# Hyperactivation of the Silencing Proteins, Sir2p and Sir3p, Causes Chromosome Loss

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ABSTRACT

The *SIR* gene products maintain transcriptional repression at the silent mating type loci and telomeres in *Saccharomyces cerevisiae*, although no enzymatic or structural activity has been assigned to any of the Sir proteins nor has the role of any of these proteins in transcriptional silencing been clearly defined. We have investigated the functions and interactions of the Sir2, Sir3, and Sir4 proteins by overexpressing them in yeast cells. We find that Sir2p and Sir3p are toxic when overexpressed, while high Sir4p levels have no toxic effect. Epistasis experiments indicate that Sir2p-induced toxicity is diminished in strains lacking the *SIR3* gene, while both Sir2p and Sir4p are required for Sir3p to manifest its full toxic effect. In addition, the effects of Sir2 or Sir3 overexpression are exacerbated by specific mutations in the N-terminus of the histone H4 gene. These results are consistent with a model in which Sir2p, Sir3p and Sir4p function as a complex and interact with histones to modify chromatin structure. We find no evidence that toxicity from high levels of the Sir proteins results from widespread repression of transcription. Instead, we find that high levels of Sir2p and/or Sir3p cause a profound decrease in chromosome stability. These results can be appreciated in the context of the effects of Sir2p in histone acetylation and of chromatin structure on chromosome stability.

HAPLOID *Saccharomyces cerevisiae* contains three loci that code for mating-type information. Mating type is determined by genes present at the expressed *MAT* locus, while similar or identical genes present at the *HML* and *HMR* are expressed only when transposed to *MAT* following mating type switching. Repression at *HML* and *HMR* is achieved by a position effect mechanism known as silencing, which extends to other yeast genes placed at *HML* or *HMR* (for review see HERSKOWITZ *et al.* 1992; LAURENSON and RINE 1992; HOLMES *et al.* 1996). A similar position effect is exerted on genes artificially placed at yeast telomeres (APARICIO *et al.* 1991; GOTTSCHLING *et al.* 1990). Silencing is likely to involve the formation of the yeast equivalent of heterochromatin, a repressive chromatin structure that underlies the phenomena of *X* chromosome inactivation in mammals and position effect variegation in Drosophila (THOMPSON *et al.* 1993; BRAUNSTEIN *et al.* 1996a,b).

Silencing at *HML* and *HMR* depends on sequences flanking each locus, known as the E and I silencers, as well as several *trans*-acting factors, including the products of the four *SIR* genes. Each of the *SIR* genes was initially identified by genetic screens for loss of repression at the silent mating type loci (HABER and GEORGE 1979; KLAR *et al.* 1979; RINE *et al.* 1979; RINE and HERSKOWITZ 1987). None of the *SIR* genes is essential for growth, but null mutations in *SIR2, SIR3,* or *SIR4* lead to complete expression of the silent mating type loci. Deletion of the *SIR1* gene leads to an intermediate phenotype in which some cells are repressed and others derepressed (PILLUS and RINE 1989). An understanding of the function of the *SIR* genes is central to determining the mechanism of silencing in yeast.

Each of the four *SIR* genes has been cloned, but their sequences have not suggested clear functions for their protein products. *SIR1* codes for a novel protein (STONE *et al.* 1991) while *SIR2* codes for a zinc finger protein that is a member of a gene family with four other members in yeast and at least one similar member in mammals (SHORE *et al.* 1984; BRACHMANN *et al.* 1995). The *SIR3* gene codes for a protein of unknown function that has similarity to Orc1p, part of the six subunit complex that recognizes DNA replication origins in yeast (SHORE *et al.* 1984; BELL *et al.* 1995). Finally, *SIR4* codes for a large protein that shows similarity to nuclear lamins (MARSHALL *et al.* 1987; DIFFLEY and STILLMAN 1989). None of the Sir proteins have been shown to interact directly with the silencer sequences. If individual Sir proteins are fused to the Gal4p DNA binding domain and recruited to the silent mating type loci using a *GAL4* UAS sequence, Sir1p can promote weak

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silencing; no silencing is observed by recruitment of Sir2p, Sir3p, or Sir4p (CHIEN *et al.* 1993).

Several independent approaches have suggested that the Sir proteins interact with silencer-binding factors and histone proteins to promote transcriptional repression. MORETTI *et al.* (1994) used a two hybrid screen to find proteins that interact with the silencer-binding factors and identified an association of Rap1 with Sir3p and Sir4p. The Rap1p-Sir3p association may be direct, as these proteins physically interact *in vitro*. MORETTI *et al.* (1994) also used the two hybrid assay to show that the Sir3 and Sir4 proteins interact *in vivo*. In an independent study HECHT *et al.* (1995) investigated the ability of Sir2p, Sir3p, and Sir4p to interact with the N-terminal tails of histone H3 and H4 *in vitro*. While they found no evidence for a Sir2p-histone association, they showed that both Sir3p and Sir4p bind the tails of H3 and H4 (HECHT *et al.* 1995). Using protein affinity chromatography, MOAZED and JOHNSON (1996) found that Sir2p and Sir3p associate with Sir4p. Finally, immunofluorescence experiments using antibodies to Sir3p, Sir4p, and Rap1p suggest that these proteins colocalize in discrete foci associated with the nuclear periphery (PALLADINO *et al.* 1993; COCKELL *et al.* 1995).

Genetic approaches to determining the function of the Sir proteins have been limited by the identical null phenotype of the *SIR2, SIR3,* or *SIR4* genes and the absence of alleles with reduced or novel functions. The *SIR* genes, particularly *SIR1, SIR2,* and *SIR4,* are transcribed at low levels, which may reflect their highly specific role in the cell (IVY *et al.* 1986). We have examined the effects of overexpressing the *SIR* genes in hopes of observing phenotypes that would help us understand their normal function. We have previously shown that overexpression of *SIR4* leads to a dominant disruption of silencing, the "anti-*SIR*" effect (MARSHALL *et al.* 1987), and that overexpression of *SIR2* leads to a global deacetylation of histone molecules in the cell (BRAUNSTEIN *et al.* 1993). Here we report that overexpression of *SIR2* or *SIR3* is toxic to yeast cells. Using the overexpression phenotypes in epistasis analysis, we find evidence for the functional interaction of the Sir proteins in a complex that interacts with histones and, at high levels, interferes with the mitotic transmission of chromosomes.

