMR*.** The results presented above suggest that the *HML* locus, at which deletion of both flanking regions is required for derepression, has a different organization of its regulatory sites than *HMR*, at which deletion of the centromere-proximal, or E, site yields nearly complete derepression. However, a major difference in the two sets of observations is that the *HML* locus we have examined carries α-mating information, whereas the *HMR* locus examined previously carries **a**-mating information. This difference is potentially significant, since the α-allele-specific information contains a RAP1 binding site that is not present in the **a**-allele-specific information (8). Since RAP1 binding within E is associated with repression at *HMR*, we were interested in determining whether the observed differences in regulatory organization of *HML* versus *HMR* could be attributed to the differences in the mating information present at the two tested loci.

By the procedure described in Materials and Methods, we converted the α-mating information resident at the *HML* locus to **a**-mating information. We then examined the effect of deletion of flanking sequences on expression of the resident **a**-mating information by both mating assays and RNase protection assay. Results of this analysis (Fig. 3; Table 3) show that effects of deletion of flanking sequence on regulation of *HML* is independent of the mating information present at the locus. The ability of a *MAT*α *HML***a**E$^-$I$^+$ or a *MAT*α *HML***a**E$^+$I$^-$ strain to mate as an α is undiminished

TABLE 3. Quantitative mating assays of *HML***a** strains with E and I mutations

Strain[a]	Mating efficiency (%)[b]
DMY53 *HML*αE$^+$I$^+$	1.0
DMY112 *HML***a**E$^+$I$^+$	1.0
DMY108 *HML***a**E$^+$I$^-$	0.98
DMY110 *HML***a**E$^-$I$^+$	0.87
DMY113 *HML***a**E$^-$I$^-$	6×10^{-6}
YAB61 *HMR***a**E$^-$I$^+$	2×10^{-6}

[a] All strains are *MAT*α and carry the indicated *HML* or *HMR* allele. The full genotypes are provided in Table 1, and the *HML* alleles are diagrammed in Fig. 1.

[b] Quantitative mating assays were performed with tester strain DC14**a**. Assays were performed as described in Materials and Methods and are expressed as percent mating-competent cells relative to DMY53 (*HML*αE$^+$I$^+$ *MAT*α).

compared with a *MAT*α *HML***a**E$^+$I$^+$ strain, whereas a *MAT*α *HML***a**E$^-$I$^-$ strain is completely sterile. In addition, no **a**-specific mRNA is detectable in our *HML***a**E$^+$I$^+$ (DMY 112), *HML***a**E$^+$I$^-$ (DMY108), or *HML***a**E$^-$I$^+$ (DMY110) strains, while full levels of **a***1* mRNA are present in an *HML***a**E$^-$I$^-$ strain (DMY113). In contrast to these results with *HML* and consistent with previous studies of *HMR*, data in Fig. 3 confirm that deletion of the E site from *HMR***a** yields full production of **a***1* mRNA, even though the *HMR* I site is still intact (strain YAB61). Thus, we conclude that the regulatory organizations of *HML* and *HMR* are different.

The *HML* E and I regulatory domains can function as silencers of heterologous gene expression. One hallmark of a silencer region is its ability to repress expression of a heterologous gene placed in its vicinity. To examine the silencer properties of the E and I regions of *HML*, we inserted *URA3* at various sites within the *HML* locus and measured its level of expression as a function of the presence of either of the two regulatory regions (Fig. 4). Expression of the inserted *URA3* gene was assessed by determining the specific activity of OMP-DCase in the various strains and by examining the phenotype of the strains. Both assays of *URA3* expression were consistent: strains that were sensitive to the analog FOA always had high levels of OMP-DCase specific activity and strains that were FOA resistant had low but measurable levels of OMP-DCase activity.

