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Identification of a Novel Allele of *SIR3* Defective in the Maintenance, but Not the Establishment, of Silencing in *Saccharomyces cerevisiae*

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ABSTRACT

Using a screen for genes that affect telomere function, we isolated *sir3-P898R*, an allele of *SIR3* that reduces telomeric silencing yet does not affect mating. While *sir3-P898R* mutations cause no detectable mating defect in quantitative assays, they result in synergistic mating defects in combination with mutations such as *sir1* that affect the establishment of silencing. In contrast, *sir3-P898R* in combination with a *cac1* mutation, which affects the maintenance of silencing, does not result in synergistic mating defects. *MAT***a** *sir3-P898R* mutants form shmoo clusters in response to α-factor, and *sir3-P898R* strains are capable of establishing silencing at a previously derepressed *HML* locus with kinetics like that of wild-type *SIR3* strains. These results imply that Sir3-P898Rp is defective in the maintenance, but not the establishment of silencing. In addition, overexpression of a C-terminal fragment of Sir3-P898R results in a dominant nonmating phenotype: *HM* silencing is completely lost at both *HML* and *HMR*. Furthermore, *HM* silencing is most vulnerable to disruption by the Sir3-P898R C terminus immediately after S-phase, the time when new silent chromatin is assembled onto newly replicated DNA.

IN eukaryotes with large chromosomes that are easily analyzed in the light microscope, heterochromatin was originally defined as chromosomal regions that stain darkly and appear to remain condensed during interphase. Heterochromatin replicates late in S-phase, often localizes to the periphery of the nucleus during interphase, and is less accessible to enzymes and DNA binding proteins (reviewed in Pardue and Hennig 1991). In the budding yeast *Saccharomyces cerevisiae*, the silent mating loci (*HM* loci) and telomere-adjacent sequences are organized into specialized domains of the genome that share these characteristics of heterochromatin (Grunstein 1998) and have a number of features in common. The four core histones and the silent information regulator proteins Sir2p, Sir3p, and Sir4p are structural components of both telomeric and *HM* locus heterochromatin (reviewed in Grunstein 1998). The two *HM* loci (*HML* and *HMR*) and telomere-adjacent regions compete with each other for the same silent chromatin components, presumably because the available pool of these proteins is limiting (Buck and Shore 1995; Marcand *et al.* 1996).

The process of forming heterochromatin is thought to proceed by a similar process at both the *HM* loci and at telomeres (reviewed in Grunstein 1998; Lustig 1998). First, DNA binding proteins bind to "silencer" sequences adjacent to the *HM* loci or to $TG_{1\text{-}3}/C_{1\text{-}3}A$ tracts at the telomeres. The *HM* silencers bind Rap1p, Abf1p, and the origin recognition complex (ORC). At telomeres, Rap1p binds the the terminal ($TG_{1\text{-}3}$) repeats. Second, at the *HM* loci, but not at telomeres, Sir1p associates with proteins bound to the silencer sites (Triolo and Sternglanz 1996; Fox *et al.* 1997). Third, the DNA binding proteins recruit (at *HM* loci with the help of Sir1p) the Sirp complex, which is composed of Sir2p, Sir3p, and Sir4p (Chien *et al.* 1993; Moretti *et al.* 1994; Lustig *et al.* 1996; Marcand *et al.* 1996). Fourth, the Sirp complex propagates a higher-order chromatin structure along the DNA, rendering it inaccessible to transcription factors and other enzymes (Hecht *et al.* 1996; Strahl-Bolsinger *et al.* 1997).

Three mechanistic processes, establishment, maintenance, and inheritance, play important roles in silencing. Miller and Nasmyth (1984) first demonstrated that *de novo* silencing of a derepressed *HM* locus could occur if cells pass through S-phase. Pillus and Rine (1989) observed that *sir1* cells exist in two epigenetically distinct subpopulations: one in which *HM* silencing is normal and the cells are mating competent and one in which the *HM* loci are derepressed and the cells are mating defective. The *de novo* repression of an *HM* locus in cells previously carrying a derepressed *HM* locus, termed the process of *establishment*, is very inefficient in *sir1* cells. Furthermore, the silent, mating-competent state is heritable in *sir1* cells that were previously carrying a silent *HM* locus. This ability to promote the transmission of the silent state from mother to daughter cells is defined as *inheritance.*

The *maintenance* of silencing is defined as the process

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required after the formation of silent chromatin that retains the silent state during the same cell cycle. Defects in the maintenance of silencing were first detected experimentally by exposing *MAT***a** *HML*α cells to α-factor. Wild-type cells arrest and form mating projections (shmoos) in response to α-factor while *cac1* cells alternately shmoo (respond to α-factor, indicating that they can establish the silent state) and then divide (fail to respond to α-factor, indicating that they are no longer in the silent state). This alternation between responding and not responding to α-factor gives rise to "shmoo clusters," microcolonies of cells with a shmoo morphology that are indicative of a defect in the maintenance of silencing (Enomoto and Berman 1998). Defects in the inheritance of silencing have been studied by monitoring the state of excised chromosomal *HM* loci containing or lacking the adjacent silencer sequences (Holmes and Broach 1996; Bi and Broach 1997; Cheng *et al.* 1998; Ansari and Gartenberg 1999). The inheritance of the silent state into the next generation (involving passage of S-phase) required functional silencers *in cis* (Holmes and Broach 1996; Cheng *et al.* 1998). If silencers were excised from the silent DNA, *HML* silencing was maintained during arrest on α-factor (Holmes and Broach 1996) or *in vitro* (Ansari and Gartenberg 1999). *HM* silencing was not maintained well in cells moving through the cell cycle (Bi and Broach 1997; Cheng *et al.* 1998). However, in a small fraction (5%) of the cells, the silent state was inherited in the next cell cycle despite the absence of silencer sequences (Holmes and Broach 1996). This was interpreted to indicate that it is the silent chromatin itself, rather than the silencer sequences, that is inherited. The silencer sequences, however, improve the efficiency of that inheritance.

Distinguishing between establishment, maintenance, and inheritance is complicated by the fact that these three aspects of silencing appear to be interdependent and partially redundant processes. One can easily imagine that, in the presence of a highly efficient maintenance and inheritance, a defect in establishment will not manifest as a silencing defect. Conversely, if maintenance is defective, there will be no silent structure to inherit, and silencing will be highly dependent upon strong establishment.

Role of Sir3p in silencing: Sir3p is an important structural component of silent chromatin that is required for silencing at both *HM* loci and at telomeres. Sir3p interacts physically with Sir4p, Rap1p, Rad7p, the N termini of histone H3 and histone H4, and with other molecules of Sir3p (reviewed in Stone and Pillus 1998). Most of these interactions occur via the Sir3p C terminus (Sir3-C). The Sir3p N terminus (Sir3-N) modulates the activity of Sir3-C: interactions of Sir3p with Sir3-C and with Sir4p are increased when the Sir3p N terminus is deleted (Moretti *et al.* 1994; Gotta *et al.* 1998; Park *et al.* 1998). Sir3p is post-translationally modified into multiple phosphorylated forms (Stone and Pillus 1996). The classic experiment done by Miller and Nasmyth (1984) used an allele of *SIR3* (*sir3-8*^{ts}) to demonstrate that Sir3p is required for the maintenance of silencing throughout the cell cycle and that the *de novo* establishment of silencing requires functional Sir3p during S-phase.

High-copy expression of full-length Sir3p leads to increased silencing and increased spreading of telomeric silencing: silent chromatin extends farther inward from the telomere (Renauld *et al.* 1993) and Sir3p is physically associated with more centromere-proximal chromatin than in strains expressing wild-type levels of the Sir proteins (Strahl-Bolsinger *et al.* 1997). On the other hand, high-level expression of Sir3p domains affects the nuclear distribution of the Sirp complex and influences silencing in different ways: high-copy expression of the N-terminal domain of Sir3p (Sir3-N) enhances telomeric silencing and redistributes more Sirp complex to the telomeres (Gotta *et al.* 1998); high-copy expression of the C-terminal fragment of (Sir3-C) decreases telomeric silencing by promoting the localization of the Sirp complex to the nucleolus (Gotta *et al.* 1998), where it acts to reduce the silencing of, and frequency of recombination between, rDNA repeats (Smith *et al.* 1998). However, high-copy expression of Sir3-C does not have an obvious effect on *HM* silencing, suggesting that the *HM* loci compete effectively with the nucleolus for the Sirp complex while the telomeres do not. Thus different domains of Sir3p have markedly different effects on the cellular distribution of the Sir proteins and on silencing at the *HM* loci and telomeres.

Role of Sirp complex localization in silencing: Rap1p, Sir3p, and Sir4p co-localize with telomeric DNA to a small number of punctate foci near the nuclear periphery of wild-type cells (Gotta *et al.* 1996). This localization pattern often, but not always, correlates with the silent state of the *HM* loci and telomeres (Konkel *et al.* 1995; Gotta *et al.* 1996). *RLF* (Rap1 localization factor) genes were isolated by their effect on the segregation of TEL + CEN plasmids (circular plasmids carrying both centromere and telomere sequences; Enomoto *et al.* 1994a,b) and their effect on the nuclear distribution of Rap1p. *rlf* mutants alter both the segregation of TEL + CEN plasmids and the localization of Rap1p (Enomoto *et al.* 1994b, 1997). *RLF2* is identical to *CAC1*, which encodes the largest subunit of the chromatin assembly factor I complex and causes a significant loss of silencing at telomeres but no obvious loss of silencing at the mating loci (Enomoto *et al.* 1997; Kaufman *et al.* 1997; Monson *et al.* 1997). *RLF4* is identical to *NMD2/UPF2* (Lew *et al.* 1998), a component of the nonsense-mediated mRNA decay pathway. Both *rlf2* and *rlf4* mutants disrupt telomeric silencing, are mating competent, yet have subtle defects in the maintenance of *HM* silencing (Enomoto and Berman 1998; Lew *et al.* 1998).

In this report we characterized a *rlf3* mutant, a mating-competent allele of *SIR3* that is defective in telomeric silencing. Like *rlf2* and *rlf4* alleles, *sir3^rlf3^* mutants are mating competent, yet they exhibit synergistic mating defects in combination with mutations in silencer sequences or with loss of *SIR1* function. The relevant mutation in *sir3^rlf3^* alters the proline codon at position 898 to an arginine codon. The C-terminal fragment of Sir3^rlf3^p exhibits a novel anti-Sir phenotype when overexpressed: it causes loss of *HM* silencing as well as the loss of telomeric silencing. Furthermore, *HM* locus chromatin is most vulnerable to this anti-Sir activity during and immediately after S-phase, the time when chromatin is assembled onto newly replicated DNA.

