Silencing of genes at nontelomeric sites in yeast is controlled by sequestration of silencing factors at telomeres by Rap1 protein

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Rap1p binds to silencer elements and telomeric repeats in yeast, where it appears to initiate silencing by recruiting Sir3p and Sir4p to the chromosome through interactions with its carboxy-terminal domain. Sir3p and Sir4p interact in vitro with histones H3 and H4 and are likely to be structural components of silent chromatin. We show that targeting of these Sir proteins to the chromosome is sufficient to initiate stable silencing either at a silent mating-type locus lacking a functional silencer element or at a telomere in a strain in which the Rap1p carboxy-terminal silencing domain has been deleted. Silencing by Sir protein targeting can also be initiated at a telomere-proximal site (*ADH4*), but is much weaker at an internal chromosomal locus (*LYS2*). Strikingly, deletion of the Rap1p silencing domain, which abolishes telomeric silencing, improves targeted silencing at *LYS2* by both Sir3p and Sir4p, while weakening the silencing activity of these proteins at or near a telomere. This effect may result from the release of Sir proteins from the telomeres, thus increasing their effective concentration at other chromosomal sites. We suggest that telomeres and Rap1p serve a regulatory role in sequestering Sir proteins at telomeres, controlling silencing at other loci in *trans* and preventing indiscriminate gene silencing throughout the genome.

[Key Words: Sir proteins; mating type; silencing; Rap1; telomere position effect]

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The yeast *Saccharomyces cerevisiae* uses a form of position-effect regulation, commonly referred to as transcriptional silencing (Brand et al. 1985), to control the expression of genes that determine mating type (for review, see Laurenson and Rine 1992). A very similar but less stable form of repression is also observed when genes are placed near telomeres in yeast (Gottschling et al. 1990; Aparicio et al. 1991), suggesting that silencing may play a more general role in regulating gene expression in this organism (for review, see Shore 1995). Many of the properties of silencing in yeast are reminiscent of position-effect variegation in *Drosophila*, and recent molecular studies (Hecht et al. 1995) suggest that silencing in yeast may be analogous to heterochromatin formation in more complex eukaryotes.

A number of *cis*- and *trans*-acting elements involved in silencing have now been identified and characterized in some detail. The silencer elements that flank the two silent mating-type loci (*HML* and *HMR*) all contain an autonomously replicating sequence (ARS) consensus se-

quence (ACS, or A site), which is bound by the origin recognition complex (ORC) (Bell and Stillman 1992; Difley and Cocker 1992). In addition, each silencer contains binding sites for Rap1p (the E site), Abf1p (the B site), or both (Brand et al. 1987; Shore and Nasmyth 1987; Shore et al. 1987; Buchman et al. 1988; Kimmerly et al. 1988; Mahoney et al. 1991; Boscheron et al. 1996). On the other hand, telomeres contain multiple Rap1p-binding sites within the terminal poly(C₁₋₃A) tracts (Longtine et al. 1989; Gilson et al. 1993) that play a role in telomeric silencing (Kyrion et al. 1993; Buck and Shore 1995). Telomere-associated repeat sequences also contain ACS elements and Abf1p-binding sites (Biswas and Biswas 1990; Louis et al. 1994), but their role in telomeric silencing, if any, is unclear. Genetic studies have demonstrated that individual ORC subunits (Bell et al. 1993, 1995; Foss et al. 1993; Micklem et al. 1993) and Abf1p (Loo et al. 1995) do play a role in silencing at *HM* mating-type loci, whereas Rap1p functions in silencing at both *HM* loci and telomeres (Kurtz and Shore 1991; Sussel and Shore 1991; Kyrion et al. 1993; Moretti et al. 1994).

In addition to the genes encoding silencer- and telomere-binding proteins, three silent information regulator genes (*SIR2*, *SIR3*, and *SIR4*) are required for silencing at both *HM* loci and telomeres (Rine and Herskowitz

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1987; Aparicio et al. 1991). A fourth *SIR* gene, *SIR1*, is required for stable silencing at *HM* loci (Pillus and Rine 1989; Stone et al. 1991), but plays no role at telomeres where position-effect repression is unstable or variegated (Aparicio et al. 1991). Sir1p appears to act upstream of the other Sir proteins at *HM* silencers, through interactions with ORC, to ensure that silencing is reestablished efficiently after each cell division (Chien et al. 1993). Both Sir3p and Sir4p interact with Rap1p in the two-hybrid system (Moretti et al. 1994), suggesting a model in which silencing is initiated by the recruitment of a complex of these Sir proteins to the chromosome by Rap1p. This complex may polymerize along the chromatin fiber through a network of Sir-Sir interactions (Chien et al. 1991; Moretti et al. 1994), leading to the formation of a heterochromatin-like structure through direct interactions of Sir3p and Sir4p with the basic amino-terminal tails of histones H3 and H4 (Hecht et al. 1995).

One prediction of this model for the initiation of silencing is that direct targeting of either Sir3p or Sir4p to the chromosome might be sufficient to bypass the requirement for Rap1p and other silencer-binding proteins and establish repression. However, previously we found that Gal4p DNA-binding domain (Gbd) hybrids with either Sir2p, Sir3p, or Sir4p failed to establish significant repression at an *HMR* locus containing Gal4p DNA-binding sites, whereas a Gbd-Sir1p hybrid did do so (Chien et al. 1993). We noted, although, that expression of the Gbd-Sir3p and Gbd-Sir4p hybrids had a strong derepressing effect on a normally silenced telomeric reporter gene that contained no Gal4p-binding sites. Because it is known that transcriptional silencing can be very sensitive to the dosage of *SIR* genes (Marshall et al. 1987; Sussel and Shore 1991; Renaud et al. 1993; Sussel et al. 1993), we tested the effect of the identical Gbd-Sir3p and Gbd-Sir4p fusions when expressed at a lower level, using the *RAP1* promoter instead of the strong *ADH1* promoter, and a low copy number centromere-containing plasmid. Here we show that Gbd-Sir3p and Gbd-Sir4p hybrids can act as strong initiators of silencing when targeted to the *HMR* locus or to sites at or near a telomere, even in cells containing a *RAP1* carboxy-terminal deletion that abolishes normal telomeric silencing. At an internal (nontelomeric) site, silencing was observed only with Gbd-Sir4p in wild-type (*RAP1*⁺) cells, but it was weak. Silencing by Gbd-Sir hybrids at an internal site was greatly improved, however, by deletion of the Rap1p carboxy-terminal domain. We argue that Rap1p bound at telomeres acts as a "sink" or "reservoir" for Sir proteins in the nucleus, thus preventing the inappropriate silencing of genes elsewhere in the chromosome. This model may have important implications for the effect of telomere length and telomeric silencing on gene regulation in higher eukaryotes.

