MOLECULAR AND CELLULAR BIOLOGY, June 2005, p. 4514–4528 Vol. 25, No. 11
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.11.4514–4528.2005

A Nonhistone Protein-Protein Interaction Required for Assembly of the SIR Complex and Silent Chromatin†

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Received 25 August 2004/Returned for modification 21 October 2004/Accepted 22 February 2005

Budding yeast silent chromatin, or heterochromatin, is composed of histones and the Sir2, Sir3, and Sir4 proteins. Their assembly into silent chromatin is believed to require the deacetylation of histones by the NAD-dependent deacetylase Sir2 and the subsequent interaction of Sir3 and Sir4 with these hypoacetylated regions of chromatin. Here we explore the role of interactions among the Sir proteins in the assembly of the SIR complex and the formation of silent chromatin. We show that significant fractions of Sir2, Sir3, and Sir4 are associated together in a stable complex. When the assembly of Sir3 into this complex is disrupted by a specific mutation on the surface of the C-terminal coiled-coil domain of Sir4, Sir3 is no longer recruited to chromatin and silencing is disrupted. Because in *sir4* mutant cells the association of Sir3 with chromatin is greatly reduced despite the partial Sir2-dependent deacetylation of histones near silencers, we conclude that histone deacetylation is not sufficient for the full recruitment of silencing proteins to chromatin and that Sir-Sir interactions are essential for the assembly of heterochromatin.

Silent chromatin, or heterochromatin, is a specialized chromatin structure that is refractory to transcription and recombination, that replicates late, and that is found at both centromeric and telomeric regions, where it plays crucial roles in the structure and segregation of chromosomes (13, 24). In the budding yeast *Saccharomyces cerevisiae*, silent chromatin exists at three loci: the silent mating type cassettes (*HML* and *HMR*, or the *HM* loci), telomeres, and the repetitive rRNA gene loci (5, 12, 17, 51).

The assembly and inheritance of heterochromatin are thought to be governed primarily by changes in histone modifications (21, 44). The assembly of silent chromatin in budding yeast requires histones H3 and H4. Deletion of the N-terminal tail of histone H3 or H4 compromises silencing at both the *HM* loci and telomeres (22, 25, 64). Mutation of lysine 16 of histone H4, a major site of acetylation in budding yeast, to glutamine or glycine also causes severe defects in silencing (23, 33).

In addition to histones, the protein deacetylase Sir2 and the histone binding proteins Sir3 and Sir4 are required to assemble silent chromatin. Sir2 is the founding member of a conserved family of NAD-dependent protein deacetylases (20, 30, 52). The primary target of budding yeast Sir2 is thought to be the N-terminal tails of histones H3 and H4; therefore, Sir2 may be the enzyme responsible for creating the regions of hypoacetylated nucleosomes that are observed in silent chromatin (4, 57). Sir3 and Sir4 bind to the N-terminal tails of histones H3 and H4 in vitro, with a preference for the hypoacetylated rather than the acetylated tail (6, 14). In addition, mutations in histone H4 that disrupt silencing can be suppressed by second-site suppressors in *SIR3*, suggesting that Sir3 and histone H4 may interact directly in vivo (23).

In other eukaryotes, the assembly of silent chromatin requires modifications of histones which then also are bound by specific silencing proteins. For example, in the fission yeast *Schizzosaccharomyces pombe*, lysine 9 of histone H3 is deacetylated by Sir2 and then methylated by Clr4 (40, 42, 49). This modified nucleosome forms a binding site for the chromodomain proteins Swi6 and Chp1 which, although not homologous to Sir3 and Sir4, play functionally similar roles (2, 13, 41).

In addition to modifying and binding to histones, the Sir proteins engage in extensive protein-protein interactions with one another. Sir4 tightly binds to Sir2, and this interaction is thought to be essential for the recruitment of Sir2 to the *HM* loci and telomeres (9, 18, 36, 55). Sir4 also interacts with itself, suggesting that it functions as a dimer in vivo (7, 8, 39). Lastly, Sir4 binds to Sir3 in the absence of Sir2 and independently of chromatin (15, 55). These observations suggest that the three Sir proteins form a soluble complex, which has been named the SIR complex (15, 36, 37, 55). The existence of a stable SIR complex in solution, however, has not been demonstrated. Native purification of these proteins from yeast has yielded separate Sir4/Sir2 and Sir3 fractions (18, 62).

The assembly of silent chromatin is a stepwise process (18, 32, 47). First, recruitment proteins, including Sir1, Abf1, Rap1, Ku dimers, and the ORC complex, bind to regions called the E and I silencers, which initiate the assembly of silent chromatin at the *HM* loci, or to telomeres (46). These proteins then recruit the SIR complex by binding to Sir4 and perhaps Sir3 (32, 38). Once initiated, the spreading of silent chromatin requires all three Sir proteins as well as the enzymatic activity of Sir2 (18, 32, 47). Therefore, it has been proposed that successive cycles of histone deacetylation by Sir2 and recruitment of additional SIR complexes cause the spread of silent chromatin emanating from silencers and telomeres (18, 34, 46). This spreading is thought to involve the polymerization of

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† Supplemental material for this article may be found at http://mcb.asm.org/.

the SIR complex, yet little is known about which Sir-Sir interactions are required for nucleation and growth of the SIR polymer. Similar stepwise models for heterochromatin assembly have been proposed in other organisms. For example, the histone binding proteins Swi6 and HP1, from fission yeast and mammals, respectively, may also polymerize as they spread along chromatin (13), although even less is known about this process.

Although Sir3 interacts with Sir4 in the SIR complex, a number of experiments have suggested that Sir3 can function independently of Sir4 and Sir2. First, the overexpression of Sir3 extends regions of silent chromatin, yet only Sir3 is found in these regions, suggesting that it can spread along chromatin alone (15, 43, 55). Second, a fragment of Sir3 binds to the nonacetylated N-terminal tail of histone H4 in vitro with a K_d of 35 nM (6), an affinity that may allow Sir3 to bind to hypoacetylated chromatin independently of Sir4 in vivo. Accordingly, deletion of the acetylase gene *SAS2* results in hypoacetylation of lysine 16 on histone H4 and allows significant spreading of Sir3 in the absence of Sir2 at telomeres (26, 56). It has been difficult, however, to assess the importance of these interactions in vivo, because mutations that selectively disrupt the pairwise interactions of Sir proteins have not been reported.

Here we show that large portions of the cellular Sir4, Sir3, and Sir2 proteins form a stable and soluble SIR complex and that the integrity of this complex is required for the proper assembly of silent chromatin. To directly test the physiological significance of the interaction between Sir4 and Sir3 in vivo, we created mutations in Sir4 that selectively abolish the binding of Sir4 to Sir3. These mutations interfere with silencing at both the *HM* loci and telomeres by specifically blocking the recruitment of Sir3 to chromatin, despite significant deacetylation of histones near silencers. Our results therefore demonstrate that the association of Sir3 with silent chromatin depends on both the direct interaction of Sir3 with Sir4 and Sir3-histone interactions.

MATERIALS AND METHODS

Strain and plasmid construction. Table 1 lists the strains used in this work. All strains except the mating testers (57/2 and m31; gifts from Fred Winston, Harvard Medical School) are derivatives of the strain W303 background (W303-1a; Rodney Rothstein, Columbia University, New York, NY). Standard genetic techniques were used to manipulate yeast strains (50), and standard protocols were used for DNA manipulation (48). All deletions and replacements were confirmed by PCR and by mutant phenotype analysis. The sequences of all of the primers used in this study are available upon request. Bacterial strain DH5α was used for the amplification of DNA.

SIR3 was tagged and *SIR4* was deleted by the PCR targeting method. Cells were transformed with cassettes containing the bacterial Kan^r or Nat^r gene, which confers G418 or nourseothricin resistance in W303, respectively. The cassettes were amplified by PCR from pFA6a-13myc-kanMX6 (31) or pAG25-natMX4 (11) with primers containing sequences that flank the stop codon of *SIR3* or sequences immediately upstream of the start codon and downstream of the stop codon of *SIR4*. *SIR3-myc13* was crossed to *sir*Δ strains that were either *hml*Δ::*TRP1* or complemented by *SIR-CEN* plasmids. *HML* was deleted with pKK294, *SIR2* was complemented by pRS315-SIR2, and *SIR4* was complemented by pRS315-SIR4 (kindly provided by Jasper Rine, University of California, Berkeley).

SIR2-H364Y was made by two-step gene replacement. A C-terminal fragment of *SIR2* was cloned into pRS306 to create pDM638 (kindly provided by Jason Tanny). pDM638 was cut with BglII and transformed into yeast. Transformants were screened for nonmaters and were assumed to contain *SIR2-H364Y* followed by *URA3* and a C-terminal fragment of *SIR2*. Nonmaters were grown on plates containing 5-fluoroorotic acid (5-FOA), and the resultant Ura$^-$ cells were again screened for nonmaters. Nonmaters were screened by Western blotting for the expression of full-length Sir2 and by PCR for the presence of the H364Y substitution (which destroys an NcoI restriction site).

sir4 coiled-coil mutants were also made by two-step gene replacement. The BamHI/XhoI restriction fragment from pGST-Sir4-C2-M1307N, pGST-Sir4-C2-I1311N, pGST-Sir4-C2-E1310R, pGST-Sir4-C2-K1324D, and pGST-Sir4-C2-K1325D (7) was cloned into pRS306 to create pAR417, pAR416, pAR423, pAR424, and pAR425, respectively. These plasmids were cut with MfeI and transformed into yeast. Transformants were assumed to contain a mutated full-length *SIR4* gene followed by *URA3* and a short C-terminal duplication of *SIR4*. URA$^+$ transformants were grown on 5-FOA to select for recombination between the duplicated C-terminal regions, and the resultant Ura$^-$ strains were screened by PCR for the presence of the mutation and by Western blotting for the expression of full-length Sir4. Screening by PCR was done with oligonucleotides that have different abilities to hybridize with wild-type *SIR4* and mutant *SIR4*. *SIR4-M1307N* and *SIR4-I1311N*, which are mating defective, were crossed by transforming complementing plasmid pRS314-SIR4 into the appropriate strains.

TABLE 1. List of strains used in this study

Strain[a]	MAT	Relevant genotype[b]
ADR21	**a**	W303-1a
ADR22	α	Wild type
ADR2366	**a**	*pep4*Δ::*LEU2*
ADR2432	**a**	*SIR3-TAP-TRP1 pep4*Δ::*LEU2*
ADR2740	**a**	*sir2*Δ::*HIS3 SIR3-myc13-KAN*^{R} *hml*Δ::*TRP1*
ADR2743	**a**	*sir4*Δ::*HIS3 SIR3-myc13-KAN*^{R} *hml*Δ::*TRP1*
ADR2746	**a**	*SIR3-myc13-KAN*^{R} *hml*Δ::*TRP1*
ADR2749	**a**	*SIR3-myc13-KAN*^{R}
ADR2808	**a**	*SIR2-H364Y*
ADR2829	**a**	*TELVII-L*::*URA3*
ADR2831	**a**	*adh4*::*URA3*
ADR2844	**a**	*sir4*Δ::*HIS3 TELVII-L*::*URA3 hml*Δ::*TRP1*
ADR2856	**a**	*TELVII-L*::*URA3 hml*Δ::*TRP1*
ADR2860	**a**	*adh4*::*URA3 hml*Δ::*TRP1*
ADR2888	α	*sir4*Δ::*HIS3*
ADR2895	**a**	*sir4-K1324E SIR3-myc13-KAN*^{R}
ADR2898	**a**	*SIR4-M1307N SIR3-myc13-KAN*^{R}
ADR2951	**a**	*SIR4-M1307N TELVII-L*::*URA3*
ADR2959	**a**	*SIR4-I1311N TELVII-L*::*URA3*
ADR2972	**a**	*SIR4-M1307N*
ADR2973	**a**	*SIR4-I1311N*
ADR2975	α	*SIR4-M1307N*
ADR2977	α	*SIR4-I1311N*
ADR2991	**a**	*sir4-E1310R SIR3-myc13-KAN*^{R}
ADR2994	**a**	*SIR4-I1311N SIR3-myc13-KAN*^{R}
ADR2997	**a**	*sir4-K1325E SIR3-myc13-KAN*^{R}
ADR3020	**a**	*SIR2-H364Y SIR3-myc13-KAN*^{R} *hml*Δ::*TRP1*
ADR3030	**a**	*sir4-E1310R TELVII-L*::*URA3*
ADR3036	**a**	*sir4-K1324D TELVII-L*::*URA3*
ADR3042	**a**	*sir4-K1325D TELVII-L*::*URA3*
ADR3060	**a**	*sir4-E1310R*
ADR3061	α	*sir4-E1310R*
ADR3062	**a**	*sir4-K1324D*
ADR3064	α	*sir4-K1324D*
ADR3066	**a**	*sir4-K1325D*
ADR3068	α	*sir4-K1325D*
ADR3103	**a**	*sir4*Δ::*NAT*^{R} *TELVII-L*::*URA3*
ADR3112	**a**	*sir4*Δ::*NAT*^{R} *SIR2-H364Y SIR3-myc13-KAN*^{R} *hml*Δ::*TRP1*
ADR3113	**a**	*sir4*Δ::*NAT*^{R} *SIR3-myc13-KAN*^{R} *hml*Δ::*TRP1*
ADR3246	α	*hmr*Δ*E*::*TRP1*
ADR3305	α	*sir4*Δ::*NAT*^{R} *hmr*Δ*E*::*TRP1*
ADR3307	α	*SIR4-M1307N hmr*Δ*E*::*TRP1*
ADR3309	α	*sir4-E1310R hmr*Δ*E*::*TRP1*
ADR3311	α	*SIR4-I1311N hmr*Δ*E*::*TRP1*
ADR3313	α	*sir4-K1324D hmr*Δ*E*::*TRP1*
ADR3315	α	*sir4-K1325D hmr*Δ*E*::*TRP1*
JRY3289[c]	**a**	*sir3*Δ::*HIS3*
JRY3411[c]	**a**	*sir4*Δ::*HIS3*
JRY3433[c]	**a**	*sir2*Δ::*HIS3*
57/2[d]	**a**	*his1-1*
m31[d]	α	*his1-1 met1*

