DYNAMICS OF TELOMERE RECOMBINATION IN THE YEAST *KLUYVEROMYCES LACTIS*

by

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ABSTRACT

Telomeres are normally maintained by the enzyme telomerase. While telomerase is active in germline and stem cells in humans, it is inactive in most somatic cells. However, 90% of human cancers have telomerase activity that acts to immortalize cancer cells. A significant minority of cancers use another pathway, called Alternative Lengthening of Telomeres (ALT) to generate long and heterogeneous telomeres by recombination. However, the pathways which lead to ALT cancers are not well understood. Telomerase deletion mutants in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* have been extensively used as model systems to study recombination at telomeres. In our studies, using telomeric repeats known to be disrupted in Rap1p binding, we have shown that recombinational telomere elongation can occur that more closely resembled that of ALT cancers. This suggests that Rap1p acts to inhibit telomeric recombination. In a second study, we observed the fate of a single abnormally long telomere composed of mutationally tagged "Bcl" repeats in otherwise wild type *K. lactis* cells. We saw frequent truncations of this telomere to near wild type size. Our data indicate that many of these apparent truncations to wild type size actually shortened the telomere to well below wild type size prior to being re-extended by telomerase. We also demonstrated that the long Bcl

telomere could become further elongated by a recombinational process that added additional Bcl repeats onto it. Our results suggest that recombination might be more common at normal telomeres than has been previously recognized.

INDEX WORDS: telomere, recombination, *Kluyveromyces lactis*, Alternative Lengthening of Telomeres, survivor, Rap1, Telomere Rapid Deletion.

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DEDICATION

I would like to dedicate this manuscript to Matt and Jake, my little family. Matt and I met when we were both finishing up our first year of graduate school. During our PhD work, we got married and had our first child. Matt has always been such a great support during the craziest moments in graduate school and has been such a great help during the writing of this dissertation, despite trying to finish up his own PhD.

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CHAPTER 1

LITERATURE REVIEW AND INTRODUCTION

Early Studies of Telomeres

Telomeres are the natural ends of linear chromosomes and are made up of both DNA and protein. Linear chromosomes were first observed by Theodor Boveri in the late 1800's (57). While looking at the chromosomes in the roundworm *Ascaris*, he noticed that at the time that cell division begins after fertilization, the chromosomes fragmented and that these fragmented chromosomes persisted throughout the life of the animal. In the 1930's, Herman Muller and Barbara McClintock noticed independently in their studies of X-ray irradiation of *Drosophila* and maize that broken ends were unstable and would fuse, while natural chromosome ends were relatively stable (129, 143). In 1938, Muller named this free end the "telomere (143)."

After the discovery of semi-conservative DNA replication, a problem was predicted with replicating the ends of linear chromosomes (159, 217). Since DNA synthesis requires a 3′-OH group for priming, a portion of the end would be lost and telomeres would shorten over time, eventually causing loss of a section of the chromosome. Several different mechanisms were proposed in order to get around this problem. One of the first suggested that the ends of chromosomes could be temporarily fused so that priming could take place using the end of another chromosome (22). While this mechanism does occur in linear prokaryotic viruses such as T7 and T4, it is not a common method of telomere maintenance (22). Another method proposed to get around the end-replication problem was to use a covalently bound protein to the

5′ terminus, which would then substitute for the 3′-OH in order to prime lagging strand synthesis [reviewed in (12)]. Although the phage Φ^{29} and some mammalian adenoviruses use this mechanism, it is also not commonly used [reviewed in (12)].

In 1974, Cavalier-Smith proposed that telomeres were made up of a palindromic sequence (26). After incomplete replication of the 5′ end, the 3′ overhang created could loop back around on itself, creating a 3′-OH available to elongate the other strand. After filling in the gap on the opposite strand, the looped strand could be nicked to form an intact end. Bateman expanded this model in 1975 by proposing that the telomere forms a natural hairpin structure and closes off the end by a terminal loop (6). This model holds true for the Vaccinia virus and can be seen in a modified form in *Saccharomyces cerevisiae* in the absence of telomerase in cells unable to undergo recombination (4, 125).

Evidence that telomere elongation by telomerase could provide a solution to the end-replication problem came when the telomere sequence was defined for the rDNA minichromosomes in the ciliate *Tetrahymena thermophila*. In macronuclear DNA, this rDNA is composed of 10,000 copies of 21 kb palindromic linear molecules (49, 89). When the sequence was determined by *in vitro* labeling and other analytical methods, 20-70 tandem repeats of 3′-CCCCAA-5′/5′-GGGGTT-3′ were found at the ends (13, 15). There were no covalently attached proteins to the ends. These findings disproved both the hairpin model of end protection as well as the model in which a covalently bound protein substitutes for the 3′-OH needed for priming. In 1984, *S. cerevisiae* telomeres were found to have 300 bp of tandem irregular repeats with the sequence $C_{1\text{-}3}A$ (185). When the *Tetrahymena* telomeres were introduced into yeast, they acquired yeast repeats at their termini (185).

An enzyme acting as a terminal transferase was indicated in *Tetrahymena* cell free extracts when primers of the G-rich sequence of *Tetrahymena* or of *S. cerevisiae* were elongated with only the addition of dGTP and dTTP (72). Telomerase was determined to be a ribonucleoprotein in 1987 (73). After telomerase activity was discovered in humans, it was found that its activity was low in human somatic cells but high in cancer cells (38). Because it is an important factor in maintaining cancer cells, it was recognized as a potential target for cancer therapy (90).

Overview of Telomeres and Their Functions

While telomeres are most commonly composed of tandem repeats, their sequence, length, and protein compositions vary greatly among organisms. The yeast *S. cerevisiae* has irregular tandem repeats of $G_{1\text{-}3}T$ at the G-strand of telomeres (185). Humans have telomeres made up of homogeneous sequences of TTAGGG (142), while *Kluyveromyces lactis* has a homogeneous 25 bp sequence (130) (for a list of sequences from several organisms, see Table 1.1). The telomeres from the hypotrichous ciliate *Euplotes crassus* are only 42 base pairs in length on the G-strand, while *S. cerevisiae* and *K. lactis* telomeres are a few hundred base pairs in length, human telomeres are heterogeneous in length and have sizes of 5-15 kb, and telomeres in strains of laboratory mice are even longer (42, 92, 98, 185, 194).