Results

Gbd-Sir3p and *Gbd-Sir4p* hybrids restore repression when targeted to mutated *HMR-E* silencers

To test the ability of the new Gbd-Sir3p and Gbd-Sir4p

constructs to establish transcriptional silencing at *HMR*, we used strains containing Gal4p-binding sites (UAS_G) inserted at mutated silencers (Chien et al. 1993; Buck and Shore 1995). In the first two strains, the *HMR-E* silencer was deleted for the Rap1p-binding site (element E) and either the ORC-binding site (A) or the Abf1p-binding site (B). In the third strain, all three silencer regulatory sites (A, E, and B) are deleted and replaced by UAS_G sites. All three of these mutated silencers are completely defective in repression as judged by Northern analysis of steady-state *a1* mRNA levels (Buck and Shore 1995).

We first tested the ability of Gbd-Sir3p and Gbd-Sir4p hybrids to silence the *a1* gene normally present at the *HMR* locus in a strain in which two *HMR-E* silencer elements (A and E) were deleted and replaced by UAS_G sites. As shown in Figure 1, the expression of Gbd-Sir3p or Gbd-Sir4p resulted in a very strong reduction in steady-state *a1* mRNA with the UAS_G-containing silencer. The Gbd alone control plasmid failed to confer repression. A control strain lacking UAS_G sites at *HMR* also remained derepressed in the presence of Gbd-Sir3p or Gbd-Sir4p, demonstrating that the site of action for the hybrid proteins is the mutated silencer.

Figure 1. Targeted silencing by Gbd-Sir3 and Gbd-Sir4 hybrid proteins at the silent mating-type locus *HMR* lacking a functional silencer ($\Delta A\Delta E$). (A) Schematic representation of the targeted silencing strategy used in this study. The wild-type *HMR-E* silencer is shown in the top line. (B) The *HMR* locus contains the endogenous *a1* gene, whose steady-state expression is measured by Northern blotting. Actin message is shown as a control.

The effects of the two Gbd hybrids were also examined in strains containing a *TRP1* reporter gene in place of the normal *a1* and *a2* genes at *HMR*. In these strains repression is reflected by a reduction in the ability to form colonies on medium lacking tryptophan (Sussel and Shore 1991). Figure 2 shows the effect of G_{BD}-Sir3 and G_{BD}-Sir4 in the three different mutant silencer contexts ($\Delta A\Delta E$, $\Delta E\Delta B$, or $\Delta A\Delta E\Delta B$) with or without UAS_G sites. With both hybrids, the ability to form colonies on medium lacking tryptophan is reduced $>10^4$ -fold. Nonetheless, there is a clear difference observed between the two double mutant strains and the complete silencer deletion. In the double-mutant silencer strains (where either the *HMR-E* A or B sites remain) silencing is particularly strong, as judged by the absence, or near absence, of growth on synthetic complete (SC)-tryptophan plates even at the lowest cell dilution ($\sim 10^5$ cells per spot). In contrast, cell growth is seen up to the second dilution when either hybrid is targeted to the complete silencer deletion. The ability of the two Gbd-Sir hybrids to establish silencing is remarkably similar in these three different *HMR-E* mutant contexts. Consistent with the results from the *HMRa* strains, the Gbd control plasmid failed to establish silencing and the two Gbd-Sir hybrids require the UAS_G sites at the mutated silencers to function.

Figure 2. Targeted silencing of an *hmr::TRP1* reporter by Gbd-Sir3 and Gbd-Sir4 hybrid proteins with different *HMR-E* silencer deletions. Silencing is measured by comparing the ability of cells to grow in the absence (– TRP) and presence (+ TRP) of tryptophan. Each row consists of spots representing 5- μ l aliquots from a set of 10-fold serial dilutions of a liquid culture.

Figure 3. Gbd-Sir3- and Gbd-Sir4-mediated silencing depends on *SIR2*, *SIR3*, and *SIR4*, but not *SIR1*. The assays were performed as described in Fig. 2.

Silencing by Gbd-Sir3p and Gbd-Sir4p requires SIR2, SIR3, and SIR4 but not SIR1

To determine whether Gbd-Sir3p and Gbd-Sir4p silencing resembles normal silencing at *HMR*, their dependence on *SIR* gene function was examined in a series of derivatives of the $\Delta A\Delta E::3 \times \text{UAS}_G \text{ hmr}::\text{TRP1}$ reporter strain in which individual *SIR* genes were mutated by gene disruption. As shown in Figure 3, targeted silencing by either hybrid is unaffected by mutation of *SIR1*. In contrast, repression by Gbd-Sir3p or Gbd-Sir4p is completely lost in a *sir2* reporter strain. Likewise, silencing by Gbd-Sir3p is abolished completely by mutation of *SIR4*, as is silencing by Gbd-Sir4p in a *sir3* mutant. Silencing by Gbd-Sir3p is compromised severely by mutation of *SIR3*, as indicated by the *TRP1* reporter assay. In the *sir3* mutant, colony formation in the absence of tryptophan is reduced only by $\sim 50\%$, compared to the $>10^4$ -fold drop observed in the *SIR* wild-type strain. However, silencing by Gbd-Sir4p in a *sir4* mutant is affected much less severely, as the majority of cells in the culture ($>90\%$) still fail to form colonies on the plates lacking tryptophan, and those that do grow very slowly.

Targeting of Sir3p or Sir4p enhances telomeric silencing

To test the effect of Gbd-Sir3p and Gbd-Sir4p hybrids on telomere position effect, we used a strain (Lev8) with four UAS_G sites downstream of a *URA3* reporter gene placed near an artificial telomere created at the *ADH4* locus on the left arm of chromosome VII [Fig. 4A; Gottschling et al. 1990]. An isogenic control strain (Lev7) contained the same telomeric *URA3* gene but no UAS_G sites. Three plasmids, expressing Gbd alone, Gbd-Sir3p, or Gbd-Sir4p were transformed into these strains, and the transformants were then assayed for the ability to

A telomere VII L **B ADH4** **C LYS2**

Figure 4. Schematic representation of the different *URA3*-marked loci used in this study. (A) The structure of the chromosome VII-L telomere in strains Lev7 and Lev8, with and without *UAS_G* sites, respectively. The formation of the telomere deletes the 5' half of the *ADH4*-coding sequence. The orientation of the telomere repeat sequence is represented by successive arrowheads. (B) The structure of the *ADH4* locus in strain Lev113. The *URA3* marker is inserted downstream (centromere-proximal) of the *ADH4* gene. The physical distance from the adjacent telomere is indicated. (C) The structure of the *LYS2* locus, in strains Lev98 (4 × *UAS_G* sites) and Lev100 (4 + 2 × *UAS_G* sites) on the *top* line, and strains Lev116–Lev119 on the *bottom* line. Tel and leT refer to a 270-bp telomeric repeat (TEL270) inserted in either orientation. The *URA3* marker is inserted in the 3' half of the *LYS2*-coding sequence and is transcribed in the opposite direction. The physical distance from the adjacent telomere of chromosome II-R is indicated.

grow on plates containing 5-fluoro-orotic acid (5-FOA), which kills cells expressing the *URA3* gene, or on plates lacking uracil (Table 1).

