Action of a RAP1 carboxy-terminal silencing domain reveals an underlying competition between HMR and telomeres in yeast

Stephen W. Buck and David Shore

Department of Microbiology, College of Physicians and Surgeons of Columbia University, New York, New York 10032 USA

RAP1 is a sequence-specific DNA-binding protein in yeast that can either repress or activate transcription. Previous studies have demonstrated a direct role for RAP1 in silencing at HM mating-type loci and telomeres. Here, we show that a small carboxy-terminal domain of RAP1 is sufficient to establish repression when fused to the GAL4 DNA-binding domain (GBD) and targeted to mutated HMR silencers containing GAL4 DNA-binding sites. Silencing by GBD/RAP1 hybrids, like normal silencing at HMR, requires the trans-acting factors SIR2, SIR3, and SIR4. However, GBD/RAP1-mediated silencing is independent of SIR1, whose product is normally required for the establishment of repression at HMR. Targeted silencing also displays an unusual response to silencing-defective rap1 mutations. The incorporation of a rap1 missense mutation into GBD/RAP1 hybrids can improve targeted silencing, yet wild-type GBD/RAP1 hybrids fail to establish repression in strains in which the endogenous RAP1 locus carries a rapF mutation. In addition, we find that telomeric silencing is increased in rapF strains. We propose that the rapF mutation creates an HMR-specific silencing defect by shifting a balance between silencing at HMR and telomeres in favor of telomeric silencing. This balance is regulated by telomere length and by interactions between the RAP1 carboxyl terminus and both RIF1 and SIR4 proteins. In support of this model, we show that abnormally long telomeres antagonize silencing at HMR and a rap1 hybrid protein displays a strengthened interaction with SIR4 in a two-hybrid assay.

[Key Words: Transcriptional silencing; RAP1; silencer; telomere; mating type]

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Mating-type genes in the yeast *Saccharomyces cerevisiae* are subject to a position-effect repression phenomenon called silencing [Klar et al. 1981; Nasmyth et al. 1981; Abraham et al. 1984; Feldman et al. 1984; Brand et al. 1985]. When present at the MAT locus near the centromere of chromosome III, these genes [a or α] are expressed and regulate cell type [a or α haploid or nonmating a/α diploid types]. Two other copies of mating-type information are present near the telomeres of chromosome III at loci called HML and HMR. Although the HM loci contain complete structural gene and promoter sequences, they are not normally transcribed, because of the action of flanking regulatory elements called silencers and a number of trans-acting factors [for review, see Laurenson and Rine 1992].

Silencing at the HMR locus (which usually contains a information) requires a flanking silencer element called HMR-E. This silencer is comprised of three partially redundant regulatory elements, called A, E, and B [Brand et al. 1987; Kimmerly et al. 1988]. The A element is an autonomously replicating sequence (ARS) consensus sequence [ACS] recognized by a set of six proteins called the origin recognition complex (ORC) [Bell and Stillman 1992; Diffley and Cocker 1992]. Temperature-sensitive mutations in the ORC2 protein confer a silencing defect at the permissive temperature, demonstrating a role for the ORC in transcriptional silencing [Bell et al. 1993; Foss et al. 1993; Micklem et al. 1993]. The E and B sites are binding sites for two abundant and essential regulatory proteins, RAP1 and ABF1, respectively [Shore and Nasmyth 1987; Shore et al. 1987; Buchman et al. 1988a,b; Diffley and Stillman 1989]. A role for RAP1 in silencing has been demonstrated by the isolation of mutations in the protein that result in derepression of either HML or HMR [Kurtz and Shore 1991; Sussel and Shore 1991; Kyrian et al. 1993]. In contrast to ORC and RAP1, there is as yet no direct evidence for the role of ABF1 in silencing. An intriguing feature of both RAP1 and ABF1 is that they also bind to the promoters of a large number of genes, where they often function as transcriptional
activators [Shore and Nasmyth 1987; Buchman et al. 1988b].

Transcriptional silencing at HM loci also requires a number of other trans-acting factors in addition to the known silencer binding proteins. These include the products of the four SIR [silent information regulator] genes [Haber and George 1979; Klar et al. 1979; Rine et al. 1979; Shore et al. 1984; Ivy et al. 1986; Rine and Herskowitz 1987; Stone et al. 1991]. The SIR proteins are presumed to be involved in either the establishment or maintenance of an altered chromatin structure at HM loci and telomeres, although the precise role of these proteins in silencing is at present unclear. Important insights into the nature of silencing have come from studies of the SIR1 gene. Pillus and Rine [1989] showed that mutation of SIR1 creates a different phenotype than mutation of any of the other three SIR genes. In cells mutant for either SIR2, SIR3, or SIR4, silencing cannot be maintained, and these cells are uniformly derepressed. In contrast, repression at HML is only destabilized in sir1 mutant cells, leading to the production of two populations of cells [repressed and derepressed] within a clonal culture. It was proposed, therefore, that SIR1 plays a specific role in the establishment of silencing. This hypothesis is supported by recent experiments that indicate that SIR1 acts at the HMR-E silencer (Chien et al. 1993).

Telomeres in yeast can also act as silencer elements, repressing the expression of genes placed in their vicinity [Gottschling et al. 1990]. Telomeric silencing, also known as telomeric position effect [TPE], probably occurs by a process similar to silencing at the HM loci because it requires the function of three of the four SIR genes [SIR2, SIR3, SIR4]. TPE differs from HM silencing, however, in that it is inherently unstable and unaffected by mutation of SIR1 [Aparicio et al. 1991]. The instability of TPE can be explained by an inability of telomeres to utilize the SIR1 establishment function (Chien et al. 1993). At present, the only known sequence-specific DNA-binding protein that mediates TPE is RAP1 [Kyrion et al. 1993], whose binding sites occur often within the terminal C1-A repeats at telomeres [Longtine et al. 1989; Gilson et al. 1993]. It appears, therefore, that TPE is not brought about by discrete silencer elements analogous to those at HM loci but is instead mediated by long poly(C1-A) tracts (~300 bp) to which multiple copies of RAP1 are bound.

Mutational analysis has indicated that the carboxyl terminus of RAP1 is critical for both HM silencing and TPE [Sussel and Shore 1991; Kyrion et al. 1993]. For example, missense mutations in the RAP1 carboxyl terminus, referred to as rap1* alleles, result in derepression of HMR when the HMR-E silencer is weakened by mutation of the ACS [hmrAA] [Sussel and Shore 1991]. The rap1* mutations also result in elongation of the poly(C1-A) tracts at telomeres by as much as 50%. A detailed characterization of the rap1* mutations has indicated that their defect may be in the establishment of repression at HMR, rather than its maintenance [Sussel et al. 1993]. Truncations of the carboxyl terminus of RAP1 lead to a complete loss of TPE and HML silencing but fully derepress HMR only when the HMR-E silencer is mutated [hmrAA] [Kyrion et al. 1993; Moretti et al. 1994]. Severe RAP1 truncation alleles [rap1*] display dramatic increases in telomere tract length (>10-fold). However, this alteration of telomere structure does not explain the loss of TPE in rap1* mutants. In fact, when elongated telomeres are inherited by RAP1 wild-type spores after a cross with a rap1* mutant, silencing at these telomeres is found to be stronger than silencing at normal-length telomeres [Kyrion et al. 1993].