Because telomeres potentially could resemble double-strand DNA breaks, they are "capped" by several double-stranded and single-stranded telomere binding proteins in order to protect them from homologous recombination (HR) and from fusions created by non-homologous end joining (NHEJ). One unrepaired double-strand break generated by induction of the HO endonuclease or 5-10 kb of single-stranded DNA can cause cell cycle arrest in *S.*

cerevisiae (183, 192). In humans, avians, plants, mice, and multiple protists, telomeric ends appear to often be capped by a structure called a telomeric loop (t-loop), in which a 3′ overhang at the telomere end is believed to intramolecularly strand invade internal telomeric sequences (29, 74, 144, 147, 152). This could potentially act as a structure to prevent the recognition of a telomere as a double-strand break (74).

Little is known about the genetic requirements for NHEJ and HR at telomeres. At genomic double-strand DNA breaks, NHEJ joins broken ends throughout the cell cycle, but competes with HR for access to broken ends during late S/G2 phase [reviewed in (193)]. During NHEJ, the Ku70/80 complex is recruited to broken ends, which then recruits the DNA-dependent protein kinase and finally Lig4p for rejoining the two ends. HR requires extensive resectioning of the 5′ strand ends of double-strand breaks and subsequent recruitment of Rad51. A detailed description of HR in the yeast genome and at telomeres will be described in detail later in this chapter.

Telomeres develop an evolutionarily conserved "bouquet" clustering in meiosis [reviewed in (41)]. During prophase I in the leptotene/zygotene transition, telomeres cluster at the nuclear periphery near the centriole. This clustering appears to facilitate interactions between homologous chromosomes and could also play a role in meiotic recombination. Telomere clustering also appears to be important for proper chromosome segregation. In the budding yeast *Saccharomyces cerevisiae*, telomeric attachment to the nuclear envelope is dependent on *NDJ1* (36).

Another feature of telomeres is their role in Telomere Position Effect (TPE) [reviewed in (161)]. When a gene is introduced in the subtelomeric region of telomeres, it can be silenced when telomeres are of wild type length, but expressed when telomeres are short. This silencing is

dependent on the C-terminus of Rap1 (97). The Ku70/80 complex also appears to be important for silencing (137). Ku70/80 binding appears to block the inhibitory factors Rif1p and Rif 2p from binding to telomeres, since mutations in these inhibitory factors overcome the need for Ku70/80 for telomere silencing. However, binding of Sir2-4p to Rap1p promotes silencing at telomeres (140, 208). There is also evidence that TPE can occur in human cells (8, 93).

Because telomeres are subject to gradual loss due to the end replication problem, organisms have developed a variety of ways to extend their ends. While the most common method to extend telomere ends is by telomerase, some organisms employ other methods. *Drosophila* uses what is possibly the most unique of telomere maintenance mechanisms. The telomeres in *Drosophila* are made up of the long terminal repeat (LTR) retrotransposons *HeT-A*, *TART*, and *TAHRE*, which are maintained by gene conversion and transposition [reviewed in (179)]. Because the ends of *Drosophila* chromosomes are not composed of simple repeats, they are presumably not capped in the same sequence-dependent manner as telomerase-maintained telomeres. Instead, any sequence present at the end of a chromosome is capped by several proteins, including ATM and ATR, which appear to cap telomeres in humans as well.

Although the majority of mitochondrial chromosomes are circular, several organisms have shown the presence of linear mitochondrial chromosomes. In the fungal pathogen *Fusarium oxysporum*, there are linear mitochondrial plasmids that have a hairpin at one end and 5 bp repeats at the other (214). Several *Tetrahymena* species, including *T. thermophila*, have linear mtDNA which terminate in 31-53 bp repeats (141). These were proposed to be maintained by recombination as suggested by the finding that, in at least one case, the repeats were different on the two ends of the same DNA molecules. The yeasts *Candida parapsilosis*, *Pichia philodendra*, and *Candida salmanitcensis* all also appear to have linear mtDNA. The telomeres

of these molecules appear to be maintained by making use of their palindromic sequences (154), which will be described later in this chapter. Linear mitochondrial telomeres have been most studied in *C. parapsilosis*. This yeast has a 738 bp mitochondrial telomere with inverted tandem repeats. It has a 5′ single-stranded extension of 110 nt (153). It also has the first identified mitochondrial telomere binding protein, which appears to have single-strand binding activity (202). These linear mitochondrial chromosomes appear to be maintained by recombination and small circles of telomeric repeats have been isolated from *C. parapsilosis* (154, 203). A proposed mechanism for the use of these circles in recombinational telomere elongation will be described later in this chapter.

Telomerase-Mediated Telomere Maintenance

Since its discovery, telomerase-mediated telomere elongation has been found to be a widespread telomere maintenance mechanism and has been identified from many organisms including several ciliates, the plant *Arabidopsis thaliana*, the yeasts *S. cerevisiae*, *Kluyveromyces lactis*, and *Schizosaccaromyces pombe*, mice, and humans [reviewed in (21)]. Telomerase has been most thoroughly studied in *S. cerevisiae* and in humans, so telomere maintenance in these two organisms will be discussed thoroughly in this section.

In *S. cerevisiae*, core telomerase is made up of the RNA component Tlc1 and the protein components Est1p, which facilitates recruitment and activation of telomerase, Est2p, which is the catalytic component containing reverse transcriptase, and Est3p, which plays an unknown role in the telomerase complex but which plays a role in telomere replication (104, 105, 113, 190, 198). In humans, the RNA component is hTR and the reverse transcriptase is hTERT with no other known core components (55, 149). Interestingly, the yeast Tlc1 appears to be exported to the

cytoplasm to be assembled into the enzyme while the human hTR is initially localized to the Cajal body and carries a box H/ACA RNA domain, which binds sno proteins and directs pseudouridylation of spliceosomal snRNAs [reviewed in (91)].

