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

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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 (G_{BD}) and targeted to mutated *HMR* silencers containing GAL4 DNA-binding sites. Silencing by G_{BD} /RAP1 hybrids, like normal silencing at *HMR*, requires the *trans*-acting factors SIR2, SIR3, and SIR4. However, G_{BD} /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^s* mutations. The incorporation of a *rap1^s* missense mutation into G_{BD} /RAP1 hybrids can improve targeted silencing, yet wild-type G_{BD} /RAP1 hybrids fail to establish repression in strains in which the endogenous *RAP1* locus carries a *rap1^s* mutation. In addition, we find that telomeric silencing is increased in *rap1^s* strains. We propose that the *rap1^s* 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^s* 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; Kyrion 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

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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 telomere 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 $C_{1-3}A$ 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($C_{1-3}A$) 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^s* alleles, result in derepression of *HMR* when the *HMR-E* silencer is weakened by mutation of the ACS (*hmrΔA*) (Sussel and Shore 1991). The *rap1^s* mutations also result in elongation of the poly($C_{1-3}A$) tracts at telomeres by as much as 50%. A detailed characterization of the *rap1^s* mutants 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 dere-

press *HMR* only when the *HMR-E* silencer is mutated (*hmrΔA*) (Kyrion et al. 1993; Moretti et al. 1994). Severe *RAP1* truncation alleles (*rap1^t*) display dramatic increases in telomere tract length (>10-fold). However, this alteration of telomere structure does not explain the loss of TPE in *rap1^t* mutants. In fact, when elongated telomeres are inherited by *RAP1* wild-type spores after a cross with a *rap1^t* 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 (G_{BD})/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). G_{BD} /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^s* mutants, display a *hmrΔA*-specific silencing defect. In addition, *rap1^s* 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 G_{BD} /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 G_{BD} /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, G_{BD} /*rap1^s* hybrids display no defect in targeted silencing, whereas a chromosomal *rap1^s* mutation abolishes the ability of G_{BD} /RAP1 (but not G_{BD} /*rap1^s*) 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 G_{BD} (Hardy et al. 1992a) and are expressed from the *RAP1* promoter. To assay the activity of

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 ΔA and ΔB silencers are completely functional, whereas the double-mutant silencers $\Delta A\Delta E$ and $\Delta E\Delta B$ and the triple-mutant silencer ($\Delta A\Delta E\Delta B$) are derepressed.

these hybrids, we used three types of strains containing GAL4-binding sites (UAS_G) 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 $\Delta A\Delta E::(UAS_G)_n$ and $\Delta E\Delta B::(UAS_G)_n$, respectively, where n indicates the number of GAL4-binding sites inserted within the silencer deletion. In the third type, $\Delta A\Delta E\Delta B::(UAS_G)_n$, 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 UAS_G -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 re-

press 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 UAS_G 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 UAS_G -containing silencers. The G_{BD} control plasmid failed to confer repression in all cases. Control strains lacking UAS_G 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 UAS_G sites at the silencer. For example, a single site in the $\Delta A\Delta E$ 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 $\Delta A\Delta E\Delta B$ silencer requires more UAS_G sites than are needed at silencers containing either A or B. The triple-deletion silencer gives only partial repression with three copies of UAS_G . 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. The results from the RNA blot were confirmed by measuring the mating efficiency of these strains, a biological assay for silencing. We found a strict correlation between the decrease in *a1* mRNA levels in these strains and their ability to mate with a *MATa* haploid tester strain (data not shown).

