Two Classes of *sir3* Mutants Enhance the *sir1* Mutant Mating Defect and Abolish Telomeric Silencing in *Saccharomyces cerevisiae*

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ABSTRACT

Silent information regulators, or Sir proteins, play distinct roles in chromatin-mediated transcriptional control at the silent mating-type loci, telomeres, and within the rDNA repeats of *Saccharomyces cerevisiae*. An unusual collection of *sir3* mutant alleles was identified in a genetic screen for enhancers of the *sir1* mutant mating-defective phenotype. These *sir3-eso* mutants, like the *sir1* mutant, exhibit little or no mating defects alone, but the *sir1 sir3-eso* double mutants are essentially nonmating. All of the *sir3-eso* mutants are defective in telomeric silencing. In some mutants, this phenotype is suppressed by tethering Sir1p to telomeres; other mutants are dominant for mating and telomeric silencing defects. Additionally, several *sir3-eso* mutants are nonmating in combination with the *nat1* N-terminal acetyltransferase mutant. The temperature-sensitive allele *sir3-8* has an *eso* phenotype at permissive temperature, yet acts as a null allele at restrictive temperature due to loss of sir3-8 protein. Sequence analysis showed that eight of the nine *sir3-eso* alleles have mutations within the N-terminal region that is highly similar to the DNA replication initiation protein Orc1p. Together, these data reveal modular domains for Sir3p and further define its function in silencing chromatin.

TRANSCRIPTIONAL silencing is one means by which cells regulate gene expression. Silencing occurs when chromatin structure is modified at certain regions of chromosomes, inactivating the genes in those regions. Examples of silencing include the inactive mammalian X chromosome, position effect variegation in Drosophila, and the silent mating-type loci in fission and budding yeasts (for reviews, see Laurenson and Rine 1992; Weiler and Wakimoto 1995; Allshire 1996; Lyon 1999). In *Saccharomyces cerevisiae*, at least three genetic loci are subject to transcriptional silencing: the silent mating-type loci, telomeres and the rDNA. Numerous genes play a role in silencing at these loci. *SIR1*, *SIR2*, *SIR3*, and *SIR4* were originally identified in mutant strains that inappropriately expressed the silent mating-type genes, generally leading to a mating-defective phenotype (Rine and Herskowitz 1987; Laurenson and Rine 1992). *SIR2*, *SIR3*, and *SIR4* also function in silencing genes positioned near telomeres (Aparicio *et al.* 1991; Vega-Palas *et al.* 1997; Pryde and Louis 1999). Sir2p, Sir3p, and Sir4p exist in a multiprotein complex that interacts with site-specific DNA binding proteins and with nucleosomes to mediate silencing (Moretti *et al.* 1994; Hecht *et al.* 1995, 1996; Moazed *et al.* 1997; Strahl-Bolsinger *et al.* 1997). Sir proteins are likely to be targeted to silent chromatin by Rap1p, Abf1p, and/or origin recognition complex (ORC) proteins, which bind directly to DNA sites within silencer sequence elements (Shore *et al.* 1987; Buchman *et al.* 1988; Sussel and Shore 1991; Bell and Stillman 1992; Foss *et al.* 1993; Liu *et al.* 1994; Loo *et al.* 1995). Chromatin is thought to be silenced through the interaction of Sir proteins with the N-terminal tails of histones H3 and H4 (Hecht *et al.* 1995). Sir2p plays an additional role in chromatin of the nucleolar rDNA array (Bryk *et al.* 1997; Smith and Boeke 1997). However, the molecular details of how Sir protein complexes achieve silencing remain incompletely defined.

A unique role for Sir1p in the establishment of silencing was demonstrated with the discovery that *sir1* mutants exhibit a heritable yet epigenetic mating-defective phenotype (Pillus and Rine 1989). In a population of *sir1* mutant cells, two subpopulations exist: one is mating competent and normally silenced as in wild type, the other is mating defective due to derepression of the silent mating-type loci. Although *sir1* mutants do not have a defect in the maintenance of silencing, it appears that silencing is not established efficiently in the subpopulation that is transcriptionally derepressed. How Sir1p functions in silencing is unclear, but mechanistic clues come from experiments in which Sir1p is tethered to regions of DNA lacking silencer sequences. When Sir1p is fused to the DNA binding domain of Gal4p, the fusion protein can be targeted to Gal4p-binding sites engineered near reporter genes to result in transcriptional silencing (Chien *et al.* 1993; Fox *et al.* 1997). Moreover, Sir1p can be shown to interact physically with the

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DNA replication initiation subunit Orc1p (Triolo and Sternglanz 1996). A central 17-amino-acid domain of Sir1p appears to direct it to silencers and is required for the interaction with Orc1p (Gardner *et al.* 1999). Sir1p is not known to participate directly with ORC in DNA replication, however, so the mechanistic significance of the Sir1p-Orc1p interaction is not yet understood.

Sir3p is a key component of silent chromatin (reviewed in Stone and Pillus 1998). It is an integral subunit of the multiprotein complex that functions at the silent mating-type loci and at telomeres. The *sir3* null mutant is nonmating and defective in telomeric silencing (Rine and Herskowitz 1987; Aparicio *et al.* 1991). Indeed, Sir3p is a limiting factor in telomeric silent chromatin (Renauld *et al.* 1993) and when tethered to DNA appears to recruit other proteins to achieve silencing (Lustig *et al.* 1996). Several *sir3* mutants were previously identified that suppress silencing defects of mutants in the histone H4 N terminus or the Rap1p C terminus (Johnson *et al.* 1990; Liu and Lustig 1996), providing genetic evidence for Sir3p-histone and Sir3p-Rap1p interactions. A recent study revealed that an N-terminal fragment consisting of approximately half of the Sir3p protein (Gotta *et al.* 1998) is sufficient for enhanced telomeric silencing previously seen with *SIR3* overexpression (Renauld *et al.* 1993). Additionally, three broad domains were identified to have different properties in nucleating telomeric silencing by assaying the ability of tethered Sir3p fusion proteins to silence in conjunction with a *rap1* telomere-defective mutant (Park *et al.* 1998). When an N-terminal region and a C-terminal region of Sir3p are expressed simultaneously, partial complementation of the *sir3* null mutant mating defect is observed, suggesting that the two halves can function independently (Le *et al.* 1997; Gotta *et al.* 1998). From these studies and the work described in this article, a picture of distinct functional domains for Sir3p emerges.

In a genetic screen for *e*nhancers of the *sir one* mutant mating defect (Reifsnyder *et al.* 1996), we uncovered a collection of mutants including those termed the *sir3-eso* mutants to emphasize their distinct phenotypes described here. A genetic interaction between *SIR1* and *SIR3* had previously been noted, in that overexpression of *SIR1* can suppress the mating defect associated with certain *sir3* alleles (Stone *et al.* 1991). The *sir3-eso* mutants provide additional evidence for specific *SIR1-SIR3* genetic interactions. Analysis of *sir3-eso* mutations revealed that the N-terminal domain of Sir3p is critical for silencing the *HM* silent mating-type loci and telomeres in the absence of *SIR1*. From sequence and phenotypic classification, the N-terminal region of Sir3p that is highly similar to the DNA replication initiation protein Orc1p is highlighted, suggesting a functional link between Sir1p, Sir3p, and Orc1p.

MATERIALS AND METHODS

Yeast strains, growth conditions, and transformation: Genotypes of yeast strains used in this study are listed in Table 1. Yeast extract/peptone/dextrose (YPD) rich medium, supplemented synthetic medium lacking the appropriate nutrient for plasmid selection, and minimal medium were prepared as described (Sherman 1991). 5-Fluoroorotic acid (5-FOA) plates were prepared by adding 5-FOA to a final concentration of 0.1% (Sikorski and Boeke 1991) to supplemented synthetic medium. Transformations into various yeast strains were performed with lithium acetate as described (Schiestl and Gietz 1989). pLP1202 was used to delete *HML* in LPY4441. In JRY4603 and JRY4623, the *SIR3* or *SIR1* open reading frames, respectively, were deleted by standard methods (Baudin *et al.* 1993). Crosses were performed with descendants of AMR7 and RS862 to make LPY1132; LPY4441, JRY4603, and EY957 to make LPY2709; AMR27 and YDS631 to make LPY3237; RS862 and YDS631 to make LPY3238 and LPY3620; JRY4623 and LPY3620 to make LPY3320 and LPY3321; and RS862 and YDS634 to make LPY4417.

