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The *SIR1* Gene of *Saccharomyces cerevisiae* and Its Role as an Extragenic Suppressor of Several Mating-Defective Mutants

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The *SIR1* gene product of *Saccharomyces cerevisiae* is one of several proteins involved in repressing transcription of the silent mating-type genes. Strains with mutations in the genes coding for these proteins are defective in mating due to derepression of the silent loci. We have found that overexpression of the *SIR1* gene suppresses the mating defects of several of these mutants, including *nat1* and *ard1* mutants (the products of these two genes are responsible for N-terminal acetylation of a subset of yeast proteins), certain *sir3* mutants, and a histone H4 mutant. The *SIR1* gene has been sequenced and found to contain an open reading frame coding for a 678-amino-acid protein.

Cell type in the budding yeast *Saccharomyces cerevisiae* is determined by the genetic information present at the *MAT* locus on chromosome III. The **a** or α haploid cell types contain the *MAT***a** or the *MAT*α locus, respectively. Cells of opposite mating type can mate with each other to form a third cell type, the *MAT***a**/*MAT*α diploid, that cannot mate. There are two additional mating-type loci on chromosome III, *HML*α and *HMR***a**, that play a role in mating-type interconversion (10, 29). *HML* and *HMR* are called the silent loci because they are transcriptionally inactive. If the silent loci become derepressed due to a mutation that disrupts silencing, the genotypically haploid cells behave like diploids in that they can no longer mate due to the presence of both **a** and α mating-type information in the same cell. Thus, regulation of expression of the silent mating-type genes is important for maintaining the integrity of cell type-specific gene expression.

HML and *HMR* contain the same transcription units and promoters that are present at *MAT*. Transcription of the mating-type genes at the silent loci is repressed, however, by *cis*-acting sequences known as silencers that are present as far as 1 kb upstream and downstream of both *HML* and *HMR* (1, 4, 9). Silencers are analogous to transcriptional enhancers in that both are capable of acting in a distance- and orientation-independent fashion and of acting on heterologous genes (4, 16). Of course, silencers are different from enhancers in that they function in transcriptional repression rather than in transcriptional activation.

In addition to the *cis*-acting silencer elements, a number of *trans*-acting gene products are required for silencing. Some of these gene products are the four SIR proteins (11, 23, 24); NAT1 (20) and ARD1 (30), two proteins required for an N-terminal acetyltransferase activity (20); histone H4 (13); and probably RAP1 and ABF1, two abundant DNA-binding proteins (7, 28) essential for viability in yeast cells (8, 26). Mutations in any one of the four *SIR* genes lead to a mating defect in **a** or α cells. *HML* and *HMR* are completely transcriptionally active in *sir2*, *sir3*, and *sir4* mutants; these mutants therefore have a nonmating phenotype. The *sir1* mutant is also derepressed at the silent loci, though to a much lesser extent than the other *sir* mutants, leading to a weak mating-defective phenotype (22, 23). *nat1* and *ard1* mutants are also partially derepressed at the silent loci, particularly at *HML*α, leading to a partial mating defect in *MAT***a** strains. *nat1 ard1* double mutants have no greater mating defect than mutants carrying either single mutation. In fact, the *nat1 ard1* double and single mutants have identical phenotypes in all respects. For these reasons and others, Mullen et al. concluded that NAT1 and ARD1 are subunits of a multimeric N-terminal acetyltransferase responsible for the N-terminal acetylation of a number of proteins in the cell (20). Thus, the mating-defective phenotype of *nat1 ard1* mutants is presumably due to the lack of N-terminal acetylation of some protein whose acetylation is important for functioning in silencing.

The work described in this article began with an interest in uncovering the protein that must be N-terminally acetylated by NAT1 and ARD1 in order for full silencing to occur. A search was made for extragenic suppressors of the *nat1 ard1* mating defect. The *SIR1* gene, when overexpressed, was found to be such a suppressor. Overexpression of *SIR1* suppresses not only the *nat1 ard1* mating defect but also the mating defects of certain *sir3* mutants and of a histone H4 mutant with an N-terminal deletion. Here we report the sequence of the *SIR1* gene and discuss possible models for the role of SIR1 in silencing.

MATERIALS AND METHODS

Yeast strains and media. The genotype and source of the *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cells were grown at 30°C in YPD (25) unless selective pressure was required to maintain a plasmid, in which case the strains were grown in supplemented minimal medium (25).

Plasmids and yeast transformations. A multicopy genomic library, containing *Sau*3AI partial genomic fragments cloned into the 2μm-based *LEU2* vector YEpM4, was given to us by S. Powers and M. Wigler. The *SIR1* gene was cloned from

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TABLE 1. Yeast strains

Strain	Relevant genotype	Source or reference
W303-1a	*MATa ura3 leu2 ade2 his3 trp1 can1-100*	R. Rothstein
W303-1b	W303-1a *MATα*	R. Rothstein
AMR1	W303-1a *nat1-5::LEU2*	20
AMR6	*MATa leu2 ura3 nat1-3::URA3 ade2 (ade1?) trp1 lys2 his3 his7*	19
AMR7	W303-1a *nat1-3::URA3*	19
YAB102	W303-1b *ΔEhmre*	5
AMR35	YAB102 *sir1-21.4::LEU2*	This study
RTY-2c	*MATα ΔEhmre leu2 ura3 nat1-3::URA3 his3 ard1::HIS3 lys2 ade2 trp1 can1-100*	This study
RS549	W303-1b *hmrΔ77-144::TRP1 sir1*	18
YDS71	W303-1b *sir2::TRP1*	27
YDS10	*MATa leu2 trp1 gal2 prb1-111 pep4-3 prc1-407 ura3-52 sir3::LEU2*	28
YDS73	W303-1b *sir3::LEU2*	27
YDS27	*MATa leu2 trp1 ura3-52 prb1-1112 pep4-3 prc1-407 gal2 sir4::LEU2*	D. Shore
381-11-1G,91a	*MATa sir3-8 sup4-3 cry1 his4 trp1 ade2 tyr1 lys2*	S. Fields
T81A	*MATa his3 ard1::HIS3 leu2 can1*	31
T81A-6c	*MATa his3 ard1::HIS3 leu2 ura3 can1-100*	J. Mullen
YJG10	*MATa ade2-101 his3Δ200 leu2-3,112 lys2-801 trp1Δ901 ura3-52 hhf1::HIS3 hhf2::LEU2*[*URA3* plasmid with *hhf2Δ(4-23)*]	M. Grunstein
JM2	*MATα ade2 his3 leu2 trp1 ura3 hhf1::HIS3 hhf2::LEU2 (can1?)*[*URA3* plasmid with *hhf2Δ(4-23)*]	J. Mullen
RTY-1c	*MATα ura3 nat1-3::URA3 his3 ard1::HIS3 lys2 leu2 trp1 can1-100*	This study
216	*MATa his1*	K. Nasmyth
217	*MATα his1*	K. Nasmyth
AMR27	W303-1a *sir1-23.2::LEU2*	This study
AMR30	W303-1b *sir1::URA3*	This study
AMR31	W303-1a *sir1::URA3*	This study
YDS76	W303-1a *sir3::SUP4-0*	D. Shore
RS862	W303-1a *sir3::TRP1*	This study
JRY1303	*MATa ade2-101 his3Δ200 lys2-801 ura3-52 met sir3::LYS2*	J. Rine

