A novel Raplp-interacting factor, Rif2p, cooperates with Riflp to regulate telomere length in *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae* Rap1 protein binds with high affinity to sites within the poly(C_3A) tracts at telomeres, where it plays a role in both telomere length regulation and the initiation of telomeric silencing. Raplp initiates silencing at telomeres by interacting through its carboxy-terminal domain with Sir3p and Sir4p, both of which are required for repression. This same domain of Raplp also negatively regulates telomere elongation, through an unknown mechanism. We have identified a new Rapl-interacting factor (Rif2p) that plays a role in telomere length regulation. Rif2p has considerable functional similarities with a Raplp-interacting factor (Riflp) identified previously. Mutations in *RIF1* or *RIF2* result in moderate telomere elongation and improved telomeric silencing. However, deletion of both *RIF1* and *RIF2* in the same cell results in a dramatic increase in telomere length, similar to that seen with a carboxy-terminal truncation of Raplp. In addition, overexpression of either *RIF1* or *RIF2* decreases telomere length, and co-overexpression of these proteins can reverse the telomere elongation effect of overexpression of the Rap1p carboxyl terminus. Finally, we show that Riflp and Rif2p can interact with each other in vivo. These results suggest that telomere length regulation is mediated by a protein complex consisting of Riflp and Rif2p, each of which has distinct regulatory functions. One role of Raplp in telomere length regulation is to recruit these proteins to the telomeres.

*Key Words:* Raplp, Riflp, Rif2p, telomere length, transcriptional silencing, *Saccharomyces cerevisiae*

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Telomeres, the ends of eukaryotic chromosomes, are specialized protein-DNA complexes typically based upon a simple DNA-repeat structure (TTAGGG in many multicellular organisms). Telomeres in the yeast *Saccharomyces cerevisiae*, which have an irregular repeat structure commonly abbreviated as C_3A, are essential for chromosome stability (Sandell and Zakian 1993). The maintenance of telomeres in yeast (Singer and Gottschling 1994), and probably most other organisms, requires a unique reverse transcriptase, called telomerase, that can add telomeric repeats onto the chromosome ends (for review, see Blackburn 1994; Zakian 1995a). The length of telomeric repeats is variable between strains and individual clones of a given strain and can spontaneously increase or decrease (Shampay and Blackburn 1988), suggesting a balance between telomerase addition and degradation that is under complex genetic control (Lustig and Petes 1986; for review, see Greider 1996; Zakian 1995a).

The telomeric repeats in *S. cerevisiae* form high-affinity binding sites for the essential repressor/activator protein 1 (Raplp) (Shore and Nasmyth 1987; Buchman et al. 1988; Longtine et al. 1989) as often as once per 18 bp (Gilson et al. 1993). Raplp plays two roles at telomeres, both of which are mediated by its carboxy-terminal domain (for review, see Shore 1995; Zakian 1995a): It is involved in transcriptional silencing, or telomere position effect (TPE) (Kyrion et al. 1993; Moretti et al. 1994), and it controls telomere length (Conrad et al. 1990; Lustig et al. 1990; Sussel and Shore 1991; Kyrion et al. 1992; Liu et al. 1994). Raplp is also involved in silencing at the *HM* mating-type loci (Kurtz and Shore 1991; Sussel and Shore 1991; Kyrion et al. 1993). The role of Rap1p in transcriptional silencing appears to be the recruitment of specific repressor proteins [Sir3p and Sir4p] to the telomeres and *HM* silent mating-type loci, Raplp also binds to the...
upstream regions of many genes, including a large number of ribosomal protein and glycolytic enzyme genes, where the protein appears to function as a transcriptional activator (for review, see Shore 1994). Activation of transcription by Raplp may be mediated by a region immediately amino-terminal to the silencing and telomere domain [Hardy et al. 1992a] and involves, at least in some cases, specific interactions with other DNA-binding activator proteins [Tornow et al. 1993; Drizinic et al. 1996].

Point mutations [e.g., rapl'] and frameshift mutations (e.g., rap1') within the Raplp carboxyl terminus result in varying degrees of telomere tract elongation, suggesting that this domain regulates telomere elongation [Sussel and Shore 1991; Kyrion et al. 1992; Liu et al. 1994]. However, this function of Raplp does not appear to be mediated directly by the Sir proteins. In fact, mutation of SIR3 or SIR4 actually results in slight telomere shortening [Palladino et al. 1993], suggesting that the normal function of these proteins in some way supports telomere elongation. Instead, a number of studies indicate that other proteins interact with the Raplp carboxyl terminus to negatively regulate telomere growth. For example, overexpression of the Raplp carboxyl terminus in the absence of its centrally located DNA-binding domain [Conrad et al. 1990; Hardy 1991] or introduction of extra telomere repeats into cells [Runge and Zakian 1989] results in telomere elongation, consistent with the idea that titratable Raplp-interacting factors control telomere length. One candidate for such a factor is Riflp, which was identified in a two-hybrid screen as a protein that interacts with the Raplp carboxyl terminus [Hardy et al. 1992b]. Interestingly, the telomere elongation defect of rap1'' mutants may result from an inability to bind Riflp: Mutant rap1'' proteins interact weakly or not at all with Riflp in two-hybrid assays, and disruption of Rif1 results in telomere elongation similar to that observed in severe rap1'' mutants [Sussel and Shore 1991; Hardy et al. 1992b]. However, several lines of evidence point to the existence of other Raplp-interacting factors involved in telomere length regulation. First, rap1'' mutants (which completely lack the carboxyl terminus) have a much more severe telomere elongation phenotype than either rap1'' or rif1 mutants [Kyrion et al. 1992]. Second, point mutations in the Raplp carboxyl terminus with weak telomere elongation phenotypes display additive effects when present together or in combination with a Rif1 mutation [Liu et al. 1994; L. Sussel, S. Buck, and D. Shore, unpubl.]. Finally, overexpression of the Raplp carboxyl terminus causes further telomere elongation in Rif1 mutant cells, suggesting the existence of other Raplp-interacting factors that regulate telomere length [Wiley and Zakian 1995].