## MATERIALS AND METHODS

**Plasmids:** Plasmids in which *SIR2* or *SIR3* expression was placed under control of the *GAL10* promoter were constructed from the high copy expression vectors YEp51 and YEp54 (BROACH *et al.* 1983; ARMSTRONG *et al.* 1990). The *SIR2* and *SIR3* genes were subcloned using plasmids pJH20.1 and pKAN59, respectively (IVY *et al.* 1986). A *Sal*I linker was inserted into the *Acc*I site of plasmid pJH20.1, which lies 45 bp upstream of the initial ATG codon of *SIR2*. The 3.5-kb *Sal*I-*Hin*dIII fragment from the resulting plasmid was inserted into either YEp51, forming pAR14, or YEp54, forming pAR44. The 3.7-kb *Hpa*I fragment from pKAN63 was ligated into the *Sma*I site of pUC12. This plasmid (pAR3) was linearized with *Sac*I, treated with exonuclease BAL31, and reclosed in the presence of *Sal*I linkers. The 3.5-kb *Sal*I-*Bam*HI fragment from one of the recovered plasmids, in which the *Sal*I site is 7 bp upstream of the initial ATG codon of the *SIR3* open reading frame, was inserted into YEp51 and YEp54 to create pAR16 and pAR82, respectively. The *GAL10::SIR4* plasmid, pSIR4.7, was derived by insertion of a *Kpn*I-*Bam*HI fragment spanning the 2-$\mu$ circle replication and partitioning sequences of plasmid pSIR4.3 into the equivalent sites of plasmid pSIR4.6, each of which has been previously described (MARSHALL *et al.* 1987). pSAS2 was constructed by isolating a 5.0-kb *Eco*RV fragment from pAR82, containing the *GAL10::SIR3* fusion, and subcloning it into pAR14, cut with *Hin*dIII and made blunt with Klenow enzyme. pYML2 was made by first subcloning a *Cla*I-*Kpn*I fragment containing the *SIR3-R3* mutation from pLJ90 (JOHNSON *et al.* 1990) into pAR12, forming pYML1. A *Sal*I-*Bam*HI fragment containing the *SIR3-R3* mutation was then isolated from pYML1 and subcloned into pAR34 cut with *Sal*I and *Bam*HI, forming pYML2. pYML2 is identical to pAR16, except for the *SIR3-R3* mutation. pSH105, used to delete the *SIR3* gene, was made by replacing the large *Bgl*II fragment within the *SIR3* open reading frame with a *Bgl*II fragment containing the *URA3* gene.

**Strains:** Yeast strains used in this study are listed in Table 1. Plasmid C369 (SHORE *et al.* 1984) was used to make the *sir2::TRP1* deletion. Plasmid pSH105 was used to make the *sir3::URA3* deletion. Plasmid pAR59 (MARSHALL *et al.* 1987) was used to make the *sir4::URA3* deletion. Strain Y1191 was a segregant from a cross between strains PKY499 (KAYNE *et al.* 1988) and lab strain AB8-16C. Isogenic derivatives of Y1191 that differed only in the plasmid-borne *HHF2* allele were derived from strain Y1191 by a plasmid shuffle protocol. Strain Y1191 was transformed to Trp+ with plasmid pMH310 (YCp *TRP-HHF2*) and a Ura− segregant of one such transformant was recovered. This segregant was transformed to Ura+ with plasmids pPK617, pPK618, or pPK606, bearing *HHF2* alleles Δ4-14, Δ4-20, and Δ4-23, respectively (KAYNE *et al.* 1988). Trp- segregants of selected transformants were recovered to yield strains Y1186, Y1181, and Y1176, respectively. *SIR2* or *SIR3* overexpressing versions of these strains were obtained by transforming each of them to Trp+ with plasmids pAR44 or pAR82.

**Immunological procedures:** Preparation of antibodies to Sir2p and Sir3p was described previously (BRAUNSTEIN *et al.* 1993). Immunoprecipitation experiments were performed in the protease-deficient strain BJ2169. Cultures of this strain harboring the indicated plasmid or plasmids were grown at 30° in SC raffinose medium to a density of $10^7$ cells/ml, at which point galactose was added to 2% and incubation continued for 3 hr. Extracts were prepared as described (BRAUNSTEIN *et al.* 1993) and incubated with the indicated antibody (15 $\mu$l/ml extract) at 0° for 3 hr. Formalin-fixed Staph A cells (ImmunoPrecipin, BRL, 30 $\mu$l/ml extract) were added and incubation continued for 20 min. Immunoprecipitates were then harvested by centrifugation and washed twice with RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris-HCl pH 7.2). Samples were boiled in PAGE sample buffer and the presence of Sir proteins was determined by Western blot analysis as described (BRAUNSTEIN *et al.* 1993).

**Chromosome loss:** Strain YPH1015 (provided by P. HIETER) contains a *HIS3*-marked 125-kb linear chromosome fragment derived from chromosome *III* (SHERO *et al.* 1991). A *LEU2*-marked 73-kb circular derivative of chromosome *III* was introduced as described (RUNGE and ZAKIAN 1993) into an isolate of strain YPH1015 lacking the linear chromosome *III* fragment. Each strain was then transformed with appropriate *SIR*-overexpressing plasmids and controls. Chromosome loss was deter-

**TABLE 1**

**Description of yeast strains used in this study**

| Strain | Genotype | Source |
|---|---|---|
| Y2155 | *HMLa MATa HMRa ade2 leu2 lys2 trp1-289 ura3-52* | BRAUNSTEIN *et al.* (1993) |
| Y2156 | Y2155; Δ*sir2::TRP1* | This study |
| Y2157 | Y2155; Δ*sir3::URA3* | This study |
| Y2158 | Y2155; Δ*sir4::URA3* | This study |
| BJ2169 | *MATa leu2 trp1 ura3 prbl-1122,407 pep4-3* | E. JONES |
| Y1191 | *MATa ade2 his3Δ1 leu2-3,112 trp1Δ901 hhf1::HIS3 hhf2::LEU2 LYS2::GAL1-lacZ* [YCp-*URA3-HHF2*] | This study |
| Y1186 | *MATa ade2 his3Δ1 leu2-3,112 trp1Δ901 hhf1::HIS3 hhf2::LEU2 LYS2::GAL1-lacZ* [YCp-*URA3-hhf2Δ4-14*] | This study |
| Y1181 | *MATa ade2 his3Δ1 leu2-3,112 trp1Δ901 hhf1::HIS3 hhf2::LEU2 LYS2::GAL1-lacZ* [YCp-*URA3-hhf2Δ4-19*] | This study |
| Y1176 | *MATa ade2 his3Δ1 leu2-3,112 trp1Δ901 hhf1::HIS3 hhf2::LEU2 LYS2::GAL1-lacZ* [YCp-*URA3-hhf2Δ4-23*] | This study |
| Y2215 | *MATα leu2-3,112 ura3-52 ade2 trp1-289 can1 Δhis1::URA3* | This study |
| YPH1015 | *MATa ade2-101 his3Δ200 leu2Δ1 lys2-801 trp1Δ63 ura3-52* [*CEN 3L YPH985.his.sup11*] | SHERO *et al.* (1991) |
| Y2279 | Y2215 × YPH1015 | This study |

