Mutational Analysis Defines a C-Terminal Tail Domain of RAP1 Essential for Telomeric Silencing in *Saccharomyces cerevisiae*

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**ABSTRACT**

Alleles specifically defective in telomeric silencing were generated by *in vitro* mutagenesis of the yeast RApl gene. The most severe phenotypes occur with three mutations in the C-terminal 28 amino acids. Two of the alleles are nonsense mutations resulting in truncated repressor/activator protein 1 (RApl) species lacking the C-terminal 25–28 amino acids; the third allele is a missense mutation within this region. These alleles define a novel 28-amino acid region, termed the C-terminal tail domain, that is essential for telomeric and *HML* silencing. Using site-directed mutagenesis, an 8-amino acid region (amino acids 818–825) that is essential for telomeric silencing has been localized within this domain. Further characterization of these alleles has indicated that the C-terminal tail domain also plays a role in telomere size control. The function of the C-terminal tail in telomere maintenance is not mediated through the RApl interacting factor RIF1: rapl alleles defective in both the C-terminal tail and RIF1 interaction domains have additive effects on telomere length. Overproduction of SIR3, a dose-dependent enhancer of telomeric silencing, suppresses the telomeric silencing, but not length, phenotypes of a subset of C-terminal tail alleles. In contrast, an allele that truncates the terminal 28 amino acids of RApl is refractory to SIR3 overproduction. These results indicate that the C-terminal tail domain is required for SIR3-dependent enhancement of telomeric silencing. These data also suggest a distinct set of C-terminal requirements for telomere size control and telomeric silencing.

**TELOMERES**, the unique protein-DNA structures present at the ends of eukaryotic linear chromosomes, can exert repressive effects on the transcription and replication of adjacent DNA domains (Gottschling *et al.* 1990; Levis *et al.* 1985). These telomere position effects have been most extensively investigated in the yeast *Saccharomyces cerevisiae*. Genes placed adjacent to the poly(G,T) tracts present at the yeast telomere are subject to a metastable transcriptionally repressed state, with both repressed and derepressed states maintained for multiple generations (Gottschling *et al.* 1990). This effect, called telomeric silencing, is associated with a reduced accessibility of subtelomeric chromatin to modification by *Escherichia coli dam* methylase (Gottschling 1992). These data suggest that the telomere may be involved in establishing a closed chromatin state in adjacent domains. In yeast, proximity to a telomere also influences the temporal control of replication, with telomere proximal origins activated late in S phase (Ferguson and Fangman 1992). These properties resemble those of the transcriptionally quiescent and late-replicating heterochromatic regions present in higher organisms (Zakian 1989).

Several genes have been defined genetically as essential for telomeric silencing in yeast: the *NAT1* and *ARD1* genes, encoding subunits of N-terminal acetyltransferase; *HHF2*, encoding one of the two copies of histone H4; and the *SIR2*, *SIR3* and *SIR4* genes, encoding proteins of unknown function (Aparicio *et al.* 1991; Laurentson and Rine 1992). Interestingly, the SIR3 protein acts as a dose-dependent enhancer of telomeric silencing (Renauld *et al.* 1993). This finding has led to the proposal that SIR3 facilitates the spreading of heterochromatin into subtelomeric domains.

Many of the requirements for telomeric silencing are shared with silencing of the cryptic mating-type genes present at *HML* and *HMR*, located 12 and 23 kb from the left and right telomeres of chromosome III, respectively (Laurentson and Rine 1992). Although telomeric position is not required for *HM* repression, it appears to influence this process, since the requirements for trans-acting factors involved in *HMR* silencing are influenced by the proximity of *HMR* to the telomere (Thompson *et al.* 1994).

An additional similarity between telomeric and *HM* silencing is the presence at both types of loci of binding sites for the essential DNA binding protein repressor/activator protein 1 (RApl) encoded by the *RApl* gene (Shore and Nasmyth 1987). Remarkably, RApl is capable of binding to sequences at a wide variety of genomic loci, including upstream activation sequences of numerous genes, the silencer elements necessary for transcriptional repression of *HML* and *HMR*, and sites embedded within telomeric simple sequence tracts (see...
A.

![Diagram of RAP1 domain structure](image)

**Figure 1.** C-terminal tail alleles define a novel RAP1 domain. (A) Summary of RAP1 domain structure. The DNA binding (HENRY et al. 1990), activation (HARDY et al. 1992a) and the rap1" (SUSSEL and SHORE 1991) domains are shown, together with the C-terminal tail domain defined in this study. Also shown are the sites of mutation of the alleles generated by random mutagenesis in this study (indicated by the underlined alleles) and the positions of the rap1-5" (P994L) (KURTZ and SHORE 1991), rap1" (SUSSEL and SHORE 1991) and rap1" (KRON et al. 1992) alleles discussed in the text. The site of truncation produced by each nonsense mutation is indicated by an arrow. The rap1" alleles consist of four clustered alleles: rap1-11(R747S), rap1-12(G726E D727N), rap1-13 (D727A), and rap1-14 (G736E) (SUSSEL and SHORE 1991). These alleles display a loss of hmrAA silencing and increases in telomere tract lengths. The rap1" alleles consist of three alleles: rap1-17 (opal 663), rap1-18 (amber 684) and rap1-19 (ochre 665) (KRON et al. 1992). These alleles display several phenotypes including extreme telomere elongation and instability, decreased chromosome stability, abrogation of telomeric silencing, attenuation of HML silencing, and a decreased growth rate (KRON et al. 1992, 1993). (B) C-terminal sequences (amino acids 795–827) of the wild-type (RAP1), rap1-22, rap1-20 and rap1-21 alleles. Underlines refer to the sites of mutated residues.

B.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAP1</td>
<td>LKSNQEQIRKLVVKGHTGRMRRMRRFFFEKDLL</td>
</tr>
<tr>
<td>rap1-22</td>
<td>LKSNQEQIRKLVVKGHTGRMRRMRRFFFEKDLL</td>
</tr>
<tr>
<td>rap1-20</td>
<td>LKSNKNK</td>
</tr>
<tr>
<td>rap1-21</td>
<td>LKSNND</td>
</tr>
</tbody>
</table>

Laurenson and Rine 1992). At telomeres, RAP1 binding sites are present within the poly(G13) tracts at an average frequency of one site in every 20 bp of tract sequence (GILSON et al. 1993). That RAP1 associates with telomeres in vivo is indicated by both the presence of RAP1 in telomeric chromatin fractions (WRIGHT et al. 1992) and the immunolocalization of telomeric RAP1 complexes to peripheral regions of the nucleus (KLEIN et al. 1992; PALLADINO et al. 1993). At HMR, the RAP1 binding site is one of three sites for DNA binding proteins making up the HMRE silencer element (SHORE et al. 1987). The two remaining sites are an autonomously replicating sequence (ARS) element which serves as a binding site for the origin recognition complex (BELL and STILLMAN 1992), and a binding site for the multifunctional protein ABF1 (SHORE et al. 1987). These sites display functional redundancy. Elimination of any one of the three sites does not significantly impair silencing, while elimination of any two sites abolishes HMR silencing (BRAND et al. 1987). Similarly, HML is flanked by two functionally redundant silencer elements, one of which contains a high affinity RAP1 binding site (HOFMANN et al. 1989; MAHONEY and BROACH 1989; MAHONEY et al. 1991).