Here we present the identification and characterization of a novel protein, which we call Rif2p [Raplp-interacting factor 2p]. This protein interacts with the carboxyl terminus of Raplp in the two-hybrid system, and is also able to interact with Riflp. Rif2p has striking functional similarities to Riflp, despite a lack of sequence similarity. However, deletion of both Rif genes has a strong synergistic effect on telomere length, resulting in telomere elongation similar to that seen in rap1'' cells. We argue from these and other data that Riflp and Rif2p form a functional complex capable of regulating telomere length when recruited to telomeres by Raplp.

### Results

#### Isolation of Rif2

To identify proteins that interact with Raplp and play a role in mediating Raplp-dependent functions, we have used the two-hybrid system to screen yeast genomic DNA libraries with a LexA/Raplp fusion encoding amino acids 635–827 of Raplp. Previously we reported the isolation of clones containing SIR3 and SIR4, two genes directly involved in transcriptional silencing [Moretti et al. 1994]. Here we describe the isolation and characterization of a new gene from this two-hybrid screen, which we call RIF2 [Raplp-interacting factor 2]. As shown in Table 1, the GAD/Rif2p hybrid encoded by the library plasmid interacts specifically with LexA/Raplp(635–827), but not with LexA alone or an unrelated LexA hybrid [LexA/lamin]. Furthermore, when the reading frame between the GAD sequence and the RIF2 insert was disrupted (creating GAD/rif2fs) no activation of transcription was observed. Thus, the interaction with LexA/Raplp(635–827) is dependent on the GAD/Rif2 fusion protein.

LexA/Raplp(635–827) contains not only the Raplp silencing domain, but also most of a transcriptional activation domain, which spans amino acids 630–695 [Hardy et al. 1992a]. In addition, this hybrid protein has been shown to be a transcriptional activator in cells mutated for either SIR2, SIR3, SIR4, or RIF1 [Moretti et al. 1994]. Therefore, we wanted to determine whether expression of the GAD/Rif2 fusion was able to cause increased activation from other LexA fusion proteins that are themselves weak or cryptic activators. Both LexA/Gcr1(4–419) and LexA/Cln2(15–491) activate transcription from LexA operators to a low level. However, no increase in transcriptional activity of these fusions was observed in the presence of GAD/Rif2 (Table 2), further demonstrating that the interaction of GAD/Rif2 with Raplp is specific. Sequence analysis of the GAD/Rif2 plasmid revealed that the insert encoded a large open reading frame

| GAD/Rif2 interacts specifically with LexA/Rap1 | GAD | GAD/Rif2 | GAD/rif2fs* |
|-----------------------------------------------|-----|----------|-------------|
| LexA/Raplp(635–827)                           | 7.1 | 249      | 6.9         |
| LexA                                          | 3.5 | 3.7      | 3.6         |
| LexA/lamin                                    | 3.7 | 3.7      | 3.4         |

β-Galactosidase activity in CTY10-5D cells determined as described previously [Moretti et al. 1994].

*GAD/rif2fs contains the same Rif2 insert as GAD/Rif2 but has a frameshift mutation introduced between the GAL4 activation domain sequences and the RIF2 sequences.
Localization of a minimal Rif2p-interacting domain within Rap1p

Sir3p, Sir4p, and Rif1p interact with a large portion of the carboxyl terminus of Rap1p encompassing part of the putative activation domain and all of the sequences carboxy-terminal to it [Hardy et al. 1992b; Moretti et al. 1994]. To determine whether a similar region was required for interaction with Rif2p, we tested a series of LexA/Rap1p fusions with increasing amino-terminal deletions. Proteins of the expected size for all LexA/Rap1p fusions were visible by Western blotting with a LexA-specific antiserum [data not shown]. As shown in Figure 1, GAD/Rif2 was able to interact with amino-terminal truncations up to and including LexA/Rap1p(679–827) (the numbers in parentheses indicate the Rap1p amino acids present in the hybrid), but failed to interact with hybrids beginning at amino acid 691 or 702 in Rap1p. This pattern is very similar to that of GAD/Sir3p(307–978) and GAD/Rif1p(1614–1916), but unlike that of GAD/Sir4p(1204–1358), which interacts only with the larger LexA/Rap1p(635–827) hybrid (Moretti et al. 1994).

To determine the carboxy-terminal boundary of the Rif2p-interacting region of Rap1p, we used a series of LexA/Rap1p fusions with a common amino-terminal fusion point, at amino acid 667. In contrast to the results obtained with Sir3p and Rif1p hybrids [Moretti et al. 1994], in which mutation at amino acid 825 or deletion to amino acid 799 of Rap1p severely weakened the interactions with these proteins, the same LexA/Rap1p mutations actually increased the signal obtained with GAD/Rif2 [Fig. 1]. The two shorter Rap1p fusions tested, encoding amino acids 667–756 and 667–716, were unable to interact with GAD/Rif2. Thus, the region of Rap1p required for interaction with Rif2p lies between amino acids 679 and 799. In contrast, the Rap1p interaction with Rif1p or Sir3p requires amino acids 679–827, and an even larger region is required for interaction with Sir4p [Moretti et al. 1994].

