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_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" mutants. Furthermore, hybrid proteins containing rap1" missense mutations are defective in an interaction with RIF1 in the two-hybrid system. Taken together, these data support the idea that the rap1" 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" 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^v) that are specifically defective in transcriptional silencing [Sussel and Shore 1991]. Both types of rap1 mutants (rap1^ts and rap1^v) also display changes in the poly(C_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_3_A) sequences [Conrad et al. 1990; Lustig et al. 1990]. Conversely, silencing-defective rap1^v mutants have longer poly(C_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 Tomow 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
the carboxyl terminus of RAPI fused to the GAL4 DNA-binding domain (G_{BF}) 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 terminator of RAPI also results in telomere elongation (Conrad et al. 1990; Hardy 1991), another phenotype associated with rapi* mutants. Derepression by G_{BD}/RAPI hybrids occurs only when they are expressed from a strong promoter on multicopy plasmids and not when expressed from the RAPI promoter in single copy. Furthermore, this dominant-negative effect is not dependent on a DNA-binding site to target the expression by G_{IF}/1^3/RAPI hybrids occurs only when they portions of the two hybrids that tether the activation domain to the promoter. We have adapted the two-hybrid screen to isolate RAPI-interacting proteins by starting with a GAL1-lacZ reporter strain expressing the G_{BD}/RAPI(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 Sau3AI) 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 1EU2-containing plasmids were isolated from these cells and transformed back into two different reporter strains, one with and one without the G_{BD}/RAPI(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}/RAPI(653-827)-dependent activation of the reporter gene (Fig. 1). Activation requires the RAPI portion of the G_{BD}/RAPI 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}/RIF1 interaction but, instead, encodes a polypeptide that interacts with RAPI(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 BamH1 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 Ncol site and a distal BamH1 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 Xba1 site located -500 bp beyond the fusion junction abolished activation, whereas deletion of sequences beyond the distal BamH1 site had no effect (Fig. 1). These data indicate

**Results**

**Isolation of a RAPI-interacting protein by the two-hybrid system**

Expression of G_{BD}/RAPI 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 678–881). 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 tether the activation domain to the promoter. We have adapted the two-hybrid system to isolate RAPI-interacting proteins by starting with a GAL1–lacZ reporter strain expressing the G_{BD}/RAPI(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 Sau3AI) 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}/RAPI(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.

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Isolation and DNA sequence of the RIF1 gene

Using the proximal BamHI–XbaI 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 GAD/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 MluI site (ACGCGT) in the 6.5-kb sequence (at nucleotide 262), ~200 bp upstream of the putative initiator ATG of RIF1. MluI sites are often found within the promoters of genes whose mRNA levels increase at the G1/S-phase boundary (Andrews and Herskowitz 1990). Several genes encoding DNA replication proteins contain one or two MluI 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 MluI–XbaI 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 rifs::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 rifs::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 rifs::URA3 mutants are defective in silencing, the effects of the RIF1 disruption at the
Figure 2. (See facing page for legend.)
HMR locus were assayed. Because the HMLE 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 HMLE silencer contains two other regulatory sites (called A and B), either one of
Figure 3. rif1::URA3 mutants are defective in silencing at hmr\(\Delta\)::TRP1. The effect of a rif1::URA3 gene disruption on transcriptional silencing in strains containing hmr\(\Delta\)::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 mutants (in a hmr\(\Delta\)::TRP1 background) and a strain carrying the defective hmrAB 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 ABFl protein (Shore et al. 1987, Buchman et al. 1988a). We noted previously that rap1 mutants display a defect in silencing only when the A element at HMR is deleted (hmr\(\Delta\)A) [Sussel and Shore 1991]. Furthermore, the hmr\(\Delta\)A silencer is affected most severely by overexpression of G\(\Delta\)D/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, hmr\(\Delta\)A::TRP1, or hmr\(\Delta\)B::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 hmr\(\Delta\)A::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 hmr\(\Delta\)A::TRP1 caused by the rif1::URA3 mutation was complete and indistinguishable from that of the strong rap1 mutants, rap1-12 and rap1-13. Strains containing a wild-type silencer (hmr::TRP1) or a B silencer (hmr\(\Delta\)B::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 XhoI 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 \(\sim 1.2\) 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 (\(\sim 200-300\) 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\(\Delta\)D/RAP1 hybrids [Hardy et al. 1992] was used to determine which RAP1 sequences in G\(\Delta\)D/RAP1 are required for G\(\Delta\)D/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\(\Delta\)D/RAP1 and those required for an interaction with G\(\Delta\)D/RIF1. For example, G\(\Delta\)D/RAP1(653-827) and G\(\Delta\)D/RAP1(655-827) both completely derepress the hmr\(\Delta\)A::TRP1 locus and cooperate with G\(\Delta\)D/RIF1 to give comparable levels of 3-galactosidase expression. In addition, the G\(\Delta\)D/RAP1(679-827) hybrid displays a reduced ability to both derepress \(\sim 10\) to 20-fold lower than G\(\Delta\)D/RAP1(653-827) and G\(\Delta\)D/RAP1(655-827) and to participate in G\(\Delta\)D/RIF1-dependent activation (36 units, compared with 616
and 747 (Sussel and Shore 1991). The rap1" mutants cause either strong [rap1-12 and rap1-13] or weak [rap1-11 and rap1-14] derepression of the hmrΔ::TRP1 locus and variable elongation of poly[C1-3A] tracts at telomeres. A series of four G_BD/rap1"[653-827] hybrids were constructed that incorporated the mutant changes of the rap1" alleles into the G_BD/RAPI[653-827] hybrid. These G_BD/rap1" 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" alleles and the ability of the corresponding G_BD/rap1" hybrids to activate the GAL1-lacZ reporter when expressed together with G_AD/RIF1. The two strong rap1" mutants, rap1-12 and rap1-13, are both completely defective in silencing the hmrΔ::TRP1 locus and also fail to activate in conjunction with G_AD/RIF1. Perhaps more significant is the observation that the weak rap1" alleles [rap1-11 and rap1-14], with only a partial defect in silencing of hmrΔ::TRP1 display a measurable but reduced ability as G_BD/rap1" hybrids to interact with G_AD/RIF1. These results suggest that the strong rap1" mutants have a totally defective RIF1 interaction site, whereas the weak rap1" mutants retain a partially functional RIF1 interaction site. Furthermore, they signify that the defect in silencing by the rap1" mutants may be explained by a failure to interact with RIF1. Interestingly, though, the G_BD/rap1" hybrids derepress the hmrΔ::TRP1 locus to the same extent as the G_BD/ RAP1[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" hybrids