Previous molecular and genetic studies have defined three distinct domains in the RAP1 protein: a central DNA binding domain (HENRY et al. 1990), a flanking region essential for the transcriptional activation function of RAP1 (HARDY et al. 1992a), and a C-terminal silencing domain called the rap1" domain (SUSSEL and SHORE 1991) (Figure 1A). Specific missense alleles within this domain (termed the rap1" alleles) mapping to a region between amino acids 727 and 747 relieve silencing directed by a defective HMRE silencer lacking
the ARS element (hmrΔAA) (Sussel and Shore 1991). Interestingly, these alleles do not decrease the efficiency of either telomeric or HML silencing (E. Wiley and V. Zakian, L. Sussel and D. Shore, personal communications; this study). The function of this rap1 domain appears to be mediated through interactions with RIF1 (Hardy et al. 1992b), identified as a protein capable of interaction with the C terminus of RAPl in a two-hybrid system. While RIF1 is unable to associate with mutant Rap1 proteins, this association is restored in strains carrying a rif1 missense allele, suggesting that RIF1 physically interacts with the rap1 domain. While required for hmrΔA silencing, Rif1 is dispensable for telomeric silencing (Kyrion et al. 1993).

In contrast to the rap1 alleles, rap1 alleles which lack the C-terminal 144–165 amino acids (termed the rap1 alleles) abrogate telomeric silencing and attenuate HML silencing, without affecting either cell viability or DNA binding by RAPl (Kyrion et al. 1993). rap1 cells also display an enhanced accessibility of subtelomeric chromatin to exogenously introduced E. coli dam methylase (Kyrion et al. 1993), suggesting that RAPl may be involved in the formation of heterochromatin near the telomere. These studies raise the possibility that C-terminal regions of RAPl outside the rap1 domain participate in telomeric and HML silencing.

Rap1 also plays an important role in regulating the length and stability of the poly(G1,T) tracts of yeast telomeres. rap1 temperature sensitive (rap1 mutants confer a temperature-dependent reduction in telomere tract length (Conrad et al. 1990; Lustig et al. 1990). In contrast, rap1 alleles display gross telomere elongation, with telomeres attaining sizes up to 4 kb larger than the ~300-bp tract found in wild-type cells (Kyrion et al. 1992). rap1 telomeres are also highly unstable, generating telomeres differing in size by 2 kb in only 25 generations. Loss of the C-terminal 144–165 amino acids therefore appears to result in a greater susceptibility of the telomere to processes of both elongation and shortening. Overproduction of the C-terminal 330 amino acids of RAPl similarly results in telomere elongation, suggesting the titration of a factor important for telomere size control (Conrad et al. 1990). Chromosome loss is exacerbated in rap1 alleles, raising the possibility that the role of RAPl at the telomere may be important for maintaining chromosome stability.

We have used both random and site-specific mutagenesis to explore the regions of RAPl important for telomeric silencing and size control. These studies identified a novel 28-amino acid C-terminal tail domain that is essential for telomeric silencing. This domain also plays important roles in HML silencing and telomere length control. Interestingly, we find that the telomeric silencing defects of some, but not all, of these alleles are suppressed by Sir3 overproduction. These data indicate that the C-terminal tail domain is required for Sir3 enhancement of telomeric silencing and suggest the presence of functional interactions between Sir3 and the RAPl C-terminal tail.

MATERIALS AND METHODS

Plasmid constructions: pRS315/RAPl was constructed by cloning the EcoRI/XbaI fragment containing the RAPl gene into the HIS3 centromeric (CEN) vector pRS313 (Sikorski and Hieter 1989). pRS306/rap1-21 and pRS306/rap1-22 were constructed by cloning the EcoRI/XbaI fragment containing the full-length rap1-21 and rap1-22 genes into the URA3-integrating vector, pRS86 (Sikorski and Hieter 1989), pD150, which contains the RAPl gene cloned in a TRPI-CEN plasmid, has been previously described (Kurtz and Shore 1991). The high copy vector YEp351A was constructed by cloning the 3.6-kb BamHII fragment containing the ADE2 gene into the BamHII site of YEp351 (Hml et al. 1986). The high copy vector YEp351A/Sir3 was constructed by cloning the 4.5-kb SalI fragment containing the Sir3 gene (derived from pR273 kindly provided by J. Rine) into YEp351. The 3.6-kb BamHII fragment containing the ADE2 gene was then cloned into the unique BamHII site. All plasmids were transformed into E. coli strains HB101, JM105, JM109 or TOP10F (Invitrogen).

Yeast strain constructions and media: The strains used in this study are listed in Table 1. All strains (with the exception EMFY75) were derived from W303. Heterozygotes were constructed by transforming GSK23-8b with each of the pRS313-based rap1 alleles. Control strains for the heterozygotes were generated by transforming the CLY strains containing the pRS313-based rap1 allele with the TRPI-CEN plasmid pRS314 (Sikorski and Hieter 1989). In this study, rap1 alleles generated by random hydroxylamine mutagenesis are denoted by their allele numbers (i.e., rap1-20, rap1-21, rap1-22, rap1-23 and rap1-24) while those alleles generated by site-directed mutagenesis are denoted by the amino acid(s) altered by the mutation.

To generate rap1-21, rap1-22, rif1::URA3 rap1-21 and rif1::URA3 rap1-22 strains containing an ADE2-marked telomere, AJL395-1d was crossed to CLY/rap1-21 or CLY/rap1-22 and the resulting diploids (CL80 and CL81, respectively) were sporulated. The genotypes of these spore colonies were confirmed by genetic and Southern analysis.

AJL424-4b was constructed as follows: AJL 275-2a VIII-ADE, which contains a URA3 gene centromere-proximal to the telomeric ADE2 gene, was grown on adenine omission medium to select for cells expressing the telomere-proximal ADE2 gene. Once telomeric silencing spreads continuously from the telomere toward centromere-proximal sequences, selection for Ade+ cells precludes telomeric silencing at the URA3 locus (Renauld et al. 1993). Hence, all Ade+ cells should be Ura+ and sensitive to 5-fluoroorotic acid (FOA²). Cells were then plated onto 5-FOA medium to select derivatives containing spontaneously generated mutations within the subtelomeric URA3 gene. Several of the candidates that did not have detectable reversion frequencies were identified. The structure of the ade2 RAP1-marked telomeres in these strains was confirmed by Southern analysis. One of these strains was crossed to the rap1-1-containing strain AJL278-4d forming the diploid AJL442. Southern analysis demonstrated that the ADE2-marked telomere in AJL424-4b contains a poly(G1,T) tract of ~1.5 kb.

For the construction of strains carrying high copy plasmids, CLY/RAPl, CLY/rap1-21, CLY/rap1-22, CLY/R818A, CLY/F821G, CLY/F822G, and CLY/Δ820-827 were transformed with either YEp351A or YEp351A/Sir3. In each case, multiple transformants were characterized as indicated in the text. The copy
numbers of the YEp plasmids and the RAPl-containing CEN plasmids were determined by quantitative Southern analysis using the ADE2 and HIS3 genes as probes for the high copy and centromeric plasmids, respectively. The average copy numbers of the YEP plasmids and the RAPl-containing plasmids were determined by quantitative Southern analysis for the strains, respectively, were identified and characterized as indicated.

Individual strains each containing 1, 2, or 5 copies of the rap1-21 allele or 1, 2, or 3 copies of the rap1-22 allele, integrated at the rap1-17 locus, were identified and characterized as indicated in the text.