***eso* mutant screen:** A total of 259,000 colonies from 35 independent cultures of AMR27 transformed with *SIR1* plasmid pJR910 (also known as strain LPY94) and 80,000 colonies from 8 independent cultures of JRY3010 with pJR910 (also known as LPY122) were mutagenized and plated on supplemented synthetic medium (Reifsnyder *et al.* 1996). Resulting colonies were then screened at 30° for mutants that mated when the *SIR1* plasmid was present but that did not mate without the plasmid. Genetic linkage analysis and plasmid complementation tests were performed to determine if the *eso* mutants were in previously identified silencing genes *SIR2*, *SIR3*, *SIR4*, *NAT1*, and *ARD1* (Reifsnyder 1996). Twenty-nine mutants in six complementation groups were uncovered. These included 1 allele of *sas2* (Reifsnyder *et al.* 1996), 5 alleles of *sir2* (S. Garcia and L. Pillus, unpublished results), 13 alleles of *sir3*, 3 alleles of *sir4*, 2 alleles of *ard1*, and 5 alleles of *nat1*. Eight of the *sir3-eso* alleles were rescued by gap repair as described below. As preliminary mating analysis did not distinguish novel phenotypes of the 5 alleles that were not rescued (data not shown), we continued detailed analysis for only those 8 that were. The *sir3-eso* allele designations in the original mutant strains prior to gap repair and sequencing are as follows: LPY221, 1.6.o; LPY222, 2.9.a; LPY225, 3.25.a; LPY238, 6.1.b; LPY275, 10.16.a; LPY521, 2o; LPY669, H9b; LPY683, 3.i.j; and JRY188, *sir3-8*.

Plasmids: pJR910 (also known as pLP17) contains *SIR1* on a *CEN-URA3* plasmid. pLP27 (Stone and Pillus 1996) was used to complement *sir3* mutants in above crosses where appropriate. pJR273 contains *SIR3* as a 4.5-kb *Sal*I fragment in the *CEN-URA3* plasmid pSEYC58 (Emr *et al.* 1986). pRS313 and pRS315 are *CEN*-based low-copy plasmids with *HIS3* or *LEU2* markers, respectively (Sikorski and Heiter 1989). YEp351 is a *LEU2*, 2μ plasmid (Hill *et al.* 1986). pLP143 was constructed by inserting the *Sal*I *Hin*dIII 5′ *SIR3* fragment, containing an *Nde*I site engineered by site-directed mutagenesis at codons −1/+1, into pKS+ Bluescript (Stratagene, La Jolla, CA) and subsequently inserting the *Hin*dIII 3′ *SIR3* fragment from pJR273; thus pLP143 contains *SIR3* as a *Sal*I fragment flanked by a nonstandard polylinker. An *Apa*I-*Bam*HI fragment that contains the *Sal*I fragment was then inserted into pRS313 to make pLP187, or into pRS315 to make pLP190. pLP189 was made in parallel with pLP187, except that it additionally contains the *A2T* mutation at codon 2 made by site-directed mutagenesis. pLP465 and pLP468 were created by gap repair of pJR273, containing *sir3-eso* mutants *R92K* and *T135I*, respectively. The *Sal*I fragments of pLP465 and pLP468 were subcloned into pRS313 to make pLP1048 and pLP946,

TABLE 1

Yeast strains

Strain	Genotype	Source
W303-1a	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	R. Rothstein
W303-1b	W303-1a *MAT*α	R. Rothstein
AMR7	W303-1a *nat1-3::URA3*	Stone *et al.* (1991)
AMR27	W303-1a *sir1::LEU2*	Stone *et al.* (1991)
RS862	W303-1a *sir3::TRP1*	Stone *et al.* (1991)
YDS631	W303-1b *adh4::URA3-*$(C_{1\text{-}3}A)_n$	Chien *et al.* (1993)
YDS634	W303-1b *adh4::URA3-4xUAS*$_G$-$(C_{1\text{-}3}A)_n$	Chien *et al.* (1993)
JRY188	*MAT*α *his4am leu2 rme1 sir3-8 trp1am ura3-52*	J. Rine
JRY3010	AMR27 *MAT*α	J. Rine
JRY4603	W303-1b *sir3::HIS3 ADE2 lys2*	J. Rine
JRY4623	W303-1b *sir1::TRP1 ADE2 lys2*	J. Rine
381G	*MAT***a** *SUP4-3 cry1 his4-580 trp1 ade2-1 tyr1 lys2*	Hartwell (1980)
EY957	W303-1a *bar1*	E. Elion
LPY78	*MAT*α *his4*	P. Schatz
LPY142	*MAT***a** *his4*	P. Schatz
LPY221	AMR27 *sir3-R30K*	This study
LPY222	AMR27 *sir3-T135I*	This study
LPY225	AMR27 *sir3-E140K*	This study
LPY238	AMR27 *sir3-R92K*	This study
LPY275	AMR27 *sir3-L96F*	This study
LPY521	AMR27 *sir3-E140K*	This study
LPY669	AMR27 *sir3-S813F*	This study
LPY683	AMR27 *sir3-L208S*	This study
LPY1132	W303-1b *nat1-3::URA3 sir3::TRP1*	This study
LPY2709	EY957 *hml*Δ*::TRP1 sir3::HIS3*	This study
LPY3237	W303-1b *sir1::LEU2 adh4::URA3-*$(C_{1\text{-}3}A)_n$	This study
LPY3238	W303-1b *sir3::TRP1 adh4::URA3-*$(C_{1\text{-}3}A)_n$	This study
LPY3320	W303-1a *sir1::TRP1 sir3::TRP1 adh4::URA3-*$(C_{1\text{-}3}A)_n$	This study
LPY3321	W303-1b *sir1::TRP1 sir3::TRP1 adh4::URA3-*$(C_{1\text{-}3}A)_n$	This study
LPY3620	W303-1a *sir3::TRP1 adh4::URA3-*$(C_{1\text{-}3}A)_n$	This study
LPY4417	W303-1b *adh4::URA3-4xUAS*$_G$-$(C_{1\text{-}3}A)$ *sir3::TRP1*	This study
LPY4441	W303-1a *hml*Δ*::TRP1*	This study

respectively. pLP1131, pLP464, pLP675, pLP469, pLP473, and pLP472 were created by gap repair of pLP187, containing *sir3-eso* mutants *R30K*, *L96F*, *sir3-8*(*E131K*), *E140K*, *L208S*, and *S813F*, respectively. The wild-type *SIR3* gene was cloned from strains W303-1a and 381G by gap repair of pLP187 to make pLP1130 and pL1133, respectively. pLP304 is the wild-type *SIR3* gene in YEp351 (Stone and Pillus 1996); pLP535, pLP1190, pLP828, pLP681, pLP791, pLP516, pLP526, pLP534, and pLP586 contain the *sir3-eso* mutations as *Sal*I fragments in YEp351, made from the corresponding *CEN-HIS3* plasmids, in the following order: *A2T*, *R30K*, *R92K*, *L96F*, *sir3-8E131K*, *E140K*, *L208S*, and *S813F*. A *Bam*HI digest was performed to direct integration of pLP1202, an *hml*Δ*::TRP1* construct. The following constructs were previously reported (Bell *et al.* 1995): pSIR3.12 (referred to here as *ORC1N-SIR3C*, containing the first 231 amino acids of Orc1p fused to the final 677 amino acids of Sir3p), pSIR3.15 (*SIR3C*, deleting the N-terminal 241 amino acids of Sir3p), pSPB1.34 (*SIR3N-ORC1C*, containing the first 235 amino acids of Sir3p fused to the final 679 amino acids of Orc1p), pSPB1.36 (*ORC1-SIR3-ORC1*, substituting amino acids 457–680 of Orc1p with amino acids 557–779 of Sir3p), pSIR3.13 (*SIR3-ORC1-SIR3*, substituting amino acids 505–834 of Sir3p with amino acids 405–738 of Orc1p), and pSPB1.43 (*ORC1C*, deleting the N-terminal 235 amino acids of Orc1p).

Gap repair and DNA sequencing: The *sir3-eso* mutant alleles were rescued from their chromosomal locations by standard methods of gap repair (Rothstein 1991). The gap-repaired plasmids that rescued the *sir3-eso* mutants were made by introducing pJR273, digested with *Bam*HI and *Cla*I, into strains LPY238 and LPY222 for pLP465 and pLP468, respectively; and from pLP187, digested with *Cla*I and *Stu*I, into strains LPY275 and JRY188 for pLP464 and pLP675, or with *Stu*I and *Nru*I, into LPY669, LPY683, and LPY225 for pLP472, pLP473, and pLP676, or with *Sty*I into LPY521 for pLP469, or with *Hpa*I into W303-1a, LPY221, and 381G for pLP1130, pLP1131, and pLP1133. Because modestly increased gene dosage of the *sir3-eso* mutants appeared to be sufficient to restore mating in the strains from which gap repair was done, several plasmids were rescued from each strain and transformed into appropriate *sir3* single and *sir1 sir3* double null mutants to identify those with an *eso* phenotype. During the process of sequencing we discovered that the *sir3-eso* mutations often lay outside of the gapped regions, an observation that has been previously described (Rothstein 1991). Therefore the entire open reading frame was sequenced for each mutant allele. Because pLP676 and pLP469 were identical, we chose to use only one, pLP469, in further analysis. Additionally, sequencing pLP1130 and pLP1131 revealed that the wild-type *SIR3* alleles from strains W303-1a and 381G (the parental strain used in the genetic screen that yielded the *sir3-8* mutant; Hartwell 1980) contain the identical sequence to that reported in the Saccharo-

myces Genome Database (open reading frame YLR442C; http://genome-www.stanford.edu/Saccharomyces/). Note that these sequences differ at several positions from the original *SIR3* sequence reported (Shore *et al.* 1984).