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 mutant silencer contexts ($\Delta A\Delta E$, $\Delta E\Delta B$, or $\Delta A\Delta E\Delta B$) with or without UAS_G 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 $>10^3$ -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 ^a	Source
W303-1B	<i>HMLαMATα HMRa ade2-1 ura3-1 his3-11,15leu2-3,112 trp1-1 can1-100</i>	R. Rothstein (pers. comm.)
YSB1	W303-1B <u>$\Delta A \Delta E$</u> <i>hmr::TRP1 ga14::LEU2</i>	Chien et al. (1993)
YSB2	YSB1 except <u>$\Delta A \Delta E::[UAS_G]_3$</u>	Chien et al. (1993)
YSB4	YSB1 except <u>$\Delta A \Delta E::[UAS_G]_1$</u>	Chien et al. (1993)
YSB35	YSB1 except <u>$\Delta E \Delta B::[UAS_G]_3$</u>	Chien et al. (1993)
YSB44	W303-1B <u>$\Delta A \Delta E::[UAS_G]_1$</u> <i>ga14::LEU2</i>	this study
YSB46	YSB44 except <u>$\Delta A \Delta E[UAS_G]_3$</u>	this study
YSB48	YSB44 except <u>$\Delta A \Delta E[UAS_G]_4$</u>	this study
YSB50	YSB44 except <u>$\Delta A \Delta E[UAS_G]_7$</u>	this study
YSB54	YSB44 except <u>$\Delta A \Delta E[UAS_G]_2$</u>	this study
YSB62	YSB44 except <u>$\Delta A \Delta E \Delta B[UAS_G]_3$</u>	this study
YSB66	YSB44 except <u>$\Delta A \Delta E \Delta B[UAS_G]_4$</u>	this study
YSB68	YSB44 except <u>$\Delta A \Delta E$</u>	this study
YSB70	YSB44 except <u>$\Delta E \Delta B$</u>	this study
YSB72	YSB44 except <u>$\Delta A \Delta E \Delta B$</u>	this study
YDS31	<i>MATα his1</i>	K. Nasmyth (pers. comm.)
YDS655	W303-1B except <i>HIS3</i>	R. Rothstein (pers. comm.)
YSB256	W303-1B except <u>$\Delta A \Delta E[UAS_G]_3$</u> <i>hmr::TRP1 HIS3::G_{BD} ga14::LEU2</i>	this study
YSB107	YSB256 except <i>HIS3::G_{BD}/RAP1(653–827)</i>	this study
YSB114	YSB107 except <u>$\Delta A \Delta E$</u> <i>hmr::TRP1</i>	this study
YSB127	YSB256 except <u>$\Delta E \Delta B[UAS_G]_3$</u> <i>hmr::TRP1</i>	this study
YSB123	YSB127 except <i>G_{BD}/RAP1(653–827)</i>	this study
YSB140	YSB123 except <u>$\Delta E \Delta B$</u> <i>hmr::TRP1</i>	this study
YSB103	YSB107 except <u>$\Delta A \Delta E \Delta B[UAS_G]_3$</u> <i>hmr::TRP1</i>	this study
YSB102	YSB103 except <i>HIS3::G_{BD}/RAP1(653–827)</i>	this study
YSB252	YSB107 except <i>HIS3::G_{BD}/RAP1(667–827)</i>	this study
YSB253	YSB107 except <i>HIS3::G_{BD}/RAP1(679–827)</i>	this study
YSB254	YSB107 except <i>HIS3::G_{BD}/RAP1(702–827)</i>	this study
YSB255	YSB107 except <i>HIS3::G_{BD}/RAP1(653–799)</i>	this study
YSB257	YSB107 except <i>HIS3::G_{BD}/RAP1(562–827)</i>	this study
YSB258	YSB107 except <i>HIS3::G_{BD}/RAP1(588–827)</i>	this study
YSB259	YSB107 except <i>HIS3::G_{BD}/RAP1(618–827)</i>	this study
YSB260	YSB107 except <i>HIS3::G_{BD}/RAP1(630–827)</i>	this study
YDS702	W303-1B except <u>ΔA</u> <i>sir1::URA3</i>	this study
YSB160	YSB2 except <i>sir1::URA3</i> (RS1172)	C.-t. Chien and R. Sternglanz (pers. comm.)
RS1042	YSB2 <i>sir2::URA3</i>	Chien et al. (1993)
RS1061	YSB2 <i>sir3::URA3</i>	Chien et al. (1993)
RS1067	YSB2 <i>sir4::URA3 MATα</i>	Chien et al. (1993)
YSB76	YSB114 except <i>rap1-12::URA3</i>	this study
YSB300	YSB107 except <i>rif1::URA3</i>	this study
YSB302	YSB110 except <i>rif1::URA3</i>	this study
YSB110	YSB114 except <i>HIS3::G_{BD}/rap1-12(653–827)</i>	this study
YSB74	YSB110 except <i>rap1-12::URA3</i>	this study
YSB116	YSB114 except <i>HIS3::G_{BD}/rap1-12(653–827)</i>	this study
YSB80	YSB107 except <i>MATα</i>	this study
YSB282	YSB80 except <i>HIS3::G_{BD}/rap1-12(653–827)</i>	this study
AJL394-2a	W303-1B except <i>rap1-17 ADE2:URA3:TelVIII</i>	A. Lustig (pers. comm.)
AJL275-2a	AJL394-2a except <i>RAP1</i>	A. Lustig (1993)
AJL440-1c	AJL275-2a except <i>rap1-17 MATα</i>	A. Lustig (pers. comm.)
YSB237	YSB2 except <i>rif1::URA3</i>	this study
CTY10-5D	<i>MATα ade2-1 trp1-901 leu2-3, 112 his3-200gal4 gal80 URA3::lexA op-lacZ</i>	C.-t. Chien and R. Sternglanz (pers. comm.)
YSB215	W303-1B except <u>ΔA</u> <i>hmr::TRP1 adh4::URA3 rap1::LEU2 p(CEN-HIS3-RAP1)</i>	this study
YSB216	YSB215 except <i>p(CEN-HIS3-<u>rap1-12</u>)</i>	this study
YSB290	W303-1B except <u>ΔA</u> <i>hmr::TRP1</i>	this study
YSB284-7	YSB107, except derived from <i>RAP1/rap1-17</i> diploid	this study
YSB288-9	YSB110, except derived from <i>RAP1/rap1-17</i> diploid	this study
YSB291-4	YDS290, except derived from <i>RAP1/rap1-17</i> diploid	this study
YSB304	YSB291, except <i>MATα</i>	this study
YSB296	YDS290, except derived from <i>RAP1/YSB293</i> diploid	this study
YSB297	YSB290, except derived from <i>YSB293/YSB304</i> diploid	this study
YSB298	YSB290, except derived from <i>rap1'/YSB293</i> diploid	this study

