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Genetics

Genetic evidence for an interaction between SIR3 and histone H4 in the repression of the silent mating loci in *Saccharomyces cerevisiae*

(silencing/H4 mutations/*sir3* suppressors)

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ABSTRACT **Repression of transcription from the silent mating loci (*HMLα* and *HMRa*) is essential for mating ability in *Saccharomyces cerevisiae*. This silencing is known to require at least five proteins (SIR1, SIR2, SIR3, SIR4, and histone H4) and is accompanied by a change in chromatin structure. We show here that four positions of histone H4 (N-terminal residues 16, 17, 18, and 19) are crucial to silencing. *HMLα* and *HMRa* are efficiently repressed when these positions are occupied by basic amino acids but are derepressed when substituted with glycine. These results suggest that acetylation of Lys-16 would lead to derepression of the silent mating loci. Three strong extragenic suppressors of the latter H4 mutations were isolated and determined to be located in *SIR3*. These suppressors allow high mating efficiencies in cells expressing either wild-type H4 or H4 containing single amino acid substitutions. They did not allow efficient mating in a strain that contained an H4 N-terminal deletion. These results indicate that the *SIR3* mutations do not bypass the requirement for the H4 N terminus but, rather, allow repression in the presence of a less than optimal H4 N terminus. This provides a link between one of the SIR proteins and a component of chromatin.**

In *Saccharomyces cerevisiae*, the determination of mating type relies on the permanent repression of genetic information. There are three mating loci in yeast [*HMLα*, *MAT* (**a** or *α*), and *HMR***a**] that encode regulatory proteins that control haploid-specific functions (1). However, despite the presence of identical promoter sequences at the silent loci and MAT (e.g., *HMLα* and *MATα* or *HMR***a** and *MAT***a**), only the information located at the *MAT* locus is expressed and determines the mating type of the cell (**a** or *α*). *MAT***a** and *MATα* cells can mate to form a diploid cell, which expresses both **a** and *α* information. The regulatory proteins expressed at *MAT***a** and *MATα* act together to shut down haploid functions and prevent further mating (2). Similarly, a haploid cell that expresses both **a** and *α* information is also inhibited from further mating (1). Therefore, the stable expression of haploid functions depends on the efficient repression of *HMLα* and *HMR***a**.

Extensive studies have revealed that four nonessential proteins are required for repression of *HMLα* and *HMR***a**: SIR1, SIR2, SIR3, and SIR4 (1). Two cis-acting sites, the E and I silencers, located outside the region of homology are also essential for repression (3). Identification of proteins that bind to these DNA sites revealed that two additional proteins, RAP1 and ABF1, may be involved in silencing. No evidence of SIR proteins binding to these DNA sites could be found (4, 5).

How these proteins and DNA sequences interact to inhibit transcription in this region is yet unclear. Although there are chromatin structure differences between *MAT* and the silent loci (6), it is unknown whether they are the cause or result of expression at *MAT*. Recently, deletions in the N terminus of histone H4, a component of the nucleosome, were found to lead to transcriptional derepression of the silent loci (7). This was, to our knowledge, the first direct evidence that a change in chromatin structure resulted in a change in expression at *HMLα* and *HMR***a**. We have extended these studies to investigate which residues in the N terminus of H4 are important for silencing. The H4 deletion, removing amino acids 4–14, reduces mating efficiency only 3-fold, whereas deletion of amino acids 4–19 reduces mating efficiency >4 orders of magnitude. This larger deletion removes all four acetylatable lysines (located at positions 5, 8, 12, and 16) as well as a highly basic region extending from amino acids 16 to 20. Is the reversible acetylation of the lysine residues crucial for repression of the mating loci or is the highly basic region the only requirement? Is one or more of the SIR proteins functioning through H4 to modulate chromatin structure? To address these questions, we have made single amino acid changes in H4 and found four residues that are essential for repression. In addition, three independent suppressors of these mutations were found to be *SIR3* mutations, suggesting that an interaction between histone H4 and SIR3 is important for repression of the silent mating loci.

