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A Position Effect on the Expression of a tRNA Gene Mediated by the *SIR* Genes in *Saccharomyces cerevisiae*

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The *SIR* genes of *Saccharomyces cerevisiae* are responsible for the position-dependent regulation of the a and α mating-type genes. Previous work by others has shown that the products of the *SIR* genes prevent the accumulation of stable transcripts of the a and α genes at *HML* and *HMR*. Results of this study establish that this regulation is a region-specific effect rather than a gene-specific effect since expression of a tRNA gene placed at *HMR* is repressed by the products of the *SIR* genes.

In a number of organisms it has been shown that the expression of a gene can be profoundly influenced by the position of that gene in the genome (21, 26, 31). However, in no case has the molecular basis of a position effect on gene expression been established. One of the more experimentally accessible examples of a position effect on gene expression is the negative regulation of the silent mating-type genes of the yeast *Saccharomyces cerevisiae* (for a review, see reference 19). The mating type of this yeast is controlled by codominant alleles of a single locus, known as *MAT*, located near the middle of chromosome III. **a** cells have the *MAT***a** allele, and α cells have the *MAT*α allele. Each *MAT* allele encodes two genes, **a**1 and **a**2 in *MAT***a** or α1 and α2 in *MAT*α, that are transcribed divergently from a central promoter region. The same genetic information that is present at *MAT***a** or *MAT*α is found at two additional loci known as *HML* and *HMR*, which are located on the left and right arms of chromosome III, respectively. Typically, *HML* contains an unexpressed copy of *MAT*α sequences (*HML*α) and *HMR* contains an unexpressed copy of *MAT***a** sequences (*HMR***a**). In addition, *HML***a** and *HMR*α alleles have been described as natural variants and have been derived in laboratory strains (2, 18; L. C. Blair, Ph.D. thesis, University of Oregon, Eugene, 1979). The DNA sequence of the genes encoded by the *MAT* locus, including their promoter regions, is identical to the sequence of these same genes at *HML* and *HMR* (3). Yet these genes are expressed at *MAT* but are not expressed at *HML* or *HMR*. The regulation of the silent mating-type genes involves both *trans*- and *cis*-acting functions (1, 13, 17, 23, 33). A mutation in any of four unlinked *SIR* genes results in the constitutive expression of the **a** and α genes at *HMR* and *HML* and has no direct effect on expression of genes at *MAT*. In addition, *cis*-dominant mutations adjacent to *HML* and *HMR* have defined sites, known as *E* and *I*, approximately 1,000 base pairs (bp) to either side of the internal promoter region that are required for *SIR*-dependent regulation of *HML* and *HMR* (1, 13). The physical basis of the *SIR* effect at *HMR* and *HML* is unknown. In principle, promoters of the **a**1, **a**2, α1, and α2 genes may have some special feature that allows them to be sensitive to the *SIR* effect. Alternatively, the *SIR* gene products may affect *HML* and *HMR* in such a way that the expression of any gene in the region would be repressed. This possibility was tested by determining whether a gene other than **a**1, **a**2, α1, or α2, when placed at *HMR***a**, could be regulated by the products of the *SIR* genes. Specifically, we determined whether expression of the tRNA gene *SUP3am* would be repressed when that gene is present at *HMR***a** and whether this repression is dependent on the products of the *SIR* genes. Because this gene is transcribed in vivo by RNA polymerase III, whereas the **a**1 and **a**2 genes normally present at *HMR* are apparently transcribed by RNA polymerase II (37), this experiment provides a stringent test of the generality of *SIR* regulation.

MATERIALS AND METHODS

Strains, media, and genetic methods. The *S. cerevisiae* strains used in this study are listed in Table 1. *Escherichia coli* MC1061 was used for the propagation of plasmids (10).

Yeast rich medium (YPD), minimal medium (YM), and sporulation medium were prepared as described previously (5). Amino acid supplements were added, when needed, at a concentration of 30 μg/ml. Hypertonic media were prepared by supplementing YPD with ethylene glycol at either 1.5 or 2.0 M, as noted.

Standard genetic manipulations were performed as described previously (29). Yeast cells were transformed by the method of Hinnen et al. (20), except that spheroplasts were prepared with lyticase, which was a gift from the laboratory of R. Scheckman. The segregation of *sir2-1*, *sir3-8*, and *sir4-9* was followed by determining their effect on mating phenotype. *sir1-1* segregation was monitored by determining its effect on sporulation as described previously (33).

Plasmids and plasmid constructions. The plasmid pJR1 consists of the *HMR***a** *Hin*dIII fragment in YRp14 (35, 44). pJR10 consists of the *MAT***a** *Hin*dIII fragment in pBR322 (7). Plasmids consisting of either the wild-type *SUC2* gene (pRB58) or the mutant allele *suc2-215am* (pRB55) in YEp24 were obtained from M. Carlson (8, 9). The yeast *SUP3am* gene on plasmid mWJ64 was provided by R. Rothstein.

To construct the *hmra*::*SUP3am* allele, the yeast *SUP3am* gene was isolated on a 137-bp *Bam*HI fragment from mWJ64 and inserted into pJR1 at the unique *Bgl*II site of *HMR***a** (Fig. 1). An asymmetrically positioned *Sma*I site within the *SUP3am* gene and a *Xho*I site within *HMR***a** were used to determine the orientation of the insert. Plasmids with inserts in both orientations were obtained. The plasmid chosen for this study (pJR351) contains the *SUP3am* gene inserted into the 3′ end of the **a**1 gene of *HMR***a** with the orientation of transcription of the *SUP3am* gene opposite that of **a**1 (28).

