Dependence of the Regulation of Telomere Length on the Type of Subtelomeric Repeat in the Yeast *Saccharomyces cerevisiae*

Rolf J. Craven and Thomas D. Petes

Department of Biology, Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, North Carolina 27599-3280

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, chromosomes terminate with ~400 bp of a simple repeat poly(TG_{1-3}). Based on the arrangement of subtelomeric X and Y′ repeats, two types of yeast telomeres exist, those with both X and Y′ (Y′ telomeres) and those with only X (X telomeres). Mutations that result in abnormally short or abnormally long poly(TG_{1-3}) tracts have been previously identified. In this study, we investigated telomere length in strains with two classes of mutations, one that resulted in short poly(TG_{1-3}) tracts (*tel1*) and one that resulted in elongated tracts (*pif1*, *rap1-17*, *rif1*, or *rif2*). In the *tel1 pif1* strain, Y′ telomeres had about the same length as those in *tel1* strains and X telomeres had lengths intermediate between those in *tel1* and *pif1* strains. Strains with either the *tel1 rap1-17* or *tel1 rif2* genotypes had short tracts for all chromosome ends examined, demonstrating that the telomere elongation characteristic of *rap1-17* and *rif2* strains is Tel1p-dependent. In strains of the *tel1 rif1* or *tel1 rif1 rif2* genotypes, telomeres with Y′ repeats had short terminal tracts, whereas most of the X telomeres had long terminal tracts. These results demonstrate that the regulation of telomere length is different for X and Y′ telomeres.

MOST eukaryotic chromosomes terminate with simple repeats in which the GT-rich strand extends 3′ to the chromosome end (Greider 1996). In the yeast *Saccharomyces cerevisiae*, telomeres contain ~400 bp of poly(TG_{1-3}) (Shampay *et al.* 1984; Walmsley and Petes 1985). In addition to the TG_{1-3} tracts, yeast chromosomes have telomere-associated repeats called X and Y′ (Chan and Tye 1983; Walmsley *et al.* 1984). Both the "core" X (~500 bp in length) and Y′ elements (two types, 5.2 and 6.7 kb) have ARS sequences (Louis *et al.* 1994). Although all telomeres have a single X element, telomeres can lack Y′ elements or have one or more copies (Louis 1995). The arrangement of sequences (telomere to centromere) is TG_{1-3} tract: $(Y')_{0->2}$: X.

In a population of yeast cells, the telomere lengths, even for a single chromosomal telomere, are heterogeneous (Walmsley and Petes 1985). This length heterogeneity is consistent with models of telomere replication in which telomere length is in a dynamic equilibrium between forces that reduce and extend the lengths of poly(TG_{1-3}) tracts (Lustig and Petes 1986). The primary mechanism to extend telomeric tracts is telomerase, an RNA-protein complex that adds telomeric repeats to the 3′ end of the GT-rich strand (Greider and Blackburn 1985). Synthesis of the CA-rich strand presumably requires the normal DNA synthetic apparatus using the GT-rich strand as a template. Loss of telomeric repeats may occur as a consequence of loss of the RNA primer on the CA-rich strand or as a consequence of exonucleases. Single-stranded poly(TG_{1-3}) "tails," longer than those expected from removal of an RNA primer, are observed late in S phase (Wellinger *et al.* 1993), suggesting an active role of exonucleases in telomere metabolism.

Mutations that result in either elongated or shortened poly(TG_{1-3}) tracts have been identified. Mutations in the genes *EST2* (encoding one of the protein subunits of telomerase; Lingner *et al.* 1997), *TLC1* (encoding the RNA subunit of telomerase; Singer and Gottschling 1994) *EST1*, *EST3*, and *EST4*/*CDC13* lead to telomeres that continually shorten during subculturing until most of the mutant cells die (Lundblad and Szostak 1990; Lendvay *et al.* 1996). A second class of mutation results in chromosomes with short, but stable, poly(TG_{1-3}) tracts. Several of these mutations (*hdf1*/*yku70*, *yku80*, *rad50*, *mre11*, and *xrs2*) are required for nonhomologous ("end-joining") recombination in yeast (Boulton and Jackson 1996, 1998; Nugent *et al.* 1998; Porter *et al.* 1996; reviewed by Kanaar and Hoeijmakers 1998; Polotnianka *et al.* 1998). Mutations in the *TEL1* and *TEL2* genes also result in shortened telomeres (Lustig and Petes 1986). The *TEL1* gene encodes a very large (322 kD) protein that shares homology with a family of lipid/protein kinases involved in DNA repair and/or telomere replication including the human ATM gene and the yeast *MEC1* gene (Greenwell *et al.* 1995; Morrow *et al.* 1995; reviewed by Zakian 1996).

Several types of mutations leading to elongated telomeres have also been observed (reviewed by Greider

Corresponding author: Thomas D. Petes, Department of Biology, Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC 27599-3280. E-mail: tompetes@email.unc.edu

1996). Mutant alleles of *RAP1* that result in C-terminal truncation of the protein (*rap1-17*, *rap1-12*) have elongated terminal tracts (Sussel and Shore 1991; Kyrion *et al.* 1992). In addition, mutations that eliminate Rif1p or Rif2p, proteins that interact with the C-terminal portion of Rap1p, result in elongated telomeres (Hardy *et al.* 1992; Wotton and Shore 1997). To explain these and other observations, Marcand *et al.* (1997) suggested that telomere length is negatively regulated by Rap1p bound to the telomeric poly($TG_{1\text{-}3}$) tract; this negative regulation requires interaction between the C terminus of Rap1p and Rif1p and Rif2p. Mutations in the *PIF1* gene also result in elongated telomeres (Schulz and Zakian 1994).

One method of classifying mutations into groups that are likely to reflect functional pathways is epistasis analysis, the comparison of the phenotypes of two strains bearing single mutations to the phenotype of a single strain with both mutations. If the double-mutant strain has a phenotype that is different from either single mutant, it is likely that the two mutations involve genes in different pathways. If the phenotype of the double mutant is identical to that of one of the single mutants, it is likely that the two mutations affect the same pathway. To extend our understanding of the genetic regulation of telomere maintenance, we performed epistasis analysis with *tel1* mutants (short telomeres) and four mutants with elongated telomeres (*rap1-17*, *rif1*, *rif2*, and *pif1*). We analyzed the effects of single- and double-mutant combinations on both X and Y′ telomeres. We found that *tel1* is epistatic to *rap1-17* and *rif2* for both X and Y′ telomeres. The *tel1* mutation is also epistatic to *rif1* for Y′ telomeres, but not for most X telomeres. These results demonstrate that the regulation of telomeric length is affected by the type of subtelomeric repeat.