The results described above indicate that Rif2p can interact with a smaller region of the Rap1p carboxyl terminus than that required by either Sir3p, Sir4p, or Rif1p, suggesting that the Rif2p–Rap1p interaction does not require these other Rap1-interacting factors. To test this idea more directly, we examined the GAD/Rif2 interaction with LexA/Rap1p(647–827) in strains containing mutations in either SIR3, SIR4, or Rif1p. We chose this particular LexA/Rap1p hybrid because it still interacts with Sir3p and Rif1p hybrids but does not become a transcriptional activator when SIR genes or Rif1p are mutated [Moretti et al. 1994]. As shown in Table 3, mutation of SIR3 or SIR4 results in a 12- and 16-fold increase, respectively, in the apparent interaction between Rap1p and Rif2p. However, mutation of Rif1p results in a much more dramatic (>100-fold) increase in the Rap1p–Rif2p interaction. These data clearly indicate that the Rap1p–Rif2p interaction is not dependent upon native Sir3p, Sir4p, or Rif1p, and provide further support for the idea that this is a direct interaction. In addition, they raise the possibility that all three proteins (Sir3p, Sir4p, and Rif1p) compete with Rif2p for binding to the Rap1p carboxyl terminus.

Figure 1. GAD/Rif2 interacts with amino acids 679–799 of Rap1p. Two series of LexA/Rap1p fusions with increasing amino- and carboxy-terminal truncations were assayed for interaction with GAD/Rif2 in CTY10-5D cells. β-Galactosidase activities were determined from liquid cultures as described previously [Moretti et al. 1994]. Each fusion was also tested with GAD alone as a control.
Rif2p interacts with Rif1p

An alternative explanation of the results described above (Table 3) is that Rif2p normally interacts with the Sir proteins or with Rif1p [in addition to Rap1p], and that the binding of GAD/Rif2 to these proteins somehow prevents or weakens a functional interaction with LexA/Rap1 in the two-hybrid system. In this case, mutation of the SIR or RIF1 genes might leave more GAD/Rif2 hybrid free to interact productively with LexA/Rap1. To explore this possibility we created a LexA/Rif2 fusion protein containing amino acids 2–395 of Rif2p. This fusion protein did not activate transcription on its own and no significant increase in $\beta$-galactosidase activity was observed upon coexpression of either GAD/Sir3 or GAD/Sir4, suggesting that these proteins do not interact with Rif2p (Table 4). However, a GAD/Rif1 fusion was able to interact with LexA/Rif2[2–395], giving a signal $>20$-fold over the background with GAD alone (Table 4). Thus, it appears that Rap1p, Rif1p, and Rif2p are all able to interact with each other. In light of these results, the simplest interpretation of the effect of SIR mutations on the Rif2p–Rap1p interaction (Table 3) would be that Sir3p and Sir4p compete for binding to the Rap1p carboxyl terminus with Rif2. In the case of Rif1p, because of the observed interaction with Rif2p, we favor the idea that the RIF1 mutation improves the Rap1p–Rif2 interaction by freeing GAD/Rif2 to interact more productively with the LexA/Rap1 hybrid. However, we cannot rule out the possibility that Rif1p also competes with Rif2p for binding to Rap1p.

Rif2p and its interaction with Rif1p and Rap1p

Analysis of the amino-acid sequence of Rif2p (Fig. 2A), reveals that it encodes a protein of 395 amino acids with a predicted molecular mass of 46 kD. [The RIF2 gene is on chromosome XII-R, hypothetical protein YLR453c, GenBank accession no. U22382.] Western analysis of epitope-tagged Rif2p, expressed from its own promoter, revealed a protein of the expected size [data not shown]. This protein has no significant homology to other known sequences and no convincing structural motifs. The only notable features of the amino acid sequence of Rif2p are the presence of a lysine-rich region (21.3% lysine) spanning amino acids 15–75 and a second basic region at the carboxyl terminus (17.1% lysine and 14.3% arginine over 35 amino acids).

To determine which region of Rif2p was responsible for the interaction with Rap1p and Rif1p, we created a series of LexA/Rif2 fusions and tested their interactions with GAD/Rap1 and GAD/Rif1. As shown in Figure 2B, truncation of the carboxyl terminus of Rif2p to amino acid 388 did not affect the interaction with Rap1p or Rif1p, but further truncation (to amino acid 332) abolished the interaction with both proteins. Both GAD/Rap1 and GAD/Rif1 were able to interact with Rif2p fusions starting at amino acid 2 or 18. However, deletion of the first 49 amino acids of Rif2p abolished the interaction with Rap1p (Fig. 2B). In contrast, the LexA/Rif2[50–395] hybrid interacted strongly with GAD/Rif1, in fact giving more than twice as many $\beta$-galactosidase units as the LexA/Rif2[2–395] hybrid, which interacts with both Rap1p and Rif1p. A further deletion of 40 amino-terminal residues, giving LexA/Rif2[90–395], resulted in a hybrid unable to interact with either the Rap1p or the Rif1p hybrid. Thus, Rap1p and Rif1p interact with largely overlapping regions of Rif2p, although there does appear to be a difference between the regions of Rif2p required for interaction with these two proteins. The fact that GAD/Rif1 interacts with LexA/Rif2[50–395], whereas GAD/Rap1 is unable to, suggests that the Rif1p–Rif2p interaction is independent of Rap1p.