[a] Strains originated in this study unless otherwise indicated.
[b] All strains are isogenic to W303-1a (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*), except for strains 57/2 and m31.
[c] The source was Jasper Rine.
[d] The source was Gerry Fink.

pDM538 (pRS313-SIR4) was made by cloning the XhoI/SacII fragment of pRS315-SIR4 into pRS313. The BsrGI/XhoI fragment of pDM538 was replaced with a BsrGI/XhoI *SIR4* fragment that was amplified by PCR from *SIR4* (ADR21), *SIR4-M1307N* (ADR2972), *sir4-E1310R* (ADR3060), *SIR4-I1311N* (ADR2973), and *sir4-K1324D* (ADR3062) to create pAR450 to pAR454, respectively. The BsrGI/XhoI fragment contains the *SIR4* sequence from the natural BsrGI site to 216 nucleotides downstream of the stop codon, followed by an XhoI site introduced during PCR. The correct sequence of this region of *SIR4* (and each of the *sir4* mutants) was confirmed by sequencing of all five plasmids. The image of the Sir4 coiled coil (see Fig. 2) was made by using WebLab Viewer Lite 3.2 (protein database code 1NYH).

The *TEL-VII-L*::*URA3* and *adh4*::*URA3* strains were made by using pTEL::URA3 and pADH4::URA3, respectively (kindly provided by Dan Gottschling, Fred Hutchison Cancer Research Center). Wild-type strains ADR2856 and ADR2860 were crossed to the appropriate strains.

The *hmrΔE* (58) strains were constructed by crossing derivatives of CCFY100 (kindly provided by Kurt W. Runge, The Lerner Research Institute) (45) to the appropriate strains.

The *sir2*Δ::*HIS3* (JRY3433), *sir3*Δ::*HIS3* (JRY3289), and *sir4*Δ::*HIS3* (JRY3411) strains were kindly provided by Jasper Rine, University of California, Berkeley.

Immunoprecipitation and Western blotting. Yeast extracts for immunoprecipitation and Western blotting were made by bead beating (multitube bead beater; Biospec) frozen cell pellets in 350 μl lysis buffer (50 mM HEPES-KOH [pH 7.6], 500 mM sodium acetate, 5 mM magnesium acetate, 0.1 mM EDTA, 0.25% NP-40, 5% glycerol and, added just before use, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], leupeptin at 1 μg/ml, pepstatin at 1 μg/ml, bestatin at 1 μg/ml, and 1 mM benzamidine) with an excess of glass beads (Biospec) for two pulses of 30 s, with incubation on ice for 5 min between the pulses. A typical extract was made from cells harvested from a 50-ml culture at an optical density at 600 nm (OD_{600}) of 1.0 to 1.5. The resulting lysate was separated from the glass beads and centrifuged at 14,000 rpm for 5 min to remove insoluble material. The protein concentration of each lysate was determined by using Bradford reagent (Bio-Rad), and samples were normalized based on these measurements. A portion of the lysate was mixed with an equal volume of 2 × sodium dodecyl sulfate (SDS) sample buffer (4% SDS, 160 mM Tris-HCl [pH 6.8], 20% glycerol, 20 mM EDTA, 0.04% bromophenol blue, and 1 mM PMSF) for Western blotting.

Standard methods were used for polyacrylamide gel electrophoresis and protein transfer to nitrocellulose (Schleicher & Schuell). Typically, samples were run on 10% or 12.5% polyacrylamide gels (80:1 and 120:1 acrylamide:bis, respectively, with no added SDS). Blots were stained with Ponceau S to confirm transfer and equal loading of samples and then were blocked for 30 min in blocking buffer (4% nonfat dried milk [Carnation] in TBST [20 mM Tris-HCl {pH 7.5}, 150 mM NaCl, 0.1% Tween 20]). All antibodies were incubated overnight at 4°C or for 2 h at 25°C. After being washed with TBST, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Amersham) at a 1:5,000 dilution in blocking buffer for 30 min at 25°C, washed again, incubated with Western Lightning reagents (Perkin-Elmer) according to the manufacturer's instructions, and then exposed to X-Omat film (Kodak).

The following antibodies were used for Western blotting. Anti-myc antibody 9E10 (Covance) was used at a dilution of 1:1,000 in TBST-0.02% NaN_3. Affinity-purified rabbit polyclonal anti-Sir2 and anti-Sir4 antibodies (36) were used at a dilution of 1:5,000 in antibody storage buffer (autoclaved 4% nonfat dried milk, TBST, 5% glycerol, 0.02% NaN_3).

The remaining lysate was used for immunoprecipitation with 9E10 or Sir4. A total of 0.5 to 3 μg of antibody was added to the lysate, and the mixture was incubated on ice for 30 min. Samples then were centrifuged at 14,000 rpm for 5 min at 4°C and transferred to 10 to 15 μl of protein A–Sepharose CL-4B beads (Pharmacia) which had been equilibrated in lysis buffer. The beads were rotated end over end at 4°C for 2 to 4 h. After immunoprecipitation, the beads were washed quickly three times with lysis buffer (the beads were transferred to fresh tubes after the second wash) and two times with low-salt wash buffer (50 mM HEPES-KOH [pH 7.6], 150 mM sodium acetate, 5 mM magnesium acetate, 5% glycerol and, added just before use, 1 mM DTT and 1 mM PMSF). All washes were performed on ice. After the final wash, residual buffer was removed from the beads with a Hamilton syringe, and the beads were resuspended in 1.5× SDS sample buffer, heated to 65°C for 15 min, and processed for Western blotting as described above.

Silver staining was performed as described previously (18).

Centrifugation at 100,000 × *g* was performed with a TLA 100.3 rotor in a tabletop ultracentrifuge (Beckman).

In some experiments, the lysis buffer contained an additional 0.5 M, 1.0 M, or 1.5 M sodium acetate or 100 μg/ml ethidium bromide and 0.1 μg/ml DNase I (750 U). When the beads were washed more extensively, the third wash with lysis buffer was extended, and a fourth wash with tube transfer was added after the extended wash.

Calf intestinal phosphatase (New England Biolabs [NEB]) was used for phosphatase treatment. After lysis buffer washes, the beads were washed with phosphatase buffer (NEB buffer 3) one time, incubated with 10 U of phosphatase in NEB buffer 3 (or no addition) at 37°C for 15 min, and then washed two times with low-salt wash buffer before being processed for Western blotting.

ChIP. Typically, 200 ml of yeast was grown to an OD_{600} of 1.5 at 30°C. Yeast lysates were prepared as described above, except that before harvesting of yeast cells, cultures were fixed with 1% formaldehyde for 15 min at 25°C. Cross-linking was quenched by the addition of glycine to 125 mM followed by 5 min of incubation. The cells were harvested, washed with 20 mM Tris-HCl (pH 7.5)–150 mM NaCl, and split into three tubes; yeast pellets were frozen in liquid nitrogen. The cells were lysed with 600 μl chromatin immunoprecipitation (ChIP) lysis buffer (50 mM HEPES-KOH [pH 7.6], 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and, added just before use, 1 mM PMSF, leupeptin at 1 μg/ml, pepstatin at 1 μg/ml, bestatin at 1 μg/ml, and 1 mM benzamidine). Prior to normalization of various lysates, the chromatin in the lysates was sheared by sonication with three 20-s pulses at 40% power (Branson digital Sonifier 450 with a microtip), with 5 min on ice between pulses. The DNA was sheared to an average size of 400 to 500 bp. After sonication, the lysates were processed as described above. Sir3, Sir4, and histones H4 and H3 were immunoprecipitated with 0.5 μl affinity-purified rabbit anti-Sir3 polyclonal antibody, 1.0 μl affinity-purified rabbit anti-Sir4 antibody (36), 2 μl antibody to histone H4 acetylated at K5, K8, K12, and K16 [histone H4-Ac(K5, K8, K12, K16)], and 2 μl rabbit anti-histone H3-Ac(K9, K14) antibody (the latter two from Upstate Biotechnology). Beads were washed three times with ChIP lysis buffer (the beads were transferred to fresh tubes after the second wash), one time with Li buffer (10 mM Tris-HCl [pH 8.0], 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and one time with 50/10-TE (50 mM Tris-Cl [pH 8.0], 10 mM EDTA). Protein and cross-linked DNA were eluted in 100 μl 50/10-TE–1% SDS at 65°C for 15 min. After the eluate was removed from the beads, the beads were washed with 150 μl 50/10-TE–0.67% SDS; the wash was pooled with the first eluate. The mixture was incubated for 6 h to overnight at 65°C to reverse the cross-links, incubated with 50 μg/ml proteinase K for 2 h at 55°C, extracted with an equal volume of 1:1 unbuffered phenol:chloroform, and extracted with chloroform alone. The remaining associated DNA was precipitated by the addition of 400 mM LiCl, 10 μg glycogen, and 2 volumes of ethyl alcohol, washed with 75% ethyl alcohol, air dried, and resuspended in 50 μl of 10/1-TE (10 mM Tris-Cl [pH 8.0], 1 mM EDTA) containing 2 μg/ml RNase A.

Input DNA was prepared by mixing 50 μl of the starting lysate (after sonication) with 200 μl 50/10-TE–1% SDS. The lysate was processed in a fashion similar to that of the immunoprecipitates, except that a second phenol:chloroform extraction was performed and the final DNA pellet was resuspended in 50 μl 10/1-TE containing 20 μg/ml RNase A.

The precipitated DNA and the input DNA were analyzed by PCR with either 5 μl of a 1:10 dilution of the immunoprecipitated material or 5 μl of a 1:10,000 dilution of the input material. PCR amplifications were performed with 12.5-μl reaction mixtures containing PCR buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl), 0.1 mM deoxynucleoside triphosphates, 0.1 mCi of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham), 1 μM each JH301 and JH302 internal *ACT1* primers, and 1 μM each silent-locus primer. Reaction conditions were 1 cycle of 95°C for 2 min, 55°C for 30 s, and 72°C for 1 min; 25 or 26 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final step of 72°C for 4 min. The primers used for ChIP are listed in Table 2 and were described previously (18, 19, 32); they yielded products ranging from 150 to 400 bp. After completion of the PCR amplifications, 1.5 μl of loading dye (50 mM Tris-HCl [pH 8.0], 50% sucrose, 4 M urea, 0.05% bromophenol blue) was added to each reaction mixture, and reactions were run on 6% acrylamide (30:1 acrylamide:bis)–Tris-borate-EDTA gels for 45 min at 100 V. Gels were dried and subjected to autoradiography and quantification by phosphorimaging (Personal FX and QuantityOne; Bio-Rad).

