A Unique Class of Conditional *sir2* Mutants Displays Distinct Silencing Defects in *Saccharomyces cerevisiae*

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ABSTRACT

Silencing provides a critical means of repressing transcription through the assembly and modification of chromatin proteins. The NAD^+-dependent deacetylation of histones by the Sir2p family of proteins lends mechanistic insight into how *SIR2* contributes to silencing. Here we describe three locus-specific *sir2* mutants that have a spectrum of silencing phenotypes in yeast. These mutants are dependent on *SIR1* for silencing function at the *HM* silent mating-type loci, display distinct phenotypes at the rDNA, and have dominant silencing defects at the telomeres. Telomeric silencing is restored if the mutant proteins are directly tethered to subtelomeric regions, via a *G*al4p DNA-*b*inding *d*omain (GBD), or are recruited by tethered GBD-Sir1p. These *sir2* mutations are found within conserved residues of the *SIR2* family and lead to defects in catalytic activity. Since one of the mutations lies outside the previously defined minimal catalytic core, our results show that additional regions of Sir2p can be important for enzymatic activity and that differences in levels of activity may have distinct effects at the silenced loci.

SILENCING is a process by which transcriptional repression occurs in a regional, promoter-nonspecific manner. Chemical modifications to DNA, histones, and other nuclear proteins can lead to specific alterations in chromatin structure that may disrupt or promote transcriptional silencing (reviewed in Wolffe and Guschin 2000; Rice and Allis 2001). In *Saccharomyces cerevisiae* at least three loci are subject to silencing: telomeres, the silent mating-type loci (*HMR* and *HML*, the *HM* loci), and the ribosomal DNA (rDNA) repeats. Although many *cis*- and *trans*-acting factors have been implicated in silencing in yeast (reviewed in Gartenberg 2000), few factors appear required at all three loci; among these is the *s*ilent *i*nformation *r*egulator, Sir2p.

The Sir2 protein is an NAD^+-dependent protein deacetylase that is conserved across all kingdoms of life (reviewed in Gottschling 2000; Guarente 2000; Shore 2000; Moazed 2001). The Sir2 family of proteins is the first example of enzymes that couple NADase and deacetylase activities. This unique activity may be what renders cells with aberrant levels of Sir2p defective in a wide range of cellular processes such as silencing, chromosome segregation, DNA repair and recombination, cell cycle checkpoints, and senescence (reviewed in Shore 2000). Sir2p is found in at least two complexes, the *r*egulator of *n*ucleolar *s*ilencing and *t*elophase exit (RENT) complex and the Sir2/4 complex, which appear, respectively, to act within the rDNA array or at telomeres (Shou *et al.* 1999; Straight *et al.* 1999; Ghidelli *et al.* 2001; Hoppe *et al.* 2002). The Sir2, Sir3, and Sir4 proteins also mediate silencing at the *HM* loci with the aid of the targeting protein Sir1p.

In contrast to the variegated silencing states observed for PolII transcribed genes at the telomeres and within the rDNA array, the *HM* loci are distinct in that they normally appear completely repressed. This difference may be due in part to the interactions between Sir1p and Orc1p and to their participation in silencing at the *HM* loci (Pillus and Rine 1989; Triolo and Sternglanz 1996; Fox *et al.* 1997; Gardner *et al.* 1999). One puzzle about *sir1*Δ mutant phenotypes has been that a subpopulation of cells maintains normal silencing. This suggests that other *SIR1*-independent mechanisms are involved in the establishment of silencing at *HML* and *HMR*. To gain insight into these mechanisms, a genetic screen to isolate enhancers of the *sir1*Δ mating defect was performed, yielding several alleles of genes known to function in silencing, including *SIR2*, *SIR3*, and *SIR4* (Reifsnyder *et al.* 1996; Stone *et al.* 2000). The work presented here describes the characterization of the *sir2*^{eso} (***e**nhancers of **s**ir-**o**ne*) mutants.

In previous studies, several *sir2* alleles were demonstrated to have a wide range of silencing defects that could be locus specific, dominant, and in some cases correlated with decreases in enzymatic activity (Sherman *et al.* 1999; Tanny *et al.* 1999; Cuperus *et al.* 2000; Imai *et al.* 2000; Perrod *et al.* 2001; Armstrong *et al.* 2002; Hoppe *et al.* 2002). In this study, we have characterized a unique class of conditional *SIR2* alleles that can silence the *HM* loci only in the presence of Sir1p. Al-

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though two of the mutants contain mutations within the conserved catalytic core domain, all are dominantly defective in silencing at the telomeres, but can function if targeted to the locus as *G*al4p DNA-*b*inding *d*omain (GBD) fusion proteins or via GBD-Sir1p. We demonstrate that robust enzymatic activity is not necessary for silencing telomeric and rDNA reporter genes and propose that wild-type levels of activity may be required for initiating stable silencing at the telomeres but not within the rDNA array.

MATERIALS AND METHODS

Yeast methods and strains: Yeast strains and plasmids are listed in Table 1or described below. Strains were grown at 30° and standard manipulations were performed as described (Rose *et al.* 1989). Yeast extract/peptone/dextrose (YPD), synthetic selective media, and minimal media were prepared as described (Sherman 1991).

Plasmids: The pRS313 (pLP60) and pRS315 (pLP62) vectors were used for subcloning (Sikorski and Hieter 1989). Inserting a 2.7-kb *Bst*NI genomic fragment of *SIR2* into the *Sma*I site of pLP60 created the plasmids pLP284 and pLP285. pLP1102, pLP1110, and pLP1112 are described below in *Gap repair and sequencing*. Inserting an *Eag*I-*Sal*I fragment of pLP285 into pLP62 created the plasmid pLP1237. The plasmids pLP1187, pLP1188, and pLP1189 result from inserting *Pvu*II fragments of the corresponding gap-repaired plasmids pLP1102, pLP1110, and pLP1112, respectively, into pLP62. The pJR1061/pKL5 (*GAL4(1-147)-SIR1*) vector is also known as pLP114 (Chien *et al.* 1993). The plasmid pLP118 is vector pYSR102 containing *SIR1*. The plasmid pLP762 is a 6.6-kb *Sac*II-*Eco*RI genomic fragment of *SIR4* inserted into pRS424 (Sikorski and Hieter 1989). The pGBD-C1 vector (James *et al.* 1996) is also known as pLP956. The plasmid pLP1073 contains the *SIR2* core domain (*Bcl*I-*Nru*I sites) cloned into the *Sma*I site of pLP956. The plasmid pLP1074 contains the *Cla*I fragment of *SIR2* from pLP285 inserted into pGBD-C3 (James *et al.* 1996), also known as pLP958. The plasmids pLP1369, pLP1370, and pLP1371 were created by inserting a *Sac*I-*Nco*I fragment from pLP1102, pLP1110, and pLP1112, respectively, into pLP1074. The pGex-4T-1 is also known as pLP1334. The plasmid pDM111a contains wild-type *SIR2* inserted into pGex-4T-1 (Straight *et al.* 1999) and is also known as pLP1275. The plasmids pLP1335, pLP1336, and pLP1337 contain a *Sac*I-*Nco*I fragment from pLP1102, pLP1110, and pLP1112, respectively, inserted into pLP1275. Mutations in pLP1187, pLP1369, and pLP1335 were confirmed by sequence analysis using primer no. 56 listed below. Mutations in pLP1188, pLP1189, pLP1370, pLP1371, pLP1336, and pLP1337 were confirmed by sequence analysis using primer no. 164 listed below.

The *eso* screen: Details of the mutagenesis are described elsewhere (Reifsnyder *et al.* 1996; Stone *et al.* 2000). Thirty-nine independent mutants were isolated on the basis of their ability to mate in the presence of a *SIR1* plasmid but not in its absence. Several of the *eso* mutants contained mutations in *SIR2* as assessed by standard linkage analysis and complementation tests. Five alleles of *sir2* were isolated from this screen and were cloned by gap repair as described below. The original *sir2*^{eso} allele designations prior to gap repair were: LPY655, 6.2k; LPY667, G16a^{-}; LPY727, J9a; LPY733, M5b^{-}; and LPY1418, Gi.

Gap repair and sequencing: To identify the mutations within *SIR2* we performed gap repair analysis (Rothstein 1991). The *SIR2* wild-type gene on a plasmid was digested in three different ways: Gap1 (G1), pLP284 with *Cla*I and *Eco*NI; Gap2 (G2), pLP285 with *Nco*I and *Nru*I; Gap3 (G3), pLP285 with *Nru*I and *Blp*I. The mutation within the open reading frame (ORF) of the *sir2*^{eso} LPY655 was cloned using gaps G1 (pLP1101) and G3 (pLP1102). The mutations in LPY667, LPY733, and LPY1418 were cloned with G2, pLP1112, pLP1111, and pLP1110, respectively. The mutation in LPY727 was cloned with G3 (pLP1137). Plasmids bearing the lesions that resulted in the original *eso* phenotype were isolated and the entire open reading frames of the gap-repaired plasmids were sequenced. In the course of this sequencing, we identified several polymorphisms between the GenBank sequence data for the S288C background and the W303 background of these studies. Four silent changes were shown with numbering relative to the ORF start: ACA–ACG at nucleotide (nt) 339; CCA–CCG at nt 624; UUG–UUA at nt 774; and CCT–CCC at nt 1404. Four polymorphisms resulted in amino acid substitutions. These included P65T (CCA–ACA, nt 458), K320N (AAC–AAA, nt 1224), M424V (GTG–ATG, nt 1534), and T527A (ACG–GCG, nt 1848). The number of polymorphisms observed from comparative analyses is within the range predicted between these two strain backgrounds (Primig *et al.* 2000). The oligonucleotides used for sequencing were:

No. 55 5′-ATCGCTTCGGTAGACAC-3′
No. 56 5′-AACGTCTTGGGGATCAT-3′
No. 87 5′-AACGTCTTGGGGATCAT-3′
No. 88 5′-GAAGGAACCAAGCTTACGATTTC-3′
No. 163 5′-TCCTTAACTCATATGGCG-3′
No. 164 5′-TGAAACTATGCAATGGAG-3′.

Mutations were identified by comparison to the wild-type *SIR2* sequence reported in the *S. cerevisiae* Genome Database (http://genome-www.stanford.edu/Saccharomyces/).

Qualitative and quantitative mating assays: For qualitative mating assays, cells were patched onto YPD plates for 12–18 hr and then replica plated to YPD to assay for growth and onto a lawn of cells of the opposite mating type, LPY78 or LPY142, on minimal medium to assay for successful diploid formation. The *sir2*^{eso} mating efficiencies were determined by performing quantitative assays as described (Stone *et al.* 2000). Briefly, *sir2*Δ (LPY1557 and LPY4627), *sir1*Δ *sir2*Δ (LPY3439 and LPY3440), wild-type (LPY5 and LPY79), and *sir1*Δ (LPY6 and LPY80) strains were transformed with vector control, pLP60; wild-type *SIR2*, pLP285; *sir2-R139K*, pLP1102; *sir2-G270E*, pLP1110; or *sir2-F296L*, pLP1112 (see supplemental data for transformed yeast strain numbers at http://www.genetics.org/supplemental/). Cells were grown to mid-logarithmic phase under selection, serially diluted, and plated onto plates lacking histidine, to assay for growth, or mixed with tester cells of the opposite mating type and plated onto minimal plates, to evaluate mating. Colonies were counted and mating efficiencies were determined by dividing the number of colonies on minimal plates by the number of colonies on selective plates. Mating efficiencies were calculated by averaging three independent experiments done in duplicate. Standard deviations were determined using preset options on Microsoft Excel.