METHODS

Plasmid Constructions. The plasmid pLJ438T was constructed by cloning a *Bam*HI–*Hinc*II restriction fragment containing *HHT2* (histone H3 copy II) in place of the *Bam*HI–*Nru* I fragment in pUK499 (7). An *Eco*RI–*Bgl* II restriction fragment encoding *TRP1* was then cloned in place of the *Mst* II–*Bam*HI fragment (destroying the *Eco*RI and *Mst* II sites). This vector then contains copy II H3–H4 genes with the *TRP1* gene inserted between the two histone genes.

The *SIR3* single-copy vectors (pLJ87, *SIR3*; pLJ88, *sir3R1*; pLJ90, *sir3R3*) were constructed by ligation of a 4.5-kilobase (kb) *Sal* I restriction fragment encoding the entire *SIR3* gene into the *Sal* I site in SEYC58 (*ARS1 CEN4 URA3*; ref. 8). The plasmids pJR742 (*HMLα* in SEY8), pJR63 (*SIR1* in YEP24), pJR68 (*SIR2* in pSEY8), pJR104 (*SIR3* in YEP24), and pJR643 (*SIR4* in pSEY8) were gifts from J. Rine.

Mutagenesis of HHF2. To efficiently create a number of mutations in the N terminus of H4-2, two derivatives of the plasmid pUK499 (*URA3 CEN4 ARS1 HHF2*) were constructed: one contained a deletion of amino acids 6–19 and created the restriction sites *Bsp*MII and *Afl* II (pPK626) and the other deleted amino acids 12–24 and created an *Xba* I site and an *Afl* II site (pLJ9). Oligonucleotides were then synthesized to span this region, encoding amino acids 6–19 or 12–24 and containing the mutation of interest. Ligation of these oligonucleotide cassettes recreated the H4-2 N terminus, which was then directly sequenced using double-

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Abbreviation: EMS, ethyl methanesulfonate.

stranded sequencing protocols and the Sequenase kit (United States Biochemical). The plasmids resulting from this procedure (see Table 1) were then used to transform the yeast strain UKY403 [*MAT***a** *ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2/pUK421 (TRP1 GAL-HHF2)*], followed by loss of pUK421. The *TRP1* versions of these plasmids were made by insertion of the *Eco*RI–*Stu* I restriction fragment encoding *TRP1* into the *Eco*RV restriction site in the *URA3* gene, resulting in a TRP^+ URA^- vector (indicated by T in plasmid name, for example pLJ305T).

Strain Constructions. Yeast strain LJY438I was constructed as follows: PKD2-5C (*MATα ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52*) was mated to UKY412 (*MAT***a** *ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2/pUK499 (URA3 CEN4 ARS1 HHF2)*) and sporulated, and the resulting tetrads were dissected. A spore that was HIS^+ LEU^- and URA^- was recovered, indicating that it was *hhf1::HIS3 HHF2* and had lost the plasmid pUK499. This strain was then transformed with the plasmid pLJ438T that had been digested with *Eco*RI and *Csp*I45, releasing a fragment containing *hhf2del4-19-TRP1-HHT2*. Transformants that were TRP^+ were selected and screened for the absence of pheromone production using halo assays (phenotype expected for *hhf2del4-19*). One such transformant was found and Southern analysis revealed that it had integrated the *TRP1* gene adjacent to *HHF2*. This strain was then named LJY438I (*MAT***a** *ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2del4-19*).

PKY501, LJY305, and LJY305T are all isogenic to UKY412 with pUK499 replaced with pPK301 (pUK499 expressing *HHF2* with one codon change, leaving the amino acid sequence identical. This change is the result of the introduction of the *Afl* II restriction site used in the oligonucleotide mutagenesis. All mutant *HHF2* also have this codon change), pLJ305 (pUK499 *HHF2-gly16*), and pLJ305T (pUK499 *TRP^+ URA^- HHF2-gly16*).