Relative fold enrichment values for each strain were calculated as follows: [silent locus (immunoprecipitate)/*ACT1* (immunoprecipitate)]/[silent locus (input)/*ACT1* (input)]. The average and standard deviation for three independent experiments were determined. For clarity, these values were scaled (see Fig. 6 and 7) so that the average values of *sir3*Δ in the Sir3 ChIP and of *sir4*Δ in the Sir4 ChIP were set to 1; the average values were scaled (see Fig. 8) so that the wild-type values in both histone H3 and histone H4 ChIPs were set to 1.

TABLE 2. List of PCR primers used in ChIP experiments in this study

Amplified locus	Primer pair	Sequence
HMR-E[a]	DM490	TGCAAAAACCCATCAACCTGG
	DM491	ACCAGGAGTACCTGCGCTTA
*HMR***a**	OAR161	TCCGCCGATTTATTTTTCTG
	OAR162	CAGTTTCCCCGAAAGAACAA
HML-E	OAR167	AAAGGACGAGACACCAGAAGA
	OAR168	AGTCCGTGCCGAAAACTTTA
*HML*α[a]	GH81	AGACGGCCAGAAACCTC
	GH82	TCGCCTACCTTCTTGAAC
TEL0.07[b]	OAR149	CATGACCAGTCCTCATTTCCATC
	OAR150	ACGTTTAGCTGAGTTTAACGGTG
TEL0.6[a]	DM241	CAGGCAGTCCTTTCTATTTC
	DM242	GCTTGTTAACTCTCCGACAG
ACT1[c]	JH301	GCCTTCTACGTTTCCATCCA
	JH302	GGCCAAATCGATTCTCAAAA

[a] The primers were used previously (18).
[b] The primers were used previously (32).
[c] The primers were used previously (19).

Nicotinamide release assays. Nicotinamide release assays were adapted from Landry et al. (29). Sir4 was immunoprecipitated as described above. After the beads were washed with lysis buffer, they were washed two times with deacetylase reaction buffer (50 mM Tris-Cl [pH 7.5], 100 mM NaCl, 1 mM DTT); residual buffer was removed with a Hamilton syringe. To the beads was added a 350 μM concentration of a 16-amino-acid N-terminal peptide of histone H4-Ac(K5, K8, K12, K16) and 0.025 μCi [carbonyl-^{14}C]NAD (54.0 mCi/mmol; Amersham) in deacetylase reaction buffer. Reaction volumes were 10 μl. The beads were incubated for 2 h at 30°C, and the reaction was stopped by the addition of 73.5 μl of quench buffer (370 mM sodium borate [pH 8.0], 50 mM glycine [pH 9.0]). The supernatant was removed from the beads and extracted with 0.5 ml ethyl acetate. Nicotinamide release was quantified by scintillation counting of 0.45 ml of the ethyl acetate phase in 2 ml scintillation fluid. Typically, 2 to 10% of the NAD is converted to nicotinamide in these reactions. The histone H4 N-terminal peptide was synthesized and purified by reverse-phase high-pressure liquid chromatography at the Tufts University core facility.

Quantitative mating. Tested strains and tester strains were placed on YEP-2% glucose plates supplemented with 0.005% adenine and tryptophan and grown overnight at 30°C. On the following morning, the cells were scraped from the plates, inoculated into YEP-2% glucose liquid medium containing 0.005% adenine and tryptophan, and grown for 2 to 4 h at 25°C to an OD_{600} of 1 to 2 for the tested strains and to an OD_{600} of 4 to 6 for the tester strains. Quantities of 10^6, 10^5, 10^4, 10^3, and 10^2 cells of the tested strain (in 100 μl) were mixed with 10^7 to 5×10^7 cells of the tester strain (in 300 μl). The mixture was plated directly on synthetic minimal-2% glucose plates and grown at 25°C for 3 days, and then counting was done. A quantity of 10^2 cells of the tested strain was also plated on synthetic complete-2% glucose plates to accurately determine the number of viable cells that were mated in the experiment. Each mating was performed in triplicate. Mating efficiencies were typically between 50 and 100% for wild-type cells (ADR21 and ADR22), and the mating defect of *sir4*Δ was reproducibly more severe for *MAT***a** cells than for *MAT*α cells (see Fig. 4A).

Silencing assays. Cells were grown in YEP-2% glucose liquid medium to an OD_{600} of 1 to 2, and 10-fold serial dilutions were spotted on synthetic complete, 5-FOA, or tryptophan-deficient plates containing 2% glucose. Plates were incubated for 2 to 3 days at 30°C and then photographed.

RESULTS

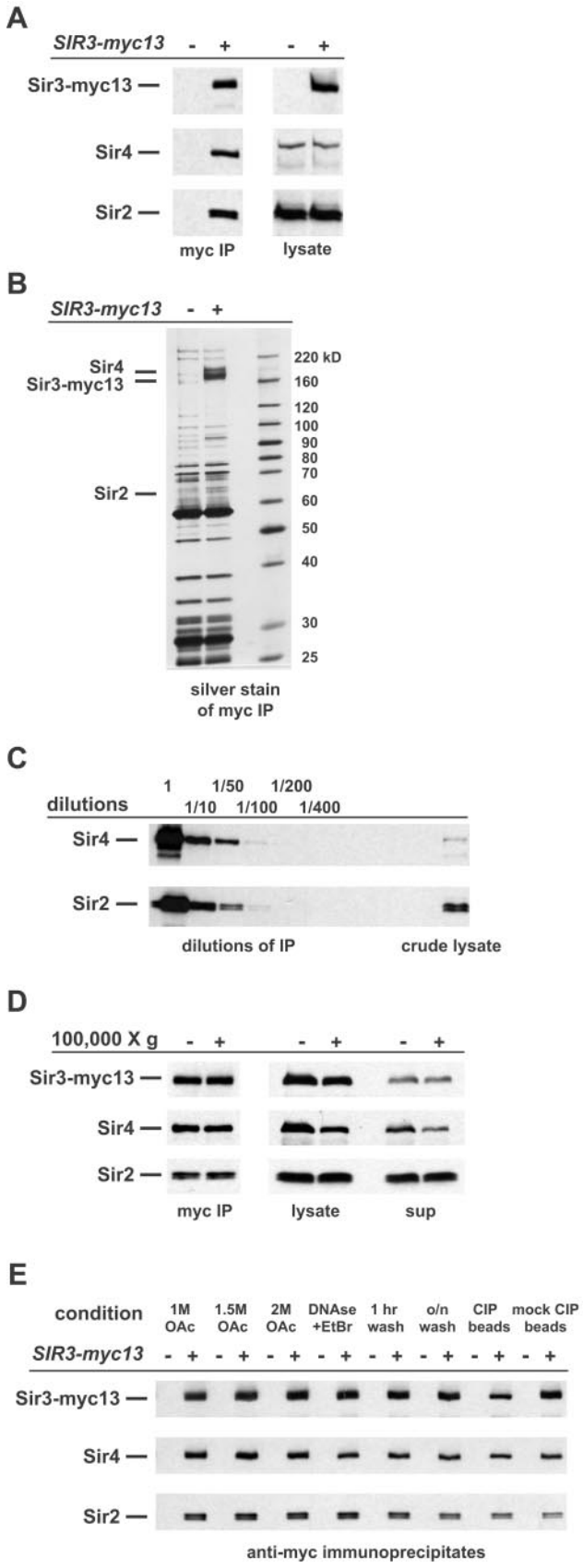
The SIR complex contains Sir2, Sir3, and Sir4. Previous work has shown that affinity tagged versions of Sir3 bind to Sir4 and to Sir2 in immunoprecipitations (15, 36, 55). These interactions between the Sir proteins were independent of chromatin (15), lending support to the proposal that the Sir proteins exist in a soluble SIR complex. It was unclear, however, whether stoichiometric amounts of Sir4 and Sir2 coprecipitated with Sir3 in these experiments. In contrast, tandem affinity purification (TAP) purifications of the Sir proteins yielded two distinct SIR subcomplexes (18), one that contained Sir4 and Sir2 and another that contained Sir3 alone. Although trace amounts of Sir4 could be found in Sir3-TAP purifications (see Fig. S1B in the supplemental material) and trace amounts of Sir3 could be found in TAP-Sir4 purifications (62), the Sir proteins did not appear to assemble into a homogeneous complex of all three Sir proteins.

In order to resolve the discrepancy between these observations and to test whether Sir2, Sir3, and Sir4 form a soluble SIR complex, we quantified the relative amounts of Sir4 and Sir2 present in Sir3 complexes that could be immunoprecipitated from whole-cell extracts by a different affinity purification strategy. We first created a strain that replaces the endogenous *SIR3* with a fusion of *SIR3* and 13 myc epitopes (*SIR3-myc13*). Sir3-myc13 is fully functional for silencing and is expressed in yeast at wild-type levels (see Fig. S2 in the supplemental material). Precipitation of Sir3-myc13 with an anti-myc antibody resulted in coprecipitation of Sir4 and Sir2 (Fig. 1A), consistent with the results of earlier studies with affinity-tagged Sir3 (15, 55). Large portions of Sir4 and Sir2 coprecipitated with Sir3-myc13 (Fig. 1B) and, based on serial dilutions of the precipitates, we estimated that 25% of total Sir4 and 13% of total Sir2 were precipitated (Fig. 1C). Although the Sir2 protein appeared to be present in the precipitate at lower levels than Sir4 (Fig. 1B), it has been shown that virtually all Sir4 is bound to Sir2 (36) and that Sir2 stains poorly with silver (data not shown). It is therefore likely that equal amounts of Sir2 and Sir4 coprecipitate with Sir3.

The SIR complex remained soluble after centrifugation of up to 100,000 × *g* for 1 h (Fig. 1D) and could be precipitated from extracts made in buffers containing up to 2 M sodium acetate (Fig. 1E). In addition, extensive treatment of the lysate with DNase I and ethidium bromide did not disrupt the integrity of the complex (Fig. 1E). These treatments will degrade DNA associated with the SIR complex and disrupt DNA-protein interactions (28). The activity of Sir3 is regulated by phosphorylation (1, 54). Treatment of the immunoprecipitation with alkaline phosphatase eliminated phosphorylation of Sir3 but did not disrupt the interaction among Sir3, Sir4, and Sir2 (Fig. 1E). We therefore conclude that substantial portions of Sir2, Sir3, and Sir4 exist in a stable and soluble SIR complex.

Why do TAP purifications of Sir3-containing complexes apparently behave differently from the anti-myc precipitates? Our current and previous TAP purifications of both Sir3 and Sir4 did not yield a SIR complex that contained stoichiometric amounts of each protein (18, 62). We found that although the complete SIR complex was present in the starting TAP lysate, it failed to elute from the affinity resin during the first step of the purification (see Fig. S1C in the supplemental material), perhaps as a result of aggregation or extensive multimerization of one of its components. This was not an issue in the anti-myc immunoprecipitations because the bound material was eluted from the affinity resin by SDS (Fig. 1B).

Using coprecipitation experiments, we examined the archi-



tecture of the SIR complex. As was reported previously by Grunstein and colleagues (15, 55), Sir3 interacts with Sir4 independently of Sir2 (see Fig. S3 in the supplemental material); because silent chromatin does not form in *sir2*Δ cells, these data confirm that the interaction between Sir3 and Sir4 occurs independently of silent chromatin. In addition, the SIR complex can form independently of Sir2 enzymatic activity. *SIR2-H364Y*, which renders Sir2 inactive and destroys silencing in vivo (20, 61), does not affect the stability of the SIR complex (see Fig. S3 in the supplemental material). As shown previously, the interaction between Sir2 and Sir3 depends on the presence of Sir4 (55), suggesting that in yeast extracts, Sir2 and Sir3 cannot form a stable complex in the absence of Sir4.

Sir4 coiled-coil mutants cannot bind to Sir3 in vivo. Recently, two crystal structures of C-terminal fragments of Sir4 (Sir4-C) were reported (7, 39); these showed that the extreme C terminus (amino acids 1272 to 1335) self-associates to form a parallel coiled coil. This domain lies within the region of Sir4 that binds to Sir3 (36, 37), and C-terminal coiled-coil fragments of Sir4 bind tightly to C-terminal fragments of Sir3 in vitro (7). Three amino acids (M1307, E1310, and I1311) located on the exposed surface of the coiled coil were shown to be critical for the interaction between the C-terminal fragments of Sir3 and Sir4 in vitro, while neighboring surface sites (K1324 and K1325) had more modest effects on this interaction (7). Although these substitutions blocked the interaction between fragments of Sir3 and Sir4-C, they did not affect the ability of Sir4-C to self-associate into a coiled coil. The cluster of three residues was proposed to be the specific binding site for two Sir3 proteins on the Sir4 coiled coil (7).