MATERIALS AND METHODS

Strains and plasmids: Yeast strains used in this study are listed in Table 1. The temperature-sensitive *sir3-8* allele was introduced into the W303 strain background by digesting pSH135 (Holmes and Broach 1996) with *NruI* and performing two-step gene replacement (Rothstein 1991).

Plasmids used in this study are listed in Table 2. pSE615 was constructed by *in vivo* recombination of pSE562 and pLL550. pSE562 was constructed by inserting the *Eco*RI fragment of *sir3^rlf3^* [amino acids (aa) 439–972] from pSE393 into pACT-II (Li *et al.* 1994). pLL550 was constructed by inserting the *Bgl*II-*Sal*I fragment of *sir3^rlf3^* (aa 307–979) from pSE393 into pGAD424.

pSE647 and pSE856 were constructed by gap repair of pSE615 digested with *Bsi*W1 + *Nde*I and transformed into a *SIR3* strain to replace the *sir3-P898R* allele. DNA sequencing confirmed that the wild-type allele replaced the mutant allele in pSE856 and that in pSE647 the insertion of an adenine immediately after codon 853 led to a frameshift mutation generating 35 additional amino acids prior to a termination codon. pSE853 was constructed by gap repair of pM393 (digested with *Bsi*WI and *Nde*I; Moretti *et al.* 1994) in *sir3^rlf3^* strain (YJB966). The presence of the mutant allele was confirmed by sequencing. pSE1072 was constructed by recombination between pSE1033 (digested with *Xho*I and *Sph*I) and pSE438 (digested with *Hpa*I) such that the *sir3^rlf3^* allele was replaced by the wild-type *SIR3* allele pSE438. pSE1071 was constructed by recombination between pSE1033 (digested with *Xho*I and *Sph*I) and pSE425 (digested with *Hpa*I) to ensure that the *Xho*I-*Sph*I fragment was the wild-type *SIR3* allele. pSE1033 was constructed by recombination between pAR16 (digested with *Kpn*I and *Eco*RV; Holmes and Broach 1996) and pSE715 (digested with *Xba*I) such that the P_{GAL}-SIR3-1-979 gene was inserted into the P_{GAL}-SIR3-C (439–987) region of pSE715. pSE332 was constructed by recombination between pJR273 (digested with *Pvu*II) and pJkmf (−) (digested with *Hin*dIII; Kirschman and Cramer 1988). pSE334 is an *in vivo* recombination product of pSE332 (digested with *Pvu*II) and YCplac111 (digested with *Hin*dIII; Gietz and Sugino 1988) such that *SIR3* was inserted into the *lacZ* gene in YCplac111. pSE338 was constructed by inserting the 3′ region of (*Eco*RI-*Sal*I fragment) *SIR3* from pSE332 into YIPlac128 (Gietz and Sugino 1988). pSE650 was made by inserting the pSE615 *Bam*HI-*Pst*I fragment into the *Bgl*II-*Pst*I-digested pSE497. pSE497 is analogous to pRSET-C (Invitrogen, Carlsbad, CA) with a kanamycin resistance marker replacing the β-lactamase gene (S. Enomoto, unpublished results). pSE912 was made by ligation of pSE856 (digested with *Bam*HI and *Pst*I) and pSE497 (digested with *Bam*HI and *Pst*I). pSE715 was made by recombination of pSE650 (digested with *Xba*I) and YGALSET351 (digested with *Xho*I; Enomoto *et al.* 1998). pSE481 is an *in vivo* recombination product of pSE438 (digested with *Pvu*II) and YIPlac211 (digested with *Hin*dIII; Gietz and Sugino 1988).

Isolation of *rlf3* alleles: The TEL + CEN plasmid screen used to isolate *sir3^rlf3^* and methods used to isolate complementing genes were described previously (Enomoto *et al.* 1994a,b, 1997; Lew *et al.* 1998). Quantitative mating, telomeric silencing, and *HMR::TRP1* derepression assays were performed as previously described (Enomoto *et al.* 1997; Enomoto and Berman 1998). For the quantitative mating assays, four matings were performed for each strain. The median values were compared by the rank sum test (Snedecor and Cochran 1980).

Different *sir3^rlf3^* alleles were subcloned by gap repair. pSE334 was digested with *Hpa*I and transformed into a *SIR3*/*sir3^rlf3^* strain (YJB276 × YJB497). The *Hpa*I sites flank the *SIR3* open reading frame (ORF) at nucleotide (nt) −206 and nt 3485 relative to the ORF. A total of 10 independent plasmids with the appropriate restriction map were recovered and functionally tested for complementation of the telomeric silencing phenotype in YJB1033 (*sir3* null). Two classes of plasmids were obtained and we chose one of each type for further characterization. pSE392 restored telomeric silencing to wild-type levels; pSE393 did not restore telomeric silencing to wild-type levels and thus contained the *sir3^rlf3^* allele.

Mapping the lesion in the *sir3^rlf3^* alleles: To map the mutations within the *sir3^rlf3^* allele, pSE393 was digested at nt 487 with *Cla*I or at nt 2502 with *Kpn*I and cotransformed with pSE332 digested with different restriction enzymes whose sites span the *SIR3* gene. The telomeric silencing phenotype was checked for several transformants for each plasmid pair. This analysis indicated that the lesion in *sir3^rlf3^* was located between the *Nru*I site (nt 2283) and the *Xho*I site (nt 2833).

To reintegrate *sir3* alleles, pSE481 was linearized by digestion with either *Kpn*I (at nt 2502) or *Xho*I (at nt 2833), and two-step gene replacement of these alleles was performed into wild-type *SIR3* strains. Candidate strains were assayed for TEL + CEN antagonism [by crossing them to YJB499 carrying p49K (Enomoto *et al.* 1994a,b)] and for telomeric silencing by monitoring expression from URA3 inserted at the left end of chromosome VII. Strains for assaying telomeric silencing were generated by transformation with pVIIL-URA3-TEL (Gottschling *et al.* 1990) or by genetic crosses to strains carrying chromosome VIIL-URA3-TEL.

Sequencing of *sir3^rlf3^* alleles: DNA sequencing of the entire *SIR3* gene in both pSE392 and pSE393 was performed by the University of Minnesota microchemical facility, using the following primers:

acaggagatggtaccacgct, agcgtggtaccatctcctgt, tttatgcggcgtcc aaaa,
gtaaatagtcatttccttc, ttccggattttgtattaa, agtttattttgggaagac, caaa ccggtctaaaatta,
tgcttcatcagaactttc, ggtgatgtgagcgcagaa, gtttgggttccatttcct, tag atctggcctgaattg,
gtaatgataacttgccaa.

Mating assays: For the mating establishment assay, cells were pregrown on synthetic complete medium lacking leucine (SC-Leu; Sherman *et al.* 1986) containing 2% glucose (to prevent expression of P_{GAL}-*SIR3* or P_{GAL}-*sir3-P898R*) at 25° and shifted to 37° for 18 hr (to inactivate Sir3-8p). Cells were transferred to SC-leu containing 0.5% galactose and 2% raffinose (to induce P_{GAL}-*SIR3* or P_{GAL}-*sir3-P898R* expression) that was preheated to 37° and maintained at 37° (to continue inactivation

TABLE 1

Yeast strains used

Strains	Genotype	Source
Isogenic to S150B-2		
YJB276	*MAT*a *leu2-3, 112 ura3-52 trp1-289 his3Δ ade2Δ [cir+]*	J. Berman
YJB199	YJB276 *MATα ADE+*	J. Berman
YJB487	YJB276 *SUP*[a] *adh4::URA3-TEL [cir0]*	J. Berman
YJB497	YJB276 *MATα SUP*[a] *cyh2 sir3-rlf3 [cir0]*	This study
YJB499	YJB276 *SUP*[a] *cyh2 sir3-rlf3 adh4::URA3-TEL*	This study
YJB998	YJB276 *SIR3-LEU2*	This study
YJB1033	YJB276 *MATα sir3Δ::TRP1 adh4::URA3-TEL*	This study
YJB1250	YJB276 *HIS+ trp1::URA3*	J. Berman
YJB1267	YJB276 *SUP*[a] *HIS+ lys2 adh4::URA3-TEL cyh2 sir3-P898R [cir0]*	This study
YJB2670	YJB276 *SUP*[a] *sir3-P898R, -5μ adh::URA3-TEL [cir0]*	This study
YJB2728	*YJB276 SUP*[a] *SIR3-5μ adh::URA3-TEL [cir0]*	This study
Isogenic to W303		
YJB195	*MAT***a** *ura3-1 ade2-1 his3-11 leu 2-3,112 can 1-100 trp1-1*	J. Berman
YJB209	*MATα ade2-1 his3-11 leu2-3,-112 can1-100 trp1-1 ura3-1*	J. Berman
YJB955	YJB195 *HMRΔA::TRP1*	J. Berman
YJB958	YJB195 *HMRΔA::TRP1 cac1::LEU2*	J. Berman
YJB959	YJB195 *HMR::TRP1*	J. Berman
YJB1104	YJB195 *HMRΔE::TRP1*	J. Berman
YJB1143	YJB195 *HMRΔB::TRP1*	J. Berman
YJB1504	YJB209 *sir3-P898R*	This study
YJB1532	YJB195 *HMRΔE::TRP1 sir3-P898R*	This study
YJB1544	YJB195 *HMR::TRP1 sir3-P898R*	This study
YJB1563	YJB195 *HMRΔA::TRP1 sir3-P898R*	This study
YJB1578	YJB209 *cac1::LEU2*	J. Berman
YJB1539	YJB195 *HMRΔB::TRP1 sir3-P898R*	This study
YJB1633	YJB209 *HMRΔA::TRP1 VII::URA3-TEL*	J. Berman
YJB1838	YJB195 *cac1::LEU2*	J. Berman
YJB1940	YJB195 *sir1::HIS3*	J. Berman
YJB1941	YJB209 *sir1::HIS3*	J. Berman
YJB2001	YJB195 *HMRΔA::TRP1 sir1::HIS3*	J. Berman
YJB2315	YJB195 *sir3-P898R sir1::HIS3*	J. Berman
YJB2316	YJB209 *sir3-P898R sir1::HIS3*	J. Berman
YJB2352	YJB195 *sir3-P898R*	J. Berman
YJB2353	YJB209 *sir3-P898R cac1::LEU2*	J. Berman
YJB2354	YJB195 *sir3-P898R cac1::LEU2*	J. Berman
YJB2769	YJB195 *sir3-8*	This study
YJB2930	YJB195 sir3-8 sir1::HIS3	This study
YJB5094	YJB209 *rap1-17* VII::*URA3/ ADE2 LexAS3*	This study
YJB5093	YJB209 *sir3-P898R rap1-17* VII::*URA3/ ADE2 LexAS3*	This study
Miscellaneous		
YJB4 (A364A)	*MAT***a** *ade1 ura1 gal1 ade2 tyr1 his7 lys2*	L. Hartwell
YJB5 (B364B)	*MATα ade1 ura1 gal1 ade2 tyr1 his7 lys2*	L. Hartwell
YJB905 (CTY10-5d)	*MAT***a** *ade2-1 trp1-901 leu2-3,112 his3-200 leu2 gal4* gal80 *URA3::lexA op-lacZ*	S. Fields

[a] *trp1-289* is suppressed by an unlinked, uncharacterized suppressor mutation.

of Sir3-8p) for the times indicated in Figure 4A. All subsequent manipulations were performed on plates prewarmed to 37° and incubated at 37°. Mating competence was assayed by streaking these cells across tester strain B364B (Table 1) on rich medium containing glucose (YPAD; which represses P_{GAL}-*SIR3* or P_{GAL}-*sir3-P898R* expression) and allowing the cells to mate for 18 hr. The efficiency of mating was determined by analyzing the growth of cells on SC-his medium containing glucose, which selected for diploids formed in the test cross.