^aUnderlined part of genotype refers to the deletion mutation at the *HMR-E* silencer.

Figure 2. Targeted silencing by the $G_{BD}/RAP1(653-827)$ hybrid protein at two double-deletion *HMR-E* silencers ($\Delta A\Delta E$ and $\Delta E\Delta B$) and the triple-deletion silencer ($\Delta A\Delta E\Delta B$) is GAL4-binding site (UAS_G)- and RAP1-dependent. 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 number above each lane indicates the number of UAS_G sites present at the silencer deletion. (+) The presence of the $G_{BD}/RAP1$ plasmid; (–) the presence of a plasmid expressing G_{BD} only.

RAP1 sequences in the hybrid protein and GAL4-binding sites at the mutant silencer. Unlike the case for the *HMRa* strain, where partial repression was observed, we did not find evidence for repression of *TRP1* by $G_{BD}/RAP1(653-827)$ in a strain with a triple-deletion silencer containing three copies of UAS_G . 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.

$G_{BD}/RAP1$ 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 $G_{BD}/RAP1(653-827)$ 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 $\Delta A\Delta E::[UAS_G]_3$ silencer linked to the *TRP1* reporter gene. As shown in Figure 3B, all four of these $G_{BD}/RAP1$ 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 $G_{BD}/RAP1(653-827)$ hybrid. Removal of 14 amino acids from the RAP1 junction to create $G_{BD}/RAP1(667-827)$ had no effect on silencing, whereas two larger deletions, $G_{BD}/RAP1(679-827)$ and $G_{BD}/RAP1(702-827)$, 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, $G_{BD}/RAP1(653-799)$ 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).

