A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation

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The yeast RAP1 protein is a sequence-specific DNA-binding protein that functions as both a repressor and an activator of transcription. RAP1 is also involved in the regulation of telomere structure, where its binding sites are found within the terminal poly($C_{1-3}A$) sequences. Previous studies have indicated that the regulatory function of RAP1 is determined by the context of its binding site and, presumably, its interactions with other factors. Using the two-hybrid system, a genetic screen for the identification of protein–protein interactions, we have isolated a gene encoding a RAP1-interacting factor (RIF1). Strains carrying gene disruptions of *RIF1* grow normally but are defective in transcriptional silencing and telomere length regulation, two phenotypes strikingly similar to those of silencing-defective *rap1*^{s} mutants. Furthermore, hybrid proteins containing *rap1*^{s} missense mutations are defective in an interaction with RIF1 in the two-hybrid system. Taken together, these data support the idea that the *rap1*^{s} phenotypes are attributable to a failure to recruit RIF1 to silencers and telomeres and suggest that RIF1 is a cofactor or mediator for RAP1 in the establishment of a repressed chromatin state at these loci. By use of the two-hybrid system, we have isolated a mutation in *RIF1* that partially restores the interaction with rap1^s mutant proteins.

[*Key Words*: Transcriptional silencing; telomere structure; *Saccharomyces cerevisiae*; RAP1; protein–protein interactions; two-hybrid system]

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Repressor/activator protein 1 (RAP1) is an essential regulatory protein in yeast whose DNA-binding sites are found at three types of chromosomal elements: promoters, silencers, and telomeres. Analyses of *rap1* mutants have revealed that the protein acts at all three types of loci. Temperature-sensitive lethal mutations in *RAP1* (*rap1*^{ts}) are defective in transcriptional activation, an apparently essential function of RAP1 (Giesman et al. 1991; Kurtz and Shore 1991). Although one *rap1*^{ts} strain is also partially defective in silencing (Kurtz and Shore 1991), the repression function of RAP1 can be genetically separated from essential activation functions, as demonstrated by the isolation of several viable mutants (*rap1*^{s}) that are specifically defective in transcriptional silencing (Sussel and Shore 1991). Both types of *rap1* mutants (*rap1*^{ts} and *rap1*^{s}) also display changes in the poly($C_{1-3}A$) sequences at telomeres, which contain RAP1-binding sites approximately every 40 bp (Longtine et al. 1989). When grown at semipermissive temperatures, where RAP1 function is limiting, *rap1*^{ts} mutants undergo a progressive loss of poly($C_{1-3}A$) sequences (Conrad et al. 1990; Lustig et al. 1990). Conversely, silencing-defective *rap1*^{s} mutants have longer poly($C_{1-3}A$) tracts, the lengthening effect being proportional to the strength of the silencing defect (Sussel and Shore 1991).

Several observations suggest that the opposite regulatory functions of RAP1 are not intrinsic to its binding sites but, instead, result from interactions with different factors at promoters and silencers. For example, the particular DNA sequence of a RAP1-binding site does not determine its regulatory function: Promoter-derived binding sites function in place of a normal silencer site and vice versa (Brand et al. 1987; Shore and Nasmyth 1987; Buchman et al. 1988b). Furthermore, silencers and promoters containing RAP1-binding sites typically appear to be complex regulatory sites in which at least one other regulatory element is required for either proper repression or activation (Rotenberg and Woolford 1986; Brand et al. 1987; Kimmerly et al. 1988; Stanway et al. 1989). Several candidates exist for RAP1-interacting factors at silencers (e.g., SIR1–SIR4 and ABF1) (Rine and Herskowitz 1987; Shore et al. 1987; Buchman et al. 1988a; Diffley and Stillman 1988) and at promoters (e.g., GCR1 or GAL11/SPT13) (Fassler and Winston 1989; Nishizawa 1990; Santangelo and Tornow 1990). No biochemical or genetic data, however, directly indicate that any of these proteins physically interacts with RAP1.

Recently, we have shown that high-level expression of

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the carboxyl terminus of RAP1 fused to the GAL4 DNA-binding domain (G_{BD}) causes a dominant-negative effect on silencing, leading to the derepression of normally silent genes at the *HMR* locus (Hardy et al. 1992). Overexpression of the carboxyl terminus of RAP1 also results in telomere elongation (Conrad et al. 1990; Hardy 1991), another phenotype associated with *rap1*^{s} mutants. Derepression by G_{BD}/RAP1 hybrids occurs only when they are expressed from a strong promoter on multicopy plasmids and not when expressed from the *RAP1* promoter in single copy. Furthermore, this dominant-negative effect is not dependent on a DNA-binding site to target the hybrids to the silencer. We reasoned, therefore, that derepression results from the titration of limiting silencer factors. The part of RAP1 responsible for this dominant-negative effect on silencing was mapped to a region from amino acid 667 to the carboxyl terminus (amino acid 827), the same region of the protein to which the silencing-defective *rap1*^{s} mutants map. Our results indicated that this putative titrated factor is probably neither RAP1 itself nor the other silencer binding factor ABF1 (Hardy et al. 1992).

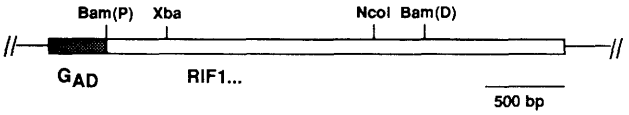
On the basis of these results, we have used a novel genetic system (Fields and Song 1989; Chien et al. 1991) to identify a protein that interacts with the RAP1 carboxyl terminus. We report the cloning and characterization of a gene encoding such a protein, designated *RIF1* (for RAP1-interacting factor 1). Strains carrying disruptions of the *RIF1* gene are defective in silencing and have elongated poly($C_{1-3}A$) tracts at telomeres, both phenotypes characteristic of *rap1*^{s} mutants. By use of the two-hybrid system, we have shown that the region of RAP1 required for the silencer derepression effect (amino acids 667–827) is also necessary for the RAP1–RIF1 protein–protein interaction. Furthermore, hybrids made with the different rap1^s mutants display a defect in interacting with RIF1 that is proportional to the corresponding *rap1*^{s} silencing defects. These results suggest that one function of RAP1, defective in the rap1^s mutants, is to direct the binding of RIF1 to silencers and telomeres. Interestingly, two rap1^s hybrids that appear to be completely unable to interact with RIF1 in the two-hybrid system still lead to silencer derepression when overexpressed, suggesting that the RAP1 carboxyl terminus may also interact with other factors at silencers and telomeres not yet unidentified.

Results

Isolation of a RAP1-interacting protein by the two-hybrid system

Expression of G_{BD}/RAP1 hybrids from a strong promoter results in derepression of the silent *HMR* locus, presumably as the result of protein–protein interactions that either sequester or titrate another factor important for silencing (Hardy et al. 1992). In an attempt to identify this putative RIF, we have used the two-hybrid system, a genetic method to identify protein–protein interactions (Fields and Song 1989; Chien et al. 1991). This method utilizes the separable domains of the transcriptional activator GAL4, its amino-terminal DNA-binding domain (G_{BD}, amino acids 1–147), and its carboxy-terminal transcriptional activation domain (G_{AD}, amino acids 768–881). Fields and co-workers showed that coexpression in yeast of two interacting proteins (X and Y) as G_{BD}/X and G_{AD}/Y hybrids resulted in the activation of a *GAL1–lacZ* reporter gene. Activation occurs presumably by a protein–protein interaction between the X and Y portions of the two hybrids that tethers the activation domain to the promoter. We have adapted the two-hybrid screen to isolate RAP1-interacting proteins by starting with a *GAL1–lacZ* reporter strain expressing the G_{BD}/RAP1(653-827) hybrid. This strain was then transformed with a library of plasmids in which yeast genomic fragments (produced by partial digestion with the enzyme *Sau*3A) were fused to sequences encoding the SV40 T antigen nuclear localization signal followed by the G_{AD} (a generous gift of P. Bartel and S. Fields). Approximately 120,000 transformants were replica plated onto X-gal plates, and 11 blue colonies were identified and purified (for details, see Materials and methods). Potential activation domain hybrids encoded on *LEU2*-containing plasmids were isolated from these cells and transformed back into two different reporter strains, one with and one without the G_{BD}/RAP1(653-827) hybrid plasmid. Of the 11 plasmids, 10 activated in both reporter strains and, by restriction mapping, appeared to contain all or portions of the *GAL4* gene.