LJY33-305T was constructed by mating JRY1264 (*MATα ura3-52 trp1 his3 gal-can-1 cir0 lys2 sir3::LYS2*; gift from J. Rine) with PKY501, sporulating the diploid and dissecting the resulting tetrads. One spore was obtained that was HIS^+ LEU^+ URA^+ LYS^+ and did not form a halo on **a** or α tester strains. Southern analysis of the mating type loci showed that this strain was an α strain. HIS^+ is indicative of the presence of *hhf1::HIS3* and LYS^+ indicates the presence of *sir3::LYS2*. LEU^+ may be due to either *LEU2* in the chromosome or *hhf2::LEU2*. To distinguish between these possibilities, we looked at the stability of the *URA3* marker. *URA3* is expressed from the plasmid pPK301 (*URA3 CEN4 ARS1 HHF2*) and is required for viability if both chromosomal copies of histone H4 are deleted in the chromosome but should be relatively unstable if only one copy has been deleted. One spore was found that had a completely stable *URA3*; this strain was named LJY33 [*MATα his3 trp1 ura3-52 ade2 lys2 sir3::LYS2 hhf1::HIS3 hhf2::LEU2/pPK301 (URA3 HHF2)*]. To construct isogenic strains expressing mutant *hhf2*, the plasmid pPK301 was exchanged with either pLJ305T (LJY33-305T), pLJ912T (LJY33-912T), pLJ921T (LJY33-921T), or pLJ933T (LJY33-933T).

sir3R1 was integrated into the chromosome as follows: a 4-kb *Hpa* I restriction fragment containing *sir3R1* was cotransformed with YRP17 into LJY305 and transformants were selected on synthetic yeast medium lacking tryptophan. The transformants were then screened for the ability to mate with an α tester strain (indicative of the integration of *sir3R1* into the chromosome). Two transformants were found that were able to mate, and one of these was grown under nonselective conditions and screened for loss of YRP17 (Trp^-). This strain was then renamed LJY305R1.

SIR3 was integrated into LJY305TR1 and LJY305TM1 (isogenic to LJY305R1 and LJY305M1 with hhf2-gly16 on a TRP1 vector) by transformation of pLJ97 (YIP5 containing a 4.5-kb *Sal* I–*Sal* I fragment encoding *SIR3*) digested at the *Xho* I site within *SIR3* and selecting for URA^+. A stable URA^+ transformant of each strain was isolated and shown to contain an integrated plasmid at the *SIR3* locus by Southern analysis, creating strains LJY305WR1 and LJY305WM1.

Gene Conversion of *HMLα* to *HML*a. A plasmid encoding HO under control of the galactose promoter (pGAL-HO; ref. 9) was introduced into LJY4381 and LJY305T, and HO synthesis was induced by growth on galactose. After 22 hr, cells were plated for single colonies on rich glucose medium. To detect gene conversion events at the silent loci we measured the frequency at which these strains gained the ability to mate with a *MAT***a** tester strain. Approximately 10% of the cells were found to mate with the tester strain. Southern analysis on the parental strains and strains that were now able to mate was performed, using *MAT***a** probe. In each case, the ability to mate correlated with a switch in the information located at *HML* from α to **a**.

Ethyl Methanesulfonate (EMS) Mutagenesis. Logarithmic-phase cultures of either LJY305 or LJY912 were washed with H_2O and resuspended in 50 mM Tris (pH 7.5) containing 3% EMS. After 30 min at 22°C, the cells were washed again with H_2O and resuspended in 6% sodium thiosulfate. After 10 min, the cells were washed once more in H_2O, diluted, and plated for single colonies on rich medium.

Sequence Analysis. The first 1070 base pairs (bp) of *SIR3* was sequenced using double-stranded sequencing protocols and the Sequenase kit (United States Biochemical). Oligonucleotide primers were synthesized ≈250 bp apart and overlapping sequences were clearly read. The following changes were found in our sequence of the wild-type *SIR3* and in each of the mutants: G to A, base 24; T to C, base 165; C to A, base 444; A to G, base 459; C to T, base 523; A to G, base 876; G to A, base 969; A to G, base 990; T to C, base 991; C to A, base 1003; G to T, base 1026.

