Rap1p telomere association is not required for mitotic stability of a C₃TA₂ telomere in yeast

Mary Kate Alexander and Virginia A.Zakian

Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

1Corresponding author
e-mail: vzakian@molbio.princeton.edu

Telomeric DNA usually consists of a repetitive sequence: C₁₋₃A/TG₁₋₃ in yeast, and C₃TA₂/T₂AG₃ in vertebrates. In yeast, the sequence-specific DNA-binding protein Rap1p is thought to be essential for telomere function. In a tcl1h mutant, the templating region of the telomerase RNA gene is altered so that telomerase adds the vertebrate telomere sequence instead of the yeast sequence to the chromosome end. A tcl1h strain has short but stable telomeres and no growth defect. We show here that Rap1p and the Rap1p-associated Rif2p did not bind to a telomere that contains purely vertebrate repeats, while the TG₁₋₃ single-stranded DNA binding protein Cdc13p and the normally non-telomeric protein Tb9p did bind this telomere. A chromosome with one entirely vertebrate-sequence telomere had a wild-type loss rate, and the telomere was maintained at a short but stable length. However, this telomere was unable to silence a telomere-adjacent URA3 gene, and the strain carrying this telomere had a severe defect in meiosis. We conclude that Rap1p localization to a C₃TA₂ telomere is not required for its essential mitotic functions.

Keywords: RAP1/RIF2/telomerase/telomere/yeast

Introduction

Telomeres, the DNA–protein complexes found at the ends of linear eukaryotic chromosomes, usually consist of a short repeated sequence that is G-rich on the strand running 5’ to 3’ towards the telomere. For example, telomeres in the budding yeast Saccharomyces cerevisiae contain about 350 bp of C₁₋₃A/TG₁₋₃ DNA, while the telomere sequence in all vertebrates, including humans, is C₃TA₂/T₂AG₃. In diverse organisms, including yeast and humans, the G-rich strand of the telomere extends to form a 3’ single-strand overhang (reviewed in Shore, 2001).

Telomeres have several important functions. They promote the complete replication of chromosome ends through the addition of repeated sequences by telomerase, a specialized reverse transcriptase. Telomeres have an essential role in capping chromosome ends, preventing the degradation and end-to-end fusions seen at double-stranded DNA breaks. In S.cerevisiae, loss of even a single telomere results in a cell-cycle arrest mediated by the DNA damage checkpoint (Sandell and Zakian, 1993). Cells that recover from this arrest without replacing the missing telomere lose the broken chromosome at a very high rate (Sandell and Zakian, 1993). In many species, from yeasts to mammals, a gene placed next to a telomere can be reversibly silenced, a phenomenon known as telomere position effect, or TPE (reviewed in Tham and Zakian, 2002). Telomeres also play an important role in chromosome pairing during meiosis. In many species, telomeres cluster early in meiosis, a process known as bouquet formation, which may help align homologous chromosomes (reviewed in Scherthan, 2001). In fission yeast, the telomere-associated proteins Tat1 and Rap1p are required for efficient meiosis (Cooper et al., 1997; Chikashige and Hiraoa, 2001; Kanoh and Ishikawa, 2001). In S.cerevisiae, the meiosis-specific telomere-associated protein Nd1p, promotes chromosome pairing and is required for bouquet formation (Chua and Roeder, 1997; Conrad et al., 1997; Trelles-Sticken et al., 1999).

Several proteins are known to associate with Saccharomyces telomeres. Rap1p, an essential protein that binds to duplex S.cerevisiae telomeric DNA (Berman et al., 1986; Longtime et al., 1989), is the major in vivo telomere binding protein present in 10–20 copies per telomere (Conrad et al., 1990; Wright et al., 1992; Gilson et al., 1993). Rap1p recruits two groups of proteins, the Sir proteins that mediate TPE (Aparicio et al., 1991; Moretti et al., 1994) and the Rif proteins that inhibit both telomerase- and recombination-mediated telomere lengthening (Bourns et al., 1998; Teng et al., 2000). Cdc13p, which binds single-stranded TG₁₋₃ DNA in vitro (Lin and Zakian, 1996; Nugent et al., 1996) and chromosome ends in vivo (Bourns et al., 1998; Tsukamoto et al., 2001), is essential because it helps protect chromosome ends from degradation (Garvik et al., 1995). Cdc13p also plays a positive role in regulation of telomerase (Nugent et al., 1996; Qi and Zakian, 2000). Saccharomyces cerevisiae telomerase includes the catalytic subunit, Est2p, as well as an RNA subunit, TLC1, which has a short template region consisting of the C-stand telomere sequence (reviewed in Nugent and Lundblad, 1998).

Rap1p is also required for transcriptional repression at the silent mating-type loci (Shore et al., 1987) and annealing of telomeres (Liu et al., 1994; Moretti et al., 1994). However, since neither mating-type silencing nor TPE is essential, silencing is therefore not an essential function of Rap1p. Rap1p is also a transcriptional activator at many loci in Saccharomyces, binding in the upstream regions of ~5% of yeast genes, which together account for ~37% of all yeast mRNA transcripts (Huet et al., 1985). Since many Rap1p-regulated genes are essential, the role of Rap1p as a transcriptional activator is almost certainly essential.