Sequencing was performed using an Applied Biosystems (Foster City, CA) automated facility. The oligonucleotides used for sequencing were

SIR3-10: 5′GAGACTGCATGTGTACATAGGC3′
SIR3-12: 5′GCAGCCCTTTCATCACCTTCC3′
SIR3-131: 5′TAACTGCTGAGCTATCAGAGAT3′
SIR3-14: 5′CAGAGGAAATACCAATAAACTC3′
SIR3-15: 5′TTTAGACCGGTTTGCACCAG3′
SIR3-16: 5′AGAAAATATGGTTCGCCATTTC3′.

Immunoblot analysis: Preparation of protein extracts, SDS-PAGE electrophoresis, and immunoblotting for Sir3p detection were performed as described (Stone and Pillus 1996). High-copy 2μ plasmids were used to facilitate detection of Sir3p from wild-type and mutant strains. For all parameters tested previously, results with high-copy plasmids and endogenous genes were identical (Stone and Pillus 1996).

Quantitative mating and telomeric silencing assays: For quantitative mating assays, cells were grown to midlogarithmic phase in a supplemented synthetic medium for plasmid selection. They were then diluted appropriately to obtain ~100–300 colonies per plate and plated on the same medium to quantitate total number of cells. At the same time, appropriate dilutions for testing mating were mixed with *MAT***a** or *MAT*α mating-type testers LPY142 or LPY78, respectively, and plated onto minimal medium for diploid selection to quantitate the number of mating-competent cells. The mating efficiency is defined as the number of cells that mated per total number of cells. At least two experiments were performed for each strain, for which mean values were determined and the range of each of those values was indicated.

For monitoring telomeric silencing from a *URA3* reporter gene (Aparicio *et al.* 1991), cells were grown to saturation for 2–3 days at room temperature in a supplemented synthetic medium for plasmid selection. These cultures were then plated in serial fivefold dilutions onto the same medium either lacking or containing 5-FOA, and plates were incubated at room temperature until colony growth was visible. Colonies resistant to 5-FOA are silenced for *URA3*.

RESULTS

A genetic screen identifies enhancers of the *sir1* mutant mating-defective phenotype: The *SIR1* gene functions in establishing stable and heritable patterns of gene expression at the silent mating-type *HM* loci (Pillus and Rine 1989). The *sir1* null mutant exhibits a partial mating-defective phenotype due to an epigenetic phenomenon in which the silent mating-type genes in some cells of the population are completely derepressed yet in other cells the *HM* genes are fully repressed (Pillus and Rine 1989). To identify genes that contribute to silencing in the population of transcriptionally repressed *sir1* mutant cells, we performed a genetic screen for *e*nhancers of the *sir one* mutant mating phenotype (*eso*) mutants (Reifsnyder *et al.* 1996). Mutants were identified that were completely mating defective in the absence of *SIR1*, but mating competent in its presence. This was done by replica-plating mutagenized *sir1* mutant cells on two media, either selecting for or against a *SIR1* plasmid, then testing for mating in the presence or absence of the plasmid (Reifsnyder *et al.* 1996). By looking for mutants that were mating defective only in the absence of *SIR1*, we sought to avoid isolating previously characterized mutants that were completely silencing defective.

Six complementation groups were found to affect mating in *sir1* mutant cells. One of the *eso* mutants was in *SAS2*, a gene that encodes a member of a conserved family of acetyltransferases (Reifsnyder *et al.* 1996). Five other complementation groups contained mutant alleles of genes known to be involved in silencing: *ARD1*, *NAT1*, *SIR2* (S. Garcia and L. Pillus, unpublished results), *SIR3*, and *SIR4* (see materials and methods for details). Alleles of *ARD1* and *NAT1* were predicted to arise from the *eso* screen, as null mutants were previously shown to be mating defective in combination with *sir1* mutants (Whiteway *et al.* 1987; Stone *et al.* 1991). This report focuses on characterization of the *sir3-eso* mutants.

Eight *sir3-eso* mutant alleles were rescued on plasmids by gap repair (see materials and methods). In addition to these eight alleles from the *eso* screen, two other independently isolated *sir3* mutants were found to have an *eso* phenotype and thus were included in our analysis. One mutant, called *A2T*, was a site-directed mutant in which the alanine residue at codon 2 was changed to threonine for a separate study (E. M. Stone and L. Pillus, unpublished data). The other was the *sir3-8* allele, previously described to be temperature sensitive for mating (Hartwell 1980; Rine and Herskowitz 1987). The *sir3-8* allele was recovered on a plasmid by gap repair and, similar to the behavior of *sir3-8* at its chromosomal locus, *sir3* null mutant strains bearing the *sir3-8* plasmid were temperature sensitive for mating. Transformants containing the *sir3-8* plasmid were completely mating defective at the restrictive temperature of 37° (data not shown) but fully mating competent at the permissive temperature of 23° in a *SIR1 sir3*Δ strain. The plasmid was tested for its ability to complement the mating-defective phenotype of a *sir1*Δ *sir3*Δ double mutant at the permissive temperature and was found to be unable to fully complement the mating defect (see below). Therefore *sir3-8* was classified as an *eso* mutant. Other previously identified *sir3* alleles, however, were not *eso* mutants. The *SIR3R1* and *SIR3R3* alleles, originally recovered as suppressors of the mating defect of histone H4 N-terminal mutants (Johnson *et al.* 1990), did not enhance the mating defect of the *sir1* mutant (T. Lewis and L. Pillus, unpublished results). Moreover, $SIR3^{L31}$ (and $SIR3^{N205}$, identical to *SIR3R3*), isolated as suppressors of the telomeric silencing defect of *rap1* C-terminal mutants (Liu and Lustig 1996), did not exhibit an *eso* phenotype (data not shown). Thus the *sir3-eso* mutants are distinguished by their *SIR1*-specific phenotype.

Altered residues in *sir3-eso* mutant alleles cluster at the N terminus: The entire *SIR3* open reading frame

TABLE 2

The *sir3-eso* mutations

Allele name	Codon mutated	Amino acid substitution
sir3-A2T	2: GCT → ACT	Alanine → threonine
sir3-R30K	30: AGA → AAA	Arginine → lysine
sir3-R92K	92: AGA → AAA	Arginine → lysine
sir3-L96F	96: CTC → TTC	Leucine → phenylalanine
sir3-8(*E131K*)	131: GAG → AAG	Glutamic acid → lysine
sir3-T135I	135: ACT → ATT	Threonine → isoleucine
sir3-E140K	140: GAG → AAG	Glutamic acid → lysine
sir3-L208S	208: TTG → TCG	Leucine → serine
sir3-S813F	813: TCT → TTT	Serine → phenylalanine

was sequenced for each gap-repaired allele to identify the changes that conferred the *eso* phenotype. Only 1 bp was found to be mutated for each allele. The alleles were thus renamed to reflect the nature of the mutations (Table 2). One mutation, leading to the *E140K* substitution, was independently isolated twice, but all others were distinct. Therefore, a total of nine different altered residues were identified in the collection of *sir3-eso* mutants. Interestingly, eight of these nine substitutions clustered at the N terminus of Sir3p. This is the 214-amino-acid region most similar to Orc1p (50% identical, 63% similar; Bell *et al.* 1995). Seven of the mutated residues that mapped to this region were identical in wild-type Sir3p and Orc1p, and one was conserved (L96 in Sir3p; V96 in Orc1p). The remaining allele, S813F, was found in a larger region encoding the C terminus that is also conserved in Sir3p and Orc1p, although not as extensively as is the N-terminal region. Five *sir3-eso* mutations led to changes in conserved residues in the recently defined BAH domain (*b*romo-*a*djacent *h*omology, at Sir3p N-terminal amino acids 48–189; Callebaut *et al.* 1999). The BAH module is widely conserved among diverse proteins, including Sir3p and Orc1p, DNA methyltransferases, and DNA replication proteins; it has been suggested to be important for protein-protein interactions in processes that link methylation, replication, and transcriptional regulation. Thus, mutations that lead to changes in conserved residues within an N-terminal domain of Sir3p resulted in the *eso* phenotype and may serve to define a functionally significant domain.

The protein encoded by the *sir3-8* mutant is thermolabile: Because changes in Sir3 protein levels might account for silencing defects seen in *sir3-eso* mutants, immunoblot analysis was performed to determine if steady-state levels of Sir3 mutant proteins were similar to those of wild-type Sir3p. Immunoblot analysis of whole-cell protein extracts, using a polyclonal Sir3p antiserum, demonstrated that Sir3p levels and electrophoretic mobility were comparable to wild type for all of the *sir3-eso* mutants (data not shown). The mobility of *sir3-eso* mutant proteins was also evaluated during the pheromone and starvation responses, previously shown to result in Sir3p hyperphosphorylation and associated mitogen-activated protein (MAP) kinase pathway modulation of silencing (Stone and Pillus 1996). None of the alleles examined appeared defective in pheromone- or starvation-induced Sir3p hyperphosphorylation (data not shown). However, when protein was examined from a *sir3-8* mutant culture grown at the restrictive temperature of 37°, little to no protein was detected (Figure 1). When grown at the permissive temperature of 23°, sir3-8 mutant protein migrates normally (compare first and third lanes, Figure 1), but it disappears when shifted for 3 hr or more to 37° (final lane on right, Figure 1). When grown for as many as 16 generations after shifting to the restrictive temperature, sir3-8p remains undetectable (data not shown).