Experiments employing GAL4 DNA-binding domain [GBD/RAP1 hybrid proteins have provided further insights into the function of the RAP1 carboxyl terminus, indicating that it plays a role in both silencing and transcriptional activation [Hardy et al. 1992a]. GBD/RAP1 hybrids containing residues 630–695 of RAP1 function as transcriptional activators when targeted to a GAL1-lacZ reporter gene, suggesting that this region of RAP1 may function as a transcriptional activation domain in the native protein. On the other hand, hybrids containing sequences from residue 678 to the end of the protein [amino acid 827] interfere with silencing when overexpressed, suggesting that this carboxy-terminal domain of RAP1 may normally play a role in repression. This dominant-negative effect on silencing produced by overexpression suggests that the RAP1 carboxyl terminus mediates its effects at silencers and telomeres through interactions with other proteins. One such protein, RIF1, was identified in a two-hybrid screen [Hardy et al. 1992b]. rif1 mutants, like rap1* mutants, display an hmrAA-specific silencing defect. In addition, rap1* proteins are defective in RIF1 binding, suggesting that one function of RAP1 at HMR is to recruit RIF1 to the silencer.

Here, we have extended our studies of the role of the RAP1 carboxyl terminus in silencing at HMR. Using GBD/RAP1 hybrid proteins, we have identified a small carboxy-terminal domain of RAP1 that is sufficient to establish repression when targeted to an HMR-E silencer deleted for all three of the normal regulatory sites [A, E, and B]. Targeted silencing by GBD/RAP1 hybrids requires the function of SIR2, SIR3, and SIR4 but differs from normal silencing at HMR in that it is independent of SIR1 function. Surprisingly, GBD/rap1* hybrids do not silence in targeted silencing, whereas a chromosomal rap1* mutation abolishes the ability of GBD/RAP1 (but not GBD/rap1*) hybrids to repress at HMR. We explain these results in terms of a model in which the HMR silencer and telomeres compete for limiting silencing factors.

Results

To investigate the transcriptional silencing functions of RAP1 in more detail, we developed a system in which RAP1 hybrid proteins substitute for endogenous silencer binding factors at the HMR-E silencer [see Fig. 1]. The hybrid proteins consist of carboxy-terminal sequences of RAP1 fused to the GBD [Hardy et al. 1992a] and are expressed from the RAP1 promoter. To assay the activity of
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Figure 1. Schematic representation of the targeted silencing strategy employed in these studies. The wild-type HMR-E silencer is shown on the top line. As noted in the text, both AA and AB silencers are completely functional, whereas the double-mutant silencers AAAE and AEd~B and the triple-mutant silencer AAAEAB are derepressed.

these hybrids, we used three types of strains containing GAL4-binding sites [UASG] inserted at mutated silencers [see Materials and methods, Table 1]. In the first two sets of strains, the HMR-E silencer is deleted for the RAP1 binding site (E) and either the ORC-binding site (A) or the ABF1-binding site (B). These silencers are referred to as ΔAAΔE::[UASG]n and ΔEΔB::[UASG]n, respectively, where n indicates the number of GAL4-binding sites inserted within the silencer deletion. In the third type, ΔΔEΔB::[UASG]n, all three known silencer regulatory sites have been deleted and are replaced by binding sites for the GAL4 protein. All three of these mutated silencers are completely defective in repression [Brand et al. 1987; Kimmerly et al. 1988]. To assay the function of G_{BD}/RAP1 hybrids in these strains, hybrid gene constructs are either integrated in single copy at the HIS3 locus or expressed from low-copy-number centromere (CEN) plasmids. All relevant strains contain a gal4::LEU2 gene disruption to avoid competition by native GAL4 for binding to the UASG-containing silencers.

G_{BD}/RAP1 hybrids restore repression when targeted to mutated HMR-E silencers

We first tested the ability of G_{BD}/RAP1 hybrids to repress the a1 gene normally found at the HMR locus. A CEN plasmid expressing a G_{BD}/RAP1 hybrid containing the carboxy-terminal 175 amino acids of RAP1 [G_{BD}/RAP1(653–827)] or a control plasmid expressing G_{BD} alone were introduced into strains with or without UASG sites at the HMR-E silencer. We first assayed for transcriptional silencing directly by measuring steady-state a1 mRNA levels in these strains. As shown in Figure 2, the expression of G_{BD}/RAP1(653–827) resulted in either a reduction or a complete loss of detectable a1 mRNA with all three types of UASG-containing silencers. The G_{BD} control plasmid failed to confer repression in all cases. Control strains lacking UASG sites at HMR also remained derepressed in the presence of G_{BD}/RAP1(653–827), demonstrating that the site of action for the hybrid protein is the mutated silencer. In addition, we noticed that the level of repression is influenced by the number of UASG sites at the silencer. For example, a single site in the ΔΔEΔB silencer gave partial repression, whereas multiple tandem sites cloned into this deletion resulted in full repression. Although the A and B sites at HMR-E appear to increase the level of repression by G_{BD}/RAP1(653–827), they are not absolutely required, because repression can occur in their absence. However, full repression at the ΔΔEΔB silencer requires more UASG sites than are needed at silencers containing either A or B. The triple-deletion silencer gives only partial repression with three copies of UASG. Full repression is only achieved when four copies are present. These results closely parallel mutational analyses of the HMR-E silencer element [Brand et al. 1987; Kimmerly et al. 1988] that demonstrated that full repression required the E site [the RAP1 binding site] plus either the A or B sites.

To further characterize silencing mediated by G_{BD}/RAP1(653–827), we examined its effect in strains containing a TRP1 reporter gene at HMR in place of the normal a1 and a2 genes. In these strains repression is reflected by a reduction in the ability to form colonies on medium lacking tryptophan. Previous studies have shown that the TRP1 reporter can provide an accurate and sensitive assay for silencing [Sussel and Shore 1991]. Because this assay measures the properties of individual cells in a culture, it can reveal a loss of repression in a small fraction of cells within a population that might go undetected in an mRNA assay. Figure 3A shows the effect of the G_{BD}/RAP1(653–827) hybrid on TRP1 expression in three different mutator silencer contexts [ΔΔEΔE, ΔΔEΔB, or ΔΔEΔB] with or without UASG sites. The hybrid gene in these experiments is integrated in single copy at the HIS3 locus. In both of the double-mutant silencer strains the ability to form colonies on plates lacking tryptophan is reduced >10⁻¹-fold by expression of the hybrid protein. Consistent with the results from the HMRa strains, repression of TRP1 was dependent on
Table 1. Yeast strains