The effects of the E and I regions on *URA3* expression are summarized in Fig. 4. Insertion of the *URA3* gene at the *Hin*dIII site centromere proximal of *HML* placed the gene under control of the I site but not the E site. In those strains in which the I site was intact, *URA3* expression was diminished. In those strains in which the I site was deleted, *URA3* was expressed at high levels. Expression of the *URA3* gene at this location proved to be unaffected by the E site. The presence of an intact E site at its normal position to the left of the *HML* did not repress expression in an I$^-$ background of *URA3* inserted to the right of *HML* (compare strains DMY5 and DMY9). Similarly, deletion of the E site in an I$^+$ background does not alleviate repression of *URA3* located at the rightward *Hin*dIII site (compare strains DMY7 and DMY10).

In contrast to these results, *URA3* positioned centromere distal of *HML* is responsive to regulation by both E and I. In these constructions, the *URA3* gene is expressed at high levels only when both E and I sites are deleted. In strains in which either the E or the I site is intact, expression from the leftward *URA3* gene is diminished. Thus, each silencer can influence expression of a heterologous gene placed in its

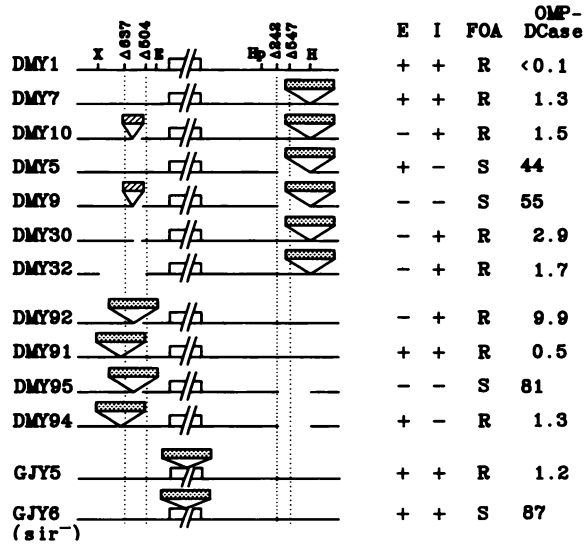

FIG. 4. Repression of heterologous gene expression by the E and I silencers at *HML*. The expression of *URA3* inserted at various sites flanking different *HML* alleles was determined by direct assay of the encoded product, OMP-DCase, and by examination of resistance or sensitivity to the analog FOA. Normal levels of *URA3* expression result in FOA sensitivity (S), and reduced expression yields resistance (R). The structure of the *HML* allele in each strain is diagrammed on the left, showing the position of insertion of *URA3* (stippled box), *SUP4* (hatched box), and relevant restriction sites and deletion endpoints (Fig. 1). The orientation of the *URA3* insert is the same in each construction, with transcription of the *URA3* gene proceeding from left to right. Deletions within E in strains DMY92 and DMY95 render the locus fully E^- (Mahoney and Broach, unpublished observations). Values for OMP-DCase activity are averages of assays performed in triplicate. In all cases, individual vales differed from the average value by <10%. Restriction abbreviations are as in the legend to Fig. 1.

immediate vicinity. In addition, I, but not E, can exert its influence on a gene even when it is placed on the opposite side of *HML*.

As is true of the *HMR* E site, the silencers associated with *HML* can affect expression from genes transcribed by RNA polymerase III as well as those, like *URA3*, transcribed by RNA polymerase II. These results are presented in Fig. 5.