SIR1 overexpression was previously shown to partially suppress the mating defect of *sir3-8* mutant cells (Stone *et al.* 1991). This suppression appears not to result from stabilization of sir3-8p, however, as the mutant protein levels are not restored by *SIR1* overexpression (data not shown). Our results identifying sir3-8p as a thermolabile protein, together with previous data demonstrating that

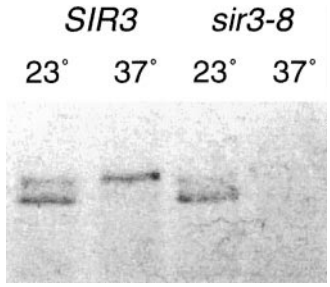

Figure 1.—The sir3-8 protein is thermolabile at the restrictive temperature. Immunoblot of whole-cell lysates was probed with an anti-Sir3p antiserum. Transformants of *sir3* null mutant strain LPY2709 contained wild-type *SIR3* plasmid pLP304 or *sir3-8* mutant plasmid pLP791. Cultures were grown at 23°, and one-half of each culture was shifted to 37° for 3 hr before harvesting. Note that the temperature shift causes a change in Sir3p mobility (compare lanes 1 and 2) due to hyperphosphorylation as previously described (Stone and Pillus 1996).

the *sir3-8* mutant is completely mating defective at 37° (Hartwell 1980; Rine and Herskowitz 1987), support the interpretation that *sir3-8* behaves as a conditional null allele.

Two classes of *sir3-eso* alleles exhibit different mating-defective phenotypes: To quantitate *sir3-eso* mutant mating phenotypes, each *sir3-eso* allele was introduced into appropriate strains on a centromeric plasmid expected to be present in approximately one or two copies per transformed cell. Plasmids were used in quantitative experiments to ensure that the strain backgrounds were isogenic. The mutants behaved similarly when present on plasmids or at their endogenous chromosomal locus (data not shown). The *sir3-eso* plasmids were tested for their ability to complement the chromosomal null *sir3* mutant, and mating phenotypes were compared for wild-type *SIR1* and *sir1* mutant strains in *MAT*α and *MAT***a** backgrounds. Because the *sir3-8*(*E131K*) mutant is temperature sensitive for mating, all assays were performed at the permissive temperature of 23°. Strains carrying the other alleles exhibited no defect at 37° and behaved similarly at 23° and 30°.

Quantitative mating assays revealed that strains carrying the *sir3-eso* plasmids were severely mating defective in a *MAT*α *sir1 sir3* background but had little or no mating defect in a *MAT*α *SIR1 sir3* background (Table 3). Thus, the *sir3-eso* mutants clearly did not represent null or complete loss-of-function alleles. For seven of the *sir3-eso* plasmids, transformants in the *sir1 sir3* mutant background mated with 10^{-5}–10^{-6} efficiency or less, similar to the vector control transformant lacking wild-type *SIR3* altogether. The two remaining *sir3-eso* mutants, *sir3-A2T* and *sir3-8*(*E131K*), were partially mating impaired in *sir1 sir3* strains. These results are in contrast to the *sir1* mutant carrying a wild-type *SIR3* plasmid, which exhibited only a mild decrease in mating compared to the *SIR1* strain (Table 3). Thus, all of the *sir3-eso* mutants significantly enhance the *sir1* mutant mating-defective phenotype in the *MAT*α background.

Quantitative mating analysis revealed that all nine *sir3-eso* mutants also showed severely decreased mating efficiency in a *MAT***a** *sir1 sir3* strain (Table 4). Interestingly, a subset of strains carrying the *sir3-eso* alleles exhibited a mating defect in the presence of *SIR1* in the *MAT***a** *sir3* background. Two strains bearing different alleles, *sir3-T135I* and *sir3-E-140K*, mated 100-fold less efficiently than wild-type *SIR3* strains. A third mutant, *sir3-L208S*, was completely nonmating in a *MAT***a** strain. It should be noted that this allele would not have been recovered in the *eso* screen in the *MAT***a** background, although it clearly fits the definition of an *eso* mutant when present in a *MAT*α strain. Because none of these mutants was mating defective in the *MAT*α strain (Table 3) and because *sir3-T135I*, *sir3-E140K*, and *sir3-L208S* cluster near one another within the region encoding the N terminus of Sir3p, they define a *MAT***a**-specific class of alleles that may identify an N-terminal functional subdomain important for silencing *HML*α but not *HMR***a** in a wild-type *SIR1* strain background (see discussion).

We determined whether the *sir3-eso* alleles were dominant or recessive by quantitative mating assays in a *MAT*α *sir1* strain that was wild type for *SIR3*. The *MAT*α background was used to avoid the *MAT***a**-specific effects of some of the alleles noted above. This analysis revealed that three of the alleles, *sir3-T135I*, *sir3-E140K*, and *sir3-L208S*, had partially dominant phenotypes, exhibiting a decreased mating efficiency of 10^{-3} (Table 3). These

TABLE 3

The *sir3-eso* phenotype is characterized by a mating defect in the *sir1* mutant background

Strain: / Relevant genotype: / *SIR3* allele[b]	Mating efficiency[a] LPY3238 *MAT*α *sir3*Δ	LPY3321 *MAT*α *sir1*Δ *sir3*Δ	LPY3237 *MAT*α *sir1*Δ
SIR3	$7 \times 10^{-1} \pm 0.7$ (1)	$2 \times 10^{-1} \pm 0.3$ (10^{-1})	$2 \times 10^{-1} \pm 0.1$ (10^{-1})
sir3-A2T	$7 \times 10^{-1} \pm 0.6$ (1)	$4 \times 10^{-4} \pm 4$ (10^{-3})	$4 \times 10^{-2} \pm 2$ (10^{-1})
sir3-R30K	$8 \times 10^{-1} \pm 0$ (1)	$2 \times 10^{-5} \pm 0.9$ (10^{-5})	$4 \times 10^{-2} \pm 2$ (10^{-1})
sir3-R92K	$9 \times 10^{-1} \pm 0.8$ (1)	$1 \times 10^{-5} \pm 0.7$ (10^{-5})	$2 \times 10^{-2} \pm 0.6$ (10^{-2})
sir3-L96F	$9 \times 10^{-1} \pm 0.4$ (1)	$3 \times 10^{-5} \pm 0.4$ (10^{-5})	$4 \times 10^{-2} \pm 0$ (10^{-1})
sir3-8(*E131K*)	$7 \times 10^{-1} \pm 0.2$ (1)	$4 \times 10^{-3} \pm 4$ (10^{-2})	$2 \times 10^{-1} \pm 0.5$ (10^{-1})
sir3-T135I	$5 \times 10^{-1} \pm 0.3$ (1)	$\leq 2 \times 10^{-6} \pm 0.5$ (10^{-6})	$3 \times 10^{-3} \pm 0.3$ (10^{-3})
sir3-E140K	$7 \times 10^{-1} \pm 0.7$ (1)	$4 \times 10^{-6} \pm 0.4$ (10^{-5})	$2 \times 10^{-3} \pm 0.8$ (10^{-3})
sir3-L208S	$3 \times 10^{-1} \pm 0.8$ (10^{-1})	$\leq 3 \times 10^{-6} \pm 1$ (10^{-6})	$3 \times 10^{-3} \pm 2$ (10^{-3})
sir3-S813F	$1 \times 10^{-1} \pm 0.2$ (10^{-1})	$7 \times 10^{-6} \pm 3$ (10^{-5})	$3 \times 10^{-2} \pm 0.9$ (10^{-2})
Vector only	$1 \times 10^{-5} \pm 0.4$ (10^{-5})	$4 \times 10^{-6} \pm 1$ (10^{-5})	$2 \times 10^{-1} \pm 0$ (10^{-1})

[a] Mating efficiency is expressed as a mean of two experimental values, with the range indicated. In parentheses, each efficiency is presented relative to this wild-type plasmid control, rounded to the nearest exponent.

[b] Plasmids used were pLP187, pLP189, pLP1131, pLP1048, pLP464, pLP675, pLP946, pLP469, pLP473, pLP472, and pRS313, in descending order.

