MOLECULAR AND CELLULAR BIOLOGY, Feb. 1993, p. 727–738
0270-7306/93/020727-12$02.00/0

Vol. 13, No. 2

Conditional Silencing: The *HMRE* Mating-Type Silencer Exerts a Rapidly Reversible Position Effect on the Yeast *HSP82* Heat Shock Gene

SEEWOO LEE AND DAVID S. GROSS*

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, P.O. Box 33932, Shreveport, Louisiana 71130

Received 18 August 1992/Returned for modification 6 October 1992/Accepted 26 October 1992

The *HMRE* silencer of *Saccharomyces cerevisiae* has been previously shown to transcriptionally repress class II and class III genes integrated within the *HMR* silent mating-type locus up to 2.6 kb away. Here we study the ability of this element to repress at an ectopic position, independent of sequences normally associated with it. When integrated 750 bp upstream of the *HSP82* heat shock gene, the silencer represses basal-level transcription ~5-fold but has no effect on chemical- or heat-shock-induced expression. Such conditional silencing is also seen when the *HMRE/HSP82* allele is carried on a centromeric episome or when the entire *HMR*a domain is transplaced 2.7 kb upstream of *HSP82*. Notably, the a1 promoter within the immigrant *HMR*a locus remains fully repressed at the same time *HSP82* is derepressed. The position effect mediated by the *E* silencer is absolutely dependent on the presence of a functional *SIR4* gene product, is lost within 1 min following stress induction, and is fully reestablished within 15 min following a return to nonstressful conditions. Similar kinetics of reestablishment are seen in *HMRE/HSP82* and *HMRa/HSP82* strains, indicating that complete repression can be mediated over thousands of base pairs within minutes. DNase I chromatin mapping reveals that the ABF1, RAP1, and autonomously replicating sequence factor binding sites within the silencer are constitutively occupied in chromatin, unaltered by heat shock or the presence of *SIR4*. Similarly, the heat shock factor binding site upstream of *HSP82* remains occupied under such conditions, suggesting concurrent occupancy of silencer and activator binding sites. Our results are consistent with a model in which silencing at the *HMRE/HSP82* allele is mediated by direct or indirect contacts between the silencer protein complex and heat shock factor.

It has long been appreciated that the expression of a eukaryotic gene can be influenced by its relative position on the chromosome (39). Such phenomena, known as position effects, have been described for a diversity of organisms ranging from insects to mammals (reviewed in references 16 and 71). A typical position effect results from the juxtaposition of euchromatin with heterochromatin, in which the former is phenotypically repressed by the latter. Such an outcome can be seen following an autosomal-to-X translocation in mammals or following translocation of euchromatic genes to near or within the centromeric heterochromatin of *Drosophila* cells (66). Formation of heterochromatin in *Drosophila* cells is believed to be mediated by specific proteins that bind chromatin over large distances (31, 53), with the extent of heterochromatic spreading defined by boundary constraints (69).

Saccharomyces cerevisiae possesses two loci on chromosome III that exhibit analogous *cis*-inactivating properties. These loci, the silent cassettes *HML* and *HMR*, have been shown to exert a negative position effect on the transcription of nearby genes (36, 49). They contain full copies of α and **a** mating-type genes and their complete promoter sequences, which are not activated until they are translocated to the *MAT* locus (35). Inactivating position effects have also been demonstrated to occur near the ends of chromosomes in yeast cells (20). Both telomeres and the *HM* loci are repressed in *trans* by the products of *SIR2*, *SIR3*, *SIR4* (silent information regulators), *HHF2* (histone H4), and two genes encoding subunits of an N-terminal acetyltransferase, *ARD1* and *NAT1* (3, 33, 47, 54, 70). In addition, *SIR1* is required for silencing at the *HM* loci (55) and has been implicated in the establishment of repression at *HML* (50). Additional *trans*-acting regulators of silencing at *HMR* have been identified and include *CDC7* (3a), *SUM1* (37a), and *RIF1* (26a).

Repression of the silent mating-type genes is mediated in *cis* by sequences, termed *E* and *I*, disposed respectively to the left and to the right of each silent locus (1, 18). These silencers each encompass a relatively short region (<150 bp) and are located ~1 kb from the promoters that they regulate. The most thoroughly studied silencer is the centromere-proximal, or *E*, site of *HMR*, which has been shown to mediate *SIR* repression of the mating-type genes in an orientation- and position-independent fashion (8). In addition, the *E* silencer has been found to fully repress the transcription of two heterologous class II genes, *LEU2* and *TRP1*, and strongly (>15-fold) repress the expression of a class III tRNA gene, *SUP3*, when these loci are integrated within the *HMR* domain (8, 61). Three functional domains have been identified within *HMRE* (9): an 11-bp autonomously replicating sequence (ARS) core consensus sequence (element A) that serves as a binding site for one or more proteins (5a, 28, 60), a binding site (E) for an abundant DNA-binding protein termed RAP1 or GRF1 (11, 37, 63), and a binding site (B) for a protein, ABF1, that binds near consensus ARS elements (11, 14, 15). Surprisingly, there is no evidence that SIR proteins themselves bind to DNA (11, 64).

While *HMRE* is necessary to repress the transcription of genes near or within the *HMR* locus, it is not clear whether

* Corresponding author.

TABLE 1. Yeast strains used

Strain	Genotype	Source
IV16-17A	*MATα can1 his4 leu2-3,112 trp1-1 ura3-52 sir4-351*	J. R. Broach
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. J. Rothstein
W303-1B	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. J. Rothstein
B-7056	*MATα leu2-3,112, ura3-52, cyc1::CYH2^s, cyc7-67, cyh2^r*	F. Sherman
SLY101	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 cyh2^r*	This study
SLY102	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 hsp82Δ::CYH2^s cyh2^r*	This study
SLY103	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 hsp82Δ::CYH2^s sir4Δ-2::HIS3 cyh2^r*	This study
SLY105	*MATα can1 his4 leu2-3,112 trp1-1 ura3-52 sir4-351 HMRE/HSP82*	This study
SLY106	*MATα can1 leu2-3,112 trp1-1 ura3 HMRE/HSP82*	This study
SLY107	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HMR***a***/HSP82 sir4Δ-2::HIS3 cyh2^r*	This study

this sequence by itself is sufficient. For example, whereas the *E* silencer is capable of exerting full *SIR* repression upon chromosomal *HMR* loci in which the *I* element has been deleted, plasmid-borne *HMR* copies containing *I* deletions are slightly derepressed (1, 8). Thus, it is possible that additional sequences are required for the *E* silencer to exert its function in the genome. Also unclear is whether regulation of the intact silencer is altered when it is placed elsewhere in the genome.

To establish that the *E* silencer per se is capable of exerting a position effect, we have introduced *HMRE*-bearing fragments into the upstream region of the *HSP82* heat shock gene, located on the left arm of chromosome XVI. We demonstrate that *HMRE* can in fact exert *SIR4*-dependent repression outside of its natural chromosomal context. However, in contrast to previous studies, silencing is conditional: the *E* silencer affects basal but not heat shock-induced transcription of *HSP82*. When the entire 5-kb *HMR***a** domain is integrated upstream of *HSP82*, a virtually identical phenotype results, arguing against a role for other *HMR* elements in mediating silencing. Notably, *HMRE* rapidly losses its ability to repress *HSP82* when cells are stressed and rapidly regains its function when cells are returned to nonstressful conditions. In contrast to this flexibility in function, the protein-DNA interactions within the upstream region of the *HMRE/HSP82* allele apparently remain unchanged, irrespective of stress or the presence of *SIR4*.

MATERIALS AND METHODS

Materials. *S. cerevisiae* strains used in this study are listed in Table 1. Restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were purchased from New England Biolabs, Inc. (Beverly, Mass.). Sequenase (version 2.0) was obtained from United States Biochemical Corp. (Cleveland, Ohio), RNasin was obtained from Promega (Madison, Wis.), lyticase was obtained from Sigma (St. Louis, Mo.), and oxalyticase was obtained from Enzogenetics (Corvallis, Ore.). SP6 RNA polymerase and GeneScreen were purchased from NEN Research Products (Boston, Mass.). [α-^{32}P]UTP and [α-^{32}P]dATP were obtained from either NEN or ICN Biomedicals (Costa Mesa, Calif.).

Plasmid constructions. To construct the *HMR/HSP82* donor alleles, the 2.9-kb *Eco*RI *HSP82* fragment spanning positions −1300 to +1601, borne on pUTX20 (a gift of D. B. Finkelstein, Panlabs Inc.), was cloned into the *Eco*RI site of p102 to create p103. Plasmid p102 is a derivative of the yeast integrating vector YIp5 in which *Ava*I and *Cla*I sites have been destroyed; it contains the selectable marker *URA3*. A 353-bp *Xba*I-*Aha*III fragment containing *HMRE* was subcloned from pJA82.6 (a gift of J. Abraham, California Biotechnology, Inc.) and inserted into the *Cla*I site of p103, located 673 bp upstream of *HSP82*. The resultant *HMRE/HSP82* transplacement construct was termed p111. To construct the *HMR***a***/HSP82* donor allele, a 4.92-kb *HMR***a** fragment, also subcloned from pJA82.6, was inserted into the *Cla*I site of p103, generating p110. The orientation of each *HMR* fragment was determined by restriction mapping; each was integrated in the inverse of its conventional orientation with respect to the gene (Fig. 1A).

To construct the episomal *HMRE/HSP82* allele, a 1.5-kb *Eco*RI-*Cla*I fragment containing the 3′ end of *HSP82* (spanning +1601 to +3125 and harbored on pUTX17; kindly provided by D. B. Finkelstein) was subcloned into *Eco*RI-*Cla*I-digested YCp50, a centromeric yeast shuttle vector bearing the *URA3* selectable marker (36a), creating p203. The *HMRE/HSP82 Eco*RI fragment of p111, spanning −1653 to +1601, was then subcloned into the *Eco*RI site of p203, reconstructing the full-length *HMRE/HSP82* allele and creating plasmid p205 (see Fig. 3A).