All transformations were carried out by the LiOAc method (Sherman et al. 1986). YPD, synthetic complete (SC) medium, SC/low adenine medium, SC omission medium and 5-FOA-containing medium were prepared as described (Kyon et al. 1992; Sherman et al. 1986).

Scheme for the identification of suppressors: To generate random mutations within the C-terminus of RAPl, the 1.2-kb Spml/XbaI fragment, encoding the C-terminal 292 amino acids, was subcloned into pUC19, and the plasmid was mutagenized with 1 M hydroxylamine for 30 min at 75°C. The mutagenized fragment was purified and used to replace the reporter YEp plasmids and the RAPl-containing plasmids in the wild type, rap1-21, rap1-22, A820-827, R818A and F821G F822G strains, respectively.
nics through repression of the URA3 gene at the VII telomere. FOA+ candidates capable of rapid growth were further analyzed. DNA recovered after transformation of genomic DNA into E. coli was retransformed into the rapl-17 strain to ensure the absence of genomic mutations responsible for the phenotypes. The sequence of the entire SphI/XbaI fragment was determined from each candidate using double-strand sequencing methods (Ausubel et al. 1987). Sequence analysis of these alleles revealed that the rapl-20 allele contains a base pair deletion at nucleotide 3159. In addition, a second base pair deletion at nucleotide 3337 is present in the 3' untranslated region. The rapl-21 gene contains a unique C to T transition at nucleotide 3192. The rapl-23 allele contains a base pair deletion at nucleotide 3159. In addition, a second base pair deletion at nucleotide 3337 is present in the 3' untranslated region. The rapl-21 gene contains a unique C to T transition at nucleotide 3192. The rapl-23 and rapl-24 alleles contain a G to T transversion at nucleotide 2427 and a C to A transversion at nucleotide 2976, respectively.

To test the phenotype of each allele as the sole source of RAP1, the pRS313 CEN plasmids containing the candidate alleles were transformed into GR28-3b, and a plasmid shuffle was performed to eliminate the wild-type allele, giving rise to the CLY series of strains (Kwon et al. 1992) (Table 1). For each shuffled strain, conferring a defect in telomeric silencing, the production of a RAP1 species of the expected size and abundance was confirmed by Western analysis (see Figure 3). The ability of Rapl-21, Rapl-22, and Rapl-23 to bind DNA with wild-type efficiencies was demonstrated using mobility shift assays by measuring the percentage of oligonucleotide probe bound to RAP1 in extracts isolated from wild-type and mutant cells over a range of extract concentrations (Kwon et al. 1992). All four mutants are capable of growth at all temperatures tested (25°, 30° and 37°), although the rapl-22 mutant displays a slight decrease in relative growth rate at 30° and 37°.

Oligonucleotide-directed mutagenesis: The 1.2-kb SphI/XbaI fragments of the wild-type RAP1 and rapl-22 genes were cloned into pBS(-) (Stratagene), and the resulting plasmids were transformed into the ung- dut- strain CJ236 in the presence of the helper phage M13K07. Single-stranded DNA was isolated and utilized as template for oligonucleotide-directed mutagenesis (Ausubel et al. 1987). Oligonucleotides were used to prime elongation by Klenow fragment, and the extension mix was transformed into E. coli strains HB101 or JM109. DNA was isolated from the transformants and Southern blots of undigested DNA were probed with the mutant oligonucleotide. Blots were sequentially washed at increasing stringencies to detect clones carrying the mutant sequence. The structure of candidate plasmids was confirmed by Southern analysis of undigested DNA. In each case, the sequence of the nucleotides denoted in the 5' to 3' orientation were used as primers for synthesis on wild-type template DNA (the sites of mutation are shown in bold):

CM1 (K805A):
GGAAATGAGGAGGAGATTTTTTGAGAAGGAC;
CM2 (K808A K809A):
GGAAATGGCGAACAAAAAGATTTTTGAGAAGGAC;
CM3 (K819A):
GGAAATGAGGGAAGATTTTTTGAGAAGGAC;
CM5 (rapl-12; G726E D727N):
GTATTTCAGAAAAATATAGGCCATACAGGC;
CM6 (K824R):
GGAAAAGATTTTTTGAGAAGGCGACCTG;
CM7 (K824A):
GGAAAAGATTTTTTGAGAAGGCGACCTG;
CM8 (R820A):
GGAAAATGAGGAAAGATTTTTTGAGAAGGAC.

The oligonucleotide CM5 frequently generated rearrangements within the primer region. One of these (820-827) resulted in a stop codon truncating the terminal 8 amino acids of RAP1. The oligonucleotide CM5 was also used as a primer on rapl-22 template DNA to generate the rapl-12, 22 allele. The SphI/XbaI fragment was purified from each plasmid and used to replace the corresponding wild-type fragment of pRS313/RAP1. The resulting plasmids were transformed into both the rapl-17 strain AJL369-4d and the wild-type strain GR28-3b. In the latter case, a plasmid shuffle was used to generate strains (the CLY series; Table 1) containing the mutant rapl gene as the sole source of RAP1. For each mutant exhibiting a defect in telomeric silencing, the production of a full-length protein of expected abundance was confirmed by Western analysis using antibodies directed against RAP1.
suspended in YPD medium and appropriate dilutions plated onto both 5-FOA-containing synthetic medium and synthetic medium lacking 5-FOA under selection for the plasmids. Statistical significance between the distribution of FOA\(^\text{'}\) frequencies generated by the various alleles was determined by the rank sum test (Snedecor and Cochran 1980). The repression of the ADE2-marked VII, telomere was monitored by measuring the frequency of red colonies or sectors (Gottschling et al. 1990; Kirion et al. 1993). As expected for repression due to telomeric silencing, the \(\text{Ade}^-\) phenotype was reversible as indicated by the production of white sectors among red colonies.

The derepression of HML\(\alpha\) was monitored by quantitative mating assays of wild-type and mutant HML\(\alpha\) MAT\(\alpha\) HMR\(\alpha\) strains (Dutcher and Hartwell 1982; Kirion et al. 1993) using the MAT\(\alpha\) strain EMPIY 75 as the tester strain. Previous studies have confirmed that the decreased mating efficiency of \(\text{rapl}-17\) HML\(\alpha\) MAT\(\alpha\) HMR\(\alpha\) strains is the consequence of derepression of \(\alpha1\) and \(\alpha2\) transcription at the HML\(\alpha\) locus rather than an indirect effect on mating functions (Kirion et al. 1993).

**Determination of telomere tract sizes:** Subculturing of cells prior to telomere tract size determination was performed by multiple rounds of growth on solid medium, each round representing \(\approx 25\) generations (Lustig and Petes 1986). Telomere tract size was determined following digestion of genomic DNA with \(\text{XhoI}\), which cleaves 870 bp from the junction between the Y' element and the poly(G\(_{12}\)T) tract. Blots of \(\text{XhoI}\) digested DNA were probed with poly(dG)\(\cdot\)poly(dC). Telomere tract length was determined by measuring changes in the length and heterogeneity both of these Y'-containing terminal fragments and of individual telomeric fragments from termini that lack the Y' element.