TABLE 4

A subset of *sir3-eso* mutants exhibits *MAT*a-specific mating defects

Strain: Relevant genotype: *SIR3* allele[b]	Mating efficiency[a] LPY3620 *MAT***a** *sir3*Δ	LPY3320 *MAT***a** *sir1*Δ *sir3*Δ
SIR3	$2 \times 10^{-1} \pm 0.5$ (1)	$3 \times 10^{-2} \pm 2$ (10^{-1})
sir3-A2T	$3 \times 10^{-1} \pm 1$ (1)	$6 \times 10^{-6} \pm 3$ (10^{-5})
sir3-R30K	$2 \times 10^{-1} \pm 0.8$ (1)	$\leq 3 \times 10^{-6} \pm 0.7$ (10^{-5})
sir3-R92K	$7 \times 10^{-2} \pm 0.5$ (10^{-1})	$\leq 5 \times 10^{-6} \pm 1$ (10^{-5})
sir3-L96F	$2 \times 10^{-1} \pm 0.5$ (1)	$\leq 3 \times 10^{-6} \pm 0.5$ (10^{-5})
sir3-8(*E131K*)	$2 \times 10^{-1} \pm 0.2$ (1)	$2 \times 10^{-5} \pm 1$ (10^{-4})
sir3-T135I	$2 \times 10^{-3} \pm 0.4$ (10^{-2})	$\leq 3 \times 10^{-6} \pm 0.6$ (10^{-5})
sir3-E140K	$4 \times 10^{-3} \pm 2$ (10^{-2})	$4 \times 10^{-6} \pm 0.1$ (10^{-5})
sir3-L208S	$6 \times 10^{-6} \pm 2$ (10^{-5})	$\leq 3 \times 10^{-6} \pm 0.3$ (10^{-5})
sir3-S813F	$3 \times 10^{-2} \pm 0.5$ (10^{-1})	$\leq 3 \times 10^{-6} \pm 0.1$ (10^{-5})
ORC1N-SIR3C	$8 \times 10^{-2} \pm 3$ (10^{-1})	$2 \times 10^{-2} \pm 0.3$ (10^{-1})
Vector only	$3 \times 10^{-5} \pm 0.4$ (10^{-4})	$\leq 3 \times 10^{-6} \pm 0.2$ (10^{-5})

[a] Mating efficiency is expressed as a mean of two experimental values, with the range indicated. In parentheses, each efficiency is presented relative to this wild-type plasmid control, rounded to the nearest exponent.

[b] Plasmids used were pLP187, pLP189, pLP1131, pLP1048, pLP464, pLP675, pLP946, pLP469, pLP473, pLP472, pSIR3.12, and pRS313, in descending order.

three alleles also constitute the class with *MAT***a**-specific mating-defective phenotypes. We grouped these alleles, calling those that are primarily recessive class I and those three alleles that exhibit *MAT***a**-specific and dominant *eso* mating defects class II. None of the *sir3-eso* mutants had dominant mating defects in a wild-type *SIR1* mutant background, as wild-type *MAT***a** and *MAT*α strains carrying plasmids containing the *sir3-eso* alleles mated with normal efficiency (data not shown).

To determine if *sir3-eso* mutants, like *sir1* mutants, inherit alternate states of *HM* locus gene expression, consistent with a role in establishing silencing, we performed pedigree analysis on several cell lineages (Pillus and Rine 1989). Two *sir3-eso* mutants, *R30K* and *S813F*, exhibited lineages in which cells transcriptionally silenced at the *HM* locus gave rise to transcriptionally active cells in subsequent generations (data not shown). Thus, these two alleles appear to be defective in maintenance of silent chromatin, and an establishment defect is not a general property of *sir3-eso* mutants.

Sir3p has been hypothesized to dimerize (Moretti *et al.* 1994), and partial *trans*-complementation between an N-terminal coding region and C-terminal coding region of *SIR3* has been observed (Le *et al.* 1997; Gotta *et al.* 1998). Therefore heterodimer formation between different sir3-eso mutant proteins might lead to interallelic complementation. When *MAT***a** *sir1* or *MAT*α *sir1* strains bearing the alleles *sir3-R30K*, *sir3-R92K*, *sir3-L208S*, and *sir3-S813F* at their endogenous chromosomal locus were transformed with the entire panel of *sir3-eso* plasmids, mating ability was not restored (data not shown). The failure of different *sir3-eso* mutants to function in interallelic complementation may reflect a requirement for Sir1p in heterodimer formation, or may be due to the inability of sir3-eso heterodimers to achieve an appropriate tertiary structure.

Mating defects are observed in a subset of *nat1 sir3-eso* double mutants: *NAT1* and *ARD1* encode subunits of an N-terminal acetyltransferase (Mullen *et al.* 1989; Park and Szostak 1992). Null *nat1* and *ard1* single and double mutants have identical phenotypes, including a partial mating defect in a *MAT***a** strain background (Mullen *et al.* 1989) and synergistic loss of mating in the absence of *SIR1* (Whiteway *et al.* 1987; Stone *et al.* 1991). Thus *nat1* mutants have an *eso* phenotype in both *MAT***a** and *MAT*α strains since they enhance the *sir1* mating defect and in fact were identified in the *eso* screen described here (see materials and methods). Because both *sir3-eso* mutants and *nat1* mutants enhance the mating defect of *sir1* mutants, we asked if *sir3-eso* mutants enhance the *nat1* mating defect in a wild-type *SIR1* background. The *sir3-eso* plasmids were introduced into a *MAT*α *nat1 sir3* strain, as *MAT*α *nat1* mutants and *MAT*α *sir3-eso* mutants are completely mating competent, in contrast to either of these mutants in a *MAT***a** background.

Quantitative mating data revealed that some but not all of the *sir3-eso* alleles have more severe phenotypes in combination with *nat1* mutants (Table 5). Strains with the *sir3-T135I* and *sir3-L208S* alleles exhibited an ~100-fold decreased mating efficiency and are found among the class II mutants with *MAT***a**-specific and dominant mating defects. The third class II mutant, *sir3-E140K*, did not exhibit worsened mating in the *nat1* mutant. Moreover, two additional *sir3-eso* mutants, *sir3-8*(*E131K*) and *sir3-S813F*, were completely mating defec-

TABLE 5

Synergistic interactions occur between *sir3-eso* alleles and the *nat1* mutant

Strain:	LPY1132
Relevant genotype:	*MAT*α *nat1*Δ *sir3*Δ
SIR3 allele[b]	Mating efficiency[a]
SIR3	$2 \times 10^{-1} \pm 0.9$ (1)
sir3-A2T	$2 \times 10^{-1} \pm 1$ (1)
sir3-R30K	$5 \times 10^{-2} \pm 3$ (10^{-1})
sir3-R92K	$3 \times 10^{-2} \pm 2$ (10^{-1})
sir3-L96F	$3 \times 10^{-2} \pm 2$ (10^{-1})
sir3-8(*E131K*)	$2 \times 10^{-5} \pm 0$ (10^{-4})
sir3-T135I	$8 \times 10^{-3} \pm 6$ (10^{-2})
sir3-E140K	$3 \times 10^{-2} \pm 2$ (10^{-1})
sir3-L208S	$6 \times 10^{-3} \pm 5$ (10^{-2})
sir3-S813F	$2 \times 10^{-5} \pm 0.8$ (10^{-4})
Vector only	$4 \times 10^{-5} \pm 0.1$ (10^{-4})

[a] Mating efficiency is expressed as a mean of two experimental values, with the range indicated. In parentheses, each efficiency is presented relative to this wild-type plasmid control, rounded to the nearest exponent.

[b] Plasmids used were pLP187, pLP189, pLP1131, pLP1048, pLP464, pLP675, pLP946, pLP469, pLP473, pLP472, and pRS313, in descending order.

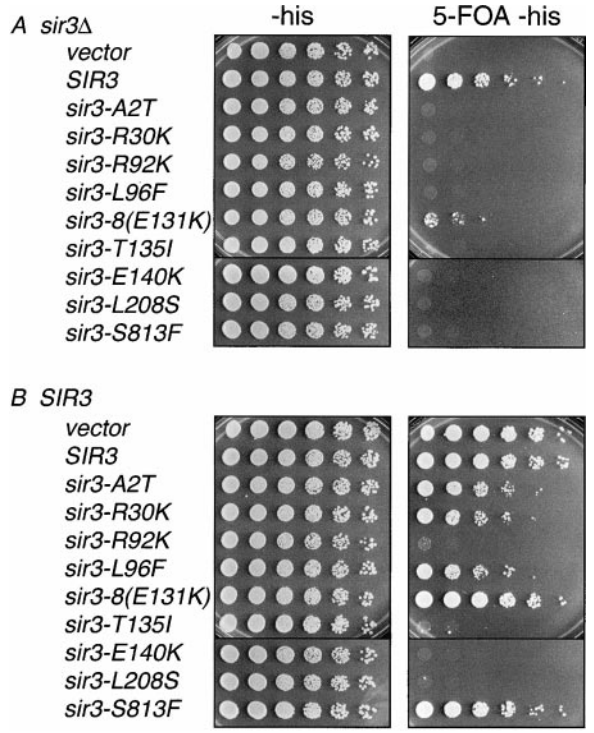

Figure 2.—*sir3-eso* mutants exhibit telomeric silencing defects. Serial fivefold dilutions were plated on supplemented synthetic medium selecting for plasmid maintenance to monitor growth (-his, left) or the same medium containing 5-FOA to monitor silencing of the telomere-proximal *URA3* reporter gene (right). Transformants of *sir3* mutant strain LPY3238 for complementation test (A), or of *SIR3* wild-type strain YDS631 for dominance test (B), contained the following *CEN*-based plasmids ordered from top to bottom: vector control pRS313; *SIR3* wild-type control pLP187; and *sir3-eso* alleles pLP189, pLP1131, pLP1048, pLP464, pLP675, pLP946, pLP469, pLP473, and pLP472.

tive in the absence of *NAT1*. The remaining mutants had little or no effect on mating efficiency in combination with *nat1* mutants. In addition, mating defects were tested in *sir3-eso sas2* double mutants. *SAS2* is also a member of a gene family with acetyltransferase activity and, like *sir3-eso* mutants, the *sas2* mutant is severely mating defective only in the absence of *SIR1* (Reifsnyder *et al.* 1996). Mating efficiency for all *sir3-eso sas2* double mutants was comparable to that of the single mutants (data not shown), suggesting a specific interaction between the *NAT1* and *SIR3* genes.