A single G_{AD} plasmid was found to confer G_{BD}/RAP1(653-827)-dependent activation of the reporter gene (Fig. 1). Activation requires the RAP1 portion of the G_{BD}/RAP1 hybrid; no activation is observed when the plasmid is transformed into a strain expressing the G_{BD} alone. The hybrid is therefore not targeted to the reporter gene through a direct G_{BD} interaction but, instead, encodes a polypeptide that interacts with RAP1(653-827), which will be referred to hereafter as RIF1. Subsequent analysis (see below) indicated that the plasmid contained a carboxy-terminal region of the *RIF1* gene fused in-frame to G_{AD}-coding sequences. To demonstrate that the activation effect required the production of a G_{AD}/RIF1 fusion protein, the following experiments were done. First, a frameshift mutation was created between G_{AD} and RIF1 sequences (at a *Bam*HI site) to truncate the fusion protein just beyond the G_{AD} sequences. This frameshift mutation in the G_{AD}/RIF1 hybrid abolishes activation, demonstrating that the RIF1 portion of the hybrid is required for activation of the *GAL1–lacZ* reporter gene (Fig. 1). However, two downstream frameshift mutations, at a unique *Nco*I site and a distal *Bam*HI site, had no effect on activation by the G_{AD}/RIF1 plasmid, indicating that any coding sequences beyond these two sites are not necessary for the interaction. To determine the 3′ end point of *RIF1* sequences required for activation, two other mutations in the insert DNA were constructed. Deletion of sequences 3′ to an *Xba*I site located ~500 bp beyond the fusion junction abolished activation, whereas deletion of sequences beyond the distal *Bam*HI site had no effect (Fig. 1). These data indicate



DNA Binding Domain Hybrid	Activation Domain Hybrid	βgal Units
G_{BD}	—	<1
G_{BD}/RAP1	—	<1
G_{BD}/RAP1	G_{AD}/RIF1	616
—	G_{AD}/RIF1	<1
G_{BD}	G_{AD}/RIF1	<1
G_{BD}/RAP1	G_{AD}/RIF1-Bam(P) fs	<1
G_{BD}/RAP1	G_{AD}/RIF1-NcoI fs	681
G_{BD}/RAP1	G_{AD}/RIF1-Bam(D) fs	750
G_{BD}/RAP1	G_{AD}/RIF1(Bam(P)-XbaI)	<1
G_{BD}/RAP1	G_{AD}/RIF1(Bam-Bam)	770
G_{BD}/RIF1(Bam-Bam)	—	<1
G_{BD}/RAP1	RIF1 2μm	<1

Figure 1. Isolation of a plasmid encoding a RAP1-interacting protein using the two-hybrid system. A plasmid (G_{AD}/RIF1) was isolated from a library of fusions in the expression vector pGAD2 on the basis of its ability to activate a *GAL1–lacZ* reporter gene in the presence of the G_{BD}/RAP(653-827) hybrid (see Material and methods). A partial restriction map of G_{AD}/RIF1 is shown above. Quantitative β-galactosidase assays were conducted on a variety of strains transformed with the designated plasmids, and the average of four independent transformants is given. All the strains contain 2-μm-based high copy number plasmids. The G_{BD} hybrids are expressed from the strong *ADH1* promoter, whereas the G_{AD} hybrids are expressed from a cryptic promoter, perhaps lying within the *ADH1* terminator fragment. G_{AD}/RIF[*Bam*(P)–*Bam*(D)] and G_{AD}/RIF[*Bam*(P)–*Xba*I] contain the corresponding restriction fragments from the original G_{AD}/RIF1 isolate cloned into the pGAD2 vector (see Materials and methods). RIF1 2 μm contains the full-length *RIF1* gene, including putative upstream promoter sequences, on a 2-μm-based *LEU2*-containing plasmid.

that the RIF1 sequences necessary and sufficient for an interaction with the RAP1 carboxyl terminus are located upstream of the *Nco*I site and that the sequences contained on the small *Bam*HI–*Xba*I fragment are not sufficient for this interaction. Activation by G_{AD}/RIF1 is also dependent on the G_{AD} portion of the hybrid because a G_{BD}/RIF1 hybrid, which targets the carboxy-terminal part of RIF1 from the original G_{AD}/RIF1 hybrid to the reporter gene, does not result in activation (Fig. 1). Furthermore, overexpression of full-length RIF1 (from a 2-μm plasmid containing the complete gene) in the presence of the G_{BD}/RAP1(653-827) hybrid does not result in activation of the reporter gene (Fig. 1; RIF1 2 μm).

Isolation and DNA sequence of the RIF1 *gene*

Using the proximal *Bam*HI–*Xba*I *RIF1* fragment to probe a yeast genomic library, a large overlapping clone of ~8.0 kb was obtained. The DNA sequence of 6.5 kb of this fragment was determined and is shown in Figure 2. The sequence contains a single large open reading frame that could encode a protein of 1916 amino acids with a molecular mass of 219 kD. Although this open reading frame contains the splicing signal 5′-TACTAAC-3′ (at nucleotide 4947), we found no nearby match to the conserved 5′ splice site sequence (5′-GTATGT-3′) (Guthrie 1991 and references therein). Northern analysis of both total and poly(A)-selected RNA shows that this 8.0-kb fragment hybridizes to an ~6.3-kb RNA, consistent with the 5.8-kb open reading frame present in the fragment (data not shown). The RIF1 sequence present in the G_{AD}/RIF1 hybrid begins at amino acid 1614 of the predicted protein, indicating that a carboxy-terminal portion of RIF1 is sufficient to interact with RAP1. We failed to find any strong similarities between the predicted RIF1 protein and sequences present in available protein data bases (for details, see Material and methods), and the protein sequence does not appear to indicate any distinctive structural features. Interestingly, there is a single *Mlu*I site (ACGCGT) in the 6.5-kb sequence (at nucleotide 262), ~200 bp upstream of the putative initiator ATG of *RIF1*. *Mlu*I sites are often found within the promoters of genes whose mRNA levels increase at the G_1/S-phase boundary (Andrews and Herskowitz 1990). Several genes encoding DNA replication proteins contain one or two *Mlu*I sites in their upstream regions, and these sites appear to be at least in part responsible for their cell cycle-regulated transcription (Pizzagalli et al. 1988; Bauer and Burgers 1990; Brill and Stillman 1991; Lowndes et al. 1991).

