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Genetics

Separation of transcriptional activation and silencing functions of the *RAP1*-encoded repressor/activator protein 1: Isolation of viable mutants affecting both silencing and telomere length

(silencers/gene regulation/DNA-binding protein/mating type/*Saccharomyces cerevisiae*)

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ABSTRACT **The repressor/activator protein 1 (RAP1) binds to the upstream activating sites of many genes, the silencer elements flanking the unexpressed mating-type loci *HMR* and *HML*, and the poly($C_{1-3}A$) sequences at telomeres, suggesting that RAP1 might have three distinct regulatory functions. To determine the *in vivo* role of RAP1 in repression of the *HMR* silent locus, we developed a screen to isolate rap1 mutants specifically defective in silencing. Fifteen independent mutants defining four different *rap1* alleles were isolated. These alleles are defective to different extents in repression of an *HMR* locus containing a mutated, but fully functional, silencer. All four alleles are missense mutations in only three codons within a small C-terminal region of the gene. These silencing-defective mutants have no apparent growth defects, indicating that expression of the large number of essential genes that have promoters containing RAP1-binding sites is normal. A transcriptional silencing function of RAP1 can therefore be genetically separated from its presumably essential activation functions. Surprisingly, three of the silencing-defective *rap1* alleles have significantly longer telomeres, suggesting that the function of RAP1 in both transcriptional silencing and telomere-length regulation may be related. In addition, we have demonstrated that increased gene dosage of either *SIR1* or *SIR4*, two other factors required for silencing, suppresses the silencing defect of the rap1 mutants. The properties of *SIR4* dosage suppression suggest that SIR4 protein may interact directly with RAP1 at silencers.**

Regulation of transcription in eukaryotes often involves a complex interplay of repressors and activators. In some cases a single protein may, in different contexts, display either activity. For example, the adenovirus E1A protein is a transcriptional activator of some cellular genes, yet a repressor of others (for a review, see ref. 1), and the glucocorticoid receptor can also mediate both positive and negative control of transcription (2). In the yeast *Saccharomyces cerevisiae*, the *RAP1* gene encodes an essential protein that appears to be capable of both activating and repressing (silencing) transcription (3). Binding sites for repressor/activator protein 1 (RAP1), which is probably identical to the TUF protein (4), are found in the promoter regions of many genes, including ribosomal protein genes, the *MATα* genes, and several glycolytic enzyme genes (5), where they appear to be UAS elements. Temperature-sensitive (ts) rap1 mutants are defective in their ability to activate the *MATα* genes (6), suggesting that RAP1 is an activator of these genes and, by inference, of the large set of genes that have UASs containing RAP1-binding sites. RAP1 also binds *in vitro* with high affinity to specific sequences within the poly($C_{1-3}A$) repeat region of telomeres (5, 7), and studies of *rap1*^{ts} alleles indicate that RAP1 is also involved in telomere-length regulation (8, 9). Finally, RAP1-binding sites are also found at the silencers flanking the unexpressed mating-type gene loci *HMR* and *HML* (10, 11).

Studies of the silencer found to the left of the *HMR* locus (called *HMRE*) have shown that it can act in a distance- and orientation-independent manner (12) and is comprised of multiple redundant regulatory elements, called A, E, and B (13, 14). Mutations in either the A or the B element individually have no effect on silencing, whereas a mutation of the central E element (a RAP1-binding site) results in a partial loss of silencer function. Disruption of any two of the three elements leads to a complete loss of silencing. The B element is a binding site for autonomously replicating sequence (ARS) binding factor 1 (ABF1) (initially referred to as SBF-B) (3, 10, 11), whereas the A element is an ARS consensus sequence (15). Although repression mediated by *HMRE* also requires the products of four *SIR* genes (16), it is unclear whether the SIR proteins bind directly to sequences at or near the silencer or interact with or modify other proteins that bind to the silencer, such as RAP1 or ABF1.