***sir3-eso* mutants are defective in telomeric silencing:** In addition to its requirement for silencing at the *HM* loci, *SIR3* is essential for telomeric silencing. The *URA3* reporter gene placed proximal to telomeric sequences is transcriptionally repressed, and this telomeric silencing, observed as sensitivity to 5-FOA, is abolished in a *sir3* null mutant (Aparicio *et al.* 1991). Resistance to 5-FOA is a measure for silencing of the telomere-positioned *URA3* gene, because cells expressing *URA3* are sensitive to 5-FOA and only silenced cells are resistant and able to form colonies. We tested whether *sir3-eso* mutants functioned in telomeric silencing by plating a dilution series of transformants on growth medium selecting for plasmid maintenance (-his) and on 5-FOA-containing medium to monitor silencing and maintain selection for the plasmid (5-FOA-his). Control transformants of a *sir3*Δ mutant strain showed no growth with vector only and good growth with a wild-type *SIR3* plasmid (Figure 2A). Transformants containing the *sir3-eso* plasmids were completely defective for eight of the *sir3-eso* mutants, and the *sir3-8*(*E131K*) mutant exhibited a partial defect in telomeric silencing. The telomeric silencing phenotype was apparent in the presence of *SIR1*, and additional telomeric defects were not detected in a *sir1* mutant background (data not shown). Therefore the *sir3-eso* mutants are unable to function in telomeric silencing.

Because several *sir3-eso* mutants exhibited dominant effects in silencing at the *HM* loci, the panel of mutants was also examined for dominant effects on telomeric silencing. Plasmids bearing each mutant allele were transformed into a telomeric reporter strain that was wild type for *SIR3* and tested for silencing as above. Four mutants showed a striking loss of telomeric silencing in the presence of wild-type *SIR3* (Figure 2B). Three of these dominant mutants, *sir3-T135I*, *sir3-E140K*, and *sir3-L208S*, are class II mutants, which exhibit a dominant mating-defective phenotype in the absence of *SIR1*, whereas one, *sir3-R92K*, is a class I mutant, among those that are recessive for the *eso* mating defect. The mutants with dominant telomeric silencing defects presumably

form nonfunctional complexes with other silencing proteins, thereby interfering with wild-type *SIR3* silencing at telomeres.

The Orc1p N terminus functionally replaces that of Sir3p in mating-type silencing in the absence of Sir1p, but not in telomeric silencing: Given the high degree of sequence similarity between the N termini of Sir3p and Orc1p, a chimeric protein between the first 231 amino acids of Orc1p and the Sir3p C-terminal 677 amino acids was created and tested for silencing function (Bell *et al.* 1995). This *ORC1N-SIR3C* construct is capable of substituting for Sir3p in mating-type silencing (Bell *et al.* 1995). Because the *sir3-eso* mutations affected Orc1p-conserved residues, we hypothesized that the N terminus of Orc1p might also functionally replace the Sir3p N terminus in the absence of Sir1p. Indeed, the Orc1N-Sir3Cp chimera was mating proficient in both *MAT***a** and *MAT*α *sir1* mutant backgrounds (Table 4 and data not shown). The Orc1N-Sir3Cp chimera was also tested for complementation of the *sir3* mutant telomeric silencing defect. In contrast to its efficient mating, the Orc1N-Sir3Cp chimera was defective in telomeric silencing (Figure 3A), exhibiting only partial function reflected by its intermediate growth on 5-FOA-containing medium. The Orc1N-Sir3Cp telomeric silencing defect was comparable in both *sir1* mutant and *SIR1* wild-type strain backgrounds (data not shown). Moreover, the Orc1N-Sir3Cp chimera appeared to be partially dominant, inhibiting telomeric silencing in *SIR3* strains (Figure 3B). Thus, the N terminus of Sir3p is distinguished from that of Orc1p by its function at the telomere, perhaps through interactions with telomere-specific silencing proteins.

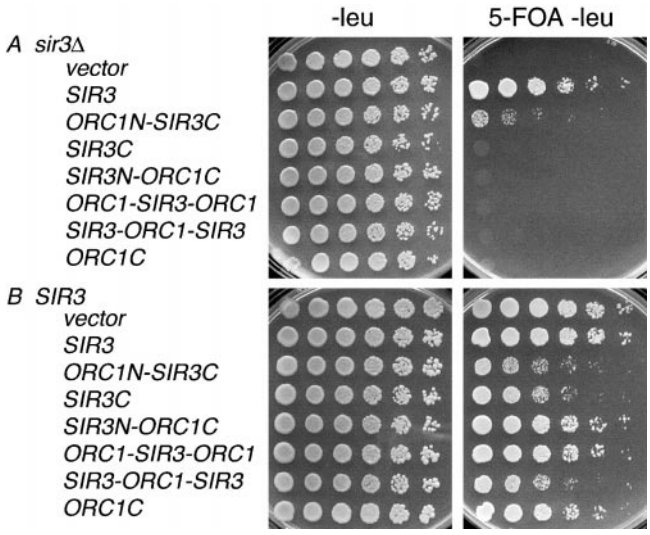

Figure 3.—The Orc1p N terminus cannot substitute for that of Sir3p in telomeric silencing. Serial fivefold dilutions were plated on supplemented synthetic medium for plasmid selection to monitor growth (-leu, left) or the same medium containing 5-FOA to monitor silencing of the telomere-proximal *URA3* reporter gene (right). Transformants of *sir3* null mutant strain LPY3238 for complementation test (A), or of *SIR3* wild-type strain YDS631 for dominance test (B), contained the following plasmids: vector control pRS315; *SIR3* wild-type control pLP190; and *ORC1N-SIR3C* (pSIR3.12), *SIR3C* (pSIR3.15), *SIR3N-ORC1C* (pSPB1.34), *ORC1-SIR3-ORC1* (pSPB1.36), *SIR3-ORC1-SIR3* (pSIR3.13), or *ORC1C* (pSPB1.43) (Bell *et al.* 1995). Original plasmid designations are noted in parentheses.

To further dissect the effect of *SIR3* and *ORC1* sequences on telomeric silencing in *sir3* mutant and *SIR3* wild-type strain backgrounds, the remaining chimeric and deletion constructs in the series reported by Bell *et al.* (1995) were examined. The additional constructs tested were a *SIR3N-ORC1C* chimera, which is the reciprocal chimera to the *ORC1N-SIR3C* noted above; two sandwich chimeras in which internal *ORC1* and *SIR3* regions were swapped to make *ORC1-SIR3-ORC1* and *SIR3-ORC1-SIR3*; and N-terminal deletions to result in *SIR3C* and *ORC1C*. None of the constructs supported telomeric silencing in the *sir3* mutant strain (Figure 3A), consistent with their inability to promote mating-type silencing (Bell *et al.* 1995). However, partial dominance was observed with the *SIR3C* and *SIR3-ORC1-SIR3* constructs (Figure 3B), in addition to the *ORC1N-SIR3C* chimera described above. None of the other constructs exhibited dominant effects on telomeric silencing. As the C terminus of Sir3p was unable to complement the *sir3* mutant, the partial telomeric silencing seen for the Orc1N-Sir3Cp chimera must be due to a low level of function of the Orc1p N-terminal region. In contrast, the partial dominance of Orc1N-Sir3Cp, Sir3Cp, and Sir3-Orc1-Sir3p may be due to Sir3p sequences common to all three constructs, which might sequester other silencing proteins into nonfunctional complexes.

Tethered Sir1p suppresses the telomeric silencing defect of *sir3-eso* mutants: *SIR1* does not appear to function in silencing telomeric reporter genes in standard assays, although it may play a modest role at native telomeres (Aparicio *et al.* 1991; Pryde and Louis 1999). However, strong silencing is achieved by tethering Sir1p directly to telomeric sequences via the Gal4p DNA binding domain (GBD; Chien *et al.* 1993). Because silencing at the *HM* loci occurs only in the presence of *SIR1* in *sir3-eso* mutant strains, we asked if the telomeric silencing defect of the *sir3-eso* mutants could be suppressed by tethering Sir1p to the telomere. A *sir3* null mutant strain with the *GAL4*-upstream activating sequence positioned near a telomeric reporter gene was transformed with either the *GBD* control vector or *GBD-SIR1* plasmid and the *sir3-eso* genes or appropriate controls on high-copy 2μ plasmids.