For mating assays with Sir3-R898P-C, strain YJB905 (*ade2 leu2*) containing *LEU2*-marked plasmids pSE856, pSE647, pSE615, or pACT II was mated with tester strain YJB199 (*ADE2 leu2*) and the formation of diploids was determined by selection on SC-leu-ade.

β-Galactosidase assays: β-Galactosidase assays were performed using standard methods (Ausubel *et al.* 1989). A minimum of four independent transformants were assayed at least twice each. All measurement values were normalized to that of P_{ADH}-LEX$_{BD}$-*GAL4*$_{AD}$ = 10,000 units. The rank sum test (Snedecor and Cochran 1980) was used to assess the statisti-

TABLE 2

Plasmids used

Name	Relevant feature	Source
p184.4	*GST HHT* (aa 1–46)	Hecht *et al.* (1995)
p107.1	*GST HHF* (aa 1–34)	Hecht *et al.* (1995)
pGEX-B Globin	*GST* β-globin	Johnston *et al.* (1996)
pACT II	P_{ADH} GAL4$_{AD}$ vector	Moretti *et al.* (1994)
pSE647	pACT II-*SIR3*$_{438–852}$	This study
pSE615	pACT II-*sir3*-P898R$_{438–979}$	This study
pSE856	pACT II-*SIR3*$_{438–979}$	This study
pSE1072	P_{GAL}-*sir3-P898R*	This study
pSE1071	P_{GAL}-*SIR3*	This study
pBM116	P_{ADH}-LEX$_{BD}$ vector	Moretti *et al.* (1994)
pBTM-R7	P_{ADH}-LEX$_{BD}$-*RAD7*$_{1–565}$	Paetkau *et al.* (1994)
PM26	P_{ADH}-LEX$_{BD}$-*RAP1*$_{635–827}$	Moretti *et al.* (1994)
PM250	P_{ADH}-LEX$_{BD}$-*RAP1*$_{647–827}$	Moretti *et al.* (1994)
PM398	P_{ADH}-LEX$_{BD}$-*SIR4*$_{839–1358}$	Moretti *et al.* (1994)
pSE853	P_{ADH}-LEX$_{BD}$-*sir3-P898R*$_{307–979}$	This study
pM392	P_{ADH}-LEX$_{BD}$-*SIR3*$_{2–979}$	Moretti *et al.* (1994)
PM393	P_{ADH}-LEX$_{BD}$-*SIR3*$_{307–979}$	Moretti *et al.* (1994)
PM395	P_{ADH}-LEX$_{BD}$-*GAL4*$_{AD}$	Moretti *et al.* (1994)
pSE49K	TEL+CEN *ADE2*	Enomoto *et al.* (1994b)
pSH135	*sir3-8*^{ts} YIP *URA3*	Holmes and Broach (1996)
pSE393	*sir3*^{rlf3} YCP *LEU2*	This study
pSE332	*SIR3* Kan	This study
pSE334	*SIR3* YCP *LEU2*	This study
pSE392	*SIR3* (S150B-2) YCP *LEU2*	This study
pSE338	homology 3′ to *SIR3* locus YIP *LEU2*	This study
pSE650	T7 *sir3-P898R*$_{438–979}$	This study
pSE912	T7-*SIR3*$_{438–979}$	This study
pSE408	T7-*RAP1*	Enomoto *et al.* (1998)
pSE715	P_{GAL10} T7 *sir3-P898R*$_{438–979}$	This study
pVIIL-URA3-TEL	VIIL::*URA3*-TEL	Gottschling *et al.* (1990)
pSE481	*sir3*^{rlf3} YIP *URA3*	This study
pVIIL-*URA3-ADE2-LexAS3-TEL*	VIIL-*URA3-ADE2-LexAS3-TEL*	Lustig *et al.* (1996)

AD, activation domain; BD, DNA binding domain; YIP, yeast integration plasmid; YCP, yeast centromere plasmid.

cal significance of the values obtained between wild-type and *sir3-P898R* strains.

GST pull-down assay: Sir3p, Sir3-P898Rp, and Rap1p were produced *in vitro* in the presence of [^{35}S]methionine using the TNT-coupled transcription and translation system (Promega, Madison, WI). GST-β-globin$_{1–123}$, GST-histone H3$_{1–46}$, and GST-H4$_{1–34}$ were produced in *Escherichia coli* essentially as described (Smith and Johnson 1988; Hecht *et al.* 1995). Briefly, recombinant protein production was induced in *E. coli* strain BL21 carrying the plasmids by the addition of isopropyl thiogalactoside to 1 mm and incubation at 25° for 1 hr. Cells were collected by centrifugation and lysed by sonication in TGD$_{150}$ [20 mm Tris-Cl (pH 8.0), 150 mm NaCl, 1 mm EDTA, 0.1% Triton X-100, 1 mm glutamate, 1 mm DTT, 1 mm PMSF]. Cell debris was removed by centrifugation and the resulting supernatant was incubated with 40 μl of a 50% slurry of glutathione-agarose (Sigma, St. Louis) for 45 min at room temperature. The resin was washed twice with TGD$_{150}$ and then incubated with ^{35}S-labeled protein in 100 μl buffer for 1 hr at room temperature. The resin was washed with buffer three times before all proteins were eluted by the addition of 20 μl of SDS loading buffer. Proteins were separated by SDS-PAGE and detected by autoradiography.

Arrest/release anti-SIR shmooing assay: Cells were grown in SC-leu containing 2% glucose and arrested by the addition of α-factor (0.1 μg/ml), hydroxyurea (400 mm), or nocodazole (10 μg/ml) for 4 hr at 25°. Arrested cells were collected by centrifugation and resuspended in SC-leu medium containing 2% galactose and 2% raffinose and the appropriate cell cycle inhibitor and incubated for 18 hr. Cells were then washed three times with fresh medium containing glucose and after a 20-min recovery were placed on SC-leu medium containing α-factor. Arrest and cycling of the cells was monitored by examination of cell morphology.

RESULTS

Identification of a novel allele of *SIR3*: Circular plasmids carrying both telomeric and centromeric DNA (TEL + CEN plasmids) are highly unstable (Longtine *et al.* 1992), a phenotype termed TEL + CEN antagonism. Furthermore, these TEL + CEN plasmids are stabilized by mutations that alter telomeric chromatin structure and function (Enomoto *et al.* 1994a,b). A

screen for mutants that lost TEL + CEN antagonism initially identified a large number of mutants that were defective in mating and could be restored to wild-type mating competence by transformation with a copy of *SIR2*, *SIR3*, or *SIR4* (Enomoto *et al.* 1994a,b). We focused our attention on mutants that also perturbed the nuclear localization of Rap1p [from the punctate, perinuclear foci that co-localize with a majority of telomeric DNA foci (Gotta *et al.* 1996) to a more diffuse distribution of Rap1p]. These mutants, which segregated as single genes and altered the localization of Rap1p, were termed Rap1p localization factor mutants and the mutated genes were designated "*RLF* genes." Analyses of *RLF2*, which is allelic with *CAC1*, and of *RLF4*, which is allelic with *NMD2/UPF2*, have been reported elsewhere (Enomoto *et al.* 1997; Enomoto and Berman 1998; Lew *et al.* 1998).

Here we describe the characterization of *rlf3*, a novel mating-competent allele of *SIR3.* Strains carrying the *rlf3* allele are defective in TEL + CEN antagonism, Rap1p localization, and telomeric silencing (see below). To identify the gene mutated in the *rlf3* strain, we used a low-copy (CEN) library and isolated clones that restored the TEL + CEN antagonism phenotype and the telomeric silencing of the *rlf3* strain. We isolated >80 clones, all of which contained the *SIR3* gene. Because an extra copy of *SIR3* can complement some nonallelic mutations (Enomoto *et al.* 1998), the isolation of *SIR3* was not sufficient to indicate that the *rlf3* mutation is an allele of *SIR3*, especially since the *rlf3* strain was mating competent (see below) and all other reported *sir3* alleles were defective in *HM* silencing and mating (Rine and Herskowitz 1987). To test the allelism of *rlf3* and *SIR3*, *rlf3* strain YJB497 was crossed to strain YJB998, which contained a *LEU2*-marked *SIR3* allele. In five complete tetrads, the *SIR3-LEU2* allele always segregated away from the *rlf3* mutation, detected using TEL + CEN antagonism assays (Enomoto *et al.* 1994a,b), consistent with the idea that *rlf3* is allelic to *SIR3.*

The *rlf3* allele of *SIR3* (*sir3*^{rlf3}) was cloned by gap repair of a wild-type, plasmid-borne copy of *SIR3.* The sequence of the mutant allele identified six different missense mutations, five of them near the *Bgl*II site and one 3′ of the *Kpn*I site (Figure 1A). The gap repair strategy mapped the mutant phenotype to the single cytosine-to-guanine transversion at nt 2693, which causes a proline-to-arginine substitution at amino acid 898. We confirmed that the single P898R substitution is responsible for the restoration of the TEL + CEN antagonism phenotype when expressed from a plasmid in a *sir3* null strain. This allele is called *sir3-P898R.* We refer to the original allele as *sir3*^{rlf3} and to the five missense mutations near the *Bgl*II site collectively as *sir3-5*μ.