Telomeric and rDNA silencing assays: Telomeric and rDNA silencing assays were performed as described previously (Gottschling *et al.* 1990; Smith and Boeke 1997). Silencing of a *URA3* reporter gene placed proximal to telomere VII was assayed as described (Gottschling *et al.* 1990), with the modification that the strains tested were grown in liquid medium for 4 days at 30° instead of on solid medium prior to testing. For telomeric silencing assays, LPY1953 and LPY1954 were transformed with pLP62, pLP1237, pLP1187, pLP1188, and pLP1189. For telomeric *SIR1*-tethering silencing assays,

TABLE 1

Yeast strains used in this study

Strain[a]	Genotype	Source
LPY5	W303-1a *MAT***a** *ade2-1 can1-100 his3-11,15 leu2,3,112 trp1-1 ura3-1*	R. Rothstein
LPY6	AMR27 W303-1a *sir1*Δ*::LEU2*	Stone *et al.* (1991)
LPY11	W303-1a *sir2::HIS3*	
LPY12	AMR50 W303-1a *sir1::URA3*	R. Sternglanz
LPY78	*MAT*α *his4*	P. Schatz
LPY79	W303-1b *MAT*α *ade2-1 can1-100 his3-11 leu2,3,112 trp1-1 ura3-1*	R. Rothstein
LPY80	W303-1b *sir1*Δ*::LEU2*	
LPY142	*MAT***a** *his4*	P. Schatz
LPY655	6.2k W303-1b *sir1*Δ*::LEU2 sir2^{eso}-R139K*	
LPY667	G16a$^-$ W303-1a *sir1*Δ*::LEU2 sir2^{eso}F296L*	
LPY733	M5b$^-$ W303-1a *sir1*Δ*::LEU2 sir2^{eso}G270E*	
LPY1029	YDS631-W303-1b adh4::*URA3* (C1-3)n	Chien *et al.* (1993)
LPY1030	YDS634-W303-1b adh4::*URA3*-1XUAS (C1-3)n	Chien *et al.* (1993)
LPY1398	W303-1b *sir2::HIS3* + pLP37	
LPY1418	JRY3003-3i W303-1a *sir1*Δ*::LEU2 sir2^{eso}G270E*	
LPY1557	W303-1a *sir2*Δ*::TRP1*	
LPY1953	YCB652 *MAT***a** *his3*Δ*200 leu2*Δ*1 lys2*Δ*202 trp1*Δ*63 ura3-52 sir2*Δ*2::TRP1* ADH4::*URA3*-TEL	Brachmann *et al.* (1995)
LPY1954	YCB647 *MAT***a** *his3*Δ*200 leu2*Δ*1 lys2*Δ*202 trp1*Δ*63 ura3-52 ADH4::URA3-TEL*	Brachmann *et al.* (1995)
LPY2446	JS128 (S6) *MAT*α *his3*Δ*200 leu2*Δ*1 ura3-52 RDN:: Ty1 -mURA3*	Smith and Boeke (1997)
LPY2447	JS163 (S6) *sir2*Δ*2::HIS3 RDN:: Ty1 -mURA3*	Smith and Boeke (1997)
LPY3439	W303-1a *sir1*Δ*::LEU2 sir2*Δ*::TRP1*	LPY1557 × LPY80
LPY3440	W303-1b *sir1*Δ*::LEU2 sir2*Δ*::TRP1*	LPY1557 × LPY80
LPY3712	W303-1a *sir2^{eso}G270E*	LPY1418 × LPY79
LPY4595	W303-1a *sir2*Δ*::TRP1* (LPY1557) + pLP285	
LPY4621	WY53-W303-1b *net1::Myc9-Net1/LEU2*	Straight *et al.* (1999)
LPY4624	W303-1a *sir2::TRP1* adh4::*URA3*-1XUASGBD (C1-3A)n	LPY3439 × LPY1030
LPY4627	W303-1b *sir2*Δ*::TRP1*	LPY3439 × LPY1030
LPY4724	W303-1a *sir2::HIS3 net1::Myc9-Net1/LEU2*	LPY4621 × LPY1398
LPY5378	GCY62-W303-1b *sir2::KanMX4* RDN1::4XUASg-*mURA3-HIS3*	Cuperus *et al.* (2000)
LPY5611	W303-1a *sir2::HIS3* adh4::*URA3*-1xUASGBD(C1-3A)n	LPY11 × LPY1030
LPY5615	W303-1b *sir2::TRP1 net1::Myc9-Net1/LEU2*	LPY4595 × LPY4724
LPY6400	W303-1b *sir1::URA3 sir2::TRP1 net1::Myc9-Net1/LEU2*	LPY5615 × LPY12

[a] Except where indicated, strains used were constructed in the course of these experiments or are part of the lab collection. The LPY numbers for transformed strains used in this study can be found in the supplemental data at http://www.genetics.org/supplemental/.

LPY4624 and LPY1030 were cotransformed with pLP114 and the same set of plasmids listed above (pLP62-pLP1189). For rDNA silencing assays, LPY2446 and LPY2447 were transformed with the same set of plasmids listed above (pLP62-pLP1189). All transformed strains were grown for 4 days at 30°, serially diluted fivefold, and plated onto selective plates to assay growth. Diluted cells were also plated onto the following silencing test plates: plates lacking leucine supplemented with 0.1% 5-fluoroorotic (5-FOA), plates lacking leucine and histidine containing 0.1% 5-FOA for telomeric silencing assays, and plates lacking uracil for assaying rDNA silencing.

Targeting assays: Yeast strains LPY1030, LPY5611, and LPY5378 were transformed with pLP956, pLP1073, pLP1074, pLP1369, pLP1370, and pLP1371 and assayed for telomeric and rDNA silencing as described above. All transformed strains were grown for 4 days at 30° and serially diluted fivefold on plates lacking tryptophan to assay growth and onto test plates lacking tryptophan and containing 0.1% 5-FOA. To be certain that the mutants expressed at high-copy (2μ) levels retained their *eso* phenotype, the strains were tested for mating ability in a *sir1*Δ *sir2*Δ and *SIR1 sir2*Δ background. As expected, they were able to mate only in the presence of Sir1p (data not shown).

Immunoblot and immunofluorescence analyses: Levels of Sir2p and mutant sir2^{eso} proteins were detected by immunoblot analysis as described (Stone and Pillus 1996). Cell extracts from ~2.0×10^7 cells were loaded per well. Sir2p was detected using a 1:1000 dilution of the polyclonal antiserum (2916/8) raised to a C-terminal peptide of Sir2p (Smith *et al.* 1998). Goat anti-rabbit secondary antibody was used at 1:10,000 and Western blots were developed using a standard alkaline phosphatase detection system (Promega, Madison, WI).

Centromeric plasmids bearing the *sir2^{eso}* mutations and wild-type *SIR2* were transformed into *sir2*Δ (LPY1557) and *sir1*Δ *sir2*Δ (LPY3439) strains. Immunofluorescence using affinity-purified antibody raised to the C terminus of Sir2p (2916/8) was performed as described (Stone and Pillus 1996; Gotta *et al.* 1997; Ersfeld 1999). The other antibody used was anti-Nop1p (D77; Aris and Blobel 1988). Fluorescein-conjugated anti-rabbit and Texas-red-conjugated anti-mouse secondary antibodies (Jackson Immunoresearch, West Grove, PA) were preadsorbed against spheroplasted mutant and wild-type yeast cells. Microscopy was performed on an Applied Precision optical sectioning microscope to collect images spaced at 0.2-μm increments. The images were deconvolved using the Delta Vision deconvolution software as previously described (Pogliano *et al.* 1999).

NAD$^+$ hydrolysis assays: Glutathione S-transferase (GST; pLP1334), GST-Sir2p (pLP1275), GST-sir2-R139Kp (pLP1335), GST-sir2-G270Ep (pLP1336), and GST-sir2-F296Lp (pLP1337)

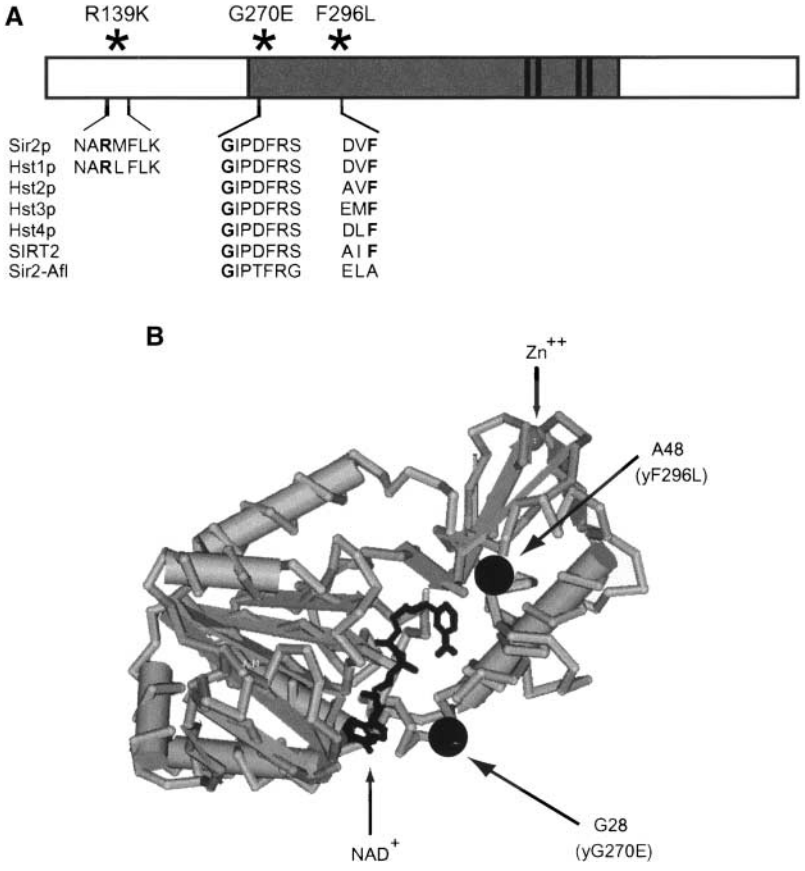

FIGURE 1.—The *sir2*^{eso} mutations result in substitutions of highly conserved amino acids, including a subset within the catalytic core domain of Sir2p. (A) The *sir2*^{eso} alleles were cloned onto plasmids using gap repair (pLP1102-R139K, pLP1110-G270E, and pLP1112-F296L). Sequence analysis of each *sir2*^{eso} ORF revealed mutations altering amino acids that lie within conserved residues of the Sir2p family. Two mutations (G270E and F296L) changed amino acids within the highly conserved core of *SIR2* (dark shading), a domain essential for silencing function. (B) The crystal structure of *A. fulgidus*-Sir2 with *sir2*^{eso} amino acid changes mapped using Cn3D, the National Center for Biotechnology Information Entrez structure retrieval and analysis program (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). The R139K substitution is not shown since the Sir2-Afl enzyme lacks the N-terminal domain of ySir2p.

fusion proteins were expressed in *Escherichia coli* BL21 (DE3) during a 4- to 5-hr induction with 0.5 mM isopropyl β-D-thiogalactoside at room temperature. Proteins were purified on glutathione Sepharose beads as directed by the manufacturer (Pharmacia, Piscataway, NJ). Purified proteins were dialyzed against 50 mM sodium phosphate (pH 7.2) and stored at 4° in 50 mM sodium phosphate (pH 7.2), 0.5 mM dithiothreitol (DTT), and 10% glycerol (LANDRY *et al.* 2000b). Protein concentration was deduced from extinction coefficient measurements as described (GILL and VON HIPPEL 1989) and by SDS-PAGE, comparing Coomassie brilliant blue staining of purified GST-protein samples and various concentrations of the BSA protein standard. NAD^+-hydrolysis assays to measure histone deacetylation were performed as described (LANDRY *et al.* 2000a). Reactions were carried out in 1 ml with 50 mM glycine (pH 9.0), 0.5 mM DTT, 5 mM tetrasodium pyrophosphate (NaP_2O_7), 0.1 mg/ml BSA, 1 mg calf thymus histones (Sigma, St. Louis), 2 μCi $^{[4\text{-}3H]}NAD^+$ (Amersham TRA298; 4.3 Ci/mmol, 1 mCi/ml), and 3.7 μg of purified proteins. The reactions were performed in triplicate and incubated at 30°. After 10 min and 3, 7, 24, and 33 hr, 185 μl of the total reaction was transferred to tubes containing 135 μl 0.5 M boric acid (pH 8.0) to quench the reaction. A total of 1 ml of ethyl acetate was added and vortexed for 5 min and 700 μl of the ethyl acetate phase was transferred to 3 ml Ecoscint fluid (National Diagnostics, Atlanta) and analyzed by scintillation counting. Radioactivity released from Sir2p wild-type control reactions lacking histones was subtracted. The slope, or rate of change, for activities through five time points was calculated and shown as percentage of wild-type Sir2p activity with standard deviations indicated.