| Strain     | Genotype* | Source                                      |
|------------|-----------|---------------------------------------------|
| W303-1B    | HMLaMATα | R. Rothstein (pers. comm.)                  |
| YSB1       | W303-1B  | Chien et al. (1993)                         |
| YSB2       | YSB1 except ΔAE [UAS]   | Chien et al. (1993)                         |
| YSB4       | YSB1 except ΔAE [UAS]   | Chien et al. (1993)                         |
| YSB35      | YSB1 except ΔAE [UAS]   | Chien et al. (1993)                         |
| YSB44      | W303-1B  | this study                                  |
| YSB46      | YSB44 except ΔAE [UAS] | this study                                  |
| YSB48      | YSB44 except ΔAE [UAS] | this study                                  |
| YSB50      | YSB44 except ΔAE [UAS] | this study                                  |
| YSB54      | YSB44 except ΔAE [UAS] | this study                                  |
| YSB62      | YSB44 except ΔAE [UAS] | this study                                  |
| YSB66      | YSB44 except ΔAE [UAS] | this study                                  |
| YSB68      | YSB44 except ΔAE [UAS] | this study                                  |
| YSB70      | YSB44 except ΔAE [UAS] | this study                                  |
| YSB72      | YSB44 except ΔAE [UAS] | this study                                  |
| YDS31      | MATα his1 | K. Nasmyth (pers. comm.)                    |
| YDS655     | W303-1B except HIS3 | R. Rothstein (pers. comm.)                  |
| YSB256     | W303-1B except ΔAE [UAS] | this study                                  |
| YSB107     | YSB256 except HIS3: G_{RB}/RAP1 (653–827) | this study                                  |
| YSB114     | YSB107 except ΔAE [UAS] | this study                                  |
| YSB127     | YSB256 except ΔAE [UAS] | this study                                  |
| YSB128     | YSB127 except G_{RB}/RAP1 (653–827) | this study                                  |
| YSB140     | YSB123 except ΔAE [UAS] | this study                                  |
| YSB103     | YSB107 except ΔAE [UAS] | this study                                  |
| YSB102     | YSB107 except HIS3: G_{RB}/RAP1 (653–827) | this study                                  |
| YSB252     | YSB107 except HIS3: G_{RB}/RAP1 (667–827) | this study                                  |
| YSB253     | YSB107 except HIS3: G_{RB}/RAP1 (679–827) | this study                                  |
| YSB254     | YSB107 except HIS3: G_{RB}/RAP1 (702–827) | this study                                  |
| YSB255     | YSB107 except HIS3: G_{RB}/RAP1 (706–799) | this study                                  |
| YSB257     | YSB107 except HIS3: G_{RB}/RAP1 (652–827) | this study                                  |
| YSB258     | YSB107 except HIS3: G_{RB}/RAP1 (588–827) | this study                                  |
| YSB259     | YSB107 except HIS3: G_{RB}/RAP1 (618–827) | this study                                  |
| YSB260     | YSB107 except HIS3: G_{RB}/RAP1 (630–827) | this study                                  |
| YDS702     | W303-1B except ΔAE [UAS] | this study                                  |
| YSB160     | YSB2 except sir1: URA3 | C.-t. Chien and R. Sternberg (pers. comm.) |
| RS1042     | YSB2 sir2: URA3 | Chien et al. (1993)                         |
| RS1061     | YSB2 sir3: URA3 | Chien et al. (1993)                         |
| RS1067     | YSB2 sir4: URA3 MATa | Chien et al. (1993)                         |
| YSB76      | YSB114 except rap1-12: URA3 | this study                                  |
| YSB300     | YSB107 except rif1: URA3 | this study                                  |
| YSB302     | YSB110 except rif1: URA3 | this study                                  |
| YSB110     | YSB114 except HIS3: G_{RB}/rap1-12 (653–827) | this study                                  |
| YSB74      | YSB110 except rap1-12: URA3 | this study                                  |
| YSB116     | YSB114 except HIS3: G_{RB}/rap1-12 (653–827) | this study                                  |
| YSB80      | YSB107 except MATa | this study                                  |
| YSB282     | YSB80 except HIS3: G_{RB}/rap1-12 (653–827) | this study                                  |
| AJL394-2a  | W303-1B except rap1-17 ADE2: URA3:TelI17 | A. Lustig (pers. comm.)                     |
| AJL275-2a  | AJL394-2a except RAP1 | A. Lustig (1993)                            |
| AJL440-1c  | AJL275-2a except rap1-17 MATa | A. Lustig (pers. comm.)                     |
| YSB237     | YSB282 except rif1: URA3 | this study                                  |
| CTY10-5D   | MATα ade2-1 trp1-901 leu2-3, 112 his3-200 gal4 gal80 | C.-t. Chien and R. Sternberg (pers. comm.) |
| YSB215     | W303-1B except ΔAE [UAS] | this study                                  |
| YSB216     | YSB215 except p(CEN–HIS3–rap1-12) | this study                                  |
| YSB290     | W303-1B except ΔAE [UAS] | this study                                  |
| YSB284-7   | YSB107, except derived from RAP1/rap1-17 diploid | this study                                  |
| YSB288-9   | YSB110, except derived from RAP1/rap1-17 diploid | this study                                  |
| YSB291-4   | YDS290, except derived from RAP1/rap1-17 diploid | this study                                  |
| YSB304     | YSB291, except MATa | this study                                  |
| YSB296     | YDS290, except derived from RAP1/YSB293 diploid | this study                                  |
| YSB297     | YSB290, except derived from YSB293/YSB304 diploid | this study                                  |
| YSB298     | YSB290, except derived from rap1-1/YSB293 diploid | this study                                  |

*Underlined part of genotype refers to the deletion mutation at the HMR-E silencer.
RAP1 sequences in the hybrid protein and GAL4-binding sites at the mutant silencer. Unlike the case for the HMRα strain, where partial repression was observed, we did not find evidence for repression of TRPI by GBD/RAP1(653-827) in a strain with a triple-deletion silencer containing three copies of UASc. This could reflect subtle differences in the sensitivities of the two genes (TRPI and a1) to the silencer or a very low threshold of TRPI expression required for growth on -Trp medium.

GBD/RAP1 hybrids were used previously to define a transcriptional activation domain in RAP1 that extends from amino acid 630 to 695 (Hardy et al. 1992a). The GBD/RAP1 hybrid, though it contains part of this region, is incapable of activating transcription. To ask whether RAP1 hybrids that can function as activators are also capable of bringing about repression when targeted to HMR, we tested a series of larger hybrids with RAP1 amino-terminal endpoints at amino acids 630, 618, 588, and 562. Constructs expressing these hybrids were integrated at the HIS3 locus in a strain containing a Δ∆AE::[UASc]Δ silencer linked to the TRPI reporter gene. As shown in Figure 3B, all four of these GBD/RAP1 activators can act as repressors when targeted to HMR. This result indicates that the opposing functions of these hybrids (activation and repression) are regulated by the context of the GAL4-binding sites.

We then mapped the sequences in RAP1 required for silencing by the GBD/RAP1(653-827) hybrid. Removal of 14 amino acids from the RAP1 function to create GBD/RAP1(667-827) had no effect on silencing, whereas two larger deletions, GBD/RAP1(679-827) and GBD/RAP1(702-827), showed partial or no activity, respectively (Fig. 3C). We do not yet know whether these hybrids are defective as a result of the loss of sequences required for silencing or because they fail to produce a stable hybrid protein. A small deletion of the carboxyl terminus, GBD/RAP1(653-799) was also completely defective in repression, yet probably produces at least some stable protein because a related LexA/RAP1 hybrid functions as a transcriptional activator (Moretti et al. 1994).