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TABLE 1. Strains and crosses used in this study

Strain	Genotype or parents	Source[a]
Haploid		
JRY50	**a** *his3-532 trp1-289 ura3-52 sir4-9*	
JRY188	α *trp1am his4 ura3-52 leu2 rme1 sir3-8*	
JRY225	**a** *ade6 arg4-17 leu2 trp1am sir1-1*	
JRY547	**a** *hmra::SUP3am* (Isogenic with JRY50)	
JRY801	α *hmra::SUP3am his3-532 trp1-289 ura3-52*	
JRY802	**a** *hmra::SUP3am his3-532 trp1-289 ura3-52*	
JRY804	α *hmra::SUP3am his3-532 ura3-52 leu2-3 leu2-112 trp1-289*	
JRY805	**a** *ade6 leu2-3 leu2-112 trp1-289 sir2-1*	
JRY902	α *suc2-Δ9 trp1-289 ura3-52 sir4-9*	
JRY903	**a** *hmra::SUP3am his4-519 leu2-3 leu2-112 suc2-Δ9 ura3-52*	
JRY904	**a** *hmra::SUP3am suc2-Δ9 ura3-52*	
JRY905	**a** *hmra::SUP3am his3-532 suc2-Δ9 ura3-52 sir4-9*	
JRY906	**a** *hmra::SUP3am his3-532 his4-519 suc2-Δ9 ura3-52 sir4-9*	
JRY907	α *hmra::SUP3am his3-532 his4-519 leu2-3 leu2-112 ura3-52*	
JRY908	α *his4-519 leu2-3 leu2-112 trp1-289 suc2-Δ9 ura3-52*	
SEY2102	α *ura3-52 leu2-3 leu2-112 suc2-Δ9 his4-519*	S. Emr et al. (12)
Diploid		
XBE4	JRY225 × JRY804	
XBE6	JRY802 × JRY188	
XBE7	JRY805 × JRY801	
XRS13	JRY547 × JRY188	
XRS19	JRY547 × SEY2102	
XRS24	JRY902 × JRY906	
XRS25	JRY902 × JRY903	

[a] Unless otherwise noted, all strains were stocks from our laboratory or were constructed in the course of this study.

Replacement of *HMR*a with *hmra::SUP3am*. The *HMR*a allele of JRY50 was replaced with the *hmra::SUP3am* hybrid allele by the one-step gene replacement method (34). Specifically, the plasmid pJR351 (5 μg) was digested with *Hin*dIII, and mixed with 2 μg of intact plasmid YCp50 which contains the yeast *URA3* gene (22). This mixture was used to transform JRY50 (*trp1-289am*, *ura3-52*, and *sir4-9ts*) by coselecting for Trp^+ and Ura^+ at 34°C.

The insertion of the *SUP3am* gene into the 3′ end of the **a**1 gene alters the last codon of **a**1 and extends its open reading frame by 28 amino acids (28; R. Rothstein, personal communication). To determine whether this disruption destroyed the function of the **a**1 gene product, the mating phenotype of the segregants from XRS19 (*MAT***a**/*MAT*α *sir4-9*/*SIR4 hmra::SUP3am*/*HMR***a**) was analyzed. If the **a**1 gene product encoded by *hmra::SUP3am* is nonfunctional, all *MAT*α segregants carrying *hmra::SUP3am* will mate normally, regardless of their *SIR* genotype (expression of functional **a**1 and α2 genes is required for the nonmating phenotype of *sir* mutants). This hypothesis predicts fewer *MAT*α mating-defective segregants than *MAT***a** mating-defective segregants from XRS19. However, if the *hmra::SUP3am* allele encodes a functional **a**1 gene product, the normal mating ability of both *MAT***a** and *MAT*α segregants requires the *SIR4* genotype. Therefore, half of the segregants from XRS19 would be mating defective, and the mating defect would be evenly distributed between *MAT***a** and *MAT*α segregants (*SIR4* and *MAT* are unlinked). Twice as many *MAT***a** mating-defective segregants as *MAT*α mating-defective segregants (141 versus 70, respectively) were observed from a total of 138 tetrads. This result confirms that the **a**1 gene product of *hmra::SUP3am* is defective.

Hybridization analysis. Yeast chromosomal DNA was isolated from cells grown to mid-log phase (A_{600} = 1-2) by the method of Holm et al. (C. Holm, D. Wagner, W. Fangman, and D. Botstein, manuscript in preparation). The DNA was digested to completion with the indicated restriction enzymes, electrophoretically separated on 1% agarose gels, and transferred to nitrocellulose in the presence of 20× SSPE (3.6 M NaCl, 0.2 M sodium phosphate, 20 mM sodium EDTA [pH 7]) as described previously (39). The filters were baked for 2 h at 80°C in vacuo and prehybridized at 42°C for at least 2 h in 50% formamide–5× SSPE–BFP (0.02% [wt/vol] each of bovine serum albumin, Ficoll [molecular weight, 400,000; Pharmacia Fine Chemicals, Piscataway, N.J.] and polyvinylpyrrolidone)–0.1 mg of denatured, sonicated salmon sperm DNA per ml–1% glycine. Hybridizations to probes radiolabeled by nick translation (32) were carried out for at least 12 h at 42°C in 50% formamide–5× SSPE–BFP–0.1 mg of salmon sperm DNA per ml–0.3% sodium dodecyl sulfate (SDS). Filters were washed at least four times in 2× SSPE at room temperature for 15 min and at least once at 65°C.