To construct the recipient *hsp82*Δ::*CYH2^s* allele, the −174 to +535 *Xba*I fragment of *HSP82* in p103 was replaced with a 1.6-kb *Pst*I fragment bearing the *CYH2* gene and promoter (pAB220; a gift of F. Sherman, University of Rochester), creating p106. In this construct, the orientation of the *CYH2* transcription unit is opposite that of *HSP82*.

Strain constructions. Yeast strains used in this study are derivatives of three parental strains, IV16-17A, W303-1A, and B-7056 (Table 1). The *HMRE* silencer was introduced into the *HSP82* locus by using the two-step transplacement procedure of Scherer and Davis (59) as modified by Boeke et al. (6). Yeast transformations were performed by using the spheroplast technique of Beggs (5) as modified by Burgers and Percival (12). Spheroplasts were generated as previously described (2). To construct SLY105, IV16-17A was transformed with p111, linearized at the unique *Ava*I site (corresponding to position −914 of the *HSP82$^+$* allele [Fig. 1B]), to site direct its integration within the *HSP82* locus. *URA3$^+$* transformants were selected on uracil-deficient (Ura$^-$) medium (62), replica plated to rich YPD medium (1% yeast extract, 2% Bacto Peptone, 2% dextrose) to facilitate homologous recombination between tandemly duplicated sequences, and then counterselected on a medium containing 1.25 mg of 5-fluoro-orotic acid per ml for excision of plasmid and duplicated genomic sequences (44). Successful gene transplacement was demonstrated by Southern analysis (Fig. 1C), confirming construction of SLY105.

A similar strategy was used to replace *HSP82* with the *hsp82*Δ::*CYH2^s* allele. The recipient in this transformation was SLY101. To make this strain, W303-1A (*MAT***a** *CYH2^s*)

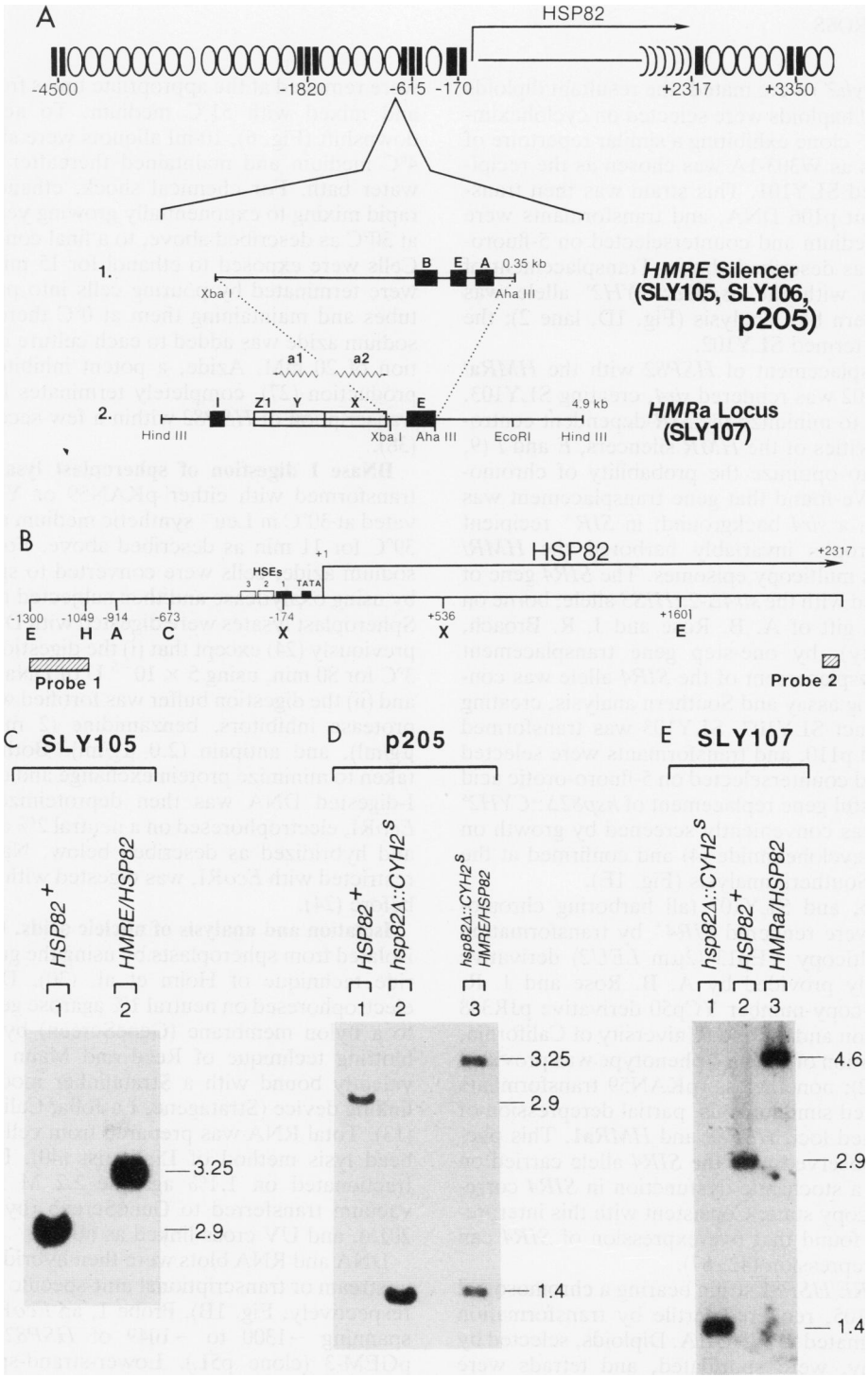

FIG. 1. Chromatin structure of the *HSP82* heat shock locus and confirmation of site-directed integrations of the *HMR* loci. (A) The chromatin map of the *HSP82* domain is shown at the top. The locations of nucleosomes (ovals), half-nucleosomes (half-ovals), DNase I-hypersensitive regions (vertical bars; gaps correspond to internal footprints), and disrupted chromatin structures (horizontal line), mapped with respect to the underlying DNA sequence (24, 38, 68), are indicated. Below are shown physical maps of the two *HMR* fragments integrated into the −673 *Cla*I site: 1, the 0.35-kb *Xba*I-*Aha*III fragment harboring the *E* silencer; and 2, the 4.9-kb *Hin*dIII fragment bearing the *HMR***a** locus with flanking *I* and *E* silencers. Note that each *HMR* fragment is integrated within *HSP82* in the inverse of its conventional orientation. Divergent **a1**/**a2** transcriptional units under *SIR* regulation are depicted by wavy lines. (B) Physical map of the *HSP82* locus, located on the left arm of chromosome XVI (46). The transcriptional unit (arrow), whose 5′ and 3′ ends have been defined by S1 nuclease mapping (17), is indicated, as are putative *cis*-regulatory sequences, including HSE1, HSE2, and HSE3, centered at positions −167, −198, and −225, respectively, and the TATA box at position −79. Also shown are the probes used in DNA and RNA blot hybridization experiments (probes 1 and 2, respectively) and the location of pertinent restriction sites (E, *Eco*RI; H, *Hin*dIII; A, *Ava*I; C, *Cla*I; X, *Xba*I). Filled horizontal boxes in panels A and B represent regulatory sites demonstrated to bind sequence-specific DNA-binding proteins in intact cells, in isolated nuclei, or in whole cell extracts (11, 24, 44, 48; this report). (C to E) Southern analyses of *HMR*/*HSP82* strains. (C) *Eco*RI-digested genomic DNA isolated from strains W303-1B (lane 1) and SLY105 (lane 2); (D) DNA isolated from strains W303-1B (lane 1), SLY102 (lane 2), and p205-transformed SLY102 (lane 3); (E) DNA isolated from SLY103 (lane 1), W303-1B (lane 2), and SLY107 (lane 3). In each of the three panels, bands specific to the *HSP82*$^+$, *HMRE*/*HSP82*, *hsp82*Δ::*CYH2*^{s}, and *HMR***a**/*HSP82* alleles are indicated (2.9-, 3.25-, 1.4-, and 4.6-kb fragments, respectively). DNA fragments were detected by using probe 1.

and B-7056 (*MATα cyh2^r*) were mated, the resultant diploids were sporulated, and haploids were selected on cycloheximide. The *MATα cyh2^r* clone exhibiting a similar repertoire of auxotrophic markers as W303-1A was chosen as the recipient strain and termed SLY101. This strain was then transformed with *Ava*I-cut p106 DNA, and transformants were selected on Ura$^-$ medium and counterselected on 5-fluoro-orotic acid medium as described above. Transplacement of the wild-type locus with the *hsp82*Δ::*CYH2^s* allele was confirmed by Southern blot analysis (Fig. 1D, lane 2); the resultant strain was termed SLY102.

To facilitate transplacement of *HSP82* with the *HMR***a**/*HSP82* allele, SLY102 was rendered *sir4*, creating SLY103. This step was taken to minimize the *SIR*-dependent centromeric and ARS activities of the *HMR* silencers, *E* and *I* (9, 34), in an attempt to optimize the probability of chromosomal integration. We found that gene transplacement was greatly facilitated in a *sir4* background; in *SIR*$^+$ recipient cells, uracil prototrophs invariably harbored the *HMR*/*HSP82* fragments as multicopy episomes. The *SIR4* gene of SLY102 was replaced with the *sir4*Δ-*2*::*HIS3* allele, borne on plasmid pMM7.1 (a gift of A. B. Rose and J. R. Broach, Princeton University), by one-step gene transplacement (56). Successful transplacement of the *SIR4* allele was confirmed by both mating assay and Southern analysis, creating SLY103. To construct SLY107, SLY103 was transformed with *Ava*I-linearized p110, and transformants were selected on Ura$^-$ medium and counterselected on 5-fluoro-orotic acid as before. A successful gene replacement of *hsp82*Δ::*CYH2^s* by *HMR***a**/*HSP82* was conveniently screened by growth on medium containing cycloheximide (4) and confirmed at the molecular level by Southern analysis (Fig. 1E).