this library as an extragenic suppressor on the plasmid pSUP1, which contains an approximately 8.5-kb genomic insert. An approximately 5.5-kb *Hin*dIII fragment located downstream of *SIR1* was deleted from pSUP1 to make pES11. pES11 contains the *SIR1* gene on a 2.9-kb *Bam*HI-*Hin*dIII fragment. pES13B contains the same 2.9-kb *Bam*HI-*Hin*dIII *SIR1* fragment in a 2μm-based *URA3* vector (YEp352). pES13 contains a 2.2-kb *Pst*I-*Hin*dIII *SIR1* fragment in YEp352. pES13 lacks the first 135 codons of the *SIR1* gene. pES14 was constructed by inserting the 2.9-kb *Bam*HI-*Hin*dIII *SIR1* fragment into the vector YCp50L. YCp50L was made by inserting the *Xho*I-*Pst*I *LEU2* fragment from YEp13 into the *Sal*I-*Nsi*I sites of YCp50, thereby disrupting the *URA3* gene of YCp50. A frameshift mutation was created in the *SIR1* gene by filling in the *Acc*I site in pES14 to make pES21. pKL1 contains the 2.9-kb *SIR1* fragment on a 2μm-based *TRP1* vector (pYSK102, given to us by Wai-kwong Eng). p16-2 is a 2μm-based *LEU2* plasmid that contains the *SIR2* gene (from A. Klar). pJR104 and pJR643 contain the *SIR3* and the *SIR4* genes, respectively, in 2μm-based *URA3* vectors (provided by L. Pillus).

The 2.2-kb *Pst*I-*Hin*dIII *SIR1* fragment was cloned into M13mp18 and M13mp19 (New England BioLabs) to make pES18 and pES19, respectively. The 0.7-kb *Bam*HI-*Pst*I *SIR1* fragment was cloned into M13mp18 and M13mp19 to make ARp18 and ARp19, respectively.

The following *SIR1* fragments were inserted into the pSK$^+$ vector (Stratagene) for making riboprobes (also see Fig. 2A): the 0.7-kb *Bam*HI-*Pst*I fragment, pES22; the 1.1-kb *Pst*I-*Bam*HI fragment, pES16; the 0.6-kb *Pst*I-*Bgl*II fragment, pKL4; the 1.1-kb *Bam*HI-*Hin*dIII fragment, pES23; and the 0.5-kb *Xho*I-*Hin*dIII fragment, pES25.

Yeast transformations were done by standard spheroplasting or lithium acetate methods (25).

Null mutations and gene replacement. The *sir1::URA3* null mutation was created by deleting an *Acc*I-*Xho*I fragment of the *SIR1* gene, which removes the entire *SIR1* coding region with the exception of the first 40 codons. The *Acc*I and *Xho*I sites were filled in with Klenow fragment, and a blunt-ended *URA3* fragment was inserted to create plasmid pES17, carrying the null mutation. The plasmid was cut with *Bam*HI and *Hin*dIII to yield the linear fragment used for the gene replacement. The *sir1-21.4::LEU2* and *sir1-23.2::LEU2* disruptions were made with the use of the plasmids pJI21.4 and pJI23.2, respectively (11). The *sir3::TRP1* null mutation was made by deleting an *Hpa*I-*Eco*RI fragment from the *SIR3* gene and inserting a 0.8-kb *Stu*I-*Eco*RI *TRP1* fragment to create plasmid pKL12. The only remaining *SIR3* coding sequences in this mutation are the final eight C-terminal amino acids. Plasmid pKL12 was cut with *Sal*I and *Xho*II to yield the linear fragment used for the gene replacement. All of these *sir1* and *sir3* disruption mutations were introduced into the chromosome by the one-step gene replacement technique (25).

Phenotype tests and screening for suppressors. α-Factor sensitivity tests and quantitative mating tests were performed as described before (20). α-Factor sensitivity was quantitated by determining the fraction of cells that formed mating structures (shmoos). α-Factor sensitivity tests were used to determine the suppression of *nat1* mating defects as well as complementation of *sir1* mutants. A population of *MATa sir1* mutant cells display a partial α-factor response phenotype (22). Complementation of this mutant phenotype is demonstrated by return of complete response to such a

population of cells. We also measured *sir1* complementation by transforming the plasmid to be tested into strain RS549. This strain has a *sir1* mutation and is phenotypically Trp^+ due to derepression of *hmr::TRP1*. RS549 becomes phenotypically Trp^- when a plasmid carrying the *SIR1* gene complements the mutant phenotype.

Quantitative mating efficiency is expressed as number of cells that mated per total number of cells. Qualitative mating tests were performed by patch mating. A patch of the strain to be tested was made on a supplemented minimal medium plate to maintain the plasmid, grown at 30°C for 1 to 2 days, and then replica plated onto SD plates (25) containing a lawn of mating-type tester strain 216 or 217. The replica plates were then scored for mating by the growth or absence of diploids. All replica plates were also incubated at 30°C except those containing strains with the temperature-sensitive *sir3-8* allele. To test *SIR1* suppression of *sir3-8* strains, the master plate was incubated at 30°C and the mating-type tester replica plate was incubated at 37°C.