A

B

C

Figure 3. Targeted silencing of an *hmr::TRP1* reporter by $G_{BD}/RAP1$ hybrid proteins. 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. (A) Effect of the $G_{BD}/RAP1(653-827)$ hybrid at *hmr::TRP1* loci with different *HMR-E* silencer deletions. The UAS_G -containing $\Delta A\Delta E$ strains are YSB256 and YSB107 [with G_{BD} alone and $G_{BD}/RAP1(653-827)$, respectively], the corresponding $\Delta E\Delta B$ strains are YSB127 and YSB123, and the corresponding $\Delta A\Delta E\Delta B$ strains are YSB103 and YSB102. The control strains lacking UAS_G sites at the $\Delta A\Delta E$ and $\Delta E\Delta B$ silencers are YSB114 and YSB140, respectively. (B) $G_{BD}/RAP1$ hybrids containing a functional RAP1 activation domain can repress transcription when targeted to a mutated *HMR-E* silencer. The strains used in these experiments are YSB256, YSB260, YSB259, YSB258, and YSB257 (top to bottom). (C) Mapping of the amino- and carboxy-terminal boundaries of a RAP1 silencing domain using a series of $G_{BD}/RAP1$ hybrids in strain YSB256, YSB252, YSB253, YSB254, and YSB255 (top to bottom).

Several missense mutations that abolish the silencing function of the G_{BD} /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 G_{BD} /RAP1 hybrid is dependent on SIR2, SIR3, and SIR4, but not on SIR1

To determine whether G_{BD} /RAP1(653–827)-mediated silencing resembles normal silencing at *HMR*, we examined its dependency on *SIR* gene function. A series of derivatives of the $\Delta A\Delta E::(UAS_G)_3$ *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 G_{BD} /RAP1(653–827) hybrid and tested for silencing. As shown in Figure 4, repression by G_{BD} /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 *hmr* ΔA silencer, is completely abolished by mutation of *SIR1* (Fig. 4, cf. rows 3 and 4). We conclude from this that G_{BD} /RAP1(653–827) is somehow able to circumvent the normal requirement for *SIR1*.

G_{BD} /rap1^s hybrids display no defect in targeted silencing

Because the silencing-defective *rap1^s* mutations (Sussel and Shore 1991) fall within the sequences contained in all of the functional G_{BD} /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 G_{BD} /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 G_{BD} /rap1-12(653–827), was integrated in single copy into the *HIS3* locus in a strain containing a $\Delta A\Delta E::(UAS_G)_3$ *hmr::TRP1* reporter gene to give strain YSB110. We chose the $\Delta A\Delta E$ 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 G_{BD} /rap1-12(653–827) hybrid to a $\Delta A\Delta E::(UAS_G)_3$ silencer was expected to mimic the action of full-length rap1-12 at a ΔA silencer. Surprisingly, we found that this G_{BD} /rap1-12 hybrid displayed no defect in silencing (Fig. 5A, cf. rows 4 and 6).

We also showed that a set of longer G_{BD} /rap1-12 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 G_{BD} /rap1-12(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 G_{BD} /RAP1 but not G_{BD} /rap1^s hybrids

The ability of the G_{BD} /rap1-12 hybrids to establish repression when targeted to *HMR* led us to investigate if the chromosomal *rap1-12* mutation could affect G_{BD} /RAP1-dependent silencing. To do this, we crossed a strain containing a marked *rap1-12* allele (*rap1-12::URA3*) to a $\Delta A\Delta E::(UAS_G)_3$ *hmr::TRP1* reporter strain containing the G_{BD} /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 G_{BD} /RAP1-mediated 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 outline above, we examined the effect of the *rap1-12* mutation on silencing established by G_{BD} /rap1-12(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 G_{BD} /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 *hmr* ΔA (YDS702) for purposes of comparison. The Trp plating assay was performed as in Fig. 3.

Figure 5. Effects of the *rap1-12* and *rif1::URA3* mutations on $G_{BD}/RAP1(653-827)$ - and $G_{BD}/rap1-12(653-827)$ -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 $G_{BD}/RAP1(653-827)$ but not $G_{BD}/rap1-12(653-827)$ is abolished by a chromosomal *rap1-12* mutation. Targeted silencing by $G_{BD}/RAP1(653-827)$ (rows 4,5) or $G_{BD}/rap1-12(653-827)$ (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 UAS_C sites at the silencer. Row 1 shows native silencing at an *hmr $\Delta A::TRP1$* 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 $G_{BD}/RAP1(653-827)$ (rows 4,5) or $G_{BD}/rap1-12(653-827)$ (rows 6,7) targeted silencing. The effect of the *rif1::URA3* mutation on native silencing at *hmr $\Delta A::TRP1$* (rows 1,2) is shown for comparison.