To generate a strain carrying a genomic copy of the *rlf3* allele, we performed two-step gene replacements using different restriction enzyme digests that targeted replacement of different domains of the wild-type *SIR3* allele with domains of the *rlf3* allele. Replacement of almost all of *SIR3* with sequence encoding aa 1–945 from the *rlf3* allele resulted in abrogation of telomeric silencing (at least 10,000-fold lower than wild-type levels), similar to that seen with the original *rlf3* mutant strain (Figure 1C, *sir3-P898R*, 5μ). In contrast, replacement of *SIR3* with aa 834–978 (the C terminus of Sir3p) from *sir3*^{rlf3} resulted in telomeric silencing levels 1000- to 10,000-fold lower than wild-type levels (Figure 1C, *sir3-P898R*). Replacement of the five upstream mutations created a strain that had a very modest reduction in telomeric silencing (Figure 1C, *sir3-5*μ). These replacement experiments indicated that the major telomeric silencing defect was due to the P898R mutation and that the 5μ mutations enhanced the telomeric silencing defect. The studies described below were conducted primarily with strains carrying the *sir3-P898R* allele.

***sir3-P898R* mutations have subtle mating defects:** To ask if the different mutations within the alleles contributed differently to the mating competence of these strains, we performed quantitative mating assays. We observed no significant difference in mating competence between wild-type, *sir3*^{rlf3}, *sir3-R898P*, *sir3-5*μ, and *sir3-R898P*, *5*μ strains (Figure 1B). While subtle defects in *HM* silencing are not detected by these mating assays, the results confirm that all of these *sir3* alleles are mating competent.

There are several genes that, when mutated, significantly reduce telomeric silencing and have only very subtle effects on *HM* locus silencing. For example, strains lacking CAF-I subunit genes (*CAC1, CAC2*, and *MSI1/CAC3*) do not have an obvious mating defect in quantitative mating assays, unless they are combined with *sir1* mutations (Enomoto and Berman 1998; Kaufman *et al.* 1998). We constructed both *MAT***a** and *MAT*α strains carrying *sir3-P898R* alone or together with *sir1* or *cac1* mutations and analyzed the mating ability of the strains in patch mating assays (Figure 2A). Similar to results seen previously with *cac1 sir1* strains, *MAT***a** *sir3-P898R sir1* mutants were defective for mating and *MAT*α *sir3-P898R sir1* mutants exhibited reduced mating ability. In contrast, *sir3-P898R cac1* double mutants (both *MAT***a** and *MAT*α) mated as efficiently as wild-type and single-mutant strains (Figure 2A). These results suggest that the *sir3-P898R* mutation and the *cac1* mutations may affect a similar aspect of silencing (*e.g.*, the maintenance of silencing) that is distinct from *SIR1* function [*e.g.*, the establishment of silencing (Pillus and Rine 1989)].

***sir3-P898R* mutations enhance defects in the *HMR* silencer:** To measure the effect of *sir3-R898P* at *HMR*, we utilized an *HMR::TRP1* construct, in which the *a1* and *a2* genes at *HMR* were replaced by the *TRP1* gene (Hardy *et al.* 1992). Assays that measure expression of *HMR::TRP1* are more sensitive to low levels of *HMR*

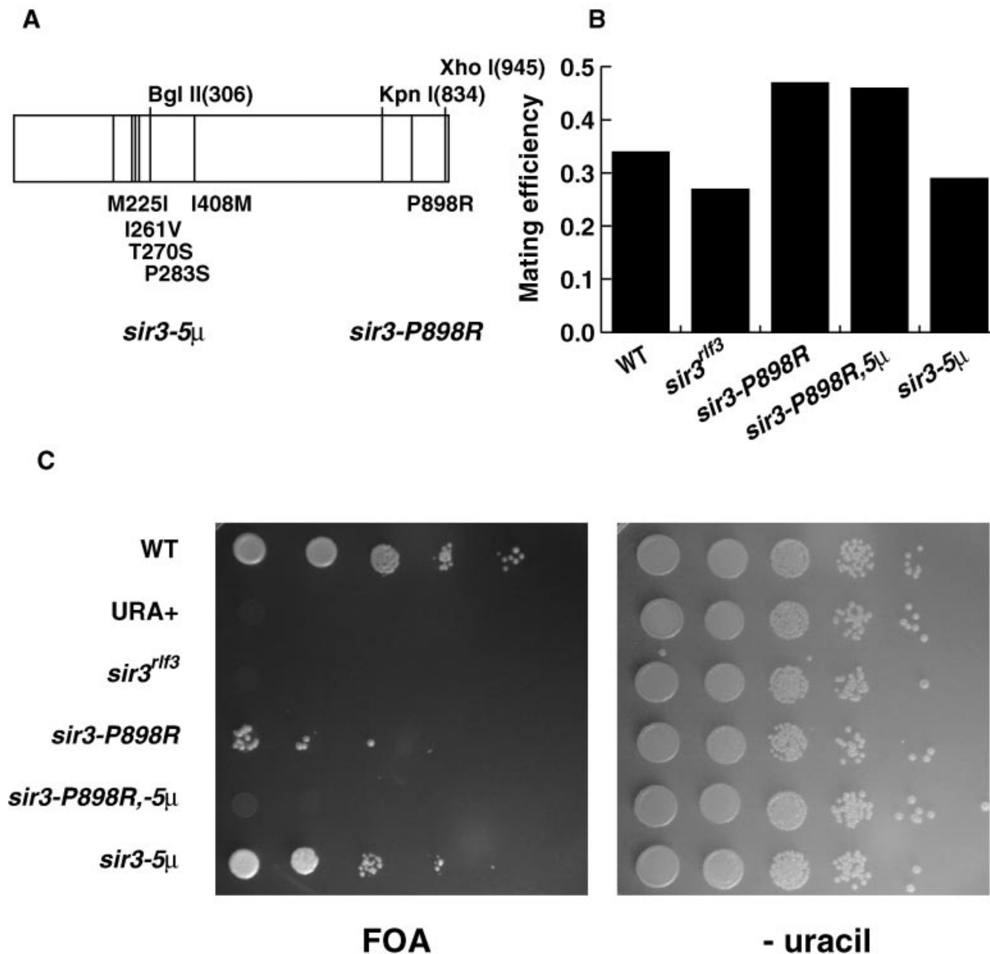

Figure 1.—A novel allele of *SIR3* that affects telomeric silencing but does not affect mating efficiency. (A) Map of the missense mutations identified within the original *sir3*^{rlf3} allele. Numbers indicate the amino acid positions within Sir3p. Restriction sites used for gap repair experiments are indicated above the gene. Below the gene, the mutations found within the *sir3*^{rlf3} allele are indicated, using the one-letter amino acid code. The *sir3-5*μ allele contains the five mutations near the *Bgl*II site and the wild-type proline at position 898. The *sir3-P898R* allele is wild type for all amino acids except the proline-to-arginine missense mutation at position 898. (B) Quantitative mating assays of the different *sir3*^{rlf3} alleles. Values shown are the median of at least four individual mating assays. The differences between the strains were not statistically significant as determined by the rank sum test (Snedecor and Cochran 1980). Strains used were as follows: WT, YJB487; *sir3*^{rlf3}, YJB499; *sir3-P898R*, YJB1267; *sir3-P898R*, *-5*μ, YJB2670; *sir3-5*μ, YJB2728. (C) Telomeric silencing assays of the different *sir3*^{rlf3} alleles. Tenfold serial dilutions of each strain were plated onto medium containing 5-fluoro-orotic acid (5-FOA) and SDC (complete) medium lacking uracil. Strains used were as follows: WT, YJB487; URA+, YJB1250; *sir3*^{rlf3}, YJB499; *sir3-P898R*, YJB1267; *sir3-P898R*, *-5*μ, YJB2670; *sir3-5*μ, YJB2728.

derepression than are mating assays. We constructed a series of isogenic strains carrying the *sir3-R898P* allele and either *HMR::TRP1* (including the intact silencer) or derivatives missing binding sites for either ORC, Abf1p, or Rap1p and compared the ability of these strains to grow on medium lacking tryptophan with the growth of isogenic *SIR3* strains (Figure 3). As previously reported (*e.g.*, Sussel and Shore 1991), of the *SIR3* strains, only the strain missing the *HMR E* Rap1p site grew on medium lacking tryptophan (Figure 3). Of the *sir3-P898R* strains, the strain with the wild-type *HMR::TRP1* allele and the strain with the *HMR e*-ΔAbf1 did not grow in the absence of tryptophan. However, the *sir3-P898R HMR e*-ΔORC site strain grew in the absence of tryptophan. Similarly, the *sir3-P898R HMR e*-ΔRap1 site strain grew very well in the absence of tryptophan. In this case the *TRP1* gene was more derepressed than in the corresponding *SIR3 HMR e*-ΔRap1 strain, as evidenced by the larger size of the Trp^{+} colonies as well as by the increased frequency of Trp^{+} colonies. Because we observed a synergistic loss of silencing when we combined *sir3-P898R* with mutations that affect the establishment of silencing [*sir1* and *HMR e*-ΔORC site (Sussel *et al.* 1993)], these results are consistent with the hypothesis that the *sir3-P898R* allele is defective in the maintenance, but not the establishment, of silencing.

***sir3-P898R* is not defective in the establishment of silencing:** At the *HM* loci, the *de novo* establishment of silencing is critically dependent on Sir1p. In *sir1* mutant cells, *HML* exists in one of two epigenetic states: silent (off) or derepressed (on; Pillus and Rine 1989). Furthermore, the process of restoring the silent state to derepressed cells is very inefficient, requiring >40 generations (Pillus and Rine 1989).