In order to screen for extragenic suppressors of the *nat1* mutant mating defect, the triple mutant RTY-2c was transformed by the spheroplasting method with the multicopy *LEU2* library described above. The transformation yielded approximately 2,000 transformants per plate. Each plate was divided into 8 to 12 sectors. The top agar was removed from each sector and then chopped up and mixed vigorously in sterile water by vortexing for 2 to 4 min. The transformed cells that were released from the top agar were then diluted appropriately and spread on a plate containing supplemented minimal medium that lacked leucine to give approximately 200 colonies per plate. After 2 days of growth, the plates were replica plated as described above for patch mating. Colonies that were able to mate with tester strain 216 were picked, purified, and tested again for mating ability.

Sequencing the *SIR1* gene. A series of deletions were made (with the Cyclone I Biosystem kit from International Biotechnologies, Inc.) of single-stranded DNA from the plasmids pES18, pES19, ARp18, and ARp19 described above. The original plasmids and the deletions were then sequenced by the dideoxy method with the use of a Sequenase Version 2.0 kit and protocols (United States Biochemical Corporation).

Computer search. Several computer programs were used for the *SIR1* homology search. These include WordSearch, from the Genetics Computer Group (GCG), University of Wisconsin, Biotechnology Center, 1710 University Avenue, Madison, WI 53705; and SEARCH, from the Protein Identification Resource, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, DC 20007. The data bases that were searched are listed as follows: GenBank, Release 64; EMBL, Release 23; and NBRF-protein, Release 25.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated as described previously (6). For large-scale isolation of polyadenylated [poly(A)$^+$] selected RNA, a similar protocol was used (3). Northern blot analysis was performed as described before (6). The plasmids described above for making single-stranded antisense RNA riboprobes were linearized with the following restriction enzymes: *Bam*HI for pES22 and pES23; *Pst*I for pES16 and pKL4; and *Xho*I or *Cla*I for pES25. Radioactive RNA was synthesized from these linearized plasmids with the appropriate RNA polymerase (T3 or T7).

Nucleotide sequence accession number. The sequence shown in Fig. 1 has been given GenBank accession number M38524.

TABLE 2. Effect of *SIR1* overexpression on mating efficiency

Strain	Relevant genotype	Plasmid[a]	Mating efficiency[b]
W303-1	Wild type, *MAT*α or *MAT***a**		1×10^{-1}
RTY-2c	*MAT*α *nat1 ard1* Δ*Ehmre*	YEpM4 (vector)	$\leq 2 \times 10^{-6}$
		pES11 (YEpSIR1)	1×10^{-2}
		pES14 (YCpSIR1)	2×10^{-3}
YDS10	*MAT***a** *sir3::LEU2*	YEp352 (vector)	$\leq 2 \times 10^{-5}$
		pES13B (YEpSIR1)	3×10^{-3}
		pJR104 (YEpSIR3)	6×10^{-2}
		pES13 (YEpsir1)	$\leq 1 \times 10^{-5}$
YJG10	*MAT***a** *hhf2*Δ*(4-23)*	pYSK102 (vector)	$\leq 8 \times 10^{-6}$
		pKL1 (YEpSIR1)	2×10^{-4}
JM2	*MAT*α *hhf2*Δ*(4-23)*	pYSK102 (vector)	1×10^{-2}
		pKL1 (YEpSIR1)	2×10^{-1}

[a] Descriptions of the plasmids are in parentheses. YEp plasmids are 2μm-based multicopy plasmids and are present at 10 to 60 copies per cell. YCp plasmids contain centromeric sequences and are present at 1 to 3 copies per cell.

[b] Mating efficiency was determined by quantitative mating as described in Materials and Methods. Briefly, mating efficiency is expressed as the number of cells that mated per total number of cells tested.

RESULTS

***SIR1* suppresses *nat1* and *ard1* mating defects.** In order to screen for extragenic suppressors of the *nat1 ard1* mating defect, a completely mating-defective triple-mutant strain was constructed. The triple mutant has mutations in *NAT1*, *ARD1*, and the silencer at *HMR*. None of the mutations by themselves lead to significant mating defects in *MAT*α strains (5, 20, 36). Our work was based on the discovery that a *MAT*α *ard1 hmr* silencer double mutant (that contains an 8-bp deletion of the RAP1-binding site in the E silencer at *HMR*, designated Δ*Ehmre*) is a nonmater due to complete derepression of *HMR* (3a). Subsequently it was shown that the corresponding *MAT*α *nat1* Δ*Ehmre* double mutant is also a nonmater due to complete derepression at *HMR* (18a). As expected, the *MAT*α *nat1 ard1* Δ*Ehmre* triple mutant, like the double mutants, is also a nonmater. A multicopy genomic library was introduced into this triple mutant, and transformants were screened for mating ability. The triple mutant was used to prevent recloning the *NAT1* or *ARD1* gene.

Screening of about 6,000 transformants led to the identification of two plasmids that reproducibly restored mating proficiency to the triple mutant. The two suppressing plasmids were found to be identical and to have a restriction map that matched that of the *SIR1* gene published previously (11). The plasmid complemented known *sir1* mutants, confirming that it contained the *SIR1* gene. As shown in Table 2, quantitative mating experiments revealed that the multicopy plasmid containing the *SIR1* gene (YEpSIR1) improved the mating of the triple mutant (RTY-2c) at least 5,000-fold and in fact restored mating to a value within 10-fold of that of a wild-type strain.

Since the mating defect of the triple mutant is due to more than one mutation, it was of interest to determine whether a multicopy *SIR1* plasmid could suppress the mating defect of the *nat1* or *ard1* single mutants. These mutants are partially derepressed at *HML*α, and thus in *MAT***a** strains they exhibit an α-factor resistance phenotype (20, 30). *SIR1* on a multi-

TABLE 3. Effect of *SIR1* overexpression on α-factor sensitivity

Strain	Relevant genotype	Plasmid	% Shmoos[a]
W303-1a	Wild type		90–100
AMR1	*nat1*	YEp352 (vector)	0
		pES13B (YEpSIR1)	52
		pJR104 (YEpSIR3)	0
		pJR643 (YEpSIR4)	0
		pES13 (YEpsir1)	0–5
T81A-6c	*ard1*	YEp351 (vector)	0
		pES11 (YEpSIR1)	42
		p16-2 (YEpSIR2)	0
AMR7	*nat1*	YCp50L (vector)	0
		pES11 (YEpSIR1)	50
		pES14 (YCpSIR1)	14
		pES21 (YCpsir1fs)	0
AMR27	*sir1*	YEp352 (vector)	35
		pES13B (YEpSIR1)	92
		pES13 (YEpsir1)	94
AMR31	*sir1*	YCp50L (vector)	25
		pES14 (YCpSIR1)	88
		pES21 (YCpsir1fs)	26

[a] Percentage of cells that responded to α-factor. See Materials and Methods for description of the α-factor sensitivity test.

copy plasmid (YEpSIR1) suppressed the α-factor resistance of *nat1* and *ard1* single mutants (Table 3). This *SIR1* suppression effect is specific, since multicopy *SIR2*, *SIR3*, or *SIR4* plasmids did not suppress the *nat1* or *ard1* mating defect. These suppression data also provide the information that *SIR1* suppression is not specific to either of the silent mating cassettes, since suppression can take place both in α cells (in the *MAT*α *nat1 ard1* Δ*Ehmre* triple mutant) and in **a** cells (in the *MAT***a** *nat1* or *MAT***a** *ard1* single mutants).