The Gbd alone control yielded 46% FOA^R colonies (72% Ura⁺ colonies) in Lev8, and 60% FOA^R (55% Ura⁺) in Lev7, suggesting a slight derepressing effect attributable to the presence of *UAS_G* sites and the binding of Gbd. In the absence of telomeric *UAS_G* sites, the Gbd–Sir3p hybrid derepresses the telomere, as indicated by a drop to 11% FOA^R (and a rise to 75% Ura⁺ cells). In contrast, Gbd–Sir4p expression caused a slight improvement of telomeric silencing in this strain (see below). More important, in the strain that contains telomeric *UAS_G* sites, both Gbd–Sir3p and Gbd–Sir4p enhance telomeric repression of *URA3* significantly, compared with the Gbd alone control. This enhancement is partic-

ularly dramatic with Gbd–Sir4p; virtually all the cells became FOA^R. After 4 days at 30°C, only very few cells were able to grow on medium lacking uracil, forming minicolonies. With Gbd–Sir3p, the proportion of FOA^R colonies rose to 73%, almost a sevenfold improvement compared to the strain without telomeric *UAS_G* sites expressing this hybrid.

Gbd–Sir3p and Gbd–Sir4p silencing was also examined in two derivatives of the tel::4 × *UAS_G*::*URA3*::*adh4* strain in which either *SIR3* or *SIR4* were mutated by gene disruption. In *sir3* and *sir4* mutants, telomeric silencing is lost (Aparicio et al. 1991). Consistent with the results obtained at *HMR*, Gbd–Sir3p or Gbd–Sir4p failed to establish silencing in *sir4* or *sir3* mutant cells, respectively (Table 1). The situation in the corresponding *sir* mutant cells was also similar to that observed at

Table 1. *Gbd-Sir3* and *Gbd-Sir4* enhance silencing at telomere VII-L

UAS _G	SIR ⁺		Δ <i>sir3</i>		Δ <i>sir4</i>	
	0 [FOA ^R (Ura ⁺)]	4	0	4	0	4
Gbd	60 (55) ^a	46 (72) ^a	<10 ⁻⁴	<10 ⁻⁴	<10 ⁻⁴	<10 ⁻⁴
Gbd-Sir3	11 (75) ^a	73 (37) ^a	<10 ⁻⁴	<10 ⁻⁴	<10 ⁻⁴	<10 ⁻⁴
Gbd-Sir4	66 (33) ^a	100 (.3) ^{a,b}	<10 ⁻⁴	<10 ⁻⁴	.074 (100) ^a	82 (31) ^a

Gbd is expressed from pSB362, Gbd-Sir3 is from sp131, and Gbd-Sir4 is from sp138. All of the reporter strains contain the *URA3* immediately adjacent to a telomere created at the *ADH4* locus on chromosome VII-L, with or without an intervening UAS_G cassette (Gottschling et al. 1990; Chien et al. 1993). *SIR*⁺ strains are Lev7 (0 × UAS_G) and Lev8 (4 × UAS_G). Δ *sir3* strains are Lev102 (0 × UAS_G) and Lev103 (4 × UAS_G), and Δ *sir4* strains are Lev104 (0 × UAS_G) and Lev105 (4 × UAS_G).

^aThe percentage of 5-FOA^R cells and the percentage of Ura⁺ cells were calculated by averaging three samples.

^bCells grew slowly on media lacking uracil, forming minicolonies after 4 days at 30°C.

HMR. In *sir3* mutant cells, Gbd-Sir3p failed to complement the telomeric silencing defect (<10⁻⁴ FOA^R cells), even in the presence of UAS_G sites at the marked telomere. This result suggests that the Gbd-Sir3p hybrid protein may fail to function as a constituent of silent chromatin, instead acting only to initiate telomeric silencing. (This conclusion is consistent with the dominant-negative effect of Gbd-Sir3p on telomeric silencing in wild-type cells; see above.) In contrast to the results with Gbd-Sir3p in a *sir3* mutant, Gbd-Sir4p was able to complement partially the telomeric silencing defect caused by a *sir4* mutation in a strain lacking telomeric UAS_G sites (a rise from <10⁻⁴ to 0.07% FOA^R colonies, compared with 66% in a *SIR4* wild-type strain expressing Gbd-Sir4p). Furthermore, in a *sir4* mutant strain with UAS_G sites at the telomere, Gbd-Sir4p restored a high level of resistance to 5-FOA, reflecting strong repression of the telomeric *URA3* gene. These results suggest that Gbd-Sir4p retains some degree of native Sir4p function, and indicate that in strains in which Gbd-Sir4p is the only functional version of Sir4p, silencing at a telomere without UAS_G sites is limited by some defect in the action of Gbd-Sir4p at the telomere (silencer) itself.

Targeting of *Sir3p* or *Sir4p* restores telomeric silencing in *rap1* mutant cells

The results described above indicate that Gbd-Sir3p and Gbd-Sir4p hybrids, when targeted to a telomere, can significantly improve repression there. However, these experiments do not address the question of whether these hybrids are by themselves sufficient to establish repression at a telomere, where a carboxy-terminal silencing "domain" of Rap1p (amino acids 667–827) is normally required (Kyrion et al. 1993; Liu et al. 1994; Moretti et al. 1994; Buck and Shore 1995). Therefore, we tested the effect of Gbd-Sir3p and Gbd-Sir4p hybrids on telomeric repression in *rap1* mutant cells where the silencing domain of Rap1p has been deleted from amino acids 670–807 (*rap1*Δ670–807). This mutation abolishes completely transcriptional repression of a telomeric *URA3*

gene, as indicated by full sensitivity of the strain to 5-FOA (Table 2).

In these *rap1* mutant cells, and in presence of telomeric UAS_G sites, the expression of Gbd-Sir3p or Gbd-Sir4p (but not Gbd alone) restored resistance to 5-FOA and lowered the proportion of Ura⁺ cells (Table 2). However, targeted silencing induced by Gbd-Sir3p or Gbd-Sir4p at a telomere is slightly weaker in a *rap1* mutant than in a wild-type cell, as revealed by a lower proportion of FOA^R colonies. Nonetheless, targeted silencing by Gbd-Sir4p in the *rap1* mutant exceeds the level of native silencing at the same marked telomere in a *RAP1* wild-type strain (92% versus 66% FOA^R). These results indicate that targeted silencing by the Gbd-Sir hybrids is independent of a functional Rap1p silencing domain, but that these hybrids (particularly Gbd-Sir4p) can act synergistically with wild-type Rap1p to establish very stable repression at a telomere.

Action of *Gbd-Sir3p* and *Gbd-Sir4p* at two loci not normally subject to silencing

The experiments described above indicate that Gbd-Sir3p and Gbd-Sir4p can establish transcriptional silencing at *HMR* and at a telomere, two loci where silencing normally occurs in the cell. Is the targeting of Sir3p and

Table 2. *Gbd-Sir3* and *Gbd-Sir4* restore silencing at telomere in *rap1*Δ670–807 mutant cells

UAS _G	0	4
Gbd	<10 ⁻⁴	<10 ⁻⁴
Gbd-Sir3	<10 ⁻⁴	33 (66) ^a
Gbd-Sir4	<10 ⁻⁴	92 (25) ^{a,b}

The *RAP1* wild-type allele, expressed from pRAP1-Sup4-0 in Lev7 (0 × UAS_G) and Lev8 (4 × UAS_G), has been replaced by a *rap1*Δ670–807 mutant allele expressed from sp18.