Experimentally, the establishment of *HML* silencing can be observed by monitoring the α-factor response of *MAT***a** cells (Pillus and Rine 1989). Wild-type *MAT***a** cells arrest in response to α-factor, forming cells with a single shmoo. *MAT*a cells carrying mutations that completely derepress *HML* (*e.g.*, *sir3*Δ strains or *sir3-8*^{ts} cells held at 37°) do not respond to α-factor, continue dividing, and form colonies of cells. *MAT***a** *sir1* cells respond in one of two ways: those that are silent at *HML* form

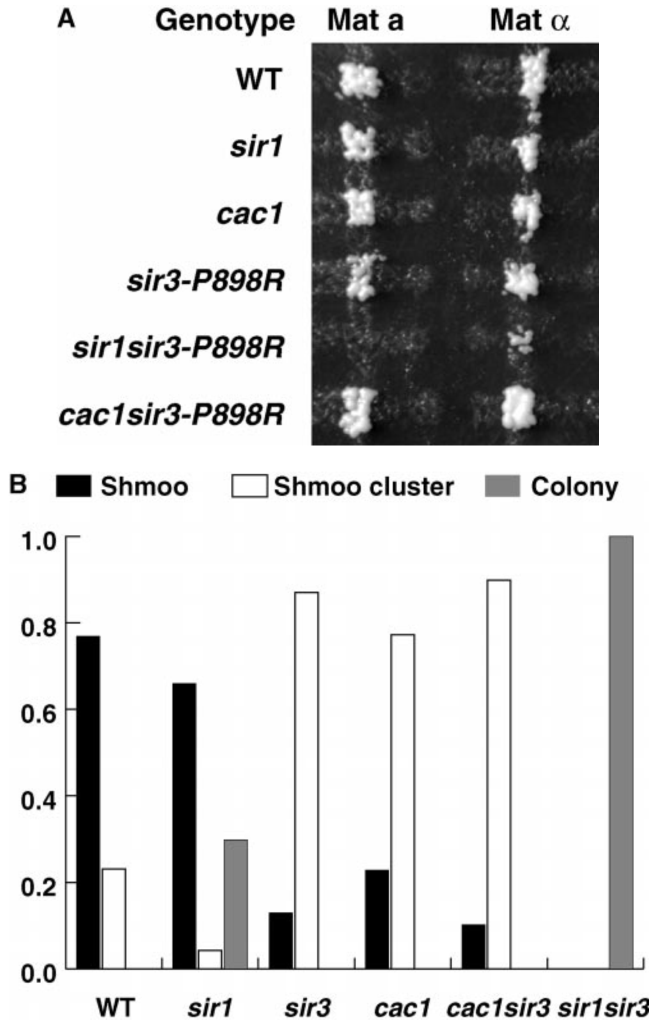

Figure 2.—*SIR1*, but not *CAC1*, is required for mating in *sir3-P898R* strains. (A) *sir3-P898R sir1* mutants have an obvious mating defect. Patch mating assays were performed using tester strains A364A and B364B. Strains used (*MAT***a** and *MAT*α, respectively) were as follows: WT, YJB195 and YJB209; *sir1*, YJB1940 and YJB1941; *cac1*, YJB1838 and YJB1578; *sir3*, YJB2352 and YJB1504; *sir1 sir3*, YJB2315 and YJB2316; *cac1 sir3*, YJB2354 and YJB2353. All *sir3* alleles were *sir3-P898R.* (B) *sir3-P898R* strains, like *cac1* strains, form shmoo clusters in response to α-factor. Cells were plated on α-factor at 23° for 18 hr and cell morphology was assayed. WT, YJB195; *sir1*, YJB1940; *cac1*, YJB1838; *sir3*, YJB2352; *sir1 sir3*, YJB2315; *cac1 sir3*, YJB2354. Shmoos, single cells with a single mating projection; shmoo clusters, cells or groups of cells with two or more elongated mating projections; colonies, groups of round cells that did not appear to respond to α-factor.

shmoos and those that are derepressed at *HML* form colonies. To ask if a strain carrying the *sir3-P898R* allele, like *sir1* strains, is defective in the establishment of silencing, we analyzed the α-factor responses of a *sir3-P898R* strain and a *sir1 sir3-P898R* strain. Unlike *sir1* strains [but similar to the response of *cac1* strains (Enomoto and Berman 1998)], the entire population of *sir3-P898R* cells initially formed shmoos that subsequently gave rise to shmoo clusters (Figure 2B). This indicates that *HML* in the *sir3-P898R* cells cannot be considered to be in different epigenetic states. Furthermore, the shmoo cluster phenotype is indicative of a defect in the maintenance of the silent state, since cells initially form shmoos (and thus have a silent *HML* locus), but the silent state is not as persistent in the mutant cells as it is in wild-type cells (Enomoto and Berman 1998). In contrast, *sir1 sir3-P898R* double-mutant cells are completely derepressed at *HML*, growing as a relatively uniform population of α-factor resistant colonies (Figure 2B). This result also supports the idea that *sir1* and *sir3-P898R* do not affect the same aspect of silencing.

We previously noted that limiting the amount of Sir3p alone resulted in increased levels of shmoo clusters in otherwise wild-type cells (Enomoto and Berman 1998). All of the *sir3-P898R* phenotypes could be explained if Sir3-P898Rp was less stable than wild-type Sir3p. Immunoblot analysis detected similar steady-state levels of Sir3p in strains carrying the wild-type and any of the other *sir3*^{rlf3} mutant alleles including *sir3-P898R* as shown in Figure 1 (M. McClellan and S. Enomoto, unpublished data). Thus, the silencing phenotypes observed in the *sir3*^{rlf3} mutants are not due to a significant reduction in the stability of the mutant Sir3 proteins.

A second way to examine the role of *SIR1*-dependent *de novo* silencing is to monitor the kinetics of the restoration of mating competence by providing Sir3p to cells that were derepressed because they lacked Sir3p function. *SIR1*-dependent *de novo* establishment of silencing must include the recruitment of Sir3p to the *HML* locus and propagation of a silent chromatin structure that includes Sir3p. If Sir3-P898Rp is defective in being recruited by Sir1p, then we would expect a delay in the *de novo* formation of silent chromatin in a strain expressing *sir3-P898R.* We used the *sir3-8*^{ts} allele (Miller and Nasmyth 1984) to generate cells in which *HML* was completely derepressed. Wild-type or mutant forms of Sir3p were provided by inducing P_{GAL}-*SIR3* or P_{GAL}-*sir3-P898R* expression on medium containing galactose. Cells were pregrown at 37° on glucose to eliminate all silencing (Figure 4A, time 0). The cells were then streaked to medium containing galactose (to induce the expression of P_{GAL}-*SIR3* or P_{GAL}-*sir3-P898R*) held at 37° (to keep *sir3-8*^{ts} inactive) for different pulse time periods. Then the restoration of mating competence was tested by streaking across a tester strain on glucose medium (to repress P_{GAL}-*SIR3* or P_{GAL}-*sir3-P898R* expression) held at 37°. The crosses were then replica-plated to glucose medium held at 37° that was selective for diploids resulting from successful mating (Figure 4A).

SIR1 strains expressing either P_{GAL}-*SIR3* or P_{GAL}-*sir3-P898R* displayed similar mating kinetics: they restored mating ability to the *sir3-8*^{ts} strain within 1.5–2.5 hr (Figure 4A). The *sir1* strains did not restore mating for up to 4 hr and the kinetics of the appearance of mating competence was similar in the strains expressing P_{GAL}-*SIR3* or P_{GAL}-*sir3-P898R.* The efficiency of mating at the

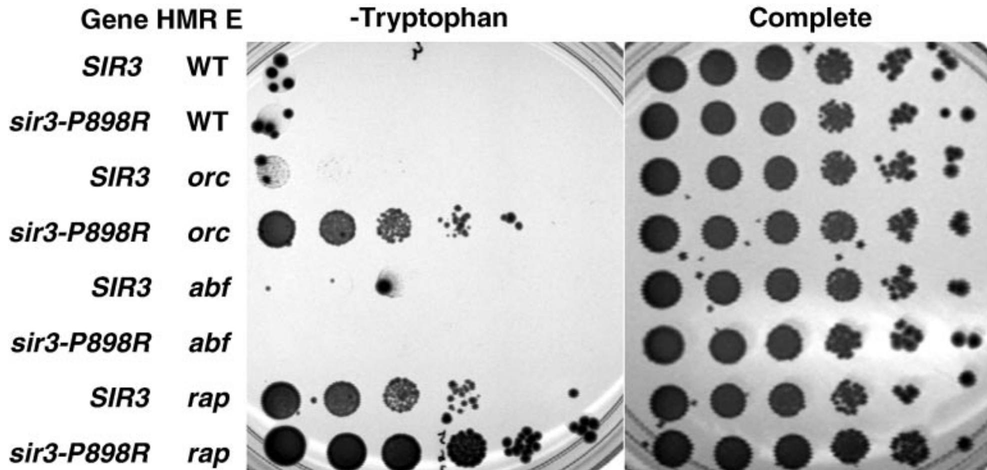

Figure 3.—At *HMR* E, deletion of the ORC or Rap1p binding sites compromises *HMR* silencing in *sir3-P898R* strains. Assay for the expression of *TRP1* inserted at *HMR* E [wild type or missing the ORC binding site (orc), the Abf1p binding site (abf), or the Rap1p binding site (rap)] was performed by plating 10-fold serial dilutions onto medium lacking tryptophan or complete medium. Colonies were photographed after 2 days of growth at 30°. Strains used were as follows: *SIR3 WT*, YJB959; *sir3-P898R* WT, YJB1544; *SIR3 orc*, YJB955; *sir3-P898R orc*, YJB1563; *SIR3 abf*, YJB1143; *sir3-P898R abf*, YJB1539; *SIR3 rap*, YJB1104; *sir3-P898R rap*, YJB1532.

earliest time points was slightly lower for both of the *sir3-P898R* strains relative to the *SIR3* strains. However, the kinetics of the appearance of some mating-competent cells were similar when either P_{GAL}-*SIR3* or P_{GAL}-*sir3-P898R* was expressed (Figure 4A). Thus, *Sir3-P898R*, like wild-type *SIR3*, was sufficient to restore silencing to chromatin that was previously in a transcriptionally active state. The fact that the kinetics of the appearance of mating competence was similar in the *SIR3* and *sir3-P898R* strains suggests that *sir3-P898R* is not defective in the ability to be recruited to *HML* and to initially form silent chromatin.

In the *sir1*Δ strains, silencing and mating competence only appeared after much longer periods of time (∞ in Figure 4A), indicating that we can detect the *sir1*-independent establishment of silencing in this assay. The amount of time required in our assay was similar to that required (∼2 days, >30 generations) for the subpopulation of *HML*-derepressed *sir1* cells to become silenced as monitored by arrest and shmooing in response to α-factor (Pillus and Rine 1989; S. Enomoto, unpublished data).

***sir3-P898R* strains are defective in the maintenance and/or inheritance of telomeric silencing:** The inheritance of silencing is defined as the ability of silent mother cells to produce silent daughter cells. Inheritance can only be monitored if the silent state is maintained during the previous cell cycle. Cells that inherit silent chromatin do not need to assemble the silent chromatin *de novo*, in part because the components of silent chromatin are already present at the silent loci. Monson *et al.* (1997) examined the "inheritance" of the silent state of a telomere-adjacent *URA3* gene by pregrowing cells on 5-fluoroorotic acid (5-FOA) to enrich for those that were silent. They then examined the proportion of cells that formed daughter cells in which the telomeric *URA3* gene remained silent. Because 5-FOA is toxic to any cell that becomes derepressed, cells that continue to divide are those that both maintained and inherited silent chromatin. Based on the assumption that any cell that failed to maintain the silent state would die on 5-FOA prior to the establishment of a *de novo* silent state, this assay measures only establishment-independent contributions to silencing. It cannot, however, distinguish between defects in the maintenance or the inheritance of silencing.