Because *rif1* mutants share many of the phenotypes of *rap1^s* mutants and $G_{BD}/rap1^s$ 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 $G_{BD}/RAP1(653-827)$ -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 $G_{BD}/rap1-12(653-827)$ 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, $G_{BD}/rap1-12$ 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 $G_{BD}/RAP1(653-827)$] despite the absence of a RAP1 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 RAP1 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; Kyrion et al. 1992, 1993). Furthermore, *rap1^s* 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 *RAP1* cells containing elongated telomeres, we took advantage of the *rap1-17* mutation, a *rap1^s* allele that results in a truncation of the RAP1 carboxyl terminus and a pronounced telomere elongation phenotype (Kyrion 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 *RAP1* 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 (Kyrion et al. 1993). To increase telomere length in strains undergoing targeted silencing at HMR, we crossed strain AJL394-2a (*rap1^s*) to both YSB80 [expressing $G_{BD}/RAP1(653-827)$] and YSB282 [expressing $G_{BD}/rap1-12(653-827)$]. The resulting diploid strains were sporulated, and haploid segregants were obtained that had a wild-type *RAP1* allele and both the *cis* and *trans* components of the targeted silencing system. Approximately half of the poly($C_{1-3}A$) 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 $G_{BD}/RAP1(653-827)$ hybrid in strains containing elongated telomeres when compared with isogenic strains derived from an identical genetic cross to a *RAP1* control strain

(Fig. 6A, cf. rows 3–5 with row 2). This result suggests that an increase in the length of telomeric poly(C_{1–3}A) tracts is by itself sufficient to decrease the efficiency of targeted silencing. However, we found that targeted silencing by the GBD/rap1-12(653–827) hybrid is only weakly affected by telomere elongation (Fig. 6A, cf. rows 6 and 7) and is considerably stronger than that of the wild-type hybrid in the presence of elongated telomeres (Fig. 6A, cf. row 7 with rows 3–5). This observation indicates that the mutant hybrid is more resistant to the

effect of telomere elongation than the wild-type hybrid and is consistent with the previous observation (Fig. 5A) that the *rap1-12* mutant hybrid suppresses the defect caused by the chromosomal *rap1-12* allele.

We also examined the effect of elongated telomeres on normal silencing at *HMR*. The *rap1-17* strain was crossed to strains containing a *hmr::TRP1* reporter with either a wild-type, *hmr* Δ A, or *hmr* Δ B silencer. All *RAP1* *hmr* Δ A haploid segregants from these crosses displayed a defect in silencing (Fig. 6A, rows 10–12; data not shown).

Consistent with the characterization of the *rap1-12* mutation, elongated telomeres do not affect either the wild-type or the ΔB *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($C_{1-3}A$) 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($C_{1-3}A$) 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 $C_{1-3}A$ 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($C_{1-3}A$) 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 $G_{BD}/RAP1$ 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- μ m origin) plasmids. No improvement in the ability of the $G_{BD}/RAP1(653-827)$ hybrid to silence in a *rap1-12* strain was observed (data not shown). Furthermore, elevated *SIR4* dosage abolished targeted silencing by $G_{BD}/RAP1(653-827)$ and $G_{BD}/rap1-12(653-827)$ hybrids in a *RAP1* background and by the $G_{BD}/rap1-$

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 $G_{BD}/RAP1(653-827)$ silencing (in a *RAP1* background) to the level produced by native RAP1 at a *hmr Δ* 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 $G_{BD}/RAP1(653-827)$ to give ~ 50 - to 100-fold repression in the *TRP1* 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 ($C_{1-3}A$) 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 $\sim 50\%$ 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^R , 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 $G_{BD}/RAP1(653-827)$ -mediated silencing in a *RAP1* strain (YSB107). *SIR4*-CEN, but not *SIR1*-CEN, partially suppresses the targeted-silencing defect of $G_{BD}/RAP1(653-827)$ 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 ^a (%)	Growth on FOA ^a (%)
YSB215	<i>RAP1</i> ⁺	37.6 (7.2)	56.4 (13.2)
YSB216	<i>rap1-12</i>	9.8 (2.7)	95.4 (6.3)

^aCompared to an equal aliquot of cells plated on YEPD (rich) medium. The values in parentheses are standard deviations. The values are averages from measurements of six independent colonies from each strain.

duced growth in the absence of uracil. This improvement of telomeric silencing is accompanied by a complete loss of ΔA *hmr TRP1* repression, as evidenced by the full growth of this strain in the absence of tryptophan (data not shown).