We further analyzed these interactions using cells in which either RIF1 or RIF2 had been deleted. A RIF2 disruption was created by replacing the sequence encoding amino acids 18–389 of Rif2p with the HIS3 gene. Cells lacking the RIF2 gene were viable and appeared to grow normally. Deletion of either RIF1 or RIF2 did not result in loss of any of the interactions described above between Rap1p, Rif1p, and Rif2p, suggesting that none of these proteins is bridging the interactions between any other pair [data not shown].

RIF2 mutation affects silencing at both HMR and telomeres

The known Rap1p-interacting proteins, Sir3p and Sir4p on the one hand, and Rif1p on the other, clearly play different roles in transcriptional silencing. Null mutations of either SIR3 or SIR4 completely abolish silencing at both telomeres and HMR loci [Ivy et al. 1986; Rine and

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**Table 3.** Deletion of genes encoding known Rap1p-interacting factors increases the interaction of LexA/Rap1 with GAD/Rif2

|          | GAD   | GAD/Rif2 |
|----------|-------|----------|
| Wild type| 3.6   | 20.9     |
| sir3::HIS3| 5.7   | 259      |
| sir4::HIS3| 5.9   | 332      |
| rif1::HIS3| 4.9   | 2244     |

For determination of $\beta$-galactosidase activity, see Table 1 footnote. In all cases, the DNA-binding domain hybrid is LexA/Rap1[647–827]. The wild-type strain is CTV10-5D, and the mutants were derived from this strain by gene disruption [Moretti et al. 1994].
Herskowitz 1987; Aparicio et al. 1991). RIF1 mutations, however, actually improve silencing at telomeres while weakening repression at HMR loci containing a mutated HMR-E silencer (Hardy et al. 1992b; Kyrian et al. 1993). It is thought, therefore, that Sir3p and Sir4p are absolutely required for silencing, consistent with the observation that these proteins appear to be structural components of silent chromatin (Hecht et al. 1995, 1996). Riflp appears to play a regulatory role in silencing by affecting the balance between telomeric and HM locus silencing, a conclusion that is supported by studies of the unusual properties of rap1 mutations, which create a defect in the Raplp-Riflp interaction (Buck and Shore 1995).

We therefore examined the effect of RIF2 mutation on silencing at HMR and at a genetically marked telomere. To examine silencing at HMR, we used a sensitive reporter gene (TRPI) and an HMR-E silencer containing a mutation of the origin recognition complex (ORC) binding site [HMRΔA::TRP1] (Sussel and Shore 1991). rap1 and rif1 mutations result in derepression at HMR only in the context of this weakened silencer (Sussel and Shore 1991; Hardy et al. 1992b). In an otherwise wild-type strain background, the hmrΔA::TRP1 reporter is strongly repressed, as indicated by the absence of growth on medium lacking tryptophan (SC-Trap, Fig. 3A). Little or no effect of a rif2 mutation alone is observed, whereas deletion of RIF1 results in a clear increase in the expression of the TRP1 reporter gene at HMRΔA, as reported previously (Hardy et al. 1992b). Interestingly, despite the absence of a clear effect of the rif2 mutation, introduction of the rif2 disruption into a rif1 HMRΔA::TRP1 strain led to a significant further derepression of the reporter gene, as indicated by the ability of essentially all of the cells to grow in the absence of tryptophan (SC-Trap, Fig. 3A). Thus, although a rif2 mutation alone has little or no effect on HMR, in combination with a rif1 mutation it has a synergistic effect on silencing at this locus.

To examine the effects of RIF mutants on telomere
position effect (TPE), we assayed the expression of a telomeric \textit{ADE2} reporter gene by observing colony color. Expression of \textit{ADE2} results in white colonies, whereas in cells that do not express the \textit{ADE2} gene red colonies are observed on plates containing minimal adenine. Colonies containing red and white sectors represent a mixed population of cells in which the \textit{ADE2} gene is repressed as well as expressed. For each strain, a single white colony was picked and grown overnight in rich medium, after which 200–300 cells were spread onto YPD plates and the colony color observed. In wild-type cells very few sectored or red colonies were observed (<1%), suggesting that the \textit{ADE2} gene was expressed in most cells, and that this derepressed state was relatively stable (Fig. 3B). Deletion of \textit{RIF2} resulted in a small but reproducible increase in the number of sectored colonies to ~11% (Fig. 3B). Mutation of \textit{RIF1} has been shown previously to increase TPE (Kyrion et al. 1993), and as shown in Figure 3B, this effect is larger than that seen in \textit{rif2} mutants (~34% sectored colonies). In cells lacking both \textit{Rif1p} and \textit{Rif2p}, a large proportion of the colonies was either completely red or contained red sectors (>50%; Fig. 3B). Thus, although mutation of \textit{RIF2} increased telomeric silencing only slightly, it had a synergistic effect when combined with a \textit{rif1} mutation.

In summary, the \textit{rif1} phenotype is stronger than that of \textit{rif2} in both \textit{HMR} silencing and TPE assays. Furthermore, despite the relatively weak effect of a \textit{rif2} mutation in either assay, it has a strong synergistic effect together with a \textit{rif1} mutation on the balance between telomeric and \textit{HMR} silencing, significantly strengthening the former and weakening the latter.