**RESULTS**

**In vitro mutagenesis identifies a C-terminal tail domain of RAP1 essential for telomeric silencing:** To define the sites within RAP1 that are involved in telomeric silencing, we generated random mutations in vitro within the C-terminal 292 amino acids. The resulting mutant alleles were then introduced on a centromeric (CEN) plasmid into a \(\text{rapl}-17\) strain containing the URA3-marked VII, telomere (see MATERIALS AND METHODS). The Rap1-17 protein, which lacks the terminal 165 amino acids, results in both complete derepression of telomere-adjacent genes and growth at rates \(\approx 2\)-fold slower than wild type (Kirion et al. 1992, 1993). Since the \(\text{rapl}-17\) defects in growth rate and telomeric silencing are recessive, we reasoned that \(\text{rapl}-17\) strains carrying plasmid-borne \(\text{rapl}\) alleles specifically defective in telomeric silencing would grow at or near wild-type rates, but be incapable of repressing telomere-adjacent genes. Such alleles would be expected to be defective in the telomeric silencing function, but not the essential function, of RAP1. For these studies, telomeric silencing was assayed by measuring the ability of cells to grow on 5-FOA-containing medium, which allows the growth of FOA\(^\text{'}\) strains (Boeke et al. 1987). In the wild-type strain used in these studies, \(\approx 50\%\) of cells are FOA\(^\text{'}\), while \(\text{rapl}-17\) strains generate FOA\(^\text{'}\) colonies at frequencies of less than \(1 \times 10^{-7}\) (Kirion et al. 1993).

Five candidates containing unique mutations in RAP1 were identified that grew at or near wild-type rates, but were severely defective in their ability to form colonies on 5-FOA medium. \(\text{rapl}\) alleles that are specifically defective in telomeric silencing are expected to produce similar phenotypes when present as the sole source of RAP1. To test this, each candidate was introduced into a strain containing both a URA3-marked VII, telomere and a \(\text{rapl}-1::\text{LEU2}\) disruption at its genomic locus. Four of the five candidates, designated \(\text{rapl}-20, \text{rapl}-21, \text{rapl}-22\) and \(\text{rapl}-24\) confer similar defects both in heteroallelic combination with \(\text{rapl}-17\) and as the sole source of \(\text{RAP1}\) (see Figures 2A and 4; data not shown).

Remarkably, three alleles (\(\text{rapl}-20, \text{rapl}-21\) and \(\text{rapl}-22\)) conferring the most severe defects in telomeric silencing mapped within the terminal 28 amino acids of RAP1 (Figures 1 and 2, Table 2A). The two strongest alleles, \(\text{rapl}-20\) and \(\text{rapl}-21\), fully derepress...
the transcription of telomere-adjacent genes. Both alleles result in small C-terminal truncations. The *rapl-20* allele contains a frameshift mutation that produces a protein terminating in a novel four amino acid tail and lacking the terminal 28 amino acids as the consequence of an ochre stop codon at amino acid 803. Similarly, the *rapl-21* allele contains an ochre stop codon at amino acid 800, producing a protein lacking the terminal 26 amino acids. Western analysis confirmed the presence of short truncations in both *rapl-20* and *rapl-21* strains (Figure 1). The third allele mapping within this region, *rapl-22*, confers a strong defect in telomeric silencing. As expected, the defect in telomeric silencing is independent of the telomere-proximal gene: both alleles result in small G-terminal truncations. The *rapl-20* allele contains a frameshift mutation that produces a protein terminating in a novel four amino acid tail and lacking the terminal 25 amino acids as the consequence of an ochre stop codon at amino acid 803. Similarly, the *rapl-21* allele contains an ochre stop codon at amino acid 800, producing a protein lacking the terminal 28 amino acids. Western analysis confirmed the presence of short truncations in both *rapl-20* and *rapl-21* strains (Figure 3). The third allele mapping within this region, *rapl-22*, confers a strong defect in telomeric silencing.

### Table 2: FOA' frequencies in mutants isolated by hydroxylamine mutagenesis

<table>
<thead>
<tr>
<th>Allele*</th>
<th>Mutation site</th>
<th>FOA' frequency×</th>
<th>Normalized frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> RAP1</td>
<td>wt</td>
<td>(2.9 \times 10^{-1}) (1.2–5.8; 20)</td>
<td>(1.0)</td>
</tr>
<tr>
<td>rapl-20</td>
<td>(\Delta 803-827^{d})</td>
<td>(&lt;1.2 \times 10^{-7}) (10)*</td>
<td>(&lt;4.1 \times 10^{-7})</td>
</tr>
<tr>
<td>rapl-21</td>
<td>(\Delta 800-827)</td>
<td>(&lt;1.7 \times 10^{-7}) (10)*</td>
<td>(&lt;5.9 \times 10^{-7})</td>
</tr>
<tr>
<td>rapl-22</td>
<td>H810Y</td>
<td>(1.8 \times 10^{-4}) (0.4–2.5; 10)*</td>
<td>6.2 (\times 10^{-4})</td>
</tr>
<tr>
<td>rapl-12</td>
<td>G796E D727N</td>
<td>5.8 (\times 10^{-4}) (4.6–7.8; 7)*</td>
<td>2.0</td>
</tr>
<tr>
<td>rapl-12,22</td>
<td>G796E D727N H810Y</td>
<td>(5.9 \times 10^{-6}) (0–60; 3; 9)*</td>
<td>2.0 (\times 10^{-5})</td>
</tr>
<tr>
<td>rapl-24</td>
<td>L736M</td>
<td>(4.5 \times 10^{-2}) (0.5–11.2; 9)*</td>
<td>1.6 (\times 10^{-1})</td>
</tr>
<tr>
<td><strong>B</strong> RAP1/pRS314</td>
<td>wt/vector</td>
<td>1.7 (\times 10^{-1}) (0.55–4.1; 10)</td>
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</tr>
<tr>
<td>RAP1/RAP1</td>
<td>wt/wt</td>
<td>1.4 (\times 10^{-1}) (0.96–3.9; 7)</td>
<td></td>
</tr>
<tr>
<td>rapl-21/RAP1</td>
<td>(\Delta 800-827/\text{vector})</td>
<td>(&lt;1.9 \times 10^{-7}) (10)*</td>
<td></td>
</tr>
<tr>
<td>rapl-21/pRS314</td>
<td>(\Delta 800-827/wt)</td>
<td>1.9 (\times 10^{-1}) (1.2–3.6; 7)</td>
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<tr>
<td>rapl-22/pRS314</td>
<td>H810Y/vector</td>
<td>4.1 (\times 10^{-5}) (0.8–13; 10)*</td>
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</tr>
<tr>
<td>rapl-22/RAP1</td>
<td>H810Y/wt</td>
<td>2.6 (\times 10^{-1}) (1.4–4.6; 6)</td>
<td></td>
</tr>
<tr>
<td><strong>C</strong> RAP1/rapl-17</td>
<td>wt/(\phi 663-827)</td>
<td>6.4 (\times 10^{-1}) (5.1–8.3; 7)*</td>
<td>(1.0)</td>
</tr>
<tr>
<td>rapl-23/rapl-17</td>
<td>D555V/(\phi 663-827)</td>
<td>2.1 (\times 10^{-5}) (0–18; 7)*</td>
<td>3.3 (\times 10^{-3})</td>
</tr>
</tbody>
</table>

*In parts A and B, each allele is present on a *CEN* plasmid in the CLY series of strains. In part C, each allele is present on a *CEN* plasmid in a strain containing an integrated copy of *rapl-17*. In part A, strains carrying a single *rapl* allele were grown on YPD. In part B, heterozygous strains or single mutants carrying a control plasmid were grown under selection (SGtrpHis) for both plasmids. In part C, strains were grown under selection for the *CEN* plasmid (SGhis).