Rap1p homologs have been identified in many species, including other yeasts (Larson et al., 1994; Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001) and humans (Li et al., 2000). Unlike the budding yeast Rap1p, the Schizosaccharomyces pombe and human Rap1p homologs
(spRap1 and hRap1) do not bind DNA directly, but are recruited to the telomere through interactions with telomeric DNA-binding proteins (Li et al., 2000; Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). In addition, spRap1 and hRap1 have not been shown to have any non-telomeric functions, such as transcriptional activation (Li et al., 2000; Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). Although S. pombe rap1+ is not essential, a rap1− mutant has long telomeres, lacks TPE and has several meiotic defects, including loss of telomere clustering, reduced sporulation efficiency and low spore viability (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). These phenotypes are indistinguishable from those of a strain lacking Tel1, the telomere-binding protein that recruits spRap1 to the telomere (Cooper et al., 1997, 1998; Nimmo et al., 1998).

Saccharomyces cerevisiae strains with rap1′ alleles that reduce the ability of Rap1p to bind DNA exhibit telomere shortening at semipermissive temperatures (Conrad et al., 1990; Lustig et al., 1990). Moreover, overexpression of Rap1p leads to increased rates of chromosome loss (Conrad et al., 1990). These data suggest that Rap1p is required not only for transcription but also for the stability function of yeast telomeres. However, the widespread role of Rap1p in transcription makes it difficult to use a standard genetic approach to determine if telomeres lacking Rap1p are functional. As an alternative, we altered the telomere sequence itself. A strain in which the yeast telomere sequence was changed to match the vertebrate sequence, the tlc1-human or tlc1h strain, is viable but has short telomeres (Henning et al., 1998). We constructed a 4.1kb strain that had one telomere consisting solely of C3TA2T2AG3 vertebrate telomeric DNA, a sequence that is not expected to bind Rap1p (Liu and Tye, 1991). As predicted, this telomere bound neither Rap1p nor the Rap1p-associated protein Rif2p, yet its role in chromosome stability was unimpaired. Thus, binding of Rap1p to a C3TA2 telomere is not essential in mitotic cells.

Results

A telomere with only vertebrate repeats can be maintained in yeast

TLC1 encodes the RNA subunit of yeast telomerase. The tlc1h gene is an allele of TLC1 in which the templating portion of the gene is modified to encode the vertebrate telomere repeat (Henning et al., 1998). Because only the distal third of yeast telomeres is subject to degradation and telomerase-mediated resynthesis (Wang et al., 1989), even after prolonged growth, telomeres in a tlc1h strain consist of a mixture of vertebrate C3TA2T2AG3 and yeast C1,3A/ TG1,3 repeats. These mixed-sequence telomeres are ~150 bp shorter than wild type (Henning et al., 1998).

Rap1p does not bind the vertebrate C3TA2 telomere sequence in vitro (Liu and Tye, 1991). To determine if vertebrate telomeric DNA can supply all telomere functions in yeast, we constructed a strain called 499UT-H (UT for URA3 at telomere, H for human telomeric DNA) in which the left telomere of chromosome VII bears only C3TA2 repeats, rather than a mixture of vertebrate and yeast repeats. The VII-L telomere in a tlc1h strain was replaced with the URA3 gene and 60 bp of vertebrate telomere sequence that was lengthened in vivo by the addition of C3TA2 repeats (Figure 1A). The wild-type control strain for 499UT-H was 499UT-Y (Y for yeast telomeric DNA), an otherwise isogenic strain that contained a wild-type TLC1 gene, fully wild-type telomeres and URA3 adjacent to the VII-L telomere.

To confirm that the VII-L telomere in 499UT-H contained only vertebrate telomeric DNA, telomeres from three independent transformants obtained using the UT-H construct were cloned and sequenced ~125 cell divisions after each received the modified telomere (Figure 1B). The amount of telomeric DNA present at individual VII-L telomeres from the tlc1h strain ranged from 210 to 240 bp. For comparison, VII-L telomeres cloned from the control 499UT-Y strain contained 320–370 bp of C1–3A DNA. None of the 499UT-H clones contained yeast C1–3A repeats but rather had up to 35 pure C3TA2 repeats. The VII-L telomere consisting solely of vertebrate telomeric DNA will hereafter be called the C3TA2 telomere.

Replication and length regulation of the C3TA2 telomere

As reported previously (Henning et al., 1998), the mixed vertebrate and yeast sequence telomeres in a tlc1h strain were ~150 bp shorter than telomeres in a TLC1 wild-type strain (Figure 1C, center and right panels). A similar shortening was seen at the C3TA2 telomere (Figure 1C, left panel). The lengths of both the C3TA2 and the mixed-sequence telomeres were stable over at least 500 cell divisions (Figure 1; data not shown).