RESULTS

Single Amino Acid Changes in Histone H4 Greatly Reduce the Mating Efficiency. The N terminus of H4 undergoes several posttranslational modifications (e.g., acetylation and phosphorylation) and has also been implicated in binding directly to the DNA wrapped around the nucleosome (10). The lysines that undergo reversible acetylation in histone H4 are evolutionarily invariant and are found at positions 5, 8, 12, and 16 in all species studied, from the slime mold *Physarum* to calf (11). Nelson (12) has shown that yeast H4 can be tetraacetylated and, based on the extreme conservation of the N terminus, it has been assumed that the same positions are acetylated in yeast as in other eukaryotes. The smallest deletion in H4 that decreased mating efficiency removed the four lysines presumed to undergo reversible acetylation. To address whether acetylation of H4 was required for silencing, we changed different combinations of these lysine residues to either a glycine or an arginine. Glycine was chosen to mimic the acetylated form of lysine because it is a neutral amino acid and the N terminus of H4 is already highly enriched in glycine (40% of the first 20 amino acids). Arginine was chosen to mimic the nonacetylated lysine (positively charged). Mutants constructed using oligonucleotide cassettes are shown in Table 1. Changing the first three lysines (5, 8, and 12) to glycine or arginine had little effect on mating efficiency (a 2-fold decrease was observed in PKY502). However, changing the fourth lysine (Lys-16) to glycine (Gly-16) or glutamine (Gln-16) decreased mating efficiency at least 4 orders of magnitude. Changing Lys-16 to Arg-16 had only a minor effect on mating efficiency (4-fold

decrease). Hence, of the lysines that undergo acetylation, only Lys-16 appears to be necessary for repression of the silent loci. High efficiency mating occurs when this position contains a positively charged amino acid, either lysine or arginine, but not when it contains a neutral amino acid (glycine or glutamine).

Amino acids 17–20 are all positively charged; in addition, His-18 has been shown to undergo reversible phosphorylation and Lys-20 has been shown to undergo methylation in higher eukaryotes (13). To address whether any of these adjacent amino acids are also necessary for repression of the silent loci, each was changed to either a glycine or arginine. Substitution of glycine at positions 17 and 18 essentially eliminates mating (efficiency is reduced 4 orders of magnitude), indicating a high level of derepression of the silent loci (Table 1). Glycine at position 19 results in a decrease of mating efficiency of ≈3 orders of magnitude. Substitution of arginine at position 17, 18, or 19 results in a <10-fold decrease in mating efficiency. Lys-20 does not appear to be strongly involved in repression as substitution with glycine reduced mating efficiency only 4-fold. Therefore, not only is Lys-16 involved in silencing but also Arg-17, His-18, and Arg-19 are involved. These four positions must be occupied by a positively charged amino acid to obtain efficient repression of the silent mating loci.

To determine whether expression of the silent mating loci was the only cause of the decrease in mating efficiency, the *HMLα* locus was converted to *HML***a**, creating strains that were *HML***a** *MAT***a** *HMR***a** (see *Methods*). In both LJY438I (H4del4-19) and LJY305T (H4 Gly-16), mating ability was restored upon gene conversion of *HML*, indicating that the mating defect is due to expression of the silent locus and not loss of expression of some other component essential for mating.