The *SIR1* gene was cloned into a centromere-based plasmid (YCp) to test whether only one to two extra copies of *SIR1* could cause suppression. As shown in Tables 2 and 3, the YCpSIR1 plasmid also suppressed the mating defect of the triple mutant and the *nat1* single mutant, although not as well as the multicopy YEpSIR1 plasmid.

nat1 and *ard1* mutants have a number of other phenotypes that are apparently unrelated to silencing. Many of these phenotypes can be attributed to a defect in G_0 arrest. For example, a saturated culture of a *nat1* mutant contains a high percentage of cells with buds due to an inability to arrest in an unbudded G_0 state. Also, a *nat1* homozygous diploid is defective in sporulation (20). Neither of these *nat1* phenotypes was suppressed by YEpSIR1 (the multicopy *SIR1* plasmid). The G_0 defects of a *nat1 ard1* double mutant (strain RTY-2c) also were not suppressed by YEpSIR1. Thus, *SIR1* suppression appears to be specific to silencing.

***SIR1* suppresses mating defects of certain *sir3* mutants and a histone H4 mutant.** The ability of a multicopy *SIR1* plasmid to suppress other mating-defective mutants was tested. YEpSIR1 was introduced into *sir2*, *sir3*, and *sir4* mutant strains. Although *SIR1* did not suppress the mating defects of *sir2* or *sir4* mutants, it did suppress the mating defects of certain *sir3* mutants (Tables 2 and 4). Overexpression of *SIR1* restored mating ability to strains carrying the *sir3*::*LEU2* allele or the *sir3-8* temperature-sensitive allele (Table 4). On the other hand, overexpression of *SIR1* did not suppress the mating defect of strains carrying the *sir3*::*SUP4-0* allele or the *sir3*::*LYS2* allele (the latter observation was originally made by L. Pillus [21a] and verified in our lab). None of the three *sir3* disruption mutants described above are true null mutants; all of them retain at least part of the *SIR3* coding region, as does the *sir3-8* mutant.

In order to understand why *SIR1* overexpression suppressed some of the existing *sir3* mutant alleles but not others, we constructed a *sir3* null mutation that removes the entire *SIR3* open reading frame (with the exception of eight C-terminal codons that remain 3′ of the *TRP1* insertion; see Materials and Methods). We found that overexpression of *SIR1* did not suppress the mating defects of this *sir3*::*TRP1* null mutant (Table 4). The implications of the *sir3* allele-specific suppression are discussed below.

Deletion of the N-terminal hydrophilic tail of histone H4 causes a severe mating defect due to complete derepression of the silent loci (13). For example, a histone H4 mutant in which N-terminal amino acids 4 to 23 have been deleted has no detectable mating ability in a *MAT***a** genetic background and mates poorly in a *MAT*α genetic background. Introduction of the YEpSIR1 plasmid into this histone H4 mutant restored weak mating ability in a *MAT***a** background (strain YJG10, Table 2) and also increased the mating efficiency of the corresponding *MAT*α strain, JM2 (Table 2). Johnson et al. also observed *SIR1* suppression of the mating defects of certain histone H4 mutants (12).

***SIR1* gene sequence and a *sir1* null mutant.** A 2.9-kb complementing *SIR1* fragment was sequenced as described in Materials and Methods. An open reading frame encoding a putative 678-amino-acid protein was deduced from the DNA sequence (Fig. 1). The putative SIR1 protein was compared with other known proteins in computer data bases in an effort to learn something about the function of SIR1. No significant similarity between SIR1 and any other protein was observed with several different computer programs (see Materials and Methods).

TABLE 4. Allele-specific suppression of *sir3* mutants

Strain	Relevant genotype	Plasmid	Mating[a]
YDS10	*MAT***a** *sir3*::*LEU2*	YEp352 (vector)	−
		pES13B (YEpSIR1)	+
		pJR104 (YEpSIR3)	+
YDS73	*MAT*α *sir3*::*LEU2*	YEp352 (vector)	−
		pES13B (YEpSIR1)	+
		pJR104 (YEpSIR3)	+
381-11-1G,91a	*MAT***a** *sir3-8*	pYSK102 (vector)	−[b]
		pKL1 (YEpSIR1)	+[b]
YDS76	*MAT***a** *sir3*::*SUP4-0*	YEp352 (vector)	−
		pES13B (YEpSIR1)	−
		pJR104 (YEpSIR3)	+
JRY1303	*MAT***a** *sir3*::*LYS2*	YEp352 (vector)	−
		pES13B (YEpSIR1)	−
		pJR104 (YEpSIR3)	+
RS862	*MAT***a** *sir3*::*TRP1*	YEp352 (vector)	−
		pES13B (YEpSIR1)	−
		pJR104 (YEpSIR3)	+

[a] Mating tests were done by patch mating (see Materials and Methods). Symbols: −, no mating; +, mating.
[b] Mating performed at 37°C; see Materials and Methods.