Yeast RNA was isolated as follows (S. Van Arsdale, G. Stetler, and J. Thorner, submitted for publication): 50-ml cultures were grown at 34°C and harvested by centrifugation during logarithmic growth. Cells were washed with 2.5% 2-mercaptoethanol, pelleted by centrifugation, and suspended in 1 ml of LETS-SDS (0.1 M LiCl, 1 mM EDTA, 0.1 M Tris hydrochloride [pH 7.5], 0.5% SDS) plus 1% diethyl pyrocarbonate. Approximately 1.5 g of acid-washed glass beads (diameter, 0.5 mm) was added, and cells were broken by vortexing at high speed for a total of 5 min, with cooling on ice every 30 s. LETS-SDS (1 ml) containing 400 μg of proteinase K (EM Reagents) per ml was added, and the lysate was incubated at 37°C for 30 min. Phenol-$CHCl_3$-isoamyl alcohol (50:50:1; 3 ml), equilibrated with LETS, was added and vortexed, and the phases were separated by centrifugation. The aqueous phase was reextracted and adjusted to 0.1 M NaCl, and the RNA was precipitated by the addition of 2.5 volumes of ethanol. Poly $(A)^+$ RNA was isolated by oligo(dT)-cellulose chromatography with minor modifications of a previously described procedure (4). mRNA samples (10 μg) suspended in 50% formamide–6% formaldehyde–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.8]–1 mM EDTA were fractionated on a 1% agarose gel in the presence of 6% formaldehyde–50 mM HEPES (pH 7.8)–1 mM EDTA (27). After several brief washes in water, the RNA was transferred to nitrocellulose (43). Hybridizations were performed as described above.

Invertase assays. Yeast cultures grown to stationary phase at 34°C in YM plus 2% glucose supplemented with histidine, tryptophan, and leucine were diluted and grown for at least an additional 4 h at 34°C to the mid-log phase. Approximately 10^7 cells were washed once in water and suspended in 1 ml of supplemented YM plus 0.1% glucose and incubated for 2.5 to 3.5 h at 34°C to derepress invertase expression. External invertase was assayed, using whole cells as described previously (15). Activity is presented as micrograms of glucose released per minute per 10^7 cells at 37°C.

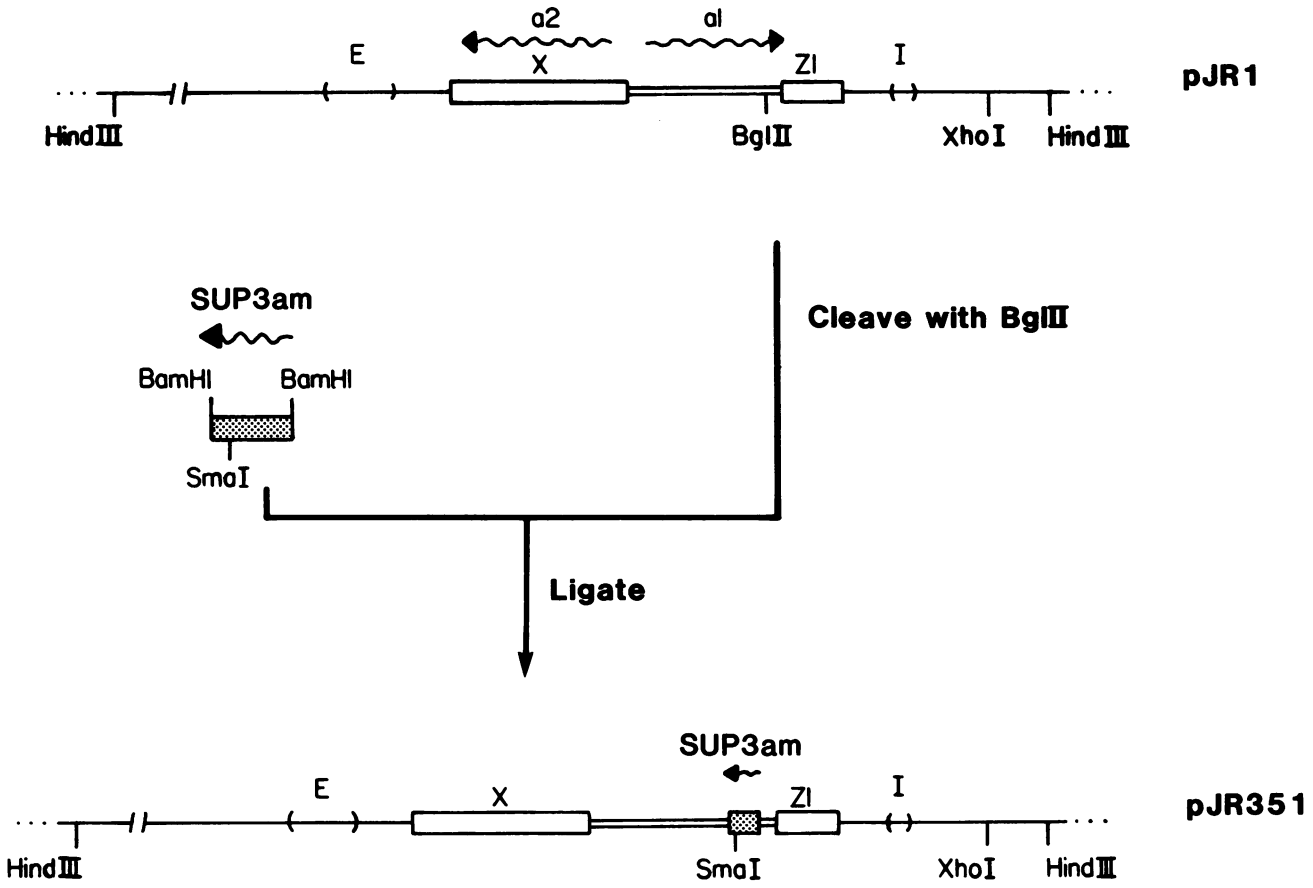

FIG. 1. Insertion of *SUP3am* into *HMR***a**. Details of the construction are given in the text. The construct is not drawn to scale.