Yeast telomeres are normally maintained by telomerase. In addition to TLC1, telomerase-mediated telomere lengthening requires at least four genes, including EST2, which encodes the catalytic subunit of telomerase (Lendvay et al., 1996; Lingner et al., 1997). In the absence of telomerase, telomeres can be maintained by RAD52-dependent homologous recombination, leading to an amplification of either sub-telomeric or telomeric repeats (Lundblad and Blackburn, 1993; Teng and Zakian, 1999). To determine which pathway, telomerase or recombination, was maintaining telomeres in the tlc1h strain, heterozygous deletions of EST2 and RAD52 were made in a diploid 499UT-H/500UT-H strain (500UT-H is identical to 499UT-H except for being of opposite mating type). The diploid was sporulated, tetrads dissected and spore products of the desired genotypes identified by replica plating.

Viability and telomere length in the 499UT-H strain were unaffected by deletion of the homologous recombination protein RAD52 (Figure 1D). In contrast, no viable est2 tlc1h spores were recovered (data not shown). The synthetic lethality observed for the est2 tlc1h strain was probably due to the fact that telomeres in the tlc1h tlc1h strain were shorter than in a wild-type strain. Strains lacking an EST gene in an otherwise wild-type background senesce after 50–100 divisions (Lundblad and Sóstak, 1989). Since the tlc1h strain started with telomeres that were ~150 bp shorter than the wild-type length, telomerase-deficient tlc1h cells should senesce after ~25–50 generations. Since it takes ~25 generations for a spore to generate a colony, even those est2 tlc1h spores that were able to form a colony after germination would...
senescence upon the replica plating that was used to identify 
\( \text{est}2\Delta \) spores. We conclude that the C1TA2 telomere is
maintained by telomerase, not recombination.

**The C1TA2 telomere does not increase the loss rate of chromosome VII**

Removal of the yeast VII-L telomere results in a dramatic increase in the rate at which this chromosome is lost (Sandell and Zakian, 1993). Although the growth rate of the \( tlc\) strain with the C1TA2 telomere was indistinguishable from wild type, loss of a single chromosome must increase by \(-1000\)-fold before it affects the overall growth rate of a strain. To determine if the C1TA2 telomere affects the mitotic stability of chromosome VII, the loss rate of this chromosome was measured using fluctuation analysis in a \( tlc\) strain that was disomic for chromosome VII, but had only one copy of each of the other 15 yeast chromosomes (Figure 2A). One copy of chromosome VII carried the C1TA2 telomere while the other copy and all other telomeres in the cell had mixed-sequence telomeres. Whereas the original disomic strain produced red colonies that die on cycloheximide plates, cells that lost the chromosome with the C1TA2 telomere generated white, cycloheximide-resistant colonies. As a control, we also determined the loss rate of chromosome VII from an otherwise isogenic \( TLC1\) strain, with completely wild-type telomeres. This analysis revealed that the loss rate of chromosome VII with the C1TA2 telomere was statistically indistinguishable from the loss rate of chromosome

**Fig. 1.** A telomere consisting solely of vertebrate telomeric DNA can be stably maintained in yeast. (A) To generate a completely vertebrate-sequence telomere at chromosome VII-L, EcoRI-Sall-digested pUT-H was integrated at the ADH4 locus on chromosome VII-L in a \( tlc\) strain, in a manner that deletes the terminal \(-20\) kb from the chromosome (Gottschling et al., 1990). The 60 bp C1TA2 sequence (black box) acts as a seed for telomere formation. The \( tlc\) telomerase can add only vertebrate repeats to this end, resulting in a VII-L telomere that contains no yeast telomeric repeats. A unique sequence (striped block) between the telomere seed and the URA3 gene was used as a PCR telomere site for telomere sequencing and chromatin immunoprecipitations. The arrow above the spotted box indicates the URA3 promoter. The indicated PstI site is located upstream of the URA3 start codon. (B) Sequencing results for modified VII-L telomere. VII-L telomeres from the 499UT-H strain were cloned and sequenced. Clones were made \(-125\) cell divisions after transformation in three independent integrants of the UT-H construct. Representative sequences for three clones, one from each integrant, are shown; the centromere-proximal end of the sequence is to the right. Seven modified telomeres were sequenced; none contained yeast telomeric DNA. While none of the sequenced telomeres contained the heterogeneous C1-L-A repeats characteristic of yeast telomeres, the VII-L telomere in the 499UT-H2 transformant contained one copy each of two variants of the vertebrate repeat: C1TA2 and C1TA1. The 499UT-H1 strain, with 245 bp of pure vertebrate repeats, was used for all subsequent experiments. (C) Telomere length is stable in the 499UT-H strain over many generations. Telomere length in the 499UT-Y and 499UT-H strains was assayed after one and ten serial restreaks on YC plates. Genomic DNA was digested with PstI and XhoI; the Southern blot was probed sequentially with URA3, C1-L-A and C1TA2. The upper band in the URA3-probed blot (*) is the endogenous ara3-52 allele. Two to three independent colonies from each strain are shown. Black arrowheads indicate the 499UT-Y VII-L telomere; white arrowheads indicate the 499UT-H C1TA2 VII-L telomere. (D) Effect of rad52A on telomere length. Southern blots of PstI-digested genomic DNA from four serial restreaks of rad52 and RAD52 versions of the 499UT-Y and 499UT-H strains were probed with URA3 which detects the VII-L telomere fragment. The upper band in the URA3-probed blot (*) is the endogenous ara3-52 allele. Arrows indicate the VII-L (URA3 probe) telomere band.
Fig. 2. The C3TA2 telomere has a wild-type loss rate. (A) Disome strain for chromosome stability assay. Loss of the test copy of chromosome VII was measured in a chromosome VII disome strain with the tlc1h mutation by selecting for white, cycloheximide-resistant colonies. *URA3* with a vertebrate telomere seed was integrated at the VII-L telomere on the test copy of chromosome VII, generating a telomere containing solely vertebrate telomeric DNA. The control strain was a VII-L disome with *URA3* at the VII-L telomere on the test copy of the chromosome and wild-type *TLC1* (Sandell and Zakian, 1993). C3TA2 repeats are in black portion of triangles; C1,3A DNA is in white. (B) Rates of chromosome loss. The average rates of chromosome loss for UT-Y (*TLC1*, wt telomeres) and UT-H (*tlc1h, C3TA2 VII-L telomere*) disome strains. Error bars are standard deviation.