Two observations suggest that the effect of the *rap1-12* mutation at *HMR* is the result of more than telomere elongation alone. First, the defect caused by the inheritance of elongated telomeres from a *rap1*⁺ strain appears less severe than that caused by *rap1-12*, although the extent of C_{1–3}A expansion appears to be greater in the former case (Fig. 6). Second, the *G_{BD}/rap1-12(653–827)* hybrid can act directly at a *HMR-E* silencer to suppress the defect caused by the *rap1-12* allele (Fig. 5A). One possible explanation of these results, based on the sensitivity to *SIR4* gene dosage described above, is that the *rap1-12* mutation leads to an altered interaction with the *SIR4* protein that can by itself contribute to a shift in the balance between *HMR* and telomeric silencing. To test this idea, we took advantage of the recent observation that the RAP1 carboxyl terminus can interact with the carboxyl terminus of *SIR4* in the two-hybrid system (Moretti et al. 1994). We therefore determined whether the RAP1–*SIR4* interaction is affected by the *rap1-12* mutation. To do this, we constructed a LexA/*rap1-12(635–827)* hybrid and compared it with the corresponding LexA/RAP1(635–827) hybrid in a two-hybrid assay with a GAL4 activation domain (*G_{AD}*)/*SIR4* hybrid. As shown in Table 3, the mutant *rap1-12* hybrid interacts much more strongly with *SIR4* than does the wild-type LexA/RAP1 hybrid, as indicated by an ~20-

fold increase in β -galactosidase activity. This experiment was repeated in a *rif1::URA3* reporter strain to test the idea that the increase in the interaction with *SIR4* is the result of a decreased interaction between *rap1-12* protein and RIF1 (Hardy et al. 1992b). The *rif1::URA3* disruption increases the ability of LexA/RAP1(635–827) to activate by itself (Moretti et al. 1994), but the *SIR4/G_{AD}* hybrid gene increases the β -galactosidase signal above this background level. Strikingly, the *rap1-12* mutation still resulted in a large (approximately fivefold) increase in the interaction with *SIR4/G_{AD}* compared with the wild-type hybrid (Table 3). This increase represents a 47-fold increase above the (*G_{AD}* only) control, whereas the wild-type hybrid displayed a 3-fold increase above the (*G_{AD}* only) control. Therefore, the increase in the interaction of *SIR4/G_{AD}* with LexA/*rap1-12(635–827)* cannot be explained by the failure of the mutant hybrid to interact with RIF1, a potential competitor for *SIR4* binding. We also noticed that the *rif1* mutation created a much smaller increase in the activation potential of the LexA/*rap1-12(635–827)* hybrid compared with the corresponding wild-type hybrid. Taken together, these results are consistent with the idea that the *rap1-12* mutation strengthens the interaction between RAP1 and *SIR4* and suggest that the phenotypes of *rap1-12* strains described above result at least in part from an improved ability of telomeric *rap1-12* protein to bind a limiting amount of *SIR4* protein. It is important to point out, however, that these two-hybrid experiments measure a RAP1–*SIR4* interaction in a different context than that of *HMR* or telomeres, as reflected by the fact, for example, that we do not detect an interaction between the *G_{BD}/RAP1(653–827)* hybrid and the small carboxy-terminal fragment of *SIR4* used in the two-hybrid assays (data not shown). We cannot be certain, therefore, that the two-hybrid results precisely reflect the interactions that occur at silencers and telomeres.

Discussion

A minimal RAP1 silencing domain

The use of *G_{BD}/RAP1* hybrids has allowed us to define a minimal domain of RAP1 (amino acids 667–827) that is

Table 3. Interaction of *G_{AD}/SIR4* with *G_{BD}/RAP1* and *G_{BD}/rap1^s* hybrids

DNA-binding domain hybrid	Activation domain hybrid ^a	β -Gal units ^b	
		<i>RIF1</i>	<i>rif1::URA3</i>
LexA/RAP1(635–827)	<i>G_{AD}</i> alone	7 (2)	94 (5)
LexA/RAP1(635–827)	<i>G_{AD}/SIR4</i>	89 (6)	270 (11)
LexA/ <i>rap1-12(635–827)</i>	<i>G_{AD}</i> alone	15 (2)	29 (2)
LexA/ <i>rap1-12(635–827)</i>	<i>G_{AD}/SIR4</i>	1913 (28)	1360 (123)

^a*G_{AD}/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 *G_{AD}/SIR4* hybrid consists of amino acids 1204–1358 of *SIR4* fused in-frame to the GAL4 activation domain (Moretti et al. 1994).