Disruption of the RIF1 *gene affects silencing*

To examine directly the in vivo function of RIF1, the cloned gene was used to construct a disruption of the chromosomal copy. An *Mlu*I–*Xba*I fragment of *RIF1* (Fig. 2, base pairs 262–5685) was deleted and replaced by a fragment containing the *URA3* gene. This results in deletion of >90% of the predicted *RIF1* open reading frame and removes all of the amino-terminal coding sequences. Initially, a *ura3*$^-$ homozygous diploid was transformed with this construct. Ura$^+$ transformants were selected and screened by Southern blotting for those in which a single copy of the gene had been replaced by the *rif1::URA3* deletion/substitution. Four different isolates with the appropriate structure were sporulated, and most tetrads yielded four viable spores with *URA3* segregating 2 : 2, indicating that *RIF1* is not an essential gene (data not shown). The growth of *rif1::URA3* cells on YEPD plates is indistinguishable from isogenic *RIF1* cells, indicating that RIF1 is not required for any essential transcriptional activation functions mediated by RAP1.

To determine whether *rif1::URA3* mutants are defective in silencing, the effects of the *RIF1* disruption at the

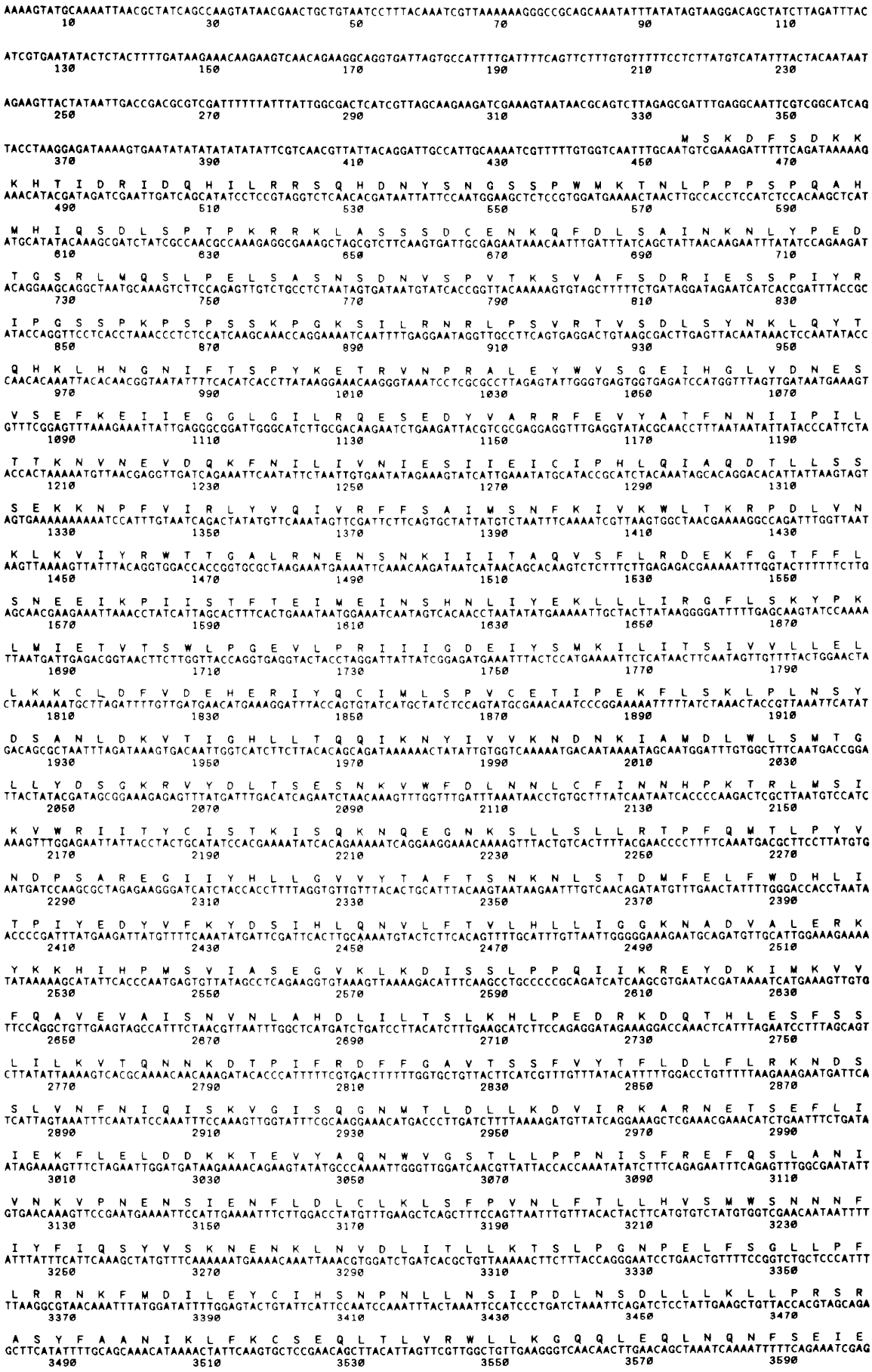


Figure 2. {*See facing page for legend.*}

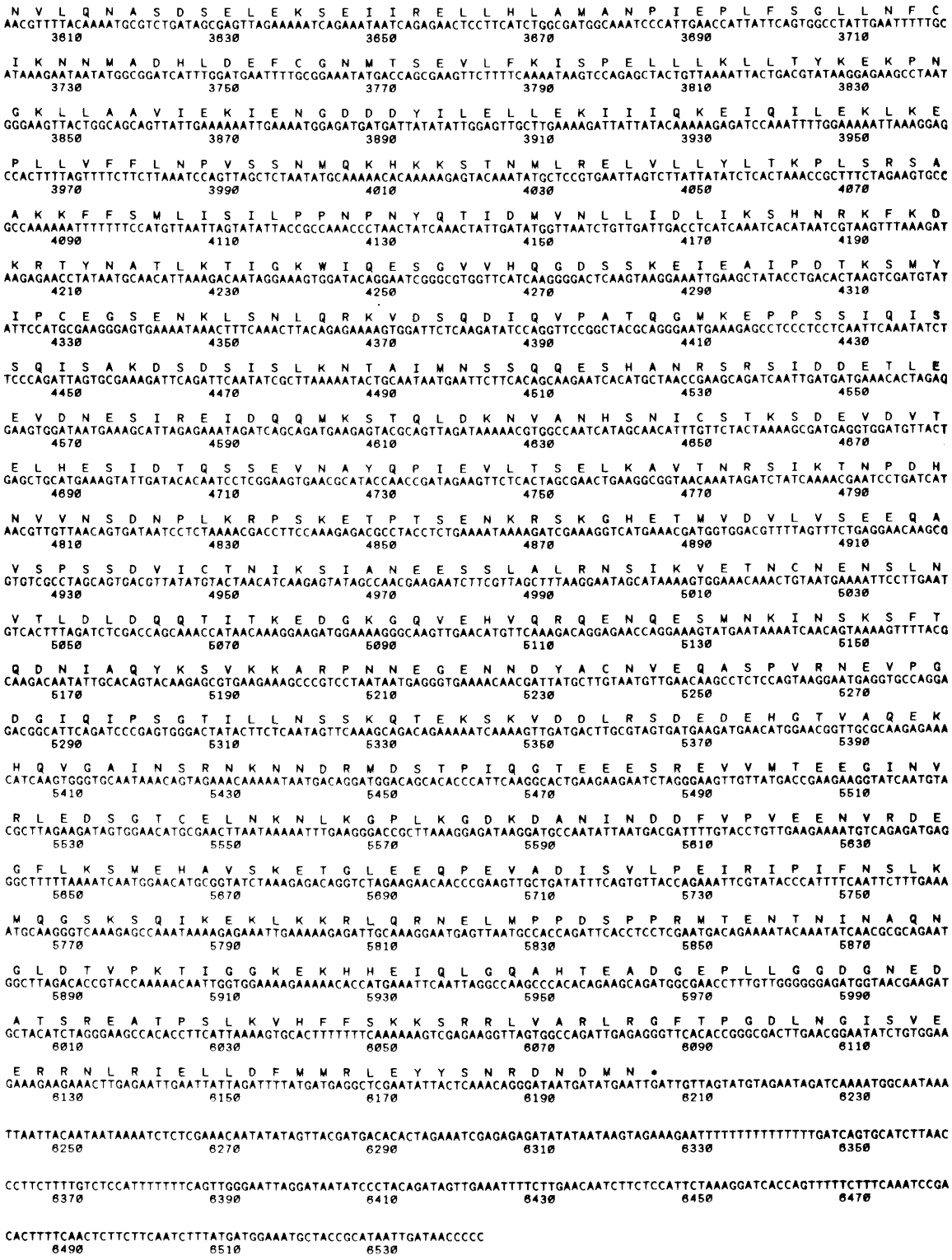


Figure 2. Nucleotide and deduced amino acid sequence of the *RIF1* gene. The DNA sequence shown begins 454 bp upstream of the putative translational start site (first methionine) of RIF1 and extends 337 bp beyond the TGA termination codon. The predicted amino acid sequence of RIF1 is shown in the one-letter code.