(Figure 3A). This loss of silencing was quantitated by comparing plating efficiency on complete medium versus FOA plates. The frequency of silencing at the C3TA2 telomere was reduced ~30 000-fold, from 0.54 ± 0.24 (average ± standard deviation) in the *TLC1* 499UT-Y strain to 1.8 ± 0.59 × 10⁻⁵ at the C3TA2 telomere (Figure 3A).

**The tlc1h strain has a severe meiotic defect**

To determine the behavior of the C3TA2 telomere in meiosis, we sporulated a diploid strain that was homozygous for *tlc1h* and the C3TA2 telomere (H/H strain) and compared it with an otherwise isogenic *TLC1/TLC1* diploid that also had *URA3* at the left telomere on both copies of chromosome VII but had only yeast sequence telomeres (Y/Y strain) (Figure 4A). We also sporulated a *tlc1h/tlc1h* diploid that had one copy of the C3TA2 telomere (H/HY strain; the rest of the telomeres were mixed-sequence telomeres) and a *tlc1h/tlc1h* diploid in which neither of the VII-L telomeres was modified by insertion of *URA3* (HY/HY strain; all telomeres were a mixture of yeast and vertebrate repeats).

Although the frequency of sporulation, as measured by comparing the number of asci with the total number of asci plus unsporulated cells, was only 27% in the H/H strain (compared with 41% for the Y/Y wild-type strain), this difference was not statistically significant (Figure 4B). A much more severe defect was seen when the percentages of asci containing complete tetrads were compared between the two strains; while 82% of asci in the Y/Y wild-type diploid contained four spores, only 11% did so in the H/H strain. Most asci in the H/H strain, 69%, contained only two spores. A similarly low fraction of four-spore asci and high fraction of dyads was seen in the H/YH and YH/YH strains, in which all telomeres contained a mixture of C3TA2 and C1,3A telomeric DNA. The viability of spores from either the rare tetrads (66% spore viability) or the more common dyads (64% viability) was modestly reduced in the H/H strain compared with wild type (90% spore viability). Spore viability was similarly reduced in H/YH and YH/YH dyads (46 and 55% viability, respectively).

**Protein composition of the C3TA2 telomere: Cdc13p and Tbf1p but not Rap1p or Ril2p are bound in vivo**

To determine which proteins bound the C3TA2 telomere in vivo, we used chromatin immunoprecipitation (ChIP) (Figure 5). In this assay, proteins are crosslinked to each other and to DNA using formaldehyde. The crosslinked DNA is sheared into small pieces by sonication, and precipitated using antibodies to the protein of interest. The crosslinks are then reversed, the DNA is deproteinized, and the precipitated DNA is PCR amplified. To examine whether telomeric sequences were preferentially associated with the proteins of interest, we used a multiplex PCR strategy that was developed to examine telomere-associated proteins (Tsukamoto et al., 2001; Taggart et al., 2002). Four sequences were PCR amplified from the immunoprecipitated DNA (Figure 5A): ARO, a 372 bp
segment of ARO1, a gene on chromosome IV that is far from either telomere and serves as a negative control for telomere binding; ADH, a 301 bp fragment from within ADH4, a sub-telomeric gene that is 5 kb from the left telomere of chromosome VII; VII-L TEL, a 252 bp sequence that is immediately adjacent to the modified VII-L telomere; and VI-R TEL, a 265 bp sequence adjacent to the VI-R telomere.