Immunoprecipitation reactions: A total of 25 ml of LPY5615 and LPY6400 transformed with pLP60, pLP285, pLP1102, pLP1110, and pLP1112 was grown in medium lacking histidine until it reached an A_{600} of 0.7–0.8. The cells were harvested and lysed as described (STRAIGHT *et al.* 1999). Three microliters of α-Sir2p polyclonal antiserum (above) or 4 μl of α-Sir4p polyclonal antiserum (7795, raised against a β-gal-Sir4 fusion protein) was used and incubated at 4° for 3–4 hr. One hundred microliters of 10% (w/v) protein-A Sepharose (Pharmacia) in lysis buffer was added and mixed at 4° for 1 hr. Immune complexes were harvested and washed as described (STRAIGHT *et al.* 1999) and resuspended in 50 μl of 2.5× SDS sample buffer. Ten microliters of sample was loaded on 12 cm 9% SDS polyacrylamide. Immunoblots were probed with a 1:20 dilution of α-myc (9E10) hybridoma supernatant, a 1:1000 dilution of α-Sir2p polyclonal antiserum (2916/8), or a 1:1000 dilution of α-Sir4p polyclonal antiserum raised against a Sir4p C-terminal peptide (2913/8; PALLADINO *et al.* 1993). Secondary antibodies, horseradish peroxidase-coupled anti-rabbit (for Sir2p and Sir4p) and anti-mouse (for 9E10; Promega) were used at 1:10,000 and detected using the enhanced chemiluminescence reagents (Pharmacia).

RESULTS

Identification of the *sir2*^{eso} mutations and characterization at the *HM* loci: Five *sir2* mutants were isolated from the *eso* mutant screen described previously (REIFSNYDER *et al.* 1996; STONE *et al.* 2000). The *sir2*^{eso} mutations were cloned onto centromeric (CEN) plasmids using standard gap repair (ROTHSTEIN 1991). The repaired plasmids were tested for their ability to confer mating in a *SIR1 sir2*Δ strain but not in a *sir1*Δ *sir2*Δ strain to confirm their *eso* phenotype. The mutations contained in the

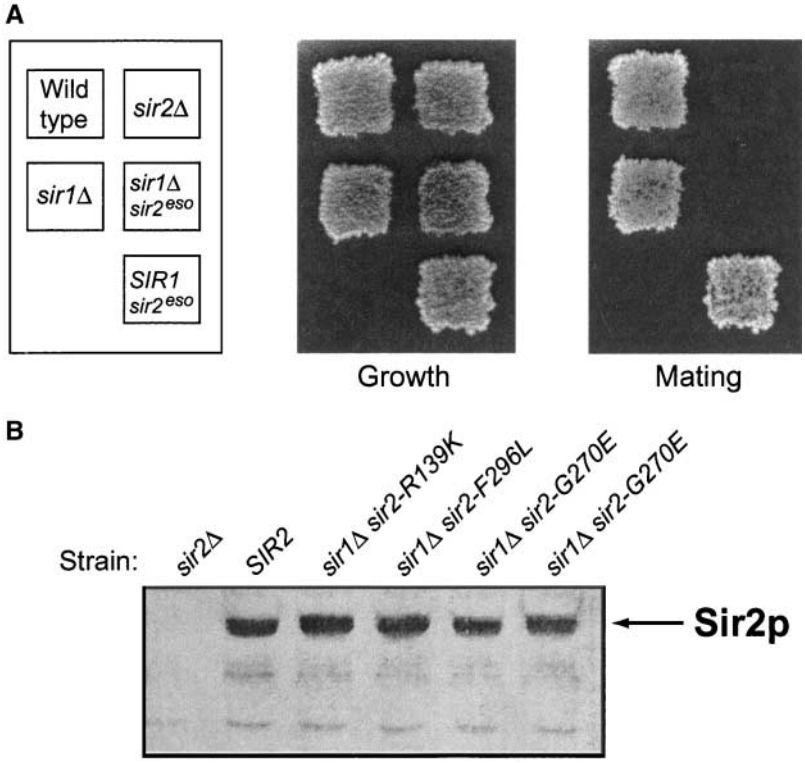

FIGURE 2.—The *sir2*^{eso} mutants enhance the *sir1*Δ defect and the mutant proteins are expressed at wild-type levels. (A) The *sir2*^{eso} mutants are completely mating defective only in *sir1*Δ cells. *MAT***a** strains were patched and replica plated onto YPD plates (growth control) and onto minimal plates top spread with cells of the opposite mating type. The *sir2-G270E* mutant, in the absence of Sir1p and in the presence of Sir1p, is shown. The strains used are wild-type, LPY5; *sir1*Δ, LPY6; *sir2*Δ, LPY11; *sir1*Δ *sir2–G270E*, LPY1418; and *sir2–G270E*, LPY3712. (B) Immunoblot analysis of *sir2*^{eso} mutant whole-cell extracts using anti-Sir2p antisera (2916/8). The sir2^{eso} mutant proteins are expressed from their chromosomal locus in a *sir1*Δ background. The three *sir2*^{eso} alleles characterized in this article are expressed at levels comparable to wild type in both the *SIR1* wild-type and *sir1*Δ backgrounds (the *sir1*Δ background is shown). The strains from left to right are *sir2*Δ (LPY11), wild type (LPY5), *sir1*Δ *sir2-R139K* (LPY655), *sir1*Δ *sir2-F296L* (LPY667), *sir1*Δ *sir2-G270E* (LPY1418), and *sir1*Δ *sir2-G270E* (LPY733).

five *sir2*^{eso} alleles were identified by sequence analysis and three are shown in Figure 1A. Two of the strains (LPY655 and LPY667) had mutations affecting amino acids that are highly conserved among Sir2p family members at positions R139K and F296L, respectively. Although the *eso* mutants were isolated from independently mutagenized cultures, two additional strains (LPY1418 and LPY733) contained an identical mutation changing a highly conserved glycine to a glutamic acid, G270E. Characterization of one strain (LPY1418) was extended as representative of both of these mutants.

Another mutant isolated from the screen, LPY727, contained a nonsense mutation at amino acid 15. The mutant protein was presumed to be translated using a downstream methionine since a truncated version of the protein was detected by immunoblot analysis (data not shown). This mutant protein also appeared to be expressed at lower levels, perhaps due to three additional mutations found in the promoter region. Previous evidence indicates that silencing is sensitive to Sir2p dosage (HOLMES *et al.* 1997; SMITH *et al.* 1998). Therefore, it is possible that this mutant's *eso* phenotype is due to some combination of decreases in levels of expression and altered N-terminal sequence. This mutant has not been pursued further. Immunoblot analysis showed that levels of the other sir2 mutant proteins were equivalent to wild type in the presence and absence of Sir1p (*sir1*Δ background shown in Figure 2B). Since the sir2 mutant proteins appear to be expressed comparably to wild type, the phenotypes observed cannot be due to decreased expression or to instability leading to grossly lowered steady-state levels. Thus, it appears that the mutant defects are due to more specific influences on silencing.

To evaluate the effects of these mutations relative to proposed enzymatic activities, we considered the structures of *Archaeoglobus fulgidus* Sir2 (Sir2-Afl) complexed with NAD^+ and of a human homolog, SIRT2 (FINNIN *et al.* 2001; MIN *et al.* 2001). The two superimposed enzymes show similarities to the regions containing the Rossman fold domain, commonly found in NAD(H)/NADP(H)-binding proteins, and to the smaller catalytic domain containing a structural zinc atom (FINNIN *et al.* 2001) reviewed in DUTNALL and PILLUS (2001). The structure of the yeast Sir2 protein has not yet been determined. However, on the basis of the structural similarities between the Sir2-Afl and SIRT2, it appears that two of the *sir2*^{eso} mutants contain amino acid changes in conserved residues within regions that contribute to the Rossman fold domain (Sir2-Afl in Figure 1B). The equivalent amino acid changes in Sir2-Afl are at amino acids G28 (yG270E) and A48 (yF296L). The fact that this domain is the postulated NAD^+-binding portion of the enzyme suggests that these two mutants might be impaired in catalytic activity. The R139K mutation lies in a region N-terminal of the conserved core domain and *a priori* was not expected to influence activity. This region is not present in Sir2-Afl or SIRT2 structures and therefore the location of R139K could not be inferred (FINNIN *et al.* 2001; MIN *et al.* 2001). The N-terminal region is also variable in members of the yeast *SIR2* family members and might be expected to influence *SIR2*-specific functions (BRACHMANN *et al.* 1995). Effects of

TABLE 2

The *sir2*^{eso} quantitative mating efficiencies

Plasmid	Strain genotype:	Mating efficiency[a]		
		*MAT***a** *sir2*Δ	*MAT***a** *sir1*Δ *sir2*Δ	*MAT***a** *sir1*Δ
SIR2 (pLP285)		1.0	0.4 ± 0.07	0.2 ± 0.02
Vector only (pLP60)		$1 \times 10^{-6} \pm 0.04 \times 10^{-6}$	$3 \times 10^{-6} \pm 0.6 \times 10^{-6}$	0.5 ± 0.06
sir2-R139K (pLP1102)		0.8 ± 0.07	$1 \times 10^{-4} \pm 0.3 \times 10^{-4}$	0.1 ± 0.02
sir2-G270E (pLP1110)		0.9 ± 0.07	$3 \times 10^{-4} \pm 0.6 \times 10^{-6}$	$2 \times 10^{-4} \pm 0.4 \times 10^{-4}$
sir2-F296L (pLP1112)		0.7 ± 0.09	$3 \times 10^{-6} \pm 0.4 \times 10^{-6}$	$2 \times 10^{-4} \pm 0.4 \times 10^{-4}$
		*MAT*α *sir2*Δ	*MAT*α *sir1*Δ *sir2*Δ	*MAT*α *sir1*Δ
SIR2 (pLP285)		1.0	0.2 ± 0.2	0.2 ± 0.08
Vector only (pLP60)		$7 \times 10^{-6} \pm 2 \times 10^{-6}$	$2 \times 10^{-4} \pm 0.1 \times 10^{-4}$	0.6 ± 0.02
sir2-R139K (pLP1102)		0.8 ± 0.04	$2 \times 10^{-4} \pm 0.4 \times 10^{-4}$	0.1 ± 0.01
sir2-G270E (pLP1110)		0.9 ± 0.02	$2 \times 10^{-4} \pm 0.3 \times 10^{-4}$	0.01 ± 0.001
sir2-F296L (pLP1112)		0.8 ± 0.01	$2 \times 10^{-4} \pm 0.2 \times 10^{-4}$	0.005 ± 0.002

[a] Mating efficiencies from three independent experiments are shown with standard deviations and normalized to either *MAT***a** *sir2*Δ or *MAT*α *sir2*Δ strains transformed with a plasmid containing wild-type *SIR2* (pLP285).

the *sir2*^{eso} mutants on catalysis were investigated as described below.

By virtue of the design of the *eso* screen, the *sir2*^{eso} mutants displayed a characteristic mating defect in the absence of Sir1p. A qualitative mating assay with one representative *sir2*^{eso} mutant is shown in Figure 2A. It is notable that a *sir1*Δ strain mates comparably to a wild-type strain by this assay despite its known epigenetic silencing defects at the *HM* loci (Pillus and Rine 1989). Therefore, it remained a possibility that the *sir2*^{eso} alleles displayed silencing defects at *HML* and *HMR* even in the presence of Sir1p at levels detectable only through quantitative analysis. Mating efficiencies for strains transformed with plasmids bearing the *sir2*^{eso} mutations, wild-type *SIR2*, or vector only were calculated and normalized to wild type. The results showed that the *sir2*^{eso} mutants were modestly defective in silencing in the presence of *SIR1* (Table 2). Although they mated at 70–90% efficiency in comparison to wild type, the *sir2*^{eso} mutants were slightly more efficient in mating than the *sir1*Δ *SIR2* mutant strain. The *sir1*Δ *sir2*^{eso} mutants are as defective as a *sir1*Δ *sir2*Δ mutant, with mating efficiencies four to seven orders of magnitude lower than those of wild-type strains. In addition, two lesions within the core domain, *sir2-G270E* and *sir2-F296L*, conferred dominantly derepressed phenotypes in *MAT***a** *sir1*Δ and *MAT*α *sir1*Δ cells but not in *SIR1* wild-type backgrounds (data not shown).

The *sir2*^{eso} mutants are defective in telomeric silencing and can be partially suppressed by tethering Sir1p to telomeres: In contrast to silencing at the *HM* silent mating-type loci, loss of Sir1p has no effect on silencing reporter genes at telomeres (Aparicio *et al.* 1991). Since the *sir2*^{eso} defects at the *HM* loci were revealed only in *sir1*Δ mutants, although *SIR1* has no apparent role in telomeric silencing, it was possible that the *sir2*^{eso} alleles would be competent in telomeric silencing. To test this hypothesis, a *sir2*Δ strain marked at telomere VII with *URA3* was transformed with CEN plasmids containing the *sir2*^{eso} alleles (pLP1187-1189), *SIR2* (pLP1237), or vector only (pLP62). Telomeric silencing was assayed on 5-FOA-containing medium as described previously (Gottschling *et al.* 1990). All three *sir2*^{eso} alleles were sensitive to 5-FOA, demonstrating that they were completely defective in silencing the telomeric reporter gene (Figure 3A).