Figure 2. Targeted silencing by the GBD/RAP1(653-827) hybrid protein at two double-deletion HMR-E silencers (ΔΔAE and ΔEAB) and the triple-deletion silencer (ΔΔΔEΔB) in strain W303-1B containing a UASc-dependent reporter by GBD alone and GBD/RAP1, respectively. (A) Mapping of the amino- and carboxy-terminal boundaries of a RAP1 silencing domain using a series of GBD/RAP1 hybrids in strain W303-1B. The results are consistent with the idea that GBD/RAP1 repressors have the ability to silence both UASc and UASg reporters.

Figure 3. Targeted silencing of an hmr::TRP1 reporter by GBD/RAP1 hybrids. 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-μl aliquots from a set of 10-fold serial dilutions of a liquid culture. (A) Effect of the GBD/RAP1(653-827) hybrid at the TRP1 locus with different HMR-E silencer deletions. The ΔΔAE::[UASc]ΔΔ silencer linked to the TRP1 reporter by GBD/RAP1(653-827) was completely derepressed by the ΔΔAE::[UASc]ΔΔ silencer linked to the TRP1 reporter by GBD/RAP1(653-827) in a strain with a triple-deletion silencer containing three copies of UASc. This could reflect subtle differences in the sensitivities of the two genes (TRP1 and a1) to the silencer or a very low threshold of TRP1 expression required for growth on -Trp medium.

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Several missense mutations that abolish the silencing function of the GBD/RAP1(653–827) hybrid map to the region deleted in this construct, further indicating the importance of the last 28 amino acids for the silencing activity of the hybrid. These mutants will be described elsewhere.

Establishment of silencing by a GBD/RAP1 hybrid is dependent on SIR2, SIR3, and SIR4, but not on SIR1

To determine whether GBD/RAP1[653–827]-mediated silencing resembles normal silencing at HMR, we examined its dependency on SIR gene function. A series of derivatives of the ΔAAE::[UASp]3 hmr::TRP1 reporter strain was constructed in which individual SIR genes were mutated by gene disruption [Chien et al. 1993]. These strains were then transformed with a CEN plasmid expressing the GBD/RAP1[653–827] hybrid and tested for silencing. As shown in Figure 4, repression by GBD/RAP1[653–827] is completely lost in sir2, sir3, and sir4 mutant cells. Strikingly, no loss of silencing was detected in a sir1 mutant. In contrast, silencing dependent on native RAP1, in strains containing a hmrΔA silencer, is completely abolished by mutation of SIR1 [Fig. 4, cf. rows 3 and 4]. We conclude from this that GBD/RAP1[653–827] is somehow able to circumvent the normal requirement for SIR1.

GBD/rapl hybrids display no defect in targeted silencing

Because the silencing-defective rap1 hybrids [Sussel and Shore 1991] fall within the sequences contained in all of the functional GBD/RAP1 hybrids described above, we decided to introduce the strongest of these mutations [rap1-12, a double mutation at positions 726 and 727] into the GBD/RAP1[653–827] hybrid and assay its effect on targeted silencing at HMR [see Materials and methods]. The construct encoding the mutant hybrid, which we will refer to as GBD/rapl-12(653–827), was integrated at the chromosomal SIR1 locus in a strain containing the AAE::[UASp]3 hmr::TRP1 reporter gene to give strain YSB110. We chose the ΔAAE silencer because the rap1-12 mutation displays its phenotype only in the context of an A site mutation [Sussel and Shore 1991]. Targeting of the GBD/rapl-12(653–827) hybrid to a ΔAAE::[UASp]3 silencer was expected to mimic the action of full-length rap1-12 at a ΔAA silencer. Surprisingly, we found that this GBD/rapl-12 hybrid displayed no defect in silencing [Fig. 5A, cf. rows 4 and 6].

We also showed that a set of longer GBD/rapl-12 hybrids [with amino-terminal endpoints at amino acid 562, 588, 618, or 630] can establish silencing in both types of double-deletion silencer, hmr::TRP1 strains [YSB2 and YSB35], as judged by at least a 500-fold decrease in the ability to form colonies in the absence of tryptophan [data not shown]. Because all of these longer hybrids are transcriptional activators [Hardy et al. 1992a], we conclude that the ability of the GBD/rapl-12(653–827) hybrid to silence cannot be explained by the absence of a potentially competing activation domain.

Chromosomal rap1-12 mutation abolishes the function of GBD/RAP1 but not GBD/rapl hybrids

The ability of the GBD/rapl-12 hybrids to establish repression when targeted to HMR led us to investigate if the chromosomal rap1-12 mutation could affect GBD/RAP1/RAP1-dependent silencing. To do this, we crossed a strain containing a marked rap1-12::URA3 allele [rap1-12::URA3] to a ΔAAE::[UASp]3, hmr::TRP1 reporter strain containing the GBD/RAP1[653–827] hybrid gene integrated at HIS3. The resulting diploid was sporulated and dissected to obtain strain YSB74. Strikingly, we found that this strain and several other isogenic segregants containing the rap1-12 allele [Ura+] were completely defective in GBD/RAP1-dependent silencing, whereas the corresponding RAP1 segregants retained the ability to carry out targeted silencing [Fig. 5A, cf. rows 4 and 5].

Using the same strategy outlined above, we examined the effect of the rap1-12 mutation on silencing established by GBD/rapl-12[653–827]. Surprisingly, the mutant hybrid protein was unaffected by the rap1-12 mutant background, silencing equally well in both the RAP1 and rap1-12 strains [Fig. 5A, rows 6 and 7]. In other words, the rap1-12 mutation in the hybrid protein was able to suppress the targeted silencing defect caused by the very same amino acid substitutions in the chromosomal RAP1 gene [Fig. 5A, cf. rows 5 and 7].

Figure 4. Targeted silencing by GBD/RAP1 [653–827] is abolished in sir2, sir3, and sir4 mutants but is unaffected by mutation of SIR1. The SIR+ control strain in the first two rows is YSB2. Mutation of SIR2, SIR3, and SIR4 in this strain background give the same fully derepressed phenotype [data not shown]. The sir1, sir2, sir3, sir4 mutant derivatives of YSB2 in rows 4–7 are YSB160, RS1042, RS1061, and RS1067, respectively. Row 3 shows the effect of the sir1 mutation on native silencing at hmrΔA [YDS702] for purposes of comparison. The Trp plating assay was performed as in Fig. 3.
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**Figure 5.** Effects of the *rap1-12* and *rif1::URA3* mutations on GHB/*RAP1(653–827)*- and GHB/*rap1-12(653–827)*-dependent silencing of an hmr:TRP1 reporter. Silencing was assayed as described in Fig. 3. The strains used in these experiments are described in the text. (A) Targeted silencing by GHB/*RAP1(653–827)* but not GHB/*rap1-12(653–827)* is abolished by a chromosomal *rap1-12* mutation. Targeted silencing by GHB/*RAP1(653–827)* [rows 4,5] or GHB/*rap1-12(653–827)* [rows 6,7] hybrids was examined in strains in which the chromosomal copy of *RAP1* was either wild-type [rows 4,6] or contained the *rap1-12* allele [rows 5,7]. Rows 3 and 8 show that neither the wild-type nor the mutant hybrid can function in the absence of UASG sites at the silencer. Row 1 shows native silencing at an hmrA::TRP1 locus, and row 2 shows the effect of the *rap1-12* allele on this reporter for purposes of comparison with targeted silencing. (B) A *rif1::URA3* gene disruption does not abolish GHB/*RAP1(653–827)* [rows 4,5] or GHB/*rap1-12(653–827)* [rows 6,7] targeted silencing. The effect of the *rif1::URA3* mutation on native silencing at hmrA::TRP1 [rows 1,2] is shown for comparison.