mined by fluctuation analysis: for the linear chromosome *III* fragment individual colonies grown on glucose medium lacking leucine were suspended in SC raffinose medium lacking leucine, induced by the addition of galactose to 2%, then grown at 30°. Cells were plated on −Leu and −Leu −His plates at the time of induction and at various times following induction to determine the initial and final percentage of cells bearing the nonessential chromosome fragment. An identical procedure was used to determine the loss rate of the circular chromosome *III* derivative, except a *TRP*-marked *SIR3* plasmid was used, and the test chromosome was followed by the *LEU2* marker. The values shown are the means of at least two independent determinations; trials deviated from the means by <20%. Chromosome *V* loss rate was determined in strain Y2279 as described (HARTWELL and SMITH 1985), except that a disruption of the *HIS1* locus was used in place of the recessive *hom3* mutation. Recombination was not sigificantly altered in strains overexpressing *SIR* genes by this assay. Loss rates reported are the means of at least three independent determinations; trials varied from the means by <25%.

## RESULTS

**Overexpression of *SIR2* or *SIR3* is toxic:** Deletion of *SIR2, SIR3,* or *SIR4* leads to an identical phenotype in which the silent mating type loci are fully derepressed, but none of the deletions yields any additional phenotypes that might provide insights into the role of the *SIR* proteins in transcriptional silencing. Since the *SIR* genes are transcribed at low levels, we reasoned that high levels of the *SIR* proteins might induce an activity normally constrained to silenced loci to act at other places on the chromosome and result in novel phenotypes. To test this hypothesis we constructed a set of plasmids allowing inducible, high level expression of the *SIR* genes. Overexpression was achieved by placing the *SIR* genes under the control of the galactose-inducible *GAL10* promoter on high copy number vectors and adding galactose to the growth medium of strains containing these plasmids. These conditions have been shown to result in a large increase in the levels of each Sir protein (MARSHALL *et al.* 1987; BRAUNSTEIN *et al.* 1993).

High levels of Sir4p have previously been shown to cause a dominant disruption of silencing, known as the anti-*SIR* effect (IVY *et al.* 1986; MARSHALL *et al.* 1987). We find that increasing the abundance of Sir2p or Sir3p does not affect mating efficiency (data not shown). However, increased levels of Sir2p or Sir3p lead to a significant decrease in cell viability. Cultures carrying different *SIR* plasmids were grown to log phase in raffinose medium, which neither induces nor represses the *GAL10* promoter. Serial dilutions of these cultures were then plated on media containing galactose, which induces high expression of the *SIR* genes. Figure 1 shows that the *SIR4* overexpressing plasmid did not reduce the ability of the culture to form colonies when compared to cells containing a vector control. However, high-level expression of Sir2p or Sir3p is toxic to yeast cells, leading to a $10^3$–$10^4$ decrease in plating efficiency. Decreased colony number and size are also observed if cultures are plated on galactose medium containing leucine (not shown), indicating that toxicity is not solely due to a *SIR2*- or *SIR3*-induced decrease in plasmid stability.

**Interactions among the *SIR* proteins:** The complete loss of silencing due to null mutations in any one of the *SIR2, SIR3,* or *SIR4* genes initially led to the proposal that they acted as a complex. In support of this proposal, MORETTI *et al.* showed that the Sir3 and Sir4 proteins associate with each other, and with Rap1p, *in vivo* (MORETTI *et al.* 1994), while Sir2p and Sir3p associate with Sir4p *in vitro* (MOAZED and JOHNSON 1996). The ability to induce a growth phenotype by expressing individual *SIR* genes allowed us to explore further possible functional interactions among the Sir proteins *in vivo.* We first overexpressed combinations of the *SIR*

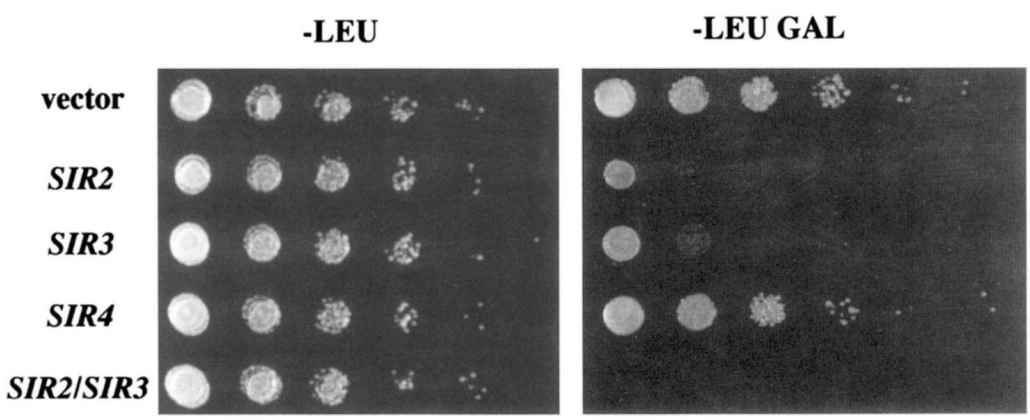


FIGURE 1.—Toxic effects of *SIR* gene overexpression. Strain Y2155 was transformed with plasmids containing galactose-inducible *SIR* genes, then grown overnight in raffinose medium lacking leucine. Sets of 10-fold serial dilutions from these cultures were spotted on glucose medium lacking leucine (−LEU) or on galactose medium lacking leucine (−LEU GAL). Photographs were taken after 2–4 days growth at 30°.

genes to assay their ability to cooperate in inducing toxic effects. We found that overexpressing *SIR4* in combination with *SIR2*, or in combination with *SIR3*, neither increased nor reduced the level of toxicity caused by *SIR2* or *SIR3* overexpression alone (Table 2). However, cells bearing a plasmid that expresses both *SIR2* and *SIR3* at high levels exhibited a decrease in viability that was substantially greater than for cells expressing either *SIR2* or *SIR3* alone (Figure 1).