We reasoned that if rap1" 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" hybrids. The G_AD/RIF1 plasmid was mutagenized by passage through an Escherichia coli mutDS 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/RAPI[653-827] hybrid and into strains coexpressing each one of the four different G_BD/rap1" 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/RAPI 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

Silencing-defective rap1" 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" alleles that contain either single or double missense mutations in the RAP1 carboxyl terminus, affecting amino acids 726, 727,
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 GAD/rif1-1 mutant hybrid are worth emphasizing. First, the mutant hybrid appears to interact less well with the wild-type GB8/RAP1 hybrid than does the wild-type GAD/RIF1 hybrid, as shown by the significantly lower β-galactosidase value. This result indicates that the GAD/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* hybrids and with the wild-type GB8/RAP1 hybrid. This property is in marked contrast to the ability of the GAD/RIF1 hybrid to distinguish between wild-type GB8/RAP1 and the two classes of rap* hybrids. The failure of the GAD/rif1-1 hybrid to distinguish between GB8/RAP1 and the different GB8/rap* 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.

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 GAD/rif1-1 mutant suppresses the activation defect of GB8/rap1* mutants

| DNA-binding domain hybrid | Activation domain hybrid |
|---------------------------|-------------------------|
| GAD/rap1                 | GAD/RIF1 | GAD/rif1-1 |
| GAD/rap1-11              | 616      | 165 |
| GAD/rap1-12              | <1       | 110 |
| GAD/rap1-13              | <1       | 177 |
| GAD/rap1-14              | 35       | 188 |
| GAD                     | 172      | ND* |
| None                     | <1       | <1 |

*Not determined.
physically associate and that this association is impor-
ting evidence that the RAPl and RIFl proteins can
depend on a physical association with the RAI^l
scriptional silencing and telomere length regulation.

The genetic experiments presented here provide comp-
pelling evidence that the RAPl and RIFl proteins can
physically associate and that this association is impor-
tant for repression at hmrΔA silencers and length regu-
lation at telomeres. Activation by GAD/RIFI would ap-
pear to depend on a physical association with the RAPl
carboxy terminus. Furthermore, using the two-hybrid
system, we have shown that GAD/rapl* mutant hybrids
interact poorly or not at all with GAD/RIFI. What is
particularly striking is the strict correlation between the
severity of the silencing defects of individual rapl* mu-
tant alleles and the ability of the corresponding GAD/
rapl* hybrids to interact with the GAD/RIFI hybrid. The
two weakest rapl* alleles lead to an intermediate level of
activation together with GAD/RIFI, whereas the two
strongest rapl* alleles fail to produce a detectable signal
in the two-hybrid system. It seems unlikely that the
GAD/rapl* defects reflect a general instability of the
rapl* carboxy-terminal domains, as the rapl* mutants
have both normal protein levels and do not display any
temperature sensitivity (Sussel and Shore 1991). In addi-
tion, the GAD/rapl* hybrids are all capable of silencer
derepression, implying that they are also stable and prop-
erty folded (data not shown).