The median value is shown together with the range of values and the number of colonies analyzed. Asterisks indicate strains that exhibit a distribution of frequencies significantly different from wild type values (P<0.01).

FOA' frequencies normalized to wild type.

This allele contains a novel 4 amino acid extension prior to the stop codon.

Two samples failed to yield any FOA' colonies in a sample size of 2.65 \(\times 10^6\) and 5.25 \(\times 10^6\), respectively, and one value was too high to measure accurately.

One sample failed to yield any FOA' colonies out of 9900 cells plated.

Identical (*rapl-24*) or only slightly reduced (*rapl-22*) relative to wild-type cells. Each allele is recessive for defects in telomeric silencing, producing wild-type frequencies of FOA' colonies in strains containing both wild-type and mutant alleles (Table 2B; data not shown). The small decrease in growth rate observed in *rapl-20* and *rapl-21* cells is also recessive (data not shown).

As expected, the defect in telomeric silencing is independent of the telomere-proximal gene: both alleles tested, *rapl-21* and *rapl-22*, also derepress an *ADE2*-marked VII telomere. *ADE2* repression was monitored by measuring the frequency of red sectors or colonies, which reflects the *ADE2* phenotype (*GOTTSHLING et al. 1990*). In wild-type cells, 10–40% of cells produce red colonies or white colonies with red sectors. In contrast, only 0.1% (2/1979; strains CLY80-10b, CLY80-13a, CLY80-17b) and 0.4% (6/1500; strains CLY81-17b, CLY81-12a) of *rapl-21* and *rapl-22* cells, respectively, give rise to red colonies or white colonies with red sectors.

Western analysis of extracts isolated from each of these alleles indicated that RAPI molecules of the expected size are produced in amounts comparable to wild-type cells (Figure 3). In addition, as determined by *in vitro* mobility shift assays, both Rapl-21 and Rapl-22 are capable of binding to their cognate site with wild-type efficiency (data not shown).

The *rapl* alleles described above are present on centromeric plasmids in a strain containing a disruption of *RAPl* at its genomic locus. To ensure that the phenotypes conferred by these mutations are not plasmid spe-
Figure 3.—Western analysis of RAP1 in extracts derived from rap1 alleles defective in telomeric silencing. (Top) Equal amounts of protein isolated from each of the indicated strains were subjected to SDS-PAGE and Western blotting using antibodies directed against RAP1. The positions of pre-stained size markers (in kilodaltons) are shown on the right. Although RAP1 has a predicted molecular weight of 93 kD, it migrates anomalously on SDS-polyacrylamide gels (Shore and Nasmyth 1987). wt and wt refer to strains containing an integrated or plasmid-borne copy of wild-type RAP1, respectively. A duplicate Coomassie Blue-stained gel is shown below the Western blot. Strains used (from left to right): AJL 2752a VIILURA, CLY/rap1-20, CLY/rap1-21, CLY/rap1-22, CLY/rap1-23, CLY/rap1-24, Δ820-827, R818A, CLY/RAPl and W303a. (Bottom) Increasing amounts of extracts isolated from CLY/F821G/F822G and wild-type (W303a) cells were subjected to SDS-PAGE and Western blotting as in the top panel. Lane b was loaded with an amount of extract identical to that used in the top panel, while lanes a and c were loaded with one-half and two times the lane b amount, respectively. The signals of these two alleles and those displayed in the top panel were comparable when analyzed in the same experiment. In both cases, the signal obtained in Western blots responds to the increasing levels of RAP1. With the exception of Rap1-23, we estimate that the levels of RAP1 in each strain do not vary by more than twofold.

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Specific, integrating plasmids containing the rap1-21 or rap1-22 gene were targeted into the rap1-17 locus of a strain containing an ADE2-marked VII₁ telomere. Strains carrying integrated copies of rap1-21 or rap1-22 grew at rates identical to rap1-17 strains that carry a plasmid-borne copy of rap1-21 or rap1-22 and displayed the predicted defect in telomeric silencing. Wild-type cells containing ADE2 or URA3-marked telomeres with poly(G,T) tracts longer than the 300-bp tract normally found in wild-type cells exhibit increased levels of repression that are proportional to the length of the telomeric tract (Kurton et al. 1993). However, despite the presence of an ADE2-marked VII₁ telomere with an elongated (~1.5 kb) poly(G,T) tract in each of the integrants, only 8/1782 and 9/4040 cells generated red colonies or sectors in the rap1-21 and rap1-22 integrants, respectively. In contrast, restoration of the wild-type RAP1 allele following recombination between the rap1-21 and rap1-17 alleles in these rap1-21 rap1-17 heteroallelic strains results in the expected hyper-repressed state: 75% of colonies produced either red colonies or white colonies with red sectors. The phenotypes of the heteroallelic strains are independent of the copy number of the integrated alleles [ranging from 1 to 5 copies for individual rap1-21 integrants (AJL442-4b/rap1-21) and 1 to 3 copies for individual rap1-22 integrants (AJL 442-4b/rap1-22)]. These results indicate that the phenotypes of the rap1-21 and rap1-22 alleles are not significantly altered in a chromosomal context.

A fifth allele identified in our screen, rap1-23, partially complements the slow growth rate of rap1-17 cells, while displaying a 300-fold decrease in the frequency of FOA' colonies compared to a wild-type RAP1 allele in the rap1-17 background (Table 2C; data not shown). However, in contrast to the other alleles generated in this study, rap1-23 cells do not exhibit a defect in telomeric silencing when present as the sole source of RAP1, possibly as the consequence of Rap1-23 overproduction (Figure 3; data not shown). Interestingly, the rap1-23 allele contains a single missense mutation at amino acid 555 within the DNA binding domain, changing an aspartic acid to a tyrosine (Figure 1). While Rap1-23 is not defective in binding DNA in vitro, rap1-23 cells display a temperature-sensitive growth defect at 37° and grow at reduced rates at both permissive and semi-permissive temperatures (data not shown), raising the possibility of an in vivo defect in DNA binding.

Fine structure analysis of the C-terminal tail: To further define the residues within the C-terminal tail essential for telomere silencing, we introduced mutations at specific sites within this region. Each mutant rap1 allele was introduced on a CEN plasmid into a strain containing either a deletion of RAP1 or the rap1-17 allele at its genomic locus as well as a URA3-marked VII₁ telomere. Initial experiments indicated that an allele...
which encodes a RAP1 species missing only the terminal 8 amino acids (Δ820-827) displays an almost complete elimination of telomeric silencing (Figures 2 and 4). These results suggest that residues within or surrounding the terminal 8 amino acids are important for telomeric silencing.

To test this possibility, we replaced each of the terminal 10 amino acids either singly or in pairs with either alanine or glycine residues (Figure 4). Two of the missense alleles confer severe defects in telomeric silencing. Replacement of the arginine at amino acid 818 with alanine or glycine residues (Figure 4) results in a 109-104-fold decrease in the frequency of FOA' colonies compared to strains carrying E823G D825G as the sole source of RAP1. In contrast, all mutant alleles are fully recessive to the wild-type (data not shown). As expected, strains containing each of these alleles grow at rates close to (Δ820-827, R820A) or at (F821G F822G) wild-type rates (Table 5). The loss of telomeric silencing is highly specific. Other mutations in this region (K819A, K819R, K824G, K824R, L826G L827G) do not influence telomeric silencing when present either as the sole source of RAP1 or in heteroallelic combination with rap1-17 (Figure 4; data not shown).