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Because *rif1* mutants share many of the phenotypes of *rap1* mutants and GHB/*rap1* hybrid proteins are defective in interacting with RIF1 in the two-hybrid system [Hardy et al. 1992b], we also tested the effect of a *rif1* mutation on targeted silencing. In a similar set of genetic crosses, targeted silencing strains containing a *rif1::URA3* mutation were generated. In this case, we found that GHB/*RAP1(653–827)*-mediated silencing was unaffected by *rif1* mutation, because it was indistinguishable from that observed in a RIF1 isolate derived from the same cross (Fig. 5B, cf. rows 4 and 5). The action of the GHB/*rap1-12(653–827)* hybrid may have been weakened very slightly by the *rif1* mutation (Fig. 5B, cf. rows 6 and 7).

*Increasing telomere length diminishes silencing at HMR*

The data described above reveal two puzzling features of the *rap1-12* mutation, both of which seem to be inconsistent with the idea that the *rap1-12* mutation has a direct effect on *HMR*. First, when targeted to an HMR-*E* silencer mutated for the A element, GHB/*rap1-12* hybrids display no silencing defect [Fig. 5A, data not shown]. Second, a chromosomal *rap1-12* mutation has a profound effect on targeted silencing by wild-type GHB/*RAP1(653–827)* despite the absence of a RAPI binding site at the HMR-*E* silencer being tested [Fig. 5A]. One explanation for the second observation is that the *rap1-12* protein exerts an indirect effect from some other sites in the chromosome that results in weakened repression at HMR. The telomeres are excellent candidates for such sites because it is known that RAPI binds to multiple sites at telomeres [Longtine et al. 1989; Gilson et al. 1993], where it controls both telomere repeat tract length and TPE (Lustig et al. 1990; Sussel and Shore 1991, Kyron et al. 1992, 1993). Furthermore, *rap1* mutations are known to increase telomere length and, presumably, the number of RAP1 molecules bound to these sites [Sussel and Shore 1991]. We thus decided to determine whether increased telomere length alone (in *RAP1* wild-type cells) is able to diminish targeted silencing at *HMR*.

To produce RAPI cells containing elongated telomeres, we took advantage of the *rap1-17* mutation, a *rap1* allele that results in a truncation of the RAPI carboxyl terminus and a pronounced telomere elongation phenotype [Kyron et al. 1992]. When a *rap1-17* strain is mated to a *RAP1* wild-type strain, the resulting diploid contains elongated telomeres that are inherited by RAPI haploid cells after sporulation. These elongated telomeres increase TPE in the *RAP1* wild-type cells, indicating that telomere length itself influences the strength of TPE [Kyron et al. 1993]. To increase telomere length in strains undergoing targeted silencing at *HMR*, we crossed strain AJL394-2a [*rap1*] to both YSB80 [expressing GHB/*RAP1(653–827)*] and YSB282 [expressing GHB/*rap1-12(653–827)*]. The resulting diploid strains were sporulated, and haploid segregants were obtained that had a wild-type RAPI allele and both the cis and trans components of the targeted silencing system. Approximately half of the poly(C3A) telomere tracts in these strains should be extremely elongated, and this was confirmed by Southern blotting [Fig. 6A].

We noticed two different effects on targeted silencing in these strains. First, there was a reproducible 10- to 100-fold decrease in targeted silencing by the GHB/*RAP1(653–827)* hybrid in strains containing elongated telomeres when compared with isogenic strains derived from an identical genetic cross to a RAPI control strain.
Figure 6. Elongated telomeres antagonize hmr::TRP1 silencing in a reversible and dosage-dependent manner. Silencing was assayed as described in Fig. 3. Elongated telomeres are inherited from a rap1⁻ parent strain in a genetic cross, and the dosage of elongated telomeres is predicted from the genotype of the parent strains (see text). Telomeres of relevant strains were analyzed by Southern blotting (shown at right of the silencing assays), and in many cases genomic DNA was treated with the exonuclease BAL31 before restriction digestion to demonstrate that bands with altered mobilities actually represent telomeric sequences. Numbers above the lanes correspond to the numbered rows in the silencing assay. Size markers are shown at left of the Southern blot (in kilobase pairs). (A) Elongated telomeres antagonize both Gap/RAP1(653–827) targeted silencing (rows 3–5) and hmrΔA silencing (rows 10–12) but have little effect on Gap-RAP1(653–827) targeted silencing (row 7). (*) Targeted silencing by Gap/RAP1(653–827) in a rap1-12 strain is shown (row 8) for purposes of comparison (see also Fig. 5A, row 5). In this strain all of the telomeres are elongated because of the rap1-12 mutation. The new strains used in this figure are as follows: Rows 2–7 are YSB284, YSB285, YSB286, YSB287, YSB288, and YSB289, respectively; rows 9–12 are YSB290, YSB291, YSB292, and YSB293, respectively; lane C is AJL275-2a (a RAP1 control), and the rap1⁻ lanes are AJL294-2a. [B] Derepression of hmrΔA by elongated telomeres is reversed by continuous subculturing and is sensitive to the dosage of elongated telomeres. YSB291 was grown in liquid YEPD medium at 30°C and at various time points assayed for silencing and telomere length (rows 2–7). Elongated telomere dosage of YSB293 was altered by genetic crosses (see text) to create strains YSB296, YSB297, and YSB298 (rows 8–10, respectively).
Consistent with the characterization of the rap1-12 mutation, elongated telomeres do not affect either the wild-type or the AB HMR-E silencer (data not shown). We performed two additional experiments to test the idea that telomere elongation, and, hence, increased amounts of RAP1-binding poly(C3A) sequence, is responsible for diminished silencing at HMR. First, we continuously subcultured strain YSB291, shown in Figure 6A, which results in a gradual (~2 bp per generation) shortening of the elongated telomeres derived from the rap1+ parent (Kyrion et al. 1992). During the course of this subculturing, we observed a continuous improvement in targeted silencing at HMR that correlated with the progressive loss of telomeric poly(C3A) sequences [Fig. 6B, rows 2–7]. In a separate experiment, we performed genetic crosses to generate strains containing different numbers, on average, of elongated telomeres. If telomeric C3A repeats act in trans to derepress HMR, then increasing the dosage of elongated telomeres would be expected to create a more severe phenotype, whereas a reduction of elongated telomeres should have the opposite effect. To increase the dosage of elongated telomeres, strain YSB293 [in which approximately one half of the telomeres should be elongated] was crossed to AJL440-1c (rap1+) and sporulated to generate haploid segregants containing, on average, a 0.75 dosage of elongated telomeres. All nine such segregants examined displayed a more severe silencing defect than the parent strain [YSB293] or a strain derived from a control cross [YSB297] (Fig. 6B, row 10; data not shown). Similarly, YSB293 was crossed to a RAP1 strain, and haploid segregants were obtained that should have an average 0.25 contribution of elongated telomeres. All 11 such segregants examined displayed clearly stronger repression than the YSB293 parent [Fig. 6B, row 8; data not shown].