The identification of a RAP1-binding site as a functional element of the *HMRE* silencer is consistent with the notion that RAP1 is involved in silencing. However, there are several examples of multiple proteins recognizing a common DNA sequence (for a review, see ref. 17), so it is possible that a second, unidentified protein binds to and functions at the silencer *in vivo*. We speculated that RAP1 does act at silencers and, furthermore, that a part of RAP1 plays a distinct role in silencing and is not required for essential activation functions. To test this hypothesis, we sought to isolate viable rap1 mutants specifically defective in their ability to repress transcription at *HMR*. We concluded that *RAP1* had not been previously identified as a *SIR* gene due to the redundancy of the silencer elements. For this reason we decided to look for such mutants in strains carrying partially deleted silencers, where the RAP1-binding site is essential for repression (13, 14). Here we describe the isolation and initial characterization of rap1 mutants defective in silencing.

MATERIALS AND METHODS

Strain Construction. *hmr*::*TRP1* strains, in which the *TRP1* gene is under the control of *HMRE* (18), were constructed by inserting an 850-base-pair (bp) *Eco*RI–*Bgl* II DNA fragment of the *TRP1* gene into an *Xba* I–*Bgl* II deletion of *HMR* that removes all of the *a2* gene and most of the *a1* gene (see Fig. 1*a*). These constructions were used to replace the normal chromosomal *HMR* locus in strain W303-1B (*HMLα MATα*

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Abbreviations: RAP1, repressor/activator protein 1; SIR, silent information regulator; UAS, upstream activation site; ARS, autonomously replicating sequence; ts, temperature sensitive.

HMRa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11, 15 ura3) as described (13). Four isogenic strains were constructed, each with a different form of the *HMRE* silencer: wild-type (*AEB*), *hmrΔA* (del 352–358), *hmrΔB* (del 274–256), *hmrΔE* (del 331–324), and *hmrΔEΔB* (del 331–324 and del 274–256) (13). To confirm that the repression of the *TRP1* gene is *SIR*-mediated, we showed that *sir3*::*LEU2* or *sir4*::*LEU2* derivatives of these strains (19, 20) are phenotypically Trp$^+$. Strains used in S1 nuclease protection analyses and quantitative mating assays were constructed by replacing the chromosomal *RAP1* gene with *rap1* mutant alleles in strains containing the wild-type, ΔA, and ΔB silencers (described above) and an otherwise intact *HMRa* locus.

Isolation and Mapping of rap1 Mutants. Hydroxylamine (21) or *mutD5* (22) mutagenized copies of *RAP1* were introduced into the *hmr*::*TRP1* strains by the plasmid shuffle technique (6, 21) and were screened for their ability to grow in the absence of tryptophan (due to expression of the *hmr*::*TRP1* locus) by replica plating. rap1 mutants were mapped by constructing *in vitro* recombinants between the mutant and wild-type genes. These hybrids were then transformed into the *hmrΔA*::*TRP1* strain and assayed for the ability to grow in the absence of tryptophan. Fragments that conferred the mutant phenotype were subcloned into M13mp18 and M13mp19, and their DNA sequence was determined (23).

RESULTS

Isolation of Silencing-Defective rap1 Mutants. To select for rap1 mutants defective in silencing, we constructed strains in which the *TRP1* gene is controlled by the *HMR* silencer in a *SIR*-dependent manner. These strains are phenotypically Trp$^-$ in a *SIR*$^+$ background and Trp$^+$ in a *sir*$^-$ background. Because RAP1 function is redundant at the wild-type *HMR* silencer, we screened for rap1 mutants in strains carrying deletions of either the A or the B silencer regulatory elements (*hmrΔA*::*TRP1* or *hmrΔB*::*TRP1*). In the absence of either of these elements the silencer remains functional but becomes dependent upon the E element, a RAP1-binding site (13). We therefore expected that these mutant silencers would enhance the phenotype of rap1 mutants defective in silencing. To avoid isolating mutations in previously identified trans-acting regulators of the silent loci (e.g., *SIR1-4*), the selection was done in a diploid strain. To allow for the isolation of recessive mutations, we disrupted both chromosomal copies of *RAP1* in the diploid and maintained a wild-type copy of the gene on a counter-selectable plasmid (6). Because we looked for mutants that would fail to silence *hmr*::*TRP1* but would grow normally at 30°C, our selection demanded that rap1 silencing mutants retain essential activation functions.