MATERIALS AND METHODS

Strain constructions and plasmids: All strains were isogenic with W303a (***a*** *leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100*) except for changes introduced by transformation. The derivation and relevant genotypes of the strains are described in Table 1. Deletions of the *TEL1* gene were made using pPG47, as described previously (Greenwell *et al.* 1995). Deletions removing all of the open reading frames of *PIF1*, *RIF1*, and *RIF2* were made using the PCR-disruption method described by Wach *et al.* (1994). For the *pif1* and *rif1* deletions, oligonucleotides were synthesized with the 5′ ends homologous to the gene to be disrupted and the 3′ ends homologous to the *kanMX* gene. These oligonucleotides were used to amplify the selectable *kanMX* gene of the transposon Tn903; yeast transformants were selected on solid medium containing 150 μg/ml Geneticin (Wach *et al.* 1994). To construct the *rif2* deletions, we used the same procedure but with oligonucleotides allowing amplification of the *HIS3* gene of pRS303 (Sikorski and Hieter 1989); transformed strains with the *rif2::HIS3* disruption were selected on medium lacking histidine. The sequences of oligonucleotides used for the disruptions are shown in Table 2. Gene disruptions were confirmed by PCR and Southern analysis of each transformant.

Using methods described by Gottschling *et al.* (1990), we also constructed strains in which the "normal" chromosomal telomere was replaced by the *URA3* gene and poly($TG_{1\text{-}3}$) sequences. To replace the chromosomal telomere V_R (Y′ telomere in Stanford Genome Database) with *URA3* and poly($TG_{1\text{-}3}$), the progenitor strains were transformed with *Eco*RI-treated pV-UCA DNA, a plasmid identical to pV-R URA3-TEL (Gottschling *et al.* 1990).

Southern analysis of telomere length: DNA was prepared according to standard protocols (Guthrie and Fink 1991) and digested with an appropriate enzyme (usually *Xho*I or *Sal*I). The resulting DNA fragments were separated by electrophoresis in 1% agarose and transferred to HyBond N+ membranes (Amersham, Arlington Heights, IL). Standard conditions of hybridization were used. The hybridization probe for Y′ telomeres contained sequences centromere-distal to the *Xho*I site in Y′, but did not contain poly($TG_{1\text{-}3}$) sequences. This probe was prepared by PCR amplification of pYT14 (Shampay *et al.* 1984) using the primers Y′F and Y′R (Table 2). Because the terminal DNA fragments representing the Y′ telomeres form a broad band of hybridization, we measured (relative to a 1-kb ladder) the size of telomeres in the middle of the band. The probe for telomeres in which the normal sequences were replaced by *URA3* (*TELVRΔ::URA3*) was the *URA3*-containing vector YIp5.

Hybridization probes for the X telomeres I_L, XI_R, and XV_L were generated by PCR amplification using the oligonucleotides shown in Table 2. These oligonucleotides were designed using information derived from the Stanford Genome Database. Although the probe for the chromosome I_L telomere hybridizes only to I_L, the probe for XI_R shares homology to III_L, and the probe to XV_L shares homology to III_R. The identity of telomeres defined by these two probes was established by reprobing the blots with hybridization probes specific to III_L (the plasmid pKB1 described by Kyrion *et al.* 1992 or a PCR fragment derived from this plasmid) and a PCR fragment derived from single-copy III_R sequences (coordinates 311,940 and 312,590 on chromosome III). The size of telomeric fragments identified for all probes was that predicted by the Stanford Genome Database, and the telomeric nature of the hybridizing DNA fragment for the XI_R and XV_L was demonstrated by Bal-31 analysis (as described below).

Bal-31 analysis of telomeric repeats: Yeast genomic DNA was purified using the Qiagen (Valencia, CA) Genomic Tip 100/g kit according to the manufacturer's instructions. A total of 10 μg of purified DNA was digested with Bal-31 (Promega, Madison, WI) at 30°. Bal-31 was then inactivated by addition of EGTA to a final concentration of 20 mm, followed by incubation at 65°. The DNA was precipitated with ethanol and digested with *Xho*I. The resulting DNA samples were examined by Southern analysis, using poly(TG/CA) (Boehringer Mannheim, Indianapolis) as a hybridization probe (Walmsley *et al.* 1984).

Statistical analysis: Telomere lengths for different chromosome ends and different strains were measured multiple times. The mean lengths and the 95% confidence limits on the means were calculated using the InStat 1.12 program. We also used this program to compare some samples by the Mann-Whitney nonparametric test.

RESULTS

Rationale: Epistasis analysis is often useful in grouping genes, defined by mutations, into functional pathways. We examined telomere lengths in strains with two

TABLE 1

Strain names, strain constructions, and relevant genotypes

Strain name	Strain construction or reference	Relevant genotype[a]
W303a	Thomas and Rothstein (1989)	Wild type
SPY40	Porter *et al.* (1996)	*tel1::URA3*
SPY40FR	Isolation of 5-fluoroorotate-resistant SPY40 derivative	*tel1::ura3*
AJL278-4d	Kyrion *et al.* (1992)	*rap1-17*
RCY3	One-step transplacement of W303a with PCR fragment containing *kanMX* cassette generated by primers (*PIF1*-F and *PIF1*-R)[b]	*pif1*Δ*::kanMX*
RCY7	One-step transplacement of RCY3 with *Eco*RI fragment of pPG47[c]	*tel1::URA3 pif1*Δ*::kanMX*
RCY11	One-step transplacement of AJL278-4d with *Eco*RI fragment of pPG47[c]	*tel1::URA3 rap1-17*
RCY12	One-step transplacement of RCY28 with *Eco*RI fragment of pPG47[c]	*rif1*Δ*::kanMX tel1::URA3*
RCY22	One-step transplacement of W303a with *Eco*RI fragment of plasmid pV-UCA[d]	*TELVR*Δ*::URA3*
RCY23	One-step transplacement of SPY40FR with *Eco*RI fragment of plasmid pV-UCA[d]	*tel1::ura3 TELVR*Δ*::URA3*
RCY24	One-step transplacement of SPY40FR with PCR fragment containing *kanMX* cassette generated by primers (*RIF1*-F and *RIF1*-R)[b]	*tel1::ura3 rif1*Δ*::kanMX*
RCY25	One-step transplacement of RCY23 with PCR fragment containing *kanMX* cassette generated by primers (*RIF1*-F and *RIF1*-R)[b]	*tel1::ura3 rif1*Δ*::kanMX TELVR*Δ*::URA3*
RCY26	One-step transplacement of RCY22 with PCR fragment containing *kanMX* cassette generated by primers (*RIF1*-F and *RIF1*-R)[b]	*rif1*Δ*::kanMX TELVR*Δ*:: URA3*
RCY28	One-step transplacement of W303a with PCR fragment containing *kanMX* cassette generated by primers (*RIF1*-F and *RIF1*-R)[b]	*rif1*Δ*::kanMX*
RCY55	One-step transplacement of RCY22 with PCR fragment containing *HIS3* generated by primers *RIF2*-F and *RIF2*-R[b]	*rif2*Δ*::HIS3 TELVR*Δ*::URA3*
RCY56	One-step transplacement of RCY23 with PCR fragment containing *HIS3* generated by primers *RIF2*-F and *RIF2*-R[b]	*tel1::ura3 rif2*Δ*::HIS3 TELVR*Δ*::URA3*
RCY57	One-step transplacement of RCY25 with PCR fragment containing *HIS3* generated by primers *RIF2*-F and *RIF2*-R[b]	*tel1::ura3 rif1*Δ*::kanMX rif2*Δ*::HIS3 TELVR*Δ*::URA3*
RCY58	One-step transplacement of RCY26 with PCR fragment containing *HIS3* generated by primers *RIF2*-F and *RIF2*-R[b]	*rif1*Δ*::kanMX rif2*Δ*::HIS3 TELVR*Δ*::URA3*
RCY59	One-step transplacement of RCY11 with PCR fragment containing *kanMX* cassette generated by primers (*RIF1*-F and *RIF1*-R)[b]	*tel1::URA3 rap1-17 rif1*Δ*::kanMX*