SLY103, SLY105, and SLY107 (all harboring chromosomal *sir4* alleles) were rendered *SIR4*$^+$ by transformation with either the multicopy YEp13 (2μm *LEU2*) derivative pKAN59 (generously provided by A. B. Rose and J. R. Broach) or the low-copy-number YCp50 derivative pJR368 (a gift of P. Laurenson and J. Rine, University of California, Berkeley). Confirmation of the Sir$^+$ phenotype was provided by mating assay (62); nonetheless, pKAN59 transformants occasionally exhibited simultaneous, partial derepression of both *HMRE*-regulated loci, *HSP82* and *HMR***a1**. This phenomenon was not observed with the *SIR4* allele carried on pJR368, suggesting a stochastic dysfunction in *SIR4* correlated with the multicopy state. Consistent with this interpretation, others have found that overexpression of *SIR4* can interfere with *SIR* repression (42, 67).

To create an *HMRE*/*HSP82* strain bearing a chromosomal *SIR4*$^+$ allele, SLY105, rendered fertile by transformation with pKAN59, was mated to W303-1A. Diploids, selected by histidine auxotrophy, were sporulated, and tetrads were dissected. Spores were cured of pKAN59 by extended growth on nonselective (Leu$^+$) medium, and *SIR4*$^+$ *HMRE*/*HSP82* segregants were screened by mating assay and Southern hybridization. A *MATα* spore meeting these criteria was identified and termed SLY106.

Cell cultivation and heat/chemical shock. Strains were cultivated at 30°C in minimal synthetic medium (0.67% yeast nitrogen base without amino acids, 2% dextrose) supplemented with the appropriate bases and amino acids (62). In a typical heat shock experiment (Fig. 2, 3B, and 4), an early- to mid-log-phase culture (2×10^7 to 5×10^7 cells per ml) was shifted to 39°C by rapid mixing with an equivalent volume of medium prewarmed to 51°C. Cells were maintained in a 39°C shaking water bath throughout the course of the heat shock. In the kinetics experiment depicted in Fig. 5, 10-ml aliquots were removed at the appropriate times from a 100-ml culture and mixed with 51°C medium. To achieve temperature downshift (Fig. 6), 10-ml aliquots were similarly mixed with 4°C medium and maintained thereafter in a 30°C shaking water bath. For chemical shock, ethanol was added with rapid mixing to exponentially growing yeast cells, cultivated at 30°C as described above, to a final concentration of 8.5%. Cells were exposed to ethanol for 15 min. All experiments were terminated by pouring cells into prefrozen centrifuge tubes and maintaining them at 0°C thereafter. In addition, sodium azide was added to each culture at a final concentration of 20 mM. Azide, a potent inhibitor of cellular ATP production (27), completely terminates heat shock-induced transcription of *HSP82* within a few seconds of its addition (38).

DNase I digestion of spheroplast lysates. SLY105 cells, transformed with either pKAN59 or YEp213, were cultivated at 30°C in Leu$^-$ synthetic medium and heat shocked at 39°C for 11 min as described above. Following addition of sodium azide, cells were converted to spheroplasts at 30°C by using oxalyticase and then subjected to hypotonic shock. Spheroplast lysates were digested with DNase I as described previously (24) except that (i) the digestion was conducted at 3°C for 80 min, using 5×10^{-5} U of DNase I per μg of DNA and (ii) the digestion buffer was fortified with three additional protease inhibitors, benzamidine (2 mM), leupeptin (2.0 μg/ml), and antipain (2.0 μg/ml). Both precautions were taken to minimize protein exchange and degradation. DNase I-digested DNA was then deproteinized, restricted with *Eco*RI, electrophoresed on a neutral 2% agarose gel, blotted, and hybridized as described below. Naked control DNA, restricted with *Eco*RI, was digested with DNase I at 37°C as before (24).

Isolation and analysis of nucleic acids. Genomic DNA was isolated from spheroplasts by using the guanidine hydrochloride technique of Holm et al. (30). DNA samples were electrophoresed on neutral 1% agarose gels, capillary blotted to a nylon membrane (GeneScreen) by using the alkaline blotting technique of Reed and Mann (51), and then covalently bound with a Stratalinker model 1800 UV cross-linking device (Stratagene, La Jolla, Calif.) set at 120,000 μJ (13). Total RNA was prepared from cells by using the glass bead lysis method of Lindquist (40). RNA samples were fractionated on 1.1% agarose–2.2 M formaldehyde gels, vacuum transferred to GeneScreen (by using LKB model 2016), and UV cross-linked as above.

DNA and RNA blots were then hybridized with an *HSP82* upstream or transcriptional unit-specific probe (probe 1 or 2, respectively; Fig. 1B). Probe 1, an *Eco*RI-*Hin*dIII fragment spanning −1300 to −1049 of *HSP82*, was cloned into pGEM-3 (clone p5L). Lower-strand-specific radiolabeled RNA transcripts were synthesized in the presence of ~75 μCi of [α-^{32}P]UTP with SP6 RNA polymerase as previously described (23). Probe 2 was generated by primer extension of a synthetic oligonucleotide spanning nucleotides +2226 to +2287, using Klenow fragment in the presence of ~75 μCi of [α-^{32}P]dATP (3,000 Ci/mmol). Additional RNA probes were synthesized from pGEM-3 constructs containing either the **a1** gene (971-bp *Xba*I fragment subcloned from pJA82.6; clone pSL901) or the *ACT1* gene (1.6-kb *Bam*HI-*Hin*dIII fragment; clone pACT1, a gift of M. Hampsey of this institution). Hybridizations were conducted by using a rotating hybridizer (Polytech Products, Somerville, Mass.) at either 45°C (*HSP82* probe 2) or 55°C (all others), and blots were stringently washed at 45°C (probe 2), 58°C (probe 1), 60°C (**a1**), or 65°C (*ACT1*) essentially as previously described

(13, 23). Indirect end labeling of genomic DNA samples isolated from transformed strains (Fig. 7A) was conducted in the presence of denatured vector DNA (20 μg per blot) that served as the competitor, effectively eliminating cross-hybridization.

Hybridized membranes were autoradiographed, stripped, and rehybridized as before (2). Hybridization signals were quantified within the linear response range of the film, using an LKB Bromma UltroScan laser densitometer and employing multiple exposures when necessary. *HSP82* and **a1** transcript levels cited in the text and shown in Fig. 2A, 2B, 3B, and 4 are based on internal normalization to *ACT1* levels.

Recovery and sequencing of plasmid DNA from transformed yeast cells. Total DNA from p205-transformed SLY102 cells was purified as described above. One microgram of this DNA was then used to transform *Escherichia coli* DH5α to ampicillin resistance, and plasmid DNA was recovered. The *Hin*dIII fragment containing *HMRE*, spanning −1402 to −458 of the *HMRE/HSP82* allele, was subcloned into M13mp18, and the region between −850 and −650 was sequenced on both strands, using the chain termination method of Sanger et al. (58).

RESULTS

Construction of *HMR/HSP82* alleles. To test whether the *E* silencer could function in an ectopic chromosomal context, we integrated DNA fragments containing *HMRE*, either alone or as part of the larger *HMR***a** locus, upstream of the *HSP82* gene. This gene was selected as the recipient locus since it is transcriptionally regulated at both basal and induced levels (19), and *cis*-acting sequences responsible for this regulation have been identified (21, 22, 44). Equally important, *HSP82* is dispensable for growth under both control and heat-shocked conditions, since a functionally homologous protein (97% identical at the amino acid level) is encoded by the constitutively expressed cognate gene, *HSC82* (7).

As schematically illustrated in Fig. 1A, integration at a site 673 bp upstream of *HSP82* situates the *HMR* loci just 5′ of a nuclease-hypersensitive region centered at position −615 (24, 44). These repressor sequences are positioned well upstream of *cis*-regulatory elements defined by recent genomic footprinting and in situ mutagenesis experiments (Fig. 1B, filled boxes) (21, 22, 24, 44). Furthermore, integration of the *HMRE* silencer at this site configures its three functional subdomains 750 bp 5′ of the gene, closely approximating their native position relative to the bidirectional **a1/a2** gene promoter (~900 bp [1]). While *E* is disposed ~2.7 kb away from *HSP82* in the *HMR***a** insertion (Fig. 1A), it is within its previously reported functional range (8) and, as discussed below, appears to function equivalently in the two hybrid alleles.

Site-specific integration of *HMRE* into the yeast genome was accomplished by using two-step gene transplacement (6, 59). Successful replacement of the wild-type *HSP82* allele (2.9-kb *Eco*RI fragment) with the *HMRE/HSP82* allele (3.25-kb fragment), thereby creating strain SLY105, was confirmed by Southern analysis (Fig. 1C). Construction of strains carrying an episomal copy of *HMRE/HSP82* or a chromosomal copy of *HMR***a***/HSP82* was similarly confirmed (Fig. 1D and E; see below).

The *HMRE* element conditionally silences *HSP82* on chromosome XVI. To assess the activity of the ectopic *HMRE* element, we measured *HSP82* transcript levels both before and after an 11-min 30-to-39°C heat shock. As can be seen in Fig. 2A, insertion of the *HMRE* fragment per se has no effect on promoter function in a *sir4* background, since basal and induced mRNA levels are virtually identical to wild-type levels (lanes 2 versus 1 and 5 versus 4). As expected, the *HMR***a** cassette has been fully derepressed (illustrated by the nascent appearance of **a1** transcript not seen in a wild-type, *SIR4*$^+$ strain). However, following introduction of a functional allele of the *SIR4* gene by transforming SLY105 with pKAN59, a 2μm-based plasmid, *HSP82* basal transcription is repressed ~5-fold (lane 3 versus 2). Induced expression, on the other hand, is unaffected (lane 6 versus 5). The **a1** promoter within the *HMR***a** locus is strongly repressed (≥100-fold) in both states (lanes 3 and 6), confirming the function of the *trans*-acting proteins mediating *HMRE* repression (see introduction). In particular, this observation confirms that *SIR4* is present and functional in ≥99% of the cells.