The properties of the compensating GAD/rifl-l mu-
tant argue strongly that there is a specific protein–pro-
tein interaction between RIFl and the small carboxy-
terminal region of RAPl affected by the rapl* alleles. The
GAD/rifl-l hybrid appears to interact less well with wild-type
GAD/RAPl than does GAD/RIFI, implying that the
tifl-1 mutation does not work by increasing the affin-
y of RIFl for both wild-type RAPl and rapl* pro-
teins. Instead, we suggest that the.tifl-1 mutation alters
a specific interaction with RAPl defined by the rapl* alle-
es, such that the rifl-1 protein can no longer distin-
guish between wild-type RAPl and the four different
rapl* mutants and thus interacts equally well with all of
them. The properties of the rifl-1 mutation are similar to
those described for loss-of-contact mutations in se-
quence-specific DNA-binding proteins (Ebright et al.
1987). A simplified model for the RAPl–RIFl interac-
tion, based on the mutant data, is shown schematically
in Figure 6. The model predicts that the rapl* alleles (at
amino acids 726, 727, and 747) define a specific interac-
tion site on the RAPl carboxy terminus and that strong
rapl* alleles (rapl-12 and rapl-13) create a repulsive in-
teraction with RIFl at this site that is abolished by the
rifl-1 mutation. Further experiments, involving site-di-
rected mutagenesis of both proteins, will test the valid-
ity of this proposal.

 Rifl: a co-factor or mediator for RAPl
 at silencers and telomeres

Previous studies have identified a large number of trans-
acting regulators of silencing, including the four SIR

genomes [Haber and George 1979; Klar et al. 1979; Rine and
Herskovitz 1987], HHF2 (encoding histone H4) [Kayne
et al. 1988; Megue et al. 1990], NATl and ARDl (encod-
ing an amino-terminal protein acetyltransferase) [White-
way 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], RAPl (Shore and Nasmyth
1987; Kurtz and Shore 1991; Sussel and Shore 1991), and
presumably the gene encoding the other silencer binding
factor ABFl [Difffley and Stillman 1989; Halter et al.
1989, Rhode et al. 1989, Francesconi and Eisenberg
1991]. It is worth noting that in searching directly for a
RAPl-interacting factor involved in silencing, we have
identified a novel gene. Our results suggest that the con-

Figure 6. A model for the RAPl–RIFl 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 RAPl and RIFl, defined in
part by amino acids 726, 726, and 747 in RAPl and amino acid
1906 in RIFl. The complementary rectangular surfaces represen-
t the sum of all other RAPl–RIFl interactions. The rapl-12
mutation creates an unfavorable interaction that signifi-
cantly reduces or abolishes binding [B]. The rifl-1 mutation creates
an unfavorable contact with rapl-12, allowing rifl-1 to inter-
act equally well with rapl-12 and wild-type RAPl protein [C,D].
In the weaker alleles, rapl-14 and rapl-11, we imagine that the
repulsive rapl-1–RIFl interaction seen for the rapl-12–RIFl com-
bination [B] is either minimal [rapl-14] or essentially nonexis-
tent [rapl-11].

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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 [Kelcher 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 HMR region. 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 rap1Δ 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 Mlu1 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 rap1Δ and rif1 mutations may not function at telomeres or it may also be redundant there. This may explain why rap1Δ 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(C1- C4) tracts. It remains to be seen whether telomeric silencing is also independent of RIF1. The reason for telomere elongation in both rap1Δ 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 rap1Δ silencing defects and the extent of telomere elongation [Sussel and Shore 1991] suggests that these two phenomena are related at some level. Perhaps mechanisms controlling telomere length are more sensitive to the effects of rap1Δ 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 GαG12/RAPI 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 GαG12/RAPI hybrid on these silencers (data not shown). Because the
GBD/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 GBD raped 13 hybrids are all effective derepressors, including the GBD rap 1-12 and GBD rap 13 hybrids, which fail to interact with GAD/RIF1 in the two-hybrid system. Although the simplest model would propose that RIF1 is the factor titrated by the overexpression of GBD/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 GAD 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 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 GYY:171 [leu2-2, 112 his3 D200 gal4 Dgal80 GAL1- lacZ]. A library of partial Sau3A-digested yeast genomic sequences in the vector pGAD2 [Chien et al. 1991] was generously provided by P. Bartell and S. Fields. Plasmid DNAs were rescued from the vector pGAD2 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 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.

Isolation of RIF1 using the two-hybrid system

The yeast GAL1-lacZ reporter strain GGY:171, containing a plasmid expressing the GAD/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 S-C-His-Leu medium at 30°C. After 3–5 days of growth, colonies [1- to 1.5-mm diam.] were replica-plated onto S-C-His-Leu plates containing X-gal. After 1–5 days, blue colonies were identified, purified by restreaking on S-C-His-Leu plates, and retested by replica plating onto X-gal plates. Positive colonies were picked from the S-C-His-Leu plates and grown overnight in 10 ml of S-C-Leu liquid medium. DNA prepared from these cultures was transformed into the E. coli strain BA1, and Ampρ Leuρ 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 GBD/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 GAD/rif1 mutant

The original GAD/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 medium 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 GAD/rap1-12 hybrid. Transformants were selected on S-C-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 GBD/rap1 or GAD/RAP1 hybrids. The mutation was localized to a small restriction fragment of the GAD/rif1-1 plasmid by exchanging corresponding fragments with the original plasmid encoding the GAD/RIF1 hybrid. An XbaI–NcoI fragment from the mutagenized plasmid conferred the mutant phenotype when placed in the GAD/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 Xhol-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 sequence marked "advertisement" in accordance with 18 USC sections 1734 solely to indicate this fact.

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