The C-terminal tail domain is a highly basic region with lysine and arginine residues comprising 32% of the terminal 28 amino acids. The lysine-rich nature of this region raised the possibility that reversible modification of lysine residues may serve as a means for regulation of RAP1 function. This possibility appears unlikely, however, since mutations of each of the lysine residues in this region to alanine or glycine do not influence telomeric silencing (Figure 4). Nonetheless, we cannot rule out the possibility that multiple lysine residues play redundant functions in this process.

**Truncation of the C-terminal tail attenuates HMLα silencing:** The rap1-17 mutation, truncating the termi-
Table 3

Quantitative mating assays of wild-type, rap1-17, rap1-21, rap1-22 and rap1-24 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Allele</th>
<th>Mating efficiency a (± range of values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G126-3b</td>
<td>Rap1/rap1::LEU2</td>
<td>77.0 ± 10.3</td>
</tr>
<tr>
<td>AJY309-4d</td>
<td>rap1-17</td>
<td>1.9 ± 0.77</td>
</tr>
<tr>
<td>CLY/rap1-21</td>
<td>rap1-21/rap1::LEU2</td>
<td>2.4 ± 0.88</td>
</tr>
<tr>
<td>CLY/rap1-22</td>
<td>rap1-22/rap1::LEU2</td>
<td>63.6 ± 9.2</td>
</tr>
<tr>
<td>CLY/rap1-24</td>
<td>rap1-24/rap1::LEU2</td>
<td>68.2 ± 2.3</td>
</tr>
</tbody>
</table>

a The efficiency of quantitative mating is presented as the mean value of two independent trials together with the range of values observed.

b The first and second alleles refer to the plasmid-borne and chromosomal rap1 alleles, respectively.

d The value of contrast, neither the repressed Gterminal tail is required for full silencing of non-mating phenotype. Our results indicate that the type levels of mating (data not shown). Hence, the mating assays using gesting that the functions of RAPl in telomeric and pared to the excessive: transformation of rap1-21, rap1-22, rap1-24 or wild-type alleles (Table 3). In this assay, cells expressing the normally repressed HMLa information in a MATa cell exhibit a non-mating phenotype. Our results indicate that the rap1-21 allele, truncating the terminal 28 amino acids, confers a 40-fold decrease in mating efficiency compared to the RAPl allele, similar to the effect seen with the rap1-17 allele. This defect in HML silencing is recessive: transformation of rap1-21 cells with a CEN plasmid containing the wild-type RAPl gene restores wild-type levels of mating (data not shown). Hence, the C-terminal tail is required for full silencing of HML. In contrast, neither the rap1-24 (L736M) nor rap1-22 (H810Y) alleles display silencing defects at HML, suggesting that the functions of RAPl in telomeric and HML silencing, while overlapping, may have some distinct features. The rap1 allele, rap1-12, also does not decrease the efficiency of HML silencing (see Table 5), confirming previous studies indicating that the rap1 domain is not necessary for HML silencing (L. Susset and D. Shore, personal communication).

Mutations in the C-terminal tail domain disrupt telomere size control: We next tested the length and stability of telomeric tracts in the alleles generated in this study. The majority of telomeres in yeast can be visualized as a heterogeneous group of XhoI fragments capable of hybridization to a telomeric probe. This is the consequence of a conserved XhoI site in the Y subtelomeric element adjacent to the majority of telomeric poly(G13) tracts. In wild-type cells, these fragments cluster at sizes of ~1.2 kb, corresponding to a tract length of ~300 bp (Kirjon et al. 1992). Each of the silencing-defective alleles identified in this study display similar defects in telomere elongation and stability, resulting in ~250–350 bp increases in poly(G13) tract lengths (Figures 4 and 5; see Table 5). The degree of heterogeneity generated after 25 generations of growth was also increased both in Y class telomeres (large arrow, Figure 5), and at individual telomeres lacking the Y element (small arrows, Figure 5). With the exception of rap1-24, each allele is recessive for defects in telomere elongation and stability. In contrast, strains heterozygous for rap1-24 display a semi-dominant increase in telomere tract size, with telomeric tracts attaining sizes 100 bp greater than wild-type (data not shown). These data suggest that Rap1-24 is capable of more effectively interfering with the function of wild-type RAPl in telomere size control. Interestingly, with the exception of the 110-bp increase in telomere size conferred by the K808A K809A mutation, none of the remaining alleles generated in the C-terminal tail deviated from wild-type tract lengths. These data suggest a mechanistic link between the generation of defects in telomeric silencing and telomere size control.

The rap1 and C-terminal tail domains act independently in telomere size control: Susset and Shore (1991) have characterized a domain, defined by the rap1 alleles, that reduces or eliminates silencing conferred by an hmrA silencing element (Figure 1A). Each allele also results in a 200–300-bp increase in telomere tract size. Subsequent studies have demonstrated that the rap1 phenotypes are mediated through loss of interactions with the RAPl interacting protein RIF1 (Hardy et al. 1992b). Indeed, rifi null alleles confer phenotypes similar to those displayed by rap1 strains (Hardy et al. 1992b).

To explore the potential interactions between the rap1 and C-terminal tail domains in telomere size control, we compared the telomere tract lengths in isogenic strains carrying either the rap1 allele, rap1-12, which eliminates association with RIF1 in a two-hybrid system (Hardy et al. 1992b), or the rap1-22 allele, mapping within the C-terminal tail domain, with those of an allele (rap1-12,22) encoding a protein defective in both domains. Interestingly, the rap1-12,22 allele reproducibly confers tract length increases of 500–600 bp, close to the sum of the length increases found in the individual alleles (Figure 6). A qualitatively similar result was observed in rifi::URA3 rap1-22 and rifi::URA3 rap1-21 double mutants, although the increased heterogeneity displayed by rifi alleles precluded a quantitative analysis of these data (data not shown). These studies suggest that the C-terminal tail acts independently of the rap1 domain and RIF1 in telomere size control and indicates that the C-terminal tail is unlikely to play a role in stabilizing RAPl/RIF1 association. Furthermore, neither the rap1-12,22 allele nor double mutants between rifi and rap1-21 or rap1-22 recreate the rap1-17 phenotypes (Figure 6; data not shown), indicating that additional regions within the RAPl C terminus must be involved in regulating telomere size.
We have previously demonstrated that rif1 null alleles enhance the frequency of telomeric silencing (Kurion et al. 1995), raising the possibility that RIF1 may act as an antagonist of telomeric silencing. One explanation for the loss of telomeric silencing observed in the C-terminal tail alleles is the elimination of an association site for a factor that interferes with RIF1 antagonism. The prediction of this hypothesis is that rap1 alleles defective in both domains would confer a hyper-repressed state identical to the rif1 null phenotype. To test this possibility, we analyzed the telomeric silencing phenotypes conferred by rap1-12, rap1-22 and rap1-12,22 alleles (Table 2). As expected, the rap1-12 allele produces FOA\(^+\) frequencies higher than observed in wild-type cells. In contrast, the rap1-12,22 allele confers phenotypes comparable to the rap1-22 allele (Table 2). Similarly, rap1-22 strains have identical phenotypes regardless of the presence or absence of RIF1 (data not shown). These data suggest that the function of the C-terminal tail domain is not mediated solely through interference with RIF1 function.