HMR locus were assayed. Because the *HMRE* silencer is a redundant regulatory element, it was necessary to examine a wild-type silencer and two different mutated silencers that retain complete function. In addition to the RAP1-binding site, the *HMRE* silencer contains two other regulatory sites (called A and B), either one of

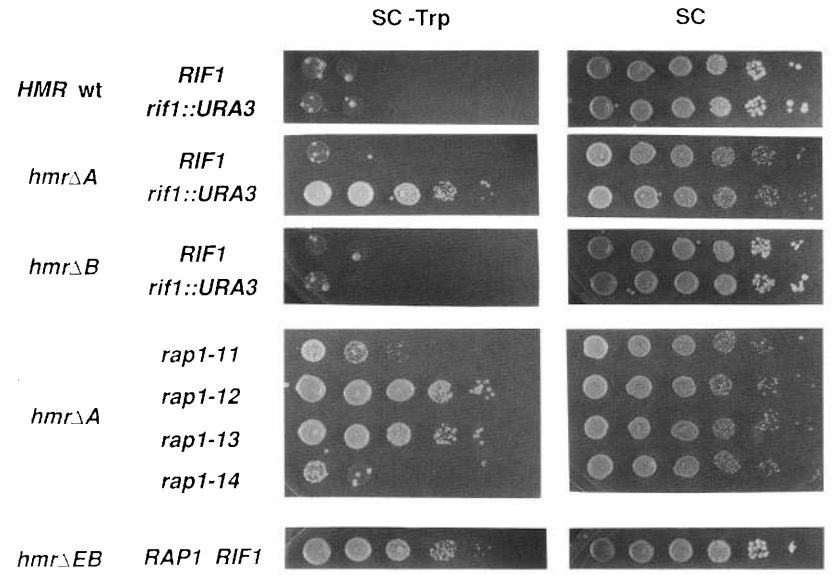


Figure 3. *rif1::URA3* mutants are defective in silencing at *hmrΔA::TRP1*. The effect of a *rif1::URA3* gene disruption on transcriptional silencing in strains containing *hmr::TRP1* loci with either wild-type or mutated silencer elements is shown. Tenfold serial dilutions of overnight liquid cultures grown in rich medium (YEPD) were spotted onto plates containing synthetic medium lacking tryptophan (SC − Trp, *left*) and synthetic complete medium (SC, *right*). The plates were incubated at 30°C for 2–3 days before being photographed. For purposes of comparison, the growth of the four different *rap1*^{s} strains (in a *hmrΔA::TRP1* background) and a strain carrying the defective *hmrΔEB* silencer are shown below.

which, together with the RAP1 site (E), is sufficient for complete repression (Brand et al. 1987; Kimmerly et al. 1988). The A element is an autonomously replicating sequence (ARS) consensus element, whereas the B element is a binding site for the ABF1 protein (Shore et al. 1987; Buchman et al. 1988a). We noted previously that *rap1*^{s} mutants display a defect in silencing only when the A element at *HMR* is deleted (*hmrΔA*) (Sussel and Shore 1991). Furthermore, the *hmrΔA* silencer is affected most severely by overexpression of G_{BD}/RAP1 hybrids (Hardy et al. 1992).

To assay for silencer function, strains in which the *TRP1* gene has been placed at *HMR* (Miller et al. 1984; Brand et al. 1985) were used. The ability of such strains to grow in the absence of tryptophan is a sensitive and accurate assay for the loss of silencer function (Sussel and Shore 1991). Haploid strains containing *hmr::TRP1*, *hmrΔA::TRP1*, or *hmrΔB::TRP1* silencers were transformed with *rif1::URA3* DNA and screened by Southern blotting for the correct chromosomal disruption. Analysis of these strains showed that only the *hmrΔA::TRP1* silencer strain was affected by the chromosomal disruption of *RIF1* and was thus able to grow in the absence of tryptophan (Fig. 3). Furthermore, the derepression of *hmrΔA::TRP1* caused by the *rif1::URA3* mutation was complete and indistinguishable from that of the strong *rap1*^{s} alleles, *rap1-12* and *rap1-13*. Strains containing a wild-type silencer (*hmr::TRP1*) or a ΔB silencer (*hmrΔB::TRP1*) showed normal repression of the *TRP1* reporter gene in the presence of the *rif1::URA3* disruption.

RIF1 *affects telomere length regulation*

To determine whether RIF1 plays a role at telomeres, as does RAP1, Southern blot analysis was used to measure the average length and heterogeneity of telomeres from the *rif1::URA3* mutant strains. Total yeast genomic DNA was digested with *Xho*I and probed with radiolabeled poly[d(G-T)] · poly[d(A-C)], which hybridizes with the terminal poly($C_{1-3}A$) sequences (Shampay et al. 1984; Walmsley et al. 1984). A prominent heterogeneous band of ~1.2 kb results from the large number of yeast telomeres that contain a subtelomeric Y′ element. As shown in Figure 4, *rif1::URA3* mutants display a significant increase in the average length of this terminal fragment (~200–300 bp), as compared to the wild-type parent strain. We presume that this results from an increase in the length of the poly($C_{1-3}A$) sequences within these fragments. In addition, the heterogeneity in length of the chromosome ends appears to increase in *rif1::URA3* strains relative to their wild-type parent. This effect is very similar to the telomere lengthening displayed by the silencing-defective *rap1-12* and *rap1-13* mutants (Fig. 4).

A carboxy-terminal domain of RAP1 required for RIF1 binding

A previously characterized series of G_{BD}/RAP1 hybrids (Hardy et al. 1992) was used to determine which RAP1 sequences in G_{BD}/RAP1 are required for G_{AD}/RIF1-dependent activation of the *GAL1–lacZ* reporter gene. Figure 5 shows that there is a very close correlation between the sequences required for derepression by G_{BD}/RAP1 and those required for an interaction with G_{AD}/RIF1. For example, G_{BD}/RAP1(653-827) and G_{BD}/RAP1(655-827) both completely derepress the *hmrΔA::TRP1* locus and cooperate with G_{AD}/RIF1 to give comparable levels of β-galactosidase expression. In addition, the G_{BD}/RAP1(679-827) hybrid displays a reduced ability to both derepress [~10- to 20-fold lower than G_{BD}/RAP1(653-827) and G_{BD}/RAP1(655-827)] and to participate in G_{AD}/RIF1-dependent activation (36 units, compared with 616