Telomerase binds to a short 3′ overhang at the telomere end. In *S. cerevisiae*, this overhang is 10-15 nucleotides throughout the cell cycle, but it increases to ~50-100 nucleotides in late S phase when telomerase is active (11). In *S. cerevisiae*, telomerase only elongates the shortest telomeres in each cell division, although the signal for this preference has yet to be discovered (199). Evidence indicates that telomerase can form dimers and higher-order multimers, potentially to elongate sister chromatids (169). In contrast to *S. cerevisiae*, human telomeres have a longer 100-250 nucleotide overhang (120, 226). However, the shortest telomeres in human cells are also preferentially elongated (182). Interestingly, this preference does not appear to exist in telomerase-positive human cancer cell lines (230).

In general, the RNA component of telomerase contains a sequence precisely complementary to the telomere end. A short alignment region prior to the template, which is 11 nt long in the human telomerase RNA hTR, allows telomerase to bind in a sequence-specific manner to the 3' overhang of the telomere at the G-rich strand (33). Telomerase then reverse transcribes sequence onto the telomere end, thus elongating the telomere. Telomerase is also capable of translocating to the new end of the telomere, where it reverse transcribes another repeat onto the end [reviewed in (160)]. In *S. cerevisiae*, the helicase Pif1p negatively regulates telomerase addition and is thought to aid in unwinding telomerase from the template (18). After elongation, the lagging strand DNA polymerases can elongate the C-rich strand.

Telomerase is recruited to the telomeres and activated through the actions of several proteins. Most of what is known about telomerase recruitment has been determined in *S.*

cerevisiae. Telomerase is active at the telomere only in late S phase [reviewed in (160)]. Although the catalytic subunit of telomerase Est2p in combination with the RNA subunit Tlc1 is constitutively present at the telomere, its protein levels peak in G1 and late S phase (198). The peak association in G1 is dependent on the interaction of a stem loop of Tlc1 with the Ku70/80 complex (10). During telomerase extension in late S phase, the longer telomere overhangs are generated and the single-strand telomeric binding protein Cdc13p becomes phosphorylated by the cell-cycle regulator Cdk1p (108, 221). This phosphorylation initiates an increased recruitment of Est1p to the telomere. Est1p brings the telomerase holoenzyme to the telomere for extension (31).

The Role of Telomere Binding Proteins in Telomere Maintenance

Both the double-stranded region of the telomere and the single-stranded overhang are protected from the cell's double-strand break repair pathways by several proteins that appear to "cap" the telomere end. The proteins at telomeres also regulate telomerase recruitment, telomere length, the generation of the telomere overhang, telomere replication, and telomere-telomere recombination.

Double-strand telomeric binding proteins are found in many organisms throughout evolution, though different proteins play this role depending on the organism. In *S. cerevisia*e and *K. lactis*, the N-terminus of the transcription factor Repressor Activator Protein 1 (Rap1p) binds double-stranded telomeric repeats at a specific binding site on each repeat (65, 94, 115, 118). It is maximally present at the telomere during late S phase and G2 and is partially dislodged during G2/M (100, 191). Its binding protects telomeres by preventing telomere-telomere fusions by the Non-Homologous End-Joining (NHEJ) pathway (162, 210). While it

participates in telomeric silencing and in the formation of G-quadriplexes at telomeres (63), its main role is in regulating telomere length.

Rap1p negatively regulates telomere length by limiting telomerase extension at a telomere (94, 97, 123, 172). The C-terminus of Rap1 is important in this negative regulation (97). Rap1 is thought to bind to each telomeric repeat of a telomere. The length of the telomeres appears to inversely correlate with the number of Rap1 binding sites available at that telomere in a protein counting mechanism (123). The proteins Rif1p and Rif2p, which bind to the C-terminus of Rap1p, also appear to negatively regulate telomere length by the same pathway (106). Because Rap1p has the ability to bend DNA, one hypothesis is that Rap1p causes the telomere to loop around on itself and cause an interaction between the Rap1p proteins as well as between the Rif1p and Rif2p proteins, thus negatively regulating telomere length (62, 173). It has been proposed that Rap1p could have a role in telomere recombination because its binding can contribute to the unwinding of duplex DNA (82). During DNA damage situations, Rap1p and is released from the telomere in a *RAD9*-dependent manner (126). Interestingly, Est2p binding to telomeres appears to influence the amount of Rap1p present at *S. cerevisiae* telomeres (87). In another study, it appeared that Rap1p's C-terminus also regulated the amount of telomeric repeat turnover at telomere ends (95).

The proteins that are known to bind the 3′ overhang at telomeres also have a substantial role in telomere length regulation and form an RPA-like complex at telomeres ends. Cdc13p has been shown to bind sequence-specific single-stranded $TG_{1\text{-}3}$ telomeric DNA at yeast telomeres (83, 110, 156). A loss of Cdc13p at the telomere causes telomere degradation and cell cycle arrest in G2/M, which is dependent on *RAD9* (119, 219, 220). Several mutations have highlighted Cdc13's role at telomeres. One of these is the temperature sensitive *cdc13-1*

mutation, which results in the accumulation of single-stranded DNA at telomeres at 37 °C and extensively elongated telomeres at permissive temperatures (60, 70). This phenotype implicates Cdc13p in the negative regulation of telomere length and also in the coordination of telomerase elongation with telomere replication by the lagging strand replication machinery. Another mutation, called *cdc13-5*, has separated Cdc13p's role in coordinating the lagging strand replication machinery from its role in negatively regulating telomerase addition, causing a defect in Cdc13p's interaction with Polymerase α (32). A third mutation, called *cdc13-2*, shows a progressive telomere shortening phenotype and highlights the function of Cdc13p in telomerase recruitment by its interaction with Est1 (10, 112, 156, 163, 171). The molecular chaperone Hsp82p also appears to play a role in Cdc13's interaction with Est1 (44). Cdc13's role in telomere length appears to be regulated by its phosphorylation status. Tel1p/Mec1p phosphorylation of Cdc13p has been shown to be important for telomerase recruitment (207). Telomere length is also regulated through a Cdk1p-dependent phosphoylation of Cdc13p, which causes specific telomerase recruitment to telomeres during S/G2 phase. This phosphorylation of Cdc13p appears to block binding of the inhibitory Stn1p/Ten1p complex (108). This Cdk1p-dependent phosphorylation also regulates the degradation of Cdc13p after late S/G2. Another role of Cdc13 is its role in the prevention of homologous recombination at telomeres, which will be discussed later in this chapter (69). In humans and in *S. pombe*, the protein POT1 appears to fulfill the role of a single-strand binding protein (7). However, a mammalian Ctc1-Stn1-Ten1 complex has been discovered in higher eukaryotes, which appears to play a role in telomere metabolism (138). In *Arabidopsis* and humans, a depletion of CTC1 causes telomere instability and increased G overhangs (197).