**High copy suppression by SIR3 displays partial allele specificity:** Previous studies have indicated that SIR3 acts as a dose-dependent enhancer of telomeric silencing. Overproduction of SIR3 leads to increased frequencies of cells capable of repressing subtelomeric gene transcription (Renault et al. 1993). To test the RAP1 requirements for SIR3 enhancer function, we introduced high copy plasmids carrying the SIR3 gene into strains carrying either the wild-type RAP1 allele or the various missense and nonsense alleles defined in this study (Figure 7, Table 4). All missense alleles were suppressed by overproduction of SIR3 to varying extents.

The strongest suppression was observed in rap1-22 (H810Y) cells, which were suppressed \(\approx 150\)-fold, with \(\approx 3\%\) of the cells forming FOA\(^+\) colonies. Similarly, the F821G F822G allele was suppressed to levels 50-fold higher than observed in control cells, with \(\approx 14\%\) of the cells forming FOA\(^+\) colonies. The R818A allele was also partially suppressed by overproduction of SIR3 but attained FOA\(^+\) frequencies of only \(\approx 10^{-4}\), lower than that observed for the rap1-22 and F821G F822G alleles, while the A820-827 allele showed variable levels of suppression among different transformants.

In contrast, rap1-21, eliminating the terminal 28 amino acids, was not suppressed by SIR3 overproduction, indicating that the C-terminal tail is required for telomeric silencing even in the presence of a high copy of SIR3. Suppression does not extend to the telomere phenotypes observed in these alleles. The telomere tract lengths of neither the wild-type, rap1-21 nor rap1-22 alleles are affected by SIR3 overproduction (data not shown).

**DISCUSSION**

In this report, we demonstrate that the 28-amino acid C-terminal tail of RAP1 is essential for telomeric and HML silencing and additionally participates in telomere tract size control. Fine structure analysis of the C-terminal tail has further identified an 8-amino acid region (amino acids 818–825) within the C-terminal tail that is essential for telomeric silencing. A summary of the phenotypes of the alleles generated in this study is shown in Table 5.

Several lines of evidence argue that these phenotypes are the consequence of an alteration of RAP1 function.
of cells carrying these alleles are not substantially reduced, suggesting that the essential function of RAPl alleles, which result in a decrease, rather than increase, in telomere tract size. Second, the growth rate is not significantly impaired. Third, rupl-1 alleles (amino acids 727-747) that derepress silencing conferred by an hmrΔA silencing element and increase telomere tract lengths by 200-300 bp. However, these alleles do not influence either HML silencing, telomeric silencing or chromosome stability (this study; L. Sussel and D. Shore, E. Wiley and V. Zakian, personal communications; A. Lustig and C. Liu, unpublished studies). Interestingly, Rap1p proteins are defective in their association with RIF1 (Hardy et al. 1992b).

In contrast, the second C-terminal domain identified in this study is essential for telomeric and HML silencing and is additionally involved in telomere size control. Indeed, loss of the C terminal tail is sufficient to explain the severe telomeric and HML silencing defects observed in the rapl-1 alleles. The C-terminal tail domain is likely to provide a function beyond simple interference with RIF1 antagonism of telomeric silencing (Kirion et al. 1993), since C-terminal tail alleles still exhibit severe defects in telomeric silencing in the absence of RIF1. While the C-terminal tail is critical for telomeric silencing, it is not the only region of RAPl important for telomeric silencing, since at least one upstream mutation (rapl-24, L736M) also confers a partial loss of telomeric silencing.

We note that not all alleles that are defective in telomeric silencing (e.g., rapl-22, rapl-24) attenuate HML silencing. The separation of these two phenotypes could be an indication that some of the factors that interact with RAPl in HML and telomeric silencing may be distinct. More likely, however, is the possibility that HML silencing is inherently more resistant to defects in RAPl function as a consequence of the redundancy present in the HML silencer that is absent at the telomere (Mahoney and Broach 1989; Mahoney et al. 1991).

In contrast to the effects of the C-terminal tail mutations on telomeric and HML silencing, the telomere tract size phenotypes of these alleles are far less extensive than observed in rapl-1 cells. While the average increase in telomere tract size in rapl-1 cells is 1-2 kb, telomeric tracts increase on average only ~200-350 bp in C-terminal tail alleles (Table 5). Similarly, the C-terminal tail alleles display a less severe decrease in poly(G1-3T) tract stability than observed in rapl-1 cells, which can generate telomeres differing by 2 kb after only 25 generations of growth. This finding suggests that more than one C-terminal region may play a role in telomere size control. Consistent with this notion, a variety of alleles containing mutations within the
Additional regions of the C terminus of RAPl are also wild-type cells (C. Lru and A. Wild type A800-827 observed following overproduction of SIR3 is significantly higher than observed in control transformants (Figure 1A; parenthesis). Asterisks indicate that the range of FOA’ frequencies (rnpl-21) are 150-bp increase in telomere size when grown at permissive temperatures (KYRION et al. 1992), since defects in the C-terminal tail domain result in chromosome loss rates elevated only 3-4 fold relative to wild-type cells (C. Liu and A. Lustig, unpublished studies).

The median FOA’ frequency is given for each independent transformant together with the number of colonies analyzed in parentheses. Asterisks indicate that the range of FOA’ frequencies observed following overproduction of SIR3 is significantly higher than observed in control transformants (P<0.05). No., transformant number.

One interpretation of the telomere size regulation data is that the RIF1 interacting region and the C-terminal tail participate in redundant functions, sufficient for maintaining telomere size control. This model appears unlikely, however, since both the rapl-12,-22 allele and rlf1 rapl-22 double mutants produce telomere size increases that are approximately the sum of the increases observed in the single mutants. Neither the overall telomere length nor heterogeneity are comparable to the phenotypes of rapl-17 cells.

Given the apparent independent action of the rapl1 and C-terminal tail domains in telomere addition, what is responsible for the extreme telomere length alteration and instability observed in the rapl1 alleles? We propose that telomeric silencing and size control have distinct C-terminal requirements. Unlike the discrete C-terminal tail domain required for telomeric silencing, factors involved in telomere size control may functionally interact with a larger segment of the RAPl C terminus. Mutations within the rapl1 or C-terminal tail domains may result in a localized perturbation of RAPl structure having two distinct effects: (a) the elimination of localized association of factors important for HMR, HML or telomeric silencing (e.g., RIF1, SIR3) and (b) interference with the association (or function) of factors involved in telomere size control that interact with a multiplicity of C-terminal sites. This latter effect may be mediated through changes in the nature of RAPl association with telomeric chromatin. While small perturbations in RAPl C-terminal structure elicited by the rapl1 or C-terminal tail alleles may have only a minor influence on telomeric chromatin structure, more extensive changes in RAPl structure (e.g., in strains containing the rapl-12rapl-22 or rapl-17 alleles) may create correspondingly more severe effects on chromatin.