Rap1p and Rif2p, a Rap1p-interacting protein involved in telomere length regulation (Wotton and Shore, 1997), were precipitated using polyclonal sera. Tbp1p, an essential yeast protein of unknown function, binds to duplex C1TA2 DNA in vitro (Liu and Tye, 1991). Cdc13p, a known in vivo single-stranded TGI2 DNA binding protein (Bourns et al., 1998; Taggart et al., 2002), also binds single-stranded T2AG1 DNA in vitro, albeit with ~10-fold lower affinity (Lin and Zakian, 1996; Nugent et al., 1996). The endogenous TBF1 locus was tagged with nine tandem Msc epitopes, while Msc-tagged Cdc13p was expressed under its own promoter from a centromere plasmid. The Msc-tagged proteins were precipitated with Msc monoclonal antibodies. The fold TEL enrichment was determined by first normalizing each TEL band to the ARO control band, and then comparing the normalized TEL band for each sample with a negative control immunoprecipitated with a non-telomeric polyclonal antibody (for Rap1p and Rif1p samples) or from an untagged strain using Msc monoclonal antibody (for the Tbp1p and Cdc13p samples).

When crosslinked chromatin from the TLC1 499UT-Y strain was immunoprecipitated with α-Rap1p polyclonal antibodies, a strong enrichment (44 ± 3.5-fold; average ± standard deviation) of the VII-L telomere-adjacent TEL sequence was observed (Figure 5B and C). In contrast, no significant enrichment of the VII-L TEL band was observed at the C1TA2 telomere (1.6 ± 1.0) in the
499UT-H strain. To confirm the Rap1p result, the Rap1p-interacting factor Rif2p was also immunoprecipitated. In the TLC1 strain, Rif2p co-immunoprecipitated the VII-L TEL DNA (5.6 ± 3.5-fold enrichment), confirming earlier work using a one-hybrid assay which showed that Rif2p interacts with wild-type telomeres in vivo (Bourns et al., 1998). Consistent with the Rap1p results, the C3TA2 VII-L TEL was not precipitated by the α-Rif2p serum (1.1 ± 0.16). The inverse result was obtained when precipitating myc-Tbf1p. The C3TA2 VII-L TEL band was enriched 82 ± 22-fold in the 499UT-H strain, but was at background levels (1.5 ± 0.30) in the 499UT-Y strain that contained only yeast telomeric DNA. In contrast, Cdc13p- myc co-precipitated the VII-L TEL DNA in both the 499UT-Y and 499UT-H strains, although the enrichment was even greater in the 499UT-H strain (44 ± 3.8-fold) than in the wild-type control (8.8 ± 1.9-fold). For each protein, the telomere association was dependent on in vivo crosslinking, demonstrating that these associations occurred in vivo (data not shown). The enrichments for Rap1p in 499UT-Y and Tbf1p in 499UT-H are minimal estimates, as these TEL bands were outside the linear range for quantitation.

In the 499UT-H strain, the VI-R telomere contained a mixture of vertebrate and yeast telomeric DNA, and thus was expected to bind Rap1p, Rif2p, Cdc13p and Tbf1p. Consistent with this expectation, Cdc13p and Tbf1p bound well to both the VI-R and VII-L telomeres in the 499UT-H strain (Figure 5B and C). In contrast to the results with the C3TA2 telomere, Rap1p and Rif2p both localized to the VI-R telomere in the 499UT-H strain.

**Nuclear localization of Rap1p and Tbf1p is unchanged in a tlc1h strain**

Immunolocalization reveals that several proteins needed for telomeric silencing, including Rap1p, are concentrated in 6–8 foci that localize to the nuclear periphery (Gotta et al., 1996). In situ hybridization shows that many telomeres localize to these Rap1p foci (Gotta et al., 1996). In contrast, Tbf1p has a punctate staining throughout the yeast nucleus (Koering et al., 2000). Given the altered telomere binding of Rap1p and Tbf1p in the tlc1h strain, we determined if the nuclear localization of either protein was also affected. To ensure that telomeres had high levels of vertebrate telomere repeats, the tlc1h strain was examined after >150 cell divisions of growth. Localization of Rap1p and Tbf1p in nuclei was analyzed by immunofluorescence using either an affinity purified anti-Rap1p polyclonal serum or anti-myc serum (Figure 3B). As reported previously (Koering et al., 2000), Tbf1p exhibited punctate staining in the TLC1 strain, a pattern that was unaltered in the tlc1h strain (Figure 3B, right). Rap1p was concentrated in foci in both the TLC1 and tlc1h strains (Figure 3B, left panels). This analysis demonstrates that the nuclear organization of Rap1p foci, and by inference of telomeres, is not grossly altered in the tlc1h strain.

**Discussion**

The essential protein Rap1p was thought to be critical both to protect chromosome ends from degradation (Conrad et al., 1990; Lustig et al., 1990) and, in concert with the associated Rif proteins, for telomere length homeostasis (Marcand et al., 1997). We show here that a telomere consisting solely of C3TA2 T2AG3 repeats (Figure 1B) did not bind Rap1p or Rif2p (Figure 5). Nonetheless, the length of this telomere was stable for >500 cell divisions (Figure 1C), suggesting that yeast also has a Rap1p-independent mechanism for monitoring telomere length. The absence of Rif proteins, which limit telomerase action (Teng et al., 2000), is normally associated with telomere lengthening (Hardy et al., 1992; Wotton and Shore, 1997). The fact that the C3TA2 telomere that lacked Rif2p (and presumably Rif1p) stabilized at a length shorter than that of a wild-type telomere suggests that Rap1p does help protect telomeres from degradation.