^bThe numbers are an average of the values obtained for three independent transformants. The values in parentheses are the standard deviations.

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^s*) and overexpression of the wild-type domain cause derepression of *HMR* (Sussel and Shore 1991; Hardy et al. 1992a) and that truncations of this domain abolish silencing at *HML* and telomeres (Kyrion et al. 1993). The dependence on *SIR2*, *SIR3*, and *SIR4* function indicates that the effect of G_{BD} /RAP1 hybrids is bona fide silencing rather than a nonspecific effect on the *HMR* locus. It is worth pointing out that the G_{BD} /RAP1(653–827) 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 G_{BD} /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 G_{BD} /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 G_{BD} /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 G_{BD} /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 G_{BD} /RAP1 hybrids at *HMR* is completely independent of *SIR1* function. Using the same targeting strategy described here, it was shown previously that a G_{BD} -*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 G_{BD} /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 G_{BD} /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 (Sussel 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 G_{BD} /RAP1 hybrids that differs from native RAP1 must explain this observation. Several possibilities can be considered. The G_{BD} /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 G_{BD} /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 G_{BD} /RAP1 hybrids.

Whatever the explanation for the *SIR1* independence of G_{BD} /RAP1 silencing, it is clear that the role of G_{BD} /RAP1 hybrids is not to recruit *SIR1* to the silencer. In fact, G_{BD} /RAP1 and G_{BD} -*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 G_{BD} -*SIR1* possesses an establishment mechanism distinct from the function of the carboxyl terminus of RAP1. Several properties of the two silencing systems are consistent with the latter model. Overexpression of G_{BD} /RAP1 hybrids has a dominant-negative effect on silencing at *HMR*, probably by titration of *SIR3* and *SIR4*, whereas G_{BD} -*SIR1* has no such effect. In addition, although the *rap1-12* mutation is antagonistic to G_{BD} /RAP1 silencing, it has no effect on silencing by G_{BD} -*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 of *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^s mutations reveal an underlying competition between HMR and telomeric silencing

The *rap1-12* mutation is one of four *rap1^s* 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* (Sussel 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^s* 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

(Hardy et al. 1992b). Consistent with this idea, the $G_{BD}/rap1^s$ hybrids display a decrease in RIF1 binding in the two-hybrid system that is proportional to the severity of the corresponding *rap1*^s defect in silencing.

Characterization of the *rap1-12* mutation reported here resulted in several unexpected findings that challenge this interpretation of the *rap1*^s and *rif1* mutations. First, we showed that targeting of the $G_{BD}/rap1-12(653-827)$ 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 $G_{BD}/RAP1$ hybrid (Figs. 5 and 6). Second, the presence of the *rap1-12* mutation in the chromosome prevents silencing by $G_{BD}/RAP1(653-827)$, 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 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 $C_{1-3}A$ repeats, which contain a high density of RAP1-binding sites (Gilson et al. 1993), increase in length by ~50% (Sussel and Shore 1991). Previous studies (Kyriou et al. 1993) showed that telomere elongation in wild-type cells can result 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($C_{1-3}A$) 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 $G_{BD}/rap1-12(653-827)$ 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 $G_{BD}/rap1-12(653-827)$ hybrid can successfully compete for a limiting silencing factor, most likely SIR4 protein (see below). Even silencers containing only a single UAS_G probably allow for the binding of a $G_{BD}/RAP1$ 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 UAS_G sites at *HMR-E*. A testable prediction of this model is that multiple RAP1 binding sites would improve silencing at *HMR* in *rap1*^s 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- μ m plasmid) are sufficient to suppress the silencing defect of *rap1*^s mutants (Sussel and Shore 1991; Sussel et al. 1993). As shown here, the defect in $G_{BD}/RAP1(653-827)$ 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*^s mutants suggests, in turn, a different view of the role of RIF1 in silencing (Hardy et al. 1992b). We propose, as suggested previously (Kyriou 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*^s strains (Kyriou 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 *rap1-12* 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 $G_{BD}/RAP1(653-827)$ and $G_{BD}/rap1-12(653-827)$ hybrids in cells lacking RIF1. In addition, a *rif1* mutation has a significantly weaker effect on tar-