^aThe percentage of 5-FOA^R cells and the percentage of Ura⁺ cells were calculated by averaging three samples.

^bCells grew slowly on media lacking uracil, forming minicolonies after 7 days at 30°C.

Sir4p sufficient to establish silencing at loci that are not normally silenced? To answer this question, we tested the hybrid proteins at two other positions in the genome, the *ADH4* locus (~20 kb from the telomere on the left arm of chromosome VII), and the *LYS2* locus (approximately in middle of chromosome II, ~360 kb from the II-R telomere).

To determine the repressive activity of Gbd-Sir3p and Gbd-Sir4p at *ADH4*, we used strain Lev113, in which a *URA3* reporter gene with four downstream UAS_G sites has been placed adjacent to the *ADH4* gene (Fig. 4B). Lev113 is fully sensitive to 5-FOA, indicating that *URA3* is expressed normally at this position, as expected (Renauld et al. 1993). This strain was then transformed with three plasmids, expressing either Gbd alone, Gbd-Sir3p, or Gbd-Sir4p, and assayed for 5-FOA resistance and growth on plates lacking uracil (Table 3). The expression of Gbd-Sir4p induced strong repression of *URA3*, as indicated by an average of 57% FOA^R in these cells. The Gbd-Sir3p hybrid caused a weaker silencing effect, as indicated by the appearance of 2.5% of FOA^R cells. The Gbd alone control failed to repress, and in an isogenic strain lacking UAS_G sites, *URA3* remained derepressed in the presence of Gbd-Sir3p or Gbd-Sir4p (data not shown).

To test the effect of Gbd-Sir3p and Gbd-Sir4p at an internal (nontelomeric) locus, we constructed strain Lev98, in which the same *URA3* reporter gene with four downstream UAS_G sites is inserted within the *LYS2* gene on chromosome II (Fig. 4C). Lev98 by itself, or transformed with a plasmid expressing Gbd alone, is fully sensitive to 5-FOA, indicating that *URA3* is expressed normally at this position (Table 3). Gbd-Sir3p failed to induce any apparent silencing on *URA3* at this position, as a transformant of Lev98 expressing this hybrid still failed to grow on plates containing 5-FOA (Table 3). However, Gbd-Sir4p caused weak repression of *URA3* in this strain, as indicated by a small proportion of FOA^R colonies (0.5%; Table 3).

Table 3. Action of Gbd-Sir3 and Gbd-Sir4 at two loci not normally subject to silencing

UAS _G	<i>ADH4</i> (4)	<i>LYS2</i>	
		4	4 + 2
Gbd	<10 ⁻⁴	<10 ⁻⁴	<10 ⁻⁴
Gbd-Sir3	2.5 (92)	<10 ⁻⁴	<10 ⁻⁴
Gbd-Sir4	57 (39) ^{a,b}	.51 (100) ^a	5.7 (95) ^a

The strain with four UAS_G sites inserted at *ADH4* is Lev113. The strains with four or a cassette of UAS_G sites inserted at *LYS2* are Lev98 and Lev100, respectively. The control strains lacking UAS_G sites at *ADH4* and *LYS2* (Lev112 and Lev96, respectively) are fully sensitive to 5-FOA in the presence of Gbd-Sir3 or Gbd-Sir4.

^aThe percentage of 5-FOA^R cells and the percentage of Ura⁺ cells were calculated by averaging three samples.

^bCells grew slowly on media lacking uracil, forming minicolonies after 4 days at 30°C.

In an attempt to improve this poor silencing at the *LYS2* locus, we created a new strain (Lev100) with two more UAS_G sites inserted 500 bp upstream of the *URA3* promoter. In this strain, Lev100, *URA3* is flanked by a set of UAS_G sites, two upstream of the promoter, and four sites downstream of the gene (Fig. 4C). In the presence of Gbd-Sir4p, the two new UAS_G sites caused an 11-fold increase in the proportion of FOA^R colonies (from 0.5% to 5.7%), but failed to cause repression in the presence of Gbd-Sir3p (Table 3).

Mutation of the Rap1p carboxyl terminus improves Sir3p and Sir4p targeted silencing at an internal site, but not near a telomere

We were curious as to why targeted silencing of *URA3* at the *LYS2* locus on chromosome II appears to be so much weaker than that which occurs at or near the telomere on the left arm of chromosome VII. One possibility that we considered is that the assembly of silent chromatin at *LYS2* is limited by the availability of Sir proteins, which might be sequestered at telomeres. This idea is supported by previous studies (Buck and Shore 1995) demonstrating that telomeres can compete with *HMR* for limiting silencer factors (Sir4p and perhaps others). Furthermore, cytological observations suggest that Sir3p and Sir4p are concentrated at telomeres in wild-type strains but not in strains in which telomeric repression is abolished by a mutation that deletes the Rap1p carboxy-terminal silencing domain (Palladino et al. 1993; Cockell et al. 1995).

Therefore, we tested the effect of the *rap1Δ670–807* mutation on Gbd-Sir targeted silencing at both *ADH4* and *LYS2*. Similar to what we found for silencing at the artificial chromosome VII-L telomere, targeted silencing of *URA3* at *ADH4* was slightly weakened by the *rap1* mutation, although this effect may be significant only in the case of Gbd-Sir4p-mediated repression (compare the proportion of Ura⁺ cells in Tables 3 and 4). However, we observed a significant and opposite effect on the action of Gbd-Sir3p and Gbd-Sir4p at *LYS2* in a *rap1Δ670–807* mutant strain. In cells expressing Gbd-Sir4p in the presence of either four UAS_G sites (4) near *URA3* or the flanking cassettes of UAS_G sites (4 + 2), the *rap1Δ670–807* mutation caused a fivefold increase in the proportion of FOA^R colonies (and a significant decrease in the proportion of Ura⁺ colonies). In the strain in which *URA3* is flanked by cassettes of UAS_G sites, the *rap1Δ670–807* mutation actually allowed detectable silencing by Gbd-Sir3p, although this repression is still weak (0.009% FOA^R).

Internal telomeric repeats boost Gbd-Sir3p and Gbd-Sir4p silencing at the LYS2 locus in a Rap1p-dependent manner

The experiments described above indicate that the *rap1Δ670–807* mutation, which abolishes telomere position effect, improves targeted silencing by both Gbd-Sir3p and Gbd-Sir4p at the nontelomeric *LYS2* locus, but not at the *ADH4* locus, which is only ~20 kb from the

Table 4. At the internal locus *LYS2*, but not at the telomeric locus *ADH4*, *Gbd-Sir3* and *Gbd-Sir4* activity is improved in *rap1* mutant cells

UAS _G	<i>ADH4</i>		<i>LYS2</i>			
	4	Δ/wt^a	4	Δ/wt^a	4 + 2	Δ/wt^a
Gbd	$<10^{-4}$	N.A.	$<10^{-4}$	N.A.	$<10^{-4}$	N.A.
Gbd-Sir3	1.9 (97) ^b	0.76	$<10^{-4}$	N.A.	.009 (100) ^b	>90
Gbd-Sir4	52 (65) ^b	0.91	2.6 (96) ^b	5.1	26 (63) ^b	4.6

The data shown here are from strains containing a *RAP1* carboxy-terminal deletion, all of which were generated from the parent strains (Table 3) by replacement of the pRAP1-Sup4-0 plasmid with sp18, which expresses the *rap1* Δ 670–807 mutant allele.