FIG. 1. The SIR complex is stable and soluble. (A) Sir4 and Sir2 are coprecipitated with Sir3-myc13. *SIR3-myc13* (+, ADR2749) or untagged (−, ADR21) cells were grown overnight at 30°C in YEP-2% glucose to the late log phase, harvested, and lysed. Sir3-myc13 was immunoprecipitated with anti-myc antibodies. The immunoprecipitates (IP) and the starting lysate were subjected to Western blotting with anti-myc, anti-Sir4, and anti-Sir2 antibodies. (B) The immunoprecipitates from panel A were run on a polyacrylamide gel and silver stained. Note that Sir2 stained poorly with silver. (C) Large portions of Sir4 and Sir2 in cells are bound to Sir3-myc13. The immunoprecipitates from panel A were serially diluted and compared to the starting protein lysate. Quantities of 1/75 and 1/40 of the immunoprecipitates contained the same amounts of Sir4 and Sir2 as the crude lysate, respectively. Equal volumes of immunoprecipitates and the crude lysate were loaded on the gel; 300-fold more crude lysate was used in the immunoprecipitation than is shown here. Greater than 90% of Sir3-myc13 was precipitated in this experiment (data not shown). We therefore estimate that 25% and 13% of Sir4 and Sir2, respectively, are associated with Sir3-myc13. (D) The SIR complex is soluble. Protein extracts of *SIR3-myc13* cells were prepared as described for panel A and either left untreated (−) or centrifuged at 100,000 × *g* for 1 h (+). After centrifugation, Sir3-myc13 was immunoprecipitated with anti-myc antibodies. The immunoprecipitates, the starting lysate, and the supernatants (sup) after immunoprecipitation were subjected to Western blotting as described for panel A. (E) The SIR complex is stable. Immunoprecipitations were performed as described for panel A in the presence of 1 M, 1.5 M, or 2 M sodium acetate (OAc) or 0.1 μg/ml (750 U) DNase I and 100 μg/ml ethidium bromide (EtBr). After immunoprecipitation, some samples were washed for 1 h or overnight (o/n) or were treated with calf intestinal alkaline phosphatase (CIP). The immunoprecipitates then were subjected to Western blotting as described for panel A.

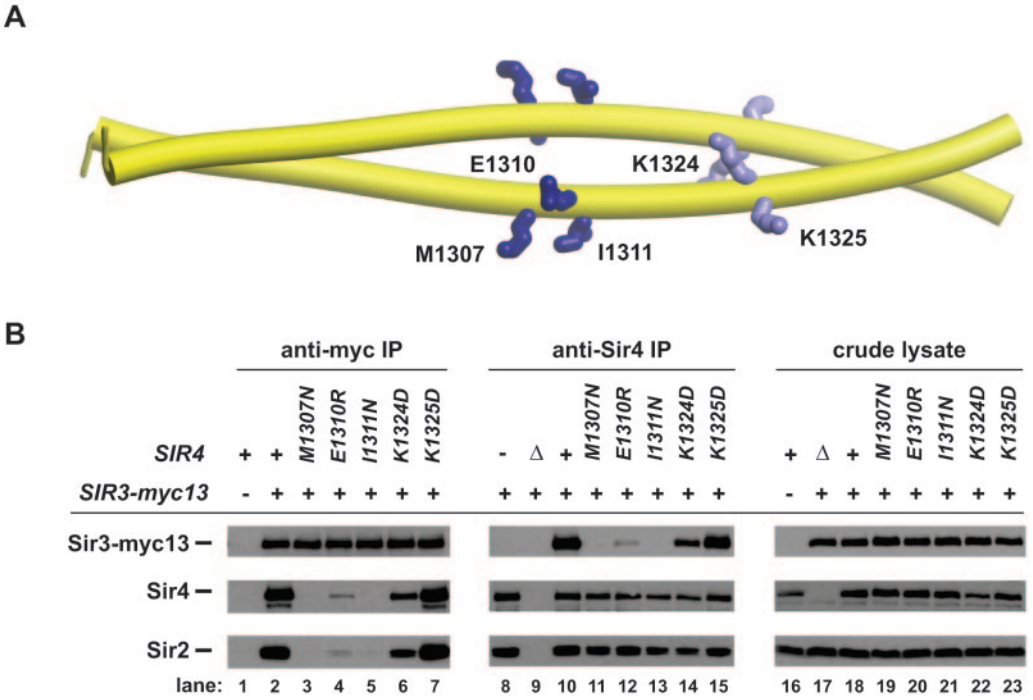


FIG. 2. *SIR4* coiled-coil mutants are defective for binding to Sir3 in vivo. (A) Surface residues on the C-terminal coiled-coil of Sir4 form a binding site for Sir3. Residues M1307, E1310, I1311, K1324, and K1325 are highlighted on the parallel coiled coil and were mutated in the endogenous *SIR4* gene. (B) *sir4* mutants are defective for binding to Sir3 but not Sir2. Wild-type (ADR21), *SIR3-myc13* (ADR2749), *sir4-M1307N SIR3-myc13* (ADR2898), *sir4-E1310R SIR3-myc13* (ADR2991), *sir4-I1311N SIR3-myc13* (ADR2895), *sir4-K1324D SIR3-myc13* (ADR2994), *sir4-K1325D SIR3-myc13* (ADR2997), and *sir4*Δ::*HIS3 SIR3-myc13* (ADR2743) cells were grown overnight in YEP-2% glucose to the late log phase, harvested, and lysed. Sir3-myc13 and Sir4 were immunoprecipitated with anti-myc and anti-Sir4 antibodies. The immunoprecipitates (IP) and the starting lysate were subjected to Western blotting with anti-myc, anti-Sir4, and anti-Sir2 antibodies.

We were interested in understanding the physiological significance of the interaction between Sir3 and Sir4. We therefore tested if mutations in the coiled coil of Sir4 tested in vitro also disrupted the interaction between full-length Sir4 and Sir3 in vivo and if this interaction played a role in the assembly of silent chromatin. We replaced individual surface residue in the Sir4 coiled coil to create five mutants, *sir4-M1307N*, *sir4-E1310R*, *sir4-I1311N*, *sir4-K1324D*, and *sir4-K1325D* (Fig. 2A). These *sir4* mutants replaced the endogenous *SIR4* gene. Four of the mutants were expressed at wild-type levels (Fig. 2B, lanes 18 to 23, middle row), whereas *sir4-K1324D* consistently produced less Sir4 protein than wild-type *SIR4* (Fig. 2B, lane 22; see also Fig. 5B, lane 18). These mutations did not affect the overall structure of Sir4 because each of these mutant proteins interacted normally with Sir2 (Fig. 2B, lanes 10 to 15, bottom row), and the Sir2 associated with the mutant Sir4 proteins had wild-type levels of deacetylase activity (Fig. 3).

These *sir4* mutants disrupted the interaction between Sir3 and Sir4 measured either by immunoprecipitating Sir3-myc13 or Sir4. For example, when Sir3-myc13 was precipitated with anti-myc antibodies, Sir4-M1307N and Sir4-I1311N did not associate with Sir3 (Fig. 2B, lanes 3 and 5, middle row), while Sir4-E1310R and Sir4-K1324D both still interacted with Sir3, although less well than wild-type Sir4 (Fig. 2B, lanes 4 and 6 compared to lane 2, middle row). These defects in binding also could be seen when Sir4 was precipitated from these strains (Fig. 2B, lanes 10 to 15). Sir4-M1307N and Sir4-I1311N precipitated no detectable Sir3 (Fig. 2B, lanes 11 and 13, top row),

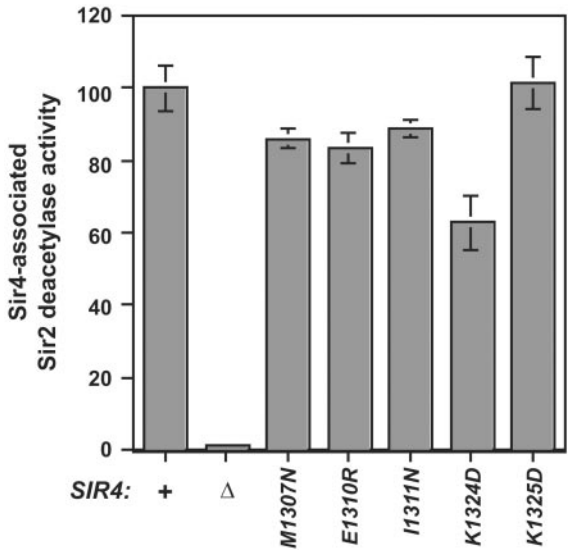


FIG. 3. Sir2 associated with mutant Sir4 proteins is fully active. The NAD-dependent protein deacetylase activity of the anti-Sir4 immunoprecipitates shown in Fig. 2B was measured by using nicotinamide release assays (29). The average and standard deviation of three experiments are shown. The average activity of the anti-Sir4 immunoprecipitates of *SIR3-myc13* (ADR2749) cells in each experiment was arbitrarily set to 100%. The reduced activity of Sir2 associated with Sir4-K1324D likely was due to the reduced levels of Sir4-K1324D and the Sir4-K1324D/Sir2 complex in these cells (Fig. 2B, lanes 14 and 22).

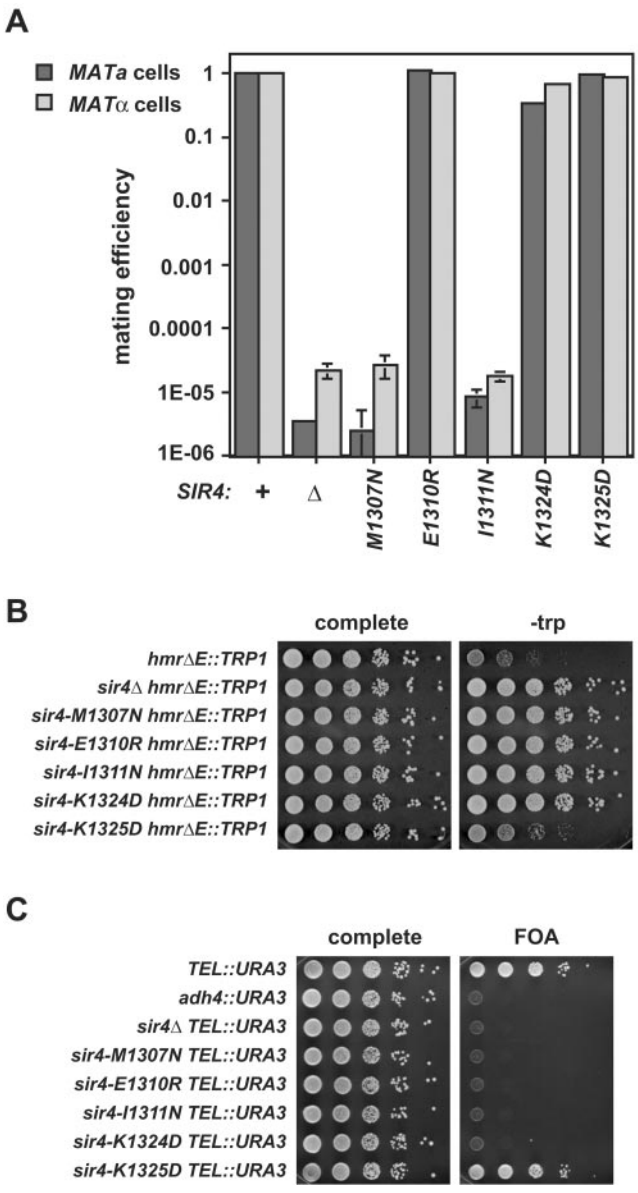


FIG. 4. *sir4* coiled-coil mutants are defective for silencing. (A) *sir4-M1307N* and *sir4-I1311N* are defective for *HM* locus silencing. Silencing at *HMR* and *HML* was measured by quantitative mating. *MAT***a** and *MAT*α wild-type (ADR21 and ADR22), *sir4*Δ::*HIS3* (JRY3411 and ADR2888), *sir4-M1307N* (ADR2972 and ADR2975), *sir4-E1310R* (ADR3060 and ADR3061), *sir4-I1311N* (ADR2973 and ADR2977), *sir4-K1324D* (ADR3062 and ADR3064), and *sir4-K1325D* (ADR3066 and ADR3068) strains were mated to *MAT*α *his1* (m31) and *MAT***a** *his1* (57/2) tester strains. The average and standard deviation of three matings are shown. (B) *sir4-E1310R* and *sir4-K1324D* are defective for silencing at *hmr*Δ*E*. All strains contained *hmr*Δ*E*::*TRP1*, which deletes the E element of the *HMR-E* silencer and inserts a *TRP1* reporter gene within *HMR*. Wild-type (ADR3246), *sir4*Δ::*NAT*^{R} (ADR3305), *sir4-M1307N* (ADR3307), *sir4-E1310R* (ADR3309), *sir4-I1311N* (ADR3311), *sir4-K1324D* (ADR3313), and *sir4-K1325D* (ADR3315) cells were grown overnight to the late log phase in YEP-2% glucose at 30°C. Tenfold serial dilutions were spotted onto plates of complete synthetic medium-2% glucose (left) or medium lacking tryptophan (−trp) but containing 2% glucose (right). The plates were incubated at 30°C for 2 days. The most dense spots contained approximately 5×10^5 cells. (C) Defects in Sir3 binding to Sir4 derepress telomeric silencing. All strains contained *URA3* at either *TEL-VIIL* or the *ADH4* locus (which is 20 kb from *TEL-VIIL*). Wild-type (ADR2856 and ADR2860), *sir4*Δ::*HIS3* (ADR2844), *sir4-M1307N* (ADR2951), *sir4-E1310R* (ADR3030), *sir4-I1311N* (ADR2959), *sir4-K1324D* (ADR3036), and *sir4-K1325D* (ADR3042) cells were grown overnight to the late log phase in YEP-2% glucose at 30°C. Tenfold serial dilutions were spotted onto complete synthetic medium-2% glucose (left) or 5-FOA–2% glucose (right) plates. The plates were incubated at 30°C for 2 days or 3 days, respectively. The most dense spots contained approximately 5×10^4 cells.