We performed a time course experiment to examine the consequences of *SIR2* and *SIR3* coexpression further. Strains carrying plasmids expressing a single *SIR* gene, or both *SIR2* and *SIR3* were grown to log phase in raffinose medium, induced with galactose, and plated for viability. For convenience these results are presented in Table 2 as the ratio of viable cells following growth in galactose for 24 hr *vs.* the viable cells present following growth of the same strain for 24 hr in the absence of galactose. The values presented accurately reflect the behavior of the strains throughout the growth curve. The viability of strains overexpressing both *SIR2* and *SIR3* was at least 300 times less than that of the same strain expressing either *SIR2* or *SIR3* alone. This synergistic effect in reducing cell viability suggests that Sir2p and Sir3p share a common or related function, and is consistent with a physical association.

**TABLE 2**

**Viability of cultures overexpressing *SIR* genes**

| Plasmid | *SIR* gene overexpressed | Relative viability |
|---|---|---|
| YEp51/YEp54 | None | 1.1 |
| pAR14/YEp54 | *SIR2* | 0.07 |
| pAR82/YEp51 | *SIR3* | 0.067 |
| pSIR4.7/YEp54 | *SIR4* | 0.78 |
| pAR14/pAR82 | *SIR2/SIR3* | 0.00028 |
| pAR14/pSIR4.7 | *SIR2/SIR4* | 0.048 |
| pAR82/pSIR4.7 | *SIR3/SIR4* | 0.14 |

Cultures of strain Y2155 carrying the indicated plasmids were grown at 30° to log phase in raffinose medium lacking leucine and tryptophan, when the cultures were divided and galactose was added to half to a concentration of 2%. Cultures were incubated for an additional 24 hr. The number of viable, plasmid-bearing cells was determined at this time by plating appropriate culture aliquots on selective glucose media. Relative viability represents the ratio of the number of viable, plasmid-bearing cells in the induced culture at the end of 24 hr to that in the uninduced culture at the end of 24 hr.

We further explored the functional interactions among the Sir proteins by overexpressing specific *SIR* genes in backgrounds containing *SIR* gene deletions. If the Sir proteins exert toxic effects by acting as a complex, then the absence of one of the *SIR* genes might abrogate the growth defect resulting from overexpression. However, if Sir2p or Sir3p act alone to induce toxicity, the absence of the other *SIR* genes would not affect the phenotype. Accordingly, we overexpressed *SIR2* or *SIR3* in strains lacking the *SIR2* gene. As shown in Figure 2A, we found that the toxic effects

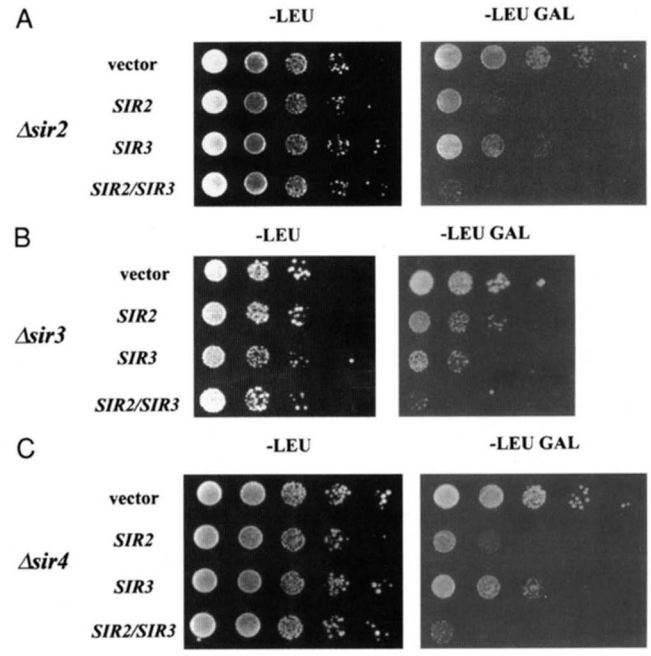


FIGURE 2.—*SIR* genes cooperate to cause toxic effects. The effects of *SIR* gene overexpression was assayed in strains lacking individual *SIR* genes. *SIR* gene deletions were created in strain Y2155, which was then transformed with a set of plasmids containing galactose-inducible *SIR* genes. Serial dilution analysis was performed as described in the legend to Figure 1. (A) Δ*sir2* strain. (B) Δ*sir3* strain. (C) Δ*sir4* strain.

of *SIR3* overexpression were diminished in this strain, suggesting that Sir2p is required to manifest *SIR3*'s overexpression phenotype. Similarly, we assayed Sir-induced toxicity in a strain deleted for the *SIR3* gene (Figure 2B). In this strain *SIR2*'s effects were nearly absent, indicating that *SIR3* is required for *SIR2* toxicity. Finally, the effects of *SIR2* or *SIR3* overexpression were assayed in a strain deleted for the *SIR4* gene (Figure 2C). *SIR3*-induced toxicity was reduced in this strain, suggesting that in addition to *SIR2, SIR3* requires *SIR4* to exert a growth defect. The toxic effect of *SIR2* overexpression was only slightly diminished in this strain, indicating that Sir2p is less dependent on *SIR4*. *SIR4* overexpression was not toxic in any of these backgrounds, indicating that a potential for *SIR4*-induced growth defects is not held in check by the presence of the other Sir proteins (not shown).

**Sir2p and Sir3p are physically associated *in vivo*:** Our functional assays are consistent with physical binding studies that indicate that *SIR3* and *SIR4* interact with each other, and suggest a previously uncharacterized interaction between *SIR2* and *SIR3*. To examine this interaction further we determined whether Sir2p and Sir3p are physically associated *in vivo*. Extracts of strains containing high levels of Sir2p, Sir3p, or both were incubated with antibodies directed against Sir2p or Sir3p. The immunoprecipitates were fractionated by SDS-PAGE, transferred to nitrocellulose, and then probed with a mixture of Sir2p and Sir3p antibodies. The results of this experiment are shown in Figure 3. Anti-Sir2p antibody did not immunoprecipitate Sir3p from extracts of a strain expressing Sir3p alone. Similarly, anti-Sir3p antibody failed to immunoprecipitate significant amounts of Sir2p from a strain expressing high levels of Sir2p alone. In contrast, each of the antibodies precipitates significant amounts of both proteins from an extract of a strain containing high levels of both. Thus, Sir2p and Sir3p form a complex when coexpressed, indicating that the two proteins are physically associated *in vivo*.