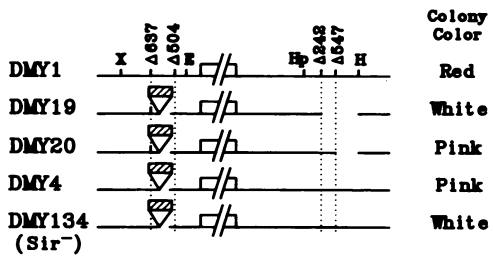

FIG. 5. Repression of $SUP4_o$ expression by the E and I silencers at *HML*. The expression of $SUP4_o$ inserted at various sites flanking different *HML* alleles was determined indirectly by examining colony color of the indicated strains after growth for 4 days on YEPD. In the absence of suppressor activity, colonies are red. Full activity of the $SUP4_o$ yields white colonies, and intermediate suppressor levels yield pink colonies. The structure of the *HML* allele in each strain is diagrammed on the left, showing the position of *SUP4* (hatched box) and relevant restriction sites and deletion endpoints (Fig. 1). Restriction abbreviations are as in the legend to Fig. 1.

We inserted centromere distal to *HML* a fragment spanning the $SUP4_o$ gene, which encodes a tyrosine-inserting ochre suppressor tRNA. To monitor $SUP4_o$ expression, the strains we constructed contained the ochre *ade2-1* allele. In the absence of nonsense suppressor activity, strains carrying *ade2-1* are Ade^- and produce red colonies when plated on rich medium, due to formation of a chromogenic substrate derived from an intermediate in purine biosynthesis that accumulates in *ade2* strains. Efficient suppression of *ade2-1* yields Ade^+, white colonies, while partial suppression of *ade2-1* yields Ade^+, pink colonies. Thus, the level of expression of $SUP4_o$ could be assessed by the color of the resulting colonies.

The effect of the I-silencer domains on expression of $SUP4_o$ mirror those obtained with *URA3*. Efficient expression of $SUP4_o$ inserted in an E deletion occurs only in the absence of I-silencer activity. The presence of I reduces efficiency of suppression of *ade2-1*, as indicated by the pink color of colonies of the strain. Thus, the I-silencer domain can reduce expression from a RNA polymerase III-transcribed gene and can do so over the distance of the intervening *HML* locus.

DISCUSSION

***HML* in the chromosome is regulated differently than it is on a plasmid.** We have shown in this report that *HML*, the silent yeast mating-type cassette resident on the left arm of chromosome III, is repressed by two separate silencers, each possessing full silencer activity. These results are quite distinct from those of a previously reported study. In the previous study of *HML* repression, silencer activity was evaluated by examining the expression of a set of plasmid-borne copies of *HML*, carrying more or less *HML* flanking sequences (13). In the current study, the activities of the silencers were assessed by examining the effects on expression of *HML*, situated in its normal chromosomal location, of deletion of sequences flanking the locus. The definitions of the boundaries of the silencer regions were identical in the two studies. However, the two silencers appear substantially more potent by the assays in the current study than they appeared in the previous study. Given that the current study involved minimal disturbance of the usual setting of *HML*, we think that the current results reflect the actual regulatory configuration of the locus more accurately.

The results we present are also at odds with observations regarding in vivo expression of a hybrid *MAT/HML* locus (56). This hybrid locus was recovered following selection for an α-mating derivative of a heterothallic *MAT***a** strain. Among the isolates Strathern et al. (56) recovered from this screen was one that carried a ring chromosome, the origin of which could be explained as an intrachromosomal homologous recombination event between the W–X regions of *MAT***a** and *HML*α. This yielded a hybrid mating-type locus containing α information and with left-hand flanking sequences from *MAT* and right-hand flanking sequences from *HML*. Thus, the hybrid locus carries the *HML* I region but not the E region. By several phenotypic criteria, the α information at this hybrid cassette is fully expressed. This indicates that, within this context, the I silencer is not capable of repressing expression of the associated mating-type locus.

