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; Laurentson 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...
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 Rap1-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, immuno-fluorescence 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 pH20.1 and pKAN59, respectively (Ivy et al. 1986). A SacI linker was inserted into the Acl site of plasmid pH20.1, which lies 45 bp upstream of the initial ATG codon of SIR2. The 3.5-kb SacI-HindIII fragments from the resulting plasmid was inserted into either YEp51, forming pPAR14, or YEp54, forming pPAR44. The 3.7-kb HpaI fragment from pKAN63 was ligated into the Smal site of pUC12. This plasmid (pPAR3) was linearized with Sall, treated with exonuclease BAL31, and recloned in the presence of Sall linkers. The 3.5-kb Salt-BamHI fragment from one of the recovered plasmids, in which the Sall site is 7 bp upstream of the initial ATG codon of the SIR2 open reading frame, was inserted into YEp51 and YEp54 to create pPAR16 and pPAR2, respectively. The GAL10::SIR4 plasmid, pSIR4.7, was derived by insertion of a KpnI-BamHI fragment spanning the 2-μ site 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 EcoRV fragment from pAR89, containing the GAL10::SIR3 fusion, and subcloning it into pPAR14, cut with HindIII and made blunt with Klenow enzyme. pYML2 was made by first subcloning a CiaI-KpnI fragment containing the SIR3-R3 mutation from pLJ90 (Johnson et al. 1990) into pPAR2, forming pYML1. A Salt-BamHI fragment containing the SIR3-R3 mutation was then isolated from pYML1 and subcloned into pPAR3 cut with Salt and BamHI, forming pYML2. pYML2 is identical to pPAR16, except for the SIR3-R3 mutation. pSH105, used to delete the SIR3 gene, was made by replacing the large EcoRI fragment within the SIR3 open reading frame with a KpnI 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 sir2::URA3 deletion. Plasmid pPAR9 (Marshall et al. 1987) was used to make the sir2::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 pMH120 (Ycp TRP1-HHF2) and a Ura− segregant of one such transformant was recovered. This segregant was transformed to Ura+ with plasmids pPK617, pPK618, or pPK610, bearing Hhf2 alleles Δ4-4, Δ4-20, and Δ4-23, respectively (Kayne et al. 1986). Trp- segregants of selected transformants were recovered to yield strains Y1186, Y1181, and Y1176, respectively. Strain Y1191 harboring plasmids pDD152 or pDD153 was transformed by tri-parental mating to Ura+ 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 B2769. Cultures of this strain harboring the indicated plasmid or plasmids were grown at 30° in SC raffinose medium to a density of 10⁶ 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 μg/ml extract) at 0° for 3 hr. Formaldehyde-fixed Staph A cells (ImmunoPrecip, BRL, 30 μl/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. Heiter) contains a HIs3-marked 125-kb linear chromosome fragment derived from chromosome III (Shero et al. 1991). A Leu2-marked 75-kb circular derivative of chromosome III was introduced as described (Runge and Zarian 1995) 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-
mained 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°C. 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 II 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 varied 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 kom3 mutation. Recombination was not significantly 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 10^6–10^7 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 genes, and then plated cells containing the SIR genes with the SIR4 plasmid and the SIR4 plasmid.
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.

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

<table>
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<tr>
<th>Plasmid</th>
<th>SIR gene overexpressed</th>
<th>Relative viability</th>
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<tbody>
<tr>
<td>YEp51/YEp54</td>
<td>None</td>
<td>1.1</td>
</tr>
<tr>
<td>pAR14/YEp54</td>
<td>SIR2</td>
<td>0.07</td>
</tr>
<tr>
<td>pAR82/YEp51</td>
<td>SIR3</td>
<td>0.067</td>
</tr>
<tr>
<td>pSIR4.7/YEp54</td>
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<tr>
<td>pAR14/pAR82</td>
<td>SIR2/SIR3</td>
<td>0.00028</td>
</tr>
<tr>
<td>pAR14/pSIR4.7</td>
<td>SIR2/SIR4</td>
<td>0.048</td>
</tr>
<tr>
<td>pAR82/pSIR4.7</td>
<td>SIR3/SIR4</td>
<td>0.14</td>
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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.

**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°.

**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) Δsir2 strain. (B) Δsir3 strain. (C) Δsir4 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, 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
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 pol1 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......
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 PKCl 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.

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 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 effect 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 Rap1p 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 Stern-glantz 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-terminal may restrict the binding of proteins such as Sir3p and Sir4p 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 underacetylated 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 (MEGE 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 Raplp, which has multiple binding sites on each telomere. Deletions of SIR2 or SIR3 delocalize Raplp 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.

**LITERATURE CITED**


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