Isolation of Chromosomal Suppressors of H4 Mutations. Having identified a region in H4 that was critical for repression of *HMLα* and *HMR***a**, we wanted to know if other proteins, perhaps the SIR proteins, could be interacting with chromatin through this region. Therefore, we isolated extragenic suppressors by mutagenizing a yeast strain expressing either the H4 Gly-16 (LJY305) or H4 Gln-16 (LJY912) and screened for the ability to mate. The H4 Gln-16 mutation was used in this study as it has a longer side chain, giving it a greater potential for interaction with another protein. LJY305 and LJY912 were treated with 3% EMS as described (see *Methods*) and plated for single colonies. Approximately 125,000 individual colonies (250 plates) were screened by replica mating and 17 colonies that could mate as **a** strains were obtained (numbered M1–M17; M1 was isolated as a suppressor H4 Gly-16 and M2–M17 were isolated as suppressors of H4 Gln-16). Upon rescreening, four mutants were subsequently discarded as they did not show a reproducible increase in mating efficiency (M2, M3, M5, and M12). The remaining suppressors were analyzed to determine if they were true revertants or intragenic suppressors (both of these would be linked to H4) or mutations in *HMLα* (an α2 mutation would cause the cell to behave like an **a** strain even though the silent loci were being expressed). To test for linkage to H4, the suppressor strains were transformed with the plasmid pLJ305T or pLJ912T, TRP1 vectors encoding the H4 Gly-16 or H4 Gln-16, respectively (Fig. 1*A*). The *URA3* vector containing the original H4 mutation was then lost from the cell by growth on nonselective medium and the strains were replica mated again. Four mutant strains were found to lose their ability to mate in the presence of the new H4 plasmid and assumed to be linked to H4 by this analysis (M4, M10, M11, M13). To screen for *HMLα* mutations, the remaining strains were transformed with a plasmid containing the entire *HMLα* locus (pJR742; Fig. 1*B*). If the suppressor restores repression of the silent mating loci, *HMLα* on the plasmid should be repressed and the cell should still be able to mate. If, however, the suppressor has not reestablished repression of the silent loci but mates because of a mutation in α2, the α2 expressed from the plasmid should complement the defective α2 protein resulting in a nonmating phenotype. By this analysis we were able to deduce that five strains had mutations in *HMLα* (M6, M7, M8, M14, M16).

A computer analysis revealed that a block of acidic residues found in both the N1 protein and nucleoplasmin, two characterized histone binding proteins, is partially conserved in SIR3 but not in any of the other SIR proteins (7). Therefore, the remaining strains were checked for linkage to *SIR3*

Table 1. Mutational analysis of the histone H4 N terminus

Strain	HHF2	Histone H4 N terminus	Mating efficiency
		10 (above L), 20 (above final K)	
		+ above R3, K5, K8, K12, K16, R17, H18, R19, K20	
		S G R G K G G K G L G K G G A K R H R K	
PKY501	+	. . . .	1.0
PKY502	Gly-5, -8, -12	. . . . G . . G . . . G	0.5
LJY305	Gly-16	. . . . G	$<10^{-4}$
LJY912	Gln-16	. . . . Q	$<10^{-4}$
LJY921	Gly-17	. . . . G . . .	$<10^{-4}$
LJY933	Gly-18	. . . . G . .	$<10^{-4}$
LJY942	Gly-19	. . . . G .	6×10^{-3}
LJY952	Gly-20	. . . . G	0.25
PKY821	Arg-5, -8, -12	. . . . R . . R . . . R	1.0
LJY902	Arg-15	. . . . R	0.04
PKY506	Arg-16	. . . . R	0.24
LJY922	Lys-17	. . . . K . . .	0.17
LJY931	Arg-18	. . . . R . .	0.38
LJY943	Lys-19	. . . . K .	0.70
LJY953	Arg-20	. . . . R	1.14

The N-terminal 20 amino acids of histone H4 are shown, with + indicating basic amino acids. The mutations described on the left are shown schematically underneath the sequence. The strains listed are all isogenic to PKY501 with pPK301 replaced by an isogenic plasmid expressing the appropriate mutation. The mating efficiencies are the average of at least four quantitative mating assays. Quantitative matings were done as described (14) using D585-11c (*MAT***a** *lys1*) as the **a** tester strain and D587-4b (*MATα his1*) as the α tester strain.