\textit{RIF1} and \textit{RIF2} mutations have a synergistic effect on telomere elongation and chromosome loss

One possible explanation for the strong effect of the \textit{rif2} mutation on silencing only when present together with a \textit{rif1} mutation is that \textit{rif2} significantly exacerbates the telomere elongation effect of \textit{rif1} mutations (Hardy et al. 1992b). Extreme telomere elongation [caused by \textit{rap1} mutations] can increase TPE in wild-type cells (Kyrion et al. 1993) while exerting an opposite effect on silencing at \textit{HMR} (Buck and Shore 1995). We therefore examined the length of the telomeric repeats in \textit{rif2} and \textit{rif1 rif2} double mutant cells. As shown in Figure 4, deletion of \textit{RIF2} alone results in an increase of ~100 bp in the average length of Y'-containing telomeres (which yield the broad lower band in the \textit{XhoI} digest shown). For comparison, a \textit{RIF1} deletion resulted in a somewhat greater increase in average length and heterogeneity of telomeres, as shown previously (Hardy et al. 1992b). In DNA from cells lacking both \textit{RIF1} and \textit{RIF2}, the length of the telomeric repeat sequences is increased by at least 600 bp, and up to 2.5 kb (Fig. 4), which is considerably greater than the increase for either single \textit{RIF} mutant. Thus, \textit{Rif1p} and \textit{Rif2p} probably perform different functions in telomere length regulation.

The fact that the telomere elongation observed in severe \textit{rap1} mutants (which are unable to interact with \textit{Rif1p} in two-hybrid assays) is similar to that seen in \textit{rif1} cells suggests that this allele of \textit{RAP1} retains some ability to regulate telomere length. Consistent with this, we found that \textit{Rif2p} is able to interact with \textit{rap1} mutants in the two-hybrid assay [data not shown]. However, this interaction was reduced to ~50% of that seen with wild-type \textit{Rap1p} fusions, indicating that the \textit{rap1} mutations do not specifically affect the \textit{Rap1p}-\textit{Rif1p} interaction, but instead may be having a more general effect on the carboxy-terminal region of \textit{Rap1p}. This reduced \textit{rap1}–\textit{Rif1p} interaction may explain why \textit{rap1} mutant double mutants have a more severe telomere elongation phenotype than either single mutant (L. Sussel, S. Buck, and D. Shore, unpubl.). An additional possibility is that some \textit{Rif1p} protein is recruited to telomeres in \textit{rap1} cells via the \textit{Rif1p}–\textit{Rif2p} interaction.

As mentioned above, previous work has shown that a large carboxy-terminal truncation of \textit{Rap1p} (in particular the \textit{rap1} allele \textit{rap1}-17, in which amino acids 663–827 are missing) results in a dramatic increase in telomere length [Kyrion et al. 1992]. The protein produced by the
To further address the functional relationships between Rif1p, Rif2p, and the Rap1p carboxyl terminus, we measured chromosome loss rates in RIF single mutants and the rif1 rif2 double mutant. Previous studies have shown that rapl' mutants display elevated chromosome loss rates, perhaps as a result of impaired telomere function (Kyrion et al. 1992). As shown in Table 5, we found that chromosome III loss rates in rif1 and rif2 mutants were elevated by 7.5- and 3.5-fold, respectively, compared with wild-type. However, just as for silencing and telomere length measurements, the rif1 rif2 double mutant displayed a synergistic effect, giving a chromosome III loss rate >30-fold greater than wild type. This relative increase is comparable to that determined for the most severe rapl' mutant (rap1-17) using a different chromosome loss assay (Kyrion et al. 1992).

Additionally, cells mutated for both RIF1 and RIF2 have a significantly longer doubling time than wild-type cells (Table 5). In contrast, mutation of either RIF gene alone does not significantly alter the growth rate, providing further evidence for synergistic action of Rif1p and Rif2p.

**Table 5. Phenotypes of cells lacking Rif1p and Rif2p**

| Genotype | Telomere length increase | Doubling time | Chromosome loss rate |
|----------|--------------------------|---------------|---------------------|
| Wild type| N.A.                     | 90            | 4.3 X 10^-7         |
| rif1     | 200-600                  | 92            | 3.2 X 10^-6         |
| rif2     | 100                      | 90            | 1.5 X 10^-6         |
| rif1 rif2| 600-2500                 | 108           | 1.5 X 10^-5         |

*All strains are isogenic to W303 except for the RIF mutations indicated.

*Derived from Figs. 4 and 5.

*Growth rates were measured for exponentially growing cells in rich medium (YPD) at 30°C.

*Based on determination of rate of loss of chromosome III [see Materials and Methods for details].
The effect of a multicopy SIR4-containing plasmid was observed in wild-type cells carrying either full-length RIF1 or RIF2 on a multicopy plasmid. DNA was prepared and analyzed as in Fig. 4. The high-copy plasmid significantly reduced telomere lengths to nearly wild-type levels, whereas RIF2 had a small effect.

We also examined the effect on telomere length of the addition of single extra copies of the RIF and SIR genes in the presence of carboxy-terminal overexpression of Raplp. The addition of RIF1 on a centromere-based plasmid was able to partially suppress the effect of the LexA/Raplp(630-827) plasmid. Strikingly, when RIF1 and RIF2 were present together on centromeric plasmids in these cells, a reduction in telomere length and heterogeneity was observed to that of wild-type cells was observed (Fig. 4, lanes 10-13). No effect of either SIR3 or SIR4 alone was observed, although some suppression of telomere elongation was observed with both together (Fig. 6, lanes 14-16). Taken together, these results suggest that the levels of RIF1p and RIF2p can have a direct effect on the regulation of telomere length, and provide further indication that these two proteins play a critical role in this process.