To measure the effect of the *sir3-P898R* allele on the inheritance and/or maintenance of telomeric silencing, we pregrew a strain YJB1267 on 5-FOA, transferred the cells to a fresh 5-FOA plate, and examined the size of the microcolonies formed after 18 hr of growth. The size of the microcolonies formed by the sir3-P898R strain were smaller (∼40–60 cells/microcolony in most cases and occasional appearance of microcolonies with ∼100 cells/microcolony) than the microcolonies formed by the *SIR3* strain (∼100–>1000 cells/microcolony; Figure 4B). In fact, colonies formed by wild-type *SIR3* cells were comparable in size to colonies formed by a *ura3* strain (Figure 4B). Since the size of the microcolonies is a function of either maintenance and/or inheritance, this indicates that the *sir3-P898R* allele is defective in at least one of these functions.

The shmoo cluster assay measures the ability of cells to maintain the silent state during a single cell cycle. This 5-FOA survival assay measures the ability of cells to maintain and/or inherit the silent chromatin state. While it is formally possible that strains carrying the *sir3-P898R* allele are defective in both the inheritance and the maintenance of silencing, a defect in maintenance alone can account for all of the observed silencing defects in strains carrying the *sir3-P898R* allele.

Interactions of Sir3-P898Rp with Rap1p, Sir3p, Sir4p,

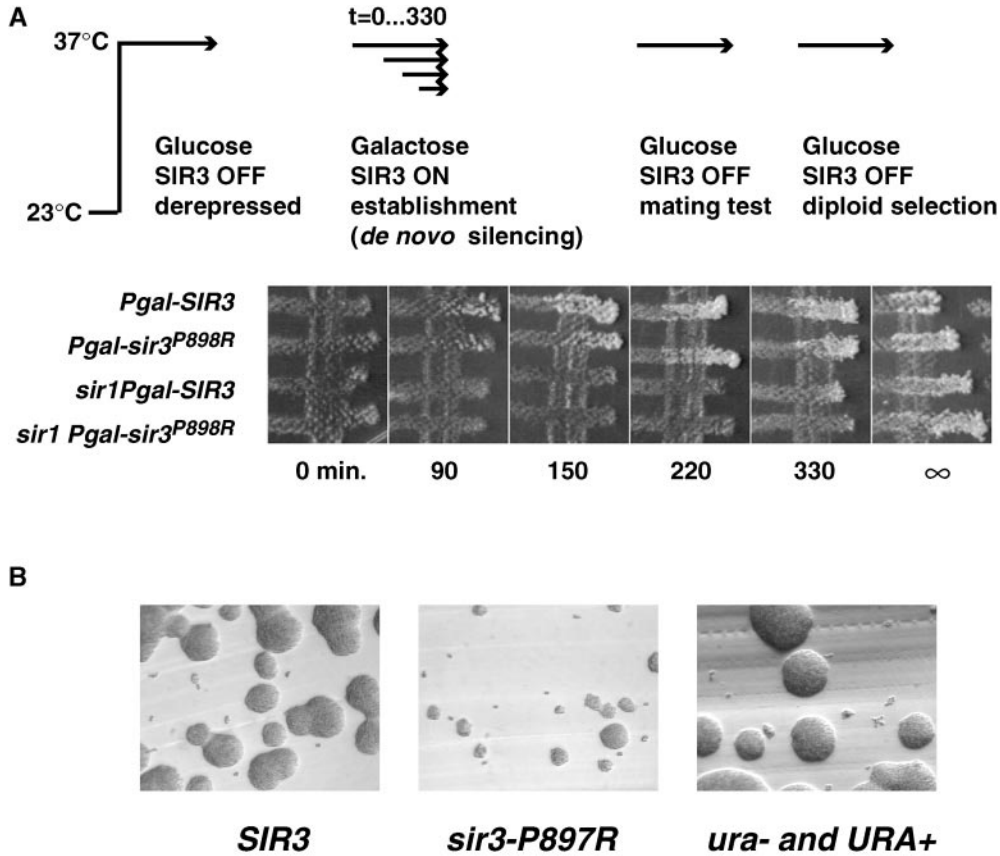

Figure 4.—*sir3-P898R* does not affect the kinetics of the establishment of HM silencing but affects the maintenance and/or inheritance of telomeric silencing. (A) Patch mating assay for the ability to establish silencing. All strains carry the $sir3\text{-}8^{ts}$ allele at the *SIR3* locus P_{GAL}-*SIR3* or P_{GAL}-*sir3-P898R*. Strains were pregrown at 23° (permissive temperature) on glucose medium and then shifted to 37° (restrictive temperature) for 24 hr. Cells were moved to medium containing galactose to induce the expression of P_{GAL}-*SIR3* or P_{GAL}-$sir3^{rlf3}$. At the indicated times following galactose induction, mating ability was assayed by streaking the strain (from left to right) across a tester strain (B364B; streaked vertically) on glucose medium at 37°. [Time (min), 0 cells were not exposed to galactose medium at all.] Diploids were identified by their ability to grow on medium lacking histidine and appear at, and to the right of, the tester strain streak. Strains used were as follows: P_{GAL}-*SIR3*, YJB2769 + pSE1071; P_{GAL}-$sir3^{rlf3}$, YJB2769 + pSE1072; *sir1* P_{GAL}-*SIR3*, YJB2930 + pSE1071; and *sir1* P_{GAL}-$sir3^{rlf}$, YJB2930 + pSE1072. (B) Microcolony assay for the inheritance and/or maintenance of silencing on FOA. *SIR3* (YJB487, left) and *sir3-P898R* (YJB1267, middle) strains carrying a telomere-adjacent *URA3* gene were pregrown on FOA to enrich for cells in which *URA3* was silent. A mixture of Ura^+ and Ura^- cells (right) was generated by growing YJB276 (*ura3*) transformed with vector YGALSET352 (*URA3*) onto complete medium to allow loss of plasmid from a proportion of the cells. The mixture of YJB276 cells (containing and not containing the plasmid) was then restreaked at low cell density onto FOA medium. All strains were photographed after 18 hr of growth on FOA at 30°.

Rad7p, and histones H3 and H4: The C-terminal domain of Sir3p (including amino acid 898) interacts with Sir3p, Sir4p, Rap1p, and Rad7p in the yeast two-hybrid system (Moretti *et al.* 1994; Paetkau *et al.* 1994). We compared the ability of the C terminus (aa 307–978) of Sir3p and Sir3-P898Rp to interact with Rap1p, Sir4p, and Rad7p using two-hybrid constructs that were originally used to reveal Sir3p interactions. In all these cases, we detected no significant difference in the interactions between the proteins and either Sir3p or Sir3-P898Rp. However, when we analyzed the Sir3p-Sir3p interactions of the mutant and wild-type proteins we found a significant difference: the Sir3-P898Rp with Sir3-P898Rp interaction was increased relative to the wild-type Sir3p with wild-type Sir3p interaction (Table 3).

Coprecipitation experiments have also demonstrated that Sir3p binds the unacetylated N-terminal tails of histones H3 and H4 *in vitro* (Hecht *et al.* 1995). Because the *sir3-P898R* mutation maps within the histone H3/H4 interaction domain of Sir3p (Hecht *et al.* 1995), we asked if the *sir3-P898R* mutation affected interactions between Sir3p and histones H3 and H4. Coprecipitation experiments were performed with either Sir3p or Sir3-P898Rp and either GST-HHT (aa 1–46) or GST-HHF (aa 1–34) (Hecht *et al.* 1995) (Figure 5). Sir3p and Sir3-

TABLE 3

Transcriptional activation of hybrid proteins

	Activation domain[a]	
DNA binding domain	pACT II-Sir3	pACT II-Sir3-P898R
LexA	3	3
LexA-Rap1	521	470
LexA-Rap1	157	100
LexA-Sir3	21	89[b]
LexA-Sir4	149	187
LexA-Rad7	554	640

[a] β-Galactosidase units were normalized against P_{ADH}-LEX-$Gal4_{AD}$ as 1000 units. The median value of at least four independent transformants is indicated.

[b] The difference between LexA-Sir3/pACTII-Sir3 and LexA-$Sir3^{rlf3}$/pACTII-$Sir3^{rlf3}$ was significant ($P < 0.05$). Differences for all other pairs were not significant at this level.

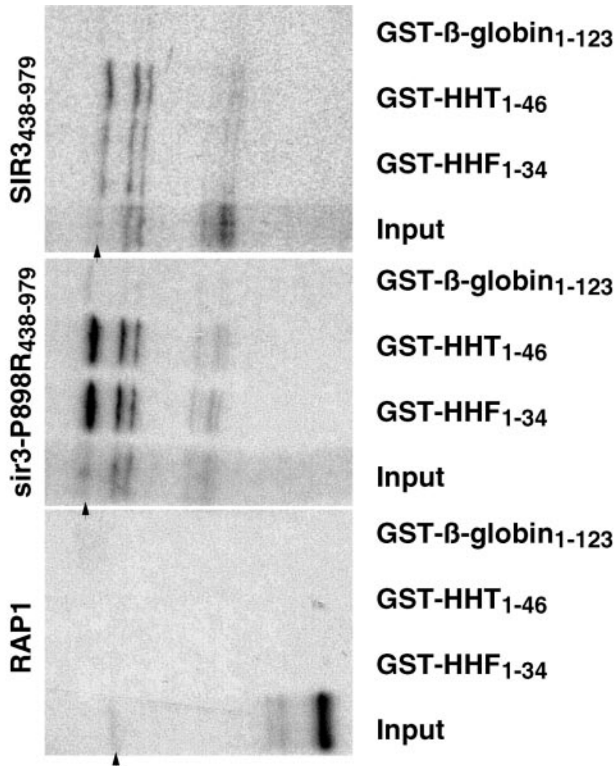

Figure 5.—Sir3-P898Rp associates with the N termini of histones H3 and H4 *in vitro.* GST-β-globin$_{1-123}$, GST-H3$_{1-46}$, and GST H4$_{1-34}$ were produced in *E. coli* and purified by glutathione affinity chromatography. Rap1p, Sir3p, and Sir3-P898Rp, produced by coupled *in vitro* transcription and translation in the presence of [^{35}S]methionine, were incubated with the immobilized GST proteins. After washing, proteins were eluted by denaturation, separated by SDS-PAGE, and detected by autoradiography. Input, 10% of total transcription and translation reaction. Other lanes represent 100% of the protein eluted from the GST proteins. Small arrows indicate full-length Rap1p, Sir3-P898R-C, and Sir3C, respectively.