Several explanations could account for the discrepancy between results obtained with the plasmid-borne copy of *HML* and those obtained with the chromosomal copy. One possibility is that the vector sequences flanking the copy of

HML carried on a plasmid interfere with, and thereby diminish, silencer activity. A second possibility is that the topological constraints of a circular plasmid versus a linear chromosome are responsible for reduced silencer activity on a plasmid. This might be significant if, as has been suggested, supercoiling or loop formation contributes to silencer function (1). However, these explanations do not account for expression of the hybrid cassette isolated by Strathern et al. (56). A third possibility is that the *HML* silencers do not function efficiently when taken out of their usual context. For instance, silencers could be optimally efficient only when resident at telomeres. Alternatively, all sequences required for optimum silencer function might not be contained within the fragment cloned onto the plasmid used in the previous studies to analyze silencer activity. A secondary silencer, or some sequence that facilitates silencing, located telomere proximal to *HML* E could account for the difference between the results presented here and those obtained with either the ring chromosome or plasmid-borne copies of *HML*.

What comprises a silencer? Three different regions of *S. cerevisiae* have now been shown to possess silencer activity: the E site of *HMR* and the E and I sites of *HML*. Those features of the *HMR* E site required for silencer function have been identified by extensive mutational analysis. Examination of the sequences of the silencers flanking *HML* suggest that a functional silencer can be composed of a variety of sets of elements, some distinct from those identified at the *HMR* silencer.

To a first approximation, the composition of the two *HML* silencers is consistent with what is required at *HMR* for silencer function. As mentioned in the introduction, three recognizable domains are present within the *HMR* silencer: an 11-bp ARS consensus sequence and two separate binding sites, one for each of two abundant DNA-binding proteins, RAP1 and ABF1. Inactivation of any one of these elements does not significantly diminish the silencing capacity of the locus, but inactivation of any two of these elements yields essentially complete derepression of *HMR*. Thus, two of the identified elements are necessary at *HMR* for imparting silencer activity, although any combination of two of the elements is sufficient (6, 24). Each of the two silencer regions of *HML* contains two of the three elements present at *HMR* E. The *HML* E site encompasses an ARS consensus sequence and a RAP1 binding site, while *HML* I spans an ARS consensus sequence and an ABF1 binding site (7, 8, 13). Thus, it would seem that any two of three defined components are sufficient to confer silencer activity.

Although composition of the two *HML* silencers and the mutational analysis of *HMR* appear to yield consistent results, further examination reveals several inconsistencies. First, although the RAP1 binding site can be deleted from *HMR* E without causing complete derepression, *HMR* E silencers lacking the RAP1 binding site are not fully functional. Deletion of the RAP1 binding site from *HMR* yields partial expression of the resident **a**-mating information, as judged by assessing phenotypic expression of *HMR***a** (24) or by measuring **a***1* transcript levels from the mutant locus (6). This would suggest that RAP1 binding is indispensable for efficient silencer function at *HMR*. The same is not true for *HML* I. As shown in this study, *HML* I acts as an efficient silencer even though it lacks a RAP1 binding site. Accordingly, at *HML* I, but not *HMR* E, silencer function is completely independent of RAP1 activity. A second distinction between *HML* and *HMR* silencers is evident from a comparison of the composition of the I sites at *HML* and *HMR*. Both sites encompass an ARS consensus sequence and an ABF1 binding site in close proximity. However, the I site at *HML* functions as an efficient silencer, while that at *HMR* does not.

These results could be explained in a number of ways. Hofmann et al. (20) detected weak RAP1 binding to a fragment encompassing *HML* I, an activity not seen in earlier studies. The presence of this RAP1 site, albeit feeble, at *HML* I but not *HMR* I might account for the difference in the activities of the two elements. Alternatively, our results could suggest that silencer elements might in some cases comprise other components in addition to those identified within *HMR* E or that features other than the mere juxtaposition of identified sequence motifs underlie silencer activity. In this light, it is worth noting that Buchman et al. (8) detected a binding site for a yeast nuclear protein distinct from ABF1 and RAP1 within the *HML* I site. Mutational analysis should indicate whether this binding protein contributes to silencer function at *HML* I.