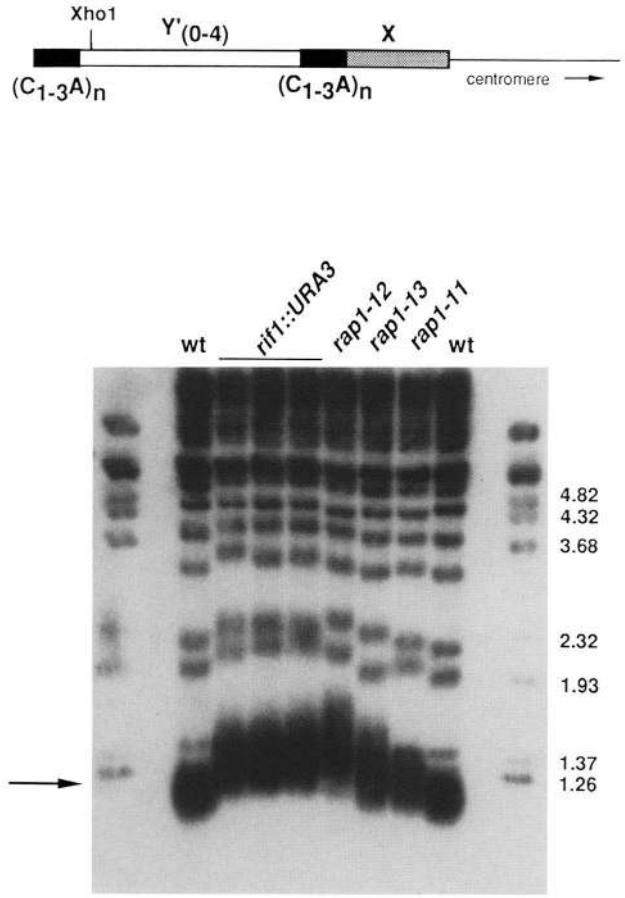


Figure 4. Telomeric poly($C_{1-3}A$) tracts are lengthened in *rif1::URA3* mutants. (*A*) Schematic representation of yeast telomeres. Note that many, but not all, telomeres contain the Y′ element, which has a *Xho*I site near the poly($C_{1-3}A$) tract. (*B*) Southern analysis. *Xho*I-digested genomic DNA from the indicated strains was probed with ^{32}P-labeled poly[d(G-T) · d(C-A)]. A heterogeneous band of ~1.2 kb in the wild-type strain (indicated by the arrow) arises from the many yeast telomeres that contain at least one Y′ element.

and 895 units). Finally, all of the additional hybrids that we examined are completely defective in both activities (Fig. 5, rows 5–11). Taken together, these results indicate that both derepression and G_{AD}/RIF1-dependent activation by G_{BD}/RAP1 hybrids require the same carboxy-terminal domain of RAP1.

Silencing-defective rap1^s mutants are defective in G_{AD}/RIF1-dependent activation

Having shown that a carboxy-terminal part of RAP1, whose overexpression causes derepression, is necessary for a RAP1–RIF1 interaction, we then asked whether silencing-defective point mutations in this region of RAP1 disrupt the RAP1–RIF1 interaction. To answer this question we made use of four different *rap1^s* alleles that contain either single or double missense mutations in the RAP1 carboxyl terminus, affecting amino acids 726, 727, and 747 (Sussel and Shore 1991). The *rap1^s* mutants cause either strong (*rap1-12* and *rap1-13*) or weak (*rap1-11* and *rap1-14*) derepression of the *hmrΔA*::TRP1 locus and variable elongation of poly($C_{1-3}A$) tracts at telomeres. A series of four G_{BD}/rap1^s(653-827) hybrids were constructed that incorporated the mutant changes of the *rap1^s* alleles into the G_{BD}/RAP1(653-827) hybrid. These G_{BD}/rap1^s hybrids were then coexpressed with the G_{AD}/RIF1 hybrid in the *GAL1–lacZ* reporter strain.

As shown in Table 1, there is a striking inverse correlation between the extent of derepression caused by the different *rap1^s* alleles and the ability of the corresponding G_{BD}/rap1^s hybrids to activate the *GAL1–lacZ* reporter when expressed together with G_{AD}/RIF1. The two strong *rap1^s* mutants, *rap1-12* and *rap1-13*, are both completely defective in silencing the *hmrΔA::TRP1* locus and also fail to activate in conjunction with G_{AD}/RIF1. Perhaps more significant is the observation that the weak *rap1^s* alleles (*rap1-11* and *rap1-14*, with only a partial defect in silencing of *hmrΔA::TRP1*) display a measurable but reduced ability as G_{BD}/rap1^s hybrids to interact with G_{AD}/RIF1. These results suggest that the strong *rap1^s* mutants have a totally defective RIF1 interaction site, whereas the weak *rap1^s* mutants retain a partially functional RIF1 interaction site. Furthermore, they signify that the defect in silencing by the *rap1^s* mutants may be explained by a failure to interact with RIF1. Interestingly, though, the G_{BD}/rap1^s hybrids derepress the *hmrΔA::TRP1* locus to the same extent as the G_{BD}/RAP(653-827) hybrid from which they were derived (data not shown; see Discussion).

Isolation of a G_{AD}/rif1 mutant that partially restores an interaction with G_{BD}/rap1^s hybrids

We reasoned that if rap1^s proteins were defective in an interaction with RIF1, then mutations in *RIF1* may suppress this defect. The two-hybrid system was utilized further to screen for a mutated G_{AD}/rif1 hybrid that could suppress the activation defect of the G_{BD}/rap1^s hybrids. The G_{AD}/RIF1 plasmid was mutagenized by passage through an *Escherichia coli mutD5* strain (see Materials and methods) and transformed into a *GAL1–lacZ* reporter strain expressing G_{BD}/rap1-12, which is completely defective in G_{AD}/RIF1-mediated activation (see Table 1). From >15,000 transformants, one plasmid was obtained that conferred a blue colony color on X-gal plates and thus appeared to suppress the G_{BD}/rap1-12 activation defect. The plasmid, encoding what will be referred to as G_{AD}/rif1-1, was retransformed into the reporter strain coexpressing the original G_{BD}/RAP1(653-827) hybrid and into strains coexpressing each one of the four different G_{BD}/rap1^s mutant derivatives of this hybrid. Interestingly, the β-galactosidase levels for each strain converged to an intermediate value between 100 and 200 units, less than one-third of the value obtained with the two wild-type hybrids, G_{BD}/RAP1 and G_{AD}/RIF1 (Table 2). The mutation was mapped to the RIF1 part of the G_{AD}/RIF1 hybrid by exchanging restriction fragments with the wild-type G_{AD}/RIF1 plasmid and