P898Rp were expressed as fusions with an N-terminal histidine$_6$-T7-gene-10-epitope tag by *in vitro* transcription and translation (see materials and methods). As a negative control, we used a histidine$_6$-T7-gene-10-epitope-tagged Rap1p (Enomoto *et al.* 1998). As expected, the wild-type Sir3p fusion protein coprecipitated with histones H3 and H4 and the Rap1p fusion protein did not coprecipitate with either of the histones (Figure 5). Like Sir3p, Sir3-P898Rp coprecipitated with histones H3 and H4 and the affinity of the wild-type and mutant Sir3 proteins for the histones was indistinguishable (Figure 5). Thus, the *sir3-P898R* mutation did not alter the ability of the protein to interact, *in vitro*, with unacetylated N-terminal tails of histones H3 and H4.

The *sir3-P898R* C terminus confers a strong nonmating phenotype: Interestingly, during our analysis of Sir3-P898Rp interactions, we found that two-hybrid plasmids (both "binding domain" and "activation domain" constructs) carrying codons 307–978 of *sir3-P898R* interfered with the mating ability of the otherwise wild-type two-hybrid reporter strain (Figure 6). Expression of these plasmids in other *MAT***a** strains resulted in a similar nonmating phenotype (data not shown). The Sir3-P898R 307-978 plasmids also conferred a nonmating phenotype on *MAT*α strains (data not shown). Similarly, a strain carrying *TRP1* within the *HMR* locus was Trp$^+$ when the Gal4-AD-*sir3-P898R* allele was expressed (data not shown). During the course of these studies, overproduction of the C terminus of wild-type Sir3p was reported to interfere with telomeric silencing (Le *et al.* 1997; Gotta *et al.* 1998; Park *et al.* 1998). We observed a similar effect with the C-terminal fragments of both Sir3p and Sir3-P898R (Figure 6). However, the mutation in *sir3-P898R* causes an increased level of derepression relative to the wild-type *SIR3* allele: the Sir3-P898Rp C-terminal fragment (Sir3-P898R-C) caused a complete loss of mating competence in patch mating assays (Figure 6). These results indicate that the *sir3-P898R* mutation enhances the ability of the Sir3p C terminus to derepress silencing at both telomeres and the *HM* loci.

The C terminus of Sir3-P898Rp disrupts silencing specifically during late S-phase: To better understand how the C-terminal fragment of Sir3-R898Pp interferes with *HM* silencing, we asked whether the disruption of silencing by the mutant protein occurred during a particular stage of the cell cycle. One possibility was that Sir3-R898Pp could interfere with silencing at any stage of the cell cycle, perhaps by titrating away a component of the normal silent chromatin complex. Another possibility was that Sir3-R898Pp could interfere with silencing by being physically assembled into the silent chromatin complex in late S-phase. For these experiments we expressed the C-terminal portion of Sir3-R898P from the galactose-inducible *GAL10* promoter. Cells were pregrown in glucose, which prevented P_{GAL}-*sir3-R898P* expression; in these cells the *HM* loci were repressed as evidenced by their sensitivity to α-factor. Cells were then arrested in G_1 by the addition of α-factor, in S-phase by the addition of hydroxyurea, or in M-phase by the addition of nocodazole. Four hours later, cells were shifted to medium containing the same cell cycle inhibitor plus galactose, to induce expression of *sir3-R898P*$_{307-979}$ during the cell cycle arrest. Cells were held under these conditions for 18 hr, washed three times with fresh glucose medium, and released into glucose medium for a brief recovery period. α-Factor was then added to the medium to monitor the mating response of the *MAT***a** cells to α-factor in the subsequent cell cycle. Most of the cells that had been arrested in G_1 with α-Factor or in M-phase with nocodazole during the induction of the C-terminal fragment of Sir3-R898P responded to α-factor in the subsequent cell cycle by arresting and forming a mating projection (Figure 7), indicating that the silent mating loci remained silent in these cells despite the presence of the *sir3-R898P* C terminus. In contrast, 66% of the cells that had been arrested with hy-

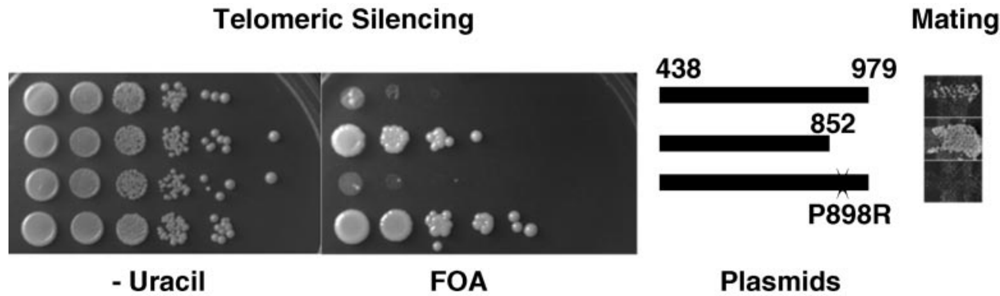

Figure 6.—High-level expression of the *sir3-P898R* C terminus disrupts both telomeric silencing and mating in a dominant negative manner. For the telomeric silencing assays (left), strain YJB1633 transformed with pSE856 (*SIR3*$_{438–979}$), pSE647 (*SIR3*$_{438–852}$), pSE615 (*sir3-P898R*$_{438–979}$), or pACTII (vector only) was plated in 10-fold serial dilutions onto SD-Ura or SD + FOA and grown for 2 days at 30°. For the mating assay (far right), YJB905 transformed with pSE856 (*SIR3*$_{438–979}$), pSE647 (*SIR3*$_{438–852}$), or pSE615 (*sir3-P898R*$_{438–979}$) was crossed to tester strain YJB199 and replica-plated to SD-Ade −Leu to select for diploids.

droxyurea were α-factor resistant, indicating that the *HML*α silencing had been perturbed in the majority of these cells. Control cells carrying only the vector and arrested with hydroxyurea continued to respond to α-factor (S. Enomoto, unpublished data). These results indicate that Sir3-R898Pp interferes with the assembly of silent chromatin during or just after replication. This could occur either by being incorporated directly into the chromatin or by interfering with the assembly of another component required for the complete silencing of the *HM* loci.

Tethering wild-type Sir3p cannot bypass the *sir3-P898R* silencing defect: Telomeric silencing is thought to be nucleated by the binding of Rap1p at the telomere repeats, recruitment of the Sirp complex (by the Rap1p C terminus interacting with Sir3p and Sir4p), and propagation of the Sirp complex onto the telomere-adjacent DNA. In a *rap1-17* strain, telomeric silencing does not occur because the Rap1-17p does not interact with Sir3p and Sir4p. However, tethering LEXA-Sir3p to telomere-adjacent lexA operator sites in a *rap1-17* strain permits silencing of a telomeric *URA3* gene (Lustig *et al.* 1996). If the major defect in *sir3-P898R* is in an early step of silencing, such as recognizing and binding to proteins like Rap1p, or being recruited to the Sirp complex, it might be possible to suppress this defect by tethering wild-type Sir3p, to weakly bypass early initiation steps. We compared the ability of wild-type Sir3p to generate silent chromatin in a *rap1-17 SIR3* strain and a *rap1-17 sir3-P898R* strain (Figure 8). As previously reported, the *rap1-17 SIR3* strain expressing pLEX-*SIR3* produced FOA-resistant colonies at a frequency of 10^{-3} (Lustig *et al.* 1996). In contrast, tethering pLEX-*SIR3* in the *rap1-17 sir3-P898R* strain did not bypass the *rap1-17* silencing defect: no FOA-resistant colonies were observed (Figure 8). Thus, the silencing defect in *sir3-P898R* strains is seen both when tethered Sir3p initiates or when normal telomere sequences initiate silencing, implying that the defect in *sir3-P898R* cannot be in an early step in silencing. Rather, the major defect must be a problem in either the propagation/assembly of the silent chromatin/Sirp complex or in the stable maintenance of the complex. This assay does not allow us to distinguish between a defect in the assembly process, which would form an unstable Sirp complex structure, and a defect in Sirp complex structure itself.

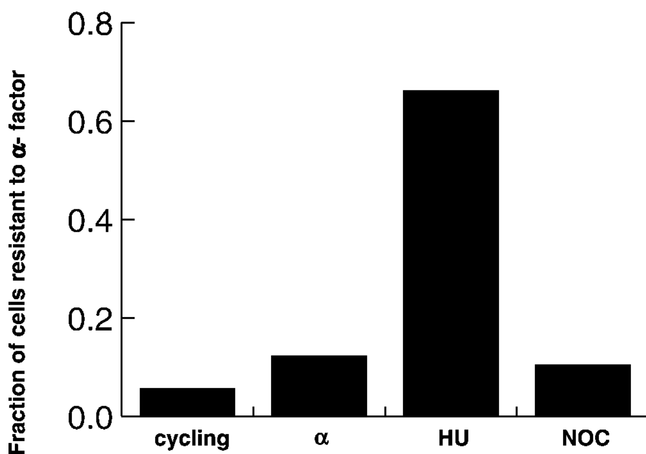

Figure 7.—High-level expression of the *sir3-P898R* C terminus preferentially disrupts silencing in cells arrested with hydroxyurea. Strain YJB276 transformed with pSE715 [P_{GAL}-*sir3-P898R*$_{438–979}$ was pregrown in glucose medium, arrested with α-factor (α), hydroxyurea (HU), or nocodazole (NOC), or allowed to cycle in the presence of galactose (to induce expression of Sir3-P898R-*C*)]. Cells were released from the cell cycle inhibitors in the presence of glucose and tested for the ability to respond to α-factor. χ^2 tests indicated that cells released from hydroxyurea arrest were significantly different from the other treatments.

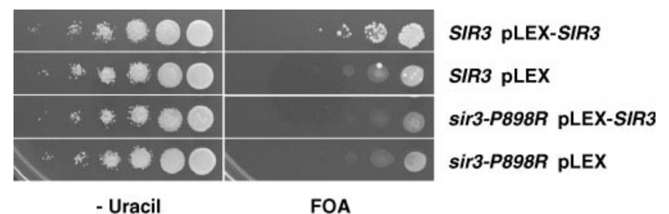

Figure 8.—Tethered LexA-Sir3p does not restore silencing to a *rap1-17 sir3-P898R* strain. Strains YJB5094 (*SIR3 rap1-17*) or YJB5093 (*sir3-P898R rap1-17*) were transformed with pBTM116 (LEX$_{BD}$) or pM392 (LEX$_{BD}$-*SIR3*). Tenfold serial dilutions were plated onto FOA and SDC-Ura plates. Cells were grown for 2 days (−uracil) or 3 days (FOA).