**Discussion**

Several lines of evidence indicate that Raplp, which binds to multiple high-affinity sites within the poly(C1−A) tracts at telomeres, plays a critical yet complex role in telomere length regulation. Temperature-sensitive RAP1 mutants, grown under semi-permissive conditions, have shorter telomeres (Conrad et al. 1990; Lustig et al. 1990), suggesting that one function of Raplp may be simply to protect the telomere from degradation by exonucleases. However, this is unlikely to be the sole function of Raplp in telomere length regulation. Lustig and colleagues have shown that carboxy-terminal truncation mutations of RAPI, which retain the DNA-binding domain and are viable, exhibit an extreme telomere-elongation phenotype (Kyrion et al. 1992). It is presumed, therefore, that this nonessential domain of Raplp, which is also required for telomere position effect and HM locus silencing (Kyrion et al. 1993; Moretti et al. 1994), negatively regulates telomere elongation. This might occur, for example, by direct inhibition of telomerase or by controlling the access of telomerase to the chromosome end. Whatever the mechanism of telomere length regulation by Raplp, it is likely to work through interactions with other proteins because overexpression of the Raplp carboxyl terminus, in the absence of its normal DNA-binding domain, results in telomere elongation (Conrad et al. 1990; Hardy 1991). These data can be most easily explained by the existence of titratable factors that interact with the Raplp carboxyl terminus to mediate telomere length regulation. The first candidate for such a factor to be identified was Riflp [Raplp-interacting factor 1], which was isolated by a two-hybrid screen using the carboxy-terminal 175 amino acids of Raplp (Hardy et al. 1992b). However, the effect on telomere length of mutating RIF1 is relatively small by comparison with the Raplp truncation mutants.

**Riflp and RIf2p are required to mediate the telomere length regulation function of Raplp**

Here we have described a new Raplp-interacting factor...
[Rif2p] with properties very similar to those of Rif1p. Both of these proteins interact with the Rap1p carboxyl terminus in two-hybrid assays, and mutation of either protein results in moderate telomere elongation and an increase in TPE. Significantly, cells lacking both of these proteins have extremely elongated telomeres that are indistinguishable from those in cells containing a rap1 mutation, which removes the Rap1p carboxyl terminus. The simplest interpretation of these observations is that the Rap1p carboxyl terminus recruits both Rif1p and Rif2p to telomeres where they are required to regulate telomere elongation. One prediction of this model, which we are at present trying to test, is that Rif proteins are localized at telomeres in vivo. Our results do not indicate whether or not Rif1p and Rif2p are sufficient to mediate the telomere length regulation function of Rap1p, but they do indicate that both are necessary. If other Rifs are involved in this regulatory function, their action must be dependent upon either Rif1, Rif2, or both. Finally, the fact that neither Rif1 nor Rif2 mutation exacerbates the telomere elongation phenotype of the rap1-17 allele suggests that the function of these two proteins in telomere length regulation requires the Rap1p carboxyl terminus.

Because Rif1p and Rif2p can interact with each other in the two-hybrid system, these two proteins may act as a complex that is recruited to the telomere. If this is the case, it might seem odd that the two proteins have distinct functions, as indicated by the synergistic effect of a rif1 rif2 double mutation on telomere length. One possible explanation for these observations is that, once recruited to the telomere, Rif1p and Rif2p interact with different proteins, and thus genetically and biochemically define two regulatory pathways for telomere length regulation. Alternatively, Rif1p and Rif2p might have a common target. In this case each protein would by itself provide partial regulation by interacting independently with this target. More information concerning the mechanism of action of both proteins will be required before these models can be distinguished. Another interesting possibility raised by the interaction of Rif1p with Rif2p is that Rif1p at one telomere may interact with Rif2p at another. Thus, the Rif proteins may play a role in the clustering of telomeres into groups that has been observed by immunolocalization of telomere-associated proteins and in situ hybridization with telomere-specific probes [Klein et al. 1992; Palladino et al. 1993; Gotta et al. 1996]. Finally, we should point out that it is unclear at present what, if any, role Rif1 and Rif2 might play in a novel mechanism of telomere length control, called “telomeric rapid deletion” [Li and Lustig 1996], which can reduce extremely elongated telomeres to wild-type lengths in what appears to be a single-division event.

*Rif and Sir proteins mediate different functions of Rap1p at telomeres*

Because the initial characterization of RIF1 revealed a complex set of effects on telomeric and HM locus silencing as well as telomere length control [Hardy et al. 1992b; Kyrion et al. 1993], the precise function of this protein was unclear. The identification and characterization of Rif2p reported here greatly clarifies this picture by providing evidence for a specialized mechanism of telomere length regulation by Rap1p, separate from its silencing function. The fact that Rif2p is required together with Rif1p for telomere length control, and that these two proteins interact with each other in two-hybrid assays, supports the idea that their primary role is to negatively regulate telomere elongation. Several lines of evidence indicate that the changes in silencing brought about by RIF mutations are likely to be secondary consequences of their effects on telomere length and structure. To begin with, telomere elongation in wild-type cells is sufficient to cause increased TPE [Kyrion et al. 1993] and decreased silencing at HMR (Buck and Shore 1995). In principle, therefore, the effects of RIF mutations on silencing could be an indirect consequence of their effects on telomere structure. Second, Rif proteins, unlike the Sir proteins, are not required for silencing, but rather affect the balance between telomeric and HMR locus silencing mediated by competition for Sir proteins [Buck and Shore 1995]. As such, the Rif proteins can be viewed as regulators of silencing, but are not required components of silent chromatin, like Sir3p and Sir4p [Hecht et al. 1995, 1996]. Finally, it is important to keep in mind that the effects of SIR3 and SIR4 mutations on telomeres are exactly opposite those of RIF1 and RIF2 mutations. In the SIR mutants TPE is abolished [Aparicio et al. 1991] and telomeres tracts become slightly shorter [Palladino et al. 1993], whereas RIF mutations improve TPE [Fig. 3B; Kyrion et al. 1993] and increase telomere length [Fig. 4, Hardy et al. 1992b].