Mutagenized *RAP1*-containing plasmids were introduced by transformation into strains containing different silencer alleles (*hmrΔA*::*TRP1*, *hmrΔB*::*TRP1*, and *hmr*::*TRP1*). From over 30,000 transformants of each strain, we isolated 15 independent silencing-defective rap1 mutants (hereafter referred to as rap1^s) in the *hmrΔA*::*TRP1* strain. These 15 mutants all mapped to the C-terminal end of the *RAP1*-coding sequence, and DNA sequencing revealed that they represented only four different alleles, all of which were missense mutations. Fig. 1*b* shows the amino acid changes of these alleles and their growth on medium lacking tryptophan. Three of these alleles contain mutations localized to two consecutive amino acids (726 and 727). The *rap1-12* allele, a double mutation that contains a base pair change in each of these codons, was repeatedly isolated from both hydroxylamine-mutagenized DNA and DNA passaged through the *Escherichia coli mutD5* strain. No rap1^s mutants were isolated in either the *hmrΔB*::*TRP1* or the wild-type silencer (*hmr*::*TRP1*) background, and none of the *rap1*^{s} alleles

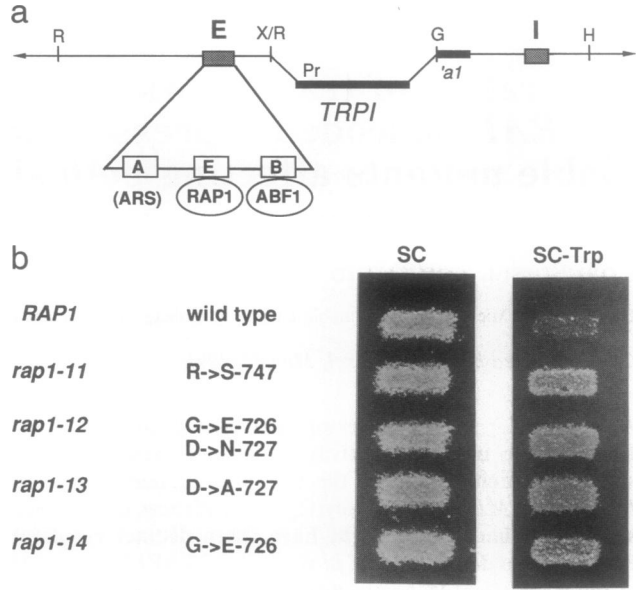

FIG. 1. Silencing-defective alleles of *RAP1*. (*a*) Schematic diagram of the *hmr*::*TRP1* locus. (R, *Eco*RI; X, *Xho* I; G, *Bgl* II; H, *Hin*dIII; Pr, promoter). (*b*) *RAP1* and *rap1*^{s} alleles and their effect on growth of *hmrΔA*::*TRP1* strains on medium lacking tryptophan. Codon changes for each allele are indicated by single-letter amino acid code followed by amino acid position number. Each strain was patched onto rich yeast extract/peptone/dextrose (YEPD) medium and replica-plated to synthetic complete (SC) medium and SC medium lacking tryptophan (SC-Trp).

caused derepression of *TRP1* when introduced into these strains.