[a] All strains are derived from W303a (***a*** *leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100*) by transformation.
[b] Disruptions were constructed using the PCR-based method described by Wach *et al.* (1994) to replace yeast genes with the *kanMX* or *HIS3* genes. The oligonucleotide sequences are shown in Table 2.
[c] The plasmid pPG47 is described in Greenwell *et al.* (1995).
[d] The plasmid pV-UCA is identical to pV-R URA3-TEL (Gottschling *et al.* 1990).

mutations, *tel1* (which results in short telomeres in single-mutant strains) and *rap1-17*, *rif1*, *rif2*, or *pif1* (each of which results in long telomeres in single-mutant strains). In these strains, we examined the lengths of Y′ telomeres and four individual X telomeres (Figure 1). For most of the strains, we also examined telomere length of a chromosomal end constructed to lack both X and Y′ sequences.

Epistasis interactions of *tel1* and *rap1-17*: We first examined telomere length in the single mutant *tel1* and *rap1-17* strains. When DNA was treated with *Xho*I and hybridized to a Y′-specific probe, the Y′ telomeres were detected as a diffuse band ~1.2 kb in size (Figure 2a). As expected from previous studies (Lustig and Petes 1986; Kyrion *et al.* 1992), the lengths of the Y′ telomeric restriction fragments were reduced ~300 bp by the *tel1* mutation and elevated by ~1 kb by the *rap1-17* mutation. The 5.2- and 6.7-kb bands in Figure 2a reflect tandemly arranged Y′ elements of two different sizes (Louis *et al.* 1994). The X telomeres, like the Y′ telomeres, were extended ~1 kb in the *rap1-17* strain AJL278-4d (Figure 2b).

Because telomere length for specific chromosome ends can show clonal variation, multiple transformants and multiple subclones were examined for all strains. Because mutations affecting telomere length often manifest a phenotypic lag, unless specified otherwise, strains with mutations introduced by transformation were grown for at least 200 generations before assaying telomere length. In Figure 3, a and b, we summarize the data from *tel1* and *rap1-17* strains, with each point representing an independent determination of telomere length. For all telomere classes within each strain, we conclude that X and Y′ telomeres are affected to similar extents by the *tel1* and *rap1-17* mutations.

When *TEL1* was deleted from a strain with the *rap1-17* mutation, the Y′ telomeres shortened to sizes that

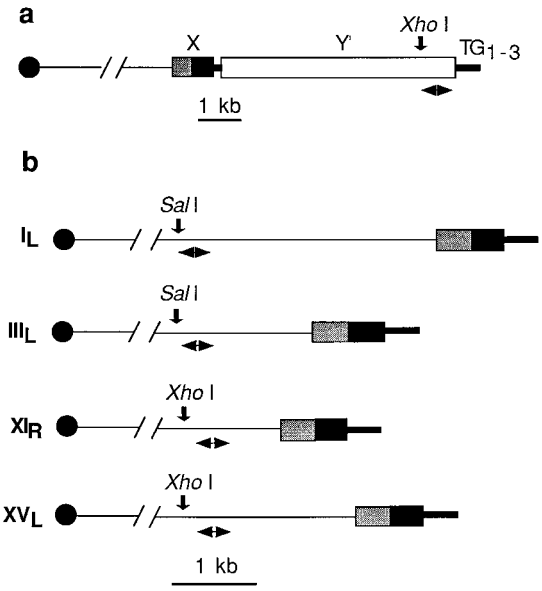

Figure 1.—Structure of (a) Y′ and (b) X telomeres. The telomere lengths were measured by treating DNA with *Xho*I or *Sal*I (as indicated) and examining the sizes of the resulting DNA fragments by Southern analysis. The positions of the probes for each telomere are shown by the double-headed arrows. Thick lines, poly($TG_{1\text{-}3}$) sequences; white rectangle, Y′ element; gray rectangle, core X; black rectangle, subtelomeric repeats; thin line, nontelomeric DNA sequences; black circle, centromere.

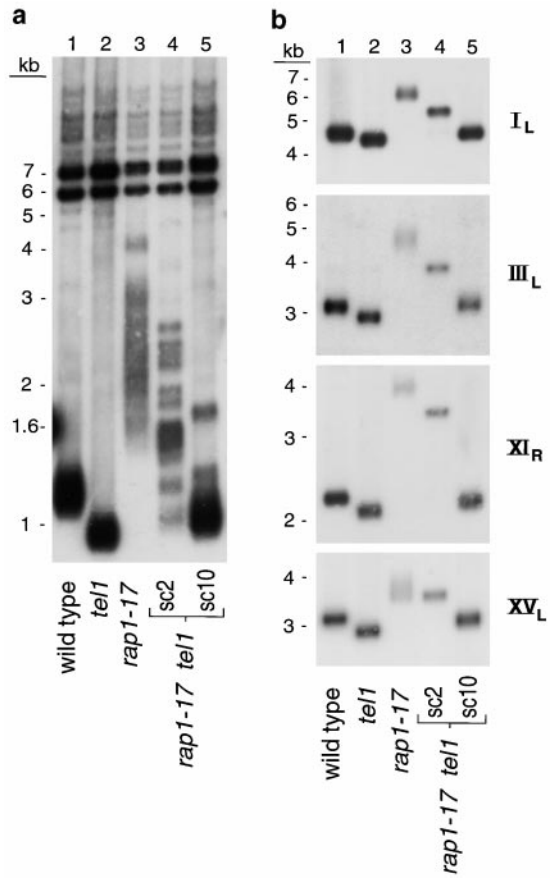

Figure 2.—X and Y′ telomere lengths in wild-type (W303a), *tel1* (SPY40), *rap1-17* (AJL278-4d), and *rap1-17 tel1* (RCY11) strains. Telomere lengths were measured by Southern analysis using restriction enzymes and hybridization probes described in Figure 1 and materials and methods. The sizes of molecular weight markers are indicated on the left of each pattern. (a) The broad bands of hybridization near the bottom of the gels represent all Y′ telomeres; the bands of ~5 and 6 kb reflect tandem Y′ elements of two different sizes. (b) A composite of four autoradiograms representing four different X telomeres. The "sc2" and "sc10" designations indicate 2 and 10 subclonings of RCY11, respectively, representing ~40 and 200 cell divisions.

were similar (although often slightly longer) than those observed in the *tel1* strain (Figures 2 and 3c). A phenotypic lag was observed for this shortening process. After 2 subclonings (~40 divisions), telomere length in the double-mutant strain was intermediate between the telomere lengths of the *rap1-17* and *tel1* strains. After 10 subclonings, the telomere lengths had reached equilibrium; an additional 12 subclonings had no further effects on telomere length (data not shown). The X telomeres also shortened in response to the *tel1* mutation in the double-mutant strain. Although both X and Y′ telomeres were slightly longer in the *rap1-17 tel1* strain than in the *tel1* strain, it is clear that the *tel1* mutation is largely epistatic to *rap1-17*.