RESULTS

Construction of a hybrid *HMR* allele. The amber suppressor tRNA gene *SUP3am* was integrated into the *HMR* locus by the one-step gene replacement scheme as described above (30, 34). Briefly, the *SUP3am* gene, present on a 137-bp *Bam*H1 fragment, was inserted into the *Bgl*II site of *HMR***a** to generate the plasmid pJR351 (Fig. 1). In this plasmid, the orientation of the tRNA gene is such that its direction of transcription is opposite the direction of transcription of the **a**1 gene into which it has been inserted. To replace the wild-type chromosomal *HMR***a** allele with the hybrid *HMR***a**::*SUP3am* allele, pJR351 was digested with *Hin*dIII to generate a fragment spanning the *SUP3am* insertion and mixed with the intact plasmid YCp50. The mixture was used to transform JRY50 coselecting for *SUP3am* expression by its ability to suppress the amber suppressible mutation *trp1-289* and for complementation of *ura3-52* by the *URA3* gene of YCp50. This cotransformation procedure avoided isolation of revertants of *trp1-289*. Transformants were selected at 34°C, the restrictive temperature for *sir4-9*, to ensure that regulation of *HMR***a** by the *SIR* gene products would not impose a selectional bias against expression of the tRNA gene at *HMR***a**.

One transformant that remained Trp^+ after more than 10 generations of nonselective mitotic growth was chosen for further analysis. A derivative of this strain that had lost YCp50 was obtained by serial subculturing in nonselective media. This Ura^- derivative was designated JRY547. In a cross with JRY188 (XRS13) the Trp^+ phenotype of JRY547 segregated 2+:2−, was linked slightly to *MAT* (PD:T:NPD = 11:35:4), and showed no linkage to *LEU2* which is on the left arm of chromosome III (PD:T:NPD = 10:29:11). From the results of this cross we conclude that the *SUP3am* gene integrated at only one site within the genome and that this site is located on the right arm of chromosome III.

The integration of the hybrid *hmr***a**::*SUP3am* allele at *HMR* was confirmed by a gel transfer-hybridization experiment. Chromosomal DNA was digested to completion with restriction enzymes, separated on an agarose gel, transferred to nitrocellulose, and probed with a radiolabeled plasmid consisting of *MAT***a** in pBR322 (pJR10; Fig. 2). In all strains carrying the integrated allele, we observed the appearance of a 1-kilobase-pair *Sma*I-*Xho*I fragment and an increase in the size of the *HMR Hin*dIII fragment by approximately 100 bp. These changes are diagnostic of the replacement of the wild-type *HMR* allele by the new hybrid allele (Fig. 2).

Insertion of *SUP3am* into *HMR*a does not disrupt regulation of *HMR*a by the products of the *SIR* genes. Insertions of additional sequences into *HMR***a** or *HML*α can eliminate or reduce the ability of the *SIR* gene products to regulate these loci. For example, a duplication of sequences within *HMR***a** that increases the overall distance between *E* and *I* by 370 bp results in partial loss of repression. In contrast, deletions within *HMR***a** have no adverse effect on regulation (1, 13). Together, these results suggest that there is an upper limit to the distance between the *E* and *I* sites that will allow repression of *HMR***a** expression, although the exact distance between *E* and *I* can vary. A comparison of the naturally occurring wild-type *HMR* alleles (*HMR***a** and *HMR*α) sug-