**Mutational alterations of histone H4 enhance *SIR2*- and *SIR3*-induced lethality:** Silencing likely involves an interaction between the Sir proteins and histones. To investigate Sir-histone interactions we examined the consequences of overexpressing *SIR2* or *SIR3* in backgrounds containing mutant forms of histone H4. Mutations in histone H4 that lead to a silencing defect map to the N-terminus of the protein (KAYNE *et al.* 1988; MEGEE *et al.* 1990; PARK and SZOSTAK 1990); this region of the protein is also required for an *in vitro* interaction with *SIR3* and *SIR4* (HECHT *et al.* 1995). We constructed strains carrying different deletion alleles of *HHF2* as well as either the high expression *SIR2* or *SIR3* plasmids. Three histone H4 deletion alleles were assayed, differing in the extent of the N-terminal deletion they contain. Wild-type cells containing the smaller Δ4-14 deletion do not exhibit a defect in silencing; however,

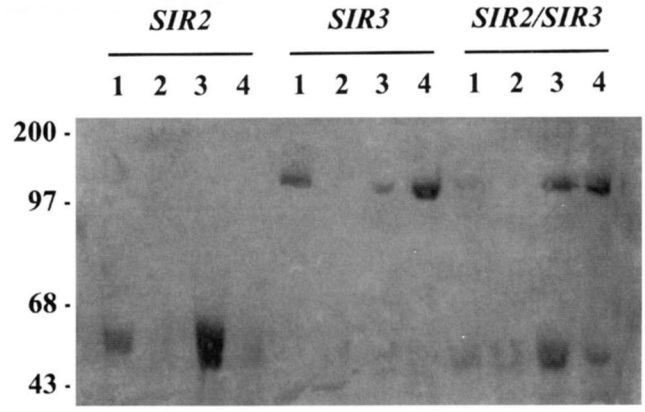


FIGURE 3.—Sir2p and Sir3p co-immunoprecipitate. Cultures of strain BJ2169 carrying pAR14 (*GAL-SIR2*), pAR82 (*GAL-SIR3*) or both plasmids were grown in SC raffinose medium to log phase then induced by the addition of galactose to 2%. Extracts of the induced strains were prepared as described in MATERIALS AND METHODS and 0.5-ml aliquots were immunoprecipitated with anti-Sir2p serum, anti-Sir3p serum, or no primary antibody. Immunoprecipitates and samples of the total cell extracts were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with a mixture of anti-Sir2p and anti-Sir3p sera. The filter was developed using an alkaline phosphatase-conjugated secondary antibody and photographed after staining for alkaline phosphatase activity. The label above each set of five lanes indicates the *SIR* gene overexpressed in those strains. Lane 1, whole cell extract; lane 2, no primary antibody; lane 3, immunoprecipitated with anti-Sir2p sera; lane 4, immunoprecipitated with anti-Sir3p sera.

strains with the Δ4-20 or Δ4-23 alleles are defective for repression (KAYNE *et al.* 1988). We examined the effects of overexpressing the *SIR2* gene in combination with these alleles (Table 3). Although Sir2p does not bind to this region of histone H4 *in vitro* (HECHT *et al.* 1995), we have previously shown that high levels of Sir2p lead to a decrease of acetylation on the four lysine residues in the N-terminus of H4 (BRAUNSTEIN *et al.* 1993). *SIR2* overexpression in strains carrying each of the *HHF2* deletion alleles showed essentially the same small but significant decrease in viability, compared to that for *SIR2* overexpression in strains carrying the wild-type *HHF2* allele.

We also examined the effects of overexpressing *SIR3* in the *HHF2* mutant backgrounds. Overexpression of *SIR3* in a background containing the Δ4-14 allele, which deletes a region that is not required for binding of Sir3p to H4 *in vitro* (HECHT *et al.* 1995), resulted in a substantial decrease in viability compared to that from overexpression of *SIR3* in the context of a wild-type H4 allele (Table 3). More extensive deletions in H4, which would be predicted not to bind Sir3p based on *in vitro* studies, suppress the increased lethality, producing a level of toxicity equivalent to a wild-type histone background. These results show that *SIR2* and *SIR3* genetically interact with histone H4 and highlight a specific interaction with *SIR3* and regions in the N-terminal tail.

Although no other function besides silencing has

**TABLE 3**

**Viability of histone deletion strains overexpressing *SIR* genes**

| *SIR* gene overexpressed | Relative viability in the presence of the *HHF2* allele | | | |
|---|---|---|---|---|
| | Wild type | Δ4-14 | Δ4-19 | Δ4-23 |
| None | 0.96 | 1.2 | 0.98 | 1.0 |
| *SIR2* | 0.062 | 0.0011 | 0.0012 | 0.00032 |
| *SIR3* | 0.091 | 0.000079 | 0.039 | 0.045 |

Strain Y1191 and its derivatives carry ARS/CEN plasmids bearing *HHF2* alleles as their sole source of histone H4. The effect of *SIR2* and *SIR3* overexpression in these backgrounds were measured by determining the relative viability, as described in the legend to Table 2.

been established for the *SIR* genes, the overexpression effects we observed might have been due to an increase in an activity that was unrelated to silencing. To examine this possibility we tested the effects of overexpressing the *SIR3-R3* allele. This allele was isolated in a screen for suppressors of a histone H4 N-terminal point mutation that was defective in silencing (JOHNSON *et al.* 1990). These suppressors were not allele specific, and their mutations did not map to the region of *SIR3* that is required for an *in vitro* association with H4 (HECHT *et al.* 1995). An independent screen for increased silencing at telomeres in the background of a hypomorphic mutation in the *RAP1* gene also yielded the *SIR3-R3* allele (LIU and LUSTIG 1996). Therefore, the *SIR3-R3* allele likely encodes a form of Sir3p that promotes more efficient silencing. If the toxic effects of *SIR3* overexpression were due to a function related to silencing, then overexpression of the *SIR3-R3* allele might be particularly toxic. To test this prediction we introduced the *SIR3-R3* mutation into our *SIR3* overexpressing plasmid and compared the effects of overexpressing the *SIR3-R3* to that of overexpressing wild-type *SIR3*. The results of this experiment are presented in Figure 4. Overexpression of the *SIR3-R3* allele was clearly more toxic than overexpression of the *SIR3* wild-type allele, suggesting that the effects of overexpression are related to Sir3p's role in silencing, and not to a novel or uncharacterized function.