For further analysis, each *rap1*^{s} mutant allele was integrated at the genomic *RAP1* locus, replacing the wild-type *RAP1* gene. The resulting strains grow normally at all temperatures tested between 17°C and 37°C and are therefore able to perform all essential RAP1 functions, presumably including transcriptional activation. Consistent with this, MATα rap1^s mutants produce normal levels of α factor in an α factor "halo" assay (data not shown). This is the most sensitive assay for expression of the *MAT*α genes, for which the UAS is a RAP1-binding site (24). In contrast, rap1^{ts} mutants with no effect on silencing have pronounced defects in α-factor production, even at permissive temperatures (6). As would also be expected from both their normal growth phenotype and the localization of the mutations outside the putative DNA-binding domain (D. Balderes and D.S., unpublished data; ref. 25), the rap1^s mutants have no apparent defect in DNA binding (data not shown). Analysis of *RAP1*/*rap1*^{s} diploids showed that three of the *rap1*^{s} alleles are recessive, whereas the *rap1-12* allele appears partially dominant to wild-type *RAP1*.

Levels of Derepression in rap1^s Mutants. To determine more accurately the degree and nature of *hmrΔA*::*TRP1* expression in each mutant, we examined the ability of mutant cells to form colonies in the absence of tryptophan. Fig. 2 shows that the RAP1$^+$ strain cannot grow without tryptophan, but rare Trp$^+$ colonies resulting from recessive mutations in one of the four *SIR* genes do arise (data not shown). The mutants all give rise to colonies, but each differs in its ability to grow on the tryptophan-deficient medium. The *rap1-12* allele (a double mutant) has the most severe phenotype: the *hmrΔA*::*TRP1* locus appears completely derepressed in every cell within the population. The other mutants, however, have a lower efficiency of colony formation on the tryptophan-deficient medium. The second strongest allele, *rap1-13*, reproducibly displays a ≈50% plating efficiency without tryptophan. The two weakest alleles, *rap1-11* and *rap1-14*, appear to allow

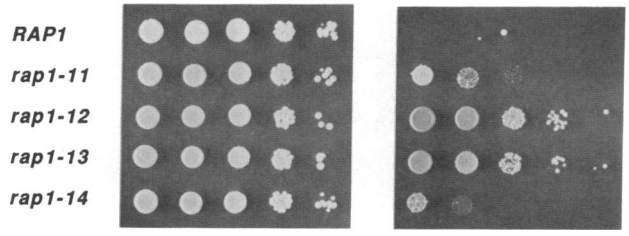

FIG. 2. The ability of *rap1*^{s}, *hmrΔA::TRP1* strains to form colonies in the absence of tryptophan. Each strain was grown overnight in liquid yeast extract/peptone/dextrose (YEPD) medium to ≈10^8 cells per ml. Serial 10-fold dilutions were spotted onto synthetic complete medium (*Left*) and synthetic complete medium lacking tryptophan (*Right*). Plates were incubated at 30°C for 48 hr.

only partial expression of *hmrΔA::TRP1*, as indicated by the smaller colony size, and this weak growth occurs in <0.1% of plated cells. The plating behavior of all but the strongest mutant suggests to us that only a fraction of the cells in the population are expressing the *hmrΔA::TRP1* locus at a level sufficient for colony formation. However, the subpopulation of cells that do form colonies on medium lacking tryptophan are not permanently derepressed, because after nonselective growth these Trp$^+$ colonies give rise to Trp$^-$ and Trp$^+$ cells in the same ratio initially seen for that particular allele (data not shown). This ability of the mutant strains to switch between Trp$^-$ and Trp$^+$ states is also evident in microscopic observation of individual colonies on medium lacking tryptophan. Each colony has a nibbled appearance resulting from the presence of many dead (Trp$^-$) cells within the growing colony.