In a separate report [Marcand et al. 1997] we present evidence that telomere length is regulated by a negative feedback mechanism that can sense the precise number of Rap1p carboxyl termini at the chromosome end. The data presented here suggest that the Rif1p/Rif2p complex, bound to Rap1p at telomeres, mediates this length-sensing and regulation function of Rap1p, which is itself antagonized by Rap1p–Sir interactions. Putting these and other data together, we propose a model in which Rap1p–Sir and Rap1p–Rif complexes are naturally partitioned to opposite ends of the telomeric Cn-A repeats [see Fig. 7]. We imagine that Rap1p–Sir interactions are favored at the proximal end of the telosome [Wright et al. 1992] (the telosome/nucleosome junction) by cooperative interactions between Sir proteins themselves [Morretti et al. 1994] and between Sir proteins and histones H3 and H4 [Hecht et al. 1995]. Because TPE is likely to result from a continuous spreading of Sir complexes along the nucleosome fiber, it follows that the Rap1p molecules at the junction between the telosome and nucleosomal DNA would be most critical for establishing silencing. On the other hand, the Rif1p/Rif2p complex is likely to act at the telomere end, and we speculate that Rif protein assembly at the distal end of the telosome might be promoted by interactions with telomere end-binding proteins. We note that a tendency of Rif complexes to assemble at the distal end of the telomere
might help to explain recent observations in Kluveromyces lactis that suggest that Raplp-binding sites nearest to the telomere end play a critical role in length regulation [Krauskopf and Blackburn 1996]. Nevertheless, we would emphasize that this model is speculative, and that the available data do not rule out a model in which Sir and Rif complexes are interdigitated along the telosome. It seems likely that a direct biochemical and structural characterization of the telosome will be required to distinguish between these two models.

Whatever the precise arrangement of Sir and Rif complexes along the telosome, the critical parameter controlling telomere length regulation would appear to be the amount of Rif complex assembled there. The shortening of telomeres caused by the overexpression of Rif proteins (Fig. 6) would thus result from an extension of the Rif complex along the telosome that results in telomere shortening, possibly by inhibition of telomerase (Fig. 7A). Conversely, mutation of one of the RIF genes may result in a decrease in the amount of the telomere bound by Rif protein, allowing telomerase activation and telomere elongation [Fig. 7B]. The removal of Sir3p or Sir4p by mutation would allow free access of the Rif1/Rif2 complex to the telosome, which would cause increased telomerase inhibition and result in telomere shortening, which is precisely what has been observed [Palladino et al. 1993]. Our model for telomere length regulation [Marcand et al. 1997] predicts that the extent of telomere shortening in Sir3 or Sir4 mutants (~50 bp) would reflect the amount of C1_{1-3}A tract occupied by Raplp-Sir complexes (thus excluding Rif1/Rif2) in wild-type cells. Assuming that Raplp binding sites occur approximately every 18 bp along the telomere, this would translate to about three Raplp-Sir complexes.

**How do Rif proteins regulate telomere length?**

Our data can be most simply explained by proposing that Rif proteins are recruited to telomeres by Raplp where they either block telomere elongation directly or promote degradation of the ends. To explain how this regulatory mechanism can be highly sensitive to the number of Raplp molecules (and ultimately Rif complexes) bound at an end, we would propose that either a stochastic process controls events at the end through interactions with a Raplp/Rif complex, as suggested previously for the yeast *K. lactis* (McCachern and Blackburn 1995), or the Raplp/Rif complex creates a length-sensitive switch to control the access of factors to the telomere end or their activity once bound there [Marcand et al. 1997]. One obvious candidate for Rif action is the telomerase enzyme itself, which has been detected recently in vitro [Cohn and Blackburn 1995; Lin and Zakian 1995], and whose RNA template is now known (Singer and Gottschling 1994). Another candidate for Rif action is the product of the PIF1 gene, a helicase that inhibits telomere elongation [Schulz and Zakian 1994]. One could imagine that Rif proteins target this enzyme to telomeres. However, genetic epistasis tests suggest that Piflp acts in a different pathway from the Raplp carboxyl terminus [Schulz and Zakian 1994]. Alternatively, the Rif proteins may interact with one or more recently identified telomere end-binding proteins to regulate telomerase. The Est1 [Virta-Pearlman et al. 1996] and Cdc13(Est4) [Lin and Zakian 1996; Nugent et al. 1996] proteins both bind to single-strand TG1_{1-3} telomeric sequences and appear by genetic criteria to be essential regulators of telomerase activity [Lundblad and Szostak 1989; Garvik et al. 1995; Nugent et al. 1996]. A newly
identified Cdc13p-interacting protein with similar func-
tions, Stnlp, is another potential Rif target [Grandin et
al. 1997]. Given our present limited understanding of
telomere replication, it seems likely that there are other
possible mechanisms of Rif1 action. Identifying factors
other than Raplp that interact with the Rif proteins, and
studying genetic and biochemical interactions between
the Rif genes and known telomere replication factors,
should provide important clues to understanding how
the Rif proteins work.