^aThe ratio is the percentage of 5-FOA^R cells with the *rap1* mutant allele divided by the percentage of 5-FOA^R cells with the *RAP1* wild-type (wt) allele (see Table 3). (N.A.) Not applicable.

^bThe percentage of 5-FOA^R cells and the percentage of Ura⁺ cells were calculated by averaging three samples.

chromosome VII-L telomere. One possible explanation for this effect might be that the larger distance of *LYS2* from a telomeric sequence limits silencing, and that this limitation is reduced by abolishing telomeric silencing through mutation of the Rap1p carboxyl terminus. To address this question, we created derivatives of the Lev98 and Lev100 strains in which a telomere repeat sequence (C_{1–3}A) of 270 bp (TEL270) is inserted in either orientation at *LYS2*, adjacent to the four UAS_G sites and the *URA3* reporter gene (Fig. 4C). The new strains (Lev116–Lev119) are fully sensitive to 5-FOA, indicating that the internal telomeric sequence is too short to act itself as a silencer, as expected (Stavenhagen and Zakian 1994). However, when Gbd-Sir4p is expressed in these cells, in the presence of four UAS_G sites or the flanking cassettes of UAS_G sites, the internal telomeric sequence increased the proportion of FOA^R colonies by an average of 36-fold or 9-fold, respectively (and decreased the proportion of Ura⁺ cells; see Table 5). Furthermore, in strains in which UAS_G cassettes flank *URA3* (4 + 2), the internal telomeric repeats had a dramatic effect on the silencing activity of Gbd-Sir3p, as indicated by >1% FOA^R, an increase of >10⁴-fold (cf. Tables 3 and 5). The orientation of the telomeric sequence relative to the *URA3* gene does not seem to affect significantly its ability to boost the silencing induced by Gbd-Sir3p and Gbd-Sir4p.

Because the telomeric sequence inserted at *LYS2* con-

tains 14 potential Rap1p-binding sites (Gilson et al. 1993), we tested its boosting effect on Gbd-Sir3p and Gbd-Sir4p targeted silencing in a *rap1* Δ 670–807 mutant background. In cells expressing Gbd-Sir3p or Gbd-Sir4p and in presence of the 4 $\times$ UAS_G cassette or the two UAS_G cassettes flanking *URA3*, and the 270-bp telomeric sequence, the *rap1* Δ 670–807 mutation returns the proportion of FOA^R colonies within twofold of the levels observed in isogenic *rap1* Δ 670–807 strains lacking the telomeric insert (Table 5, cf. Table 4). These results indicate that the augmentation of silencing by internal telomeric sequence is fully dependent on the Rap1p silencing domain, and suggest that the boosting results from interactions between this domain of Rap1p and the Sir3 and Sir4 proteins (Moretti et al. 1994).

Discussion

The role of Sir3p and Sir4p in silencing

We have shown that direct recruitment of Sir3p or Sir4p to the chromosome through the Gbd can establish transcriptional silencing on a adjacent gene. The dependence of this targeted silencing on *SIR2*, *SIR3*, and *SIR4* function indicates that it is bona fide silencing rather than a nonspecific effect on transcription. Sir3p and Sir4p have been proposed previously to be constituents of silent chromatin in yeast. Hecht et al. (1995) demonstrated

Table 5. At the internal locus *LYS2*, improvement of *Gbd-Sir3* and *Gbd-Sir4* silencing by internal telomeric repeats requires the Rap1p silencing domain

UAS _G orientation	<i>RAP1</i> wt				<i>rap1</i> Δ 670–807			
	4		4 + 2		4		4 + 2	
	Tel	leT	Tel	leT	Tel	leT	Tel	leT
Gbd	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
Gbd-Sir3	$<10^{-4}$	$<10^{-4}$	1.5 (95) ^a	.14 (97) ^a	$<10^{-4}$	$<10^{-4}$	.015 (100) ^a	.016 (100) ^a
Gbd-Sir4	20 (66) ^a	17 (90) ^a	44 (62) ^a	55 (41) ^a	5.8 (96) ^a	1.6 (99) ^a	14 (88) ^a	17 (90) ^a

The four strains Lev115–Lev119 are described in Table 6. The Tel and leT orientations of the TEL270 insert are described in Fig. 4C. The two control strains with a TEL270 insert in two different orientations and without UAS_G (Lev114 and Lev115) are fully sensitive to 5-FOA in the presence of Gbd-Sir3 or Gbd-Sir4.

^aThe percentage of 5-FOA^R cells and the percentage of Ura⁺ cells were calculated by averaging four samples.

that both Sir3p and Sir4p can interact with the amino-terminal tails of the histones H3 and H4, and inferred from this that silencing results from the polymerization of these Sir proteins along the chromatin fiber. If this model is correct, our results suggest that the localization of a small number of either Sir3p or Sir4p molecules to a site on the chromosome is sufficient to initiate the polymerization of these proteins along the chromatin, through a network of Sir–Sir interactions (Chien et al. 1993; Moretti et al. 1994), in the absence of a functional silencer element. Taken together, these studies suggest two distinct roles for the Sir3 and Sir4 proteins in silencing: (1) as constituents of the repressed chromatin and (2) as nucleation sites for initiating the assembly of silenced domains. The failure of the Gbd–Sir3p hybrid to complement a *SIR3* mutation provides indirect support for this model. We suggest that this hybrid is specifically defective in the ability to function as a component of repressed chromatin, presumably because of the fusion of Gbd to the amino terminus of Sir3p, but is still able to nucleate the assembly of Sir3p/Sir4p complexes along the chromatin.

The recruitment of Sir3p and Sir4p can establish transcriptional silencing, but do the *HM* silencers and the telomeres silence by recruiting Sir3p and Sir4p? It has been shown previously that a carboxy-terminal region of Rap1p interacts with Sir3p and Sir4p in the two-hybrid system, and with Sir3p directly in vitro (Moretti et al. 1994). A deletion of this interacting domain (amino acids 679–827) abolishes telomeric repression and derepresses *HMR* in contexts where the *HMR-E* silencer is completely dependent on the Rap1p-binding site for repression (Kyrion et al. 1993; Moretti et al. 1994). Fused to a Gbd, this region of Rap1p establishes transcriptional silencing when targeted to *HMR* or a telomere (Buck and Shore 1995; S. Marcand and D. Shore, unpubl.). Furthermore, point mutations in *RAP1* that have been isolated on the basis of a loss of silencing generally result in a weakening of the Rap1p–Sir3p interaction in the two-hybrid system (S. Buck and D. Shore, unpubl.). Taken together, these results strongly support the idea that Rap1p works at *HM* silencers and at telomeres by recruiting a Sir3p–Sir4p complex by direct protein–protein interactions with its carboxyl terminus (Moretti et al. 1994). The fact that direct recruitment of Sir3p and Sir4p brings about silencing further supports this model and suggests that the ability of Rap1p to interact with these silencing proteins may be sufficient to explain its essential role in this process.