Suppression of C-terminal tail defects in telomeric silencing by SIR3 overproduction

<table>
<thead>
<tr>
<th>Allele</th>
<th>YEp351A frequencya</th>
<th>YEp351A/SIR3 frequencya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>$5.6 \times 10^{-1}$</td>
<td>$7.6 \times 10^{-1}$</td>
</tr>
<tr>
<td>Δ A800-827</td>
<td>$1.4 \times 10^{-7}$</td>
<td>$2.3 \times 10^{-7}$</td>
</tr>
<tr>
<td>(rapl-1-21)</td>
<td>$1.4 \times 10^{-7}$</td>
<td>$1.0 \times 10^{-7}$</td>
</tr>
<tr>
<td>Δ A820-827</td>
<td>$3.2 \times 10^{-6}$</td>
<td>$2.02 \times 10^{-6}$</td>
</tr>
<tr>
<td>H810Y</td>
<td>$3.2 \times 10^{-6}$</td>
<td>$2 \times 10^{-6}$</td>
</tr>
<tr>
<td>(rapl-22)</td>
<td>$3.2 \times 10^{-6}$</td>
<td>$2 \times 10^{-6}$</td>
</tr>
<tr>
<td>R818A</td>
<td>$0.5 \times 10^{-6}$</td>
<td>$296 \times 10^{-6}$</td>
</tr>
<tr>
<td>F821G F822G</td>
<td>$0.5 \times 10^{-6}$</td>
<td>$296 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
structure. Such alterations in structure could provide greater accessibility of the telomere to factors involved in both telomere elongation and shortening. This hypothesis would help to explain the strong correlation between silencing and telomere size defects within each domain, the lack of complete allele specificity of SIR3 suppression, and the multiplicity of C-terminal alleles that cause telomere elongation. Also consistent with this model is the finding that overproduction of SIR3 suppresses the rap1-22 defect in telomeric silencing without influencing telomere tract size.

**Interactions between SIR3 and the C-terminal tail domain of RAP1:** Overproduction of SIR3 has previously been demonstrated to increase the efficiency of telomeric silencing (RENAULD et al. 1993). In this study, we demonstrate that SIR3 enhancement of telomeric silencing requires the C-terminal tail domain of RAP1. The rap1-22 allele, truncating the terminal 28 amino acids, is not influenced by overproduction of SIR3. In contrast, all of the other missense and nonsense alleles are suppressed to varying extents. These data indicate that overproduction of SIR3 can provide a function that eases the requirement for a fully functional C-terminal tail domain. This is in contrast to the telomeric effects of SIR3 overproduction in cells containing mutations in other components of the silencing machinery, which are not influenced by SIR3 overproduction (RENAULD et al. 1993). Interestingly, SIR3 overproduction does not suppress the telomere size defects of the rap1-22 allele, suggesting a distinction between the factors involved in telomeric silencing and size control.

Two general mechanisms could account for these results. First, suppression could be mediated through a SIR3-dependent bypass pathway that lowers the C-terminal tail requirements for telomeric silencing, possibly by performing a partially redundant function. Nonetheless, the C-terminal tail of RAP1 must be required for this pathway, since the rap1-21 allele abrogates telomeric silencing even in the presence of SIR3 overproduction.

Alternatively, SIR3 function in telomeric silencing may be directly mediated through physical interactions with the C-terminal tail of RAP1. In this model, truncation of the tail would result in a complete loss of contact with SIR3 and, hence, an irreversible loss of telomeric silencing. In contrast, each of the RAP1 proteins encoded by the missense alleles may vary in their ability to associate with SIR3. High copy suppression may then be

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**TABLE 5**

Summary of phenotypes exhibited by rap1 alleles characterized in this study.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Growth rate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>FOA&lt;sup&gt;b&lt;/sup&gt; frequency&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Δ tract length&lt;sup&gt;c&lt;/sup&gt;</th>
<th>HML silencing&lt;sup&gt;d&lt;/sup&gt;</th>
<th>SIR3 suppression&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAP1</td>
<td>93</td>
<td>(1.0)</td>
<td>(0)</td>
<td>(100)</td>
<td>-</td>
</tr>
<tr>
<td>Δ868-827 (rap1-17)</td>
<td>160½</td>
<td>&lt;5.0 × 10⁻⁹</td>
<td>+1500/</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>Δ800-827 (rap1-20)</td>
<td>129</td>
<td>&lt;4.1 × 10⁻⁷</td>
<td>+275</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Δ800-827 (rap1-21)</td>
<td>111</td>
<td>&lt;5.9 × 10⁻⁷</td>
<td>+315</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>Δ820-827</td>
<td>113</td>
<td>2.7 × 10⁻⁶</td>
<td>+240</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td>H810Y (rap1-22)</td>
<td>104</td>
<td>6.2 × 10⁻⁴</td>
<td>+245</td>
<td>82.6</td>
<td>++</td>
</tr>
<tr>
<td>R838A</td>
<td>110</td>
<td>1.3 × 10⁻⁴</td>
<td>+329</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>F821G F822G</td>
<td>93</td>
<td>5.5 × 10⁻⁵</td>
<td>+195</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td>L736M (rap1-24)</td>
<td>93</td>
<td>1.5 × 10⁻¹</td>
<td>+300</td>
<td>88.6</td>
<td>NT</td>
</tr>
<tr>
<td>G726E D727N (rap1-12)</td>
<td>93</td>
<td>2.0</td>
<td>+280</td>
<td>100</td>
<td>NT</td>
</tr>
</tbody>
</table>

<sup>a</sup> Generation time (in minutes) during growth in liquid YPD media presented as the mean of three independent experiments.
<sup>b</sup> FOA<sup>a</sup> frequencies derived from data shown in Figure 4 and Table 2 and normalized to wild type values.
<sup>c</sup> Median telomere tract lengths determined from data shown in Figure 4 and Table 2 and normalized to wild type values.
<sup>d</sup> HML silencing data generated in our studies normalized to wild type.
<sup>e</sup> Degree of suppression by SIR3 overproduction: -, no suppression; +, weak, but significant, suppression; ++, suppression resulting in FOA<sup>a</sup> values close to wild type.
<sup>f</sup> KIRKOR et al. (1992).
<sup>g</sup> KIRKOR et al. (1993). NT, not tested.

**FIGURE 8.—Proposed domain structure of RAP1.** The domain structure of the C-terminal 227 amino acids of RAP1 is shown together with the inferred functions of the rap1<sup>F</sup> and C-terminal tail domains. The proposed primary functions of each domain are indicated in bold, while secondary functions are indicated in plain text. The RIF1 interaction site and the proposed interaction of SIR3 with the C-terminal tail are also shown.
mediated through restoration of weak contacts between SIR3 and the C-terminal tail of RAP1. This possibility is consistent with recent molecular and biochemical evidence for an interaction between the C terminus of RAP1 and SIR3 (P. Moretti, D. Freeman and D. Shore, personal communication) and immunochemical data indicating a loss of SIR3 peripheral localization in rap1-21 cells (M. Cockell, F. Palladino, T. Laroche, G. Kirion, C. Liu, A. Lustig and S. Gasser, manuscript in preparation). Such interactions between the C-terminal tail domain and SIR3 could be directly involved in the formation of subtelomeric heterochromatin or may target SIR3 to a specific nuclear substructure necessary for the establishment or maintenance of the repressed state. Further genetic and physical studies will be necessary to distinguish between these possibilities.

Recently, the sequence of a RAP1 homolog has been determined in the related yeast Kluyveromyces lactis (Larson et al. 1994). Interestingly, each residue critical for telomeric silencing and size control identified in this study is conserved in the Kluyveromyces homolog (Figure 4). In particular, H810, R818, F821 and E823 are conserved, while E825 is replaced with an asparagine, a conservative substitution. Both K808 and K809, mutations which cause small increases in telomere tract size, are also conserved in the Kluyveromyces homolog. In contrast, other residues are replaced by non-conservative amino acids. These data reinforce the functional significance of these residues and suggest that the function of the C-terminal tail domain, and the factors interacting with this domain, may be conserved in Kluyveromyces. Hence, the missense modifications defined in this study are likely to provide valuable tools for the characterization of the factors that mediate RAP1 action at the telomere.

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