X and Y′ telomere lengths in *rif1*, *rif2*, and *rif1 rif2* strains: The Rif1 and Rif2 proteins interact with the C terminus of Rap1p, and *rif1* and *rif2* mutant strains have elongated telomeres (Hardy *et al.* 1992; Wotton and Shore 1997). Before examining interactions of the *rif* mutations with *tel1*, we first analyzed telomere lengths in strains with the *rif1*, *rif2*, and *rif1 rif2* genotypes (Figure 4). In all of these strains (as well as strains containing *tel1* in addition to these mutations), we used derivatives of W303a in which the right end of chromosome V was replaced with *URA3* and poly($TG_{1\text{-}3}$) sequences (Gottschling *et al.* 1990).

The summary of telomere lengths for *rif1*, *rif2*, and *rif1 rif2* strains is given in Figure 3, d–f. The Y′, X, and *TELVR::URA3* telomeres were elongated in the *rif1* and *rif2* single-mutant strains by ~1 kb and 500 bp, respectively. The telomeres in the *rif1 rif2* double-mutant strain were considerably longer than those in either single-mutant strain (Figures 3f and 4). Thus, as previously demonstrated by Wotton and Shore (1997), the wild-type Rif1p and Rif2p exert inhibitory effects on telomere elongation independently.

Telomere length in *tel1 rif1* strains is regulated differently for X and Y′ telomeres: The region of Rap1p required for interacting with Rif1p and Rif2p is deleted in *rap1-17* (Wotton and Shore 1997). Consequently, because *tel1* is epistatic to *rap1-17*, we expected *tel1* to be epistatic to *rif1* and *rif2*. Data from strains with the *rif1 tel1* genotype are shown in Figure 3g. The lengths of the Y′ telomeres in three independent transformants

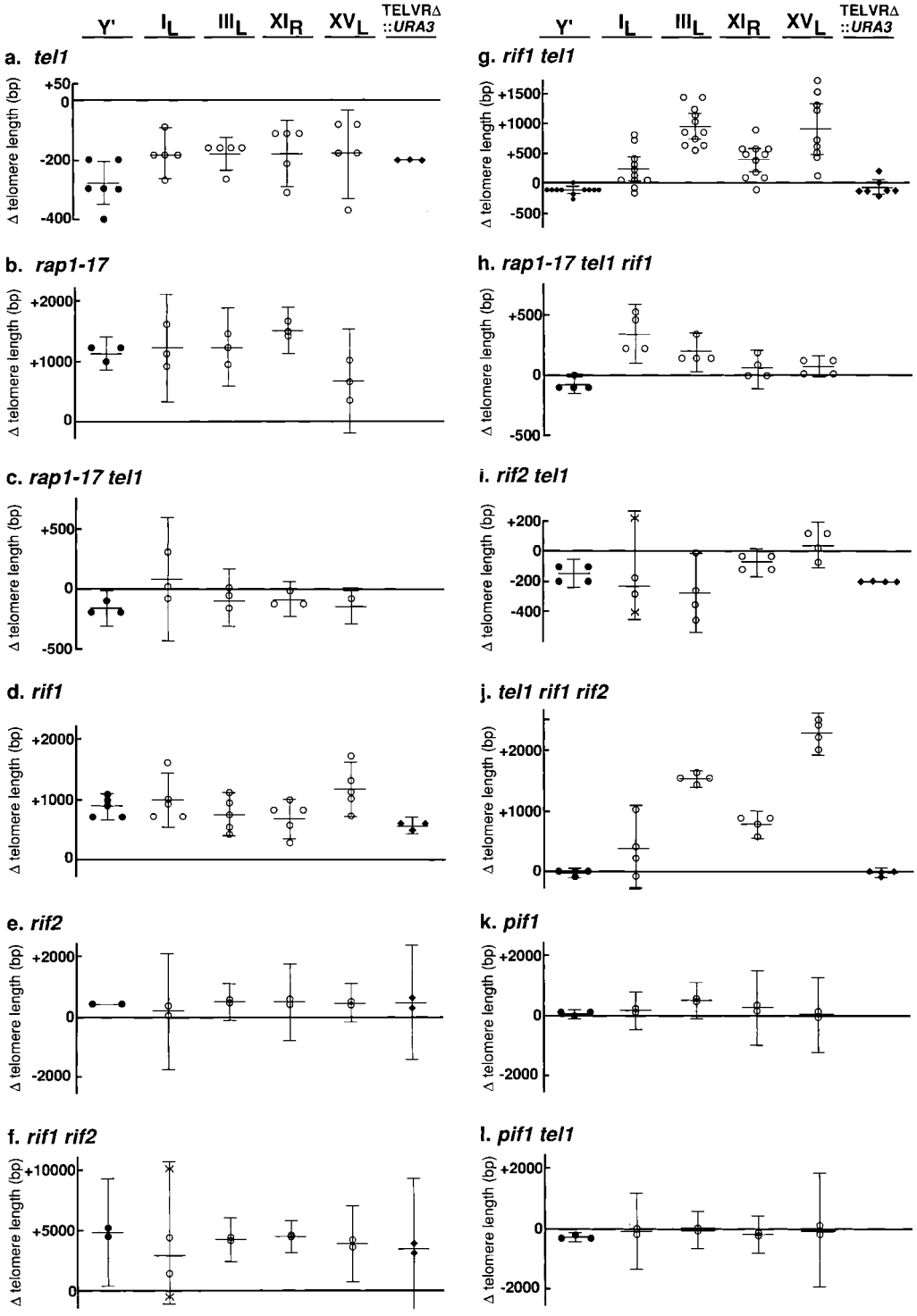

Figure 3.—Alterations in telomere length in mutant yeast strains. Telomere lengths were measured by Southern analysis in wild-type and mutant strains. All strains are isogenic with W303a or RCY22 (W303a with *TELVRΔ::URA3*) except for mutations introduced by transformation. The average lengths (in kilobases) of the terminal restriction fragments in wild-type strains (three measurements from W303a and three from RCY22) were 1.20 (Y′), 4.58 (I_L), 3.26 (III_L), 2.42 (XI_R), and 3.38 (XV_L); the average length of the terminal restriction fragment of *TELVRΔ::URA3* was 1.80. We also measured telomere lengths in various mutant strains. We subtracted the average wild-type telomere length from each individual measurement derived from the mutant strains; these values are represented as circles (●, Y′ telomeres; ○, X telomeres) or diamonds (*TELVRΔ::URA3*). Thus, the positions of the circles or diamonds on the *y*-axis represent the differences between wild-type and mutant telomere lengths for each measurement. The positions on the *x*-axis have no significance except to group telomeres of the same class; the scales on the *y*-axes are not the same for all strains. Confidence limits of 95% are indicated by short horizontal lines and the average telomere length is shown by the long horizontal line. The two examples of telomeres that altered by acquisition of a Y′ element were not included. For each genotype, we list below the strains analyzed; the designations T1, T2, etc., signify different transformants. The numbers in parentheses following the strain names show the number of individual subclones examined. (a) *tel1*: SPY40 (2); RCY23 (3). (b) *rap1-17*: AJL278-4d (3). (c) *rap1-17 tel1*: RCY11 (3). (d) *rif1*: RCY26 (3); RCY28 (2). (e) *rif2*: RCY55 (2). (f) *rif1 rif2*: RCY58 (2). (g) *rif1 tel1*: RCY12 (2); RCY24 (2); RCY25-T1 (3); RCY25-T2 (2); RCY25-T3 (2). (h) *rap1-17 tel1 rif1*: RCY59-T1 (2); RCY59-T2 (2). (i) *rif2 tel1*: RCY56-T1 (2); RCY56-T2 (2). (j) *tel1 rif1 rif2*: RCY57-T1 (2); RCY57-T2 (2). (k) *pif1*: RCY3 (2). (l) *pif1 tel1*: RCY7 (2).