Targeting of Sir4p to a telomere greatly increases the efficiency of telomeric silencing, to the point where the linked *URA3* reporter gene is repressed in virtually all of the cells within a culture. Previous studies have shown that the unusual mutation *rap1-12* results in a similar improvement in telomeric silencing and an increase in the Rap1p–Sir4p interaction, as measured by the two-hybrid system (Buck and Shore 1995). These two independent observations suggest that the efficiency of recruitment of Sir4p to a telomere may determine, at least in part, the probability that silencing will be established

at that telomere. It is possible, then, that the difference in strength between telomeric and *HM* silencing reflects a difference between the telomere and the *HM* silencers in the ability to recruit Sir4p.

Do telomeres function as regulators of chromosomal silencing?

We observed a strict correlation between the strength of targeted silencing by Sir3p and Sir4p and proximity to a telomere. Silencing established by Gbd–Sir1p and Gbd/Rap1p hybrids displays the same correlation (S. Marcand, unpubl.). These results fit well with a growing body of evidence that the strength of silencer elements is itself position dependent, in that they appear to work better when placed at or near telomeres. For example, Thompson et al. (1994) have noted that silencing at *HMR* is helped by an adjacent telomere on chromosome III (at a distance of ~25 kb), and others have shown that the *HML* and *HMR* silencers are less effective at the centromere-proximal *MAT* locus than when at their normal telomere-proximal sites (Shei and Broach 1995). In addition, the strength of $C_{1-3}A$ -based silencing is improved significantly by proximity to a telomere (Stavenhagen and Zakian 1994). Finally, the *HML* silencers do not function at *LYS2*, and this defect can be corrected by the creation of a new telomere at a distance of 12 kb from the *LYS2* locus (L. Maillet, C. Boscheron, M. Gotta, S. Marcand, E. Gilson, and S.M. Gasser, in prep.).

A particularly striking observation reported here is that targeted silencing at a nontelomeric site (*LYS2*) is improved by deletion of the Rap1p carboxyl terminus, whereas at the same time silencing at or near a telomere is either slightly weakened or unaffected. Because targeted silencing by Sir3p and Sir4p at *LYS2* does not involve Rap1p directly (to our knowledge there are no Rap1p-binding sites at this locus), we presume that the effect of the *rap1Δ670–807* mutation is indirect, and most likely attributable to events occurring at telomeres, which contain a high density of Rap1p-binding sites within the $C_{1-3}A$ repeats. We propose that the improvement of silencing at *LYS2* in the *rap1Δ670–807* mutant is attributable to an increase in the nontelomeric concentration of silencing factors (Sir3p and Sir4p, and perhaps other proteins) resulting from a release of these factors from telomeres, where they are normally concentrated through interactions with telomere-bound Rap1p (Moretti et al. 1994) and the resultant spreading of silent chromatin from these sites (Renauld et al. 1993). Five lines of evidence support this hypothesis. First, silencing is known to be extremely sensitive to the gene dosage of *SIR3* and *SIR4*. Diploid strains containing only one functional copy of the *SIR4* gene display unstable repression at *HMR* (Sussel et al. 1993), whereas extra copies of *SIR4* can suppress various silencing defects at *HMR* (Sussel and Shore 1991; Buck and Shore 1995). At telomeres, extra copies of *SIR3* increase the spreading of silent chromatin along the chromosome (Renauld et al. 1993). Second, immunolocalization studies (Palladino et al. 1993) indicate that Sir3p and Sir4p colocalize with Rap1p at the telomeres and are concentrated and sequestered in a

small number of foci at the periphery of the nucleus. This observation suggests that the local concentration of silencing factors inside the nucleus of a wild-type cell is variable, depending on the distance from one of these telomeric foci (Fig. 5A). Third, in *rap1* mutant cells where the silencing domain of Rap1p has been deleted, Sir3p appears to become delocalized in the nucleus, suggesting that its concentration may increase at nontelomeric sites, relative to wild-type strains (Cockell et al. 1995) (Fig. 5B). Fourth, efficient function of the *HML* silencers can be restored at *LYS2* when Sir3p and Sir4p are overexpressed, a situation that mimics the proposed release of these factors from telomeres in our *rap1* Δ 670–807 strains (L. Mailliet, C. Boscheron, M. Gotta, S. Marcand, E. Gilson, and S.M. Gasser, in prep.). Finally, an internal telomeric sequence, without being a silencer itself, boosts targeted silencing established by Gbd–Sir3p, Gbd–Sir4p, Gbd–Sir1p, or Gbd/Rap1p, an effect that requires the silencing domain of Rap1p (Table 5; S. Marcand, unpubl.). We propose that telomeric sequences act at *LYS2* through unstable Sir3p–Rap1p and Sir4p–Rap1p interactions, which, although by themselves are unable to establish silencing, are sufficient to increase the local

concentration of these factors at the site of the internal repeats (Fig. 5C). Similar long-range “boosting” effects on native silencers by either a telomere, telomeric repeat sequences, or individual Rap1p-binding sites have been observed at *HML* and *LYS2* (Boscheron et al. 1996; L. Mailliet, C. Boscheron, M. Gotta, S. Marcand, E. Gilson, and S.M. Gasser, in prep.). An alternative explanation is that the internal telomeric repeat interacts with a real telomere and thereby moves the internal site to within one of the telomeric foci, where silencing factor concentration is high, a situation equivalent to a subtelomeric position (Fig. 5C). Taken together, these data support the notion that silencing is very sensitive to the local concentration of silencing factors, and that the telomeres play a major role in determining silencing factor availability within the nucleus. In this view the action of telomeres is dual; negative for the whole genome by titrating away silencing factors, and positive for the subtelomeric regions by increasing locally the concentration of these factors.