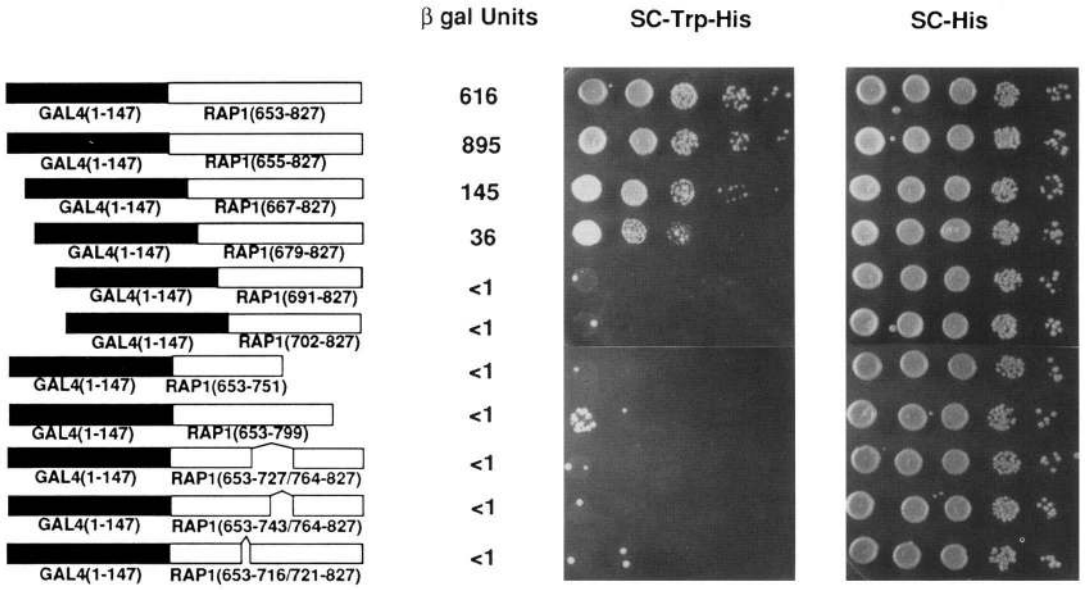


Figure 5. RIF1 interacts with a carboxy-terminal domain of RAP1. A series of G_{BD}/RAP1 hybrids was assayed for the ability to interact with the G_{AD}/RIF1 hybrid and to activate the *GAL1–lacZ* reporter gene. Quantitative β-galactosidase assays were performed for strains containing both the indicated G_{BD}/RAP plasmids and the G_{AD}/RIF1 plasmid (see Fig. 1 and Materials and methods). The G_{BD}/RAP-expressing plasmids were also assayed for their effect on silencing at the *hmrΔA::TRP1* locus (see Fig. 3; Hardy et al. 1992).

was identified by DNA sequencing as a G → A change at nucleotide 6169 (Fig. 2). This mutation would be predicted to result in a change from glutamic acid to lysine at amino acid 1906 of RIF1, only 10 codons from the carboxyl terminus of the protein.

Two features of the G_{AD}/rif1-1 mutant hybrid are worth emphasizing. First, the mutant hybrid appears to interact less well with the wild-type G_{BD}/RAP1 hybrid than does the wild-type G_{AD}/RIF1 hybrid, as shown by the significantly lower β-galactosidase value. This result indicates that the G_{AD}/rif1-1 mutant does not work by simply raising the affinity for wild-type RAP1 and thus compensating for a defect in rap1-12. Second, although the rif1-1 mutant is not allele specific, that is, it improves the interaction with both the rap1-13 and rap1-14 hybrids, it has the striking property of appearing to interact approximately equally well with all four mutant rap1^s hybrids and with the wild-type G_{BD}/RAP1 hybrid. This property is in marked contrast to the ability of the G_{AD}/RIF1 hybrid to distinguish between wild-type G_{BD}/RAP1 and the two classes of rap^s hybrids. The failure of the G_{AD}/rif1-1 hybrid to distinguish between G_{BD}/RAP1 and the different G_{BD}/rap^s hybrids suggests that the rif1-1 mutation may abolish a specific interaction with RAP1, defined at least in part by codons 726, 727, and 747 in RAP1, rather than compensating for the alteration in the *rap1-12* mutant.

Table 1. *G_{BD}/rap1^s mutant hybrids are defective in G_{AD}/RIF1-dependent activation*

DNA-binding domain hybrid	β-gal units	Silencing defect in context of full-length RAP1
G_{BD}/RAP1	616	none
G_{BD}/rap1-11	172	weak
G_{BD}/rap1-12	<1	strong
G_{BD}/rap1-13	<1	strong
G_{BD}/rap1-14	35	weak
G_{BD}	<1	

Discussion

We have isolated a novel gene, *RIF1*, on the basis of the ability of a portion of its protein product to associate with the RAP1 carboxyl terminus in vivo. Loss of RIF1 function results in derepression of an *HMR* silencer, whose ARS consensus element has been deleted, and in the elongation of telomeres, two properties characteris-

Table 2. *The G_{AD}/rif1-1 mutant suppresses the activation defect of G_{BD}/rap1^s mutants*

DNA-binding domain hybrid	Activation domain hybrid: G_{AD}/RIF1 (β-gal units)	Activation domain hybrid: G_{AD}/rif1-1 (β-gal units)
G_{BD}/RAP1	616	165
G_{BD}/rap1-12	<1	110
G_{BD}/rap1-13	<1	177
G_{BD}/rap1-14	35	188
G_{BD}/rap1-11	172	126
G_{BD}	<1	ND[a]
None	<1	<1

[a]Not determined.

tic of silencing-defective *rap1^s* alleles (Sussel and Shore 1991), confirming an involvement of RIF1 in both transcriptional silencing and telomere length regulation.

The genetic experiments presented here provide compelling evidence that the RAP1 and RIF1 proteins can physically associate and that this association is important for repression at *hmrΔA* silencers and length regulation at telomeres. Activation by G_{AD}/RIF1 would appear to depend on a physical association with the RAP1 carboxyl terminus. Furthermore, using the two-hybrid system, we have shown that G_{BD}/rap1^s mutant hybrids interact poorly or not at all with G_{AD}/RIF1. What is particularly striking is the strict correlation between the severity of the silencing defects of individual *rap1^s* mutant alleles and the ability of the corresponding G_{BD}/rap1^s hybrids to interact with the G_{AD}/RIF1 hybrid. The two weakest *rap1^s* alleles lead to an intermediate level of activation together with G_{AD}/RIF1, whereas the two strongest *rap1^s* alleles fail to produce a detectable signal in the two-hybrid system. It seems unlikely that the G_{BD}/rap1^s defects reflect a general instability of the rap1^s carboxy-terminal domains, as the *rap1^s* mutants have both normal protein levels and do not display any temperature sensitivity (Sussel and Shore 1991). In addition, the G_{BD}/rap^s hybrids are all capable of silencer derepression, implying that they are also stable and properly folded (data not shown).

The properties of the compensating G_{AD}/rif1-1 mutant argue strongly that there is a specific protein–protein interaction between RIF1 and the small carboxy-terminal region of RAP1 affected by the *rap1^s* alleles. The G_{AD}/rif1-1 hybrid appears to interact less well with wild-type G_{BD}/RAP1 than does G_{AD}/RIF1, implying that the *rif1-1* mutation does not work by increasing the affinity of RIF1 for both wild-type RAP1 and rap1^s proteins. Instead, we suggest that the *rif1-1* mutation alters a specific interaction with RAP1 defined by the *rap1^s* alleles, such that the rif1-1 protein can no longer distinguish between wild-type RAP1 and the four different rap1^s mutants and thus interacts equally well with all of them. The properties of the *rif1-1* mutation are similar to those described for loss-of-contact mutations in sequence-specific DNA-binding proteins (Ebright et al. 1987). A simplified model for the RAP1–RIF1 interaction, based on the mutant data, is shown schematically in Figure 6. The model predicts that the *rap1^s* alleles (at amino acids 726, 727, and 747) define a specific interaction site on the RAP1 carboxyl terminus and that strong *rap1^s* alleles (*rap1-12* and *rap1-13*) create a repulsive interaction with RIF1 at this site that is abolished by the *rif1-1* mutation. Further experiments, involving site-directed mutagenesis of both proteins, will test the validity of this proposal.