Recently, it was determined that there are only ~30 molecules of Sir1p per cell (Gardner and Fox 2001), suggesting that Sir1p may be limiting in the cell. Therefore, we tested whether increased *SIR1* gene dosage suppressed the *sir2*^{eso} telomeric silencing defects. Expression of *SIR1* using a high-copy 2μ plasmid did not suppress their defects; thus this simple possibility does not explain the *sir2*^{eso} phenotype (data not shown). Next, we directed Sir1p to the telomeres using a GBD-Sir1p fusion protein and a modified telomeric reporter gene containing an adjacent Gal4p DNA-binding site (UASg). Previous results demonstrated improvement in silencing upon tethering GBD-Sir1p at a telomeric reporter. However, such improved silencing is dependent on the other Sir proteins, including Sir2p (Chien *et al.* 1993). A *sir2*Δ strain marked at telomere VII with a UASg-*URA3* marker was cotransformed with GBD-Sir1p (pLP114) and with the same set of *sir2*^{eso} plasmids used for the telomeric assay shown in Figure 3A. The telomeric defects for all three alleles were suppressed by tethering Sir1p, albeit to differing degrees (Figure 3B). The *sir2–R139K* defect was fully suppressed whereas the two core mutants were only partially suppressed. Therefore, the *sir2*^{eso} mutants require Sir1p for silencing at the *HM* loci

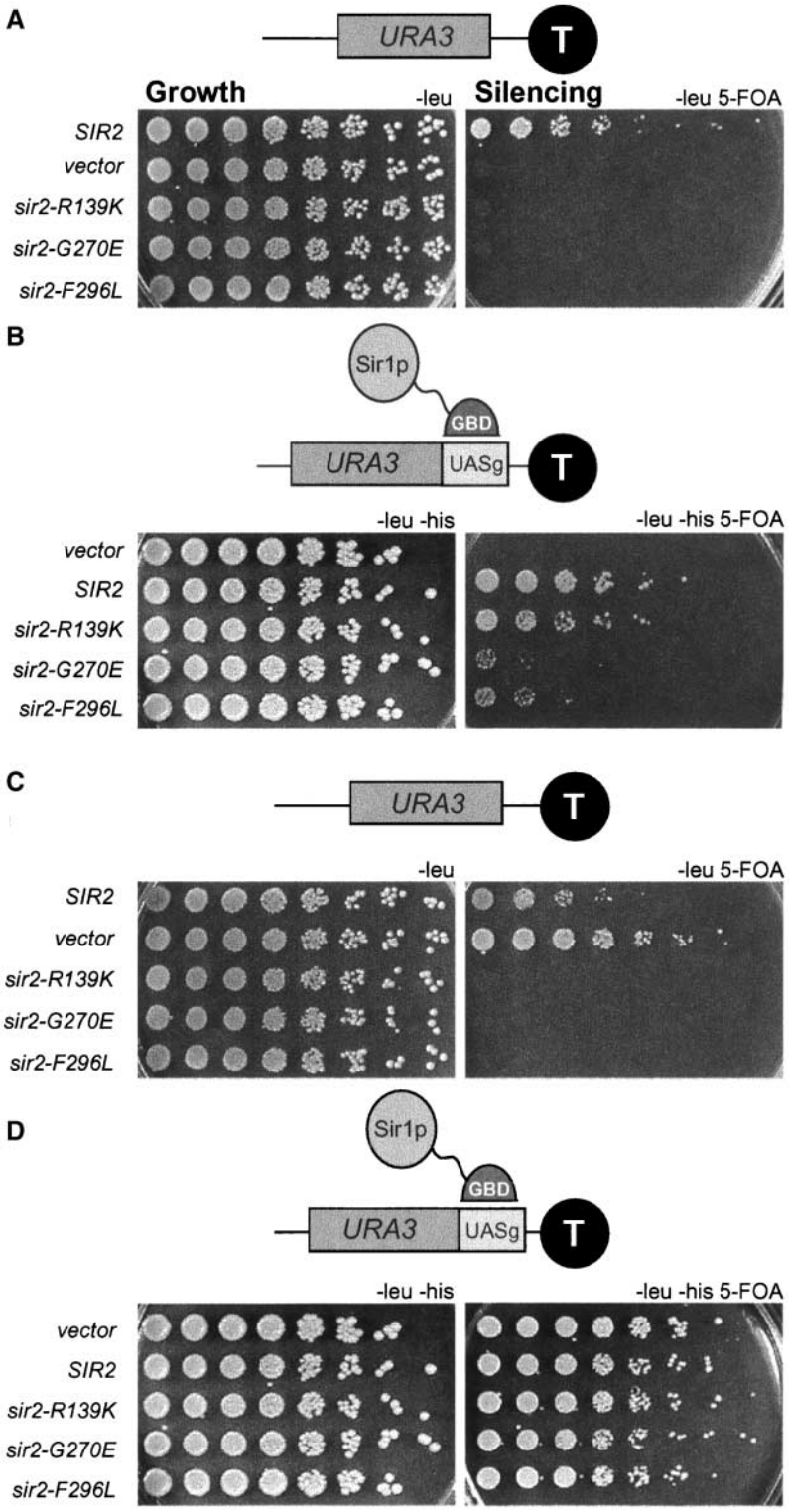

FIGURE 3.—The *sir2*^{eso} mutants show defects in TPE. (A) A *sir2*Δ strain (LPY1953) containing a *URA3* marker proximal to telomere VII was transformed with a *LEU2* CEN vector (LPY4859), *SIR2* (LPY4860), *sir2-R139K* (LPY4861), *sir2-G270E* (LPY4862), or *sir2-F296L* (LPY4863). Fivefold dilutions of the transformants were plated on 5-FOA, growth on 5-FOA indicating silencing of the *URA3* reporter, and on SC–leu plates, to control for growth differences. (B) Tethering Sir1p to a telomeric reporter partially suppresses the *sir2*^{eso} TPE defect. A *sir2*Δ strain (LPY4624) containing a *URA3* reporter at telomere VII, in addition to a copy of the Gal4p DNA-binding site, was cotransformed with a Gal4p DNA-binding domain *(GBD)-SIR1* hybrid *HIS3* 2μ vector (pLP114) and an empty *LEU2* CEN vector (pLP62) or the vector containing *SIR2* (pLP1237), *sir2-R139K* (pLP1187), *sir2-G270E* (pLP1188), or *sir2-F296L* (pLP1189). The transformants were assayed for silencing as in A. (C) The *sir2*^{eso} mutants demonstrate dominance by disrupting telomeric silencing even in the presence of Sir2p. A *SIR2* strain (LPY1954) was transformed and assayed as in A. (D) Tethering Sir1p suppresses the dominant *sir2*^{eso} phenotype. A *SIR2* strain (LPY 1030) was transformed and assayed as in B.

and can function if Sir1p is directed to the telomeres. This suggested that at some level, the sir2^{eso} mutant proteins had the capacity to function in telomeric silencing, although this function appeared limited.

To evaluate further the nature of *sir2*^{eso} function at telomeres, we performed a dominance test. In this experiment, the *sir2*^{eso} mutant genes on centromeric plasmids were transformed into a telomere-marked strain containing wild-type Sir2p. Somewhat surprisingly, the *sir2*^{eso} mutants disrupted silencing in the presence of *SIR2* (Figure 3C). However, tethering Sir1p to the telomeres overcame this dominant phenotype (Figure 3D). A potential molecular explanation for the dominance observed could be that the sir2^{eso} mutant proteins were

TABLE 3

Localization of sir2-G270Ep at telomeres and nucleolus

Strain	Total no. of cells	Telomeric staining	
		>3 foci (%)	0–3 foci (%)
SIR1 SIR2	114	44 (38)	70 (62)
sir1Δ SIR2	87	35 (38)	54 (62)
SIR1 sir2Δ	69	0 (0)	69 (100)
sir1Δ G270E	106	2 (2)	104 (98)
SIR1 G270E	138	57 (41)	75 (54)

Strain	Total no. of cells	Nucleolar staining	
		Colocalized with Nop1p (%)	No colocalization (%)
SIR1 SIR2	114	94 (83)	20 (17)
sir1Δ SIR2	87	78 (90)	9 (10)
SIR1 sir2Δ	69	0 (0)	69 (100)
sir1Δ G270E	106	23 (22)	79 (78)
SIR1 G270E	138	124 (90)	14 (10)

The *sir2^{eso}* mutant strains were analyzed by immunofluorescence in *sir1Δ* and *SIR1* strain backgrounds. The total cell number was derived from four independent experiments. Images were blinded and the number of telomeric foci counted and represented as >3 foci and 0–3 foci. Percentages are the number of cells with a specific pattern divided by the total number of cells analyzed for that sample.

mislocalized in the cell and thus titrated Sir4p or wild-type Sir2p away from the telomeres. To test this possibility sir2^{eso} mutant proteins were localized using immunofluorescence analysis.

Localization of the *sir2^{eso}* mutant proteins: Genetic and biochemical studies place Sir2p at the *HM* loci, the telomeres, and the rDNA repeats that serve as a nucleolar organizer. Consistent with this, by indirect immunofluorescence Sir2p localizes in a crescent or cup-shaped form in the nucleolus and as clustered spots at telomeric foci found at the nuclear periphery directly opposite the nucleolus (Gotta *et al.* 1997). Using a polyclonal antibody raised to a peptide specific for the C terminus of Sir2p (2916/8; Smith *et al.* 1998), we determined the localization of the sir2^{eso} mutant proteins in *sir1Δ* and *SIR1* strains. To assess nucleolar localization, we evaluated colocalization with the nucleolar marker Nop1p, the yeast homolog of fibrillarin (Aris and Blobel 1988). To assess telomeric localization, we quantitated the number of telomeric foci in the various strain backgrounds.

Two of the mutant proteins, sir2-R139Kp and sir2-F296Lp, showed localization indistinguishable from wild-type Sir2p in both *SIR1* and *sir1Δ* backgrounds (data not shown). In contrast, proper localization of sir2-G270Ep appeared to depend on the status of *SIR1*. The sir2-G270E mutant protein localized to telomeres in only ~5% of *sir1Δ* cells relative to wild-type Sir2p localization. In addition, on the basis of failure to colocalize with Nop1p, this mutant protein showed no localization to the nucleolus in ~30% of the cells analyzed relative to wild-type Sir2p (Table 3). Furthermore, the few cells with wild-type localization showed decreased staining intensity, represented in Figure 4. In marked contrast, in *SIR1* strains, the sir2-G270Ep localization was restored to telomeres and the nucleolus in a manner indistinguishable from wild type. Therefore, the localization pattern of this mutant does not account for its defects in telomeric silencing since comparable defects are observed in both *SIR1* (Figure 3) and *sir1Δ* (data not shown) backgrounds. Perhaps the sir2^{eso} mutant proteins do reach the telomeres and the nucleolus but their association to the chromatin is functionally inadequate at these silenced regions.

Sir1p has been shown to function in the establishment of silencing (Pillus and Rine 1989), serving as a recruitment factor for the other Sir proteins at the silent mating-type loci (Fox *et al.* 1997; Gardner and Fox 2001). Because Sir1p does not ordinarily serve as a recruitment protein at the telomeres, we hypothesized that Sir2p might also function in recruiting Sir3p and Sir4p to the *HM* loci and the telomeres. The *sir2^{eso}* mutants may be defective in this hypothesized recruitment function, rendering them fully dependent on other recruitment proteins. This is consistent with the *sir2^{eso}* dependence on Sir1p at the *HM* loci and GBD-Sir1p at the telomeres. To test this hypothesis, we fused the sir2^{eso} mutant proteins to the GBD to tether the mutants directly through engineered binding sites on the chromosome. If the *sir2^{eso}* mutants are defective in a recruitment function, then their defects might be suppressed if targeted to a reporter gene at the telomeres via a GBD domain. A *sir2Δ* strain marked at telomere VII with a *URA3* reporter and an adjacent Gal4p DNA-binding site was transformed with a vector expressing GBD, GBD-Sir2pcore$_{(210\text{-}440)}$, GBD-Δ73NSir2p$_{(73\text{-}562)}$, GBD-Δ73Nsir2-R139Kp, GBD-Δ73Nsir2-G270Ep, or GBD-Δ73Nsir2-F296Lp and the transformants were tested for silencing. The GBD and GBD-Sir2pcore constructs served as negative controls since it has been shown that the core domain of Sir2p is necessary but not sufficient for silencing even when tethered to a reporter (Cockell *et al.* 2000).