Even in cells where the Rap1p carboxyl terminus is absent and telomeric silencing is abolished, we still found targeted silencing by Gbd–Sir3p and Gbd–Sir4p to

Figure 5. A model for the concentration and sequestration of silencing factors by telomeres, and its effect on the establishment of silencing at internal chromosomal sites. A telomere is represented at the left, bound by several Rap1p molecules (dark shaded oval). (Right) An internal (nontelomeric) locus is represented with several UAS_G sites bound to Gbd–Sir4 hybrid molecules. The relative strength of targeted silencing at this internal position is represented at the far right. (A) In wild-type cells, telomeres recruit Sir3p and Sir4p molecules through direct Rap1p–Sir interactions, and initiate the polymerization of Sir proteins along the adjacent chromatin, titrating most of the Sir molecules away from the rest of the nucleus. At an internal position, the resultant low concentration of silencing factors compromises the strength of targeted silencing by Gbd–Sir4 hybrids. (B) In *rap1* Δ 670–807 mutant cells, the loss of Sir–Rap1p interactions abolishes telomeric silencing and liberates Sir3p and Sir4p from the telomeres, resulting in a more uniform concentration of Sir3p and Sir4p throughout the nucleus. At an internal position, this higher concentration of silencing factors improves targeted silencing. (C) In wild-type cells, the proximity of a short telomeric sequence might improve targeted silencing at an internal position, through (unstable) Sir–Rap1p interactions that cause a local increase in Sir3p and Sir4p concentration, above that created by the *rap1* Δ 670–807 mutation in B. An alternative, but not mutually exclusive, explanation is that the telomeric repeat interacts with a real telomere, and moves the internal site to an environment with a high concentration of silencing factors. These two possibilities are presented together.

be stronger at a telomere or in the proximity of a telomere (at *ADH4*) than at a more internal position on another chromosome (*LYS2*). Although there may be somewhat trivial explanations for the extent of the quantitative differences we observe, for example that the *LYS2* locus contains or is flanked by strong promoters that mitigate against the establishment of silent chromatin (Aparicio and Gottschling 1994), other possibilities should be considered. For example, the localization of telomeres to the nuclear periphery in yeast (Klein et al. 1992) may help to promote the establishment of silent chromatin. Alternatively, the late replication of telomeres and telomere-proximal regions (McCarroll and Fangman 1988) could favor the establishment of silencing, which appears to occur during the S phase of the cell cycle (Miller and Nasmyth 1984).

The biological role of telomeric silencing in yeast is still unclear. Some genes naturally positioned in the proximity of a telomere do not appear to be regulated by the *SIR* genes. For example, the expression of *SUC* and *MAL* family genes, all located next to or between subtelomeric repeats, is not affected by a loss of telomeric silencing (S. Marcand and D. Shore, unpubl.). The unstable and epigenetic nature of telomeric silencing suggests too that the function of telomeres may not be to regulate gene expression locally, although more work is clearly needed to resolve this question. On the basis of the results reported here and previously (Buck and Shore 1995), we propose instead a novel role for telomeres in regulating the susceptibility of the genome to transcriptional silencing by serving as a reservoir or molecular sink for Sir proteins, thereby controlling their effective concentration at nontelomeric loci. We suggest that this function of telomeres is important to avoid the establishment of silent chromatin at inappropriate positions, and to favor silencing at the two subtelomeric silent *HM* loci. In this regard, we note that a recent study of senescence in yeast (as measured by the average number of divisions a cell will undergo before ceasing to divide) suggests a role for some form of Sir regulation in aging, as a specific allele of *SIR4* correlates a delay in aging with a loss of telomeric silencing (Kennedy et al. 1995). This increase in life span may be a fortuitous consequence of an indiscriminate increase in silencing throughout the genome, attributable to a breakdown in its control by telomeres. Alternatively, it might reveal a natural example of regulated silencing in yeast. It will be particularly interesting, especially with regard to the second hypothesis, to identify the putative "aging" genes that are subjected to Sir regulation.

In *Drosophila*, repression induced by repeated sequences is stronger in the proximity of the centromeric heterochromatin, suggesting that centromeres in *Drosophila* play the role of telomeres in yeast in organizing the susceptibility of the genome to heterochromatinization (Dorer and Henikoff 1994). It is possible that the existence of a heterochromatin organizer is a conserved characteristic of the cell in different organisms. In humans, successive somatic cell divisions cause a progressive shortening of telomeres until cells enter into senes-

cence and stop dividing (for review, see Harley 1995). If one assumes some similarity between yeast and human telomeres, then telomere shortening in human cells may release analogs of Sir3p and Sir4p and affect expression of genes in *trans*, in much the same way a *rap1* mutation improves targeted silencing at an internal locus, or progressive telomere shortening improves silencing of the *HMR* locus (Buck and Shore 1995) in yeast. In this model, cells enter senescence when the concentration of released factors reaches a threshold sufficient to turn off genes essential for cell cycle progression.

In summary, our results highlight the dynamic nature of silencing in yeast and its extreme sensitivity to the concentrations of factors that play an essential role in its initiation and propagation. One strong inference from these results is that the concentration of Sir proteins within the nucleus varies according to the proximity of a given site from a telomere, in a manner that can be regulated by Rap1p and telomere length. It seems possible, if not likely, that this phenomenon is important not only to prevent the inappropriate silencing of genes throughout the chromosome, but to control the expression of *HM* mating-type loci (Buck and Shore 1995; L. Maillet, C. Boscheron, M. Gotta, S. Marcand, E. Gilson, and S.M. Gasser, in prep.) and as yet unidentified genes that might be normally subjected to some degree of Sir repression. Finally, we believe that this perspective may have important implications for the way in which telomere metabolism affects gene regulation in mammalian cells.

Materials and methods

Yeast strains and methods

The yeast strains used in this study are all derivatives of W303-1B (*HML α MAT α HMRA ade2-1 ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can1-100*) or its isogenic *MAT α* relative W303-1A, and are listed in Table 6. All gene disruptions or replacements (Rothstein 1991) used to generate these strains were confirmed by Southern blotting. Growth and manipulation of yeast strains were done according to standard procedures (Rose et al. 1990). Lev7 and Lev8 are derived from a cross between YLS85 and YDS631 or YDS634, respectively. Lev102 and Lev103 are derived from a cross between YLS126 and Lev7 or Lev8, respectively. Lev104 and Lev105 are derived from a cross between YLS134 and Lev7 or Lev8, respectively. Lev112, Lev113, Lev96, Lev98, Lev100, and Lev114–119 result from the transformation of YLS85 with the linearized plasmids sp163, sp164, sp107, sp109, sp156, and sp172–177, respectively.

Assays for silencing using the *hmr::TRP1* reporter were performed by spotting 10-fold serial dilutions of cultures grown in appropriate synthetic selective medium as described (Sussel and Shore 1991). Determination of the proportion of 5-FOA^R cells and Ura⁺ cells was done as follows: independent colonies were resuspended in water, diluted to an appropriate concentration, and spread on SC–His, SC–His–Ura and fresh SC–His+5-FOA plates (0.8 gram/liter of 5-FOA). The number of colonies were counted after 4 days at 30°C for the *RAP1* wild-type strains, and after 7 days for the *rap1 Δ 670–807* strains. Each percentage represents the average of three or more samples. The standard deviation between the different samples was significantly higher with the strains displaying a smaller proportion of FOA^R colonies; for a mean >30%, between 30% and 3%, or <3%, the ratio standard deviation/mean was respectively <0.30, between 0.23 and 0.72, or between 0.30 and 1.08.