Materials and methods

Plasmids

All LexA fusions were expressed from pBTM116 [2 μm origin,
TRPl, pADHl-lexA; a gift of P. Bartel and S. Fields, State Uni-
versity of New York, Stony Brook]. The LexA/Rapl fusions are
as described previously [Moretti et al. 1994], except for LexA/
Rap 1(679-756), which was created by PCR. The GAD-fusion
library screened with LexA/Rapl was created in pGAD3 [Chien
et al. 1991] and was a generous gift of P. Bartel and S. Fields.
GAD/rif2fs was created by digesting GAD/Rif2 with
MluI, end-
filling with Klenow, and religating. LexA/Rif 2 fusions were gen-
erated by PCR, except for those encoding amino acids 18-389
and 18-298, which were created by subcloning fragments from
GAD/Rif2 into pBS [Stratagene], then into pBTM116. GAD/
Rapl encoded amino acids 478-827 and was isolated by screen-
ing a two-hybrid library with LexA/Rif2(2-395). The Gbd/Rapl
fusions, GAD/Rif1, GAD/Sir3, and GAD/Sir4, are as described
previously [Hardy et al. 1992a; Moretti et al. 1994]. Wild-type
RIF1, RIF2, SIR3, and SIR4 were expressed from their own pro-
moters, and were present on CEN/ARS- or 2-μm-based pRS vec-
tors [Sikorski and Hieter 1989]. The full-length RIF2 gene, in-
cluding 495 bp of sequence 5' to the initiation codon and 120 bp
3' to the translational stop, was isolated by PCR and cloned into
pBS. The RIF2 deletion/insertion mutation was created in pBS
by replacing the sequences encoding amino acids 18-389 with
the HIS3 gene. RIF1, SIR3, and SIR4 disruption mutations have
been described previously [Hardy et al. 1992b; Moretti et al.
1994]. Sequences of oligonucleotides used in PCR-based cloning
steps and details of all plasmid constructs are available upon
request.

Yeast strains

The yeast strains used in this study are all derivatives of either
W303 [Thomas and Rothstein 1989] or the two-hybrid assay
strain CTY10-5D, and are listed in Table 6.

Yeast manipulations

Growth and manipulation of yeast strains was carried out using
standard procedures [Rose et al. 1990]. Screening of the GAD-
fusion library with LexA/Rapl was carried out in CTY10-5D, as
described previously [Moretti et al. 1994]. Spot assays were per-
fomed by spotting 5 μl aliquots of 10-fold serial dilutions of a
saturated overnight culture onto plates containing the appro-
riate selective media. Plates were photographed after 2-3 days.

Table 6. Yeast strains used in this study

| Yeast strains | genotype                  |
|---------------|---------------------------|
| W303-1A       | HMLα MATα HMRα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 |
| W303-1B       | HMLα MATα HMRα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 |
| CTY10-5D      | MATα ade2-1 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3::LexA op-lacZ |
| PM101         | CTY10-5D sir3::HIS3        |
| PM102         | CTY10-5D sir4::HIS3        |
| PM103         | CTY10-5D rif1::HIS3        |
| AIL394-1a     | W303-1A rap1-17 telVII::ADE2/URA3 |
| YLS607        | W303-1B hmrαΔ::ADE2 rif1::URA3 |
| YDW62         | CTY10-5D rif2::HIS3        |
| YDW76         | W303-1B hmrαΔ::TRP1 telVII::URA3 |
| YDW77         | YDW76 rif2::HIS3          |
| YDW123        | YDW76 rif1::URA3          |
| YDW124        | YDW76 rif1::URA3 rif2::HIS3 |
| YDW80         | W303 HMLα MATα hmrαΔ::ADE2 |
| YDW81         | YDW80 rif1::URA3 rif1::URA3 |
| YDW82         | YDW80 rif2::HIS3/rif2::HIS3 |
| YDW83         | YDW80 rif1::URA3 rif1::URA3 rif2::HIS3/rif2::HIS3 |
| YDW84         | W303-1A hmrαΔ::TRP1 telVII::ADE2/URA3 |
| YDW85         | YDW84 rif1::URA3          |
| YDW86         | YDW84 rif2::HIS3          |
| YDW87         | YDW84 rif1::URA3 rif2::HIS3 |
| YDW125        | W303 HMLα MATα hmrαΔ::TRP1 tel1-17 rif1::URA3 rif2::HIS3 |
| YDW126        | W303-1A hmrαΔ::TRP1        |
| YDW127        | W303-1A rap1-17            |
| YDW128        | YDW127 rif1::URA3         |
| YDW129        | YDW127 rif2::HIS3         |
| YDW130        | YDW127 rif1::URA3 rif2::HIS3 |
| YDW131        | YDW126 rif1::URA3 rif2::HIS3 |
β-galactosidase assays were carried out as described previously [Moretti et al. 1994].

**Chromosome loss assays**

Determination of the rate of loss of chromosome III was carried out as described previously [Chi and Shore 1995]. Briefly, single colonies were resuspended in 1 ml of YPD and sonicated. Dilutions were plated onto YPD medium to assess the number of viable cells. Half of each colony was then incubated with 100-fold excess of a MA\(\Delta\)Ta tester strain for 8 hr at 30°C. Cells were then spread onto SD plates to select for colonies that arose from a mating event. The chromosome rate loss was derived according to the following formula: \(N = F|/\log N - \log N_o\), where \(F\) = the frequency of mating events, \(N\) = the number of cells in the colony and \(N_o\) = the number of cells from which the colony arose. For this analysis, we used diploid strains that lacked any mating-type information at HMR.

**DNA sequencing**

All sequencing was carried out by use of the dideoxy chain termination method, using Sequenase (Amersham).

**Telomere blots**

DNA was isolated from overnight yeast cultures and 1 µg was digested with XhoI for 4 hr. DNA was electrophoresed through 0.8% agarose and transferred to nylon membranes (Hybond). Membranes were hybridized at 50°C in 6x SSC, 5x Denhardt’s solution, with a poly[d(G-T)] probe labeled by random priming. Membranes were washed twice in 2x SSC at 55°C for 45 min and autoradiographed with Kodak X-AR5 film.

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