One of the main roles of Cdc13p is binding the telomerase inhibitory proteins Stn1p/Ten1p to negatively regulate telomere addition (68, 70, 170). Along with its role in negative regulation of telomere length, Stn1p binds both the POL12 subunit of the Polymerase α primase and the C-terminus of Cdc13p, which highlights its role in the recruitment of the lagging strand machinery to the telomere (32, 58, 61, 76, 170). Stn1p's N-terminus binds Ten1p in order to negatively regulate telomerase addition. Stn1p and Ten1p together provide an essential capping function separate from Cdc13p's role, which depends on the recruitment of the lagging strand machinery to the telomere (165). Ten1p also enhances the DNA-binding ability of Cdc13p, participating in its capping function (228). The mRNA levels of both Stn1p and Ten1p along with those of Est1p, Est2p, and Est3p are controlled by the UPF genes, which have a role in nonsense-mediated RNA decay (40). Stn1p also appears to be important to inhibit telomere-telomere recombination, as the *stn1-M1* mutant in *K. lactis* causes extensive recombination at telomeres and leads to highly elongated telomeres and abundant telomeric circles (5, 86).

Several checkpoint and DNA repair proteins also play a role at telomeres. The Ku70/80 heterodimer plays an important role in NHEJ throughout the genome and causes telomere shortening and long 3′ overhangs if deleted and increases the instability of long telomeres (20, 71, 167, 168, 205). It has also been implicated in telomeric silencing and subnuclear localization of telomeres (19, 99). Ku70/80 also acts with Cdc13p as a positive regulator of telomerase, which is dependent on the C-terminus of Ku70p (46, 68). Ku seems to load first at telomeres, followed by Cdc13p (227). Ku is rapidly released from telomeres in the event of *RAD9*-dependent DNA damage response and also seems to control replication at telomeres (37, 126). Interesting data has shown an interaction of Ku with the Tlc1 telomerase RNA in *S. cerevisiae* (195). Data from *K. lactis* showed that loss of Ku80p caused an increase in subtelomeric

recombination and also increased 3′ overhangs (25). However, unlike *S. cerevisiae*, the *K. lactis* Ku80p was not required for normal telomere length maintenance.

The MRX complex (Mre11p-Rad50p-Xrs2p) also has an important role in telomere length maintenance by the acting in the telomerase recruitment pathway to prepare the telomere for the loading of Cdc13p (19, 45, 64, 155, 209). The MRX complex appears to function in a single pathway with the checkpoint protein Tel1p (176). Xrs2p has also been shown to function in this pathway (39). The presence of the MRX complex at telomeres is required for proper constitutive G-tails throughout the cell cycle, but not in late S phase (101).

Since several proteins bind the 3′ overhang at telomeres, proper resectioning of the 5′ end is important for telomeric capping [reviewed in (136)]. This resectioning at telomeres appears to be partially dependent on the MRX/N complex in both yeast and humans (30, 101). Another protein implicated in telomere resectioning is Exo1p, which is a member of the Rad2p family of structure-specific nucleases and contains a 5′ to 3′ exonuclease and a 5′ flap endonuclease [reviewed in (136)]. Exo1p can generate ssDNA at telomeres in the absence of both Ku and Cdc13p, but does not appear to be essential for resectioning at wild type telomeres (124, 231). The details of telomeric resectioning in *S. cerevisiae* are still elusive.

Importance of Telomeres in Human Cancers and Aging

In culture, human fibroblasts are only able to undergo about 50-80 cell divisions, which illustrates their replicative aging (79). This replicative aging is caused by replicative senescence, which is a permanent growth arrest in a cell that is still metabolically active. While there is some argument as to what directly causes senescence, the expression of telomerase in cells is enough to prevent it, so there is direct evidence that telomere length is a factor (16). A small number of

short telomeres are predictive of the timing of senescence (80). A different pathway, called STASIS, can also trigger senescence based on a cellular stress response [reviewed in (187)].

In cultured cells, short telomeres appear to initiate a DNA damage response through ATM/ATR signaling for the cell to enter mortality stage 1, or M1, which causes a cell cycle arrest [reviewed in (188)]. These senescent cells are still metabolically active and produce stimulatory and restrictive factors for tumors (43, 127, 186, 223). At senescence, 60-85% of telomere overhangs are lost (196). A loss of cell cycle regulators such as p53 bypasses M1. This allows further telomere shortening, which causes an entrance into mortality stage 2 (M2), or crisis, where mitotic catastrophe and apoptosis become dominant, resulting from breakage-fusion cycles [reviewed in (188)]. One in 10 million human cells can survive M2. This survival can occur by reactivating telomerase or upregulating its activity, although certain cancer cells can upregulate telomerase before this stage is reached (225, 226).

The observation that telomere attrition leads to senescence has been elusive in pre-cancerous cells *in vivo*, but telomere attrition can be seen readily within tissues (188). It is known that reactivation of hTERT before M1 or between M1 and M2 causes direct immortalization of cells (139). Approximately 90% of cancers have active telomerase (147). While telomerase appears to be active in most cancer cells, it takes several mutations to form a cancer cell and 20-30 cell divisions likely passes before a single mutation occurs (188). Cancer cells appear to have a different method of regulating telomere elongation than normally dividing cells, since all telomeres are accessible to telomerase instead of only the shortest telomeres (230).

While hTR the telomerase RNA can be found in all cell types, hTERT is highly expressed only in germline cells, stem cells, and immortal cancer cell lines (1, 56). In embryonic tissues, hTERT is highly expressed, but is silenced later in development (224). However, even

most of the cell types that express telomerase have gradual telomere shortening over time. Both dyskeratosis congenita and idiopathic pulmonary fibrosis can result from mutations in the telomerase pathway which cause advanced telomere attrition (59). While the role of telomerase in these diseases is well-known, the role of telomere attrition in aging is less defined. It is known that telomeres are shorter in older people than in younger and that people in chronic stress conditions on average have shorter telomeres (51, 52, 78, 111, 157).