In all cases, tethering the *sir2^{eso}* mutants restored telomeric position effect (TPE) to wild-type levels (Figure 5). This restoration was fully dependent on tethering since the constructs were unable to silence at telomeres in strains lacking the Gal4p DNA-binding site (UASg) adjacent to the *URA3* reporter (data not shown). Therefore, the *sir2^{eso}* mutants are properly localized to the telomeres by immunofluorescence, yet can fully function only if targeted to the locus either directly via a Gal4p DNA-binding domain or indirectly via GBD-Sir1p.

The *sir2^{eso}* mutants show distinct phenotypes at the rDNA: PolII-transcribed reporter genes engineered within the rDNA are subject to *SIR2*-dependent silencing. To determine whether the *sir2^{eso}* mutants function

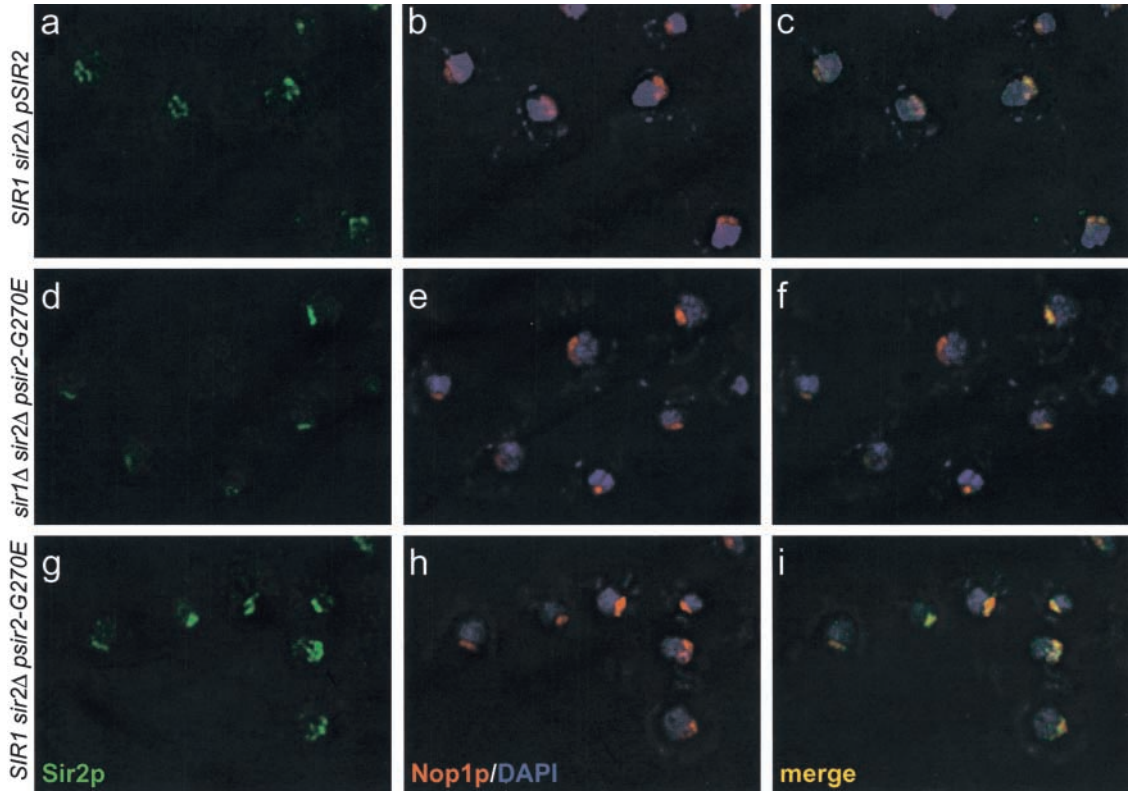

FIGURE 4.—The sir2-G270E mutant protein is mislocalized in cells lacking Sir1p. (Top: a, b, and c) *SIR1 sir2*Δ transformed with *SIR2* on a *HIS3* CEN plasmid (LPY4595). (Middle: d, e, and f) *sir1*Δ *sir2*Δ strain transformed with *sir2-G270E* on a *HIS3* CEN plasmid (LPY4602). (Bottom: g, h, and i) *SIR1 sir2*Δ transformed with *sir2-G270E* on a *HIS3* CEN plasmid (LPY4597). Strains (a, d, and g) were stained with anti-Sir2p affinity-purified antisera (2916/8) and detected by FITC-conjugated secondary antibodies (b, e, and h) with anti-Nop1p antibodies detected by a Texas-red-conjugated secondary antibody and (c, f, and i) the merge of Nop1p, Sir2p, and DNA staining with 4′,6-diamidino-2-phenylindole.

in silencing at the rDNA locus, a *sir2*Δ strain containing a *URA3* cassette inserted in the rDNA was transformed with the various *sir2*^{eso} plasmids as described above. Silencing was assayed by evaluating growth on plates lacking uracil with less growth indicating more silencing (SMITH and BOEKE 1997). The two *sir2*^{eso} mutants that carry a mutation within the conserved core domain of *SIR2* were defective in silencing the *URA3* rDNA reporter (Figure 6A). However, the mutant *sir2-R139K* functioned fully at this locus, silencing as well as or better than wild-type *SIR2*.

Transcriptional and recombinational silencing within the rDNA is distinct because it is fully dependent on *SIR2*, yet is independent of the other *SIR* genes (GOTTLIEB and ESPOSITO 1989; BRYK *et al.* 1997; FRITZE *et al.* 1997; SMITH and BOEKE 1997). Because the *sir2*^{eso} alleles

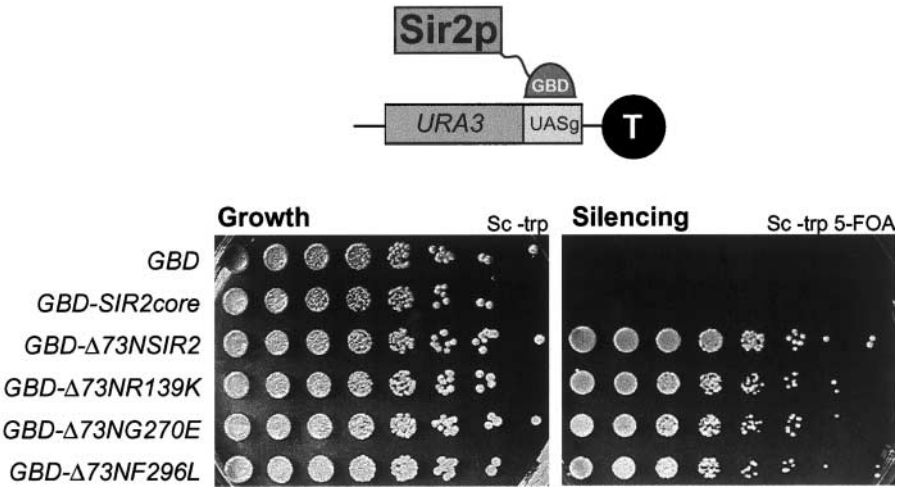

FIGURE 5.—Tethering the *sir2*^{eso} alleles directly to the telomeres rescued their telomeric silencing defects. A *sir2*Δ strain (LPY5611) marked at telomere VII with a *URA3* reporter gene with an adjacent Gal4p DNA-binding site was transformed with $GBD_{(1\text{-}147)}$ (LPY5777), GBD-Sir2-$pcore_{(210\text{-}440)}$ (LPY5778), GBD-Δ73NSir2-$p_{(73\text{-}562)}$ (LPY5779), GBD-Δ73NSir2-R139Kp (LPY5780), GBD-Δ73NSir2-G270Ep (LPY5781), or GBD-Δ73NSir2-F296Lp (LPY5782). Fivefold dilutions were plated and assayed for growth on Sc-trp or silencing on 5-FOA.

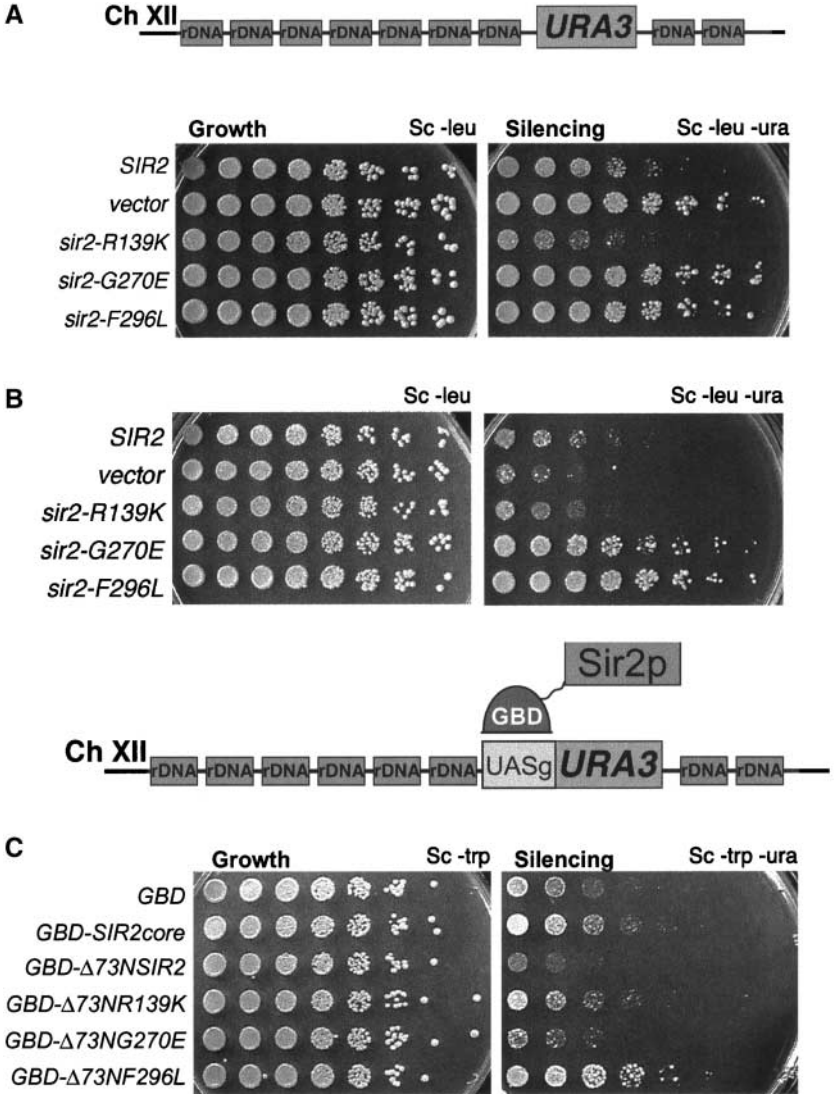

FIGURE 6.—The *sir2*^{eso} mutants have distinct silencing phenotypes within the rDNA locus. (A) A *sir2*Δ strain (LPY2447) marked at the rDNA with a *URA3* cassette was transformed with wild-type *SIR2* on a *LEU2* CEN plasmid (pLP1237), empty vector (pLP62), *sir2-R139K* (pLP1187), *sir2-G270E* (pLP1188), or *sir2-F296L* (pLP1189), and fivefold dilutions of the transformants were assayed for growth on Sc-leu or silencing on Sc-ura. The *sir2*^{eso} mutants with mutations in the conserved domain of Sir2p are defective in rDNA silencing. (B) A *SIR2* strain (LPY2446) transformed and assayed as in A. The *sir2-G270E* and *sir2-F296L* mutants are dominantly defective in rDNA silencing. (C) A *sir2*Δ strain (LPY5378) containing four Gal4p DNA-binding sites adjacent to the rDNA *URA3* reporter was transformed with GBD (LPY5637), GBD-Sir2pcore$_{210\text{-}440}$ (LPY5638), GBD-Δ73NSir2p$_{73\text{-}562}$ (LPY5639), GBD-Δ73NSir2-R139Kp (LPY5640), GBD-Δ73N-Sir2-G270Ep (LPY5641), or GBD-Δ73NSir2-F296Lp (LPY5642). Fivefold dilutions were assayed for growth and silencing. GBD alone or fused to the conserved core domain of Sir2p failed to silence the reporter. The *sir2-G270E* mutant is rescued when tethered to the rDNA; however, the *sir2-F296L* mutant remains defective. Note that mutant *sir2-R139K* becomes defective in silencing at the rDNA when tethered to the locus.

had a conditional dependence on *SIR1* at the *HM* loci, we tested whether this dependence might also exist at the rDNA. The absence of Sir1p had no effect on the *sir2*^{eso} phenotypes observed at the rDNA (data not shown). Since the *sir2-G270E* and *sir2-F296L* mutations caused dominant derepression at the *HM* loci and the telomeres, the dominance test was repeated at the rDNA locus. The *sir2*^{eso} genes on plasmids were transformed into a *SIR2* strain marked within the rDNA and, as before, transformants were assayed for growth on plates lacking uracil. The two mutants defective in rDNA silencing, *sir2-G270E* and *sir2-F296L*, also showed dominant effects at this locus (Figure 6B).