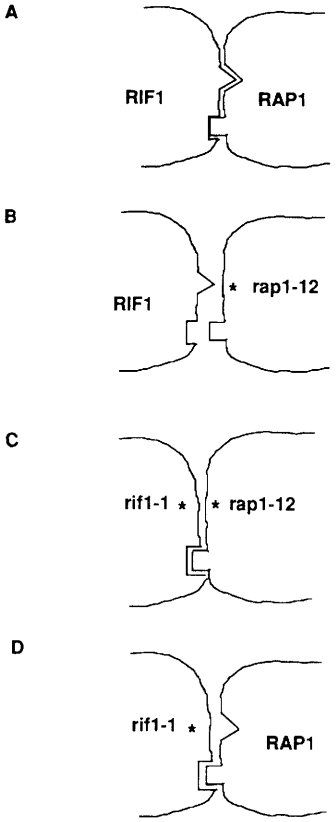


Figure 6. A model for the RAP1–RIF1 interaction based on properties of mutant proteins in the two-hybrid system. (*A*) The complementary triangular surfaces are intended to represent a proposed interaction site between RAP1 and RIF1, defined in part by amino acids 726, 726, and 747 in RAP1 and amino acid 1906 in RIF1. The complementary rectangular surfaces represent the sum of all other RAP1–RIF1 interactions. The *rap1-12* mutation creates an unfavorable interaction that significantly reduces or abolishes binding (*B*). The *rif1-1* mutation removes this unfavorable contact with rap1-12, allowing rif1-1 to interact equally well with rap1-12 and wild-type RAP1 protein (*C*,*D*). In the weaker alleles, rap1-14 and rap1-11, we imagine that the repulsive rap1–RIF1 interaction seen for the rap1-12–RIF1 combination (*B*) is either minimal (rap1-14) or essentially nonexistent (rap1-11).

RIF1: a co-factor or mediator for RAP1 at silencers and telomeres

Previous studies have identified a large number of *trans*-acting regulators of silencing, including the four *SIR* genes (Haber and George 1979; Klar et al. 1979; Rine and Herskowitz 1987), *HHF2* (encoding histone H4) (Kayne et al. 1988; Megee et al. 1990), *NAT1* and *ARD1* (encoding an amino-terminal protein acetyltransferase) (Whiteway et al. 1987; Mullen et al. 1989), *CDC7* (Axelrod and Rine 1991), *SUM1* (Klar et al. 1985; Livi et al. 1990; Laurenson and Rine 1991), *RAP1* (Shore and Nasmyth 1987; Kurtz and Shore 1991; Sussel and Shore 1991), and presumably the gene encoding the other silencer binding factor *ABF1* (Diffley and Stillman 1989; Halfter et al. 1989; Rhode et al. 1989; Francesconi and Eisenberg 1991). It is worth noting that in searching directly for a RAP1-interacting factor involved in silencing, we have identified a novel gene. Our results suggest that the con-

nection between the silencer DNA-binding factors and other proteins responsible for silencing (e.g., histone H4 and SIR proteins) may be complex, involving RIF1 and possibly other cofactors or mediators. Recent studies of transcriptional activation also point to intermediary factors in the interaction of DNA-binding activators and the basic transcriptional machinery (Dynlacht et al. 1991; Flanagan et al. 1991).

Data presented here are consistent with a model in which RAP1 targets the binding of RIF1 to silencers and telomeres. We propose that this interaction leads to the recruitment of essential silencer factors (e.g., SIR proteins), which themselves function as modifiers of chromatin structure at both silent mating-type loci and telomeres (Nasmyth 1982; Gottschling et al. 1990; Aparicio et al. 1991). It should be noted, however, that we cannot rule out a model in which RIF1 modifies RAP1 to allow it to function at silencers and telomeres but is itself not stably associated with these loci. It is possible that the proposed RAP1–RIF1 interaction is restricted to silencer and telomere loci, because deletion of *RIF1* has no effect on essential *RAP1* transcriptional activation functions and RIF1 interacts with a part of RAP1 that may be important only at silencers and telomeres. One explanation for such a restriction is that RIF1 interacts with specific DNA sequences and/or other proteins found exclusively at silencers and telomeres. Modulation of the function of a DNA-binding regulatory protein by additional protein–protein and protein–DNA interactions occurs in other systems. For example, the MATα2 repressor protein in yeast uses the MCM1 protein, itself an activator in many contexts, as a corepressor at some loci through a combination of protein–protein interactions and sequence-specific DNA binding (Keleher et al. 1988, 1989). One interpretation of our results is that RAP1 does not interact directly with any of the silencer factors identified previously (e.g., SIR1–SIR4) and that its essential function in silencing at *hmrΔA* is to target the binding of RIF1 to this locus, which then leads to the recruitment of SIR proteins. This idea can be tested in part by providing RIF1 with a heterologous DNA-binding domain and targeting the resultant hybrid protein to a silencer containing the corresponding DNA-binding site and lacking a binding site for RAP1. Whether RIF1 is a mediator or a cofactor for RAP1 at silencers and telomeres, it seems likely that it interacts with other factors at these loci (e.g., SIRs) and that such interactions will have important functional consequences.

A particularly notable feature of the *HMRE* silencer is its functional redundancy (Brand et al. 1987; Kimmerly et al. 1988; Stone et al. 1991). We noted recently that this redundancy extends to the RAP1 protein itself (Sussel and Shore 1991) because *rap1^s* mutants do not affect the ability of the protein to contribute to repression of wild-type or *hmrΔB* silencers. Because the *rif1::URA3* mutants also display a silencing defect only when the A element (an ARS consensus sequence) is deleted at *HMRE*, RIF1 could function only for this one redundant RAP1 silencing function. This RIF1-associated silencing pathway is not required for either the wild-type or *hmrΔB* silencers to work. With this in mind, one can imagine that RIF1 is required to stabilize interactions with replication factors (e.g., an ARS consensus sequence binding factor) at an *hmrΔA* silencer, where a perfect match to the ARS consensus sequence is not present. In such a scheme, RIF1 function may be needed only during the S phase of the cell cycle, when the establishment of silencing is thought to occur (Miller and Nasmyth 1984). It will be interesting to determine whether *RIF1* transcription is elevated during S phase, as suggested by the presence of the *Mlu*I site upstream of the RIF1 gene.

Genes placed near telomeres are subject to a position-effect repression (Gottschling et al. 1990) that requires many of the same *trans*-acting regulators necessary for silencing at *HML* and *HMR* (Aparicio et al. 1991). However, telomeric silencing is metastable, a phenomenon seen at silent mating-type loci only in strains with certain *cis*- or *trans*-silencing mutations (Pillus and Rine 1989; Mahoney et al. 1991; Sussel and Shore 1991; L. Sussel and D. Shore, unpubl.) and is independent of *SIR1* function (Aparicio et al. 1991). These observations have led to the suggestion that the silencers at *HML* and *HMR* have additional redundant pathways for repression not present at telomeres (Aparicio et al. 1991). The silencing pathway defined by the *rap1^s* and *rif1* mutations may not function at telomeres or it may also be redundant there. This may explain why *rap1^s* mutants appear to have no effect on telomeric silencing (B. Billington and D. Gottschling, pers. comm.), despite the occurrence of many RAP1-binding sites within the telomeric poly($C_{1-3}A$) tracts. It remains to be seen whether telomeric silencing is also independent of RIF1. The reason for telomere elongation in both *rap1^s* and *rif1* mutants remains unclear and is puzzling in light of the observation that mutations in *SIR2–SIR4*, which abolish repression at silent mating-type loci and telomeres, have no effect on telomere length (D. Gottschling, pers. comm.). However, the correlation between the strength of *rap1^s* silencing defects and the extent of telomere elongation (Sussel and Shore 1991) suggests that these two phenomena are related at some level. Perhaps mechanisms controling telomere length are more sensitive to the effects of *rap1^s* and *rif1* mutations than is the telomeric silencing machinery.