of the *rif1 tel1* genotype were short, only slightly longer than observed for Y′ telomeres in *tel1* strains. In contrast, most of the X telomeres were longer than observed in wild-type strains. Telomeric length showed considerable variation between different chromosomal ends, between different transformants at the same chromosomal ends, and between different subclones of the same transformant (Figure 3g). The III_L and XV_L telomeres in the

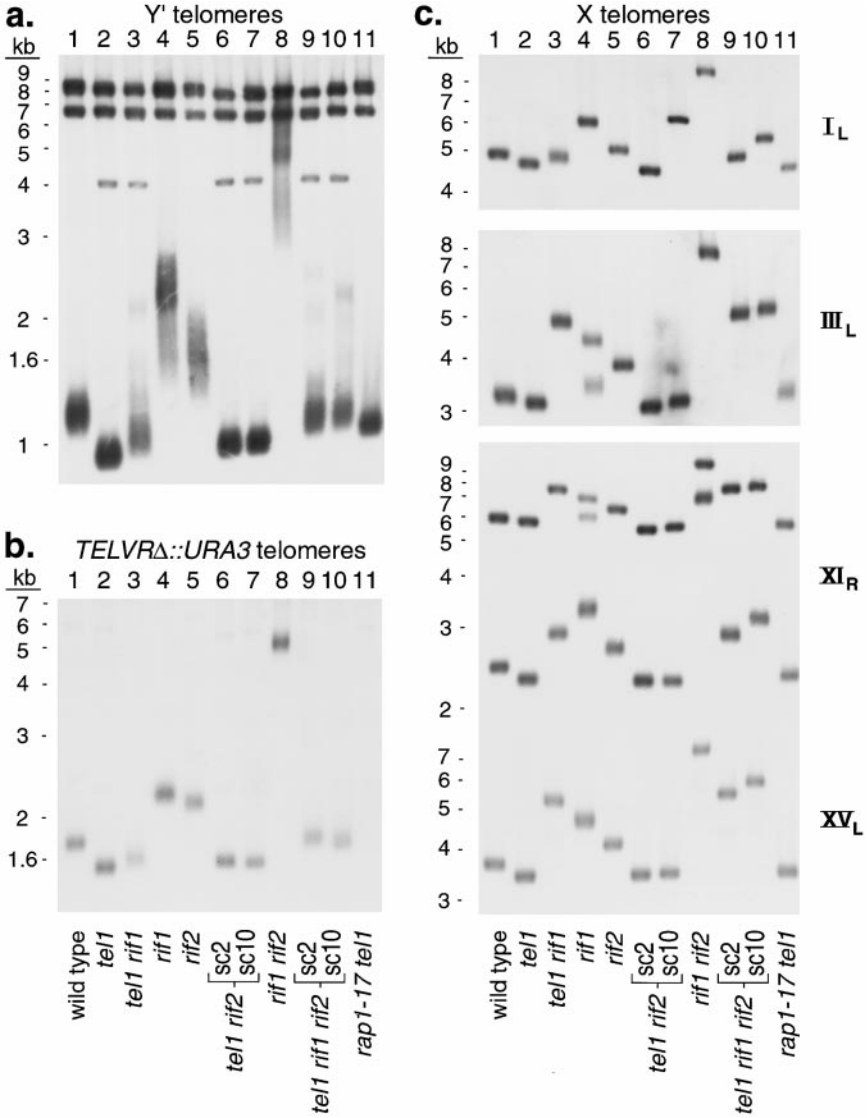

Figure 4.—(a–c) Telomere length regulation in wild type (RCY22), *tel1* (RCY23), *rif1* (RCY26), *rif2* (RCY55), *tel1 rif1* (RCY25), *tel1 rif2* (RCY56), *rap1-17 tel1* (RCY11), and *tel1 rif1 rif2* (RCY57) strains. In all strains, except RCY11, the "normal" telomeric repeats of V_R were replaced by *URA3* and poly($TG_{1\text{-}3}$) sequences (as described in materials and methods); telomere lengths for this construction (*TELVRΔ::URA3*) were analyzed using a *URA3*-specific hybridization probe. The hybridization probe for XI_R cross-hybridizes with III_L, resulting in two bands of hybridization in the XI_R blot in c (third blot from top).

rif1 tel1 strains, after 10 subclonings, were about the same length as those observed in the isogenic *rif1* strains, whereas the I_L and XI_R telomeres were intermediate in size between those found in wild-type and *rif1* strains.

By the criterion of nonoverlapping confidence limits, all X-telomere lengths were significantly different from those of Y′ telomeres, and all X telomeres (except I_L) were significantly different from *TELVR::URA3*. We also compared X telomeres to Y′ and to *TELVR::URA3* telomeres by a nonparametric test (Mann-Whitney). By this test, all X telomeres were significantly longer than the Y′ and *TELVR::URA3* telomeres ($P < 0.02$).

The XV_L telomere in the *rif1 tel1* strain RCY25-T2 was much longer than that found for the other telomeres or for the same telomere in other transformants (data not shown). This increase in length is likely to reflect an insertion of a Y′ element centromere-distal to the X repeat as has been observed previously (Louis and Haber 1990). Using one oligonucleotide specific for Y′ sequences (YPR-90R, Table 2) and a second specific for single-copy sequences located near the XV_L telomere (C15L-1300R, Table 2) in a PCR reaction with RCY25-T2 DNA, we were able to obtain an amplified fragment of a size consistent with a Y′ insertion (1.2 kb).

The analysis described above indicates that the lengths of the terminal restriction fragments in *tel1 rif1* strains are regulated differently for X and Y′ telomeres. Although these alterations presumably reflect differences in the lengths of the terminal poly($TG_{1\text{-}3}$) tracts, both X and Y′ repeats contain other simple repetitive DNA sequences (Louis 1995) that could, in principle, undergo expansions or contractions in length. To confirm that the lengths of poly($TG_{1\text{-}3}$) tracts were different for X and Y′ telomeres in *tel1 rif1* strains, we measured the lengths of these tracts by Southern analysis. As in previous studies of tract length, large (>50 kb) DNA fragments were treated for various times with Bal-31, followed by *Xho*I digestion (Walmsley and Petes 1985). The resulting fragments were examined by Southern analysis using poly(GT/CA) as a hybridization probe.