We proposed in the section above that the *sir2*^{eso} mutants could be defective in a type of recruitment function at the telomeres. To extend this hypothesis to the rDNA, we tested the ability of the *sir2*^{eso} strains to silence the rDNA if targeted directly to this locus. The *GBD-Δ73NSIR2* constructs were transformed into a *sir2*Δ strain containing a modified *URA3* reporter gene with four adjacent Gal4p DNA-binding sites (4X-UASg) within the rDNA locus (CUPERUS *et al.* 2000). Because of inherent variability in silencing assays, in each case multiple independent transformants were evaluated. We observed, as expected, that the GBD-Δ73NSir2p consistently silenced when tethered. Neither GBD-Sir2pcore nor GBD constructs alone silenced. The modest differences between these controls (Figure 6) demonstrate the occasional variability noted above. The results with the *sir2*^{eso} alleles were somewhat surprising. One of the two rDNA silencing-defective *sir2*^{eso} mutants, *sir2-G270E*, was rescued when tethered, whereas the other mutant, *sir2-F296L*, was not. Further, the rDNA silencing-competent *sir2*^{eso} mutant, *sir2-R139K*, became impaired for silencing when tethered to the rDNA reporter (Figure 6C). We observed that simply expressing these constructs in a strain with a silencing reporter but no UAS had no effect (data not shown); thus the effects observed are completely dependent on the tethering site.

Therefore, all three mutants showed a different spec-

TABLE 4

The *sir2*^{eso} mutants are defective in NADase activity

GST fusion protein	Normalized NADase activity
GST-Sir2p	1.00 ± 0.08
GST-sir2-R139Kp	0.37 ± 0.01
GST-sir2-G270Ep	0.59 ± 0.04
GST-sir2-F296Lp	0.34 ± 0.07
GST	0.05 ± 0.02

Purified proteins GST-Sir2p (pLP1275), GST-sir2R139K (pLP1335), GST-sir2G270E (pLP1336), GST-sir2F296L (pLP1337), or GST (pLP1334) were tested for their ability to convert NAD^+ to nicotinamide and ADP-ribose in a histone-dependent manner. Reactions were performed in the presence of [^{3}H]NAD^+ and 3.7 μg of purified enzymes. Radiolabeled [^{3}H]nicotinamide was detected as described by Landry *et al.* (2000a; see materials and methods). The rate of change over five time points (10 min and 3, 7, 24, and 33 hr) was calculated and normalized to wild-type Sir2p. Multiple experiments were performed. The experiment shown was performed in triplicate and is shown with standard deviations.

trum of phenotypes with respect to rDNA silencing, implying that they had different silencing defects at this locus. Although inadequate association with chromatin might explain the silencing phenotypes of the *sir2*^{eso} mutants at the *HM* loci and the telomeres, this model seemed inadequate to explain the diversity of phenotypes of the *sir2*^{eso} mutants at the rDNA. A significant element of Sir2p silencing function is its NAD^+-dependent deacetylase activity. We therefore asked if the differences between the *sir2*^{eso} silencing abilities were reflected in their catalytic activities.

The *sir2*^{eso} mutants are impaired in NADase (deacetylase) activity: Previous reports showed that the Sir2p family of proteins functions as NAD^+-dependent protein deacetylases and that decreases in deacetylase activity correlate with loss of silencing (Imai *et al.* 2000; Landry *et al.* 2000b; Smith *et al.* 2000). The role of NAD^+ in the deacetylation reaction has been investigated and has led to a deeper understanding of the mechanism of catalysis (Imai *et al.* 2000; Landry *et al.* 2000a; Borra *et al.* 2002). On the basis of the products released, the reaction is described as the hydrolysis of one NAD^+ to form nicotinamide and the novel product acetyl-ADP-ribose (AADPR), for each acetyl group removed. This results in a 1:1:1 molar ratio of acetyl-ADP-ribose (AADPR), nicotinamide, and a deacetylated peptide substrate with an enzyme-ADP-ribose intermediate (Landry *et al.* 2000a). This finding allows for a direct correlation between NADase activity and histone deacetylation for the Sir2 family members.

To determine whether the sir2^{eso} proteins retained wild-type deacetylase activity, recombinant GST-sir2^{eso} mutant fusion proteins were expressed in *E. coli*, purified, and tested for NADase activity *in vitro* (Landry *et al.* 2000a). The results of three independent NADase assays showed that the mutants were all partially impaired in enzymatic activity (Table 4 and data not shown). The GST-sir2-R139Kp and GST-sir2-F296Lp mutant proteins consistently had <50% activity when compared to wild-type GST-Sir2p. The GST-sir2-G270E mutant protein was slightly more active than the other two mutant enzymes but never achieved activities >70% of wild-type activity. The activities shown in Table 4 reflect consistent differences of mutant activity relative to wild type. Further distinctions between the GST-sir2^{eso} mutant proteins may become apparent with extensive kinetic analyses or with comparisons between NADase and deacetylase activities. Since NADase and deacetylase activities are coupled, however, the decreases in activity for the *sir2*^{eso} mutant proteins suggest that they are not significant enough to completely abolish silencing because these mutants were capable of silencing when tethered to a reporter or when tethered by Sir1p. Since the *sir2*^{eso} mutants are not catalytically dead but have only partially impaired enzymatic activity, we asked whether the silencing defects observed *in vivo* might also reflect inefficient complex formation or protein interactions.

The sir2^{eso} mutant proteins interact with Sir4p and Net1p: Sir2p is known to interact with a number of other proteins. Among these are Sir4p (Moazed *et al.* 1997) and Net1p (Shou *et al.* 1999; Straight *et al.* 1999), associations with which are correlated with telomeric and rDNA silencing, respectively. We considered the possibility that the *sir2*^{eso} phenotype was caused by impaired interactions with these proteins and tested their associations by immunoprecipitation experiments. Immunoprecipitation was performed with either anti-Sir2p or anti-Sir4p reagents. The immunoprecipitated samples were analyzed by protein immunoblotting using the relevant antisera as noted (Figure 7). The results indicated that the sir2^{eso} mutant proteins interacted with both Sir4p and Net1p, even in the absence of Sir1p (Figure 7 and data not shown). This suggests that Sir and RENT complex formation is not grossly disrupted in the *sir2*^{eso} mutants. It is also clear that Sir1p does not stabilize the components of the complexes since interactions with Sir4p and Net1p are found in both *SIR1* and *sir1*Δ backgrounds (*sir1*Δ background for Sir4p, Figure 7A; both backgrounds for Net1p, Figure 7B). One difference observed in the *sir1*Δ background was the consistent loss of an additional α-myc-reactive band that commonly migrates with the Net1p in immunoblot analyses (Straight *et al.* 1999; Figure 7B). The band could represent a protein modification of Net1p that, either directly or indirectly, requires Sir1p, although *SIR1* has not been previously implicated in *NET1* function. Further experiments will be necessary to explore these ideas, but together they suggest that the *sir2*^{eso} phenotypes do not result from gross disruptions of Sir2p-containing complexes or activity.

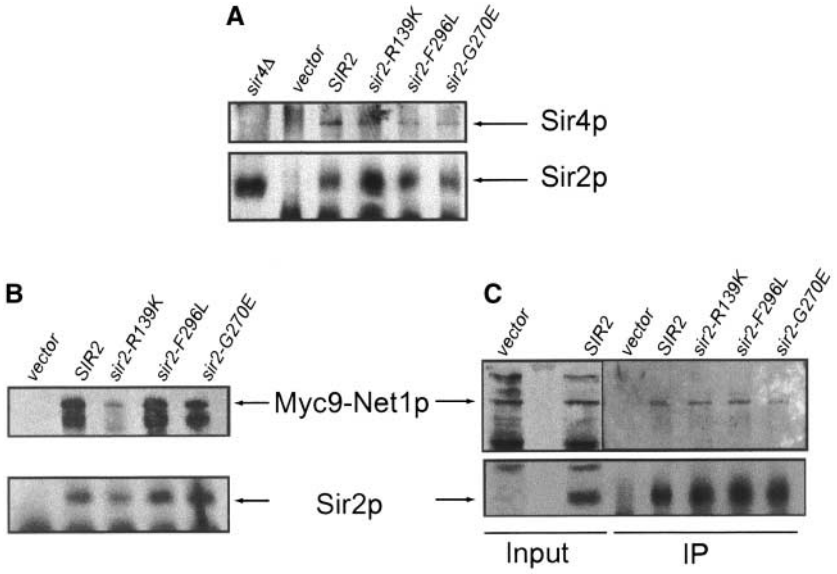

FIGURE 7.—The sir2^{eso} mutant proteins interact with Sir4p and Net1p. Extracts were prepared from *SIR1 sir2*Δ *net1*Δ*::Myc9-NET1-LEU2* and *sir1*Δ *sir2*Δ *net1*Δ*::Myc9-NET1-LEU2* strains transformed with vector (LPY6402), *SIR2* (LPY6403), *sir2-R139K* (LPY6404), *sir2-G270E* (LPY6405), or *sir2-F296L* (LPY6406). (A) Strain LPY6400 *sir1*Δ *sir2*Δ *net1*Δ*::Myc9-NET1-LEU2*. Sir4p was immunoprecipitated and tested for coimmunoprecipitation of Sir2p and sir2^{eso} mutant proteins by immunoblot analysis. Immunoprecipitations were performed with anti-Sir4p (7795) and immunoblots were probed with antisera against Sir4p (2913/8; top) and Sir2p (2916/8; bottom). (B) Strains LPY5615 *SIR1 sir2*Δ *net1*Δ*::Myc9-NET1-LEU2* (left) and LPY6400 *sir1*Δ *sir2*Δ *net1*Δ*::Myc9-NET1-LEU2* (right). Immunoblot analysis of Net1-myc9 immunoprecipitated with anti-Sir2p (2916/8) and probed with anti-myc monoclonal antibody 9E10 (top) and against Sir2p (2916/8, bottom) is shown; it is also shown in a *SIR1-sir2*Δ background (left).

DISCUSSION

Sir2p is an NAD$^+$-dependent deacetylase whose association with Sir4p and Net1p correlates with transcriptional silencing (reviewed in GARTENBERG 2000; MOAZED 2001). This study describes three new *sir2* mutants isolated as *enhancers of sir-one*Δ, the *sir2^{eso}* mutants. These mutants retain interactions with Sir4p and Net1p and are only partially compromised for catalytic activity. They do, however, display distinct phenotypes at three distinct silenced loci, including dominant effects at telomeres and within the rDNA. Many of these defects can be ameliorated by molecular targeting strategies. Thus a range of catalytic activity may be compatible with silencing functions, as long as that activity is appropriately directed to its required sites of action.

The *sir2^{eso}* mutants show mating defects in the absence of *SIR1*: The *sir2^{eso}* mutants are defective in silencing *HML* and *HMR* only in the absence of Sir1p and encode mutations in residues that are conserved in the Sir2 protein family. In quantitative mating analyses, the *sir2^{eso}* mutants were only slightly impaired in their mating ability in the presence of *SIR1*. In contrast, in a *sir1*Δ background, the *sir2^{eso}* mutants were as defective as *sir2*Δ strains. Although the mutants were not dominant in the presence of *SIR1* (data not shown), *sir2-G270E* and *sir2-F296L* displayed moderate mating defects in a *sir1*Δ *SIR2* background. Considering that Sir1p is required for targeting the Sir2/4 complex to modified synthetic silencers (FOX *et al.* 1997; GARDNER and FOX 2001), *sir2^{eso}* silencing defects may arise from unstable targeting to the *HM* loci. Such instability, when coupled with the *sir1*Δ establishment defect, may synergistically lead to the complete loss of silencing at *HML* and *HMR*.

The *sir2^{eso}* mutants are impaired, yet not totally defective, in enzymatic activity. Therefore, it is possible that in the absence of Sir1p, wild-type activity levels are required to maintain a silenced state at the *HM* loci or to initiate a stable silenced chromatin structure that can then be propagated. Our data do not yet distinguish these possibilities.