geted silencing than does the *rap1-12* mutation, indicating again that the *rap1-12* defect is not a result simply of the loss of the RIF1 interaction. Both of these results suggest that the *rap1-12* protein has an intrinsically higher affinity for SIR4 or that this interaction is affected in vivo by other RAP1-interacting proteins, such as SIR3 (Moretti et al. 1994). Furthermore, because *rif1* and *rap1-12* mutants have nearly identical telomere lengths (Hardy et al. 1992b), the different effect of these mutations on targeted silencing provides additional support for the claim that the effect of the *rap1-12* mutation on *HMR* cannot be completely accounted for by telomere elongation.

Previous work indicated that SIR1 acts at *HM* loci to raise repression above a basal level seen at telomeres (Pillus and Rine 1989; Aparicio et al. 1991; Chien et al. 1993), creating a hierarchy of silencing in which *HM* loci are more efficiently repressed than telomeres. Our characterization of the *rap1-12* 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 UAS_C oligonucleotide (TCGACGGAGCAGTCCTCCG and its complement TC-GACGGAGGACTGTCCTCCG) was used in the construction of all UAS_C-containing mutated silencers. Details of the silencer deletions (all of which are marked by a *Xho*I linker) can be found in Brand et al. (1987). UAS_C insertions were confirmed by DNA sequencing 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 (Sussel and Shore 1991).

Plasmid DNA

All G_{BD}/RAP1 constructs contained the native *RAP1* promoter upstream of *GAL4* coding sequences. G_{BD}/RAP1 fusions in the *HIS3* integrating vector pRS303 (Sikorski and Hieter 1989) were described previously (Hardy et al. 1992a). The same G_{BD}/RAP1

constructs were transferred to the *HIS3/CEN* plasmid pRS313 (Sikorski and Hieter 1989) as *Eco*RI-*Xba*I fragments. LexA/RAP1 and LexA/*rap1-12* hybrid proteins were expressed from plasmid pBTM116 (2- μ m origin, *TRP1*, *pADH1-LexA*; a gift of P. Bartel and S. Fields, State University of New York, Stony Brook). The *SIR4* 2- μ m plasmid (LSD357) consists of an *Eco*RI-*Sal*I fragment of *SIR4* cloned into pRS426 (Sikorski and Hieter 1989). *SIR4/CEN* and *SIR1/CEN* plasmids consist of *Eco*RI-*Sal*I and *Kpn*I-*Hind*III fragments, respectively, cloned into pRS316 (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 *Xho*I-*Bgl*II DNA fragment from D401 (*Xho*I linker mutant number 238; Abraham et al. 1984). The blot was reprobed with a 500-bp *Eco*RI-*Hind*III fragment from the actin gene.

Detection of poly(C₁₋₃A) telomeric repeats

Telomeric poly(C₁₋₃A) repeats were detected by Southern blotting using standard techniques (Ausubel et al. 1987) with a probe made by nick-translation of poly(dGdT) (Pharmacia). Yeast genomic DNA (20 μ g) 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 μ l. Reactions were stopped by the addition of 16 μ l of 0.2 M EGTA. DNA was precipitated by adding 50 μ l of 3.5 M ammonium acetate and 150 μ l of isopropanol. The DNA pellet was washed twice with 70% ethanol, resuspended in TE, and digested with *Xho*I. DNA fragments were resolved on 0.8% agarose gels and transferred to nitrocellulose for hybridization.

β -Galactosidase assays

LexA/RAP1(635–827) and LexA/*rap1-12*(635–827) plasmids were cotransformed with a G_{AD}/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 μ l of Z buffer (60 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 0.27% β -mercaptoethanol) and permeabilized by vortexing 3 $\times$ 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 (Breedon and Nasmyth 1985).

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