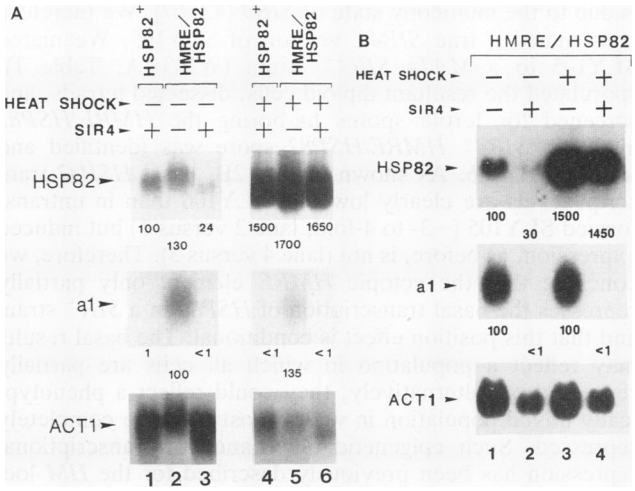

FIG. 2. Functional analysis of genes subject to *HMRE* control in SLY105 and SLY106. (A) Northern analysis of total RNA isolated from early-log-phase cultures of W303-1B (lanes 1 and 4) and SLY105 (lanes 2, 3, 5, and 6) before (−) and after (+) an 11-min 30-to-39°C temperature shift (lanes 1 to 3, 50 μg of RNA each; lanes 4 to 6, 20 μg of RNA each). The membrane was sequentially hybridized with probes for *HSP82*, **a1**, and *ACT1*; *HSP82* and **a1** transcript levels were quantitated by laser densitometry and internally normalized to *ACT1* levels. Data for each transcript are derived from a single autoradiographic exposure; similarly constructed composites are shown in Fig. 3B and 4. Control and heat-shocked *HSP82* RNA levels were quantitated relative to those of the wild-type strain, which were assigned values of 100 and 1,500, respectively, on the basis of a previous analysis (44). **a1** transcript levels were quantitated relative to those in lane 2, which was arbitrarily assigned a value of 100. All quantitations are based on multiple exposures in the linear response range of the film. The *SIR4*$^+$ derivative of SLY105 (*sir4-351*) was generated by introduction of a multicopy *SIR4* gene on plasmid pKAN59 (lanes 3 and 6). Its *sir4* counterpart was transformed with YEp213 (lanes 2 and 5). Results essentially identical to those seen in lanes 3 and 6 were obtained when SLY105 was transformed with pJR368, a centromeric *SIR4* vector (see Fig. 5, 6A, and 6B). (B) Northern analysis of total RNA isolated from untransformed strains SLY105 (lanes 1 and 3) and SLY106 (lanes 2 and 4). RNA samples (20 μg of each) were isolated from control (−) and heat-shocked (+) cultures as indicated and processed as described above.

As the requirements for silencing at *HSP82* may be more stringent than at *HMR***a**, it was necessary to rule out the possibility that the partial, conditional repression of *HSP82*

is due to the multicopy state of *SIR4* (42, 67). We therefore constructed a true *SIR4*$^+$ version of SLY105. We mated SLY105 to a *MAT***a** *SIR4*$^+$ strain (W303-1A; Table 1), sporulated the resultant diploid cells, dissected tetrads, and screened for fertile spores harboring the *HMRE/HSP82* allele. A *SIR4*$^+$ *HMRE/HSP82* spore was identified and termed SLY106. As shown in Fig. 2B, basal *HSP82* transcript levels are clearly lower in SLY106 than in untransformed SLY105 (~3- to 4-fold; lane 2 versus 1) but induced expression, as before, is not (lane 4 versus 3). Therefore, we conclude that the ectopic *HMRE* element only partially represses the basal transcription of *HSP82* in a *SIR*$^+$ strain and that this position effect is conditional. The basal results may reflect a population in which all cells are partially repressed or, alternatively, they could reflect a phenotypically mixed population in which most cells are completely repressed. Such epigenetic inheritance of transcriptional repression has been previously described for the *HM* loci and for telomeres in *S. cerevisiae* (20, 41a, 50, 67).

The *HMRE* element conditionally silences *HSP82* on an episome. It could be argued that the diminished activity of the *HMRE* silencer reflects some novel attribute of the chromosomal environment into which it was integrated. For example, it could reflect the presence of an as yet unidentified distal regulatory element, analogous to the locus control region of the human β-globin cluster (26, 57), which may antagonize silencer function. On the other hand, the compromised function of the ectopic element may reflect intrinsic properties of the silencer and promoter sequences themselves.

To distinguish between these two possibilities, we studied the expression of the same sequences when carried on a plasmid (Fig. 3A). We used the *hsp82*Δ::*CYH2*^{s} disruption strains SLY102 (*SIR4*$^+$) and SLY103 (*sir4*) as the recipients; thus, the only *HSP82* expression in these strains derives from the episomal copy of the gene. As indicated in Fig. 1D (lane 3), SLY102 transformed with the *HMRE/HSP82* plasmid p205 harbors two mutant *hsp82* alleles, *hsp82*Δ::*CYH2*^{s} (1.4-kb *Eco*RI fragment) and *HMRE/HSP82* (3.25-kb fragment), while lacking a wild-type 2.9-kb fragment. Densitometric quantitation of lane 3 indicates that the episomal *HMRE/HSP82* allele is present in ~1.1 copies per haploid cell.

In a *sir4* background, the episomal copy of *HMRE/HSP82* functions similarly to the wild-type *HSP82*$^+$ allele in both control and heat-shocked states (Fig. 3B, lanes 1 versus 3 and 4 versus 6). (The ~2-fold enhanced expression of the episomal *HMRE/HSP82* gene in noninduced *sir4* cells is of doubtful significance, as it is not seen with either *HMR/HSP82* chromosomal allele [Fig. 2 and 4].) However, in a *SIR4*$^+$ background, basal but not induced expression is repressed and to roughly the same degree (~5-fold) as before. To rule out the possibility that the attenuated position effect at the ectopic site reflects a mutation in the *HMRE* fragment, we sequenced the pertinent region in p205 (see Materials and Methods). We found that all three functional elements, A, E, and B, exhibit wild-type sequence (Fig. 3C) (9). Therefore, we conclude that the intact *HMRE* silencer works similarly on a centromeric plasmid as it does in the chromosome; in both ectopic sites, it exerts a partial, conditional position effect over *HSP82*.

Juxtaposition of the intact *HMR*a silent mating-type cassette with *HSP82* results in a similar conditional position effect. Since *HMRE* alone elicited a partial position effect that was lost upon heat shock induction, and since a role for other sequences within the *HMR***a** cassette in mediating silencing has not been ruled out, we introduced the entire silent mating-type locus upstream of *HSP82* in an attempt to establish dominant, unconditional repression. Such a manipulation effectively juxtaposes heterochromatic and euchromatic domains, closely simulating conditions normally associated with spontaneous position effects (see introduction). Using two-step gene transplacement techniques as described above, we transformed the *hsp82*Δ::*CYH2*^{s} strain SLY103 with the *HMR***a**/*HSP82* construct p110. As depicted in Fig. 1E, Southern analysis confirms replacement of the target *hsp82*Δ::*CYH2*^{s} allele (1.4-kb *Eco*RI fragment) with the donor *HMR***a**/*HSP82* allele (4.6-kb fragment), creating strain SLY107.

To assess the impact of the immigrant mating-type domain upon the heat shock gene and vice versa, we measured *HSP82* and **a1** transcript levels by Northern (RNA) blot analysis both before and after an 11-min 30-to-39°C heat shock. Figure 4 reveals that in the absence of a functional *SIR4* product, integration of the 4.9-kb fragment has an insignificant effect on *HSP82* promoter function (lanes 2 versus 1 and 5 versus 4), further supporting the view that all pertinent regulatory elements are located downstream of the

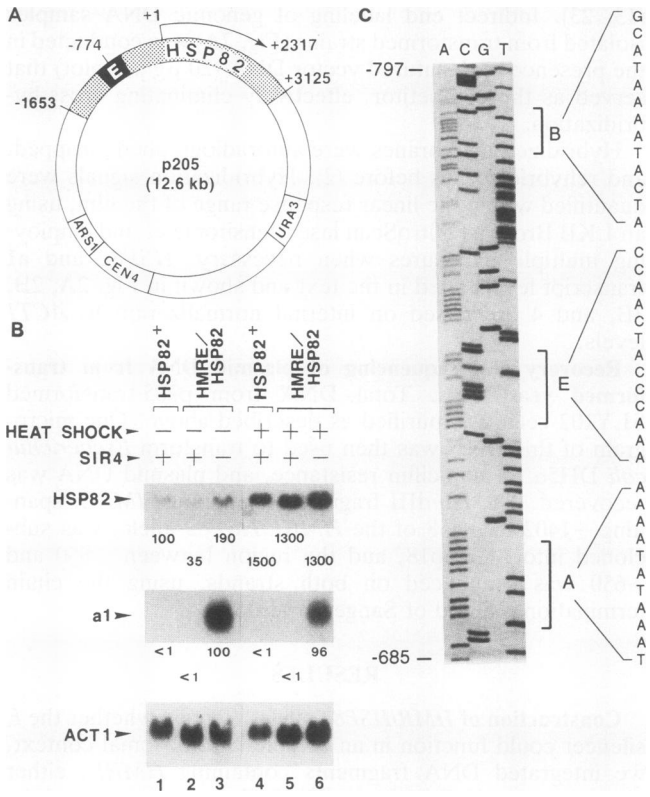

FIG. 3. Gene map, functional analysis, and sequence verification of the episomal *HMRE/HSP82* construct, p205. (A) Gene map of the YCp50 derivative p205. (B) Northern analysis of total RNA isolated from W303-1B (lanes 1 and 4) and p205-transformed SLY102 (lanes 2 and 5) and SLY103 (lanes 3 and 6) strains. RNA samples (20 μg of each) were isolated from control or heat-shocked exponential-phase cultures as indicated, fractionated, hybridized, and quantitated as described for Fig. 2. (C) DNA sequence of the three functional domains of the silencer within the *HMRE/HSP82* allele. Plasmid p205 was recovered from transformed SLY102, and the *HMRE* region was subjected to sequence analysis as described in Materials and Methods. Sequencing of both strands confirms that the *HMRE* element is wild type; the lower-strand sequence is shown.