Early Studies of Recombination at Telomeres

Before telomerase was known to be a conserved enzyme among eukaryotes, telomere maintenance was proposed to be based upon recombination (166, 216). Studies in the early 1990's placed telomeric repeats from the ciliates *Oxytricia nova* and *Tetrahymena thermophila* at telomeres in *S. cerevisiae* and saw the occasional transfer of these repeats from one telomere to another (166). However, although recombination appears to maintain the telomeres in the mosquito *Anopheles gambia* as well as the ends of certain linear mitochondrial DNAs (154, 180), telomerase was found to be the primary mode of telomere maintenance in the majority of organisms.

In 1993, recombination was shown to maintain telomeres in a deletion of the telomerase gene *EST1* in *S. cerevisiae* (116). While the majority of these cells died within 50-100 cell divisions, there were rare survivors that formed in culture. These survivors were dependent on the recombination gene *RAD52*. When the telomere structures of these survivors were observed, it was discovered that they had amplified the subtelomeric repeat tracts, called Y′ elements, that are normally interspersed within telomeric repeats in *S. cerevisiae*. This recombination evidently

acted to maintain terminal telomeric repeat tracts at the chromosome ends, though those tracts remained shorter than those in wild type cells.

Unlike *S. cerevisiae*, the yeast *Kluyveromyces lactis* has no internal telomeric repeats surrounding its subtelomeric sequences. In mutants containing a deletion in the *K. lactis* telomerase RNA gene *TER1*, rare survivors of senescence also formed (131). These survivors were also dependent on *RAD52* and had amplified terminal telomere repeat arrays, which was the first evidence of this type of amplification. This study also showed a progressive growth decline of cells during the process of senescence and showed a growth improvement when telomeres were elongated (131).

Around the same time, human systems were being developed in order to study the length polymorphisms of human telomeres. John Murnane and colleagues integrated a simian-virus 40 plasmid at the telomere on chromosome 13 in order to tag this telomere and observe potential changes in telomere length (146). While this cell line lacked telomerase, he was able to see marked increases in the size of this telomere, especially at times it had gradually shortened to less than a few hundred base pairs in size. This was the first evidence for a telomere maintenance mechanism in the absence of telomerase in human cells.

The next big discovery was that a significant minority of human cell lines did not have an active telomerase (90). In 1995, a study in Roger Reddell's lab showed that several immortalized human cell lines that had no detectable telomerase activity had telomeres that were extremely long and heterogeneous (23). These studies were the predecessors of others on the telomere maintenance mechanism in ALT cancers, which relies on recombination and will be described in detail in the next section.

Telomere Elongation by Recombination in the Absence of Telomerase

While telomerase activation appears to be the primary method used by human cancers to maintain telomere length, a significant minority of cancers, usually those of mesenchymal origin, appear to use an alternative pathway called Alternative Lengthening of Telomeres (ALT), which is thought to be primarily dependent on recombination (reviewed in (145)). The telomeres in ALT cells appear as heterogeneous smears in a gel, with many telomeres reaching limit mobility (23, 178). While many of the telomeres in these cells are long, some are very short or even have no telomeric repeats at all (164, 178, 215). In cultures of immortalized fibroblasts where ALT was discovered, long and heterogeneous telomeres only appear in a subset of cells in culture while other cells are senescent (178).

While little is known about how the ALT mechanism is triggered, several pieces of data indicate that recombination maintains the telomeres in these cells. One is that APB bodies (ALT-associated Premyleocytic leukemia bodies) cluster near the telomeres in these cells and contain telomeric DNA, the telomere-associated proteins TRF1 and TRF2, and the recombination proteins MRE11, RAD50, and NBS1 (229). Another characteristic is that ALT cancers can transfer a nontelomeric tag placed into the telomeric repeats from telomere to telomere (47). Also, when telomeric DNA in ALT cells was observed by electron microscopy, telomeric DNA in both linear and circular form was found (27, 158). Other work showed that deprotection of telomeres can cause large telomeric deletions, which could account for the extrachromosomal circles and other DNA found in ALT cells (215). Sister chromatid exchange events are common in ALT cells and indicate one potential mechanism of the telomere recombination in these cells (3). These sister chomatid exchanges are reciprocal and can be large enough to be seen using microscopy. Occasionally, an entire telomere is apparently lost when

the sister chromatid is elongated. Therefore, reciprocal sister chromatid exchange has been hypothesized to be one recombinational mechanism to generate the heterogeneity of telomere length in ALT cancer cells.

Recently, mouse telomeres have been shown to be elongated by recombination during a specific stage of early development (114). Telomeres measured about 17-18 kb in oocytes, but added 10-13 kb of sequence to the end of their telomeres during the early cleavage cycles in the embryo. Reciprocal sister chromatid exchanges appear to be common in these cells.

Yeast model systems, most notably *S. cerevisiae* and *K. lactis*, have been extensively used to determine how recombination can act at telomeres. Although yeast cells are naturally telomerase positive, deletions of any of the telomerase components cause gradual telomere shortening and eventually replicative senescence when telomeres reach a length of ~100 bp (2, 105, 117, 132, 190). Replicative senescence in these cells is dependent on the checkpoint genes *MEC1*, *DDC1*, *MEC3*, and *RAD24*, which trigger a cell cycle arrest through *RAD53* using either *RAD9* or *MRC1* (2, 50, 66).

Although most cells do not survive the senescence process, rare survivors emerge that have lengthened telomeres (116, 131, 201). The survivor formation is largely dependent on the recombination gene *RAD52* and thus is linked to recombination. Once survivors emerge, they are not stable and can redevelop a senescence phenotype (131). However, secondary senescence is often not as severe as primary senescence. It appears that a single long telomere suppresses senescence, while having telomeres that are initially short increases its severity (131).