We also examined the effect of the rap1^s mutants on expression of the wild-type *HMRa1* gene. RNA was isolated from MATα, RAP1 or rap1^s strains containing either the wild-type, *hmrΔA*, or *hmrΔB* silencer. Steady-state HMRa1 transcript levels were determined by S1 nuclease protection assays using the SIR3 transcript as an internal control (26). As predicted by the *hmr::TRP1* assays, we detect a1 transcript in all the rap1^s mutants, but only in the *hmrΔA* silencer background (Fig. 3). In addition, the relative levels of a1 transcript in the different mutants correspond closely to the phenotypes observed in the respective *hmrΔA::TRP1*-containing strains: *rap1-11* and *rap1-14* are only partially derepressed, whereas *rap1-12* and *rap1-13* have transcript levels comparable to the *hmrΔEΔB* silencer mutant and a MATa strain.

To determine the biological consequence of defective silencing at *HMR* we measured the ability of MATα strains

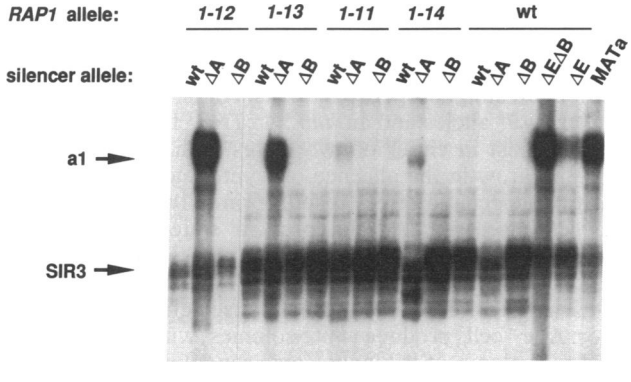

FIG. 3. Levels of *HMRa1* steady-state mRNA in rap1^s strains. Transcripts produced from *HMRa1* were measured by S1 nuclease mapping of total RNA from MATα, rap1^s or RAP1 strains containing wild-type (wt) or mutated silencers, as indicated at the top of each lane. RNA was also prepared from a MATa strain (rightmost lane). SIR3 transcript is shown as an internal control.

containing the different *rap1*^{s} alleles to mate. In MATα strains derepression of *HMRa* causes cells to assume a sterile **a**/α phenotype. Using qualitative patch mating tests, we were able to detect a significant loss of mating only with the strongest allele, *rap1-12*. Quantitative mating assays (27), however, showed that each of the *rap1*^{s} alleles led to reductions in α mating that were consistent with their respective HMRa1 transcript levels (Table 1). These results confirm that the *rap1*^{s} alleles are defective in their ability to silence an *hmrΔA* locus. Although the strongest mutant, *rap1-12*, shows a 10-fold decrease with respect to wild-type, mating is not nearly as impaired as in the completely nonfunctional silencer strain (*hmrΔEΔB*). However, HMRa1 transcript levels in these two strains appeared indistinguishable (Fig. 3). Because the mating assay, unlike the measurement of a1 mRNA or growth of *hmr::TRP1* strains on synthetic complete medium lacking tryptophan, detects cells repressed at *HMR*, this result suggests that even in the *rap1-12* strain a small subpopulation of cells is silenced.

Silencing-Defective rap1^s Mutants Have Longer Telomeres. High-affinity RAP1-binding sites occur, on average, every 40 bp within the poly(C_{1-3}A) sequences at telomeres (7), and rap1^{ts} strains have shorter telomeres when cultured at semipermissive temperatures (8, 9). To determine whether telomeres were affected in the rap1^s mutants, the average length of telomeres in these strains was measured by Southern blot analysis (28) (see Fig. 4*a*). Interestingly, in contrast to the phenotype of the *rap^{ts}* alleles, the rap1^s mutants display a significant lengthening of their telomeres (Fig. 4*b*). The degree of telomere lengthening is allele specific and roughly correlates with the level of derepression at *HMR*. The strongest allele, *rap1-12*, shows a 200- to 300-bp increase in the telomere tract length, whereas the weakest mutant, *rap1-14*, has little or no increase on tract length. Telomeres in the *RAP1/rap1*^{s} heterozygotes are nearly restored to their original (shorter) state, indicating that the mutants are only partially dominant to wild-type *RAP1* with respect to telomere length.