Telomeric silencing is disrupted in the *sir2^{eso}* mutants: Although the *sir2^{eso}* mutants were identified through a screen for silent mating-type defects, the mutants were also dominantly defective in telomeric silencing in both *sir1*Δ (data not shown) and *SIR1* backgrounds. Tethering directly to the telomeres, or indirectly through GBD-Sir1p, rescued these silencing defects and reversed the dominant effects. This raised the possibility that the mutant proteins were not properly localized in the cell. However, immunofluorescence analysis revealed that the sir2^{eso} proteins were localized indistinguishably to telomeric foci from wild-type localization in *SIR1* cells. Recent studies have also evaluated silencing protein localization by the independent technique of chromatin immunoprecipitation. In these studies, it was observed that Sir2p's enzymatic activity not only is necessary for the spread of the Sir proteins but also may influence efficient association of the Sir proteins with chromatin at the telomeres (ARMSTRONG *et al.* 2002; HOPPE *et al.* 2002; LUO *et al.* 2002). Subtle quantitative distinctions in association or dynamic occupancy may result from the decreased activity of sir2^{eso} mutant proteins. Indeed, that the mutant proteins are somehow impaired in functional association with chromatin is supported by the observation that the *sir2^{eso}* mutants became silencing competent when tethered via GBD or GBD-Sir1p, showing that their weakened enzymatic activity did not render these mutants incapable of promoting silencing.

An activity-dependent model for function at the silent mating-type loci and telomeres: Long-standing models

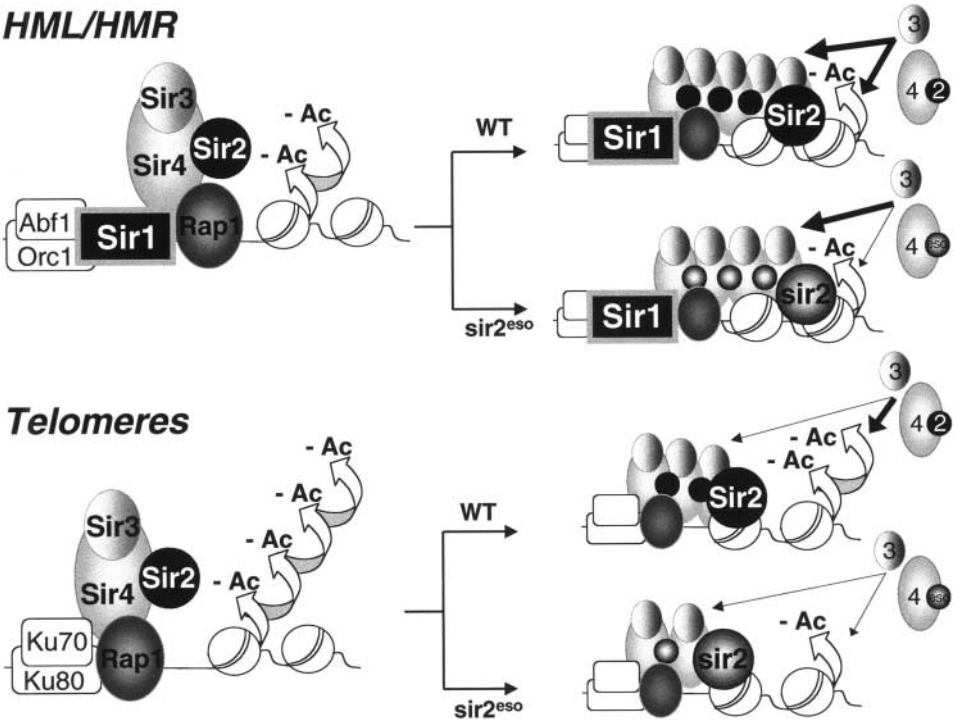

FIGURE 8.—An activity-sensitive model for *HML/HMR* and telomeric silencing. The Sir2/4 complex is recruited to the silent mating-type loci and telomeres via Rap1-Sir4p interactions (Sir1p also participates in recruitment at *HML/HMR* and Ku70p and Ku80p do so at telomeres). After initial recruitment and assembly, Sir2p activity is required to deacetylate (−Ac) histones H3 and H4, thereby recruiting additional Sir3 and Sir4 proteins leading to the spread of condensed chromatin. As the Sir proteins accumulate, subsequent spreading becomes less dependent on Sir2p activity. Although many aspects of sir2^{eso}p function support previous models of *HM* and telomeric chromatin, distinct from these models, sir2^{eso}p functions suggest that there may be locus-specific threshold requirements for NAD^+-dependent catalytic activity. Thus, robust NAD^+-dependent deacetylase activity is not necessary in all circumstances for nucleating stable silenced chromatin. For example, high levels of Sir2p enzymatic activity may not be critical when Sir1p or another targeting molecule such as GBD ensures that the Sir proteins remain associated with the locus. The *sir2^{eso}* mutants, impaired in enzymatic activity, rely heavily on a targeting factor such as Sir1p or GBD for stable retention of the Sir proteins at the locus and for propagation of silencing.

for the establishment of silent chromatin at the telomeres involve the recruitment of the Sir2/4 complex to DNA, followed by propagation of condensed chromatin through interactions among Sir3p, Sir4p, and deacetylated histone tails (Ghidelli *et al.* 2001; Carmen *et al.* 2002; Hoppe *et al.* 2002; Luo *et al.* 2002; and reviewed in Moazed 2001). The recruitment of Sir3p to the telomeres appears to be a regulated step since stable Sir3p-Sir4p interactions are detected only when the N terminus of Sir4p is removed or under conditions that strengthen Sir-nucleosome interactions (Moretti *et al.* 1994; Moazed *et al.* 1997; Strahl-Bolsinger *et al.* 1997; Ghidelli *et al.* 2001). We propose that if Sir3p is indeed also recruited through strengthened Sir-nucleosome interactions, its recruitment could become progressively less dependent on Sir2p enzymatic activity as the number of associated Sir complexes increases and spreads. This idea is consistent with the observation that overexpression of Sir3p can extend telomeric silencing into regions of chromatin that do not contain Sir2p and Sir4p (Renauld *et al.* 1993; Hecht *et al.* 1996) and provides an explanation for our observation that silencing of a reporter gene is restored by targeting enzymatically impaired sir2^{eso} mutant proteins to a single Gal4p DNA-binding site.

We suggest that at the *HM* loci, Sir1p recruits the Sir complex, thereby strengthening Sir-nucleosome interactions and serving as one method of Sir3p recruitment even when Sir2p activity is limiting. Since Sir1p is not present to strengthen Sir-nucleosome interactions at the telomeres, stable spreading of the Sir proteins may rely solely on robust Sir2p enzymatic activity. An occasional successful deacetylation event, which can then be propagated, may underlie the variegation of silencing that is the hallmark of telomeric position effects.

Our model, outlined in Figure 8, may also explain why *npt1*Δ mutants, required for the nuclear NAD^+ salvage pathway, are selectively defective in silencing, only slightly affecting the *HM* loci (Smith *et al.* 2000). Perhaps even modest decreases in enzymatic activity have amplified effects at telomeres that would be masked at *HML* and *HMR* in the presence of Sir1p.

Is dominance a threshold effect? If it is, this might explain why CEN-plasmid dosage of the *sir2^{eso}* mutants has dominant phenotypes. In these cases of marginally increased amounts of mutant proteins, a critical threshold of wild-type activity may not be met. One possibility is that even slightly increased amounts of mutant proteins are sufficient to limit availability of Sir2p interacting molecules such as NAD^+, acetylated substrates, or Sir4p, thereby interfering with proper Sir2p function. Therefore, although the *sir2^{eso}* mutants are impaired in enzymatic activity, they may be sufficiently active to initiate stable silencing if a targeting factor such as Sir1p or

GBD ensures that the Sir complex remains associated with the locus for the silenced state to be propagated. In the absence of the targeting factor, the sir2^{eso} enzymatic activity may become limiting and unable to overcome a threshold required for stable initiation of chromatin decondensation.

Distinct *sir2^{eso}* phenotypes in the rDNA underscore mechanistic differences at this locus: Although otherwise similar, the *sir2^{eso}* mutants differ from one another in their rDNA phenotypic profiles. For instance, although the *sir2-G270E* and *sir2-F296L* mutant strains were dominantly defective in rDNA silencing, they differed in their localization and their ability to rescue silencing when tethered to the rDNA array.

The *sir2-G270E* mutant was defective in rDNA silencing in the presence or absence of Sir1p but was rescued if tethered to the locus via a GBD. In addition, in the absence of Sir1p this mutant protein did not localize normally to the nucleolus. Therefore, this mutant is the only *sir2^{eso}* mutant that displays similar phenotypes at the *HM* loci, the telomeres, and the rDNA and appears to be impaired in its ability to associate with chromatin at all three silenced loci. In contrast, the sir2-F296L mutant protein localized properly to the nucleolus in the presence or absence of Sir1p and had decreased levels of silencing and increased levels of recombination (data not shown) in both backgrounds. However, it did not function in silencing at the rDNA when tethered directly to the locus. Therefore, the cause for the *sir2-F296L* defects in the rDNA likely differs from the impaired associations postulated for it at the telomeres.

In further contrast, *sir2-R139K* repressed recombination normally at the rDNA (data not shown) in the presence or absence of Sir1p and, within the limits of the silencing bioassay, was even more efficient than wild-type Sir2p at silencing the *URA3* reporter. However, synthetically tethering this mutant to the rDNA array via a GBD abrogates its function. These paradoxical effects in rDNA silencing by *sir2-R139K* may be explained by inefficient interaction with Net1p. Coimmunoprecipitation analyses showed that sir2-R139Kp consistently immunoprecipitated Net1p less efficiently than did wild-type Sir2p. Perhaps the decreased sir2-R139Kp-Net1p interaction allows function of the RENT complex at the rDNA only when the sir2-R139K mutant protein is targeted to the nucleolus exclusively through Net1p. Targeting via a GBD may abolish the already weakened interaction with Net1p and/or interactions with other RENT complex members, thereby disrupting silencing.

Together, these observations underscore and extend the growing view that there are fundamental differences between Sir2p function within the rDNA compared to the *HM* loci and telomeres. First, rDNA silencing requires a distinct (RENT) complex including Sir2p, Net1p, and Cdc14p (Shou *et al.* 1999; Straight *et al.* 1999). Also silenced rDNA repeats are in a region interspersed with highly transcribed rDNA units (reviewed in Hsieh and Fire 2000). It is not clear whether components of the RENT complex, or other proteins as yet unidentified, directly bind histones and thereby target and promote the spread of silenced chromatin at particular rDNA repeats. Such additional targeting might explain why sir2-R139Kp, which is enzymatically impaired and fails to initiate silencing at the telomeres, might still function slightly more efficiently than *SIR2* in silencing at the rDNA. The enzymatically inactive mutant sir2-H364Yp did not immunoprecipitate telomeric chromatin efficiently, yet it was able to immunoprecipitate rDNA chromatin (Tanny *et al.* 1999; Armstrong *et al.* 2002; Hoppe *et al.* 2002). Together, these mutant analyses support the idea that optimal levels of NAD^+-dependent activity appear to be required for the initiation steps of telomeric silencing but may be of less importance in initiating stable silencing within the rDNA array.

When catalysis is not enough: Other non-null *sir2* mutant alleles with locus-specific defects that raise intriguing possibilities, but leave some key unanswered questions, have been described (Sherman *et al.* 1999; Tanny *et al.* 1999; Cuperus *et al.* 2000; Imai *et al.* 2000; Perrod *et al.* 2001). One engineered mutant protein in particular, sir2-G270Ap, was shown to have enzymatic activities almost identical to those reported in this study for sir2-G270Ep (Imai *et al.* 2000; Tanny and Moazed 2001). Despite their enzymatic similarities, there are phenotypic differences between *sir2-G270E* and *sir2-G270A* mutants. Although *sir2-G270E* was defective at all silenced loci unless tethered via a GBD or Sir1p, *sir2-G270A* was mating proficient, showed partial silencing defects at the telomeres, and silenced as well as wild-type *SIR2* at the rDNA (Imai *et al.* 2000). The amino acid difference at this residue does not appear to cause major changes in relative enzymatic activity and both mutants share an isogenic background. One possible explanation for the phenotypic differences is that *sir2-G270A* is also an *eso* mutant. Thus, it might display mating defects only in a *sir1*Δ background, a condition not tested in the original report (Imai *et al.* 2000). However, this explanation does not account for the differences observed in silencing within the rDNA array and at the telomeres. Further exploration of the *sir2-G270A* mutant and its molecular associations may provide additional clues to the mechanism of silencing at the rDNA.