Remarkably, a chromosome with the C3TA2 telomere had a wild-type loss rate (Figure 2). Since loss of only one telomere is sufficient to dramatically destabilize chromosome VII (Sandell and Zakian, 1993), the C3TA2 telomere must be able to supply the critical telomere functions that ensure the stable maintenance of chromosomes. A wild-type telomere is estimated to bind 10–20 Rap1p molecules (Conrad et al., 1990; Wright et al., 1992; Gilson et al., 1993). The VII-L telomere with wild-type telomeric DNA was enriched ~30-fold more than the C3TA2 telomere in the anti-Rap1p immunoprecipitate (Figure 5), suggesting that Rap1p did not bind the C3TA2 telomere in vivo. Although we cannot rule out the possibility that the C3TA2 telomere sometimes bound one or a very few Rap1p molecules, our data suggest that the stability functions of yeast telomeres may not require Rap1p. In contrast, both Cdc13p and Tbf1p bound the C3TA2 telomere efficiently (Figure 5). Although no sequence or functional similarities have been shown between Rap1p and Tbf1p outside of the Myb-like DNA binding domain (Bilaud et al., 1996), Tbf1p may provide the stability functions of Rap1p at the modified telomere.

Although the C3TA2 telomere did not impair chromosome stability, meiosis was compromised in the tlc1h strain, as demonstrated by a paucity of four spore tetrads (Figure 4B). Because Rap1p is required for efficient meiosis in S. pombe (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001), it is tempting to speculate that the meiotic defect of the tlc1h strain is due to the lack of Rap1p at the C3TA2 telomere. However, by the criterion of ChIP, Rap1p bound as well to the mixed-sequence VI-R telomere as it did to telomeres consisting solely of yeast telomeric DNA (Figure 5). Since the YH/YH diploid, with its mixture of yeast and vertebrate telomeric DNA at all telomeres, was as defective in meiosis as the H/H strain that was homozygous for the C3TA2 telomere (Figure 4B), the meiotic defect of the tlc1h strain can not be attributed solely to the absence of Rap1p at the VII-L telomere. Ndj1p is a meiosis-specific telomere protein that affects homolog pairing and telomere clustering (Chua and Roeder, 1997; Conrad et al., 1997; Treles-Sticken et al., 2000). Although we did not examine Ndj1p binding in the tlc1h strain, an ndj1Δ strain does not have a preponderance of two-spore ascii. Thus, even if Ndj1p were improperly localized in the tlc1h strain, it would not be sufficient to explain the meiotic defects in this strain. Tbf1p bound well to both the C3TA2 telomere and the mixed-sequence VI-R telomere (Figure 5). Since Tbf1p is not normally telomere bound (Figure 5), perhaps its presence perturbs one or
more aspects of meiotic telomere behavior, even in strains that have some C<sub>1.3</sub>A (as well as C<sub>T</sub>A<sub>2</sub>) telomeric repeats.

TPE was abolished at the C<sub>T</sub>A<sub>2</sub> telomere (Figure 3A). Tbf1p, which bound efficiently to the C<sub>T</sub>A<sub>2</sub> telomere, is proposed to halt the spread of telomeric silencing within sub-telomeric DNA (Fouré et al., 1999). The absence of Rap1p, which recruits the Sir silencing proteins to the telomere (Li et al., 1994; Moretti et al., 1994), and the presence of Tbf1p are sufficient to explain the loss of TPE at the C<sub>T</sub>A<sub>2</sub> telomere (Figure 3A).

Although Cdc13p binds both single stranded TG<sub>1.3</sub> and T<sub>3</sub>A<sub>2</sub> DNAs in vitro, its affinity for the vertebrate telomeric sequence is considerably lower (Lin and Zakian, 1996; Nugent et al., 1996). Nonetheless, in the tcl1h strain, Cdc13p bound well to both the C<sub>T</sub>A<sub>2</sub> and the mixed-sequence VI-R telomere. In fact, the telomeric fragments from both the VII-L C<sub>T</sub>A<sub>2</sub> telomere and the mixed-sequence VI-R telomeres were enriched by ~5-fold more in the anti-Cdc13p precipitates from the tcl1h strain than from a TLC1 strain that had solely yeast telomeric DNA. The higher telomere enrichment in the anti-Cdc13p immunoprecipitate might reflect an altered telomere structure in the tcl1h strain, such as longer or constitutive single-stranded TG<sub>1.3</sub> tails that result in more telomere-bound Cdc13p, or an altered telomeric chromatin, that results in more efficient precipitation of telomere-bound Cdc13p. Although Cdc13p clearly associated with the C<sub>T</sub>A<sub>2</sub> telomere, the tcl1h strain was particularly sensitive to changes in Cdc13p. For example, although replacing the chromosomal CDC13 gene with the Myc-tagged CDC13 was readily accomplished in a TLC1 strain (Tsukamoto et al., 2001), we were unable to construct a tcl1h strain expressing only Myc-tagged Cdc13p. To circumvent this problem, we expressed Myc-tagged Cdc13p from a centromeric plasmid in a CDC13 background.