while Sir4-E1310R and Sir4-K1324D precipitated a fraction of the Sir3 that was precipitated from *SIR4* cells (Fig. 2B, lanes 10, 12, and 14, top row). Sir4-K1325D had no defect in its interaction with Sir3 (Fig. 2B, lane 7 compared to 2, middle row, and 15 compared to 10, top row). This was unsurprising because the K1325D substitution in Sir4-C has only minor defects in binding Sir3 in vitro (7). We conclude that the interaction between full-length Sir3 and Sir4 in vivo depends on the same residues of the Sir4 coiled coil that are required for the in vitro interaction between purified fragments of Sir3 and Sir4.

The interaction between Sir3 and Sir4 is required for silencing. Using *sir4* mutants that are selectively deficient in binding to Sir3, we were able to test whether the integrity of the SIR complex affects the assembly of silent chromatin. Silencing at *HML* and *HMR* can be monitored by the ability of haploid strains to mate (16). The presence of silent chromatin at *HML* and *HMR* is required to maintain haploid cell identity and wild-type mating efficiencies, while a loss of silencing causes cells to adopt a pseudodiploid cell identity and lose the ability to mate. Using quantitative mating assays, we determined that *sir4-M1307N* and *sir4-I1311N* are completely defective for silencing at both *HML* and *HMR* loci (Fig. 4A). *sir4-E1310R* cells, despite a strong defect in coprecipitation assays (Fig. 2B, lanes 4 and 12), maintained wild-type levels of mating. *sir4-K1324D* cells had minor mating defects, showing a two- to threefold decrease in mating efficiency. Previous studies showed that small changes in Sir4 dosage can cause a loss of silencing (59); therefore, the small mating defect could be attributed to the reduced expression of Sir4-K1324D (Fig. 2B, lane 22, and Fig. 5B) and not to the inability of Sir4-K1324D to bind to Sir3 (Fig. 2B, lanes 6 and 14). Although the *HM* loci are able to tolerate a weakening in the binding of Sir3 to Sir4, the loss of this interaction in *sir4-M1307N* and *sir4-I1311N* cells leads to a complete disruption of silencing.

Because Sir4-E1310R and Sir4-K1324D have detectable defects in their binding to Sir3, we wondered if a stronger defect in HM silencing in *sir4-E1310R* and *sir4-K1324D* might be revealed in a sensitized strain background. We therefore monitored the expression of *TRP1* gene that had been integrated at *hmr*Δ*E*, at which the E element of the *HMR-E* silencer has been mutated and can no longer bind to the Rap1 protein (58). The *TRP1* gene is effectively silenced in *SIR4* cells, and these cells grow poorly on plates lacking tryptophan (Fig. 4B, top row). In contrast, *sir4-M1307N*, *sir4-E1310R*, *sir4-I1311N*, and *sir4-K1324D* are unable to silence *TRP1* at *hmr*Δ*E*.

Silencing at telomeres can be monitored by assaying the repression of a reporter gene placed at a telomeric location. We monitored the expression of a *URA3* gene that had been integrated at the left telomere of chromosome VII, compared

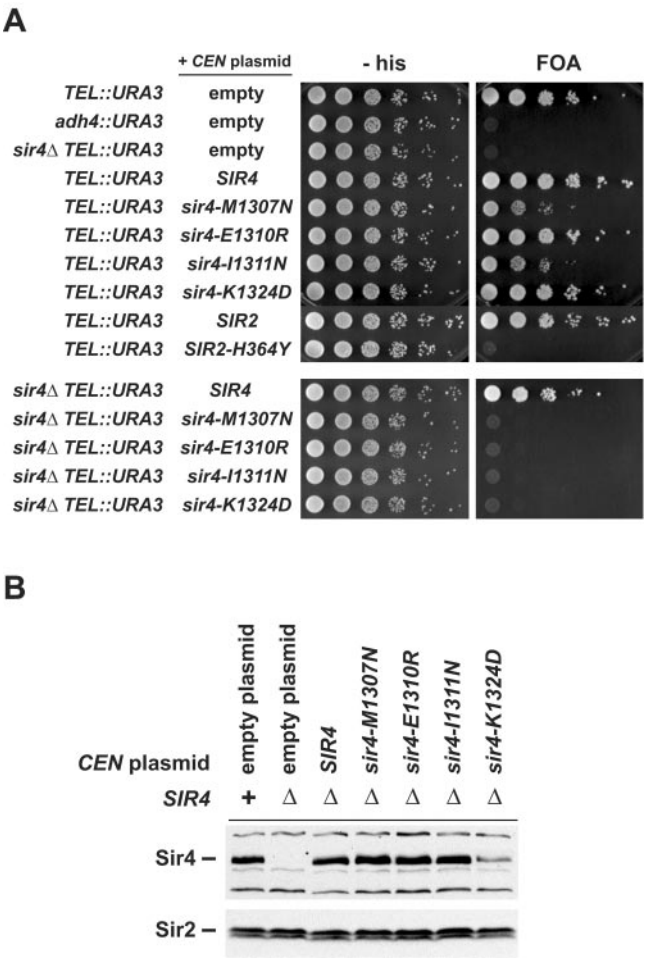


FIG. 5. *sir4* coiled-coil mutants are dominant negative. (A) *SIR4-M1307N* and *SIR4-I1311N* are dominant negative. All strains contained *URA3* at either *TEL-VIIL* or the *ADH4* locus (which is 20 kb from *TEL-VIIL*). Wild-type (ADR2829 and ADR2831) and *sir4*Δ::*NAT*^{R} (ADR3103) cells were transformed with either an empty *CEN* plasmid (pRS313) or the same plasmid containing *SIR4* (pAR450), *sir4-M1307* (pAR451), *sir4-E1310R* (pAR452), *sir4-I1311N* (pAR453), *sir4-K1324D* (pAR454), *SIR2* (pAR455), or *SIR2-H364Y* (pAR456). These strains were grown overnight to the late log phase in minimal medium lacking histidine (−his)- 2% glucose at 30°C. Tenfold serial dilutions were spotted onto plates of medium lacking histidine but containing 2% glucose (left) or medium lacking histidine but containing 5-FOA and 2% glucose (right). The plates were incubated at 30°C for 2 days or 3 days, respectively. (B) *SIR4-CEN* plasmids express Sir4 at wild-type levels. A subset of the strains tested in panel A were grown in medium lacking histidine but containing 2% glucose to the log phase, harvested, and lysed in SDS sample buffer. The protein lysates were subjected to Western blotting with anti-Sir4 and anti-Sir2 antibodies.

to an identical *URA3* gene that is integrated at the *ADH4* locus, 20 kb away (12). When the telomeric *URA3* is silenced in wild-type cells, cells can grow on 5-FOA, which is toxic to Ura$^+$ cells. The *URA3* gene integrated at *ADH4*, however, is expressed, and cells are unable to grow on 5-FOA. Like silencing at *hmr*Δ*E*, all four *sir4* mutants that are defective in the interaction with Sir3 were completely defective in telomeric silencing (Fig. 4C). *sir4-K1325D* cells showed no defects in either *HM* locus or telomeric silencing, consistent with this mutation having no effect on the interaction between Sir3 and Sir4 in coprecipitation experiments (Fig. 2B, lanes 7 and 15). Thus, the ability of Sir3 to bind to Sir4 is required for silencing at both the *HM* loci and telomeres. Furthermore, silencing at telomeres is more sensitive to mutations in Sir4 that only weaken its interaction with Sir3.

Because the *sir4* mutants are defective in binding Sir3, but not Sir2, we tested if they cause a dominant loss of silencing. We constructed low copy *CEN* plasmids containing either wild-type *SIR4*, or each of the four mutations that had telomeric silencing defects. The *sir4* on the plasmids was expressed at a similar level to the endogenous *SIR4* (Fig. 5B, except *sir4-K1324D*, which expressed at a lower level) and when transformed into *sir4*Δ cells, each of the mutants was unable to complement the silencing defect of *sir4*Δ, behaving identically to the strains tested in Fig. 4C. These plasmids were transformed into wild-type cells and the effect of simultaneous expression of *SIR4* and the *sir4* mutations on telomeric silencing was examined. *sir4-M1307N* and *sir4-I1311N* caused a partial dominant loss of silencing (Fig. 5A, approximately 100-fold less 5-FOA resistance than the wild type), while *sir4-E1310R* and *sir4-K1324D* showed no dominance. Because of the dominant phenotype of the M1307N and I1311N substitutions in *SIR4*, we will refer to these mutations as *SIR4-M1307N* and *SIR4-I1311N* in the sections that follow. The dominance of these *SIR4* alleles is similar to what was previously observed for *SIR2* alleles that produce an enzymatically inactive protein (61), although *SIR2-H364Y* causes a complete loss of silencing at telomeres when expressed simultaneously with wild-type *SIR2* (Fig. 5A).

The Sir3-Sir4 interaction is required for the recruitment of Sir3 to silencers. We next asked if the *sir4* mutants affect the recruitment of Sir4 or Sir3 to silencers or telomeres, and if the interaction of Sir3 with Sir4 is required for the spreading of silent chromatin away from these sites of initiation. Using ChIP, we examined the localization of Sir3 and Sir4 to different regions of silent chromatin. It was previously shown that low levels of Sir4 bind to silencers in the absence of the Sir3 protein (18, 32, 47). Consistent with these results, Sir4-M1307N and Sir4-I1311N, which cannot bind to Sir3, were recruited to the silencers *HMR-E* and *HML-E* to the same extent as Sir4 was recruited in a *sir3*Δ strain (Fig. 6A and B, *HMR-E* and *HML-E*, compare lanes 6 and 7 to lane 4 in Sir4 ChIP). A similar result also was obtained at a telomeric site on the right arm of chromosome VI (Fig. 6C, *TEL0.07*, compare lanes 6 and 7 to lane 4). Sir3 in *SIR4-M1307N* and *SIR4-I1311N* cells, however, did not localize to *HMR-E* and *TEL0.07*, and was present only at low levels at *HML-E* (Fig. 6, *HMR-E*, *HML-E*, and *TEL0.07*, lanes 6 and 7 in Sir3 ChIP). *sir4-E1310R*, one of the mutants that weaken the interaction between Sir3 and Sir4 (Fig. 2B), also failed to recruit Sir3 to telomeres (Fig. 6C, *TEL0.07*, lane 8 in Sir3 ChIP), but was able to recruit wild-type levels of Sir3 to *HMR-E* and *HML-E* (Fig. 6A and B, lane 8 in Sir3 ChIP). In addition, the spreading of both Sir3 and Sir4 to sites within extended silent chromatin was greatly impaired in *SIR4-M1307N* and *SIR4-I1311N* cells (Fig. 7, *HMR***a**, *HML*α, and *TEL0.6*, lanes 6 and 7), and was impaired at telomeres in *sir4-E1310R* (Fig. 7, *TEL0.6*, lane 8).

These results demonstrate that the binding of Sir3 to Sir4 is required for the localization of Sir3 to silent chromatin regions.