TABLE 2

Name, sequence, and purpose of oligonucleotides used in strain constructions or hybridization probes

Oligonucleotide name	DNA sequence (5′ to 3′)	Purpose[a]
PIF1-F	ATTTGTAATATTATCCATTGAGCGATTAGCTTACTT GTATCAATCCGTACGCTGCAGGTCGAC	Disruption of *PIF1* with *kanMX*
PIF1-R	CTATGTGTATTAATATGTACTTATTCTAAGATGTG GTCTTCGGTATCCATCGATGAATTCGAGCTCG	Disruption of *PIF1* with *kanMX*
RIF1-F	ATGTCGAAAGATTTTTCAGATAAAAAGAAACATAC GATAGATCGACGTACGCTGCAGGTCGAC	Disruption of *RIF1* with *kanMX*
RIF1-R	CAATTCATATCATTATCCCTGTTTGAGTAATATTC GAGCCTCATCATCGATGAATTCGAGCTCG	Disruption of *RIF1* with *kanMX*
RIF2-F	TACGAATATAGATATAAATACGAACGTGGTTAGTA TATAGAGACGATTGTACTGAGAGTGCACC	Disruption of *RIF2* with *HIS3*
RIF2-R	TCTCTTTGTATTGTTCGAACTCTTTCAAAAGACCT TGGTAATTTATCCTGTGCGGTATTTCACACCG	Disruption of *RIF2* with *HIS3*
YPR-90R	CACAATCGCCTAAACAGG	Test for Y′ insertion at I_L and XV_L
C1L-1200R	CGCTGATAAGTTATATTA	Test for Y′ insertion at I_L
C15L-1300R	ACATCCGCGCATATTCTTCC	Test for Y′ insertion at XV_L
Y′-F	ACACACTCTCTCACATCTACC	Y′-specific hybridization probe
Y′-R	TTGCGTTCCATGACGAGCGC	Y′-specific hybridization probe
I_L-F	GAAGCGGATGGTAATGAGAC	Telomere I_L hybridization probe
I_L-R	AGATGTATGATGCTGGGGAG	Telomere I_L hybridization probe
III_L-F	GTCCTGGCTCTGTATGTTATC	Telomere III_L hybridization probe
III_L-R	CCAATCTTTTTAAGAGTATTG	Telomere III_L hybridization probe
XI_R-F	TCATAAGCAAAGAATCTTCG	Telomere XI_R (III_L) hybridization probe
XI_R-R	GTAACCCTACGTGTCTTATAC	Telomere XI_R (III_L) hybridization probe
XV_L-F	TCTTGATGTGTCTTCACAAG	Telomere XV_L (III_R) hybridization probe
XV_L-R	ATTCTCACCATCAAAGAAG	Telomere XV_L (III_R) hybridization probe

[a] These oligonucleotides were used in pairs (F indicating forward and R indicating reverse) in PCR amplification reactions to prepare DNA fragments for plasmid/yeast strain constructions or as hybridization probes.

This probe hybridizes to both telomeric and subtelomeric poly($TG_{1\text{-}3}$) tracts and to poly(GT) tracts located throughout the genome (Walmsley *et al.* 1984). In such an analysis (Figure 5), the Y′ telomeres appear as a fuzzy band of ~1.2 kb, and the X telomeres appear as discrete bands of larger size. Telomeric poly($TG_{1\text{-}3}$) tracts can be distinguished from internal poly(GT) tracts by their sensitivity to Bal-31.

In Figure 5, four of the X telomeres are smaller than 5 kb in the wild-type strain (W303a). Two of these telomeres represent the XI_R and XV_L telomeres that we examined previously using the single-copy hybridization probes. This conclusion was based on rehybridization of the filter to single-copy probes. In the wild-type strain, the ability of the X and Y′ telomeric fragments to hybridize to the poly(GT/CA) probe was lost after 10 min treatment with Bal-31. In the *tel1 rif1* strain, after 10 min of treatment with Bal-31, most of the poly($TG_{1\text{-}3}$) sequences were also removed from the Y′ telomeres. Hybridization to the X telomeres persisted even in samples treated for 20 min with Bal-31. We conclude that the poly($TG_{1\text{-}3}$) tracts in *tel1 rif1* strains are longer on the X telomeres than on the Y′ telomeres.

In addition to examining the Y′ and X telomeres, we monitored telomere length of *TELVR::URA3*, which lacks both X and Y′ sequences (Figures 3g and 4b) in the *tel1 rif1* strains. We found that this telomere was approximately the same size as is found in strains with the *tel1* mutation. Thus, this telomere appears to be regulated in a manner similar to the Y′ telomeres rather than the X telomeres.

In summary, the *tel1* mutation appears epistatic to *rif1* for the Y′ and the *TELVR::URA3* telomeres, but not for the X telomeres. Thus, the length regulation of poly($TG_{1\text{-}3}$) tracts that occurs in *rif1* strains is Tel1p-dependent for Y′ telomeres, but largely Tel1p-independent for X telomeres. Although most of our experiments were done using *tel1 rif1* strains (RCY24, RCY25) constructed by disrupting *rif1* in a *tel1* strain, we also examined telomeres in a strain (RCY12) constructed by disrupting *tel1* in a *rif1* mutant strain. We found that Y′ telomeres shrank to approximately the lengths observed in *tel1* strains, and the X telomeres remained long (data not shown). Thus, the manner in which the double-mutant strain is constructed does not appear to affect the length of the telomeres at equilibrium.

We also examined telomere length in a *rap1-17 tel1 rif1* strain (Figure 3h). In this strain, Y′ telomeres were approximately the same size as observed in the *tel1 rif1* strains, but three of the four X telomeres were shorter

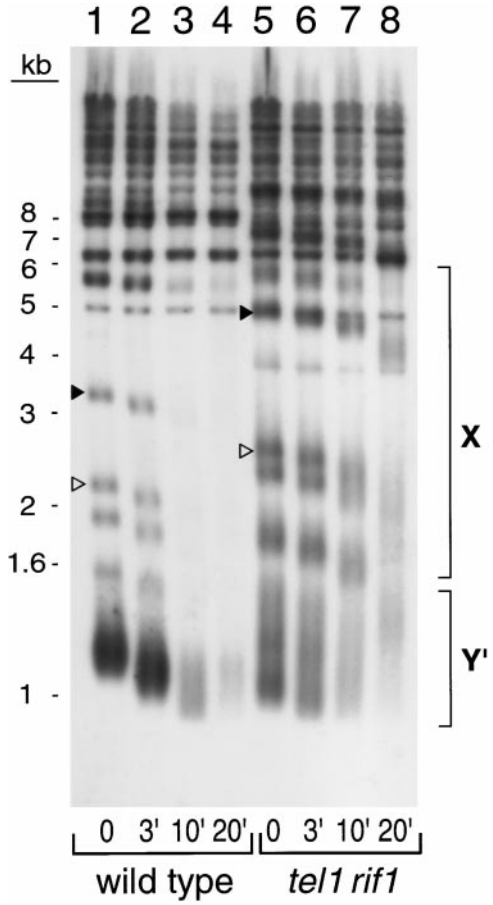

Figure 5.—Sensitivity of telomeric poly($TG_{1\text{-}3}$) tracts in a wild-type (RCY22) and a *tel1 rif1* strain (RCY25-T1 after 10 subculturings) to the Bal-31 exonuclease. As described in materials and methods, genomic DNA was treated with Bal-31 for increasing amounts of time (indicated at the bottom of the figure). The samples were then digested with *Xho*I and examined by Southern analysis using a probe poly(TG/AC) that hybridizes to telomeric poly($TG_{1\text{-}3}$) sequences as well as internal poly($TG_{1\text{-}3}$) and poly(GT) sequences. The approximate positions of the Y′ tracts and some of the X tracts are indicated at the right. The positions of tracts before digestion with Bal-31 (lanes 1 and 5) corresponding to the XV_L and XI_R X telomeres are indicated by black and white triangles, respectively. These assignments were made by reprobing of the filter with hybridization probes specific for individual telomeres.