***SIR* overexpression and transcription:** A straightforward hypothesis for Sir-induced lethality is that the *SIR* proteins are no longer constrained to establish silencing at telomeres and the silent-mating type loci, but instead cause a decrease in transcription throughout the genome and repress expression of essential genes. To investigate this possibility we examined the influence of *SIR* overexpression on the steady-state levels of a variety of mRNAs. We grew cultures to log phase, induced the *SIR* genes, then prepared RNA from induced and uninduced cultures 4 hr later. At this time cells carrying the *SIR3* and *SIR2/SIR3* overexpressing plasmids had substantially reduced plating efficiency, while cells carrying the vector control, *SIR2*, or *SIR4* overexpressing plasmids exhibited no decrease in plating efficiency. In inducing or noninducing conditions an equal number of cells yielded an equivalent amount of RNA. Therefore, Sir protein overexpression did not globally affect steady-state levels of RNA or cell integrity. We examined the effects of *SIR* gene overexpression on the levels of several specific messages by Northern analysis (Figure 5 and data not shown). We observed no effect of *SIR* overexpression on the levels of *ACT1*, *GAL1*, or *TRP1* mRNA, or on 18S rRNA. This indicates that *SIR* overexpression is not inducing a general shut down of polI or polII transcription.

RENAULD *et al.* (1993) have shown that *SIR3* overexpression causes an increase in spreading of telomere position effect. Therefore, *SIR* overexpression might not establish silencing in new locations, but rather extend silencing from the known foci of establishment into contiguous essential genes. To test if silencing nucleated at *HML* or *HMR* were spreading into essential genes, we overexpressed *SIR* genes in a strain that

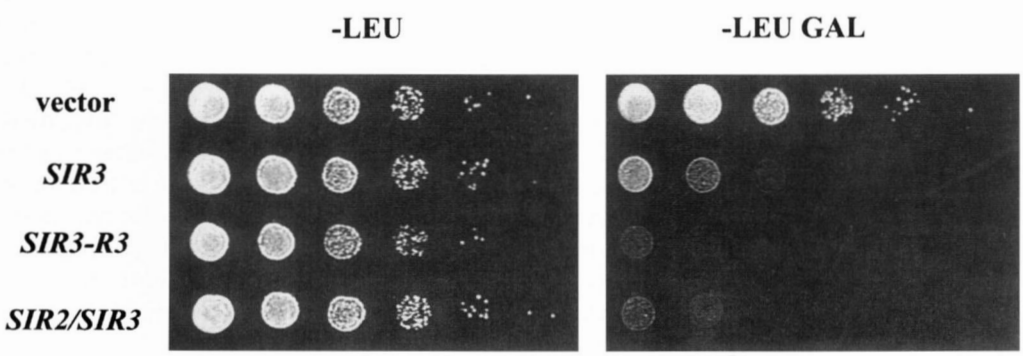


FIGURE 4.—Increased lethality is induced by overexpression of the *SIR3-R3* allele. A plasmid containing the *SIR3-R3* allele under control of the galactose promoter was introduced into strain Y2155 and tested for galactose-induced effects. Serial dilution analysis was performed as described in the legend to Figure 1.

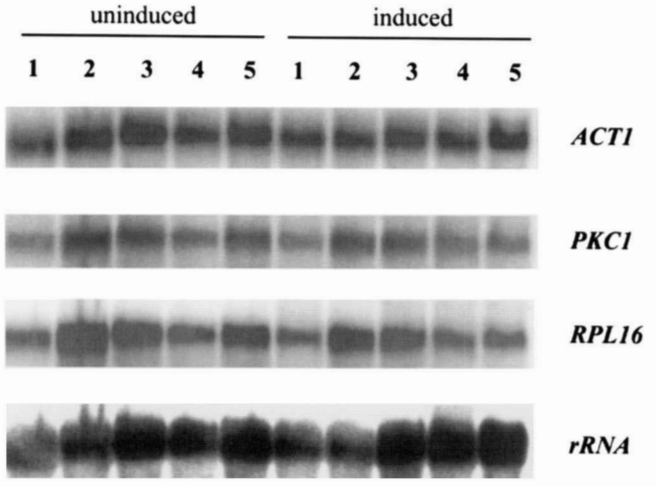


FIGURE 5.—Transcription in strains overexpressing *SIR* genes. Strain Y2155 containing *LEU2*-marked *SIR* overexpressing plasmids was grown to log phase in raffinose medium lacking leucine. The culture was divided, and galactose was added to half to a final concentration of 2%. Following 4 hr of galactose induction, growth was suspended and RNA collected from the cells, fractionated on formaldehyde/agarose gels, blotted to nitrocellulose, and probed sequentially with the indicated gene fragments. Lane 1, YEp51 (vector); lane 2, pAR14 (*SIR2*); lane 3, pAR16 (*SIR3*); lane 4, pSIR4.7 (*SIR4*); lane 5, pSAS2 (*SIR2* and *SIR3*).

lacked *HML* and *HMR*. These cells remained sensitive to Sir-induced toxicity (not shown). We also tested the possibility that high levels of *SIR* protein could extend telomere position effect into essential genes. RNA levels of *PKC1* and *SUC2*, genes tightly linked to the ends of their chromosomes, were not influenced by *SIR* overexpression (Figure 5 and data not shown). Therefore, we consider the possibility unlikely that chromosomal genes become subject to telomere position effect as a result of *SIR* overexpression.

Finally, we examined the expression of *RPL16*, a gene that is positively regulated by binding of the Rap1 protein in wild-type cells. Since Sir3p and Sir4p interact with Rap1p, *SIR* gene overexpression might exert a specific effect on genes regulated by Rap1p. In this case, we observed a twofold reduction in RNA levels regardless of the *SIR* gene overexpressed. Since the same effect is observed in the *SIR4* overexpressing strain, we conclude that this difference is not responsible for the decrease in viability observed when *SIR2* or *SIR3* is overexpressed. Therefore, we find no evidence for the proposal that a reduction in transcription is responsible for the toxic effects of *SIR* gene overexpression.