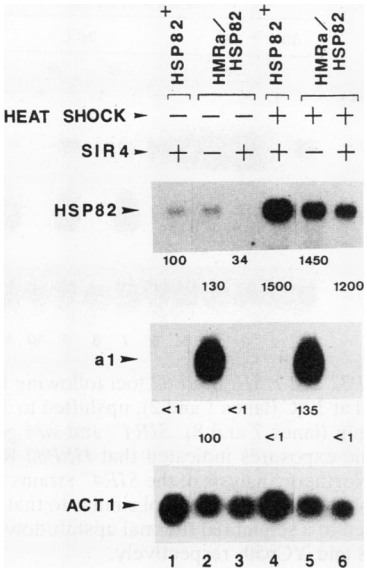

FIG. 4. Functional analysis of genes subject to *HMRE* control in SLY107. Northern analysis of total RNA isolated from W303-1B (lanes 1 and 4) and SLY107 (lanes 2, 3, 5, and 6) was performed. RNA was isolated from early-log-phase cultures, and 20 μg per lane was evaluated as described for Fig. 2. The *SIR4*$^+$ derivative of SLY107 was obtained by transformation with pJR368 (lanes 3 and 6); its *sir4* counterpart was transformed with YCp50 (lanes 2 and 5). Results similar to those found in lanes 3 and 6 were obtained following transformation of SLY107 with a 2μm-based episomal *SIR4* gene (data not shown).

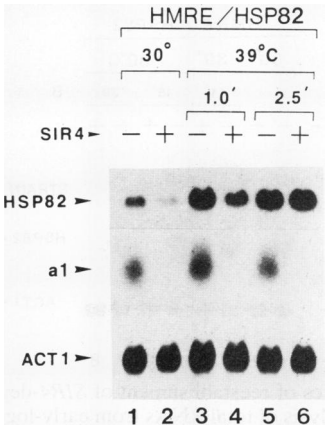

FIG. 5. Kinetics of thermal derepression at the *HMRE/HSP82* locus. Shown is Northern analysis of *HSP82* transcript levels in early-log-phase SLY105 cells cultivated at 30°C and at 1.0 and 2.5 min following a temperature upshift to 39°C. RNA samples were isolated, and 20 μg per lane was evaluated as described for Fig. 2. *SIR4*$^+$ and *sir4* derivatives of SLY105 were generated by transformation with pJR368 and YCp50, respectively.

integration site. Similarly, both native and ectopic **a1** promoters appear to be fully derepressed in the *sir4* strain, since the level of **a1** transcript in SLY107 is twice that of SLY103 (data not shown). However, following introduction of the wild-type *SIR4* gene, a 4-fold repression of *HSP82* message is seen in the non-heat-shocked state, concomitant with ≥100-fold repression of each **a1** allele (lane 3 versus 2). Therefore, the ectopic *E* silencer, alone or in combination with additional *HMR* sequences, is capable of simultaneously repressing two distant class II promoters, one partially and the other completely. While this difference in silencing may reflect the relative distances of the two promoters to *E* (2,700 bp for *HSP82* versus 900 bp for **a1**), it is unlikely, given that a similar extent of repression was obtained when *E* was disposed 750 bp to the left of *HSP82* (Fig. 2 and 3B).

As seen previously, heat shock overrides the position effect exerted by the transplaced fragment, rendering the *HMRa/HSP82* allele nearly 40-fold inducible (Fig. 4, lane 6 versus 3). Notably, the ectopic **a1** promoter remains fully silenced, indicating that the associated *SIR* complex retains function under inducing conditions. Therefore, the *HMR* domain imposes a similar conditional position effect upon *HSP82* as did the *E* silencer alone while simultaneously imposing an unconditional position effect on **a1**. Thus, our data provide no evidence for a positive position effect exerted by *HSP82* regulatory elements upon the *HMR*-repressed **a1** promoter.

Heat-induced derepression is rapid and reversible. Since the previous experiments convincingly illustrated a difference in ectopic silencer function in control and heat-shocked cells, it was of interest to determine the kinetics of derepression of *HSP82* transcription and to examine whether derepression takes place when heat-shocked cells are returned to nonstressful conditions. We therefore measured *HSP82* transcript levels in *SIR4*$^+$ and *sir4* backgrounds in the *HMRE/HSP82* strain SLY105. Because the *HSP82* message has a short half-life ($t_{1/2}$ of <2.0 min at 30°C; see Fig. 6), such an assay provides a sensitive measure of the instantaneous rate of transcription. In the absence of *SIR4* regulation, *HSP82* transcript levels rapidly increase upon temperature upshift (Fig. 5, lane 3 versus 1), with kinetics similar to those previously observed (44). In the presence of *SIR4*, *HSP82* RNA levels increase even more rapidly, exhibiting substantial derepression within 60 s of heat shock (lane 4 versus 2) and attaining a level of expression similar to that in *sir4* cells within 2.5 min (lane 6 versus 5). As before, **a1** remains strongly repressed within the *HMR* locus at either temperature (lanes 2, 4, and 6). Therefore, transcriptional derepression of the *HMRE/HSP82* gene occurs on the order of seconds.

Similarly, when SLY105 cells are sequentially subjected to an 11-min 39°C heat shock followed by a 30°C recovery, rapid *SIR4*-dependent repression of *HSP82* is seen (Fig. 6A). In fact, there is a transient reduction of *HSP82* transcripts that exceeds the repression attained in the basal state, since following a 15-min recovery from stress, *HSP82* levels in *SIR4*$^+$ cells are ≥25-fold lower than those in *sir4* cells (Fig. 6A, lane 6 versus 5). Even after 30 min, there is ≥15-fold *SIR4*-dependent repression (lane 8 versus 7). In contrast, **a1** repression is not affected by the upshift/downshift regimen (lanes 2, 4, 6, and 8). That SIR4 protein is not acting to change the half-life of the *HSP82* transcript is confirmed by the experiment shown in Fig. 6B, which reveals that *HSP82* transcript levels in the *SIR4*$^+$ *HSP82*$^+$ strain (W303-1B) are not affected by the presence of SIR4 under control, heat-shocked, or recovery conditions (compare lanes 1, 3, and 9 of Fig. 6B with lanes 1, 3, and 5 of Fig. 6A). Furthermore, this analysis indicates that following an 11-min heat shock, there is a noticable lag (≥7.5 min) before *HMRE*-dependent repression of *HSP82* transcript levels is detectable. During the next 7.5 min, a dramatic 16-fold drop in message level is seen (Fig. 6B, lane 10 versus 8). Such a biphasic response might be anticipated if, for example, remodeling of chroma-

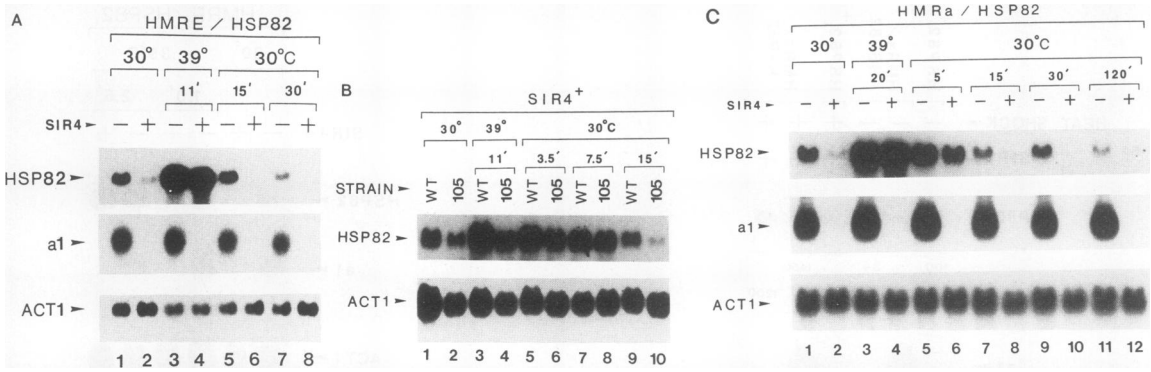

FIG. 6. Kinetics of reestablishment of *SIR4*-dependent repression at the *HMRE/HSP82* and *HMRaHSP82* loci following thermal stress. (A) Northern analysis of total RNAs from early-log-phase cultures of SLY105 maintained at 30°C (lanes 1 and 2), upshifted to 39°C for 11 min (lanes 3 and 4), and then downshifted to 30°C for either 15 min (lanes 5 and 6) or 30 min (lanes 7 and 8). *SIR4*$^+$ and *sir4* genotypes were conferred as described for Fig. 5. Densitometric quantitation of longer autoradiographic exposures indicated that *HSP82* RNA levels are repressed ≥25-fold after 15 min and ≥15-fold after 30 min in *SIR4*$^+$ versus *sir4* cells. (B) Northern analysis of the *SIR4*$^+$ strains W303-1B (wild type [WT]) and pJR368-transformed SLY105 (105), subjected to a sequential thermal upshift/downshift protocol similar to that used for panel A. (C) Northern analysis of total RNAs from early-log-phase cultures of SLY107 subjected to a sequential thermal upshift/downshift protocol as indicated. *SIR4*$^+$ and *sir4* derivatives were generated by transformation with pJR368 and YCp50, respectively.

tin is required for reestablishment of the silenced state (45). On the other hand, the lag may reflect the kinetics of deactivation of heat shock factor (HSF), the transcriptional activator of *HSP82* (1a, 4a, 22, 65).

To help distinguish between these two possibilities, we examined the kinetics of reestablishment in strain SLY107, in which the *E* silencer is disposed nearly 3 kb upstream of the heat shock gene (Fig. 1A). If remodeling of chromatin were involved in the reestablishment of silencing, then it might be anticipated that the lag would be more exaggerated in the *HMRa/HSP82* strain. However, following a 20-min heat shock, the kinetics of silencing of the *HMRa/HSP82* allele are virtually identical to those seen with *HMRE/HSP82*, with complete repression apparent as early as 15 min (Fig. 6C, lane 8). Interestingly, following a 120-min recovery, silencing is once again partial (~5-fold), as seen under continuous nonstressful conditions (Fig. 6C; compare lanes 11 and 12 with lanes 1 and 2). Taken together, the data presented in Fig. 6 indicate that *SIR4*-dependent repression of *HSP82* is due to a reestablishment of transcriptional silencing that occurs within minutes of thermal downshift. Such an effect is seen at both *HMRE/HSP82* and *HMRa/HSP82* alleles, and similar kinetics of reestablishment are seen in unrelated genetic backgrounds.