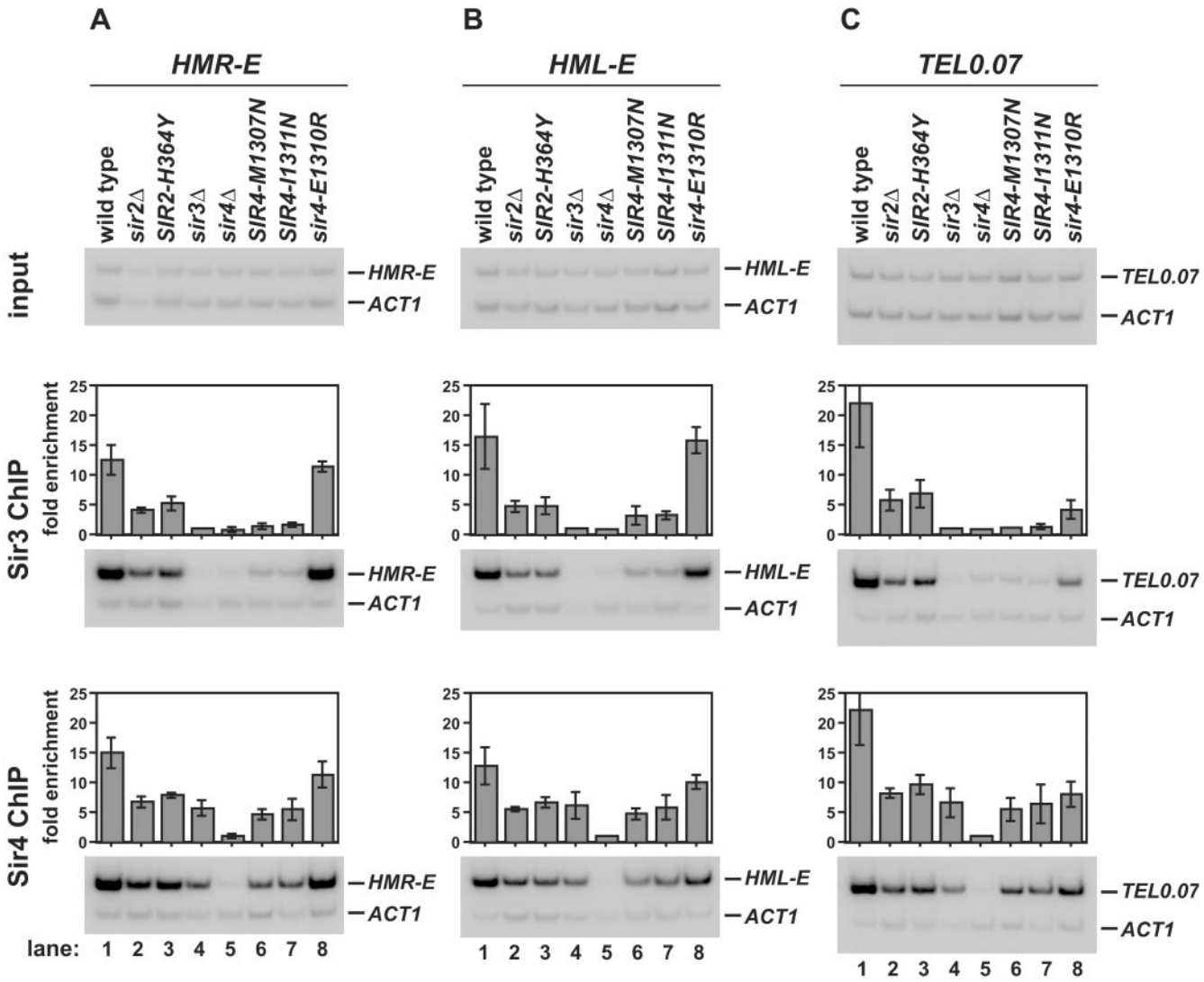


FIG. 6. *sir4* coiled-coil mutants cannot recruit Sir3 to silencers and the ends of telomeres. Wild-type (ADR21), *sir2*Δ::*HIS3* (JRY3433), *SIR2-H364Y* (ADR2808), *sir3*Δ::*HIS3* (JRY3289), *sir4*Δ::*NAT*^{R} (ADR3101), *SIR4-M1307N* (ADR2972), *SIR4-I1311N* (ADR2973), and *sir4-E1310R* (ADR3060) cells were grown overnight to the late log phase in YEP-2% glucose at 30°C and cross-linked for 15 min at room temperature with 1% formaldehyde. Cell pellets were lysed, chromatin was sheared by sonication, and Sir3 and Sir4 were immunoprecipitated with anti-Sir3 and anti-Sir4 antibodies, respectively. After the reversal of cross-links and the removal of protein from the immunoprecipitates and the starting lysate, the localization of Sir3 (Sir3 ChIP) and Sir4 (Sir4 ChIP) to *HMR-E* (A), *HML-E* (B), and a region 70 to 340 bp from the right telomere of chromosome VI, *TEL0.07* (C), was determined by analyzing the immunoprecipitated DNA by PCR with locus-specific primers (Table 2). Every PCR also contained primers to amplify a nonsilent locus, *ACT1*, as an internal control for the input DNA, the immunoprecipitation, and the PCR. For each locus, input DNA, Sir3 ChIP, and Sir4 ChIP are shown. The average and standard deviation of three independent experiments are shown above a representative panel of primary data. The *y* axis in all graphs is the fold enrichment of PCR products amplified from immunoprecipitated DNA relative to that of products from input DNA. For clarity, the enrichment of the *sir3*Δ strain in the Sir3 ChIP and the *sir4*Δ strain in the Sir4 ChIP was arbitrarily set to 1.

The absence of the entire SIR complex at silencers and the telomeres results in a lack of spreading of the Sir proteins to sites within extended silent chromatin. In addition, the ChIP data (Fig. 6 and 7) was entirely consistent with our phenotypic analysis (Fig. 4). When Sir3 was not recruited to telomeres and silencers, and Sir3 and Sir4 did not spread, no silencing was observed. Although we did not measure the localization of Sir2 in this experiment, it is likely that its localization is identical to that of Sir4 because previous ChIP experiments showed no differences in the localization of Sir2 and Sir4 (18, 47), and the tight binding of Sir2 to Sir4 is unaffected in the *sir4* coiled-coil mutants (Fig. 2B).

Previous experiments showed that the Sir2 protein that is recruited to silencers in *sir3*Δ cells is sufficient to cause partial deacetylation of the N-terminal tails of histone H3 and H4 in adjacent nucleosomes (18). Because wild-type Sir3 protein is present in *SIR4-M1307N* and *SIR4-I1311N* cells but cannot associate with Sir4, we were able to ask whether partial deacetylation of histones by silencer-bound Sir2/Sir4 is sufficient for the recruitment of Sir3 to chromatin in the absence of

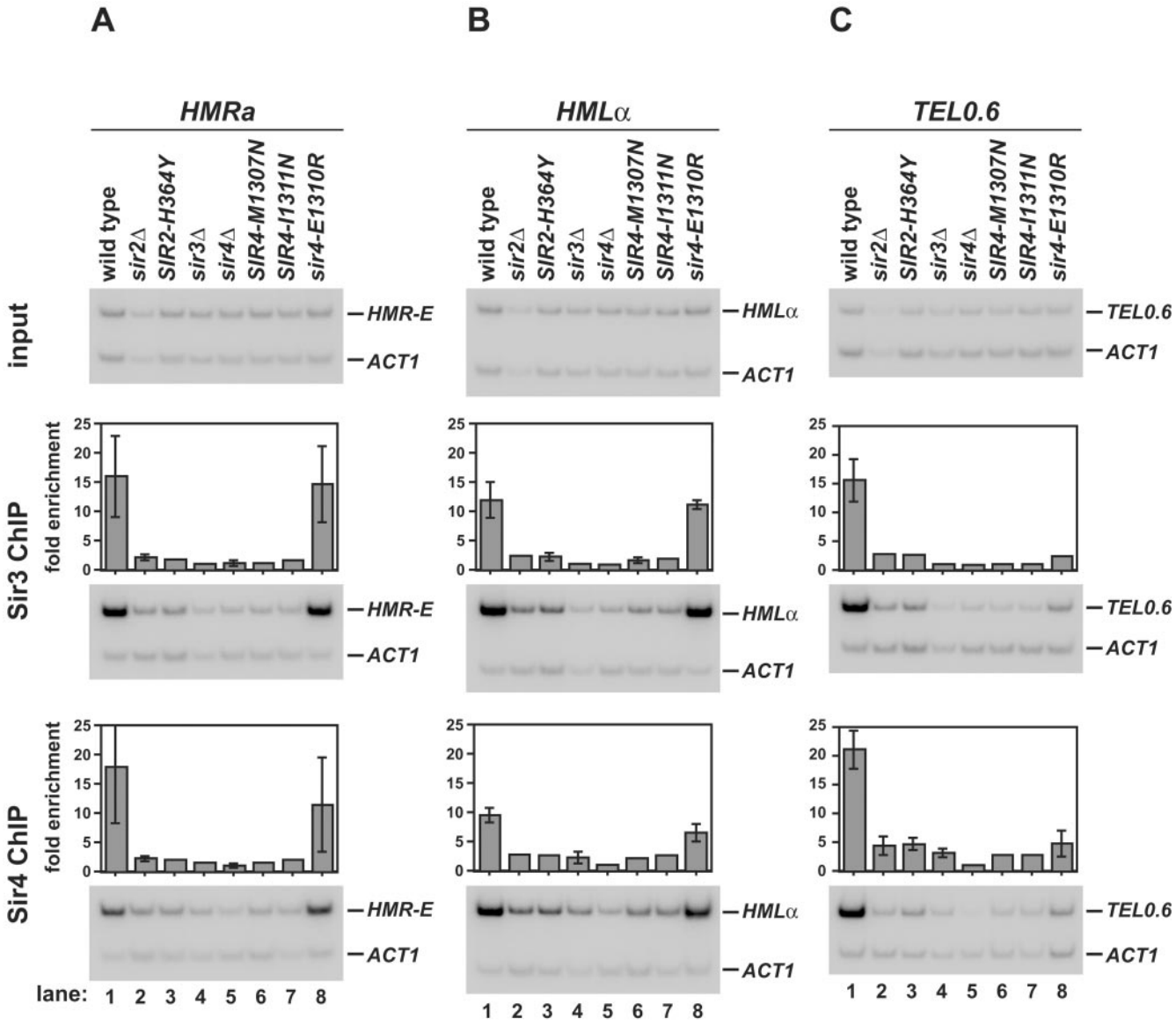


FIG. 7. Sir3 and Sir4 do not spread in *sir4* coiled-coil mutants. The localization of Sir3 (Sir3 ChIP) and Sir4 (Sir4 ChIP) to *HMR***a** (A), *HML*α (B), and a region centered 600 bp from the right telomere of chromosome VI, *TEL0.6* (C), was determined with the same samples as those used for Fig. 6. The presentation and analysis of the data are identical to those in Fig. 6.

the Sir3-Sir4 interaction. In both *SIR4-M1307N* and *SIR4-I1311N* cells, the partial deacetylation of histone H3 and H4 occurs (Fig. 8A and B, *HMR-E* and *HML-E*, lanes 6 and 7), confirming previous results that some Sir3-independent deacetylation can be observed after the recruitment of Sir2 and Sir4 to silencers. Despite this deacetylation, little Sir3 localizes to either *HMR-E* or *HML-E* (Fig. 6A and B, lanes 6 and 7, Sir3 ChIP).

We compared the localization of Sir3 and diacetylated histone H3 at the *HMR-E* silencer and of Sir3 and tetraacetylated histone H4 at the *HML-E* silencer (Fig. 9). Approximately one-fourth as much acetylated histone H3 was found at *HMR-E* in *SIR4-M1307N* and *SIR4-I1311N* cells and one-half as much acetylated histone H4 was found at *HML-E* in *SIR4-M1307N* and *SIR4-I1311N* cells relative to *sir4*Δ. Despite this change, only small amounts (10 to 20% of the wild-type level) of Sir3 localized to *HMR-E* and *HML-E* in these mutants. The amount of Sir3 recruited also was less than the amount recruited in *sir2*Δ and *SIR2-H364Y* cells, in which histone H4 is nearly fully acetylated. These results demonstrate that the binding of Sir3 to Sir4 is critical for the recruitment of Sir3 and suggest that deacetylation of the nucleosome is not sufficient for efficient Sir3 recruitment.