***HML* and *HMR* are regulated differently.** We have shown that the organization of *cis*-acting sites required for *SIR*-mediated repression at *HML* is unexpectedly but markedly distinct from that at *HMR*. *HML* is flanked by two separate and independent silencers, each capable of exerting full repression of the locus. In contrast, *HMR* is repressed by a single silencer. In addition, each of the *HML* silencers encompasses only two of the three elements that contribute to silencer activity at the *HMR* E site. Thus, either the *HML* silencers lack the internal redundancy of the *HMR* E site or they are composed of unidentified elements in addition to those found at *HMR*.

Several other apparent differences in the regulation of *HML* and *HMR* have been reported recently. First, *HML* and *HMR* exhibit differential sensitivity to inactivation of *ARD1* or *NAT1* (36, 58, 59). These two genes apparently encode two heterologous subunits of an N-terminal acetyltransferase that is responsible for N-terminal acetylation of a large number of yeast proteins. Inactivation of either gene yields essentially complete phenotypic derepression of *HML* but causes no detectable derepression of *HMR*. Similarly, Kayne et al. (23) have reported that specific deletions of the amino-terminal region of histone H4 (encoded by *HHF1* and *HHF2*) cause derepression of the silent mating-type cassettes. However, as measured by phenotypic expression, the effect on *HML* of such deletions is substantially more dramatic than their effects on *HMR*. In neither of these cases has it been shown that the differential effects are exclusively at the level of transcription and not, for example, a consequence of the different levels of **a**-cassette expression versus α-cassette expression necessary to yield a phenotypic change in the mating pattern of the cell. However, if these differences in phenotypic expression are due to differences in transcriptional activation attendant on mutation of *ARD1*, *NAT1*, or *HHF*, then the distinct regulatory organizations of the two loci could provide a source for their differential sensitivity to these mutations.

Looped domains and silencing activity. One model that has been proposed to account for silencer action postulates formation of a loop of the DNA spanning *HML*, anchored by attachments through the E and I sites. Such a loop would, on the one hand, serve to restrict repression to the region lying between the two anchor sites and could, on the other hand, be an integral component of the mechanism of silencing. For instance, subsequent supercoiling of the looped domain, made possible by restricting rotation through attachment at the base of the loop, could limit access of the region to

proteins such as RNA polymerases. Some evidence has been presented for differential in vivo supercoiling of an *HMR* plasmid in Sir^+ versus Sir^- strains, and recently loop formation of DNA spanning *HML*, promoted by proteins present in a yeast nuclear scaffold fraction, has been observed (1, 20).

The results presented in this report are not readily accommodated by a DNA loop model for silencer action. First, deletion of either E or I alone does not yield derepression of the locus. If these served as anchor points of a loop, then elimination of either site should preclude loop formation and yield derepression. Second, repression of *URA3* by the I silencer is no more pronounced when *URA3* is situated between E and I than when the gene is located outside the E-to-I interval. Accordingly, repression by the *HML* silencers is not restricted to the region lying between them, as would be expected from a model involving loop formation.

The regulatory capacity of the *HML* silencers is more consistent with models suggesting that these sites provide points of attachment of proteins that serve to constrain expression of the adjacent sequences. A number of different mechanisms can be proposed to suggest how such tethered proteins could suppress expression of the local genes. The process could be primarily a matter of nuclear architecture, in which the silencer-associated proteins restrict the DNA to a portion of the nucleus inaccessible to transcriptional complexes. Alternatively, the tethered proteins could modify local chromatin structure by specific modification of histones in the vicinity of the silencers. Finally, the tethered proteins could serve as nonproductive sinks for factors necessary for transcription activation. This could create a locally diminished concentration of essential transcription factors. Distinguishing among these models obviously requires further analysis.

ACKNOWLEDGMENTS

We thank Andrea Brand for providing strain YAB61, Alan Rose for thoughtful comments on the manuscript, and Gan-Ju Shei for excellent technical assistance.

This work was supported by Public Health Service grant GM34545 to J.R.B. from the National Institutes of Health.

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