Protein-DNA interactions within the regulatory region of *HMRE/HSP82* remain unchanged irrespective of *SIR4* or heat shock. As the foregoing experiments indicated striking flexibility in the regulation of *HMRE/HSP82* transcription, we wished to address more directly whether such changes in gene function are paralleled at the structural level. Previous studies have shown that the promoter region of *HSP82* is organized into a constitutive, nonnucleosomal DNase I-hypersensitive site in chromatin (25, 68). Similarly, *HMRE* is localized within a region of chromatin that is hypersensitive to cleavage by DNase I in both *SIR*$^+$ and *sir* genetic backgrounds (48). To address this question, we mapped sites of protein-DNA interaction within the upstream regulatory region of the *HMRE/HSP82* allele, using the spheroplast lysate technique (24). Briefly, spheroplasts were obtained from both control and heat-shocked *SIR4*$^+$ and *sir4* cells, lysed in a hypotonic buffer containing divalent cations, and mildly digested with DNase I. Purified DNA was then restricted with *Eco*RI, electrophoresed, blotted, and indirectly end labeled. The results (Fig. 7A) indicate that the promoter and silencer sequences are assembled into nucleoprotein complexes which exhibit no detectable change in structure, despite a 70-fold range in expression levels (Fig. 2). In particular, the DNase I cleavage pattern reveals the presence of several internal footprints within broad regions of hypersensitivity relative to the naked DNA control (Fig. 7A, lanes 1, 2, 4, and 5 versus 3). Protected sequences map to the TATA box and principal heat shock element (HSE1) of *HSP82* as well as to each of the three functionally defined domains of the silencer: the ARS core consensus site A, the RAP1 binding site E, and the ABF1 binding site B (Fig. 7A). Therefore, the *HMRE/HSP82* allele is organized into a chromatin structure poised for transcriptional activation, much as seen in the wild-type allele (Fig. 7B). We conclude that *HMRE*, while clearly functional and occupied by sequence-specific DNA-binding proteins, has no detectable effect on the DNase I cleavage profile of the *HSP82* promoter.

DISCUSSION

***HMRE* is functional in an ectopic chromosomal site.** The *E* silencer has previously been implicated in mediating transcriptional repression at the *HMR* silent mating-type cassette (1, 8, 61). Our results indicate that *HMRE* can, in addition, exert silencing at an ectopic chromosomal site in the absence of sequences normally associated with it. In particular, we have shown that when disposed 750 bp upstream of the *HSP82* heat shock gene in the chromosome or on a centromeric plasmid, this silencer represses *HSP82* basal activity ~5-fold. A similar level of repression is seen when the entire *HMRa* cassette is transplaced into the heat shock gene locus. Importantly, the position effect exerted by these sequences is strictly dependent on a *SIR4*$^+$ background, ruling out the possibility that the observed phenotype is a consequence of the inserted fragments disrupting *cis*-regulatory elements within the *HSP82* promoter.

Our experiments thus extend previous observations that heterologous class II and class III promoters are transcriptionally repressed when moved to the *HMRa* locus (8, 61).

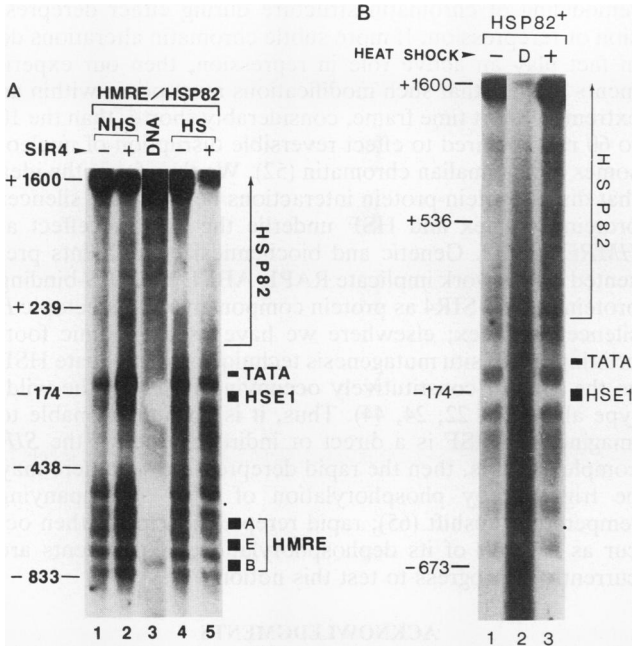

FIG. 7. DNase I chromatin footprints of the *HMRE/HSP82* and *HSP82*$^+$ alleles under transcriptionally inducing and noninducing conditions. (A) Spheroplast lysates, generated from YEp213-transformed (lanes 1 and 4) or pKAN59-transformed (lanes 2 and 5) SLY105 cells, non-heat shocked (NHS) and 11-min heat shocked (HS) as indicated, were digested for 80 min at 3°C with 5×10^{-4} U of DNase I per μg of DNA. The DNA was purified, cleaved with *Eco*RI, electrophoresed on a 2% agarose gel, capillary blotted to GeneScreen, and indirectly end labeled with probe 1 (see Fig. 1B). Naked genomic DNA (lane 3), purified from strain SLY105, was digested for 10 min at 37°C with 10^{-5} U of DNase I per μg of DNA and processed similarly. The positions of landmark *HSP82* restriction fragments are indicated on the left; the locations and identities of protected sequences relative to the naked DNA control are shown on the right. Also indicated is the location of the *HSP82* transcriptional unit (arrow), mapped with respect to the underlying DNA sequence. The strong protection at position −600 (dot), seen under all four states, has not yet been analyzed at nucleotide resolution but maps to a consensus ABF1 site (13 of 13 match between −600 and −588). It is also seen in the *HSP82*$^+$ allele (B). (B) Spheroplast lysates, generated from control (−) or 11-min heat-shocked (+) W303-1B cells (lanes 1 and 3, respectively), were digested with DNase I and processed as described above. Deproteinized genomic DNA (D; lane 2), isolated from strain W303-1B, was digested with DNase I and processed as for panel A. Sequence coordinates (left) and symbols (right) are as in panel A.

Here we have shown that *HMRE* can function in an ectopic context, repressing a heat shock gene on chromosome XVI. However, in contrast to earlier studies, we find that the basal transcription of *HSP82* is only partially repressed by *SIR*, while its heat-induced expression is completely derepressed. We do not believe that such derepression is due to thermolability of the ectopic silencer complex, as has been reported for *Drosophila* heterochromatin (16), since chemical shock (8.5% ethanol for 15 min) similarly derepresses the *HMRE/HSP82* locus (data not shown). What then is the basis for this partial, conditional position effect?

(i) It is not due to the active chromatin domain in which the heat shock gene resides. The *HSP82* locus is characterized by the presence of at least six DNase I-hypersensitive domains within an 8-kb region (Fig. 1A; see also references 25 and 68). It could be argued that one or more of these nucleosome-free regions are associated with proteins that confer position independence to genes resident in this locus, analogous to the DNase I-hypersensitive sites that constitute the human β-globin locus control region (26, 57). However, we have found that the *HMRE/HSP82* allele, when harbored on a centromeric plasmid and lacking all but 0.8 kb of flanking sequence upstream of *E* or downstream of the transcriptional unit, exhibits essentially the identical phenotype to the chromosomal copy, thus arguing against such a notion. In addition, in strain SLY107, the transplaced copy of *HMR***a** retains full silencing activity (≥100-fold) towards **a1** under both control (30°C) and inducing (39°C) conditions, identical to the *HMR***a** allele on chromosome III and inconsistent with the presence of a heat shock locus control region on chromosome XVI.

(ii) It is not due to the *E* silencer requiring additional sequences for full activity. This conclusion can be drawn from the fact that *HMRE* and *HMR***a** exert an essentially equivalent position effect upon *HSP82*. Notably, the extents of repression are similar despite the rather significant difference in distance, 0.8 versus 2.7 kb, consistent with the previously observed functional range of *HMRE* (8). This result therefore argues against a role for non-*E* sequences within *HMR***a** in mediating repression at a distance, at least for promoters not contained within the *HMR* locus proper. It is possible that the relative position of the target regulatory sequence is crucial, however, such that a promoter residing between the *E* and *I* silencers may be silenced more effectively than one located outside of them. This might be the case if, as has been previously suggested, silencers define a chromosomal loop domain within which the promoter(s) is maintained in an inactive state (29). However, Mahoney and Broach (41) have observed that *URA3* is subject to nearly complete repression when transplaced into the *HML* domain, irrespective of whether it is integrated within or is centromere distal or centromere proximal of the locus. Moreover, Brand et al. (8) have reported that a chromosomal deletion of the *HMRI* site has no discernible effect on *HMRE*-mediated repression of several class II genes, further arguing against the necessity for a promoter to be flanked by two silencers in order to be inactivated. Furthermore, the data presented in Fig. 4 tend to rule out an essential role for more distal flanking sequences, such as the telomere 23 kb away (49a), in contributing to the repressed state of the native *HMR***a** locus.

(iii) It is due to the heat shock promoter being intrinsically immune to the effects of the silencer, particularly following stress induction. It could be argued that the less potent *SIR* repression exerted upon the heat shock gene, compared with that exerted on **a1** or previously studied heterologous gene constructs, stems from the different intrinsic strengths of the respective promoters. The *HSP82* promoter is possibly one of the strongest in the cell; HSP82 protein constitutes 0.14% of the total soluble protein synthesized under nonstressed conditions and nearly 5% of protein synthesized during heat shock (43). Accordingly, when the gene is either thermally or chemically induced, *HMRE*-mediated silencing is essentially eliminated, exemplifying the tight linkage of silencer function to that of the heat shock promoter. Similar rules appear to apply to the *HMR* locus itself. When heterologous genes such as *LEU2* or *HIS3* are moved to the *HMR* silent locus, phenotypic repression is not seen (67a). While these results appear to be at odds with earlier experiments indicating that *LEU2* was fully repressed when inserted into *HMR* (8), a fragment encompassing less than the full-length *LEU2* promoter may have been used in the earlier study (e.g., see

reference 10). Thus, the ability of the *E* silencer to exert a position effect on a nearby gene appears to be inversely correlated with the strength of the target gene's promoter. Consistent with this interpretation is the stronger *SIR4*-dependent repression of *HSP82* RNA levels seen after a 30-to-39-to-30°C upshift/downshift regimen than before it, paralleling the lower level of expression of the heat shock gene (Fig. 6A, lanes 5 and 8; Fig. 6B, lanes 9 and 10; Fig. 6C, lanes 7 to 12).