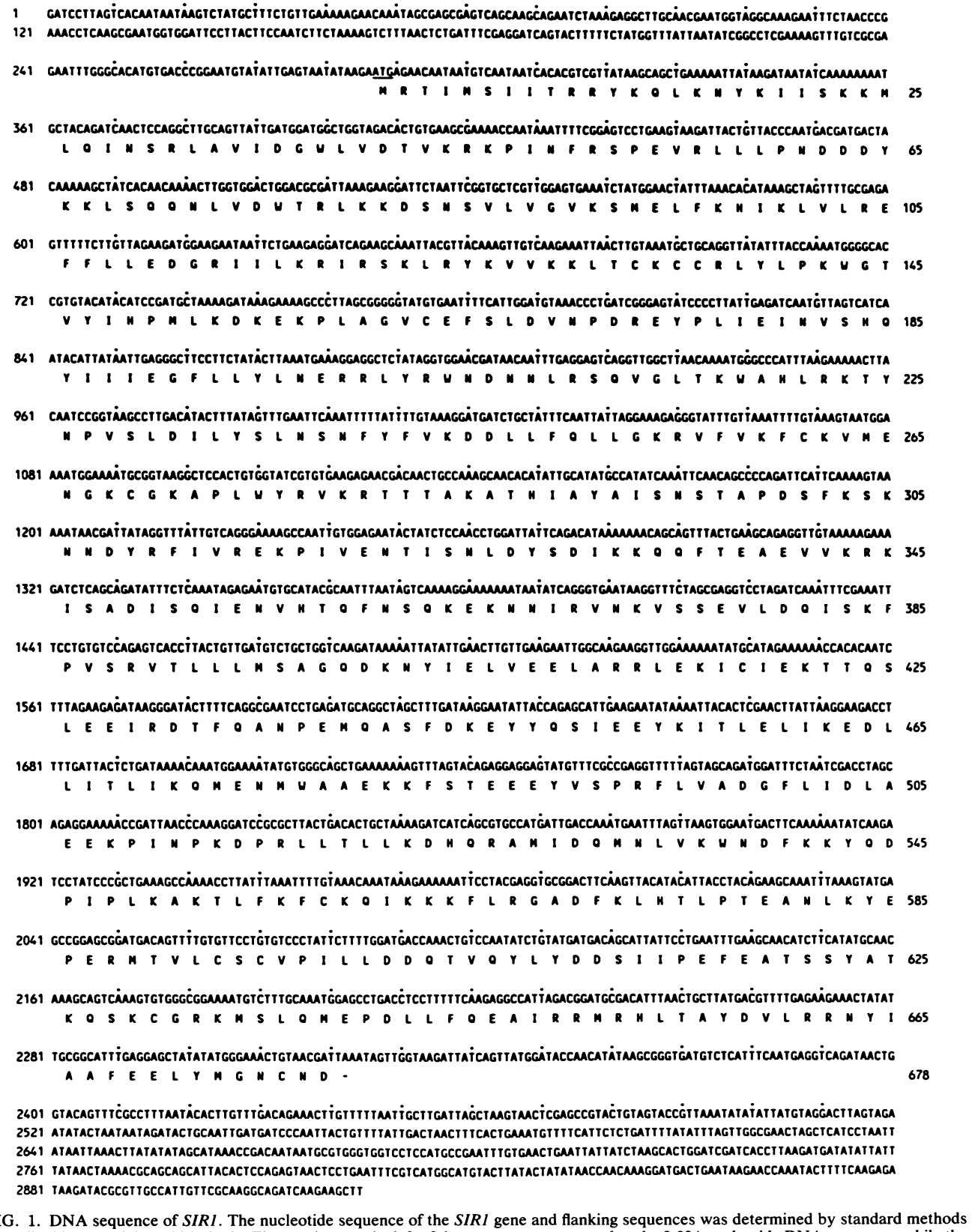

FIG. 1. DNA sequence of *SIR1*. The nucleotide sequence of the *SIR1* gene and flanking sequences was determined by standard methods as described in Materials and Methods. The numbers to the left of the sequence correspond to the 2,924-nucleotide DNA sequence, while the numbers to the right of the sequence correspond to the amino acid sequence of the putative 678-amino-acid SIR1 protein (indicated in standard one-letter code). The GenBank accession number is M38524.

A frameshift mutation was created by filling in an *Acc*I site at codon 40 of the *SIR1* open reading frame. A single-copy plasmid carrying this mutation (called YCpsir1fs in Table 3) was transformed into appropriate *sir1* or *nat1* mutant strains. Although a comparable plasmid (YCpSIR1) containing the wild-type *SIR1* gene both complemented a *sir1* mutant and suppressed a *nat1* mutant, YCpsir1fs neither complemented nor suppressed (Table 3). *SIR1* transcripts from YCpsir1fs in a strain with a *sir1* null mutation on the chromosome appeared to be identical to transcripts from YCpSIR1 in the same strain, and thus the *Acc*I site frameshift mutation does not influence transcription of the *SIR1* gene (data not shown). Therefore, the SIR1 protein must begin upstream of codon 40 of the open reading frame, and we have assumed that it begins at the methionine codon at nucleotides 287 to 289, as shown in Fig. 1. Methionine codons are also present at codons 5 and 25, and thus it is possible that the SIR1 protein is slightly shorter than indicated in Fig. 1.

Previous disruptions of the *SIR1* gene have been described (11, 14). All of these disruptions were made by insertions of selectable markers in the 3′ half of the *SIR1* coding sequence. The most extensive disruption, JI23.2 (11), involves a deletion of the 3′ half of the open reading frame and subsequent insertion of a selectable marker gene. Low levels of transcript were detected from the 5′ end of the *SIR1* gene in some of these disruptions by Northern blot analysis (data not shown). In order to determine whether deletion of the entire coding region of *SIR1* would lead to a phenotype not seen previously, we constructed a new mutation, the *sir1*::*URA3* allele (see Materials and Methods). This null mutant had no phenotype other than that seen with previous *sir1* mutants, namely, weak derepression of the silent mating-type genes.

Analysis of the two *SIR1* transcripts. Two transcripts encoded by the *SIR1* gene were reported previously (11). Northern blot analysis revealed that both transcripts disappear in the *sir1*::*URA3* null mutant (data not shown). The sizes of the two transcripts are about 1.7 and 2.4 kb, and the smaller transcript appears to be two to three times more abundant than the larger one. Northern blots of total RNA or poly(A)$^+$ selected RNA were probed with different single-stranded probes (Fig. 2). Probes for the 5′ third of the gene (pES22) and for the middle third of the gene (pES16) hybridized well to both transcripts. A probe for the 3′ end of the gene (pES23) hybridized to the larger transcript to a greater extent than to the smaller transcript (Fig. 2, lane 4). These results suggest that the two transcripts differ at their 3′ ends.

It seemed unlikely that the smaller transcript results from splicing of the longer transcript, since the sequence TAC TAAC, required for formation of the splicing branch point in yeast cells (15), is not present in the *SIR1* gene. Nevertheless, we investigated this possibility by analyzing *SIR1* RNA from an *rna2* mutant. This temperature-sensitive mutant is defective in splicing, and thus unspliced RNAs accumulate at the restrictive temperature. We found that both *SIR1* transcripts were present in a temperature-sensitive *rna2* mutant at the restrictive temperature (data not shown), and therefore the smaller transcript is not a spliced product of the larger transcript. Thus, the two transcripts must have different termination sites.