When the telomeres of survivors are observed in *S. cerevisiae*, two distinct telomere maintenance pathways can be found (Figure 1.1). The first type of survivors elongate their telomeres using a process called Type I recombinational telomere elongation (RTE), which is

dependent on the recombination gene *RAD52* (116). In culture, Type I survivors are the first to emerge, but grow slowly relative to wild type. When their telomeres are observed, they have amplified subtelomeric Y′ elements. Y′ elements are present at telomeres in wild type cells and are normally interspersed with telomeric repeat elements in *S. cerevisiae*. During Type I RTE, their amplification can cause them to equal up to 10% of the entire DNA in the cell (116). However, the terminal telomeric repeat tracts in Type I survivors are shorter than those in a telomerase positive cell (116).

After continued growth in culture, a different type of *RAD52*-dependent survivor begins to form in *S. cerevisiae*. These survivors lengthen their telomeres by a process called Type II RTE, which amplifies the telomeric repeats instead of the subtelomeric Y′ elements (116, 200, 201). While they form after Type I RTE in liquid culture, their growth quickly overtakes the culture (116). The telomeric repeat tracts can be up to 10 kb, much longer than those in wild type cells or in Type I survivors (201).

The telomere structure in the yeast *Kluyveromyces lactis* is quite different than the telomere structure in *S. cerevisiae*. *K. lactis* has homogeneous 25 bp telomeric repeats that only exist as tracts at the very termini of chromosomes (130). Data has shown that if a subtelomeric tract of telomeric repeats is introduced, separated from the true telomere by a *URA3* marker gene, then survivors amplifying the *URA3*-telomere sequence (reminiscent of the Type I pathway) can readily be found (150). However, in otherwise wild type *K. lactis* cells containing telomerase deletions, survivors using Type II RTE are the only survivors found (131). Interestingly, the telomeres in cells using the Type II pathway are rarely elongated more than 1-2 kb, which is substantially shorter than the telomeres in *S. cerevisiae* survivors using the Type II pathway (201).

Recombination Pathways Potentially Acting at Telomeres

While survivor formation in yeast appears to be reliant on recombination, recombination at telomeres is quite different from recombination throughout the genome. The majority of genomic recombination, at least that involved in the repair of double-stranded breaks (DSB), in mitotically growing yeast cells is dependent on the synthesis-dependent strand annealing pathway (SDSA) (Figure 1.2) [reviewed in (96)]. At the beginning of this pathway, a DSB is recognized by the MRX (Mre11p-Rad50p-Xrs2p) complex. At the beginning of double-strand break repair, 5′ to 3′ resectioning takes place. Deletion of the nuclease of Mre11p decreases resectioning only two-fold and Exo1p has also been implicated in this pathway [reviewed in (136)]. The cell cycle regulator Cdk1p is required for this resectioning to take place (85). RPA coats the 3′ single-stranded overhang and is then replaced by Rad51p, which is homologous to *E. coli* RecA. Rad52p appears to help in the replacement of RPA with Rad51p. Rad55/57p and Rad54p help to facilitate strand invasion and Polymerase ε, PCNA, and Polymerase δ are recruited to the invading strand to perform extension. The invaded strand is then dissociated and the broken strands are ligated back together. A secondary recombination pathway in *S. cerevisiae*, called Single-strand annealing (SSA), requires *MRX*, *RAD52* and *RAD59* (Figure 1.2) (reviewed in (96)). During this pathway, a double-strand break occurs between direct repeats. After resecting the 5′ ends, the exposed repeats can anneal together, creating a deletion.

Break-Induced Replication (BIR) is a likely candidate for recombination at telomeres (Figure 1.2) (213). BIR is a type of DSB repair that occurs when only a single end is able to participate in homologous recombination. It involves the formation of a replication fork that copies DNA of the strand-invaded chromosome up to the full length of a chromosome arm, thereby creating non-reciprocal translocations (17, 53, 54). There is a long delay before the

initiation of BIR, presumably in order to allow repair to occur via SDSA, if at all possible (121). One piece of evidence supporting that BIR acts at telomeres is that 6% of *S. cerevisiae* telomeres suffer terminal deletions in survivors of telomerase deletion mutants, which is dependent on the resectioning exonuclease Exo1p (77). These deletions are not completely restored by reactivating telomerase. In a mechanism related to that of SDSA, BIR can use the *RAD51*-dependent pathway in order to act (121). However, it can also use a pathway similar to SSA that requires *MRE11, RAD50, XRS2*, *RAD59*, and *TID1* (189). It is possible that there is a third pathway which contributes to BIR since cells with deletions in essential components of both of these pathways can still undergo BIR. It has been hypothesized that ~10% of BIR events use this third pathway (189). *RAD51*-dependent BIR appears to be more efficient than *RAD50*-dependent BIR because, unlike *RAD50*-dependent BIR, *RAD51*-dependent BIR can be initiated at less than 3 kilobases away from the original break (121, 122).

While the BIR pathway is still being defined, there are many things known about its mechanism. BIR can be initiated using homologous segments within nonhomologous chromosome arms and can use homology as small as 70 bp (17). As mentioned above, compared to gene conversion by SDSA, BIR has a long delay in its initiation, which is dependent on the formation of a replication fork (121). The *RAD50*-dependent BIR pathway seems to initiate BIR at shorter regions of homology (<100 bp) compared to the *RAD51*-dependent pathway (84).

In *S. cerevisiae*, Type I Recombinational Telomere Elongation (RTE) appears to require the *RAD51*-dependent pathway, perhaps because the Y′ elements are more homogeneous than the telomeric repeats and because this pathway usually requires >100 bp of homology to act (103, 201). However, Type II RTE appears to be dependent on MRX and *RAD59*, but unlike *RAD51*-independent BIR, does not rely on the helicase *SGS1* (34, 103, 200, 201, 209). Because

the telomeric repeats in *S. cerevisiae* are heterogeneous in length and sequence, shorter stretches of homology are present in the telomeric repeats. This could be the reason for the reliance of Type II survivors on the *RAD51*-independent pathway. The PI3K checkpoint proteins Tel1p and Mec1p appear to be important to the production of Type II survivors. If one of these proteins is missing, the production of Type II survivors is reduced and if both are missing, cells only produce Type I survivors (206). In both Type I and Type II survivors, polymerase δ appears to be responsible for elongating the telomere tracts (206). However, a mutant in polymerase ε causes quicker telomere loss and survivor formation, but only Type II survivors (206).