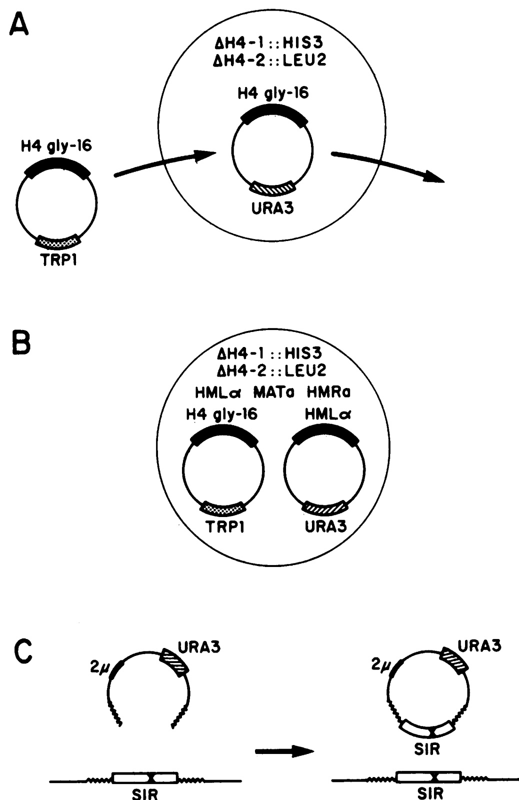

FIG. 1. Schematic diagram of strategies used to classify suppressors. (*A*) Plasmid shuffle replacing the original H4 Gly-16 plasmid (pPK305), which was exposed to EMS, with unmutagenized H4 Gly-16 plasmid (pLJ305T). (*B*) Introduction of pJR742 (*HMLα URA3*) into suppressor strains, resulting in two copies of *HMLα* in the cell. (*C*) Gap repair of *SIR* containing plasmid by chromosomal copy of *SIR* gene.

by transformation with a gapped *SIR3* plasmid and reisolation of the repaired plasmid. This procedure has been shown to be effective for recovering mutations from yeast chromosomes (ref. 14; Fig. 1*C*). The 2-μm-based vector encoding *SIR3* (pJR104) was digested with *Bgl* II and *Xho* I to create a gap in the plasmid in the *SIR3* gene. This gapped plasmid was then used to transform the remaining suppressor mutants, and plasmid DNA was isolated from URA$^+$ transformants. After passaging through *Escherichia coli*, the plasmid DNA was used to transform LJY33-305T (*sir3*::*LYS2*, H4 Gly-16) or LJY33-912T (*sir3*::*LYS2*, H4 Gln-16). Replica mating was used to screen transformants for suppressor activity. In this way we were able to isolate the suppressors from M1 (named *sir3R3*) and M9 (named *sir3R4*). The remaining two mutants (M15 and M17) did not fall into any of the above classes and await further analysis.

Directed Mutagenesis of *SIR* Genes. As the above study was not an exhaustive search for suppressors and may have missed suppressors in the other *SIR* genes, we decided to directly mutagenize plasmids encoding each of the *SIR* genes and look for plasmid-linked suppressors. This study used multicopy vectors to allow identification of mutants that were dominant when overexpressed. During this analysis we found that overproduction of wild-type *SIR1* (pJR63) resulted in weak suppression of H4 Gly-16. Further analysis revealed it also suppressed a large deletion of the H4 N terminus, H4del4-19 (Table 2). As discussed below, we believe this is due to nonspecific suppression, and *SIR1* was not further analyzed.

Plasmids encoding the *SIR2*, *SIR3*, and *SIR4* genes (pJR68, *SIR2*; pJR104, *SIR3*; and pJR643, *SIR4*) were grown in an *E. coli mutD5* strain and then used to transform the yeast strain LJY305T (H4 Gly-16, SIR$^+$). We screened for suppressors by replica mating and found 1 of ≈3000 that was linked to *SIR3* (*sir3R1*). We were unable to isolate any suppressors of our H4 mutation in *SIR2* (0/11,000) or *SIR4* (0/8000).

Characterization of *SIR3* Suppressors. There are two simple mechanisms by which *sir3* mutations could suppress the H4 mutants: either they could bypass the requirement for H4 in repression of the silent loci or they could restore function to the H4 mutant. To distinguish between these possibilities, we analyzed two of these mutations quantitatively with respect to their allele specificity. *sir3R1* was first integrated into the genome, creating strain LJY305R1 (described in *Methods*; *sir3R3* was isolated as a genomic mutant in LJY305M1). The level of suppression of both *sir3R1* and *sir3R3* was determined by quantitative mating analysis (Table 2). *sir3R1* increases the mating efficiency of LJY305 by ≈3 orders of magnitude. *sir3R3* suppresses the H4 mutation more efficiently than *sir3R1*, resulting in an increase of mating efficiency of almost 4 orders of magnitude.