The predicted stop sites of the two transcripts in relation to the *SIR1* open reading frame have been diagrammed in Fig. 2. The smaller transcript of 1.7 kb must terminate within the open reading frame. The larger transcript is estimated to terminate between the *Xho*I and *Cla*I sites as illustrated,

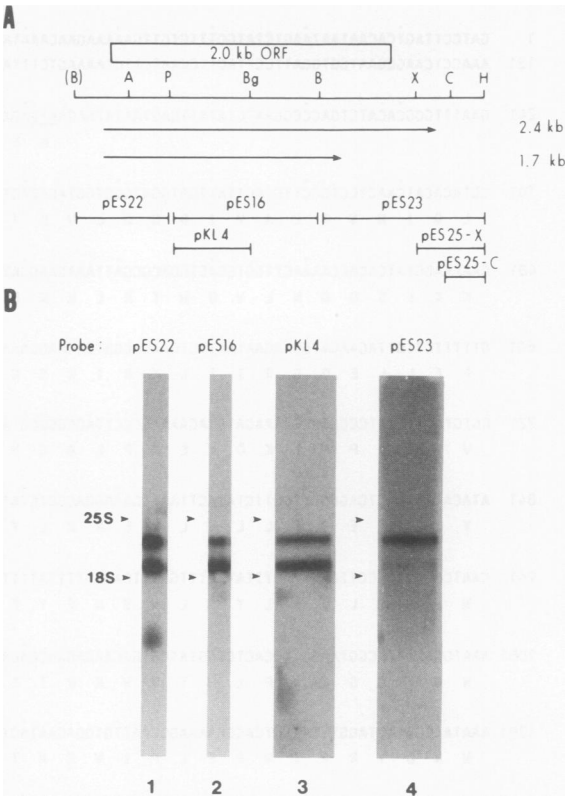

FIG. 2. Analysis of the two *SIR1* transcripts. (A) Restriction map of the *SIR1* gene, and relative positions of the SIR1 open reading frame (ORF) and riboprobes used in Northern blotting experiments (see Materials and Methods for detailed description of riboprobes). Restriction sites are abbreviated as follows: B, *Bam*HI; A, *Acc*I; P, *Pst*I; Bg, *Bgl*II; X, *Xho*I; C, *Cla*I; H, *Hin*dIII; ( ), nongenomic site. (B) Northern blots of *SIR1* transcripts. Blots were probed with the following ^{32}P-riboprobes: lane 1, pES22; lane 2, pES16; lane 3, pKL4; lane 4, pES23. Lanes 1 and 2 contain total cellular RNA, while lanes 3 and 4 contain poly(A)$^+$ selected RNA. RNA was prepared from the yeast strain W303-1a (RNA isolation procedures described in Materials and Methods). Arrowheads to the left of each blot refer to the positions of 25S and 18S rRNAs.

since a faint band of the appropriate size was seen when a Northern blot was probed with pES25-X but could not be detected with pES25-C (data not shown). At least one additional transcript was seen with the pES25 probes (and faintly with probe pES23) that is presumed to result from a gene located downstream of *SIR1*; it was not investigated further.

A strain with a single-copy plasmid containing the 2.9-kb sequenced *SIR1* gene (in a chromosomal *sir1* null mutant strain) had two *SIR1* transcripts that were the same size as the transcripts from the chromosomal copy of *SIR1* (data not shown). Thus, this 2.9-kb fragment, containing only 286 bp upstream of the first ATG codon of the open reading frame, is likely to include the *SIR1* promoter. In Fig. 2, the two transcripts are shown as having identical start sites. The data presented are consistent with this interpretation, but it is also possible that the two transcripts begin at different positions. If so, these start sites are likely to be within 0.2 kb of each other.

5′-deleted fragments of the *SIR1* gene complement a *sir1* mutant but do not suppress mating-defective mutants. Ivy and

TABLE 5. Synergism between *nat1* and *sir1*

Strain	Relevant genotype	Mating efficiency[a]
W303-1b	*MAT*α wild type	9×10^{-1}
RTY-1c	*MAT*α *nat1 ard1*	8×10^{-1}
AMR30	*MAT*α *sir1*	6×10^{-1}
EMS2	*MAT*α *nat1 sir1*	9×10^{-6}
YAB102	*MAT*α Δ*Ehmre*	6×10^{-1}
AMR35	*MAT*α Δ*Ehmre sir1*	3×10^{-2}

[a] Mating efficiency was determined by quantitative mating as described in Materials and Methods. Briefly, mating efficiency is expressed as the number of cells that mated per total number of cells tested.

co-workers cloned a *SIR1*-complementing DNA fragment (11) that does not contain the *SIR1* promoter or the first 118 amino acids of the putative SIR1 protein (as judged by a comparison of their clone with the sequence shown in Fig. 1). To confirm their observation, a plasmid (YEpsir1 in Tables 2 and 3) was constructed that contained only DNA downstream of the *Pst*I site and thus lacks the first 135 codons for the SIR1 protein. This plasmid complemented a *sir1* mutant but was unable to suppress the mating defects of a *nat1* mutant or the *sir3*::*LEU2* mutant (Tables 2 and 3). Transcription of the truncated *SIR1* gene probably occurs from a fortuitous promoter on the plasmid. It is not clear whether lack of suppression with the 5′-deleted *SIR1* gene is due to the loss of natural upstream promoter sequences (and subsequent loss of *SIR1* overexpression) or to the absence of the N-terminal protein sequences.

SIR1 is not the NAT1 substrate that is required for full silencing. It was shown previously that a *MAT***a** *ard1 sir1* double mutant mates at a frequency of 10^{-5} and thus has a much more severe mating defect than either *ard1* or *sir1* single mutants (30). A *MAT***a** *nat1 sir1* double mutant was constructed, and as expected, it too exhibited virtually no mating. The same mutation in a *MAT*α background also yielded a nonmater, even though each of the single mutations gave no detectable mating defect. Quantitative mating data for the relevant mutants are shown in Table 5. These observations illustrate strong synergism between *nat1*, *ard1*, and *sir1*. If N-terminal acetylation of SIR1 were required for complete silencing, one would expect a *nat1 sir1* double mutant to have no greater mating defect than a *sir1* single mutant. That is, in the absence of SIR1, the absence of NAT1 should not affect silencing further. Since strong synergism was observed, we conclude that SIR1 is not the crucial substrate for the NAT1 and ARD1 N-terminal acetyltransferase that is required for full silencing.