The *sir3-eso* mutants exhibited telomeric silencing defects even when present at elevated dosage on 2μ plasmids, as observed in control transformants containing *GBD* alone (Figure 4A). However, partially restored telomeric silencing was observed for several of the mutants when they were overexpressed, particularly *sir3-A2T* and *sir3-8* (compare Figures 2A and 4A). Significantly, when Sir1p was directed to telomeres with the *GBD-SIR1* plas-

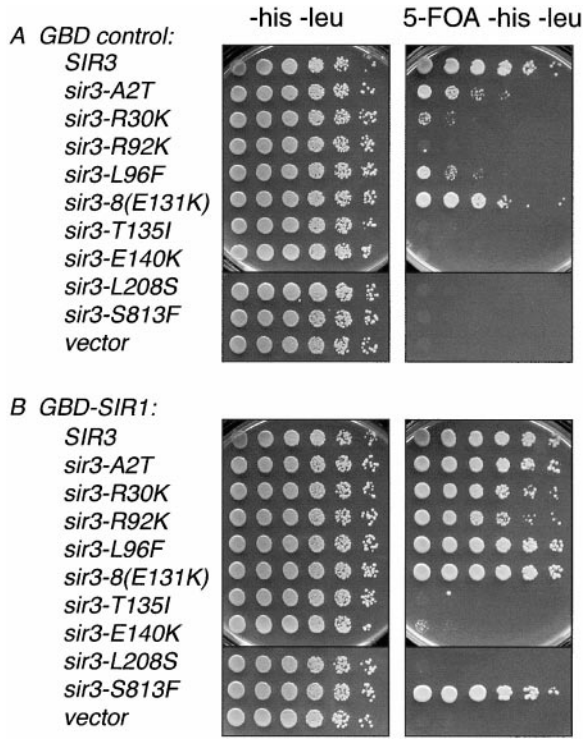

Figure 4.—Tethered Sir1p suppresses *sir3-eso* mutant telomeric silencing defects. Serial fivefold dilutions were plated on supplemented synthetic medium to monitor growth, selecting simultaneously for maintenance of two plasmids (-his -leu, left), or the same medium containing 5-FOA to monitor silencing of a telomere-proximal *URA3* reporter gene that contained *GAL4* binding sites for tethering (right). Transformants of *sir3* mutant strain LPY4417 containing control *GBD* plasmid pMA424 (A), or pKL5 Sir1p tethering plasmid *GBD-SIR1* (B), as well as the following 2μ plasmids carrying *sir3-eso* mutants and controls, are ordered from top to bottom: pLP304, pLP535, pLP1190, pLP828, pLP681, pLP791, pLP516, pLP526, pLP534, pLP586, and YEp351.

mid, six of the *sir3-eso* mutants exhibited near or complete restoration of telomeric silencing (Figure 4B). In contrast, *GBD-SIR1* did not suppress the telomeric silencing defect of any of the Class II *sir3-eso* mutants that were dominant for mating-type silencing defects, *T135I*, *E140K*, and *L208S*. A control experiment, in which *GBD-SIR1* and the *sir3-eso* plasmids were coexpressed in an isogenic strain without tethering sites, showed that *SIR1* overexpression itself did not suppress the *sir3-eso* telomeric silencing defect (data not shown). Together, these data suggest that *sir3-eso* mutant telomeric silencing phenotypes, like the mating-defective phenotypes, can also be made dependent on Sir1p function. *SIR1*-mediated suppression may occur in one of several ways. For example, suppression may occur via protein-protein interactions of tethered Sir1p and sir3-eso mutant proteins or by the ability of Sir1p to independently establish silencing at telomeres when tethered, thereby compensating for *sir3-eso* mutant defects.

DISCUSSION

In a genetic screen for enhancers of the *sir1* mutant silencing defect, we identified a collection of *sir3* mutant alleles. Unlike the *sir3* null mutant, which is completely mating defective in the presence of *SIR1*, the mating defects of these *sir3-eso* mutants are seen primarily in the absence of *SIR1*. Some of the mutants exhibit dominant effects; all of the *sir3-eso* alleles are defective in telomeric silencing. Mutated residues cluster in an N-terminal region that exhibits a high degree of sequence similarity with the N terminus of the DNA replication initiator Orc1p. The clustering of the mutations in the *sir3-eso* mutants thus identifies a domain of Sir3p that contributes to silencing in the absence of *SIR1*. The *sir3-eso* mutants also provide clues for the role of this shared domain in Orc1p and Sir3p and further evidence for a functional relationship between Sir1p and Sir3p.

sir3-eso* mutants define functional domains in the Sir3 protein:** The phenotypic profile of the *sir3-eso* mutants provides insight into the emerging picture of different functional domains within Sir3p (for review, see Stone and Pillus 1998). Sir3p can be viewed as consisting of two large domains (Figure 5): an N-terminal region with a high degree of similarity to Orc1p and an extended C-terminal region of the protein that can associate with other silencing proteins, including Sir2p, Sir4p, Rap1p, and the histones. Eight of the nine *sir3-eso* mutants identified cluster within the N-terminal domain (Figure 5), disrupting Sir3p silencing function at both *HM* loci in the absence of *SIR1*. Two predominant classes of *sir3-eso* alleles were uncovered: (1) those that are recessive for the *eso* phenotype and (2) those that are dominant and also *MATa** specific (*i.e.*, affecting *HML*α silencing) in wild-type *SIR1* strains. The class II mutants cluster in the N-terminal region between amino acid residues 135 and 208. In *SIR1* strains, both class I and II mutants function normally at *HMR*, but the class II mutants are defective at *HML*. Thus, the class II domain may be required for interaction with a silencing factor only at *HML*. The function of the N-terminal subdomain may be supplied by a redundant mechanism at *HMR*, consistent with the redundancy observed within the *HMRE* silencer (see, for example, Brand *et al.* 1985).

All of the *sir3-eso* alleles are defective in telomeric silencing. This phenotype is seen even in *SIR1* strains, consistent with previous suggestions that Sir1p does not play a major role in silencing telomeric reporter genes (Aparicio *et al.* 1991). However, Sir1p is known to promote silencing when tethered to telomeric sequences (Chien *et al.* 1993), and we showed that tethered Sir1p suppresses telomeric silencing defects of the class I *sir3-eso* mutants. Thus Sir1p function can compensate for the *sir3-eso* mutant silencing defect at the *HM* loci and when tethered at the telomeres. Therefore, Sir1p may directly recruit Sir3p and when tethered may substitute for a telomeric factor that can no longer interact nor-

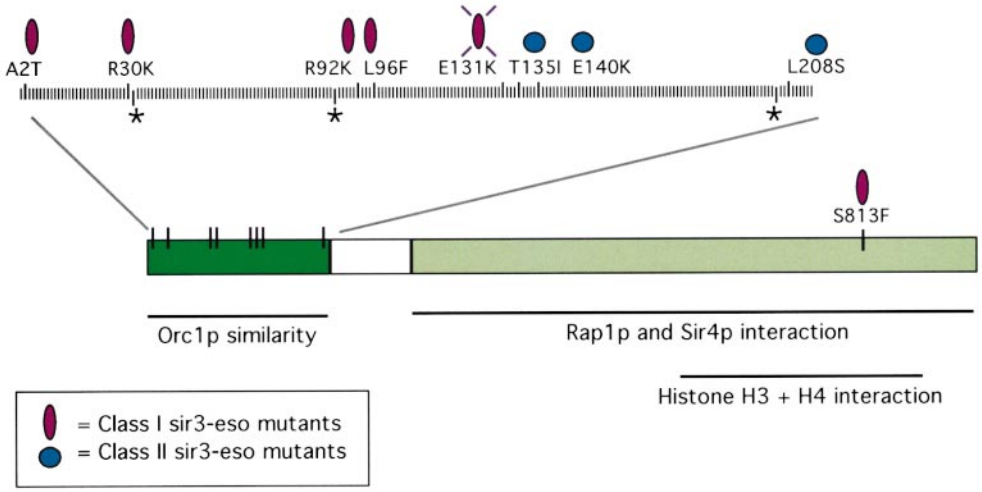

Figure 5.—Sir3p domains confer specific silencing functions. Nine different *sir3-eso* alleles defining two distinct classes of mutants were identified in this study as indicated. Eight of these cluster in the N-terminal region previously noted to be highly similar to Orc1p (in dark green, amino acids 1–214; Bell *et al.* 1995). The *sir3-8*(*E131K*) allele is distinguished in that it is temperature sensitive for mating. The C-terminal two-thirds of the protein has been implicated in a number of physical interactions with other proteins, including Rap1p, Sir4p, and histones H3 and H4 (light green; for review, see Stone and Pillus 1998). Sir2p associates indirectly with Sir3p through a mutual Sir4p interaction (Moazed *et al.* 1997; Strahl-Bolsinger *et al.* 1997). Asterisks indicate three previously identified suppressors of mutations in histone H4 or Rap1p (Johnson *et al.* 1990; Liu and Lustig 1996): *SIR3*^{L31} (S31L), *SIR3R1* (W86R), and *SIR3R3*/*SIR3*^{N205} (D205N), respectively, none of which exhibit an *eso* mutant phenotype. The *sir3-eso* mutants *R92K*, *L96F*, *E131K*, *T135I*, and *E140K* disrupt conserved residues within the BAH domain found in DNA methyltransferases and other proteins with replication and chromatin functions, including both Sir3p and Orc1p (Callebaut *et al.* 1999).

mally with Sir3p in the *sir3-eso* mutants. The telomeric silencing defect exhibited by the Orc1p-Sir3p chimera is consistent with there being a specific role for the Sir3p N terminus at telomeres, and this function might involve interaction with a silencing factor that is either redundant (with Sir1p, for example) or unnecessary at the *HM* loci. Orc1p itself may play a role at telomeres since mutations in two other *ORC* subunits, *ORC2* and *ORC5*, cause telomeric silencing defects (Fox *et al.* 1997).