Allele specificity was initially examined by exchanging the H4 Gly-16 plasmid (pLJ305) in LJY305R1 and LJY305M1 with one expressing wild-type H4 (LJY301R1 and LJY301M1) or H4del4-19 (LJY618R1 and LJY618M1). Both *sir3* suppressors were able to allow high levels of mating when the cell expressed wild-type H4 (Table 2). In a cell expressing H4del4-19, neither suppressor was able to increase the mating efficiency significantly. These results indicate that the suppressors do not bypass the need for the N terminus of H4, as SIR1 overproduction did. They also suggest that the suppressors are not strictly allele specific, in that they function in repressing transcription when either wild-type H4 is present or H4 Gly-16.

To test whether the *sir3* mutations could suppress the other H4 mutations, the single-copy vectors, pLJ87 (*SIR3*), pLJ88 (*sir3R1*), and pLJ90 (*sir3R3*), were transformed into *sir3*$^-$ strains that express H4 Gly-16 (LJY33-305T), H4 Gln-16 (LJY33-912T), H4 Gly-17 (LJY33-921T), and H4 Gly-18 (LJY33-933T). Using both replica mating and quantitative mating analysis we found that H4 Gln-16, H4 Gly-17, and H4 Gly-18 were suppressed to the same level as H4 Gly-16 (data not shown).

Dominance and recessiveness were tested by introducing a wild-type copy of the *SIR3* gene into the chromosome adjacent to the mutant *sir3* gene (see *Methods*), such that both copies were being expressed simultaneously. As can be seen from Table 2, the mating efficiency is reduced ≈30-fold in the *sir3R1* strain (LJY305WR1) and ≈7-fold in the *sir3R3*

Table 2. Quantitative mating analysis of H4 suppressors

Strain	HHF2	SIR3	Plasmid	Mating efficiency
PKY501	+	+	–	1.0
LJY305T	Gly-16	+	–	2.7×10^{-5}
LJY305T	Gly-16	+	*SIR1* (pJR63)	2.5×10^{-3}
LJY438I	Δ4-19	+	*SIR1* (pJR63)	2.2×10^{-3}
LJY305R1	Gly-16	*sir3R1*	–	7.4×10^{-2}
LJY305M1	Gly-16	*sir3R3*	–	0.14
LJY301R1	+	*sir3R1*	–	0.80
LJY301M1	+	*sir3R3*	–	0.70
LJY618R1	Δ4-19	*sir3R1*	–	6.7×10^{-4}
LJY618M1	Δ4-19	*sir3R3*	–	3.4×10^{-4}
LJY305WR1	Gly-16	*sir3R1*/+	–	2.2×10^{-3}
LJY305WM1	Gly-16	*sir3R3*/+	–	0.019

The mating efficiencies shown are the average of four quantitative mating assays and are normalized to PKY501 (the wild-type control). The *HHF2* allele is expressed from a single-copy vector and the *SIR3* allele is integrated into the chromosome. *SIR1* is expressed from a multicopy vector.

strain (LJY305WM1). These results indicate the codominant nature of the suppressor mutations with respect to wild-type *SIR3* and may reflect the formation of a multimeric complex in repressing the silent loci.

Sequence Analysis of the *SIR3* Suppressors. To determine whether suppression by *sir3R1* and *sir3R3* was due to single amino acid changes and if they were located in the same region of the protein, we determined the sequence of the suppressors. Through subcloning we found that in both *sir3R1* and *sir3R3*, suppression was associated with a region located between the promoter and the *Eag* I restriction site at amino acid 356. Oligonucleotides were synthesized that covered this region and the entire 1070 bp was sequenced for both mutants and wild-type *SIR3*. In each mutant only one amino acid change was found compared to our sequence of *SIR3*. *sir3R1* changes a tryptophan at amino acid 86 to an arginine and *sir3R3* changes an aspartate at amino acid 205 to an asparagine. Thus, both suppressors contain a single amino acid change, located in the N-terminal portion of *SIR3*. Surprisingly, neither of these mutations lies within the region of similarity with protein N1 and nucleoplasmin. In addition to these changes, we found 11 bp that were different in our sequence analysis of the wild-type *SIR3* as compared to the published sequence (see *Methods*; ref. 15).