Other RIFs

Given both the multiple functions of RAP1 in silencing and the requirement for RIF1 in only one RAP1-mediated silencing pathway, it seems reasonable to assume that other RIFs contribute to silencing at wild-type and *hmrΔB* silencers. We showed recently that overexpression of a G_{BD}/RAP1 hybrid partially derepresses *HMR* wild-type and *hmrΔB* silencers (Hardy et al. 1992). A disruption of the *RIF1* gene, however, does not derepress either the wild-type or *hmrΔB* silencers nor does it mitigate the partial derepression effect of a G_{BD}/RAP1 hybrid on these silencers (data not shown). Because the

G_{BD}/RAP1 hybrid can derepress in the absence of RIF1 activity, we propose that it may work on these silencers by titrating a different RIF. The existence of other RAP1-interacting proteins involved in silencing is highlighted further by the observation that the G_{BD}/rap1^s hybrids are all effective derepressors, including the G_{BD}/rap1-12 and G_{BD}/rap1-13 hybrids, which fail to interact with G_{AD}/RIF1 in the two-hybrid system. Although the simplest model would propose that RIF1 is the factor titrated by the overexpression of G_{BD}/RAP1 hybrids, these results raise the possibility that a protein–protein interaction (as yet unidentified) may, at least in part, underlie this phenomenon. Consequently, we are continuing to screen the library from which *RIF1* was isolated and independent libraries in which yeast sequences are joined to G_{AD} through the two other possible reading frames in search of new RIFs. It will be interesting to compare the results of these screens with pseudoreversion (extragenic suppressor) studies of *rap1^s* mutants, which are currently under way (L. Sussel and D. Shore, unpubl.).

In conclusion, using the two-hybrid system, we have identified a protein that interacts with RAP1 and functions in both transcriptional silencing and telomere length regulation. These studies provide the first direct evidence that a silencing function of RAP1 is mediated by selective interactions with another protein. RIF1 is not the product of any previously identified *trans*-acting regulator of silencing. Further study of RIF1, identification of additional RIFs, and characterization of their molecular targets should provide new insights into the regulation of silencers and telomeres.

Materials and methods

Strains and DNAs

Growth and manipulation of yeast strains was done according to standard procedures (Sherman et al. 1983). All experiments involving the two-hybrid system were performed in strain GGY:171 (*leu2-2,112 his3* Δ200 Δgal4 Δgal80 GAL1–*lacZ*). A library of partial *Sau*3A-digested yeast genomic sequences in the vector pGAD2 (Chien et al. 1991) was generously provided by P. Bartel and S. Fields. Plasmid DNAs were rescued from GGY:171 by transformation into $CaCl_2$-treated *E. coli* strain BA1 (*thr leuB6 thi thyA trpC1117 hisB str*^{R}), selecting simultaneously for ampicillin resistance and leucine prototrophy. *RIF1* gene disruptions were done in strain W303-1B (*HMLα MATα HMR***a** *ade2-1 can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura3-1*), derivatives of W303-1B containing *hmr::TRP1* loci (Sussel and Shore 1991), and an isogenic *MATα/MAT***a** diploid (W303). G_{BD}/RAP1 carboxy-terminal hybrids have been described previously (Hardy et al. 1992). G_{BD}/rap1^s hybrids were constructed by replacing a *Bgl*II–*Xba*I fragment from the original G_{BD}/RAP1(653-827) clone with the corresponding fragment from the mutant alleles. The resulting G_{BD}/rap1^s hybrids differ from the original G_{BD}/RAP1(653-827) hybrid only in those amino acids affected in the *rap1^s* mutants. Deletion derivatives of the original G_{AD}/RIF1 plasmid were constructed by placing either the *Bam*(P)–*Bam*(D) or the *Bam*(P)–*Xba*I fragment encoding *RIF1* sequences (see Fig. 1) downstream of the *Bam*HI site at the fusion junction in plasmid pGAD2 (Chien et al. 1991). The 3′ end points of the inserts are at the unique *Xba*I site in pGAD2.

Isolation of RIF1 *using the two-hybrid system*

The yeast *GAL1–lacZ* reporter strain GGY:171, containing a plasmid expressing the G_{BD}/RAP1(653-827) hybrid, was transformed with a library of genomic DNA fragments in the pGAD2 expression vector (a generous gift of P. Bartell and S. Fields) using the high-efficiency method of Schiestl and Geitz (1989). Transformants (~1000 per plate) were selected on SC-His-Leu medium at 30°C. After 3–5 days of growth, colonies (1- to 1.5-mm diam.) were replica-plated onto SC-His-Leu plates containing X-gal. After 1–5 days, blue colonies were identified, purified by restreaking on SC-His-Leu plates, and retested by replica plating onto X-gal plates. Positive colonies were picked from the SC-His-Leu plates and grown overnight in 10 ml of SC-Leu liquid medium. DNA prepared from these cultures was transformed into the Leu$^-$ *E. coli* strain BA1, and Amp^R Leu$^+$ transformants were selected. Plasmid DNA was prepared from at least six independent BA1 transformants and tested by transformation into the yeast reporter strain GGY:171 with or without the G_{BD}/RAP1(653-827)-expressing plasmid. Yeast transformants were also assayed for *lacZ* expression by replica-plating onto nitrocellulose (Breeden and Nasmyth 1985).

Isolation of a G_{AD}/rif1 mutant

The original G_{AD}/RIF1 plasmid was transformed into a *mutD5 E. coli* strain (Echols et al. 1983), selecting for ampicillin resistance on minimal M65 medium. Eight individual transformants were picked and grown to saturation in 10 ml of LB media containing 20 μg/ml of ampicillin. DNA preparations from these eight cultures were pooled and used to transform yeast strain GGY:171 containing a plasmid expressing the G_{BD}/rap1-12 hybrid. Transformants were selected on SC-His-Leu plates. After 2–3 days of growth, replicas were made onto nitrocellulose filters and tested for β-galactosidase activity. One blue colony was detected from ~15,000 transformants. Plasmid DNA was rescued from the single blue transformant and tested as described above. The rescued plasmid was shown to confer the blue colony phenotype only upon retransformation of GGY:171 strains containing G_{BD}/rap1^s or G_{BD}/RAP1 hybrids. The mutation was localized to a small restriction fragment of the G_{AD}/rif1-1 plasmid by exchanging corresponding fragments with the original plasmid encoding the G_{AD}/RIF1 hybrid. An *Xba*I–*Nco*I fragment from the mutagenized plasmid conferred the mutant phenotype when placed in the G_{AD}/RIF1 plasmid background. The sequence of the complete *RIF1*-coding sequence on this fragment was determined using synthetic oligonucleotide primers, and a single-base change was found at nucleotide 6169 (G → A).

Other methods

Liquid assays for β-galactosidase were performed as described previously (Hardy et al. 1992). The average value from at least four transformants of each plasmid construct is reported. Values for individual colonies differed by <30% from the average. Transcriptional silencing was assayed in strains containing *hmr::TRP1* loci (with wild-type or mutated silencer elements) by measuring colony-forming ability on media lacking tryptophan (Sussel and Shore 1991). Telomere tract lengths were

measured by Southern blot analysis of *Xho*I-digested genomic DNA using poly[d(G-T)] · poly[d(C-A)] probes (Shampay and Blackburn 1988). DNA sequencing was done primarily on an Applied Biosystems 373A, using the universal primer and a series of nested deletion clones generated by exonuclease III digestion or using synthetic oligonucleotide primers corresponding to sequence generated from the deletions. The sequence reported has been determined on both strands. Details of primers and deletions used in the sequencing are available on request. We compared the RIF1 sequence to sequences in data bases (SWISS-PROT 19.0, and GenPept 69.0) using the FASTA program (Pearson and Lipman 1988). In addition, Dr. M. Goebl compared the RIF1 sequence to an independent data base and failed to find any significant similarities to other proteins (M. Goebl, pers. comm.).

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