As described previously, a *MAT*α Δ*Ehmre* mutant that contains an 8-bp deletion of the RAP1-binding site at the silencer at *HMR* (5) only weakly derepresses the silent locus, and the mutant has no apparent mating defect. On the other hand, the *MAT*α Δ*Ehmre nat1* double mutant is a nonmater. Since a synergistic effect is seen between the *nat1* and *sir1* mutations and between a *nat1* mutation and the RAP1-binding site mutant, it was of interest to see whether a synergistic effect would be observed between a *sir1* mutation and the RAP1-binding site mutant. A *MAT*α Δ*Ehmre sir1* double mutant was constructed and tested for mating ability. As shown in Table 5, the *MAT*α Δ*Ehmre sir1* double mutant had only slightly decreased mating efficiency. Therefore, the effect of the two mutations, in the RAP1-binding site and in *SIR1*, is not synergistic.

DISCUSSION

The work described in this article attempts to shed light on the role of SIR1 in silencing through genetic and molecular biological analyses. We have shown that *SIR1*, when overexpressed, can act as an extragenic suppressor of several mating-defective mutants, including *nat1* and *ard1* mutants, a *nat1 ard1* Δ*Ehmre* triple mutant, certain *sir3* mutants, and a histone H4 mutant with an N-terminal deletion. The *SIR1* gene was sequenced and the deduced SIR1 protein sequence was compared with that of all known proteins in a computer data base. Unfortunately, no significant similarities were found, and thus the sequence did not offer any clues about the function of the SIR1 protein.

Since *SIR1* overexpression suppresses various *nat1* and *ard1* mutants, we considered the possibility that SIR1 itself is an acetyltransferase. A direct comparison between SIR1 and NAT1 showed no sequence similarity between the two proteins. Likewise, no similarity was found between SIR1 and ARD1. Also, *sir1* mutants exhibit no phenotypes other than the silencing defect. Thus, if SIR1 is an acetyltransferase, it is likely to have a very limited set of protein substrates from among the small group of proteins thought to be involved in silencing, namely the other SIR proteins, RAP1, ABF1, and the histones. A more general hypothesis is that SIR1 performs an unknown posttranslational modification on one or more of these proteins.

To test the possibility that histones are modified by SIR1, histones were isolated from a *sir1* mutant and from a strain carrying a plasmid that overexpressed the *SIR1* gene. The histones were electrophoresed on a Triton-acid-urea gel (20) and compared with histones from a wild-type strain. There was no change in the histone pattern in the *sir1* mutant or in the *SIR1*-overexpressing strain (unpublished data). Therefore, no evidence was obtained that SIR1 modifies histones. We cannot rule out the possibility that SIR1 modifies only a small fraction of the histones (perhaps those at the silent loci) or that SIR1 modifies the histones in a manner that cannot be detected on a Triton-acid-urea gel. It is noteworthy that overexpression of *SIR1*, either from the multicopy plasmids described in this work or even from the strong *ADH* or *GAL10* promoter, does not appear to be harmful to yeast cells.

A model for the suppression of *nat1* and *ard1* mutations by overexpression of *SIR1* must also take into account the suppression of certain *sir3* mutations (discussed in more detail below) and a histone H4 mutant. Because *SIR1* suppression is seen for these three different types of mutants, the suppression may be a fairly general phenomenon. That is, *SIR1* overexpression may strengthen one of several redundant silencing pathways. Evidence for such redundancy comes from deletion studies of silencer function at both *HML* and *HMR*. For example, deletion of any one of three sequence elements at the *HMRE* silencer has little effect on silencing, but deletion of any two of the three elements abolishes silencing (5). At *HML*, deletion of either flanking silencer (E or I) does not derepress the locus significantly, but deletion of both E and I fully derepresses *HML* (16). The observed suppression by an excess of SIR1 protein may be due to the strengthening of a weakened pathway or to the enhancement of an alternate pathway. Since only one or two extra copies of *SIR1* (YCpSIR1) can suppress the *nat1* and *ard1* mutant mating defect almost as well as overexpression of *SIR1* on a 2μm-based plasmid (YEpSIR1), SIR1 must be present in limiting quantity, and even a slight excess can improve silencing.

One way of imagining how *SIR1* overexpression can suppress the various silencing defects is by considering the work of Pillus and Rine (22) about the role of SIR1. They hypothesized that silencing can be operationally divided into two processes, establishment and maintenance. Once established, the silenced state is inherited; that is, the silenced state can be maintained as such from generation to generation. Their data supported the conclusion that SIR1 has an important role in the establishment of silencing and is less important for maintenance. We extend these ideas by suggesting that SIR1 may be a monitor of silencing; that is, its function may be to ensure that silencing is maintained. When a maintenance system fails, the role of SIR1 is to reestablish silencing. If a gene required for the maintenance system is mutated, perhaps excess SIR1 can suppress the mutant defect by reestablishing silencing with higher efficiency than normal. Thus, one might hypothesize that NAT1 and ARD1 indirectly, and SIR3 and histone H4 directly, take part in the maintenance of silencing.

Why is *SIR1* suppression of *sir3* mutations allele specific? Overexpression of *SIR1* can suppress the mating defects of strains carrying the *sir3-8* or the *sir3*::*LEU2* allele but not the *sir3*::*SUP4-0*, the *sir3*::*LYS2*, or the *sir3*::*TRP1* allele. The *sir3-8* mutant is temperature sensitive for mating ability and therefore is certainly not a null mutant. On the other hand, the *sir3*::*LEU2* disruption allele has a deletion of a *Bgl*II fragment within the gene and the insertion of *LEU2* at that position (27). The insertion is after codon 107 of *SIR3*; that is, after approximately 10% of the codons for the putative SIR3 protein of 978 amino acids, and thus this allele would be expected to cause severe disruption of SIR3 function. Interestingly, the nonsuppressible *sir3*::*SUP4-0* allele has the *SUP4-0* gene inserted at the same location in *SIR3* as the *LEU2* disruption just described. The *sir3*::*LYS2* allele was constructed by inserting the *LYS2* gene at the *Xho*I site near the 3′ end of the *SIR3* gene (14). This site occurs immediately after codon 944 of the *SIR3* open reading frame, so that approximately 95% of the codons for the putative SIR3 protein remain 5′ of the insertion.