Gene Dosage Suppression by *SIR1* and *SIR4*. We tested the ability of four known trans-acting regulators of silencing, *SIR1-4*, to suppress *rap1*^{s} mutations when present on high-copy-number plasmids (19). Fig. 5 shows the results for *rap1-12*, the most severely derepressed allele, together with a wild-type *RAP1* strain as a control. As previous studies have shown, overexpression of *SIR4* can partially derepress the silent mating-type loci (30), and in our *hmrΔA::TRP1* strain elevated *SIR4* gene dosage causes a Trp$^+$ phenotype. High gene dosage of *SIR1*, *-2*, or *-3* in this strain, however, causes no apparent phenotype. These experiments show that elevated levels of either *SIR1* or *SIR4* can suppress the silencer phenotype of each *rap1*^{s} allele. The *SIR4* suppression is particularly striking in that each altered factor (*rap1-12* or high-copy *SIR4*) alone derepresses the locus, whereas in combination they are able to restore silencing at *HMR*.

Table 1. Quantitative mating analysis of rap1^s and mutant silencer strains

RAP1 allele	Silencer allele	Mating efficiency*, %
Wild type	Wild type	100.0
Wild type	*hmrΔA*	100.0
rap1-11	*hmrΔA*	57.4
rap1-12	*hmrΔA*	6.9
rap1-13	*hmrΔA*	21.7
rap1-14	*hmrΔA*	50.8
Wild type	*hmrΔE*	60.6
Wild type	*hmrΔEΔB*	0.05

*Mating efficiencies are the average of three separate experiments and are normalized to the *RAP1* (wild-type), *hmrΔA* strain that was arbitrarily assigned a value of 100%.