***SIR* overexpression decreases chromosome stability:** Sir proteins most likely manifest their effects through an alteration of chromatin structure. Failing to observe an effect of *SIR* overexpression on transcription, we examined the effects of *SIR* overexpression on other fundamental processes of chromosome dynamics that are influenced by chromatin. To investigate the effect of high levels of Sir proteins on the mitotic stability of chromosomes, we introduced our plasmids into a haploid yeast strain bearing a marked, nonessential chromosome fragment. This strain background was also highly sensitive to *SIR* gene overexpression, with *SIR3* expression in this case showing increased toxicity compared to *SIR2*. We measured the stability of the nonessential chromosome by fluctuation analysis in inducing and noninducing conditions. These data are shown in Table 4. We observed a profound effect on the mitotic stability of the test chromosome, observing loss rates of 15–30% in *SIR2* or *SIR3* overexpressing strains, and a loss rate of 90% in the strain expressing both *SIR2* and *SIR3*.

**TABLE 4**

***SIR* gene overexpression increases chromosome loss**

| *SIR* gene overexpressed | Loss rate | | |
|---|---|---|---|
| | 125-kb linear | 73-kb circular | Chromosome *V* |
| None | 0.022 | $3.0 \times 10^{-3}$ | $3.0 \times 10^{-5}$ |
| *SIR2* | 0.12 | ND | $3.9 \times 10^{-4}$ |
| *SIR3* | 0.31 | $1.1 \times 10^{-2}$ | $3.8 \times 10^{-3}$ |
| *SIR4* | 0.016 | ND | $1.3 \times 10^{-5}$ |
| *SIR2/SIR3* | 0.92 | ND | $5.2 \times 10^{-3}$ |

Strain YPH1015 contains a nonessential chromosome fragment bearing the *HIS3* marker. *SIR* overexpressing plasmids were transformed into strain YPH1015, grown to log phase in raffinose medium lacking leucine, then divided in two, when half was induced by the addition of galactose to 2%. The number of plasmid-bearing cells that were $His^+$ was determined at the time of induction and at later times; chromosome loss rates were determined by fluctuation analysis (see MATERIALS AND METHODS). ND, not determined.

To determine if this loss of stability extended to authentic yeast chromosomes, we measured the loss rate of a marked chromosome *V* (HARTWELL and SMITH 1985) in diploid strain Y2279. In this strain *SIR3*-induced toxicity was substantially greater than that for cells overexpressing *SIR2*. Once again we observed a significant decrease in the stability of the test chromosome. The *SIR* genes mediate telomere position effect (APARICIO *et al.* 1991), and the Sir3p and Sir4p proteins may localize to the ends of chromosomes (GOLTA *et al.* 1996). As alterations in telomere metabolism are known to affect chromosome stability in yeast, it is possible that the decrease in chromosome stability we observed was due to aberrant telomere function. To examine this possibility we measured the stability of a circular chromosome derivative in a strain overexpressing *SIR3*. Once again, we observed an increase in the loss rate of the test chromosome, indicating that the effects of *SIR* overexpression are not solely due to an affect on telomeres. The increase in chromosome loss is correlated with the reduction in plating efficiency in each case, and this reduction in mitotic stability is likely to be sufficient to account for the lethal effects of *SIR* overexpression.

Eukaryotic cells have checkpoints that monitor the integrity of chromosomes and their fitness for segregation (HARTWELL and WEINERT 1989; MURRAY 1992). Given the high rates of chromosome loss we investigated whether *SIR* overexpression leads to a delay or lethality at a particular point of the cell cycle, which would indicate the activation of a checkpoint mechanism. To look for activation of a checkpoint, we grew cultures to log phase, induced high expression of the *SIR* genes, then monitored cell morphology in the cultures over a 24-hr period. For each gene tested, the proportion of cells in each phase of the cell cycle was not changed in the induced culture when compared to uninduced controls (not shown). This suggests that the defect in chromosome stability does not activate a checkpoint mechanism.

## DISCUSSION

**Protein-protein interactions and silencing:** The interactions among the various Sir proteins, silencer binding factors, and histones have been explored extensively by genetic and biochemical means. Recessive alleles of the *SIR3* or *SIR4* genes show unlinked noncomplementation with recessive alleles of *SIR1* and *SIR2* (RINE and HERSKOWITZ 1987). We previously showed that high expression of *SIR3* can suppress the disruption of silencing caused by overexpressing the *SIR4* gene (MARSHALL *et al.* 1987), which suggested an interaction between the two proteins that has been confirmed by assay in the two hybrid system (MORETTI *et al.* 1994). The two-hybrid assay also demonstrated that Sir3p and Sir4p can associate with the Rap1 protein (MORETTI *et al.* 1994). Alleles of *SIR3* can suppress silencing defects caused by mutations in the *RAP1* and histone H4 gene (JOHNSON *et al.* 1990; LIU and LUSTIG 1996), while recombinant Sir3p and Sir4p can bind to the N-terminal tails of histones H3 and H4 *in vitro* (HECHT *et al.* 1995). Sir2p and Sir3p bind to an affinity column containing the C-terminal half of Sir4p (MOAZED and JOHNSON 1996). Finally, increasing the copy number of the *SIR1* gene can suppress a variety of silencing defects, including temperature-sensitive alleles of the *SIR3* and *SIR4* genes (STONE *et al.* 1991), while Sir1p exhibits a two-hybrid interaction with Orc1p, a subunit of a silencer-binding complex of proteins (ORC) that also binds yeast ARS elements (TRIOLO and STERNGLANZ 1996).

We have investigated the functions of the Sir proteins by determining the consequences of overexpressing them in yeast cells. We report here that overexpression of *SIR2* or *SIR3* is toxic to yeast cells. The identification of specific growth phenotypes as a consequence of overexpressing the *SIR2* or *SIR3* gene has allowed us to describe a set of genetic interactions linking the Sir2, Sir3, and Sir4 proteins and histone H4. Our results complement and extend the previously identified genetic and physical interactions among the *SIR* genes and histones.

First, we observed that *SIR3*-induced toxicity is suppressed by deleting the *SIR4* gene. These results are consistent with the observation that the two proteins physically interact, and suggest that *SIR3*'s function is dependent on the *SIR4* protein. In contrast, the toxic effects of *SIR2* overexpression were not affected to the same degree in the *Dsir4* strain, indicating that *SIR2*'s function is less dependent on Sir4p.

Our experiments revealed a previously uncharacterized interaction between *SIR2* and *SIR3*. First, the two proteins act in synergy to promote inviability; second, the toxic effects induced by expressing *SIR2* or *SIR3* individually are dependent on the presence of the other protein. Finally, we have shown that the two proteins can be co-immunoprecipitated. Although these experiments do not indicate whether *SIR2* and *SIR3* associate with each other directly, they provide a framework for bringing each of the Sir proteins to the silent mating type loci.