In summary, the *sir2^{eso}* phenotypes highlight the requirement for a targeting molecule such as Sir1p or GBD to initiate stable silenced chromatin at the *HM* loci and the telomeres when Sir2p enzymatic activity is limiting. Our studies also identified a residue outside the conserved core domain of Sir2p (R139), which, when mutated, results in impaired enzymatic activity yet wild-type levels of silencing within the rDNA array. The phenotypic differences observed among the *sir2^{eso}* mutants suggest that wild-type levels of activity, although essential for stable initiation of silencing at the telomeres, are not critical for rDNA silencing and confirm

that there are mechanistic differences for Sir2p's nucleolar functions that require further investigation. Analysis of other *sir2* mutants for potential *eso* phenotypes may reveal additional mutations outside the core domain of *SIR2* that can have impaired enzymatic activity yet function in silencing when tethered to the *HM* loci or to a telomere. Such studies may help further understanding of the unique dependence on Sir1p in establishing silencing and the intricate relationships among enzymatic targeting, chromatin modification, and gene regulation.

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LITERATURE CITED

Aparicio, O. M., B. L. Billington and D. E. Gottschling, 1991 Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell **66:** 1279–1287.

Aris, J. P., and G. Blobel, 1988 Identification and characterization of a yeast nucleolar protein that is similar to a rat liver nucleolar protein. J. Cell Biol. **107:** 17–31.

Armstrong, C. M., M. Kaeberlein, S. I. Imai and L. Guarente, 2002 Mutations in Saccharomyces cerevisiae gene SIR2 can have differential effects on in vivo silencing phenotypes and in vitro histone deacetylation activity. Mol. Biol. Cell **13:** 1427–1438.

Borra, M. T., F. J. O'Neill, M. D. Jackson, B. Marshall, E. Verdin *et al.*, 2002 Conserved enzymatic production and biological effect of O-acetyl-ADP-ribose by silent information regulator 2-like NAD+-dependent deacetylases. J. Biol. Chem. **277:** 12632–12641.

Brachmann, C. B., J. M. Sherman, S. E. Devine, E. E. Cameron, L. Pillus *et al.*, 1995 The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. Genes Dev. **9:** 2888–2902.

Bryk, M., M. Banerjee, M. Murphy, K. E. Knudsen, D. J. Garfinkel *et al.*, 1997 Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. Genes Dev. **11:** 255–269.

Carmen, A. A., L. Milne and M. Grunstein, 2002 Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. J. Biol. Chem. **277:** 4778–4781.

Chien, C. T., S. Buck, R. Sternglanz and D. Shore, 1993 Targeting of SIR1 protein establishes transcriptional silencing at HM loci and telomeres in yeast. Cell **75:** 531–541.

Cockell, M. M., S. Perrod and S. M. Gasser, 2000 Analysis of Sir2p domains required for rDNA and telomeric silencing in *Saccharomyces cerevisiae*. Genetics **154:** 1069–1083.

Cuperus, G., R. Shafaatian and D. Shore, 2000 Locus specificity determinants in the multifunctional yeast silencing protein Sir2. EMBO J. **19:** 2641–2651.

Dutnall, R. N., and L. Pillus, 2001 Deciphering NAD-dependent deacetylases. Cell **105:** 161–164.

Ersfeld, K., and E. M. Stone, 1999 Simultaneous in situ detection of DNA and proteins, pp. 51–66 in *The Practical Approach Series*, edited by V. Allan. Oxford University Press, New York.

Finnin, M. S., J. R. Donigian and N. P. Pavletich, 2001 Structure of the histone deacetylase SIRT2. Nat. Struct. Biol. **8:** 621–625.

Fox, C. A., A. E. Ehrenhofer-Murray, S. Loo and J. Rine, 1997 The origin recognition complex, SIR1, and the S phase requirement for silencing. Science **276:** 1547–1551.

Fritze, C. E., K. Verschueren, R. Strich and R. Easton Esposito, 1997 Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. EMBO J. **16:** 6495–6509.

Gardner, K. A., and C. A. Fox, 2001 The Sir1 protein's association with a silenced chromosome domain. Genes Dev. **15:** 147–157.

Gardner, K. A., J. Rine and C. A. Fox, 1999 A region of the Sir1 protein dedicated to recognition of a silencer and required for interaction with the Orc1 protein in *Saccharomyces cerevisiae*. Genetics **151:** 31–44.

Gartenberg, M. R., 2000 The Sir proteins of Saccharomyces cerevisiae: mediators of transcriptional silencing and much more. Curr. Opin. Microbiol. **3:** 132–137.

Ghidelli, S., D. Donze, N. Dhillon and R. T. Kamakaka, 2001 Sir2p exists in two nucleosome-binding complexes with distinct deacetylase activities. EMBO J. **20:** 4522–4535.

Gill, S. C., and P. H. von Hippel, 1989 Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. **182:** 319–326.

Gotta, M., S. Strahl-Bolsinger, H. Renauld, T. Laroche, B. K. Kennedy *et al.*, 1997 Localization of Sir2p: the nucleolus as a compartment for silent information regulators. EMBO J. **16:** 3243–3255.

Gottlieb, S., and R. E. Esposito, 1989 A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. Cell **56:** 771–776.

Gottschling, D. E., 2000 Gene silencing: two faces of SIR2. Curr. Biol. **10:** R708–R711.

Gottschling, D. E., O. M. Aparicio, B. L. Billington and V. A. Zakian, 1990 Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. Cell **63:** 751–762.

Guarente, L., 2000 Sir2 links chromatin silencing, metabolism, and aging. Genes Dev. **14:** 1021–1026.

Hecht, A., S. Strahl-Bolsinger and M. Grunstein, 1996 Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. Nature **383:** 92–96.

Holmes, S. G., A. B. Rose, K. Steuerle, E. Saez, S. Sayegh *et al.*, 1997 Hyperactivation of the silencing proteins, Sir2p and Sir3p, causes chromosome loss. Genetics **145:** 605–614.

Hoppe, G. J., J. C. Tanny, A. D. Rudner, S. A. Gerber, S. Danaie *et al.*, 2002 Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. Mol. Cell. Biol. **22:** 4167–4180.

Hsieh, J., and A. Fire, 2000 Recognition and silencing of repeated DNA. Annu. Rev. Genet. **34:** 187–204.

Imai, S., C. M. Armstrong, M. Kaeberlein and L. Guarente, 2000 Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature **403:** 795–800.

James, P., J. Halladay and E. A. Craig, 1996 Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144:** 1425–1436.

Landry, J., J. T. Slama and R. Sternglanz, 2000a Role of NAD(+) in the deacetylase activity of the SIR2-like proteins. Biochem. Biophys. Res. Commun. **278:** 685–690.

Landry, J., A. Sutton, S. T. Tafrov, R. C. Heller, J. Stebbins *et al.*, 2000b The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc. Natl. Acad. Sci. USA **97:** 5807–5811.

Luo, K., M. A. Vega-Palas and M. Grunstein, 2002 Rap1-Sir4 binding independent of other Sir, yKu, or histone interactions initiates the assembly of telomeric heterochromatin in yeast. Genes Dev. **16:** 1528–1539.

Min, J., J. Landry, R. Sternglanz and R. M. Xu, 2001 Crystal structure of a SIR2 homolog-NAD complex. Cell **105:** 269–279.

Moazed, D., 2001 Enzymatic activities of Sir2 and chromatin silencing. Curr. Opin. Cell Biol. **13:** 232–238.

Moazed, D., A. Kistler, A. Axelrod, J. Rine and A. D. Johnson, 1997 Silent information regulator protein complexes in Saccharomyces cerevisiae: a SIR2/SIR4 complex and evidence for a regulatory domain in SIR4 that inhibits its interaction with SIR3. Proc. Natl. Acad. Sci. USA **94:** 2186–2191.

Moretti, P., K. Freeman, L. Coodly and D. Shore, 1994 Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. Genes Dev. **8:** 2257–2269.

Palladino, F., T. Laroche, E. Gilson, A. Axelrod, L. Pillus *et al.*, 1993 SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. Cell **75:** 543–555.

Perrod, S., M. M. Cockell, T. Laroche, H. Renauld, A. L. Ducrest *et al.*, 2001 A cytosolic NAD-dependent deacetylase, Hst2p, can

modulate nucleolar and telomeric silencing in yeast. EMBO J. **20:** 197–209.

Pillus, L., and J. Rine, 1989 Epigenetic inheritance of transcriptional states in S. cerevisiae. Cell **59:** 637–647.

Pogliano, J., N. Osborne, M. D. Sharp, A. Abanes-De Mello, A. Perez *et al.*, 1999 A vital stain for studying membrane dynamics in bacteria: a novel mechanism controlling septation during Bacillus subtilis sporulation. Mol. Microbiol. **31:** 1149–1159.

Primig, M., R. M. Williams, E. A. Winzeler, G. G. Tevzadze, A. R. Conway *et al.*, 2000 The core meiotic transcriptome in budding yeasts. Nat. Genet. **26:** 415–423.

Reifsnyder, C., J. Lowell, A. Clarke and L. Pillus, 1996 Yeast SAS silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases. Nat. Genet. **14:** 42–49.

Renauld, H., O. M. Aparicio, P. D. Zierath, B. L. Billington, S. K. Chhablani *et al.*, 1993 Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. Genes Dev. **7:** 1133–1145.

Rice, J. C., and C. D. Allis, 2001 Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr. Opin. Cell Biol. **13:** 263–273.

Rose, M. D., F. Winston and P. Hieter, 1989 *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Rothstein, R. J., 1991 Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. **194:** 281–301.

Sherman, F., 1991 Getting started with yeast. Methods Enzymol. **194:** 3–21.

Sherman, J. M., E. M. Stone, L. L. Freeman-Cook, C. B. Brachmann, J. D. Boeke *et al.*, 1999 The conserved core of a human SIR2 homologue functions in yeast silencing. Mol. Biol. Cell **10:** 3045–3059.

Shore, D., 2000 The Sir2 protein family: a novel deacetylase for gene silencing and more. Proc. Natl. Acad. Sci. USA **97:** 14030–14032.

Shou, W., J. H. Seol, A. Shevchenko, C. Baskerville, D. Moazed *et al.*, 1999 Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell **97:** 233–244.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae.* Genetics **122:** 19–27.

Smith, J. S., and J. D. Boeke, 1997 An unusual form of transcriptional silencing in yeast ribosomal DNA. Genes Dev. **11:** 241–254.

Smith, J. S., C. B. Brachmann, L. Pillus and J. D. Boeke, 1998 Distribution of a limited Sir2 protein pool regulates the strength of yeast rDNA silencing and is modulated by Sir4p. Genetics **149:** 1205–1219.

Smith, J. S., C. B. Brachmann, I. Celic, M. A. Kenna, S. Muhammad *et al.*, 2000 A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc. Natl. Acad. Sci. USA **97:** 6658–6663.

Stone, E. M., and L. Pillus, 1996 Activation of an MAP kinase cascade leads to Sir3p hyperphosphorylation and strengthens transcriptional silencing. J. Cell Biol. **135:** 571–583.

Stone, E. M., M. J. Swanson, A. M. Romeo, J. B. Hicks and R. Sternglanz, 1991 The *SIR1* gene of *Saccharomyces cerevisiae* and its role as an extragenic suppressor of several mating-defective mutants. Mol. Cell. Biol. **11**(4): 2253–2262.

Stone, E. M., C. Reifsnyder, M. McVey, B. Gazo and L. Pillus, 2000 Two classes of sir3 mutants enhance the *sir1* mutant mating defect and abolish telomeric silencing in *Saccharomyces cerevisiae.* Genetics **155:** 509–522.

Strahl-Bolsinger, S., A. Hecht, K. Luo and M. Grunstein, 1997 SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev. **11:** 83–93.

Straight, A. F., W. Shou, G. J. Dowd, C. W. Turck, R. J. Deshaies *et al.*, 1999 Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. Cell **97:** 245–256.

Tanny, J. C., and D. Moazed, 2001 Coupling of histone deacetylation to NAD breakdown by the yeast silencing protein Sir2: evidence for acetyl transfer from substrate to an NAD breakdown product. Proc. Natl. Acad. Sci. USA **98:** 415–420.

Tanny, J. C., G. J. Dowd, J. Huang, H. Hilz and D. Moazed, 1999 An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing. Cell **99:** 735–745.

Triolo, T., and R. Sternglanz, 1996 Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. Nature **381:** 251–253.

Wolffe, A. P., and D. Guschin, 2000 Review: chromatin structural features and targets that regulate transcription. J. Struct. Biol. **129:** 102–122.

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