Table 6. *Yeast strains*

Strain	Genotype	Source
W303-1B	<i>HMLα MATα HMRα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100</i>	Thomas and Rothstein (1989)
YSB46	W303-1B Δ AAE::3 $\times$ UAS _G <i>gal4::LEU2</i>	Buck and Shore (1995)
YSB68	YSB62 except Δ AAE	Buck and Shore (1995)
YSB1	W303-1B Δ AAE <i>hmr::TRP1 gal4::LEU2</i>	Buck and Short (1995)
YSB2	YSB1 except Δ AAE::3 $\times$ UAS _G	Buck and Shore (1995)
YSB64	YSB1 except Δ EAB	Buck and Shore (1995)
YSB35	YSB1 except Δ EAB::3 $\times$ UAS _G	Buck and Shore (1995)
YSB43	YSB1 except Δ AAEAB	Buck and Shore (1995)
YSB39	YSB1 except Δ AAEAB::2 $\times$ UAS _G	Buck and Shore (1995)
YSB41	YSB1 except Δ AAEAB::3 $\times$ UAS _G	Buck and Shore (1995)
YSB160	YSB2 except <i>sir1::URA3</i>	Buck and Shore (1995)
RS1042	YSB2 except <i>sir2::URA3</i>	Chien et al. (1993)
RS1061	YSB2 except <i>sir3::URA3</i>	Chien et al. (1993)
RS1067	YSB2 except <i>sir4::URA3</i>	Chien et al. (1993)
YLS85	W303-1B <i>MATAα rap1::LEU2 pRap1-Sup4-0</i>	Sussel and Shore (1991)
YLS126	W303-1B Δ Bhmr::TRP1 <i>sir3::LEU2</i>	Sussel and Shore (1991)
YLS134	W303-1B Δ Bhmr::TRP1 <i>sir4::LEU2</i>	Sussel and Shore (1991)
YDS631	W303-1B <i>adh4::URA3-telVII-L</i>	Chien et al. (1993)
YDS634	W303-1B <i>adh4::URA3-4</i> $\times$ UAS _G -telVII-L	Chien et al. (1993)
Lev7	YDS631 except <i>rap1::LEU2 pRAP1-Sup4-0</i>	this study
Lev8	YDS634 except <i>rap1::LEU2 pRAP1-Sup4-0</i>	this study
Lev102	Lev7 except <i>sir3::LEU2</i>	this study
Lev103	Lev8 except <i>sir3::LEU2</i>	this study
Lev104	Lev7 except <i>sir4::LEU2</i>	this study
Lev105	Lev8 except <i>sir4::LEU2</i>	this study
Lev112	YLS85 except <i>adh4::URA3</i>	this study
Lev113	YLS85 except <i>adh4::URA3 4</i> $\times$ UAS _G	this study
Lev96	YLS85 except <i>lys2::URA3</i>	this study
Lev98	YLS85 except <i>lys2::URA3-4</i> $\times$ UAS _G	this study
Lev100	YLS85 except <i>lys2::URA3 (4 + 2)</i> $\times$ UAS _G	this study
Lev114	YLS85 except <i>lys2::URA3-TEL270 (Tel)</i>	this study
Lev115	YLS85 except <i>lys2::URA3-TEL270 (leT)</i>	this study
Lev116	YLS85 except <i>lys2::URA3-4</i> $\times$ UAS _G -TEL270 (Tel)	this study
Lev117	YLS85 except <i>lys2::URA3-4</i> $\times$ UAS _G -TEL270 (leT)	this study
Lev118	YLS85 except <i>lys2::URA3-(4 + 2)</i> $\times$ UAS _G -TEL270 (Tel)	this study
Lev119	YLS85 except <i>lys2::URA3-(4 + 2)</i> $\times$ UAS _G -TEL270 (leT)	this study

Plasmid DNAs

The *rap1* Δ 670–807 allele was created by joining two *RAP1* fragments with a *XhoI* linker at each end, creating a deletion with a *XhoI* linker in the middle. The new *rap1* fragment (*EcoRI*–*XbaI*) was ligated into *EcoRI*- and *XbaI*-cut pRS313 (*HIS3 CENV1*) to give plasmid PM585. The *HIS3* gene of this new plasmid was disrupted as follows: PM585 was cut with *NdeI*, end-filled with Klenow polymerase, and religated to give plasmid sp18. Plasmid pSB362 (Gbd alone) is based on pRS313 (*HIS3 CENV1*) and contains sequences encoding Gbd fused to the *RAP1* promoter (Buck and Shore 1995). The two plasmids sp131 (encoding Gbd–Sir3p) and sp138 (encoding Gbd–Sir4p) were constructed by ligating a *HpaI* fragment from pCTC70 and pCTC41 (Chien et al. 1993), respectively, into *HpaI*- and *XbaI*-cut pSB362.

To insert *URA3* in the proximity of the *ADH4* locus, we used a genomic fragment isolated as follows: the plasmid pADH4UCA (Gottschling et al. 1990) was digested with *HindIII* and *SphI* (deleting the 3' end of *ADH4*), end-filled using Klenow polymerase, and religated to give plasmid sp190. The *ADH4* 3' fragment (*HindIII*–*SphI* filled in) was reinserted into *NotI*-cut, Klenow-treated sp190. This new plasmid (sp92) was digested with *SalI* and transformed into strain W303-1B. The genomic

DNA of this new strain was digested with *PstI*, religated and transformed into *Escherichia coli*. The rescued plasmid (sp160) contains a 5-kb genomic sequence extending from the 3' half of *ADH4* toward the centromere. The integrating plasmids (sp163 and sp164) used to place the *URA3* gene at *ADH4* were constructed by ligating *HindIII*–*BamHI* fragments containing *URA3* (from pADH4UCA) or *URA3-4* $\times$ UAS_G (from D1724; Chien et al. 1993) into *HindIII*- and *BamHI*-cut sp160. The two plasmids were integrated after digestion with *EcoRI*.

The integrating plasmids (sp107 and sp109) used to place the *URA3* gene at *LYS2* were constructed by ligating the same *HindIII*–*BamHI* fragments containing *URA3* or *URA3-4* $\times$ UAS_G into pD6P (a pUC vector ligated to a 5-kb genomic fragment including *LYS2*; Fleig et al. 1986) that was digested with *XhoI* (filled in). A cassette of UAS_G was created by inserting 2 $\times$ UAS_G (a *BamHI*–*BglII* fragment from D1716; Chien et al. 1993) into the *BamHI* site of sp109 (sp156). The TEL270 sequence (a filled-in *HindIII*–*EcoRI* fragment from pLTel; Gilson et al. 1994) was inserted into the recreated *XhoI* (filled in) site of sp107, sp109, and sp156, creating the plasmids sp172 (*Tel*), sp173 (*leT*), sp174 (*Tel*), sp175 (*leT*), sp176 (*Tel*), and sp177 (*leT*), respectively. *Tel* indicates that the telomeric insert is in the same orientation than in pADH4UCA relative to the *URA3* gene, *leT*

indicates the opposite orientation. All nine plasmids were integrated after digestion with *Bgl*III and *Eco*RI.

RNA analysis

Total yeast RNA was prepared and analyzed by Northern blotting using standard procedures. *HMR a1* mRNA was detected by probing with a random-primed 650 bp *Xho*I–*Bgl*III DNA fragment from plasmid D401 (*Xho*I linker mutant no. 238; Abraham et al. 1984). The blot was reprobed with a 500-bp *Eco*RI–*Hind*III fragment from the actin gene and analyzed with a PhosphorImager (Molecular Dynamics). The range was 14–156 counts.

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