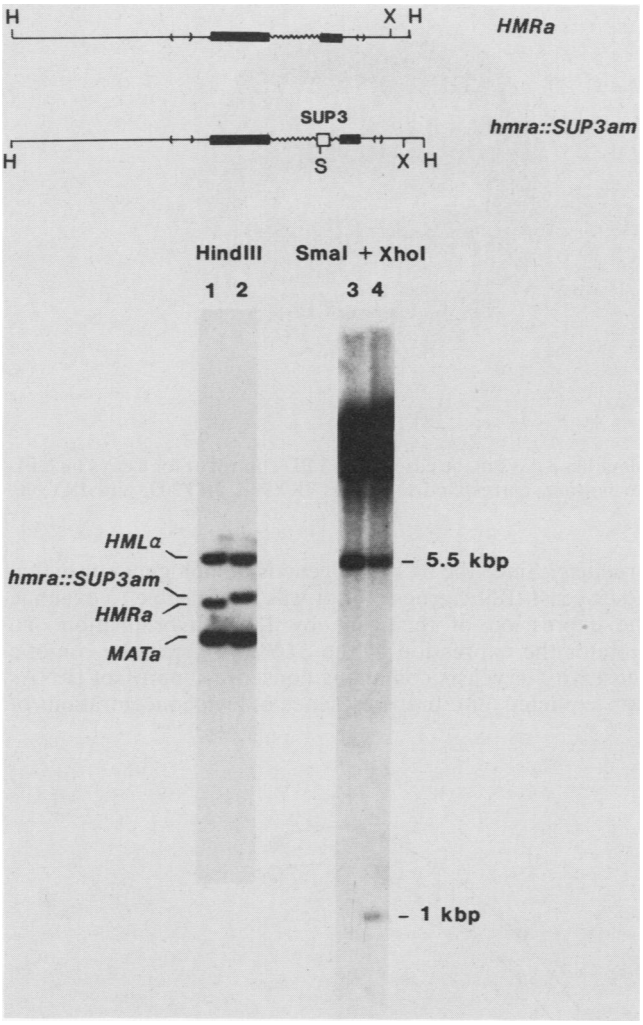

FIG. 2. Genomic DNA blot hybridization analysis. DNA from parental strain JRY50 (lanes 1 and 3) or from JRY906 (lanes 2 and 4) was digested with *Hin*dIII (lanes 1 and 2) or *Sma*I-*Xho*I (lanes 3 and 4). Fragments were electrophoretically separated on 1% agarose gels and transferred to nitrocellulose filters. The filters were hybridized to ^{32}P-labeled pBR322 containing the *MAT***a** *Hin*dIII fragment (pJR10). The appearance of the 1-kilobase pair (kbp) *Sma*I-*Xho*I fragment in lane 4 is diagnostic of the replacement of the wild-type *HMR* allele. The other portion of the *hmr***a**::*SUP3am* locus expected in lane 4 comigrated with the mixture of *MAT* and *HML* fragments that make up the higher molecular weight hybridizing bands. Abbreviations: H, *Hin*dIII; X, *Xho*I; S, *Sma*I.

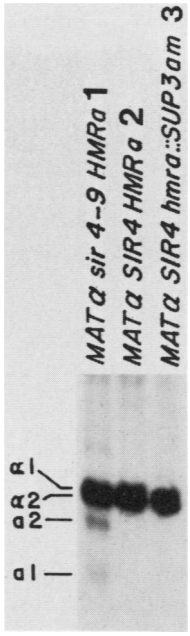

FIG. 4. RNA blot hybridization analysis. Poly(A)$^+$ RNA isolated from strains grown at 34°C was electrophoretically separated on a 1% agarose gel in the presence of formaldehyde (10 μg per lane), transferred to nitrocellulose, and hybridized with ^{32}P-labeled pBR322 containing the *MAT***a** *Hin*dIII fragment (pJR10). RNA is from strains JRY902 (*MATα sir4-9 HMR***a**; lane 1), JRY908 (*MATα SIR4 HMR***a**; lane 2), and JRY907 (*MATα SIR4 hmr***a**::*SUP3am*; lane 3).

gests that there is at least some upward flexibility in the *E*-to-*I* site distance at *HMR***a** that will still allow proper regulation (Fig. 3). *HMR***a** and *HMRα* differ only in their Y sequences (3, 41), resulting in an *E*-to-*I* distance of *HMRα* which is 105 bp greater than that of *HMR***a**. Since both of these alleles are repressed, regulation of the hybrid *hmr***a**::*SUP3am* allele, with an *E*-to-*I* site distance only 32 bp greater than that of *HMRα*, should remain functional. To test this expectation, the level of mRNAs expressed from the *hmr***a**::*SUP3am* allele was examined (Fig. 4). The level of α1 and α2 RNAs present in each lane serves as an internal control for the loading of equivalent amounts of RNA per lane. Lane 1 shows the unregulated level of **a**1 and **a**2 RNA expressed from *HMR***a** in a *sir4* strain. Lane 2, containing RNA from an *HMR***a** *SIR4* strain, demonstrates that neither the **a**1 nor the **a**2 transcript is detectable when *HMR* is repressed. The RNA in lane 3 is also from a *SIR4* strain which contains the hybrid *hmr***a**::*SUP3am* allele. Although

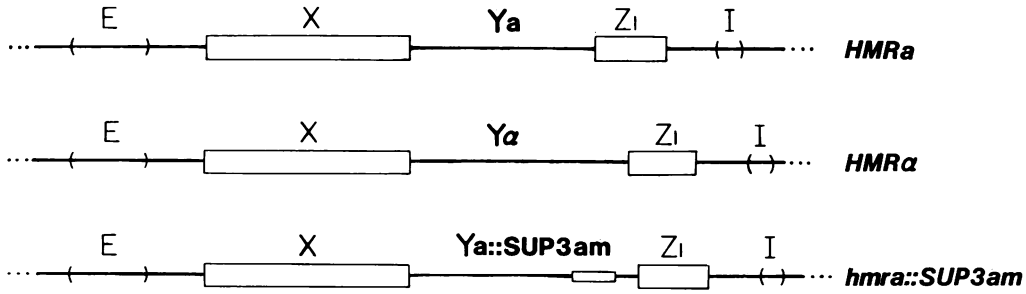

FIG. 3. Comparison of *HMR* alleles indicates that the hybrid *hmr***a**::*SUP3am* allele is approximately the same size as the naturally occurring *HMRα* allele. Specifically, the length of Y**a** is 642 bp, Yα is 747 bp, and Y**a**::*SUP3am* is 759 bp.