DISCUSSION

The interaction between modified histones and the chromatin proteins that bind to them is essential for the assembly and inheritance of epigenetic chromatin domains. Here we have shown that an additional nonhistone protein-protein interac-

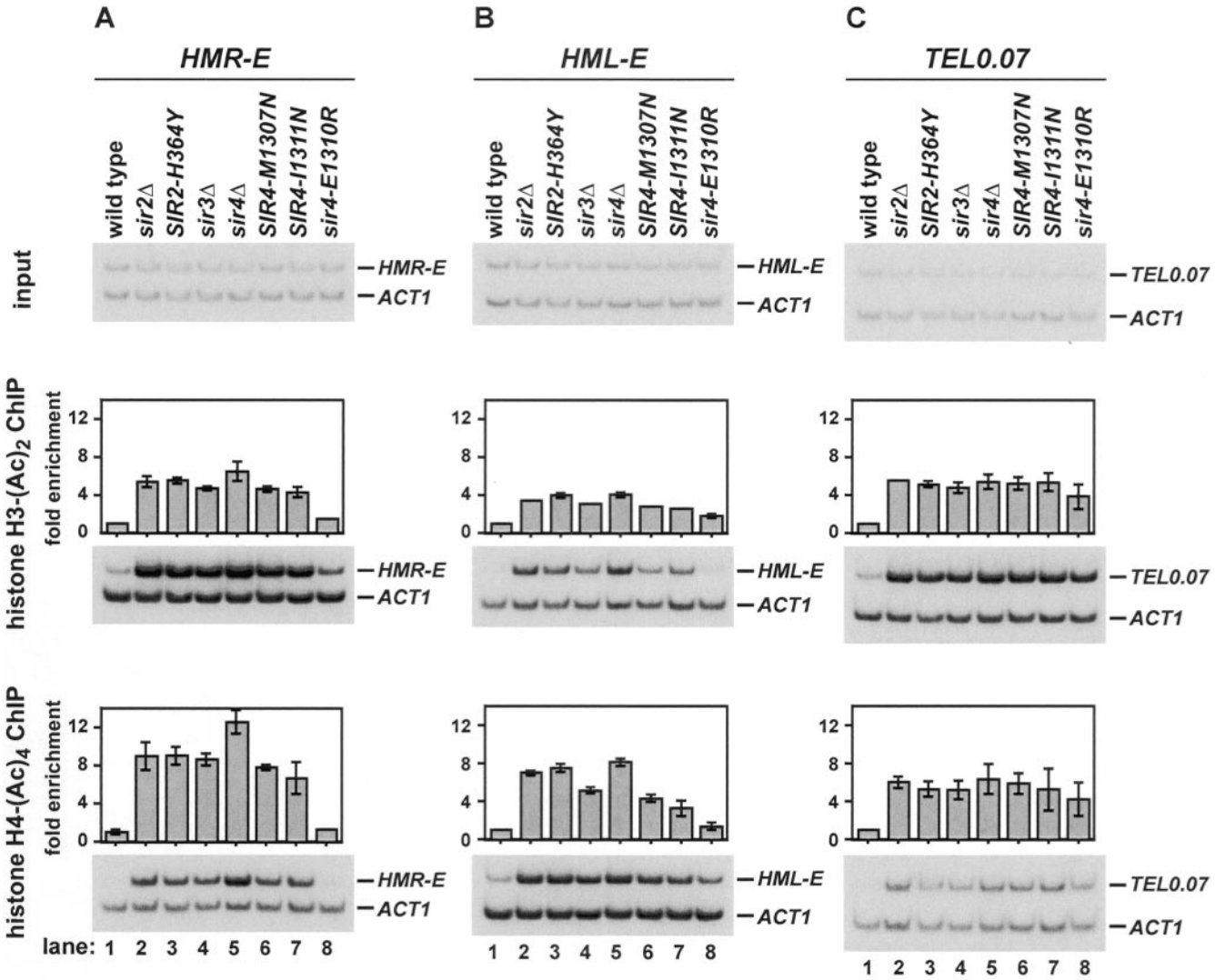


FIG. 8. The deacetylation of histones H4 and H3 correlates with the presence of Sir4. Histones H3 and H4 were immunoprecipitated with anti-diacetylated histone H3 and anti-tetraacetylated histone H4 antibodies from the same lysates as those used for Fig. 6 and 7. The acetylation state of histone H3 and H4 at *HMR-E* (A), *HML-E* (B), and *TEL0.07* (C) was determined by analyzing the immunoprecipitated DNA by PCR with locus-specific primers (Table 2). The presentation and analysis of the data are identical to those in Fig. 6. For clarity, the enrichment of the wild-type strain (which is maximally deacetylated) in both the diacetylated histone H3 ChIP and the tetraacetylated histone H4 ChIP was arbitrarily set to 1.

tion is required for the assembly of heterochromatin. Specifically, Sir3 binds to the coiled-coil region of Sir4, and this binding is required for the localization of Sir3 to chromatin. Our analysis suggests that such nonhistone protein-protein interactions play as important a role in assembly of heterochromatin as the interaction of silencing proteins with modified histones.

Assembly of the SIR complex. We have shown that Sir2, Sir4, and Sir3 form a stable and soluble SIR protein complex. Although the interactions between these three proteins were previously shown by coprecipitation and two hybrid assays (15, 36, 37, 55), earlier native biochemical purifications did not detect a complex containing all three Sir proteins (10, 18). Previous experiments suggested that this was due to the N-terminal region of Sir4 inhibiting its interaction with Sir3 (36), but because we only examined the interactions of full-length proteins, our data does not address this model. Our data show that the SIR complex is soluble and contains a large portion of the Sir4 in the cell, but does not efficiently elute from affinity resins. Nonetheless, our results are consistent with previous findings that virtually all Sir4 is bound to Sir2, and a significant fraction of Sir3 is not associated with the Sir2/Sir4 subcomplex in whole-cell extracts (18, 36, 62).

Point mutations in the coiled-coil domain of Sir4 that preclude its assembly into the SIR complex disrupt silencing at both the *HM* loci and telomeres. The specific defects in binding to Sir3 are likely the sole source of the phenotypes seen in these *sir4* mutations. The amino acid substitutions in these mutations are in the coiled-coil domain of Sir4 that fall within the previously defined Sir3 binding region and cause similar in

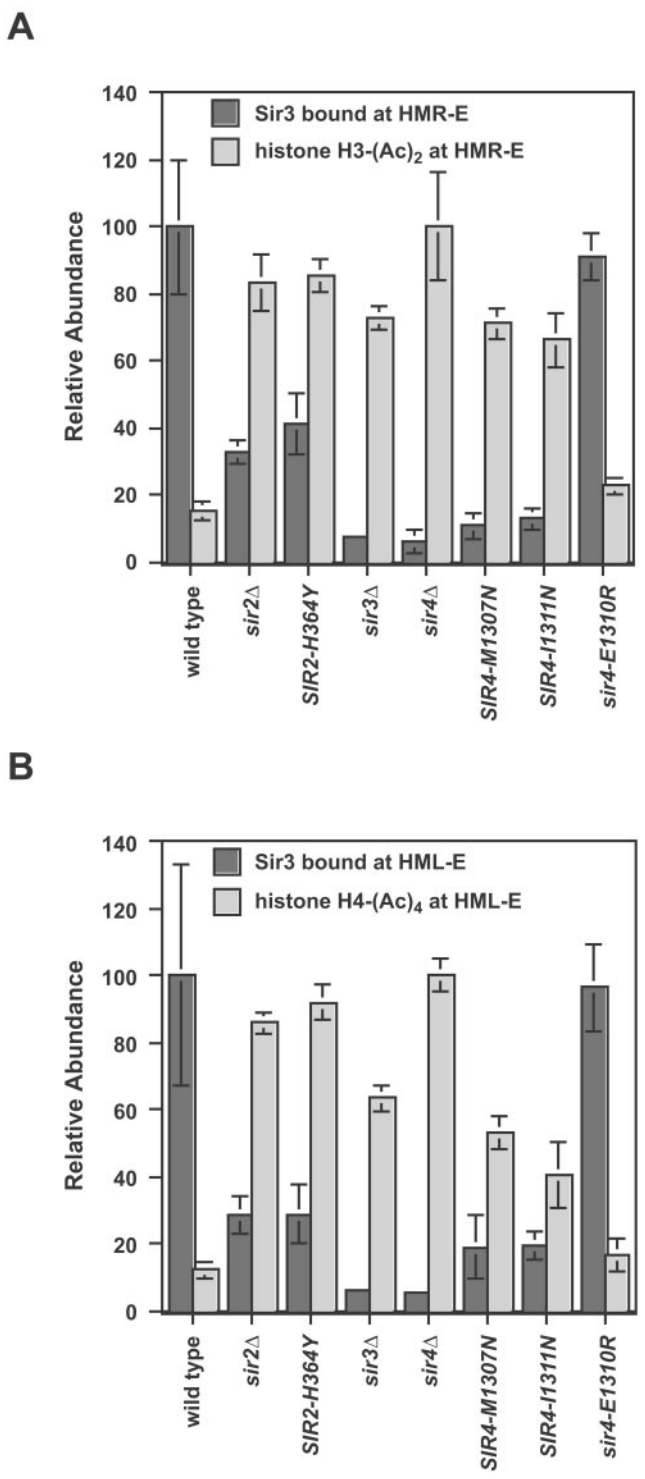


FIG. 9. Partial histone deacetylation at silencers in *SIR4-M1307* and *SIR4-I1311N* cells is not sufficient to recruit Sir3. The localization of Sir3 at *HMR-E* and *HML-E* (Fig. 6A and B, lanes 1 to 8, Sir3 ChIP), diacetylated histone H3 at *HMR-E* (Fig. 8A, lanes 1 to 8), and tetraacetylated histone H4 at *HML-E* (Fig. 8B, lanes 1 to 8) was quantified. For clarity, the values were rescaled so that the maximal recruitment of Sir3 (in wild-type cells) and the maximal acetylation of histones H3 and H4 (in *sir4*Δ cells) were arbitrarily set to 100%. Error bars show standard deviation of the mean.

vitro and in vivo disruptions of Sir3 binding to Sir4 (7). Although there is good agreement between previous in vitro pull-down experiments with purified proteins (7) and our immunoprecipitation of complexes formed in vivo, the in vitro pull-down experiments used only C-terminal fragments of both Sir3 and Sir4 and therefore could not rule out the presence of a second interacting surface between the two proteins. The results presented here show that the coiled-coil domain of Sir4 is necessary for the interaction between full-length Sir4 and full-length Sir3 in vivo.

The C terminus of Sir4 has also been shown to bind Rap1, Yku70, and Ubp3 (35, 37, 38, 65). The interactions of these proteins with full-length Sir4 are difficult to detect; therefore, it is difficult to rule out whether the *sir4* coiled-coil mutants also affect the binding of Sir4 to these proteins. We believe it is unlikely, however, that a defect in the interaction between these proteins and Sir4 contributes to the phenotype of our *sir4* mutants for the following reasons. (i) As much Sir4-M1307N and Sir4-I1311N is recruited to silencers and telomeres as wild-type Sir4 is recruited to these loci in *sir3*Δ cells (Fig. 6, compare lanes 6 and 7 to lane 4, Sir4 ChIP). We therefore conclude that the binding of Sir4-M1307N and Sir4-I1311N to recruitment factors (like Rap1 and Yku70) is normal. (ii) *yku70*Δ mutants only have telomeric silencing defects (3), while the *sir4* coiled-coil mutants also have defects at *HMR* (Fig. 4A and B). (iii) *ubp3*Δ cells have been shown to improve silencing (35), so a defect in Ubp3 binding in the *sir4* coiled-coil mutants would be predicted to improve silencing, not weaken it, as the *sir4* coiled-coil mutants do. (iv) The E element of the *HMR-E* silencer is a binding site for Rap1 (58), so if a defect in the binding of Rap1 to Sir4 was the cause of the phenotypes of the *sir4* coiled-coil mutants, than we would predict that the silencing defects of these mutants in a *hmr*Δ*E* strain would be no more severe than *hmr*Δ*E* in a *SIR4* strain. The *sir4* coiled-coil mutants, however, completely derepress silencing at *hmr*Δ*E* (Fig. 4B).

Previously we had suggested that Sir3 and the Sir4/Sir2 complex meet on chromatin either at silencers or telomeres or as they bind to deacetylated histones during spreading (34). Another possibility suggested from this study is that the SIR complex forms in solution and loads onto chromatin as a single complex, initially by interactions with DNA binding proteins and then during spreading of silent chromatin by interactions with other SIR complexes and deacetylated nucleosomes (Fig. 10).