These data further support the idea that the overall amount of telomeric poly(C3A) sequence determines the extent of derepression at HMR and argues against the possibility that the effect may be the result of some specific change at the right arm of chromosome III, where the HMR locus is ~25 kb from the telomere [Oliver et al. 1992].

**SIR4 protein is limiting in targeted silencing by Gbd1/RAP1 hybrids**

Initial characterization of the rap1-12 mutation showed that it can be suppressed by elevated gene dosage of either SIR1 or SIR4 [Sussel and Shore 1991]. This result is striking in the case of SIR4 because the effect of extra copies of this gene in a RAP1 wild-type strain is actually derepressing. To examine the effect of increased SIR1 and SIR4 gene dosage on targeted silencing in rap1-12 strains, we initially introduced the SIR1 or SIR4 genes on multicopy [2-μm origin] plasmids. No improvement in the ability of the Gbd1/RAP1[653–827] hybrid to silence in a rap1-12 strain was observed [data not shown]. Furthermore, elevated SIR4 dosage abolished targeted silencing by Gbd1/RAP1[653–827] and Gbd1/rap1-12[653–827] hybrids in a RAP1 background and by the Gbd1/rap1-12[653–827] hybrid in a rap1-12 background. The SIR1 plasmid had no effect in any of these conditions. We then examined the effect of adding the SIR4 and SIR1 genes on a low-copy [CEN] plasmid, because it had been shown previously that a single extra copy of SIR4 is sufficient to suppress the rap1-12 mutation [Sussel et al. 1993]. This modest increase in SIR4 gene dosage improved Gbd1/RAP1[653–827] silencing [in a RAP1 background] to the level produced by native RAP1 at a hmrΔA silencer [Fig. 7, row 2; cf. with 5A, row 1]. Furthermore, the SIR4/CEN plasmid partially suppressed the defect caused by the chromosomal rap1-12 mutation, enabling Gbd1/RAP1[653–827] to give ~50- to 100-fold repression in the TRPL reporter assay [Fig. 7, row 4]. The CEN–SIR1 plasmid had no effect in either of these strains [Fig. 7, row 3; data not shown].

The rap1-12 mutation improves telomeric silencing and has a strengthened interaction with SIR4

The experiments described above suggest that diminished targeted silencing in rap1+ strains may be attributed at least in part to telomere elongation. If telomere elongation in rap1-12 strains results in a competition for limiting silencing factors with HMR, one might expect to observe improved telomeric silencing in these strains. To test this idea, we constructed ΔA hmr TRP1 strains in which the telomere on the left arm of chromosome VII is altered to contain a URA3 gene followed by terminal (C3A) repeat sequences at the ADH4 locus [Gottschling et al. 1990]. The telomeric URA3 reporter gene is subject to silencing [TPE] that results in repression of the gene in ~50% of the cells in a culture. This variegated repression can be best quantified by measuring the ability of these cells to grow in the presence of 5-fluoro-orotic acid (5-FOA), which kills cells expressing the URA3 gene [FOA+ or repressed phenotype]. As shown in Table 2, telomeric repression of URA3 is significantly improved in rap1-12 strains compared with isogenic RAP1+ strains, as indicated by both an increased ability to grow in the presence of FOA and re-

**Figure 7.** The SIR4 gene on a CEN plasmid improves Gbd1/RAP1[653–827]-mediated silencing in a RAP1 strain (YSB107). SIR4-CEN, but not SIR1-CEN, partially suppresses the target-silencing defect of Gbd1/RAP1[653–827] in a rap1-12 strain (YSB143). Rows labeled vector represent strains transformed with the CEN vector alone, whereas those marked SIR1 or SIR4 are strains containing the CEN vector with the indicated SIR gene.
Table 2. Effect of the rap1-12 mutation on telomere position effect

| Strain | RAP1 allele | Growth on ~Ura* (%) | Growth on FOA* (%) |
|--------|-------------|---------------------|-------------------|
| YSB215 | RAP1+       | 37.6 [7.2]          | 56.4 [13.2]       |
| YSB216 | rap1-12     | 9.8 [2.7]           | 95.4 [6.3]        |

*Compared to an equal aliquot of cells plated on YEPA [rich] medium. The values in parentheses are standard deviations. The values are averages from measurements of six independent colonies from each strain.

Table 3. Interaction of GAD/SIR4 with GBD/RAP1 and GBD/rap1 hybrids

| DNA-binding domain hybrid | Activation domain hybrid | β-Gal units | RIF1 | rif1::URA3 |
|---------------------------|-------------------------|-------------|------|-----------|
| LexA/RAP1[635–827]        | GAD alone               | 7 [2]       | 94   [5] |
| LexA/RAP1[635–827]        | GAD/SIR4                | 89 [6]      | 270  [11] |
| LexA/rap1-12[635–827]     | GAD alone               | 15 [2]      | 29   [2] |
| LexA/rap1-12[635–827]     | GAD/SIR4                | 1913 [28]   | 1360 [123] |

*GAD/SIR4 was isolated from a library of yeast genomic partial Sau3A fragments cloned into the pGAD3 vector [Chien et al. 1991]. [The library was a generous gift of P. Bartel and S. Fields.] The GAD/SIR4 hybrid consists of amino acids 1204–1358 of SIR4 fused in-frame to the GAL4 activation domain [Moretti et al. 1994].

The use of GBD/RAP1 hybrids has allowed us to define a minimal domain of RAP1 [amino acids 667–827] that is
sufficient to establish transcriptional silencing, in the absence of any other RAP1 sequences, when targeted to the HMR locus. This conclusion is consistent with previous results showing that point mutations in this region [rap1*] and overexpression of the wild-type domain cause derepression of HMR [Susse and Shore 1991; Hardy et al. 1992a] and that truncations of this domain abolish silencing at HML and telomeres [Kyron et al. 1993]. The dependence on SIR2, SIR3, and SIR4 function indicates that the effect of GBD/RAP1 hybrids is bona fide silencing rather than a nonspecific effect on the HMR locus. It is worth pointing out that the GBD/RAP1L653-827 hybrid is incapable of acting as a transcriptional activator when targeted to the promoter of a reporter gene [Hardy et al. 1992a]. The fact that this hybrid still requires SIR2, SIR3, and SIR4 in silencing rules out a model in which the only role for one (or all) of these silencing factors is to block the activation function of RAP1 at HMR.

Several of the GBD/RAP1 hybrids described here contain a RAP1 transcriptional activation domain [Hardy et al. 1992a] yet still function as repressors when targeted to HMR. This result reinforces the conclusion from previous studies that the activity of RAP1 is context dependent [Buchman et al. 1988b; Shore and Nasmyth 1987] and shows for the first time that a small carboxy-terminal part of the protein (exclusive of its DNA-binding domain) is sufficient to carry out the correct context-dependent function [activation or silencing] when fused to a heterologous DNA-binding domain. Exactly how the HMR context influences the action of GBD/RAP1 hybrids is unknown. It seems unlikely that the A or B sites at HMR-E are strictly required to promote RAP1’s silencing function nor are they necessary to antagonize the activation function of RAP1, as GBD/RAP1 hybrids are able to establish repression in their absence. However, both the A and B sites contribute to targeted silencing when GAL4-binding sites are limiting, suggesting that they either cooperate with GBD/RAP1 or act independently to establish silencing.