We constructed the *sir3*::*TRP1* null allele (which contains no *SIR3* coding sequences except for the C-terminal eight codons to the 3′ side of the *TRP1* insertion) in order to help us understand the allele-specific suppression we observed with the existing *sir3* alleles. This null mutant was not suppressible. We therefore conclude that SIR1 suppression effects are seen only in the presence of partially functional SIR3 protein. This is easy to understand in the case of the *sir3-8* temperature-sensitive allele, which probably leads to a thermolabile protein present at all temperatures. It is more difficult to understand for the *sir3*::*LEU2* allele with an insertion after only 107 codons. Apparently this allele gives rise to a stable and partially functional protein. (Indeed, we can detect very weak mating in some strains carrying this allele.) On the other hand, inherent instability may occur in either the transcripts or the translation products of the *sir3*::*SUP4-0* and *sir3*::*LYS2* genes. We conclude that excess SIR1 can suppress a mutant SIR3 protein with as few as 107 N-terminal amino acids, as well as the temperature-sensitive sir3-8 protein.

The *sir2* and *sir4* mutant alleles that were tested were not suppressed by overexpression of *SIR1*. Perhaps the right "leaky" *sir2* or *sir4* allele might have been suppressed by excess *SIR1*. On the other hand, it is also possible that SIR2 and SIR4 are in a different silencing "pathway" from NAT1 and ARD1, SIR3, and histone H4, and hence it would never be possible to suppress *sir2* and *sir4* mutant alleles by overexpression of the *SIR1* gene.

The observed specificity of *SIR1* suppression could indicate that a direct SIR1-SIR3 protein interaction is important for silencing. SIR1 and SIR3 could form a permanent complex at the silencer, or SIR1 could be an enzyme that modifies SIR3. In either case, a partially functional SIR3 would require extra SIR1 to ensure a successful SIR1-SIR3 interaction, or the role of SIR1 could be to facilitate an interaction between SIR3 and another protein, so that a partially functional SIR3 could only form a complex with the other protein when SIR1 is in excess. There is indirect evidence that SIR3 and histone H4 do interact (12), and thus the observed SIR1 suppression of certain *sir3* and histone H4 mutants could indicate that the three proteins work together in a silencing complex. If the complex is weakened by a mutation affecting SIR3 or histone H4, then perhaps excess SIR1 can allow a functional complex to form. SIR1 could thus act as a sort of "glue" to improve the interactions of a weakened complex. Alternatively, SIR1 may not interact directly with SIR3. For example, SIR1 might modify a protein that interacts with SIR3; that protein would have to be modified maximally in order to interact with partially functional SIR3. Thus, we are left with two explanations for SIR1 suppression: SIR1 could act to strengthen an alternate pathway, or SIR1 could act like a glue to strengthen a weakened complex. It is difficult to distinguish between these two possibilities at present.

It is interesting that SIR3 itself could be the NAT1 and ARD1 substrate important for full silencing. That is, perhaps SIR3 is unacetylated at its N-terminus in *nat1* and *ard1* mutants and for that reason does not function fully. In that case, the observed suppression of *nat1* and *ard1* mutants is really another example of suppression of a poorly functioning SIR3 protein. Histone H4 is not likely to be a NAT1 and ARD1 substrate since its mobility is not changed on a Triton-acid-urea gel when it is isolated from a *nat1* mutant (20).

We determined that the two *SIR1* transcripts have different termination sites and that the smaller transcript stops within the open reading frame. There is a precedent for transcription termination within coding sequences in *S. cerevisiae*. The *CPB1* gene is a nuclear gene that codes for a mitochondrial protein (17). The *CPB1* gene codes for 1.3-kb and 2.2-kb mRNAs and has an open reading frame of 2.1 kb. The two *CPB1* transcripts have been shown to be regulated by carbon source; the 2.2-kb mRNA is predominant when cells are grown on a glucose-containing medium, whereas the 1.3-kb mRNA is predominant when cells are grown on glycerol. We have no evidence for transcriptional regulation for the *SIR1* transcripts. The ratio of the two *SIR1* transcripts does not change significantly under any of the conditions tested (unpublished data): the ratio is similar in haploid cells and diploid cells, in rich and in minimal medium, at various temperatures (25, 30, and 37°C), and when cells are grown in different carbon sources (glucose, raffinose, or galactose). The ratio does not change during meiosis, as seen when RNA was extracted at different times from a diploid culture that was undergoing synchronous meiosis. Furthermore, the ratio of the transcripts remains the same throughout the mitotic cell cycle, as observed by examining RNA at various times after release of cells from α-factor arrest. It is possible that we have not tested the appropriate conditions under which the levels of the *SIR1* transcripts might differ.

Because the smaller transcript terminates within the *SIR1*

open reading frame, the two transcripts could potentially code for two proteins. Using SIR1-specific antisera, we have been able to detect only one SIR1 protein to date, a protein of 80 kDa, the size expected of a protein coded for by the entire open reading frame (unpublished data). If the *SIR1* gene indeed codes for only one protein, then what is the role of the smaller *SIR1* transcript? We are confident that both transcripts are present in the cell, since both are polyadenylated. That is, the smaller transcript cannot be the product of nuclease cleavage, because we can detect similar amounts of both transcripts in preparations of poly(A)$^+$ RNA and total cellular RNA. It would be of interest to construct a plasmid coding for the shorter transcript but not the longer one and test whether this plasmid complements or suppresses; this has not been done. In summary, the smaller transcript may have an unknown regulatory role, or it may have no role at all, being due simply to the accidental presence of a weak transcription termination signal within the gene.

One model for silencing proposes that altered chromatin structure prevents transcription at the silent loci (2, 21). The studies described here can be considered in terms of such a model. The observed *SIR1* suppression illustrates a potential network of protein interactions and silencing pathways involving the following proteins: SIR1 itself, SIR3, histone H4, and the substrate of the NAT1 and ARD1 N-terminal acetyltransferase. The network may reflect interactions at one or more of the following levels: modifying chromatin at the silent loci, setting up a silencing complex, or maintaining the silenced state during successive cell divisions. Further characterization of the *SIR1* gene product may give important clues about the role of SIR1 and about how silencing occurs.

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ADDENDUM IN PROOF

Overexpression of SIR1 also suppresses the mating defect of strains carrying the temperature-sensitive *sir4-9* mutation.

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