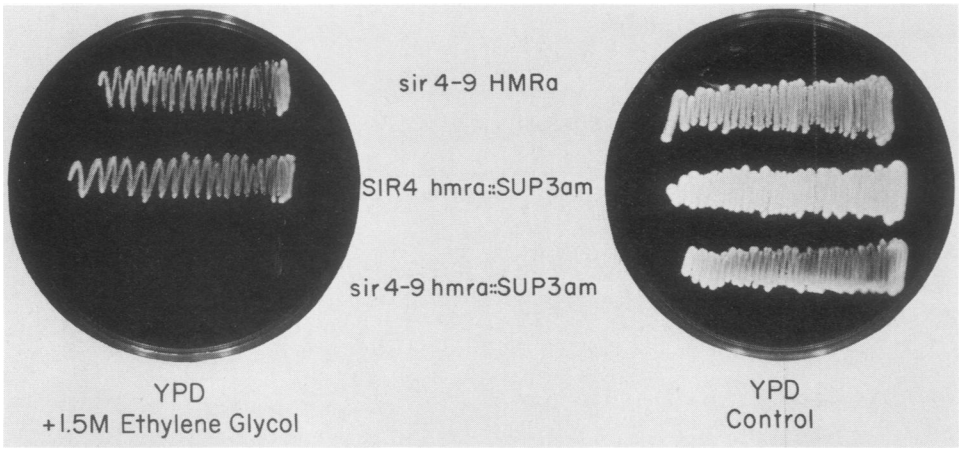

FIG. 5. Growth on hyperosmotic media. Cells were incubated at 34°C for either 3 days in the case of the YPD control or for 6 days on YPD supplemented with 1.5 M ethylene glycol. Genotypes listed, from top to bottom, correspond to strains JRY902, JRY903, and JRY906, respectively.

the stability of the hybrid transcript produced from the **a**1 promoter cannot be predicted, the absence of any detectable **a**2 transcript verifies that the locus is still repressed. Thus, insertion of 137 bp into the **a**1 gene of *HMR***a** does not disrupt the ability of this locus to be regulated.

Is *SUP3am* at *HMR* under the control of the *SIR* gene products? Since the *SUP3am* gene is homologous to several other yeast tRNA genes (16), it was not feasible to examine the expression of this gene by RNA hybridization. To monitor the expression of the *SUP3am* gene, the osmotic sensitivity of yeasts containing nonsense suppressor tRNAs was exploited (38). In the presence of high concentrations of

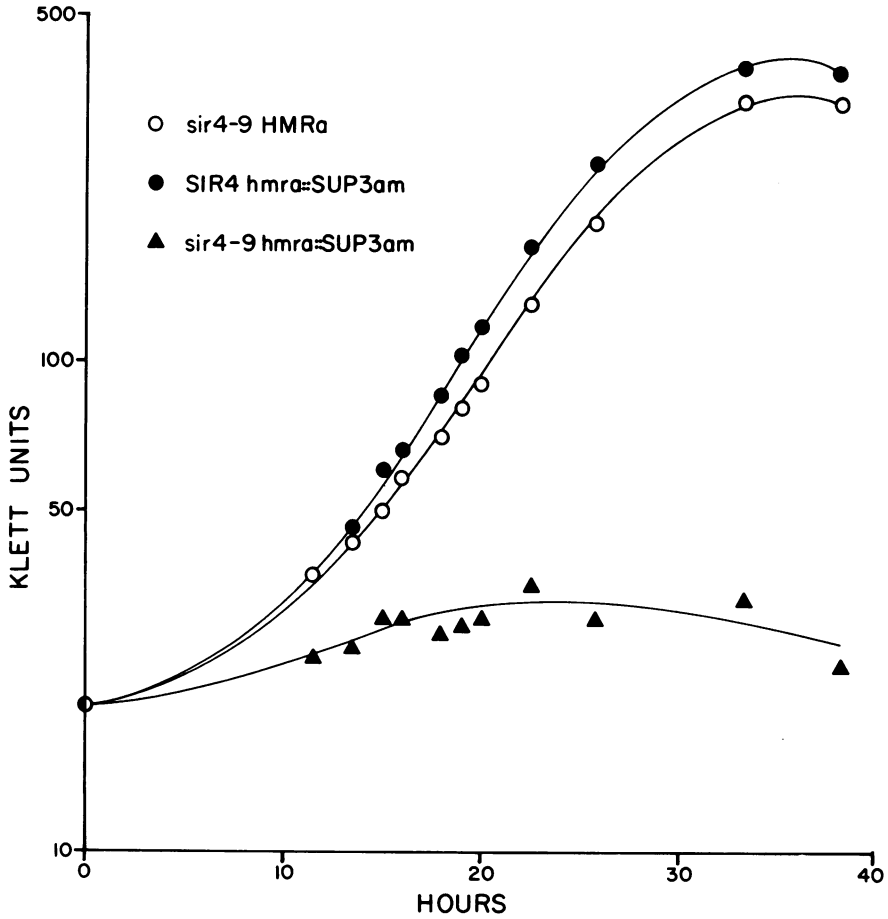

FIG. 6. Growth in YPD broth containing 2 M ethylene glycol at 34°C. All three strains showed normal growth in YPD. JRY902 (*sir4-9 HMR***a**); JRY903 (*SIR4 hmr***a**::*SUP3am*); JRY906, (*sir4-9 hmr***a**::*SUP3am*).

TABLE 2. Effect of the *SIR4* gene on the level of expression of an amber suppressor tRNA gene at *HMRa*

Strain	Genotype	Invertase activity[a]			% suppression[b]	Mean % suppression ± SD	Fold suppression
		Trial	pRB55 (*suc2-215am*)	pRB58 (*SUC2*$^+$)			
JRY902	*sir4-9 HMRa*	1	1.15	550	0.21		
		2	1.0	206	0.49		
		3	0.71	283	0.25	0.32 ± 0.15	1.0
JRY903	*SIR4 hmra::SUP3am*	1	9.5	1,458	0.65		
		2	1.62	196	0.83		
		3	3.52	654	0.54	0.67 ± 0.15	2.1
JRY906	*sir4-9 hmra::SUP3am*	1	92.9	1,070	8.7		
		2	17.8	168	10.6		
		3	73.3	690	10.6	9.97 ± 1.1	31.1

[a] Prior to harvesting, cells were derepressed in medium with a low glucose concentration at 34°C for 2.5 h in trials 1 and 2 and for 3.5 h in trial 3. Activity is expressed as micrograms of glucose formed per minute per optical density of cells at 600 nm.