The position effect is rapidly reversible at the *HMRE/HSP82* locus. A number of observations suggest that modification of local chromatin structure is involved in silencing at both *HMR* and *HML*. First, deletion of the highly conserved N terminus of histone H4 causes derepression of both silent mating-type loci (33). Second, genetic evidence suggests that SIR3 protein directly interacts with the N terminus of H4 (32), thereby implicating a role for interactions between nonhistone and histone proteins in silencing at the *HM* loci. Third, chromatin structural analysis of the three loci containing mating-type information reveals that only in the genetically active *MAT* locus (or at *HM* loci in a *sir* background) is there a DNase I-hypersensitive site corresponding to the *HO* endonuclease cleavage site; *SIR* causes the specific disappearance of such a site at *HML* and *HMR* (48). Fourth, passage through S phase is obligatory for the onset of *SIR* repression at the silent mating-type loci (45), suggesting that DNA replication and attendant remodeling of chromatin structure is required for establishment of a position effect.

In contrast, we have presented two independent lines of evidence suggesting that silencing at *HMRE/HSP82* may be either lost or regained in the absence of a substantial alteration of chromatin structure. First, DNase I footprinting analysis of the *HMRE/HSP82* allele has indicated the presence of constitutive protein-DNA interactions at all three functional domains of the *HMRE* silencer. Both elements normally occupied by sequence-specific DNA-binding proteins within the wild-type *HSP82* promoter, HSE1 and TATA (24), are likewise occupied in the *HMRE/HSP82* allele irrespective of *SIR* or heat shock, suggesting concurrent occupancy of silencer and promoter binding sites. Second, we have found that following a temperature upshift, the gene is derepressed within 1 min and, more provocatively, is rerepressed within 15 min of a compensating downshift. This repression is equally rapid in *HMRE/HSP82* and *HMRa/HSP82* strains despite the *E* silencer being disposed nearly 2 kb further upstream from the heat shock gene in the latter. Such rapid reestablishment of repression would appear to contrast with the situation prevailing at the *HM* loci, where complete inactivation of mating-type information required nearly 10 h following shift of a $sir3^{ts}$ mutant to the permissive temperature (45). However, in the experiments with the $sir3^{ts}$ mutant, a functional *SIR* complex needed to be reestablished de novo, a process requiring passage through S phase. In our experiments, the *SIR* complex, while functionally neutralized, likely retains its structural integrity during heat shock since the ectopic **a1** gene remains silenced under conditions that strongly induce *HSP82* (Fig. 4, lane 6; Fig. 6C, lane 4). We surmise that following a return to nonstressful conditions, the preexisting *SIR* complex dominates and the basal level of *HSP82* transcription is silenced immediately. Whether a similar requirement for DNA replication exists at the *HMRE/HSP82* locus is currently being investigated, but the sheer rapidity of the reversal would seem to argue against it.

In conclusion, our data appear to rule out an elaborate remodeling of chromatin structure during either derepression or rerepression. If more subtle chromatin alterations do in fact play an active role in repression, then our experiments suggest that such modifications must occur within an extremely short time frame, considerably shorter than the 10 to 60 min required to effect reversible disruption of nucleosomes in mammalian chromatin (52). We thus favor the idea that direct protein-protein interactions between the silencer protein complex and HSF underlie the position effect at *HMRE/HSP82*. Genetic and biochemical experiments presented in this work implicate RAP1, ABF1, the ARS-binding protein(s), and SIR4 as protein components of the ectopic *E* silencer complex; elsewhere we have used genomic footprinting and in situ mutagenesis techniques to implicate HSF as the protein constitutively occupying HSE1 in the wild-type allele (21, 22, 24, 44). Thus, it is not unreasonable to imagine that HSF is a direct or indirect target of the *SIR* complex. If it is, then the rapid derepression seen here may be triggered by phosphorylation of HSF accompanying temperature upshift (65); rapid rerepression might then occur as a result of its dephosphorylation. Experiments are currently in progress to test this notion.

ACKNOWLEDGMENTS

We thank Bill Garrard, Mike Hampsey, Chris Adams, and Bruce Stentz for a critical review of the manuscript; Dan Gottschling and Tina Henkin for helpful discussions; David Shore for communicating results prior to publication; Judith Abraham, Jim Broach, Mike Douglas, David Finkelstein, Mike Hampsey, Tricia Laurenson, Alan Rose, and Fred Sherman for strains and plasmids; and Lucy Tress for assistance in preparing the manuscript.

This work was supported by grants from the National Institute of General Medical Sciences (GM45842), the March of Dimes Birth Defects Foundation (5-678), and the Center for Excellence in Cancer Research at LSUMC to D.S.G. D.S.G. was the recipient of a Basil O'Connor Starter Scholar Award from the March of Dimes.

REFERENCES

1. **Abraham, J., K. A. Nasmyth, J. N. Strathern, A. J. S. Klar, and J. B. Hicks.** 1984. Regulation of mating-type information in yeast: negative control requiring sequences both 5′ and 3′ to the regulated region. J. Mol. Biol. **176:**307–331.

1a. **Abravaya, K., M. P. Myers, S. P. Murphy, and R. I. Morimoto.** 1992. The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. Genes Dev. **6:**1153–1164.

2. **Adams, C., and D. S. Gross.** 1991. Conversion of cells to spheroplasts and potent transcriptional inhibitors induce the yeast heat shock response. J. Bacteriol. **173:**7429–7435.

3. **Aparicio, O. M., B. L. Billington, and D. E. Gottschling.** 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. Cell **66:**1279–1287.

3a. **Axelrod, A., and J. Rine.** 1991. A role for CDC7 in repression of transcription at the silent mating-type locus *HMR* in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**1080–1091.

4. **Baim, S. B., and F. Sherman.** 1988. mRNA structures influencing translation in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8:**1591–1601.

4a. **Baler, R., W. J. Welch, and R. Voellmy.** 1992. Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. J. Cell Biol. **117:**1151–1159.

5. **Beggs, J. D.** 1978. Transformation of yeast by a replicating hybrid plasmid. Nature (London) **275:**104–109.

5a. **Bell, S. P., and B. Stillman.** 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature (London) **357:**128–134.

6. **Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink.** 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154:**164–175.

7. **Borkovich, K. A., F. W. Farrelly, D. B. Finkelstein, J. Taulien, and S. Lindquist.** 1989. hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol. Cell. Biol. **9**:3919–3930.
8. **Brand, A. H., L. Breeden, J. Abraham, R. Sternglanz, and K. Nasmyth.** 1985. Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. Cell **41**:41–48.
9. **Brand, A. H., G. Micklem, and K. Nasmyth.** 1987. A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell **51**:709–719.
10. **Brisco, P. R. G., and G. B. Kohlhaw.** 1990. Regulation of yeast *LEU2*: total deletion of regulatory gene *LEU3* unmasks *GCN4*-dependent basal level expression of *LEU2*. J. Biol. Chem. **265**:11667–11675.
11. **Buchman, A. R., W. J. Kimmerly, J. Rine, and R. D. Kornberg.** 1988. Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:210–225.
12. **Burgers, P. M. J., and K. J. Percival.** 1987. Transformation of yeast spheroplasts without cell fusion. Anal. Biochem. **163**:391–397.
13. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.
14. **Diffley, J. F. X., and B. Stillman.** 1988. Purification of a yeast protein that binds to origins of DNA replication and a transcriptional silencer. Proc. Natl. Acad. Sci. USA **85**:2120–2124.
15. **Eisenberg, S., D. Civalier, and B.-K. Tye.** 1988. Specific interaction between a *S. cerevisiae* protein and DNA elements associated with certain autonomously replicating sequences. Proc. Natl. Acad. Sci. USA **85**:743–746.
16. **Eissenberg, J. C.** 1989. Position-effect variegation in *Drosophila*: towards a genetics of chromatin assembly. BioEssays **11**:14–17.
17. **Farrelly, F. W., and D. B. Finkelstein.** 1984. Complete sequence of the heat shock-inducible *HSP90* gene of *Saccharomyces cerevisiae*. J. Biol. Chem. **259**:5745–5751.
18. **Feldman, J. B., J. B. Hicks, and J. R. Broach.** 1984. Identification of sites required for repression of a silent mating type locus in yeast. J. Mol. Biol. **178**:815–834.
19. **Finkelstein, D. B., and S. Strausberg.** 1983. Heat shock-regulated production of *Escherichia coli* β-galactosidase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **3**:1625–1633.
20. **Gottschling, D. E., O. M. Aparicio, B. L. Billington, and V. A. Zakian.** 1990. Position effect at *S. cerevisiae* telomeres: reversible repression of pol II transcription. Cell **63**:751–767.
21. **Gross, D. S., C. C. Adams, K. E. English, K. W. Collins, and S. Lee.** 1990. Promoter function and *in situ* protein/DNA interactions upstream of the yeast *HSP90* heat shock genes. Antonie van Leeuwenhoek J. Microbiol. **58**:175–186.
22. **Gross, D. S., C. C. Adams, S. Lee, and B. Stentz.** A critical role for heat shock transcription factor in establishing a nucleosome-free region over the TATA box of the yeast *HSP82* heat shock gene. Submitted for publication.
23. **Gross, D. S., K. W. Collins, E. M. Hernandez, and W. T. Garrard.** 1988. Vacuum blotting: a simple method for transferring DNA from sequencing gels to nylon membranes. Gene **74**:347–356.
24. **Gross, D. S., K. E. English, K. W. Collins, and S. Lee.** 1990. Genomic footprinting of the yeast *HSP82* promoter reveals marked distortion of the DNA helix and constitutive occupancy of heat shock and TATA elements. J. Mol. Biol. **216**:611–631.
25. **Gross, D. S., C. Szent-Gyorgyi, and W. T. Garrard.** 1986. Yeast as a model system to dissect the relationship between chromatin structure and gene expression. UCLA Symp. Mol. Cell. Biol. New Ser. **33**:345–366.
26. **Grosveld, F., G. B. van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51**:975–985.