DISCUSSION

sir3-P898R is an interesting allele of *SIR3* that allows us to dissect some of the separable roles of Sir3p in the processes of establishing, maintaining, and inheriting silent chromatin. Sir3p is an important component of silent chromatin at both telomeres and at the *HM* loci. Here we identified and characterized *rlf3*, an allele of *SIR3* that affects TEL + CEN antagonism, Rap1p localization, and telomeric silencing without an obvious effect on *HM* silencing. The original *sir3*^{rlf3} allele included a point mutation near the C terminus (R898P) that accounts for the majority of the phenotypes observed in the original allele. Five additional mutations slightly enhanced the telomeric silencing defect in the original *sir3*^{rlf3} allele.

We used several assays to analyze the silencing defect in *sir3-P898R* strains. In qualitative and quantitative assays, *sir3-R898P* did not cause any obvious defects in *HML* or *HMR* silencing (Figures 1B, 2A, and 3). Yet in combination with mutations that affect the establishment of *HM* silencing [*e.g.*, *sir1* or ORC site silencer mutations (Pillus and Rine 1989; Sussel *et al.* 1993)], *sir3-P898R* caused a significant loss of *HM* silencing and mating competence (Figures 2A and 3). This synergistic decrease in silencing between mutations in *sir1* (or silencer sites) and *sir3-P898R* can be interpreted in a number of ways. One possibility is that the two genes (*e.g.*, *SIR1* and *SIR3*) encode proteins that have similar, at least partially redundant, functions and when both are missing the function cannot be accomplished. An alternative interpretation is that the two genes encode proteins that have distinct functions that are dependent upon one another. We favor the latter interpretation because we do not have any data to support the idea that *sir3-P898R* affects the establishment of silencing. Using more sensitive assays, we found that *sir3-P898R* strains formed primarily shmoo clusters in response to α-factor (Figure 2B), indicating a defect in the ability to sustain the silent state of *HML*. This is very different from how *sir1* mutations affect silencing: *sir1* mutants either arrest or divide in response to α-factor and do not form shmoo clusters. In addition, the kinetics of the establishment of *de novo* silencing at *HML* was similar for Sir3-P898Rp and wild-type Sir3p (Figure 4A), indicating that initial steps of establishment were not defective in *sir3-P898R* strains, and thus implying that it is later steps in silencing that are affected. At a *URA3*-marked telomere, *sir3-P898R* strains grown on FOA had a defect in the maintenance and/or inheritance of the silent state (Figure 4B). Furthermore, while wild-type tethered Sir3p was able to initiate silencing (Lustig *et al.* 1996), it could not do so in a *sir3-P898R* strain (Figure 8), indicating that silencing in *sir3-P898R* was defective even if the initiation step was provided (by bypassing normal initiation via tethered Sir3p at the telomere). This implies that a step after the initiation of silencing is defective in *sir3-P898R* strains. The simplest explanation consistent with all of these results is that the primary defect in *sir3-P898R* strains is a defect in the maintenance of silencing. While we cannot rule out the possibility that Sir3-P898Rp has additional defects in the inheritance of a silent chromatin structure, a defect in the maintenance of silencing is sufficient to account for all of the results observed.

Sir3-R898P-C has a strong dominant negative effect on *HM* silencing, especially during S-phase: Sir3-C interacts with other proteins (*e.g.*, Rap1p, Sir4p, Sir3p, and the N termini of histones H3 and H4) that form stable silent chromatin. Like wild-type Sir3-C, Sir3-R898P-C has a dominant negative activity that interferes with telomeric silencing. The assembly of silent chromatin requires passage through S-phase (Miller and Nasmyth 1984), presumably because the state of the chromatin is reformed following passage of the replication fork. We found that cells released from HU in the presence of Sir3-R898P-C did not remain in the silent state efficiently while cells released from either α-factor or nocodazole usually remained in the silent state despite the presence of Sir3-R898P-C. This result is consistent with the idea that Sir3-R898P-C affects *HM* silencing by being assembled directly into the chromatin following early S-phase, and likely after the replication of the silent chromatin. Alternatively, Sir3-R898P-C may interfere with silencing during S-phase by associating with some factor that is required for the formation of silent chromatin especially during S-phase. In either case, the interference with silencing must have occurred sometime between early S and G_2, since expression of Sir3p-R898P-C during and after release from nocodazole did not have much effect on silencing. The interference in silencing may be due to the increased affinity of Sir3-P898R-C for other Sir3-P898R-C molecules as detected in the two-hybrid assays (Table 3).

These results differ slightly from the previous studies of silencing and cell cycle of Gottschling and colleagues, who found that silent chromatin is most accessible to transcription factors during arrest with nocodazole (which holds cells at G_2/M; Aparicio and Gottschling 1994). An important difference between these two series of experiments is that here we analyzed the maintenance of silencing after release from a cell cycle arrest, while Aparicio and Gottschling (1994) monitored transcription factor accessibility in cells during cell cycle arrest. The dynamics of chromatin accessibility are likely to be different when cells are cycling than when cells are arrested. Support for this idea comes from studies that monitored the state of excised chromosomal *HM* loci containing or lacking the adjacent silencer sequences (Holmes and Broach 1996; Bi and Broach 1997; Cheng *et al.* 1998; Ansari and Gartenberg 1999). The inheritance of the silent state into the next generation (involving passage of S-phase) requires functional silencers in *cis* (Holmes and Broach 1996;

Cheng *et al.* 1998). If silencers are excised from the silent DNA, *HML* silencing is maintained during arrest on α-factor (Holmes and Broach 1996), while chromatin is altered during arrest with nocodazole (Bi and Broach 1997). Yet *HM* silencing is not maintained well in cells moving through the cell cycle (Bi and Broach 1997; Cheng *et al.* 1998), even if the DNA is not replicated (Cheng *et al.* 1998).

sir3-P898R **may interfere with silencing by increasing the affinity of Sir3p-Sir3p interactions:** Sir3p interacts with many components of silent chromatin (reviewed in Stone and Pillus 1998) and many of these interactions are necessary for silent chromatin function. We envision three types of molecular interactions that Sir3p may use to contribute to silencing: nucleation, propagation, and stabilization.

1. Sir3p would contribute to the *nucleation* of silencing at both telomeres and the *HM* loci by interacting with proteins such as Rap1p (Moretti *et al.* 1994) and Sir4p (Moazed *et al.* 1997) that associate with the silencer sequences (Lustig *et al.* 1996). (In tethering experiments, this type of interaction is bypassed.)
2. Sir3p may interact with Sir3p and Sir4p and with nucleosomes to *propagate* the assembly of the Sirp complex (Moazed *et al.* 1997; Strahl-Bolsinger *et al.* 1997).
3. Interactions between Sir3p and the other components of the silent chromatin, including Sir proteins and nucleosomes, may *stabilize* contacts between the Sirp complex and the silenced DNA (Hecht *et al.* 1996; Strahl-Bolsinger *et al.* 1997).

Finally, we propose that the relative strength of the interactions between Sir3p and proteins at the silencer site, proteins in the Sir complex, and proteins in the nucleosomes must be balanced so that silent chromatin is appropriately organized on the DNA. If any one of these interactions is too strong [which is likely the case for *sir3-P898R-sir3-P898R* interactions (Table 3)], an aberrant structure that is less effective in overall silencing would be formed.

Since the kinetics of establishment are similar between *SIR3* and *sir3-P898R* strains (Figure 4A), our results suggest that the nucleation functions of Sir3p are not significantly affected in *sir3-P898R* mutants. The strong dominant negative effect of the Sir3-R898P-C allele on silencing is consistent with the fact that Sir3-R898P-C/Sir3-R898P-C interactions were stronger than Sir3C/Sir3C interactions in two-hybrid experiments. We propose that the stronger protein-protein interactions of *Sir3-P898Rp* relative to Sir3p perturb the stability of the Sirp complex, leading to a defect in the maintenance of silencing in *sir3-P898R* strains. That stronger protein-protein interactions can inhibit the function of complexes has been observed in other systems as well (Sandrock *et al.* 1997). Interestingly, *rap1-12* mutants also have stronger interactions with Sir4p in the two-hybrid system. In the case of *rap1-12*, however, the increased interaction between Rap1-12p and Sir4p caused reduced silencing at the *HM* loci because of a limiting supply of Sir4p that was preferentially interacting with the Rap1-12p present at higher levels at the telomeres. In the case of *sir3-P898R*, the altered Sirp complex interactions affect the maintenance of a silent Sirp complex, especially in regions of the genome where the establishment of silencing is less efficient.

Why are telomeres more vulnerable to defects in the maintenance of silencing? *sir3[rlf3]* strains have a dramatic reduction in telomeric silencing but only very subtle defects in *HM* silencing. Silencing at telomeres is less stable than *HM* silencing. This epigentic nature of telomeric silencing is likely due to less efficient establishment of silencing: tethering Sir1p to telomeres improves telomeric silencing (Chien *et al.* 1993), presumably by improving the establishment of silencing. In contrast, the establishment of silencing at *HM* loci is strong and partially redundant: two silencer sites, E and I, can nucleate silent chromatin, and Sir1p specifically improves the establishment of silencing at the *HM* loci. In fact, in *sir1* cells, *HML* behaves much like telomere-adjacent sequences in wt cells: it is silent in some cells and is actively expressed in others. And, in *sir1 sir3-P898R* strains, HM silencing is dramatically reduced.

Strains carrying mutations in *CAC1*, which encodes the large subunit of CAF-I, have many phenotypes similar to those seen in *sir3-P898R* strains. Both *sir3-P898R* and *cac1* mutations give rise to shmoo clusters and do not influence the kinetics of the *de novo* establishment of silencing (Figures 2B and 4A; Enomoto and Berman 1998), suggesting that they cause defects in the maintenance of silencing. Furthermore, *sir3-P898R* and *cac1* mutations each reduce telomeric silencing dramatically and have no obvious mating defects, yet exhibit significant reduction in mating efficiency when combined with *sir1* mutations (Figures 1 and 2). Other mutants exhibiting these characteristics include the *eso* mutants (Stone *et al.* 2000). We propose that, like *sir3-P898R*, other mutations that affect telomeric silencing, but not *HM* silencing, may do so because they affect mechanisms involved in the maintenance of silencing and because the establishment of silencing is naturally weaker at telomeres. We predict that such mutations will, like *sir3-P898R*, also affect *HM* silencing if establishment is weakened by mutations in the silencers or by mutation of *SIR1*. This is indeed the case for mutations in *CAC1/RLF2*, *CAC2*, *CAC3/MSI1*, *HHF1*, and *HHT1*: strains carrying mutations in these genes exhibit reduced *HM* silencing in combination with *sir1* mutations (Thompson *et al.* 1994; Enomoto and Berman 1998) and give rise to the shmoo cluster phenotype (Enomoto and Berman 1998).

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