The class II *sir3-eso* mutants are characterized by dominant effects on mating and telomeric silencing phenotypes, yet these mutant proteins must have some productive interactions with other silencing proteins, because they all function in silencing under some circumstances. However, these dominant mutants must interfere with the function of factors with which they interact in other situations. For example, dominant mating defects are observed in the absence of *SIR1*, but not in its presence, suggesting an inability to disrupt the silencing function that is performed by *SIR1* at the *HM* loci. Moreover, as tethered Sir1p does not suppress the class II alleles, it must not be able to function with those sir3-eso proteins at the telomeres. Interestingly, one of the class I mutants (*R92K*) is dominant for telomeric silencing but behaves like the other members of its class in that it is suppressed by tethered Sir1p.

Several *sir3-eso* mutants enhance the *nat1* mutant mating defect. Interestingly, Sir3p is a potential substrate of Nat1p/Ard1p N-terminal acetyltransferase activity, indicated by its alanine residue at codon 2 (Sherman *et al.* 1993). However, the *nat1* mutant phenotype does not appear to result from the absence of N-terminal acetylation on Sir3p, and so presumably acetylation of some other silencing protein by Nat1p must be required for normal silencing (E. M. Stone and L. Pillus, unpublished data). Because *nat1* mutants, like *sir3-eso* mutants, act as enhancers of the *sir1* mating-defective phenotype (this study and Stone *et al.* 1991), the observed *nat1 sir3-eso* interactions imply that Nat1p, Sir1p, and Sir3p provide interdependent means through which silencing may be achieved.

The temperature-sensitive mating phenotype of the *sir3-8*(*E131K*) mutant is unique among *sir3* mutants (Hartwell 1980; Rine and Herskowitz 1987). We determined that the *sir3-8* allele has the additional phenotype of enhancing the *sir1* mutant phenotype at permissive temperatures. Additionally, we discovered that *sir3-8* encodes a thermolabile protein, thereby explaining the nature of this well-known mutant in molecular terms and allowing more detailed interpretations of earlier experiments using this allele (Miller and Nasmyth 1984; Holmes and Broach 1996). For example, to evaluate the contribution of cell cycle control, silencing was abolished by raising *sir3-8* mutant cells to the restrictive temperature. Then, cells were arrested at G_1 and released at permissive temperature. Silencing was restored only during S-phase, demonstrating that establishment of silencing required DNA replication or some other S-phase event. In contrast, shifting *sir3-8* mutants from permissive to restrictive temperature revealed that maintenance of silencing can be destroyed throughout the cell cycle (Miller and Nasmyth 1984). Previously, the loss of silencing in the *sir3-8* mutant at the restrictive temperature could be explained in at least two ways: inappropriate protein folding of sir3-8p

within an intact multiprotein complex might disrupt its function, *vs.* protein instability might result in loss of function of sir3-8p within the complex or disassembly of the complex itself. We now favor the latter hypothesis, since the *sir3-8* mutant behaves as a conditional null allele.

We hypothesize that different interaction surfaces of the Sir3p molecule are disrupted by the different classes of *sir3-eso* mutations. These mutants will be valuable for extending analysis of different domains of the Sir3p molecule as detailed structural information becomes available.

How might Sir1p, Sir3p, and Orc1p functions be linked? Silencing is a heritable regulatory state, resulting from separable establishment and maintenance functions (Pillus and Rine 1989; reviewed in Rivier and Rine 1992). Sir1p functions in establishing silencing but has no apparent role in its maintenance. In contrast, other Sir proteins, including Sir3p, have clear roles in maintenance. In addition to Sir1p function in establishment, there is presumably some additional mechanism for establishing silencing since a subpopulation of *sir1* mutant cells initiates and propagates the silenced state (Pillus and Rine 1989). Candidate proteins for this function include those that promote telomeric silencing when tethered to engineered sites for ectopic DNA binding proteins, such as Sir3p, Sir4p, and Rap1p (Buck and Shore 1995; Lustig *et al.* 1996; Marcand *et al.* 1996). One goal of the screen for *eso* mutants was to find other genes that function in a manner similar to *SIR1*. Because *sir3-eso* mutant alleles were identified in the screen, they were tested for establishment defects. Pedigree analysis suggested that although *sir3-eso* mutants are weakly defective in the maintenance of silencing, they are unlikely to have an establishment defect in a *SIR1* wild-type background. The gene(s) responsible for establishment in the silencing-competent subpopulation of *sir1* mutant cells have not yet been unambiguously identified, but may include *RAP1*, or other genes uncovered in the *eso* mutant screen like *SIR2* or *SIR4*, or other as yet uncharacterized genes.

Several possibilities exist to explain the *eso* phenotype of the *sir3* alleles. The weak *sir3-eso* maintenance defect, in combination with the *sir1* establishment defect, may lead to complete derepression of the silent mating-type loci. Alternatively, *SIR1* may have an unsuspected role in maintenance of silencing that is normally redundant with that of *SIR3*, leading to the failure to maintain silent chromatin only in the *sir1 sir3-eso* double mutants. Another possibility is that *SIR3* may indeed function in establishing silencing, not constitutively, but in the absence of *SIR1*. This would be analogous to the situation seen for MAP kinase pathway genes in which *KSS1* substitutes for *FUS3* only in a *fus3* null mutant (Madhani *et al.* 1997). Finally, there may not be any other protein that functions similarly to Sir1p, but instead *SIR1*-independent establishment may occur by default at a low frequency (for example, other Sir proteins may occasionally find their way to silencers in the absence of potential recruitment by Sir1p). The molecular definition of Sir1p function and the mechanism of establishing silencing thus remain to be resolved. It is possible that Sir1p's function is distinct from the nucleation role revealed by tethering and mutant studies in which Sir3p, Sir4p, or Rap1p have been implicated in recruiting silencing factors to silent loci (Sussell *et al.* 1993; Lustig *et al.* 1996; Marcand *et al.* 1996).

Genetic interactions between *SIR1* and *SIR3* raise the possibility that the two proteins may physically associate with one another. Our studies demonstrate that *sir3-eso* mutants can enhance the *sir1* mutant mating defect and that the telomeric silencing defect of the *sir3-eso* mutants can be suppressed by tethered Sir1p. Furthermore, *SIR1* overexpression suppresses the mating defects of certain *sir3* mutant alleles (Stone *et al.* 1991). Additionally, Sir1p physically interacts with the N terminus of Orc1 (Triolo and Sternglanz 1996), and Orc1p and Sir3p share significant sequence similarity at their N termini (Bell *et al.* 1995). It is plausible that Sir1p interacts with the N terminus of Sir3p. However, physical interactions between Sir1p and Sir3p have yet to be observed in either two-hybrid analysis (Triolo and Sternglanz 1996) or coimmunoprecipitation experiments (E. M. Stone and L. Pillus, unpublished data). A transient interaction between Sir1p and Sir3p, perhaps occurring only at a specific point in the cell cycle, might be responsible for the establishment of silencing. If Sir1p does not interact with the Sir3p N terminus, perhaps another domain of the Sir3p molecule actively inhibits a potential Sir1p-Sir3p interaction.

The high degree of similarity between the Sir3p and Orc1p N termini suggests a shared function between these domains that may be imagined in several ways. For example, Sir3p may in some instances substitute for Orc1p in the traditional ORC, thereby creating an alternative complex with modified or inhibitory function in DNA replication. Conversely, Orc1p may substitute for Sir3p in a subset of the multiprotein complexes containing the other silencing proteins; such a model would then predict that Orc1p is capable of performing some function independent of the other Orc subunits. The proposal that ORC may play a role independent of DNA replication is supported by the observation that silencing and DNA replication functions of ORC are separable, although they are still *SIR* dependent (Fox *et al.* 1995; Dillin and Rine 1997). An additional possibility is that Sir2p, Sir3p, Sir4p, Rap1p, and histones may coexist with Sir1p and Orc1p in the same multiprotein complex. It is noteworthy that the N-terminal regions of both Sir3p and Orc1p contain the recently identified BAH domain, which is also found in DNA methyltransferases and other proteins thought to act in transcriptional control. The BAH domain is proposed to direct these proteins to their sites of action within chromatin

(Callebaut *et al.* 1999). Because five of the *sir3-eso* mutations are within the BAH domain, their further analysis may lead to greater understanding of the role of this domain in chromatin structure and function.

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