In summary, although Rap1p has been thought to play a central role in organizing the telomeric chromatin structure in S. cerevisiae, surprisingly, telomere binding of Rap1p is not required for the stability functions of a C<sub>T</sub>A<sub>2</sub> telomere during mitotic growth. A strain carrying an entirely vertebrate-sequence telomere that did not bind Rap1p had no growth defect and no increase in chromosome loss. Although the C<sub>T</sub>A<sub>2</sub> telomere was somewhat shorter than a wild-type telomere, its length was constant over many generations, indicating that Rap1p is also not essential for regulating telomere length. Consistent with our data, Brevet et al. (2003) found that a fully C<sub>T</sub>A<sub>2</sub> telomere could be maintained indefinitely, did not bind Rap1p by ChIP, and did not elongate in rap1t, rif1 or rif2 strains, as predicted if Rap1p did not bind this telomere (Brevet and E.Gilson, personal communication). These data suggest that transcriptional activation may be the only essential function of Rap1p, while Cdc13p and its associated proteins Stn1p and Ten1p (Grandin et al., 1997, 2001; Pennock et al., 2011) may suffice for the essential function of telomeres in end protection.

### Materials and methods

#### Plasmids and strains

pUT-H was constructed by excising 81 bp of yeast telomere sequence from pDH4U6A (mod–) (Tsukamoto et al., 2001) and replacing it with 60 bp of vertebrate telomere sequence, obtained by annealing the UT-H-5′ and UT-H-3′ oligos (Table I), which consist of the sequence (T<sub>3</sub>A<sub>2</sub>)<sub>10</sub> of (C<sub>T</sub>A<sub>2</sub>)<sub>10</sub> flanked by BamHI and EcoRI sites. The pRS304-derived TBF1 plasmid (constructs of Michaelis et al., 1997) that was used to integrate a 9-myc epitope tag at the N-terminus of the chromosomal copy of TBF1. An ~200 bp region upstream of the TBF1 gene that includes the start codon was PCR amplified using primers TBF1-N1 and TBF1-N2 (Table I), which introduced an EcoRI site at the 5′ end and a Spel site following the start codon. Another 200 bp from the beginning of the TBF1 gene was PCR amplified with primers TBF1-N3 and TBF1-N4 (Table I), which introduced a Spel site following the start codon and a SacI site at the end of the fragment. These two PCR products, which share 42 bp of sequence around the TBF1 start codon, were used as the template for a PCR with primers TBF1-N1 and TBF1-N4. The resulting 400 bp fragment was cloned into the EcoRI–SacI site of pRS304 (Sikorski and Hieter, 1989), and a 360 bp 9-myc epitope tag (Michaelis et al., 1997) was inserted into the Spel site downstream of the TBF1 start codon. A 9-myc epitope tag was integrated at the N-terminus of the chromosomal copy of TBF1 by transforming Ndr1-digested pRS304-myc-TBF1 into 499UT-Y and 499UT-H. Myc-tagged Cdc13p was expressed from the centromeric plasmid pRS314-CDC13-myc9 (Taggart et al., 2002). All strains were constructed in the YPH499 or YPH500 strain background (Sikorski and Hieter, 1989). The control strains were MATa and MATα versions of TEL::URA3/VII-L strains (Tsukamoto et al., 2001), referred to here as 499UT-Y and 500UT-Y, respectively. These strains contain URA3 adjacent to the VII-L telomere, and a 73 bp unique sequence between URA3 and the telomere; this unique sequence is in the opposite orientation to that of the strains in Tsukamoto et al. (2001).

A strain expressing a TLC1 allele in which the template region encodes the vertebrate telomere sequence instead of the yeast sequence was constructed by a two-step integration of the tcl1h allele from pTLC1h (Henning et al., 1998) into YPH500. Correct integrants were identified by colony PCR as previously described (Henning et al., 1998) and by Southern blot of Nsel-digested genomic DNA hybridized with an 800 bp C<sub>T</sub>A<sub>2</sub> probe from pSP73-Styl (from T.de Lange) to monitor incorporation of the vertebrate sequence at the telomeres. The 500UT-H strain, carrying an entirely vertebrate telomere, was constructed by integrating the 2.4 kb EcoRI–SalI fragment of pUT-H at the VII-L telomere of the YPH500 tcl1h strain (Figure 1A). The mating type of 500UT-H was switched from MATa to MATα to generate 499UT-H. Diploid strains homozygous for the tcl1h mutation and heterozygous for the UT-H telomere were made by mating 499UT-H to 500UT-H. A diploid strain homozygous for the tcl1h mutation and heterozygous for the UT-H telomere were produced by mating 499UT-H to YPH500 tcl1h.
Yeast telomeres lacking Rap1p are stable

Heterozygous deletions of EST2 were constructed by PCR amplifying HIS3 with primers containing homology to the flanking regions of the EST2 gene (est2HisA and est2HisB; Table I) and transforming the product into the 499UT-Y/500UT-Y and 499UT-H/500UT-H diploid strains. Heterozygous strains carrying a rad52::LEU2 allele were made by introducing the 5 kb BamHI fragment of pSM20 (Schild et al., 1983) into the 499UT-Y/500UT-Y and 499UT-H/500UT-H diploid strains. Haploid spore products carrying est2 or rad52 mutations were identified by replica plating spore clones of dissected tetrads to medium lacking histidine or leucine, respectively.