SIR1-dependent and SIR1-independent silencing

SIR1 is required for stable repression at HM loci [Rine and Herskowitz 1987; Pillus and Rine 1989] yet has no apparent role in TPE [Aparicio et al. 1991]. It is thus somewhat surprising that targeted silencing by GBD/RAP1 hybrids at HMR is completely independent of SIR1 function. Using the same targeting strategy described here, it was shown previously that a GBD-SIR1 hybrid can function at the HMR-E silencer to establish repression, leading to the suggestion that HM silencers have a special mechanism to recruit SIR1 that is missing from telomeres [Chien et al. 1993]. Considered in this light, the SIR1 independence of targeted silencing by GBD/RAP1 hybrids at HMR-E may be the result of a failure to recruit SIR1 protein to the mutated silencer. This notion is supported by the additional observations that elevated SIR1 gene dosage neither improves silencing by GBD/RAP1 hybrids nor does it suppress the defect in targeted silencing in a rap1-12 strain. In contrast, extra copies of SIR1 can suppress the silencing defect of a rap1-12 hmrΔA strain [Susse and Shore 1991] and several other cis- and trans-acting mutations affecting HMR [Stone et al. 1991]. Although the precise reason for this is unclear, it seems that some feature of GBD/RAP1 hybrids that differs from native RAP1 must explain this observation. Several possibilities can be considered. The GBD/RAP1 hybrids studied here may display the RAP1 carboxyl terminus in a way that is unfavorable for SIR1 binding to HMR-E. Alternatively, these RAP1 hybrids may be lacking sequences required to localize SIR1 to the HMR-E silencer. For example, SIR1 may make contacts with the RAP1 DNA-binding domain, which is not present on our GBD/RAP1 hybrids. Another possibility is that DNA bending induced by the RAP1 DNA-binding domain [Vignais and Sentenac 1989; Gilson et al. 1993] is important for SIR1 recruitment at HMR-E and is not provided by the GAL4 DNA-binding domain in the GBD/RAP1 hybrids.

Whatever the explanation for the SIR1 independence of GBD/RAP1 silencing, it is clear that the role of GBD/RAP1 hybrids is not to recruit SIR1 to the silencer. In fact, GBD/RAP1 and GBD-SIR1 hybrids seem to be functionally redundant. We consider two general models to explain these observations. The first model proposes that the two hybrids carry out the same function, that is, to recruit silencing factors [e.g., SIR3 and SIR4] to HMR [Moretti et al. 1994]. An opposing view is that GBD-SIR1 possesses an establishment mechanism distinct from the function of the carboxy terminus of RAP1. Several properties of the two silencing systems are consistent with the latter model. Overexpression of GBD/RAP1 hybrids has a dominant-negative effect on silencing at HMR, probably by titration of SIR3 and SIR4, whereas GBD= SIR1 has no such effect. In addition, although the rap1-12 mutation is antagonistic to GBD/RAP1 silencing, it has no effect on silencing by GBD-SIR1 [data not shown]. Taken together, the data in this report indicate that a special feature of the HMR-E silencer is required for SIR1 function and provide further support for the idea that a normal site of action for SIR1 is the HMR-E silencer. The targeted silencing system described here clearly lacks the ability to utilize SIR1 and therefore shares several properties in common with telomeric silencing.

rap1* mutations reveal an underlying competition between HMR and telomeric silencing

The rap1-12 mutation is one of four rap1* alleles identified originally in a screen for RAP1 mutations that result in derepression of the HMR silent locus. It only displays this phenotype when the redundancy of the HMR-E silencer is eliminated by mutation of the ACS [A element] at HMR-E [Susse and Shore 1991]. The observation that mutation of the RAP1-interacting factor RIF1 also creates an hmrΔA-specific silencing defect led to the proposal that rap1* mutations [of which rap1-12 is the most severe] create a defect in the ability of RAP1 to recruit RIF1 to silencers, which results in a loss of repression when the silencer is weakened by the ΔA mutation.
Consistent with this idea, the GBD/rap1 hybrid hybrids display a decrease in RIF1 binding in the two-hybrid system that is proportional to the severity of the corresponding rap1 mutation in silencing.

Characterization of the rap1-12 mutation reported here resulted in several unexpected findings that challenge this interpretation of the rap1 and rif1 mutations. First, we showed that targeting of the GBD/rap1-12(653-827) hybrid to HMR-E does not result in a loss of silencing as predicted by the model. In fact, targeting the rap1-12 mutant often improves the efficiency of repression relative to the wild-type GBD/RAPI hybrid (Figs. 5 and 6). Second, the presence of the rap1-12 mutation in the chromosome prevents silencing by GBD/RAPI(653-827), even though the silencers tested lack a binding site for native rap1-12 protein. Finally, we showed that the rap1-12 mutation, which abolishes repression by an hmrΔA silencer, actually improves telomeric silencing.

On the basis of these new results, we propose that the effects of the rap1-12 mutation at telomeres lead to a loss of repression at the hmrΔA silencer. Several additional observations suggest that a simple competition model may account for this shift in the balance between HMR and telomeric silencing. In rap1-12 strains, the telomeric C1-3A repeats, which contain a high density of RAPl-binding sites (Gilson et al. 1993), increase in length by ~50% (Sussel and Shore 1991). Previous studies (Kyrion et al. 1993) showed that telomere elongation in wild-type strains results in improved TPE, similar to that which we have observed in rap1-12 strains. In addition, we showed that an increase in telomere length in RAP1 cells impairs both targeted and native silencing at HMR when the ACS at the HMR-E silencer is mutated. Therefore, the telomere elongation phenotype of the rap1-12 mutation might, in principal, be sufficient to explain the effect of this mutation at HMR. Taken together, these data suggest that in rap1-12 strains telomeres may compete more effectively for silencing factors present in limiting amounts and thereby diminish repression at HMR. It is worth noting at this point that the effect of the rap1-12 mutation may result from more than just telomere elongation (see below). One indication of this is that RAP1 wild-type cells containing elongated telomeres appear to have greater amounts of poly(C1-3A) sequence than rap1-12 strains yet display a weaker HMR derepression phenotype (Fig. 6).

One apparent paradox of our results is the ability of targeted GBD/rap1-12(653-827) to silence at HMR in rap1-12 strains, a condition analogous in many respects to a normal rap1-12 strain where the mutant rap1-12 protein fails to establish repression at an hmrΔA silencer. We believe that a likely explanation for this result is that the targeting system provides multiple copies of the RAP1 carboxyl terminus in tandem at HMR-E, where the GBD/rap1-12(653-827) hybrid can successfully compete for a limiting silencing factor, most likely SIR4 protein (see below). Even silencers containing only a single UASG probably allow for the binding of a GBD/RAPI dimer, whereas a native silencer contains only one binding site for RAP1. In support of this argument, we have shown clearly that the targeting system establishes silencing more efficiently when there are multiple UASG sites at HMR-E. A testable prediction of this model is that multiple RAP1 binding sites would improve silencing at HMR in rap1 strains.