[b] The fraction of invertase activity from cells transformed with the *suc2-215am* allele (pRB55) relative to that from the same strain transformed with the wild-type *SUC2* gene (pRB58), multiplied by 100.

ethylene glycol, strains that express the *SUP3am* gene should grow more poorly than strains in which little or no suppressor tRNA is produced.

On solid or in liquid media containing ethylene glycol (Fig. 5 and 6), a control strain with no amber suppressor gene grew, albeit more slowly than on conventional media. A *sir4-9* strain carrying the hybrid *HMR* allele (JRY906) could not grow at all at 34°C in the presence of ethylene glycol since it was rendered osmotically sensitive by expression of the *SUP3am* gene at *HMR*. However, a *SIR*$^+$ strain containing the hybrid allele (JRY903) grew as well as the positive control strain (JRY902). These results indicate that wild-type *SIR4* function suppresses the osmotic sensitivity caused by expression of the *SUP3am* gene. Thus, expression of a tRNA gene at *HMR* is under the negative control of the *SIR4* gene product.

We determined the magnitude of *SIR4* repression of the *SUP3am* gene by quantitating the level of suppressor tRNA in *sir4* and *SIR4* strains. In this assay, the level of activity of an enzyme that is encoded by a gene containing an amber suppressible mutation serves to reflect the level of amber suppressor tRNA in the cell. For this experiment, the level of invertase activity encoded by an amber allele of *SUC2* [*suc2-215am*(9)] was determined. Plasmids carrying either the *suc2-215am* allele or the wild-type *SUC2* gene were introduced by transformation into each of the yeast strains listed in Table 2, each of which contained a deletion of the chromosomal *SUC2* gene; and the level of invertase activity produced in each strain was measured. The ratio of invertase activity in cells with the *suc2-215am* allele to the activity in the same cells containing the wild-type *SUC2* allele is a measure of the relative amount of amber suppressor tRNA present in each cell type. This ratio, when multiplied by 100, is defined as percent suppression. The slight amount of suppression observed in the control strain (JRY902) with no amber suppressor gene established the background level of this assay (0.32 ± 0.15%). The level of suppression in a *SIR*$^+$ strain with *SUP3am* at *HMR* (JRY903) is slightly higher (0.67 ± 0.15%). However, in the *sir4-9* strain (JRY906), the level of amber suppressor activity expressed from *hmra::SUP3am* is much higher (9.97 ± 1.1%) and is comparable to the level of suppression expected for a normally expressed suppressor tRNA gene (14, 36). Thus, in the absence of wild-type *SIR4* function, the amount of *SUP3am* tRNA expressed is 15-fold higher than in the corresponding *SIR4* strain. Although the extent of derepression varied considerably between different trials, the percent suppression did not. This result establishes that the differences in *SUP3am* expression between these strains is not due to unequal extents of derepression. Furthermore, similar results were obtained with additional strains of each *SIR4* genotype (data not shown). These results strongly argue that the repression of *SUP3am* expression at *HMRa* is due to the action of the *SIR4* gene product and is not the result of other, unrelated differences between strains.

TABLE 3. Effect of the *SIR4* gene on the level of expression of the *SUP3am* gene at *HMRa* in an a/α diploid

Strain	Genotype	Invertase activity[a]		% suppression[b]
		pRB55 (*suc2-215am*)	pRB58 (*SUC2*$^+$)	
XRS25	*MATa/MATα SIR4/sir4-9 hmra::SUP3am/HMRa*	0.2	492	0.04
XRS24	*MATa/MATα sir4-9/sir4-9 hmra::SUP3am/HMRa*	18	566	3.2

[a] Cells were grown at 30°C and derepressed in medium with a low glucose concentration at 30°C for 3.5 h. Values represent averages of at least three separate activity determinations.

[b] As defined in footnote *b* of Table 2.

Regulation of *hmra::SUP3am* parallels that of the silent mating-type genes. If the regulation of *SUP3am* at *HMRa* accurately reflects the effect of the *SIR* gene products on *HMRa*, then regulation of the *SUP3am* gene at this locus should require all four *SIR* genes. To test this hypothesis, strains carrying a mutation in each of three other *SIR* genes were crossed to a strain carrying the hybrid *hmra::SUP3am* allele (crosses XBE4, XBE6, and XBE7). For each of the segregants, ethylene glycol sensitivity was assayed as an indicator of the level of *SUP3am* expression. Suppression of *trp1am* was used to follow the segregation of the hybrid allele because even in Sir$^+$ strains expression of the *hmra::SUP3am* allele is sufficient to provide tryptophan prototrophy. In the case of *sir2-1* and *sir3-8*, as for *sir4-9*, the osmotic sensitivity conferred by the hybrid allele always cosegregated with the mutant *SIR* allele (a minimum of 15 tetrads were tested from each cross). The *sir1-1* mutation did not confer osmotic sensitivity. This last result is not surprising since even the mating phenotype of the *sir1-1* mutant is leaky, (33; data not shown). These results demonstrate that regulation of *SUP3am* at *HMRa* requires the combined action of multiple *SIR* genes, as does regulation of the silent mating-type genes themselves.