Nucleation and spreading of silent chromatin require Sir-Sir and Sir-histone interactions. Previous work showed that the recruitment of Sir3 to silencers is independent of the presence of Sir2 (18, 47). We have extended this analysis by showing that the partial deacetylation of nucleosomes adjacent to silencers (Fig. 8A and B, lanes 6 and 7) in *SIR4-M1307N* and *SIR4-I1311N* cells is unable to recruit large amounts of Sir3 (Fig. 6A and B, lanes 6 and 7). In addition, a *sas2*Δ mutation, in which lysine 16 of histone H4 is hypoacetylated, does not cause any increase in the amount of Sir3 recruited to silencers in *SIR4-M1307N* or *SIR4-I1311N* cells relative to *sir4*Δ cells, nor does it suppress any of the silencing defects in *SIR4-M1307N* and *SIR4-I1311N* cells (data not shown). These results suggest that although Sir3 has a high affinity for deacetylated N-terminal tails of histones in vitro (6), the association of

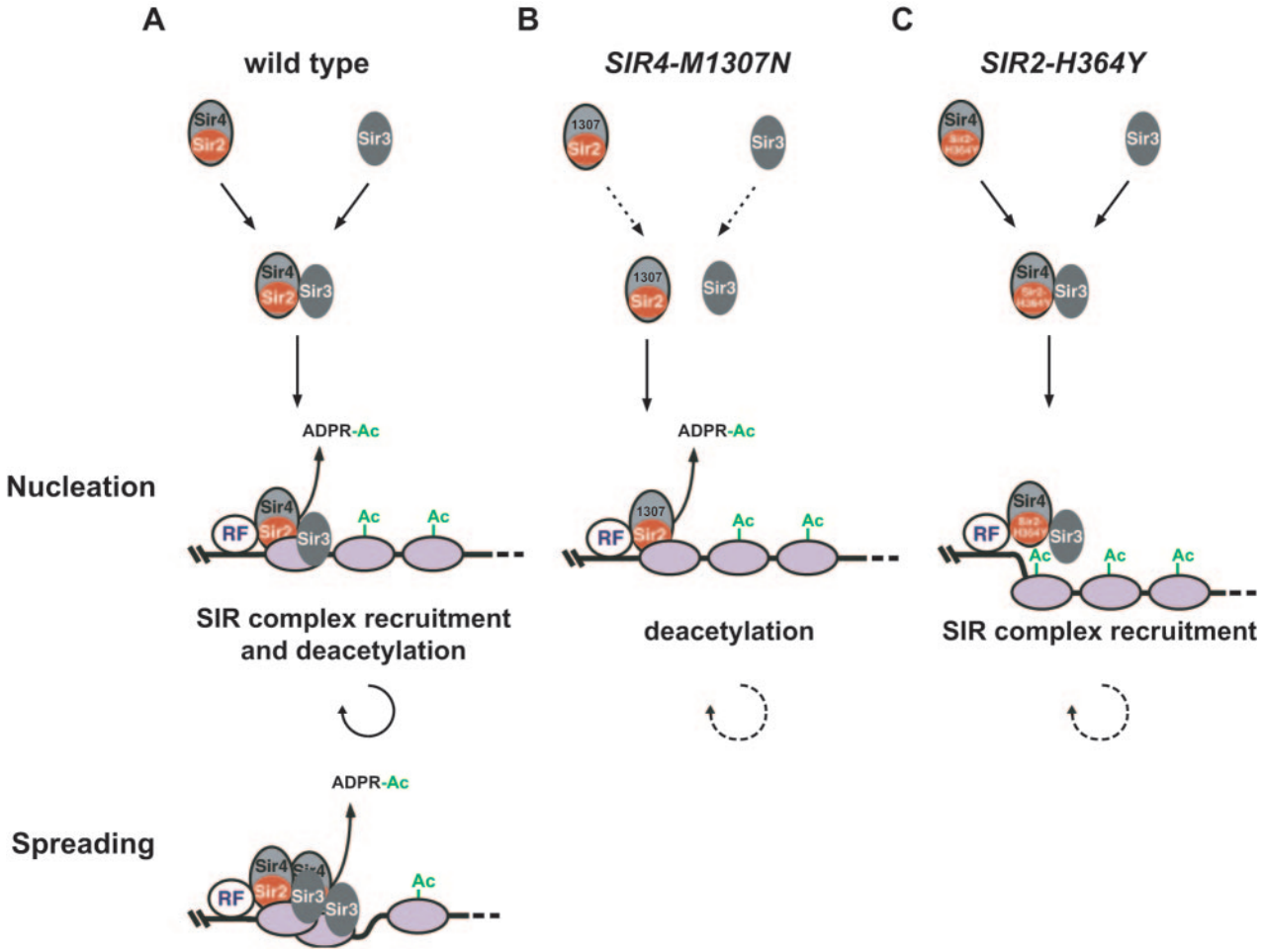


FIG. 10. Step-wise assembly of silent chromatin. Sir4/Sir2 binds to Sir3 in solution to form the SIR complex. The binding of Sir3 to Sir4 does not depend on Sir2 or Sir2 activity but depends on surface residues in the Sir4 coiled coil. The SIR complex then is recruited to silencers and telomeres by the interaction between Sir4 and recruitment factors (RF). Sir4 can be recruited in the absence of Sir2 and Sir3, while Sir2 and Sir3 recruitment depends on Sir4. (A) In wild-type cells, the nucleation and spreading of silent chromatin require both the binding of Sir3 to Sir4 and the deacetylation of nucleosomes by Sir2. Deacetylation of nucleosomes (purple spheres with green acetyl [Ac] groups) produces acetyl-ADP-ribose (ADPR-Ac) (60, 63). Spreading occurs through cycles of recruitment of the SIR complex accompanied by histone deacetylation by Sir2. (B) In *SIR4-M1307N* (or *SIR4-I1311N*) cells, a Sir4-M1307N/Sir2 subcomplex is recruited to initiation sites, and the deacetylation of proximal nucleosomes can occur. Silent chromatin, however, is not nucleated, nor does it spread because of the absence of Sir3. (C) In *SIR2-H364Y* cells, a Sir2-H364Y-containing SIR complex forms and is recruited to initiation sites, but no deacetylation of nucleosomes occurs. Silent chromatin cannot nucleate or spread because of the lack of deacetylation. For simplicity, we have shown the SIR complex as consisting of one of each of the Sir proteins, although the SIR complex may contain at least two of each of the Sir proteins. A larger SIR complex would have many additional surfaces with which to interact with adjacent SIR complexes during the spreading of silent chromatin.

Sir3 with deacetylated histone tails is not sufficient for the full recruitment of Sir3 to chromatin in vivo. Sir3 binding to deacetylated histones, however, may contribute to its recruitment to silencers. Similar amounts of Sir3 were recruited to *HML-E* in *SIR4-M1307N* and *SIR4-I1311N* cells as in *sir2*Δ and *SIR2-H364Y* cells (Fig. 6B, lanes 2, 3, 6, and 7), suggesting that Sir3 binding to either Sir4 or deacetylated histone H4 can recruit some Sir3 to this site. Although there is some Sir3 recruitment to *HML-E* in *SIR4-M1307N* and *SIR4-I1311N* cells, there is no recruitment to *HMR-E* or to *TEL0.07* (Fig. 6A and C, lanes 6 and 7, Sir3 ChIP). This difference may be caused by the greater deacetylation of histone H4 at *HML-E* compared to *HMR-E* and *TEL0.07* (Fig. 8B, lanes 6 and 7 compared to lane 4; Fig. 8A and C, lanes 6 and 7 compared to lane 4).

The assembly of silent chromatin therefore requires both the interaction of Sir3 with Sir4 and the deacetylation of histones (Fig. 10A). When either of these two steps are defective, silent chromatin cannot assemble. In *SIR4-M1307N* and *SIR4-I1311N* cells, although the Sir4/Sir2 subcomplex is recruited to silencers and telomeres and histones proximal to these sites are deacetylated, the nucleation of silent chromatin does not occur (Fig. 10B). In *SIR2-H364Y* cells, although the full SIR complex can be recruited to initiation sites, enzymatically inactive Sir2 is unable to deacetylate histones and the nucleation of silent chromatin also does not occur (Fig. 10C).

Spreading of silent chromatin is thought to require successive recruitment of additional SIR complexes followed by deacetylation of nucleosomes (Fig. 10A). However, because deacetylation alone is not sufficient to promote Sir3 binding to

chromatin (Fig. 6 to 8), we propose that the recruitment of all subsequent SIR complexes requires both histone deacetylation and lateral interactions between SIR complexes (Fig. 10A). In this model, the growth of the SIR polymer along the chromatin fiber requires two distinct interactions, one between the SIR complex and deacetylated histone tails and one between adjacent SIR complexes. We speculate that the interaction between Sir3 and the Sir4 coiled coil does not bridge adjacent SIR complexes, rather that only intact SIR complexes are able to interact during spreading of silent chromatin.

Is the Sir3-Sir4 interaction required for the spreading of silent chromatin? The dominant phenotype of *SIR4-M1307N* and *SIR4-I1311N* supports the model that the binding of Sir3 to Sir4 is required for the spreading of silent chromatin (Fig. 10B). When these mutants are expressed simultaneously with wild-type *SIR4*, silencing at telomeres is reduced by 100-fold (Fig. 5A). Under these coexpression conditions, 25% of the Sir4 dimers should contain two wild-type Sir4 molecules, 25% should contain two mutant molecules, and 50% should be dimers that contain one each wild-type and mutant Sir4 molecules. We would expect that the population containing two mutant Sir4 proteins to have little or no dominant phenotype as these mutant dimers will be unable to bind to Sir3 and incorporate into the SIR complex, and should therefore be unable to cap the spreading SIR polymer. Assuming that each Sir4 dimer binds to two Sir3 proteins (7), the dominant phenotype of *SIR4-M1307N* and *SIR4-I1311N* is likely to stem from the association of the mixed wild-type–mutant dimers with Sir3 during spreading of silent chromatin. Because these mixed dimers can only interact with one Sir3 molecule, their incorporation into silent chromatin would cap the growing SIR polymer and cause a dominant-negative phenotype.

The dominant-negative phenotype of *SIR4-M1307N* and *SIR4-I1311N* is less potent than that previously reported for *SIR2-H364Y* (61). Because *SIR2-H364Y* can still incorporate into the SIR complex (see Fig. S3 in the supplemental material), SIR complexes containing Sir2-H364Y are likely to be recruited into spreading silent chromatin with wild-type efficiency, but their lack of deacetylase activity would block the recruitment of additional SIR complexes and cap the growing SIR polymer. In contrast, the ability of mixed wild-type Sir4-M1307N or Sir4-I1311N dimers to associate with the growing SIR polymer may be impaired due to the loss of one Sir3 binding surface on the coiled coil dimer. As a result, their ability to poison the spreading of silent chromatin would be compromised compared to SIR complexes containing Sir2-H364Y.

Inheritance of silent chromatin. The N-terminal tails of histones can be acetylated, methylated, ubiquitinated, and phosphorylated (21). This wide array of possible modifications of histones has led to the proposal that different combinations of histone modifications form a "histone code" that determines the identity and function of a given chromatin domain. Because histone H3 and H4 tetramers remain associated with daughter DNA strands during DNA replication (27, 53), their modification states are also inherited and therefore may form the basis for the epigenetic inheritance of chromatin domains. In silent chromatin, the hypoacetylation of histones may be expected to form the primary epigenetic mark that ensures the stability of silent chromatin and its inheritance after DNA replication (4, 57). Our data, however, show that in addition to histone deacetylation, the tight binding of Sir3 to Sir4 at silencers and telomeres is required to recruit Sir3, suggesting that both histone modifications and interactions between silencing proteins are essential for the assembly and inheritance of silent chromatin domains.

ACKNOWLEDGMENTS

We thank Jasper Rine, Dan Gottschling, Jason Tanny, Fred Winston, and Kurt Runge for strains, plasmids, and antibodies; Mo Motamedi, Julie Huang, Erica Hong, and Andre Verdel for critical reading of the manuscript; and David Rudner, Mo Motamedi, Alex Szidon, Marc Lenburg, Dara Spatz Friedman, and Doug Kellogg for invaluable discussions and unwavering support.

This work was supported by grants from the NIH (GM61641 to D.M. and GM52504 to T.E.) and the Ellison Medical Foundation (to D.M.). A.D.R. is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research, B.E.H. is supported by a fellowship (GM06835001) from the NIH, and D.M. is a scholar of the Leukemia and Lymphoma Society.

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