The control disome strain for chromosome loss analysis was LS20xLS18 (Sandell and Zakian, 1993). The disoome strain with vertebrate-sequence telomeres was constructed by integrating the tel1H allele in LS20 (Sandell and Zakian, 1993), mating LS20 with the karl strain SLS15 (parent strain of LS18 with unmodified VII-L telomere (Sandell and Zakian, 1993)), and selecting on YC Can-Arg-Lys-Tyr low Ade to isolate a chromosome VII disoome strain. The 2.4 kbp EcoRI–SalI fragment of pUT-H was integrated at the VII-L telomere of the LS35 are2 C17H2 ADE3 copy of chromosome VII (the test chromosome). The tel1H disoome strain with the C1T4A telomere was grown for >100 generations before chromosome loss was measured. Chromosome loss assays were performed similarly to Sandell and Zakian (1993). Recombination was monitored by counting the number of red, cycloheximide-resistant colonies that arose.

Diploid strains were sporulated for 5–6 days at room temperature in 0.5% potassium acetate. Sporulated cells were incubated 15 min at 30°C with 0.5 mg/ml zymolyase in 1 M sorbitol and dissected on YC plates.

Analysis of telomeres

The VII-L telomere was PCR-amplified as in Forstemann et al. (2000), using primers dG18-Bam (Forstemann et al., 2000) and UT-Y(+) for wild-type telomeres or UT-H(+) for C1T4A telomeres (Table I). The resulting products were cloned into the pCRII-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), and sequenced using M13 primers on an Applied Biosystems 3100 automated DNA sequenceer (MWG Biotech AG, Ebersberg, Germany). Telomere length was measured by Southern blots of yeast genomic DNA digested with PstI and XhoI and probed sequentially with the URA3 gene, ~300 bp C1-A from pC7300 and 800 bp C1-T4A from pSP73-Sty11 (from T.de Lange). TPE was measured qualitatively by spotting 10-fold serial dilutions of overnight cultures onto YC, YC–Ura and FOA plates. Quantitative silencing assays were performed as in Gotschling et al. (1990).

ChIP was performed essentially as in Taggart et al. (2002) except that protein A and G Dynabeads were used (Dynam, Oslo, Norway). PCR products were quantitated using the Scion Image program (Scion Corporation, Frederick, MD). Polyclonal sera were used to precipitate Rap1p (Conrad et al., 1990) and Rtf2p (S.-C.Teng, unpublished data), while anti-Myc monoclonal antibodies (Clontech, Palo Alto, CA) were used for myc-tagged proteins. The Rtf2p antisera was raised in rabbits against full-length, glutathione S-transferase-tagged Rtf2p purified from Escherichia coli and affinity purified (S.-C.Teng, unpublished data). The negative control for samples precipitated with polyclonal antibodies was a sample immunoprecipitated with polyclonal serum to the non-telomere protein Ypr1p (provided by G.Waters).

Immunofluorescence

For immunofluorescence localization (Tham et al., 2001), diploid cells were labeled using a 1:2000 dilution of affinity-purified (W.-H.Tham, unpublished data) α-Rap1 polyclonal antibody (Conrad et al., 1990) or haploid myc-Thp1p cells were labeled with a 1:200 dilution of α-myc monoclonal antibody (Clontech). The secondary antibodies were 1:100 dilutions of Alexa Fluor 546 and Alexa Fluor 488 (Molecular Probes, Eugene, OR).

Acknowledgements

We thank P.P.Liu for the tel1H allele; S.-C.Teng, M.G.Waters and W.-H.Tham for antibodies; A.Taggart and L.Goudsouzian for help with chromatin immunoprecipitation; and M.Mondoux for help with immunofluorescence. We also thank V.Brett and E.Gilson for commenting on a previous version of the manuscript, and T.Fisher, M.Mondoux, M.Sabourin and A.Taggart for comments on the manuscript. This work was supported by National Institutes of Health grant GM43265 to V.A.Z.; M.K.A. was supported by a HHMI predoctoral fellowship and through NIH cancer training grant CA09528 to the Department of Molecular Biology at Princeton University.

References


Received November 7, 2002; revised January 9, 2003; accepted February 12, 2003

**Note added in proof**

Using chromatin immunoprecipitation, we find that Yku80p, a subunit of the heterodimeric Ku complex, binds well to the C3Ta2 telomere.