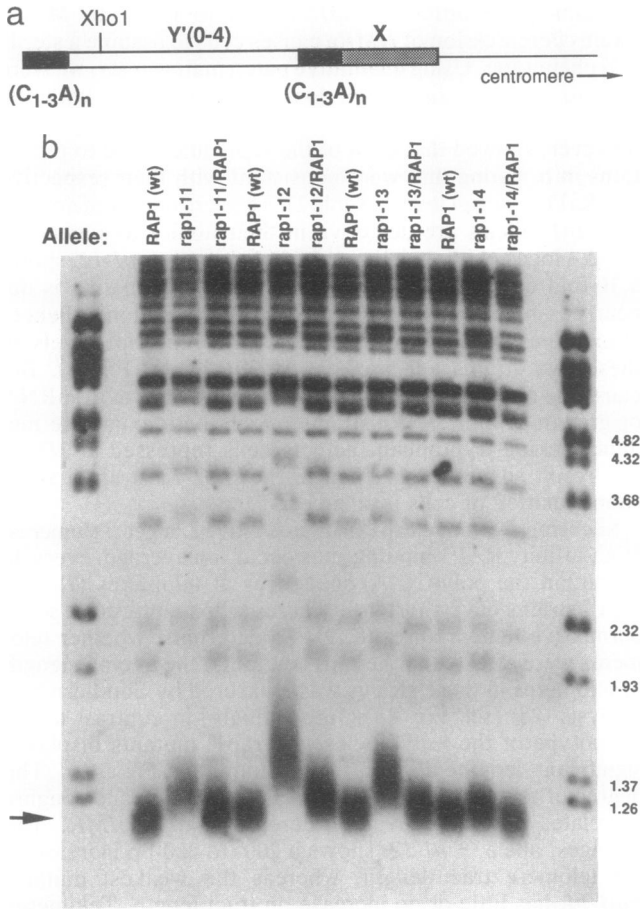

FIG. 4. Telomere tracts are longer in rap1^s strains. (*a*) Diagram of the yeast telomere. *Xho* I cuts at a unique site within the Y' telomere element. (*b*) Southern blot analysis of telomere-tract length in the rap1^s strains. Yeast genomic DNA was digested with *Xho* I and separated on a 0.8% agarose gel. The DNA was transferred to nitrocellulose and probed with ^{32}P-labeled poly[d(G-T)·d(C-A)] (29). Lanes contain DNA from wild-type (wt) *RAP1*, *rap1^s*, or heterozygous *RAP1/rap1^s* haploid strains, as designated. Telomeric $C_{1-3}A$ repeat sequences distal to the *Xho* I site are indicated by the arrow.

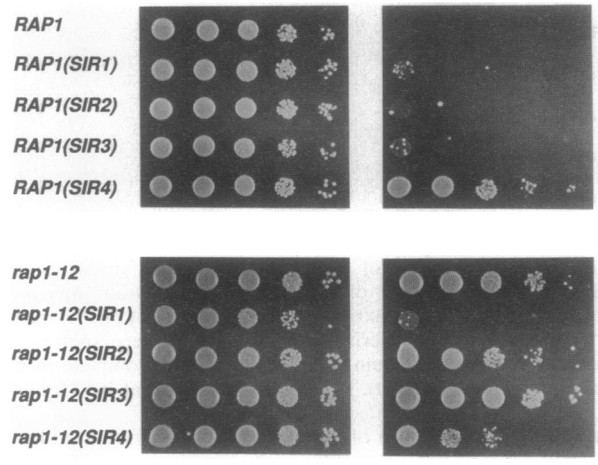

FIG. 5. Rap1^s mutants are suppressed by multicopy plasmids containing *SIR1* or *SIR4*. The assay described in Fig. 2 was used to determine the ability of *rap1^s*, *hmrΔA::TRP1* strains transformed with *SIR*-containing YEp13 (2μ,*LEU2*) plasmids (19) to grow in the absence of tryptophan. Each strain was grown overnight in liquid minimal medium lacking leucine to select for the *SIR* plasmid. Serial dilutions were then spotted onto synthetic complete medium lacking leucine to determine the total number of plasmid-containing cells plated (*Left*) and onto synthetic complete medium lacking leucine and tryptophan to determine the fraction of plasmid-containing cells expressing *hmrΔA::TRP1* (*Right*). (*Top*) Results for *RAP1* (wild-type) *hmrΔA::TRP1* strain with each *SIR* plasmid and vector (YEp13) control. (*Bottom*) Corresponding *rap1-12 hmrΔA::TRP1* strains.

DISCUSSION

In this study we present genetic evidence that the UAS and silencer-binding protein RAP1 plays a role in transcriptional repression of the *HMR* silent mating-type locus. All rap1^s mutants described here were isolated independently several times by using two different methods of mutagenesis, implying that only a limited number of single- or double-point mutations in *RAP1* would exhibit the appropriate phenotype (i.e., viable and silencing-defective). Because none of the *rap1^s* alleles display any measurable defect in growth or transcriptional activation of a specific set of genes known to be activated by RAP1 (*MATα*), we conclude that a silencing function of RAP1 can be genetically separated from transcriptional activation and DNA-binding functions.

The *rap1^s* alleles allow the expression of the *HMRa1* gene only when its associated silencer is deleted for the A site. None of the alleles has an effect on either a wild-type or *hmrΔB* silencer, nor were any rap1^s mutants isolated in either of these silencer backgrounds. In the case of the wild-type silencer, this result is not surprising because the silencer is a redundant regulatory element and deletion of the RAP1-binding site (the E site) has only a small effect on silencing. This explanation would also explain why rap1 mutants were not isolated in previous screens for silencing mutants (16, 18). However, why we isolated no mutants in the *hmrΔB* background, even in screens for ts mutants, is unclear because the double-mutant silencer *hmrΔEΔB* is completely derepressed. Possibly such mutants would be inviable at any temperature or difficult to obtain by our mutagenesis procedures.