than observed in the *tel1 rif1* strains. By the Mann-Whitney nonparametric test, the I_L, III_L, and XV_L telomeres were significantly ($P < 0.03$) longer than the Y′ telomeres in the *rap1-17 tel1 rif1* strain.

Epistasis interactions between *tel1* and *rif2*: In strains with the *tel1 rif2* genotype, the Y′ telomeres had approximately the same length as those in *tel1* strains (Figure 3, a and i). In addition, most of the X telomeres were also short, as was the *TELVR::URA3* telomere (Figure 4, b and c). One X telomere (I_L) in the *tel1 rif2* strain RCY56-T2, but not in RCY56-T1, was very long, ~1.4 kb, longer than found in the wild-type strain (Figure 4b, lane 7, I_L panel). By PCR analysis using primers derived from Y′ (YPR-90R, Table 2), and single-copy I_L sequences (C1L-1200R, Table 2), we detected a DNA fragment of the size (1.4 kb) consistent with a *de novo* insertion of Y′.

In summary, the X telomeres in the *tel1 rif2* strain, unlike those in the *tel1 rif1* strain, are regulated in the same way as the Y′ telomeres. To examine these relationships further, we also analyzed telomere lengths in strain RCY57 (*tel1 rif1 rif2*) (Figures 3j and 4). We found that lengths of the Y′ and *TELVR::URA3* telomeres were reduced to the length observed in wild-type strains, whereas the X telomeres (with the exception of I_L) maintained their elongated telomeric tracts. Thus, telomeres in the triple-mutant strain behaved in a manner similar to that observed in the *tel1 rif1* strain.

The only known function of the Rif proteins is to bind Rap1p. Because the *rap1-17* strain has a truncation of Rap1p that should prevent binding of Rif1p and Rif2p (Wotton and Shore 1997), one would expect that the phenotype of the *rap1-17 tel1* strain should be identical to that of the *tel1 rif1 rif2* strain. Because the phenotypes are different, it argues that the Rif proteins may have roles in addition to those requiring binding to Rap1p or that the Rap1p in *rap1-17* strains retains some Rif-binding activity. Alternatively, some of these phenotypic differences may reflect competitive interactions of the Rif proteins and the Sir3p/Sir4p for binding to Rap1p (Wotton and Shore 1997).

Epistasis interactions of *tel1* and *pif1*: The *PIF1* gene encodes a putative helicase that has both mitochondrial and nuclear activity, and mutations in this gene result in elongated telomeres (Schulz and Zakian 1994). In agreement with this previous study, telomere lengths of the Y′ telomeres were slightly elevated by the *pif1* mutation (Figure 3k). The X telomeres were also lengthened in the *pif1* strain RCY3. The terminal tracts for III_L and XI_R telomeres were slightly longer than the tracts for I_L and XV_R telomeres. In the *pif1 tel1* double-mutant strain, the Y′ telomeres were reduced to the length observed in the *tel1* strain (Figure 3l). The lengths of the X telomeres, however, were intermediate between those observed in the *pif1* and *tel1* strains.

DISCUSSION

In our analysis of telomere lengths in various mutant strains, we found that (1) the elongation of Y′ telomeric tracts in strains with *rif1*, *rif2*, *rap1-17*, or *pif1* mutations is largely dependent on Tel1p; (2) elongation of most X telomeres is also Tel1p-dependent for strains with *rif2* or *rap1-17* mutations, but largely Tel1p-independent in strains with a *rif1* mutation; and (3) telomeres that lack both X and Y′ repeats are regulated in the same manner as Y′ repeats. These data support the pathways shown in Figure 6.

Telomere length is substantially reduced in *tel1* strains (Lustig and Petes 1986), demonstrating that most telo-

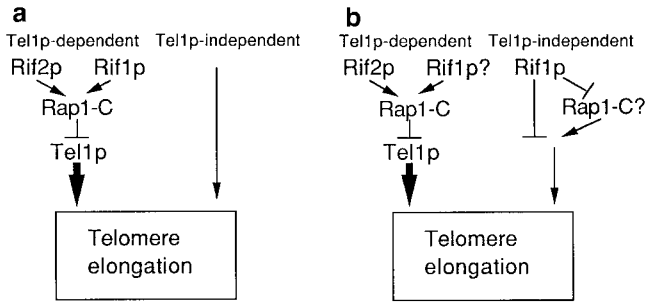

Figure 6.—Regulatory circuits for telomere length regulation for (a) Y′ and (b) X telomeres. These diagrams are based on epistasis analysis, as described in the text. As is standard, arrows indicate activation and T-junctions indicate inhibition of the pathway. Question marks represent assignments that are likely, but not proven.

mere elongation is Tel1p-dependent. Because *tel1* strains do not have a senescent phenotype, a Telp-independent pathway of telomere elongation also exists. For the Y′ telomeres and telomeres that lack both X and Y′, the simplest explanation of the epistasis results is that Rif1p, Rif2p, and Rap1p function "upstream" of the Tel1p (Figure 6a). The binding of Rif1p or Rif2p to the C terminus of Rap1p (designated as "Rap1-C") activates the telomere-elongation-suppressing function of Rap1p. The depiction of the function of Rap1p upstream of the function of Tel1p is also consistent with the results of other analyses. In wild-type cells containing high-copy-number plasmids with either long poly($TG_{1\text{-}3}$) tracts (Runge and Zakian 1989) or a *rap1* allele encoding a Rap1p deletion derivative (Conrad *et al.* 1990), telomere tracts are elongated. In strains with a *tel1* mutation, this elongation does not occur (Runge and Zakian 1996).

The pathways of telomere elongation for X telomeres are more complicated (Figure 6b). In *rif1 tel1* strains, X telomeres, unlike Y′ telomeres, are longer than those found in wild-type strains. The Rif1p, therefore, functions as a negative regulator of telomere elongation for Y′ (and, possibly, X telomeres) in a Tel1p-dependent pathway and a negative regulator of X (but not Y′) telomere elongation in a Tel1p-independent pathway. Another complication is that Rif1p acts as a negative regulator in the Tel1p-independent pathway in two different ways. First, based on the observation that X telomeric tracts are shorter in the *rap1-17 tel1 rif1* strain RCY59 than in the double-mutant *tel1 rif1* strains, we suggest that Rif1p inhibits positive activation of telomere elongation by the C-terminal domain of Rap1p. Ray and Runge (1998) previously showed that the C-terminal domain of Rap1p enhanced telomere formation by a mechanism that was independent of *TEL1*. Second, because strains with the *rap1-17 tel1 rif1* genotype have longer telomeres than those with the *rap1-17 tel1* genotype, Rif1p must also negatively regulate telomere length in a pathway that is independent of the C-terminal domain of Rap1p.