26a. **Hardy, C. F. J., L. Sussel, and D. Shore.** 1992. A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation. Genes Dev. **6**:801–814.

27. **Hewitt, E. J., and D. J. D. Nicholas.** 1963. Cations and anions: inhibitions and interactions in metabolism and in enzyme activity, p. 311–436. *In* R. M. Hochster and J. H. Quastel (ed.), Metabolic inhibitors—a comprehensive treatise. vol. 2. Academic Press, New York.
28. **Hofmann, J. F.-X., and S. M. Gasser.** 1991. Identification and purification of a protein that binds the yeast ARS consensus sequence. Cell **64**:951–960.
29. **Hofmann, J. F.-X., T. Laroche, A. H. Brand, and S. M. Gasser.** 1989. RAP-1 factor is necessary for DNA loop formation *in vitro* at the silent mating type locus *HML*. Cell **57**:725–737.
30. **Holm, C., D. W. Meeks-Wagner, W. L. Fangman, and D. Botstein.** 1986. A rapid, efficient method for isolating DNA from yeast. Gene **42**:169–173.
31. **James, T. C., and S. C. R. Elgin.** 1986. Identification of a nonhistone chromosomal protein associated with heterochromatin in *Drosophila melanogaster*. Mol. Cell. Biol. **6**:3862–3872.
32. **Johnson, L. M., P. S. Kayne, E. S. Kahn, and M. Grunstein.** 1990. Genetic evidence for an interaction between SIR3 and histone H4 in the repression of the silent mating loci in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **87**:6286–6290.
33. **Kayne, P. S., U.-J. Kim, M. Han, J. R. Mullen, F. Yoshizaki, and M. Grunstein.** 1988. Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell **55**:27–39.
34. **Kimmerly, W. J., and J. Rine.** 1987. Replication and segregation of plasmids containing *cis*-acting regulatory sites of silent mating-type genes in *Saccharomyces cerevisiae* are controlled by the *SIR* genes. Mol. Cell. Biol. **7**:4225–4237.
35. **Klar, A. J. S., and S. Fogel.** 1979. Activation of mating-type genes by transposition in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **76**:4539–4543.
36. **Klar, A. J. S., J. N. Strathern, J. R. Broach, and J. B. Hicks.** 1981. Regulation of transcription in expressed and unexpressed mating-type cassettes of yeast. Nature (London) **289**:239–244.

36a. **Kuo, C.-L., and J. L. Campbell.** 1983. Cloning of *Saccharomyces cerevisiae* DNA replication genes: isolation of the *CDC8* gene and two genes that compensate for the *cdc8-1* mutation. Mol. Cell. Biol. **3**:1730–1737.

37. **Kurtz, S., and D. Shore.** 1991. RAP1 protein activates and silences transcription of mating-type genes in yeast. Genes Dev. **5**:616–628.

37a. **Laurenson, P., and J. Rine.** 1991. *SUM1-1*: a suppressor of silencing defects in *Saccharomyces cerevisiae*. Genetics **129**: 685–696.

38. **Lee, M.-S., and W. T. Garrard.** 1991. Transcription-induced nucleosome "splitting": an underlying structure for DNase I sensitive chromatin. EMBO J. **10**:607–615.
39. **Lewis, E. B.** 1950. The phenomenon of position effect. Adv. Genet. **3**:73–115.
40. **Lindquist, S.** 1981. Regulation of protein synthesis during heat shock. Nature (London) **293**:311–314.
41. **Mahoney, D. J., and J. R. Broach.** 1989. The *HML* mating-type cassette of *Saccharomyces cerevisiae* is regulated by two separate but functionally equivalent silencers. Mol. Cell. Biol. **9**:4621–4630.

41a. **Mahoney, D. J., R. Marquardt, G.-J. Shei, A. B. Rose, and J. R. Broach.** 1991. Mutations in the *HML* E silencer of *Saccharomyces cerevisiae* yield metastable inheritance of transcriptional repression. Genes Dev. **5**:605–615.

42. **Marshall, M., D. Mahoney, A. Rose, J. B. Hicks, and J. R. Broach.** 1987. Functional domains of *SIR4*, a gene required for position effect regulation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**:4441–4452.
43. **McAlister, L., S. Strausberg, A. Kulaga, and D. B. Finkelstein.** 1979. Altered patterns of protein synthesis induced by heat-shock of yeast. Curr. Genet. **1**:63–74.
44. **McDaniel, D., A. J. Caplan, M.-S. Lee, C. C. Adams, B. R. Fishel, D. S. Gross, and W. T. Garrard.** 1989. Basal level expression of the yeast *HSP82* gene requires a heat shock regulatory element. Mol. Cell. Biol. **9**:4789–4798.
45. **Miller, A. M., and K. A. Nasmyth.** 1984. Role of DNA replication in the repression of silent mating-type loci in yeast. Nature (London) **312**:247–251.

46. **Mortimer, R. K., D. Schild, C. R. Contopoulou, and J. A. Kans.** 1991. Genetic and physical maps of *Saccharomyces cerevisiae*. Methods Enzymol. **194:**827–863.
47. **Mullen, J. R., P. S. Kayne, R. P. Moerschell, S. Tsunasawa, M. Gribskov, M. Colavito-Shepanski, M. Grunstein, F. Sherman, and R. Sternglanz.** 1989. Identification and characterization of genes and mutants for an N-terminal acetyl transferase from yeast. EMBO J. **8:**2067–2075.
48. **Nasmyth, K. A.** 1982. The regulation of yeast mating-type chromatin structure by *SIR*: an action at a distance affecting both transcription and transposition. Cell **30:**567–578.
49. **Nasmyth, K. A., K. Tatchell, B. D. Hall, C. R. Astell, and M. Smith.** 1981. A position effect in the control of transcription at yeast mating type loci. Nature (London) **289:**244–250.
49a. **Oliver, S. G., et al.** 1992. The complete DNA sequence of yeast chromosome III. Nature (London) **357:**38–46.
50. **Pillus, L., and J. Rine.** 1989. Epigenetic inheritance of transcriptional states in *S. cerevisiae*. Cell **59:**637–647.
51. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13:**7207–7221.
52. **Reik, A., G. Schutz, and A. F. Stewart.** 1991. Glucocorticoids are required for the establishment and maintenance of an alteration in chromatin structure: induction leads to a reversible disruption of nucleosomes over an enhancer. EMBO J. **10:**2569–2576.
53. **Reuter, G., M. Giarre, J. Farah, J. Gausz, A. Spierer, and P. Spierer.** 1990. Dependence of position-effect variegation in *Drosophila* on dose of a gene encoding an unusual zinc-finger protein. Nature (London) **344:**219–223.
54. **Rine, J., and I. Herskowitz.** 1987. Four genes responsible for a position effect on expression from *HML* and *HMR* in *Saccharomyces cerevisiae*. Genetics **116:**9–22.
55. **Rine, J., J. N. Strathern, J. B. Hicks, and I. Herskowitz.** 1979. A suppressor of mating type mutations in *Saccharomyces cerevisiae*: evidence for and identification of cryptic mating type loci. Genetics **93:**877–901.
56. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101:**202–211.
57. **Ryan, T. M., R. R. Behringer, N. C. Martin, T. M. Townes, R. D. Palmiter, and R. L. Brinster.** 1989. A single erythroid-specific DNase I super-hypersensitive site activates high levels of human β-globin gene expression in transgenic mice. Genes Dev. **3:**314–323.
58. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
59. **Scherer, S., and R. W. Davis.** 1979. Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. Proc. Natl. Acad. Sci. USA **76:**4951–4955.
60. **Schmidt, A. M. A., S. U. Herterich, and G. Krauss.** 1991. A single-stranded DNA binding protein from *S. cerevisiae* specifically recognizes the T-rich strand of the core sequence of ARS elements and discriminates against mutant sequences. EMBO J. **10:**981–985.
61. **Schnell, R., and J. Rine.** 1986. A position effect on the expression of a tRNA gene mediated by the *SIR* genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**494–501.
62. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
63. **Shore, D., and K. Nasmyth.** 1987. Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. Cell **51:**721–732.
64. **Shore, D., D. J. Stillman, A. H. Brand, and K. A. Nasmyth.** 1987. Identification of silencer binding proteins from yeast: possible roles in *SIR* control and DNA replication. EMBO J. **6:**461–467.
65. **Sorger, P. K., and H. R. B. Pelham.** 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell **54:**855–864.
66. **Spofford, J. B.** 1976. Position-effect variegation in *Drosophila*, p. 995–1018. *In* M. Ashburner and E. Novitski (ed.), The genetics and biology of *Drosophila*. Academic Press, Boca Raton, Fla.
67. **Sussel, L., and D. Shore.** 1991. Separation of transcriptional activation and silencing functions of the *RAP1*-encoded repressor/activator protein 1: isolation of viable mutants affecting both silencing and telomere length. Proc. Natl. Acad. Sci. USA **88:**7749–7753.
67a. **Sussel, L., and D. Shore (Columbia University).** Personal communication.
68. **Szent-Gyorgyi, C., D. B. Finkelstein, and W. T. Garrard.** 1987. Sharp boundaries demarcate the chromatin structure of a yeast heat-shock gene. J. Mol. Biol. **193:**71–80.
69. **Tartof, K. D., C. Hobbs, and M. Jones.** 1984. A structural basis for variegating position effects. Cell **37:**869–878.
70. **Whiteway, M., R. Freedman, S. Van Arsdell, J. W. Szostak, and J. Thorner.** 1987. The yeast *ARD1* gene product is required for repression of cryptic mating-type information at the *HML* locus. Mol. Cell. Biol. **7:**3713–3722.
71. **Wilson, C., H. J. Bellen, and W. J. Gehring.** 1990. Position effects on eukaryotic gene expression. Annu. Rev. Cell Biol. **6:**679–714.