DISCUSSION

We show here that the histone H4 residues involved in repression of the silent mating loci are four adjacent basic amino acids: Lys-16, Arg-17, His-18, and Arg-19. In contrast, the first three acetylatable lysines (at positions 5, 8, and 12) do not seem to be involved significantly in repression. A prediction based on our mutation analysis is that acetylation of Lys-16 would lead to derepression, but acetylation of lysine at position 5, 8, or 12 would have little effect on silencing. Independent studies reported recently by Megee *et al.* (16) have also shown that H4 mutations at positions 16 and 18 effect mating efficiency.

We isolated suppressors of the H4 Gly-16 and H4 Gln-16 mutations that restored the ability of the cell to mate and found that using two independent methods we obtained suppressors in *SIR3*. Both *sir3R1* and *sir3R3* allow repression of the silent loci in a cell expressing wild-type H4 or H4 Gln-16, Gly-16, Gly-17, or Gly-18. However, neither of these suppressors was able to efficiently suppress a deletion of the N terminus of H4. These observations indicate that the *sir3* suppressors could not bypass the need for the H4 N terminus in silencing but, rather, allowed silencing in the presence of mutated H4 N termini.

In addition to the *sir3* suppressors, we found that overproduction of *SIR1* could partially suppress both the H4 Gly-16 mutation and the H4del4-19. Overproduction of *SIR1* has also been found to allow mating in a cell that has a deletion of the RAP1 binding site and a *nat1* mutation (R. Sternglanz, personal communication). This lack of specificity of suppression suggests that *SIR1* is acting indirectly to bypass the function of histone H4 in silencing, although the precise mechanism is not clear (17).

How is this highly basic region of the H4 N terminus involved in repressing the silent mating loci and what is the role of *SIR3*? One possible model is that the *SIR3* affects posttranslational modification in this region, thereby altering the binding of the nucleosome to the DNA or possibly the condensation of the chromatin into a more compact structure. Both Lys-16 and His-18 are assumed to undergo reversible modifications (acetylation and phosphorylation, respectively). Our H4 mutation analysis indicates that both of these positions must contain a positively charged amino acid to function in repression. Thus, one would predict that inhibiting the deacetylase (or dephosphorylase) activity or stimulating the acetylase (or phosphorylase) would lead to derepression of *HMLα* and *HMR***a**—the phenotype observed with *sir*- mutations. However, since mutations in *SIR3* can suppress both an H4 Gly-16 and an H4 Gly-18 mutation, and these residues cannot be converted into a positively charged amino acid by mutation of the deacetylase (or dephosphorylase), it is highly unlikely that SIR3, at least, is involved in modification of this region of H4.

Another model is that SIR3, possibly complexed with other SIR proteins, directly binds to or interacts with the N terminus of H4. This binding could then lock the nucleosomes into place, preventing transcriptional machinery access to the region. Arguing against such a direct role are the observations that suppressor SIR3 proteins are able to function efficiently with the wild-type H4 and do not exhibit detectable differences in suppression of glycines at position 16, 17, or 18. It is possible, though, that the suppressors change the conformation of SIR3 and thus allow SIR3 binding to both wild-type and mutant H4 proteins. This model may be tested by isolating plasmid chromatin containing *HMLα* and probing for SIR3 binding.

A modification of this model, which takes into account the DNA binding properties of the H4 N terminus, is that *SIR3* enhances binding of the H4 N terminus to DNA. Thus, the amino acids that are required in H4 for repression may actually be sites that interact directly with the DNA and indirectly with SIR3. This model is particularly attractive in that it allows for the lack of specificity observed for the *sir3* suppressors. The N termini of histones H3 and H4 have been shown not to be required for formation of the nucleosome but are required for further condensation of chromatin, induced by the binding of histone H1 (18). It has been suggested that H1 may enhance the binding of the N termini to DNA or to other nucleosomes (13). In a similar manner, the binding of *SIR3* to the nucleosome could enhance the binding of the N terminus of H4 to DNA at the silent loci, resulting in repression of transcription.

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