Relationship between the Rap1p "counting" model and the function of Tel1p: Marcand *et al.* (1997) suggested that yeast cells controlled telomere length by "counting" the number of Rap1p/Rif1p and Rap1p/Rif2p molecules bound at the telomere. One version of this model is that a threshold number of Rap1p/Rif1p and Rap1p/Rif2p molecules form a complex resistant to telomere elongation which we call a *te*lomere-*c*losed (TE-C) complex. Below this value, the complex breaks down to a structure that is proficient for telomere elongation [*te*lomere-*o*pen (TE-O) complex]. Strains that lack the C terminus of Rap1p or that lack both Rif proteins are incapable of forming the TE-C complex and, consequently, constitutively elongate their telomeres. In the context of the counting model, one could postulate that Tel1p is involved in destabilizing the TE-C complex. In the absence of Tel1p, a TE-C complex involving a smaller number of Rap1p/Rif1p or Rap1p/Rif2p molecules might form, resulting in short telomeres. For this model, one would expect that mutations that disrupt the counting mechanism (*rap1-17*, *rif1*, or *rif2*) would be epistatic to *tel1*, the opposite of the observed result.

We propose an alternative model consistent with the pathway shown in Figure 6a in which there are two steps required to make telomeric sequences accessible to telomerase. The first step, as proposed by others (Marcand *et al.* 1997; Ray and Runge 1998, 1999), is the opening of the TE-C complex as the number of Rap1p/Rif1p and Rap1p/Rif2p molecules bound at the telomere is reduced. We suggest that this complex [*te*lomere-*p*re-*o*pen) (TE-PO)] is not a substrate for telomerase, but contains one or more proteins that are substrates for the Tel1p kinase activity. Phosphorylation of these proteins within the TE-PO complex results in a complex (TE-O) that is a substrate for telomerase activity.

This model is consistent with our epistasis results and assigns a function to the kinase domain of Tel1p (Greenwell *et al.* 1995; Morrow *et al.* 1995). We suggest that the Tel1p-independent pathway of telomere elongation may represent phosphorylation of the TE-PO complex by Mec1p, a protein structurally related to Tel1p (Greenwell *et al.* 1995; Morrow *et al.* 1995). This suggestion is supported by the observation that *tel1 mec1* strains or the equivalent genotype in *S. pombe* appear completely defective in telomere replication (K. Ritchie, J. Mallory, and T. D. Petes, unpublished data; Naito *et al.* 1998). Because the *tel1* mutation shortens telomeres more substantially than the *mec1* mutation, we postulate that the kinase activity of Tel1p on the TE-PO complex (Tel1p-dependent pathway) is more efficient than the activity of Mec1p on the same complex (Tel1p-independent pathway).

There are a number of other roles for Tel1p that

would be consistent with a function downstream of the Rap1 counting mechanism. For example, Tel1p could function as a cofactor required for the optimal activity of telomerase. Tel1p cannot be an essential factor for telomerase, because *tel1* mutants do not senesce. Second, Tel1p could influence telomere length indirectly by controlling the timing of telomere replication during the cell cycle. Third, Tel1p could be involved in regulating the replication of the C-rich strand of the telomere by conventional DNA polymerases.

Differential telomere length regulation of X and Y′ telomeres: Our studies demonstrate that the lengths of X telomeres are regulated differently from those of Y′ telomeres or telomeres that lack both X and Y′. Although chromosome-specific telomere length regulation has not been examined in detail previously, Liu *et al.* (1995) found variation in the effects of the *kem1* mutation on different X telomeres. In addition, the effects of *est* and *tlc1* mutations on telomere length vary somewhat for different X telomeres (Lendvay *et al.* 1996), although some of this variation might reflect stochastic events rather than telomere-specific responses to various mutations.

Given the sequence differences between X and Y′ telomeres, as well as the differences in the DNA sequences of individual X telomeres, telomere-specific responses to mutations that affect telomere length are, perhaps, not surprising. Because X telomeres, but not Y′ telomeres or telomeres that are devoid of subtelomeric repeats, had a high level of Tel1p-independent telomere elongation in the *tel1 rif1* strains, it is likely that X repeats have a *cis*-acting element responsible for this effect. One repeat found at higher density in X than in Y′ elements is TTAGGG (Brun *et al.* 1997), which is bound *in vitro* by the Tbf1p protein (Liu and Tye 1991; Brigati *et al.* 1993). It has been suggested that this protein may be involved in promoting the interaction of telomerase with telomeric repeats (Liu and Tye 1991; Kramer and Haber 1993; Ray and Runge 1998). In the context of our favored model for Tel1p function described above, binding of Tbf1p to the X telomeres may result in a telomeric complex that is more "open" for interaction with telomerase even in the absence of Tel1p. The interaction of telomerase with telomeric repeats in this complex may be facilitated by interactions with the C-terminal region of Rap1p; Ray and Runge (1998) showed that this region of Rap1p was involved in stimulating elongation of short telomeres. Because Rif1p also binds the C-terminal region of Rap1p, the negative effects of Rif1p on telomere elongation in the Tel1p-dependent pathway may reflect competing interactions between Rif1p and proteins required for telomere elongation. Because X telomeres are longer in *tel1 rif1 rap1-17* strains than in *tel1 rap1-17* strains, Rif1p also exerts a negative role on telomere elongation in the Tel1p-independent pathway that is independent of an interaction with the C-terminal sequences of Rap1p.

It is possible that the differential responses of X and Y′ telomeres to Rif1p in the Tel1p-independent pathway reflect some feature of the X elements other than interactions with Tbf1p. Among other properties that could distinguish X and Y′ telomeres are (1) the timing of telomere replication (Stevenson and Gottschling 1999), (2) the sequence of the centromere-proximal region of the poly($TG_{1\text{-}3}$) tract, and (3) the differential attachment of the X and Y′ telomeres to nuclear structures that affect telomere biosynthesis. This last possibility is consistent with evidence that the X repeats improve the segregation of circular plasmids (Longtine *et al.* 1992).

Whatever the mechanisms responsible for the variation in telomeric-tract-length regulation at X and Y′ telomeres, our results illustrate an important principle: all yeast telomeres cannot be regarded as identical substrates for the enzymes involved in telomere maintenance. In addition, our data demonstrate the complications of epistasis analysis as applied to telomere length regulation. If only the Y′ telomeres or the I_L X telomere had been examined in *tel1*, *rif1*, and *tel1 rif1* strains, we would have concluded that *tel1* was epistatic to *rif1*; if only the III_L, XI_R, and XV_L X telomeres had been examined, we would have reached the opposite conclusion.

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