Are the silent cassettes expressed in an a/α diploid? The *hmra*::*SUP3am* allele was used to resolve the question of whether *HMR***a** and *HML*α are turned on after an **a**/α diploid is formed. This hypothesis would have some biological appeal since an increase in the level of **a**1 and α2 expression might facilitate sporulation. Sir^+ and Sir^- diploid strains containing the *hmra*::*SUP3am* allele were constructed by mating the *MAT***a** strains JRY903 and JRY906, respectively, with the *MAT*α strain JRY902. The resulting diploids XRS24 and XRS25 were isolated by prototroph selection and transformed with plasmids carrying either the *suc2-215am* allele or the wild-type *SUC2* gene. To quantitate the level of *SUP3am* tRNA expressed from *hmra*::*SUP3am*, the ratio of invertase activity produced by cells with the *suc2-215am* allele to the activity produced by the same cells with the wild-type *SUC2* gene was determined. The level of *SUP3am* tRNA expressed in the Sir^+ diploid is 80-fold lower than in the Sir^- diploid (Table 3). This result demonstrates that *SIR* repression of the silent cassettes is fully maintained in an **a**/α diploid.

DISCUSSION

In the experiments presented here, our understanding of the generality of *SIR*-dependent regulation of genes at *HML* and *HMR* has been extended by the demonstration that insertion of the *SUP3am* gene into *HMR***a** results in repression of *SUP3am* expression by the products of the *SIR* genes. At least three of the *SIR* genes are required for this repression. This result indicates that *SIR*-dependent repression of *HMR***a** is not specific to a particular class of genes or promoters. The tRNA gene at *HMR***a** is in the opposite transcriptional orientation as the **a**1 gene into which it was inserted. Therefore, the regulation of *SUP3am* cannot be explained as being a consequence of the processing of a mature tRNA from an RNA polymerase II transcript that is normally repressed by the *SIR* gene products. Because *SUP3am* is not regulated by the *SIR* gene products at its normal chromosomal location nor on a centromere plasmid, and because its repression at *HMR***a** requires the products of at least three *SIR* genes, we conclude that the regulation of *SUP3am* at *HMR***a** is an accurate reflection of *SIR*-dependent regulation. Although the **a**1, **a**2, α1, and α2 promoters are located near the middle of the regulated region, the *SUP3am* gene and hence its promoter are not. Therefore, it is unlikely that there is any precise requirement for the position of a promoter between the *E* site and the *I* site for that promoter to be regulated by *SIR*.

In *SIR* cells, the *SUP3am* gene at *HMR***a** is expressed at a low level since cells containing an amber suppressible allele of *TRP1* (*trp1-289*) are tryptophan prototrophs. We cannot distinguish between the possibility that the silent mating-type genes are also expressed at a low level that is not detectable by our assays or that *SIR* is less effective at repressing *SUP3am* expression than it is at repressing **a**1, **a**2, α1, and α2 expression at *HML* and *HMR*. We attempted to increase the level of repression of the *hmra*::*SUP3am* locus by increasing the copy number of each *SIR* gene through the use of multicopy plasmid vectors containing each of the *SIR* genes. None of the plasmids was able to decrease expression of the *SUP3am* gene to the point that the *trp1-289* mutation was not suppressed (unpublished data). These results suggest that no single *SIR* product is limiting for regulation.

The effect of the *SIR* gene products on *HML* and *HMR* is not limited to transcriptional regulation. The Y-Z endonuclease cleaves at a sequence present at *MAT* but does not cleave the same sequence at *HML* and *HMR* in Sir^+ strains. In Sir^- strains, this sequence is cleaved at *HML* and *HMR* as well as at *MAT* (24, 25). Thus, the *SIR* gene products control the ability of a site-specific endonuclease to cleave its target sequence. The effect of the *SIR* gene products at *HML* and *HMR* can be viewed as a block to sequence-specific DNA-protein interactions in general. According to this model, *HML* and *HMR* are regions of genetically inert DNA, perhaps analogous to heterochromatin, in which the DNA is not accessible for recognition by sequence-specific proteins.

Additional examples of regional influences on the level of gene expression have been described in several other organisms. For instance, position effect variegation in *Drosophila melanogaster* occurs when a translocation places a euchromatic locus next to a heterochromatic region, resulting in the partial suppression of the euchromatic gene (42). In female eutherian mammals, the random inactivation of one X chromosome during development is accompanied by condensation of that chromosome to a heterochromatic condition. Evidence that this type of repression is able to influence the expression of autosomal genes positioned on the X chromosome comes from studies of a translocation in which a portion of the mouse autosome 7 has been inserted into the X chromosome. In those cells in which the X chromosome containing the insertion is inactivated, there is a coordinate inactivation of the adjacent autosomal genes (11). Regional influences can also act positively to increase the level of gene expression. For example, in *D. melanogaster*, the expression of genes located on the X chromosome is regulated to compensate for the unequal number of X chromosomes in males and females. When the autosomal gene $rosy^+$ was inserted into the X chromosome by transformation, the gene became hyperexpressed in males relative to females (40). Thus, autosomal genes inserted into the X chromosome fall under the control of the dosage compensation signals which regulate the level of chromosome X gene expression. Apparently, dosage compensation can even regulate the expression of tRNA genes that reside on X chromosomes (6). The position-dependent repression of the *SUP3am* gene at *HMR***a** may provide an opportunity to discover one mechanism by which regional control of gene expression is exerted.

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