Sir2p and Sir3p show an interesting interaction with the histone H4 N-terminus. High levels of Sir3p are synthetically lethal with the Δ4-14 H4 allele. Since this deletion does not interfere with the *in vitro* interaction of Sir3p with H4, one interpretation of this result is that this deletion increases the ability of Sir3p to bind H4. This model is consistent with *in vitro* experiments: binding of Sir3p is inhibited *in vitro* by specific point mutations at position 16; the ability of Sir3p to bind H4 containing this point mutation *in vitro* is restored by deleting positions 4–14 (HECHT *et al.* 1995).

Based on *in vitro* binding studies, Sir3p would not be expected to bind H4 containing the Δ4-19 or Δ4-23 deletions. Consistent with this, the synthetic lethal phenotype caused by the Δ4-14 mutation is suppressed by the additional deletions; in these backgrounds, *SIR3* overexpression is no more toxic than in wild-type cells. This suggests that the distal portion of the histone H4 N-terminus may restrict the binding of proteins such as *SIR3* and *SIR4* and strengthens the model that silencing involves a specific interaction between and *SIR3* and histone H4.

High levels of Sir2p also exhibit a synthetic phenotype with deletions in the H4 N-terminus, but in this case the interactions are not specific to a single H4 allele. Sir2p fails to bind this region of H4 *in vitro*. This suggests that *SIR2* interacts with histones in a less direct manner. Similar to the interaction between Sir3p and the H4 Δ4-14 allele, overexpression of both *SIR2* and *SIR3* leads to a synthetic lethality. One model to account for this relationship is that *SIR2* functions to increase the accessibility of the histone H4 N-terminal tail to *SIR3*, and thus mimics the effect of the shorter (Δ4-14) H4 N-terminal deletion.

How might Sir2p act to increase access to histones? One possibility is that Sir2p affects the acetylation levels of histone H4 and the other histone proteins. We have previously shown that overexpression of the *SIR2* gene

leads to a global decrease in the acetylation of histone molecules in the cell, and that the silent mating type loci are bound by chromatin that is underacetylated compared to the rest of the genome. Consistent with this model, we have shown that *SIR3* overexpression, either alone or in combination with *SIR2*, had no effect on histone acetylation (BRAUNSTEIN *et al.* 1993). However, as we have shown here, overexpressing *SIR3* in addition to *SIR2* has a profound effect on cell viability.

There are several ways in which a change in acetylation may be involved in silencing. First, Sir2p may induce an decrease in acetylation that is sufficient to induce transcriptional repression; the Sir3 and Sir4 proteins may then be involved in preserving the unacetylated state. Second, a lack of acetylation due to Sir2p activity may increase the accessibility of histone tails to Sir3p and Sir4p, and these proteins may induce the formation of a repressive complex. A change in the acetylation state of a nucleosome has been shown to alter the binding of a protein factor to DNA *in vitro* (LEE *et al.* 1993). This model would predict that highly acetylated histones may have reduced affinity for interactions with Sir3p and Sir4p. The *in vitro* experiments in which Sir3p and Sir4p were shown to bind the N-terminal tails of histone H3 and H4 were performed on unacetylated histones (HECHT *et al.* 1995); it is not known what the influence of acetylation is on this *in vitro* interaction. A final model would propose that Sir2p may increase access of histones to Sir3p by an unknown mechanism, and a lack of acetylation may be a consequence of silencing, rather than a cause.

**The nature of the *SIR*-induced toxic effect:** We find no evidence to support the hypothesis that *SIR* overexpression leads to widespread transcriptional silencing. First, *SIR2* or *SIR3* overexpression remains toxic in strains lacking *SIR4*, or in strains in which the histone H4 N-terminal domain is absent, backgrounds that do not support repression at the silent mating type loci or telomeres. Second, the steady-state levels of a variety of transcripts is unchanged upon induction of high levels of the *SIR* proteins. This is consistent with the high specificity of silencing in wild-type cells, and suggests that the silencer sequences provide strict controls on the choice of where to nucleate silencing.

We find a large decrease in chromosome stability in strains overexpressing *SIR2* and/or *SIR3*. The magnitude of this decrease is likely to account for the toxic phenotype we have described. Several models could account for a *SIR* induced loss of chromosome stability. Specific mutations of the acetylatable lysines in histone H4 N-terminus lead to a delay in mitosis and an increase in chromosome loss. Viability is decreased in these strains when a mutation in the *RAD9* gene is introduced, suggesting the cells have incurred DNA damage or have a defect in DNA replication (MEGEE *et al.* 1995). This defect in genome integrity was shown to be due to an alteration in the acetylation status of histone H4, and indicated the need for a dynamic equilibrium in the acetylation status of the lysine residues. We have shown that *SIR* overexpression alters the acetylation of histones and might be predicted to lead to similar defects in genome integrity. Additional processes that may be sensitive to alterations in chromatin would include kinetochore function. The formation of an inappropriate chromatin structure across centromeres could impair chromosome segregation and lead to nondisjunction. A third possibility would involve telomere metabolism. Sir3p and Sir4p likely interact with telomeres through their association with Rap1p, which has multiple binding sites on each telomere. Deletions of *SIR2* or *SIR3* delocalize Rap1p in the nucleus and lead to small decreases in chromosome stability and telomere length (PALLADINO *et al.* 1993). Finally, Sir3p has similarity to Orc1p, part of the origin recognition complex (ORC) that is essential for DNA replication in yeast cells. It has been proposed that Sir3p may substitute for Orc1p in ORC specifically at silencers (BELL *et al.* 1995). At artificially high levels Sir3p may compete Orc1p out of ORC and interfere with the initiation of DNA replication.

High levels of Sir2p and Sir3p induce a toxic effect without inducing transcriptional silencing. In addition, these effects are observed in strains deleted for the *SIR4* gene. This suggests that the Sir proteins may not function as a complex to accomplish a single task, but have independent functions of which silencing is the cumulative result. Consistent with this, *SIR2* and *SIR3* overexpression is more pronounced in combination, but some toxicity remains in the absence of the other. In addition, the *SIR2* gene has been shown to participate in suppressing recombination at the rDNA repeats, a function that does not involve any of the other *SIR* genes (GOTTLIEB and ESPOSITO 1989). Our results are consistent with a model in which the Sir proteins have independent activities yet cooperate in a complex to modify chromatin structure.

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