While Type I and Type II RTE appear to be the major players in survivor formation by recombination, some data indicates that there may be a very minor third pathway (67). Investigation into a potential third pathway led to the investigation of PAL survivors, which form in the absence of telomerase and recombination in *S. cerevisiae* mutants which lack the putative resectioning exonuclease Exo1p (125). PAL survivors rely on the creation of inverted repeats, or palindromes, at the end of the chromosome. These inverted repeats can then fold and pair with noninverted repeats more internal into the chromosome after 5′ resectioning. This pairing can then allow elongation of the 5′ overhang without the use of telomerase or recombination.

Data also shows that senescence and survivor formation can occur in the presence of telomerase. In *S. cerevisiae* and *S. pombe*, *tel1 mec1* double mutants have senescence phenotypes (175). In *S. cerevisiae*, the *cdc13-1* mutation combined with mutations in checkpoint proteins allows senescence and survivor formation to occur when telomerase was active (69). Interestingly, in *S. cerevisiae*, *cdc13-1 mec3* cells had a similar phenotype to Type II RTE despite the presence of telomerase (69). This survivor formation was dependent on *RAD50* and *RAD52*, but not *RAD51* (Figure 1.1). Type II-like survivors also appeared to arise in *cdc13-1*

yku70 mutants in telomerase-positive cells, but were more heterogeneous in length than standard Type II survivors (67).

Roll and Spread Model for Recombinational Telomere Elongation

The mechanism for Recombinational Telomere Elongation (RTE) has been extensively studied in the yeast *Kluyveromyces lactis*. Because *K. lactis* has long and homogeneous telomeric repeats that can be tagged with phenotypically silent mutations, then it is possible to observe the movement of tagged repeats to further study RTE. Currently, the evidence suggests a "Roll and Spread" model for RTE (28, 75, 135, 151, 204). During the "Roll and Spread" mechanism, a small circle of telomeric repeats (t-circle) is formed using telomeric sequence. This t-circle can act as a rolling circle template to elongate a single shortened telomere in the cell, the sequence of which can then be copied by the other telomeres in the cell using BIR-like events (see Figure 1.3).

The evidence for the Roll and Spread model comes mainly from *K. lactis*, but several different organisms also show support for this model. One important observation came from transforming a circularized DNA fragment containing telomeric repeats and a *URA3* gene into *K. lactis* cells (151). After transformation, either in the presence or in the absence of the *K. lactis* telomerase RNA gene (*TER1*), telomeres were elongated by the acquisition of tandem arrays of the telomere-*URA3* sequence at one or more telomeres. This was especially frequent in *ter1* deletion cells. A second line of evidence came upon close observation of telomeric sequence in Southern blots of a *TER1* template mutant called *ter1-16T*, which synthesized telomeric repeats which have a point mutation in the binding site for the double-stranded telomeric DNA binding protein Rap1 (75). Telomeres in this mutant are very long and heterogeneous, but a significant

portion of the telomeric signal, even from genomic DNA not digested with a restriction enzyme, ran at sizes below 0.5 kb, which indicated that it was both small and extra-chromosomal. Upon examination of this DNA by electron microscopy, it was found to be largely circular in nature. Both double-stranded and single-stranded telomeric-circles (t-circles) were found in similar abundance at sizes as small as ~100 bp. Later, much larger double stranded t-circles were also found to be present in *ter1-16T* cells (28).

Another important piece of evidence has shown that short telomeres are recombinogenic. In *ter1-Taq* cells and in other mutants which contain chronically short telomeres, there is a highly elevated rate of loss or duplication of a subtelomeric *URA3* gene through gene conversions (presumably due to BIR events) that replace one telomere with sequence from another telomere (135). More direct support for the roll and spread model comes from experiments that generated post-senescence survivors in a *TER1* mutant that initially contained a single long telomere with repeats mutated to contain a phenotypically silent *Bcl*I restriction site (204). When the tagged telomere was initially distinctly longer than other telomeres, its sequence was spread to all other telomeres in greater than 90% of the survivors. However, if the tagged telomere was initially the size of the other telomeres in the cell, the sequence from the tagged telomere was copied to all other telomeres ~10% of the time. This indicated that the amplified telomeric sequence in survivors could be generated from a single telomere source and that a single long telomere (a postulated intermediate of the roll and spread model) was preferentially copied to all other telomeres.

Some support for the Roll and Spread Model has also come from work in other organisms. In *S. cerevisiae*, a circle of telomeric repeats and a *kan* gene was introduced into cells, then long tandem arrays containing telomeric repeats and the *kan* gene could be found

(109). In human ALT cells, extrachromosomal telomeric DNA in both linear and circular form has been found (27). T-loops have been found at the ends of chromosomes and t-circles appear to be prominent. Also, t-circles have been found in the mitochondria of *C. parapsilosis*, which have linear telomeres (203). These t-circles are not seen in strains containing circular mtDNA (181).

Unregulated Telomere-Telomere Recombination Can Occur at Uncapped Telomeres

As described previously in this chapter, survivors using the Type II RTE pathway in *K. lactis* are never elongated more than 1-2 kilobases by recombination (131). They also appear to go through rounds of senescence and survivor formation, which led to the hypothesis that telomeres become capped when they are above a minimal functional length, but become uncapped and prone to recombination or telomerase addition when they become critically short (Figure 1.4).

Recently, the telomeric single-stranded capping protein Stn1p was implicated in the prevention of recombination at telomeres in *K. lactis* (86). In the *stn1-M1* mutant, which has a point mutation in the *STN1* gene, telomeres become extremely long and heterogeneous in the absence or presence of telomerase (86). Telomere length can commonly exceed 10 kb in size. Because the telomeres are much longer and more heterogeneous than those produced by Type II RTE in *K. lactis*, this type of RTE was coined Type IIR, or runaway, RTE. Interestingly, *stn1-M1* cells do not go through rounds of senescence and survivor formation. Instead, the colonies are moderately rough at all times, suggesting that telomere uncapping is chronic but not improved or worsened by passaging, unlike what occurs in *ter1* deletion mutants. This led to the hypothesis that telomere recombination can occur at any length if telomeres become uncapped

and that this would eliminate the cycle of senescence and survivor formation that occurs in telomerase deletion mutants (Figure 1.4).