The different effect of our *rap1^s* alleles on *hmrΔA* and *hmrΔB* silencers implies that the two silencers have different requirements for RAP1 activity. This functional distinction between the two silencers may be related to their ability to act as specialized origins of DNA replication. It has been shown that DNA replication, or at least passage through S phase, is necessary for the establishment of silencing at *HMR* (18, 31). The B element (an ABF1-binding site) appears neither necessary nor sufficient for the ARS activity of *HMRE* (13, 14), whereas the ARS consensus sequence (A element) is required for the ARS activity of a minimal *HMRE* silencer (13). A silencer lacking this ARS element (*hmrΔA*) may function by using one of several close matches to the ARS consensus found nearby, and this may require the presence of wild-type RAP1 at the E site. In the *hmrΔB* silencer, the intact ARS consensus element is present, and this silencer apparently does not require the RAP1 activity impaired in the mutants.

An interesting property of cells containing any of the weaker *rap1^s* alleles and an *hmrΔA::TRP1* locus is that they seem to exist in either of two states with respect to their ability to grow in the absence of tryptophan. We speculate that this epigenetic effect results primarily from switching between repressed and derepressed states rather than a graded variation in *hmrΔA::TRP1* expression. To address this question we constructed *hmr::ADE2* strains in which *ADE2* expression is monitored nonselectively by colony color: Ade$^+$ cells produce white colonies, whereas Ade$^-$ cells yield red colonies. Strikingly, some *rap1^s hmr::ADE2* strains produce colonies with distinct red/white sectors, implying clonal inheritance of two different expression states (unpublished results). Epigenetic effects on silencing have also been observed at *HML* (32, 33) and at telomeres (34) and have been attributed to a defect in the establishment, rather than the maintenance of repression (32). Because the establishment of

repression occurs during S phase of the cell cycle, it is conceivable that the rap1^s mutants affect some aspect of DNA replication or chromatin assembly required to set up silencing; this may also explain why the mutants only display a phenotype in the absence of the ARS consensus sequence at *HMR*.

The fact that high-copy-number plasmids containing either the *SIR1* or *SIR4* genes can suppress the rap1^s mutants suggests that both of these gene products become limiting in the presence of the mutant RAP1 proteins. The effect of elevated *SIR1* gene dosage is not specific to rap1^s mutants because *SIR1* overexpression suppresses other silencing defects (35). The effect of *SIR4*, however, is different and rather unusual, because elevated *SIR4* dosage in a wild-type *RAP1* strain actually results in a loss of *HMRE* silencer function. If RAP1 and SIR4 act in a complex sensitive to the stoichiometry of the two proteins, the mutant rap1 proteins, by virtue of a weakened interaction with SIR4, might require more SIR4 protein for proper complex formation than does native RAP1.

The elongation of telomeres in the rap1^s mutants was unanticipated, but the strict correlation between telomere length and derepression suggests that the two phenomena may be mechanistically related. One RAP1 function appears to be protection of chromosome ends from tract loss because rap1^{ts} mutants grown at semi-permissive temperatures have shorter telomeres (8). It is thus particularly striking that the rap1^s mutants display a telomere-elongation phenotype that, at least for *rap1-12*, is partially dominant to wild type. We conclude from this that the mutant protein binds together with native RAP1 at telomeres and alters telomere function such that tract elongation is favored. The involvement of the histone H4 in repression (36), together with evidence of *SIR*-dependent alterations of chromatin structure at *HMR* (37), suggests that repression is brought about by modifying (folding?) a region of chromatin such that it becomes inaccessible to the transcriptional machinery. Perhaps the telomere elongation seen in rap1^s mutant strains reflects an opening of the telomeric chromatin that results in increased accessibility of the telomeric repeat region to telomerase.

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