The role of SIR4 and RIF1 in the regulation of silencing at HMR and telomeres

Several lines of evidence suggest that derepression at HMR in rap1-12 strains results from an inability of the hmrΔA silencer to recruit a limiting amount of SIR4 protein, which becomes sequestered at telomeres. To begin with, silencing at HMR is extremely sensitive to the gene dosage of SIR4: Diploid strains containing only one copy of the gene display unstable repression, whereas extra copies of SIR4 (on a 2-μm plasmid) are sufficient to suppress the silencing defect of rap1 mutants (Sussel and Shore 1991; Sussel et al. 1993). As shown here, the defect in GBD/RAPI(653-827) targeted silencing in a rap1-12 strain is also relieved by increasing SIR4 dosage. Furthermore, RAP1 interacts with SIR4 in the two-hybrid system (Moretti et al. 1994), and this interaction is significantly strengthened when the RAP1 hybrid contains the rap1-12 missense mutation (Table 3). All of these data, together with the effect of telomere elongation (Sussel and Shore 1991), support the idea that SIR4 is concentrated at telomeres in rap1-12 strains, reducing by mass action the amount available for binding to HMR.

This new interpretation of the rap1 phenotype suggests, in turn, a different view of the role of RIF1 in silencing (Hardy et al. 1992b). We propose, as suggested previously (Kyrion et al. 1993), that RIF1 acts as a negative regulator of RAP1’s silencing function at telomeres by binding to the RAP1 carboxyl terminus and interfering with RAP1-SIR interactions. Consistent with this notion, rif1 mutants display improved telomeric silencing reminiscent of that seen in rap1 strains (Kyrion et al. 1993). The idea that RIF1 interferes with RAP1-SIR interactions is supported by two-hybrid results reported here and elsewhere (Moretti et al. 1994). We imagine that the effect of rif1 mutations on hmrΔA silencing is therefore the result of telomere competition for a limiting amount of SIR4, as outlined above. The SIR1-dependent form of silencing that normally operates at HM loci is apparently resistant to any negative effect of RIF1, perhaps by preventing access of RIF1 to the silencer. Alternatively, it is conceivable that RIF1 has an opposite effect at HMR, that is, to promote silencing. At present, we cannot distinguish between these two possibilities.

It is important to emphasize that although the loss of RIF1 binding and telomere elongation might in principle be sufficient to explain the rap1 phenotype, several observations suggest that the effect of this mutation is more complex. For example, in the two-hybrid assay for the RAP1-SIR4 interaction, a large difference is still observed between GBD/RAPI(653-827) and GBD/rap1-12(653-827) hybrids in cells lacking RIF1. In addition, a rif1 mutation has a significantly weaker effect on tar-
pressed chromatin. At telomeres, repression is limited to a weakened silencer, specifically the presence of the ACS, may play an important role in preventing the normal fluctuations of all UASG-containing mutated silencers. Details of the characterization of the rapl-12 mutation suggests that this hierarchy may also result from two other effects: negative regulation of RAP1 at telomeres by RIF1 and a limiting amount of SIR proteins available to establish repressed chromatin. At telomeres, repression is limited by the availability of SIR3 [Renauld et al. 1993], whereas a weakened HMR locus readily becomes limited for SIR4 protein. We suggest that yeast are restricted in the total amount of SIR-repressed chromatin that they can form. This limitation could have significance for the proper regulation of silencing, perhaps controlling the distance that a particular silenced domain spreads and preventing genes from becoming inappropriately repressed. Our results also suggest that the redundancy of the HMR-E silencer, specifically the presence of the ACS, may play an important role in preventing the normal fluctuations in telomere repeat length from causing derepression of the HMR mating-type locus.

Materials and methods

Yeast strains and methods

Standard methods of yeast genetics and molecular biology were used throughout [Rose et al. 1990]. The yeast strains used in this study are listed in Table 1. The same UASc oligonucleotide [TCGACGGAGGACAGTCCTCCCG and its complement TCGACGGAGGACTGTCCTCCCG] was used in the construction of all UASc-containing mutated silencers. Details of the silencer deletions (all of which are marked by a Xhol linker) can be found in Brand et al. (1987). UASc insertions were confirmed by DNA sequence before transplacement into the HMR locus [Brand et al. 1985]. All transplacements were confirmed by Southern blotting. Assays for silencing using the hmr::TRP1 reporter were performed by spotting 10-fold serial dilutions of cultures grown in liquid YEPD medium or appropriate synthetic (SC) selective medium as described [Susse and Shore 1991].

Plasmid DNA

All GRD/RAP1 constructs contained the native RAP1 promoter upstream of GAL4 coding sequences. GAD/RAP1 fusions in the HIS3 integrating vector pRS303 [Sikorski and Hieter 1989] as EcoRI–XhoI fragments. LexA/RAP1 and LexA/rapl-12 hybrid proteins were expressed from plasmid pBTM116 (2-μm origin, TRP1, pADH1–LexA, a gift of P. Bartel and S. Fields, State University of New York, Stony Brook). The SIR4 2-μm plasmid [LS357] consists of an EcoRI–SalI fragment of SIR4 cloned into pRS426 [Sikorski and Hieter 1989]. SIR4/CEN and SIR1/CEN plasmids consist of EcoRI–SalI and KpnI–HindIII fragments, respectively, cloned into pRS426 [Sikorski and Hieter 1989].

RNA analysis

Total yeast RNA was prepared and analyzed by Northern blotting using standard procedures [Ausubel et al. 1987]. hmr a1 mRNA was detected by probing with a random-primed 650-bp XhoI–BglII DNA fragment from D401 (Xhol linker mutant number 238, Abraham et al. 1984). The blot was reprobed with a 500-bp EcoRI–HindIII fragment from the actin gene.

Detection of poly(C;~3 A) telomeric repeats

Telomeric poly(C;~3 A) repeats were detected by Southern blotting using standard techniques [Ausubel et al. 1987] with a probe made by nick-translation of poly(CGdT) [Pharmacia]. Yeast genomic DNA (20 μg) was incubated in BAL 31 buffer at 30°C for 2 hr with or without 0.5 units of BAL 31 enzyme [New England Biolabs] in a volume of 80 μl. Reactions were stopped by the addition of 16 μl of 0.2 M EGTA. DNA was precipitated by adding 50 μl of 3.5 M ammonium acetate and 150 μl of isopropanol. The DNA pellet was washed twice with 70% ethanol, resuspended in TE, and digested with Xhol. DNA fragments were resolved on 0.8% agarose gels and transferred to nitrocellulose for hybridization.

β-Galactosidase assays

LexA/RAP1[635–827] and LexA/rapl-12[635–827] plasmids were cotransformed with a GAD/SIR4 or a pGAD control plasmid into the CTY10-5D two-hybrid reporter strain (a gift of C.-t. Chien and R. Sternglanz, State University of New York, Stony Brook). Transformants were grown in selective media with 0.05% glucose for 40 hr. Five milliliters of cells were pelleted and resuspended in 250 μl of Z buffer (60 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 0.27% β-mercaptoethanol) and permeabilized by vortexing 3 × 1 min with an equal volume of 0.45-mm glass beads. The supernatants were assayed for protein concentration and β-galactosidase activity as described previously [Breeden and Nasmyth 1985].

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S W Buck and D Shore

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