The recombination phenotype in ALT cancers appears to follow a similar pattern. In cancer cells, there is a subset of both senescent cells and growing cells and the telomeres become extremely long and heterogeneous (145, 178). One hypothesis is that ALT cancers result from a mutation in some capping function at telomeres. In our studies, detailed in this chapter, we show that a loss of Rap1p, the double-stranded telomeric binding protein in *K. lactis*, can also cause uninhibited telomere-telomere recombination.

Telomere Rapid Deletion

Telomeres are dynamic structures. While they can be lengthened by telomerase and shortened by gradual replicative loss as has been described previously, they can also be prone to damage, recombination, and other phenomena. Occasionally, telomeres will be shortened dramatically in a single cell division. Dramatic telomere shortening was first observed in the macronucleus in *Euplotes crassus* and *Tetrahymena thermophila* and during antigenic variation in *Trypanosoma brucei* (102, 148, 212), indicating that telomere shortening can occur in a variety of different organisms under different circumstances. One recombinational mechanism for dramatic telomere shortening, called TRD, was first studied in *S. cerevisiae* (107). Upon the observation of cells containing a mutation in Rap1 called *rap1-17*, which is one of the *rap1*^{t} alleles that causes abnormally long telomeres, Lustig and colleagues noticed that, occasionally, telomeres suddenly shortened considerably, much more than would be expected from gradual telomere shortening. When these cells were mated back to cells with a wild type *RAP1* allele, the long telomeres were frequently shortened to within 200 bp of the size of the wild type

telomeres, although there were some intermediate truncations (107). TRD was hypothesized to be a trimming mechanism to shorten telomeres that had become too long. When a single long telomere was tagged subtelomerically with an *ADE1* allele, shortening events were isolated at this telomere in haploid cells at a rate of 1.2×10^{-3} events/telomere/cell division. Furthermore, this shortening was decreased 3-fold upon deletion of *RAD52* (107). Therefore, it was concluded that these events were partially dependent upon recombination. Other experiments in *S. cerevisiae* concluded that TRD was partially dependent on the MRX complex and that it increased 30-70-fold in meiosis. This meiotic TRD was dependent on the meiotic bouquet protein Ndj1p (24, 88).

Further studies looking at the mechanism of TRD determined that *Hae*III sites, which were introduced into the long telomeres via the prior presence of a telomerase RNA gene template mutation, were not moved from their original location after TRD (24). Subsequently, a terminal deletion model was proposed for TRD (model shown in Figure 1.5). The first step of the proposed model was the intramolecular strand invasion of the telomeric end into internal telomeric sequences, forming a structure called a telomeric loop (t-loop). After this structure formed, it could be cleaved by a structure-specific nuclease, leaving a short telomere. One interesting possibility is that the cleaved portion could be processed to form a t-circle. Because t-circles have been shown to be important to RTE, TRD could potentially be a mechanism of how t-circles are made.

After its initial discovery in *S. cerevisiae*, TRD was also observed in *Arabidopsis thaliana* at telomeres made long by a mutation in *ku70* (but later had been complemented with the wild type *KU70*) (218). Interestingly, TRD in Arabidopsis was quite different from TRD in *S. cerevisiae*. Its appearance did not appear to be dependent on *MRE11* or several *RAD51*

paralogs, indicating that it has different genetic requirements. Also, in cells deleted for telomerase, it appeared to be able to shorten telomeres to below wild type size. In humans, deletions of telomeres have been seen in mutants of the telomere binding protein TRF2, which are dependent on NBS1, the homolog of Xrs2p in *S. cerevisiae* (215). These deletions are associated with the formation of t-circles. These results suggest that TRD could act similarly in yeast and human cells.

K. lactis is a good model system for TRD in human cells because the telomere repeats are homogeneous, but the telomere proteins resemble those of *S. cerevisiae*. Therefore, these studies could add to our knowledge of TRD and highlight important differences between TRD in *S. cerevisiae* and in humans. This manuscript will detail unique features of TRD discovered in *K. lactis*.

Focus of this Dissertation

The studies included in this manuscript focus on two specific areas of telomere recombination: how telomeric repeats defective at binding Rap1p affect telomere-telomere recombination and the role that TRD plays in *K. lactis* cells. We have shown that repeats defective at binding Rap1p are also defective at regulating RTE. We also show evidence that TRD is potentially at work at telomeres of all lengths in *K. lactis*. Both of these topics expand our knowledge of recombination at telomeres and give an interesting perspective on how recombinational mechanisms could act in ALT cancer cells.

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Table 1.1. The diversity of telomeric repeats throughout evolution (adapted from (35))

Organism	Telomeric Repeat Sequence	Reference
Homo sapiens	TTAGGG	(142)
Dictyostelium discoideum	AG_{1-8}	(48)
Trypanosoma brucei	TTAGGG	(14, 211)
Tetrahymena thermophila	TTGGGG	(15)
Arabidopsis thaliana	TTTAGGG	(174)
Caenorhabditis elegans	TTAGGC	(222)
Apis mellifera	TTAGG	(177)
Neurospora crassa	TTAGGG	(184)
Aspergillus nidulans	TTAGGG	(9)
Schizosaccharomyces pombe	$TTACAC_{0-1}G_{2-8}$	(81, 128)
Saccharomyces cerevisiae	$TG_{2-3}(TG)_{1-6}$	(185)
Candida albicans	TCTAACTTCTTGGTGTACGGATG	(134)
Kluyveromyces lactis	TGATTAGGTATGTGGTGTACGGATT	(130)

Figure 1.1. Type I RTE vs. Type II RTE in *S. cerevisiae*. A *S. cerevisiae* telomere undergoes a deletion of the telomerase RNA template Tlc1. The telomeres shorten until a critically short length, where they are elongated by either Type I RTE, which amplifies Y′ elements or by Type II RTE, which amplifies telomeric repeats. In mutants such as *cdc13-1 mec3*, Type II RTE occurs in telomerase-positive cells. Small blocks represent telomeric repeats and large blocks represent Y′ elements.

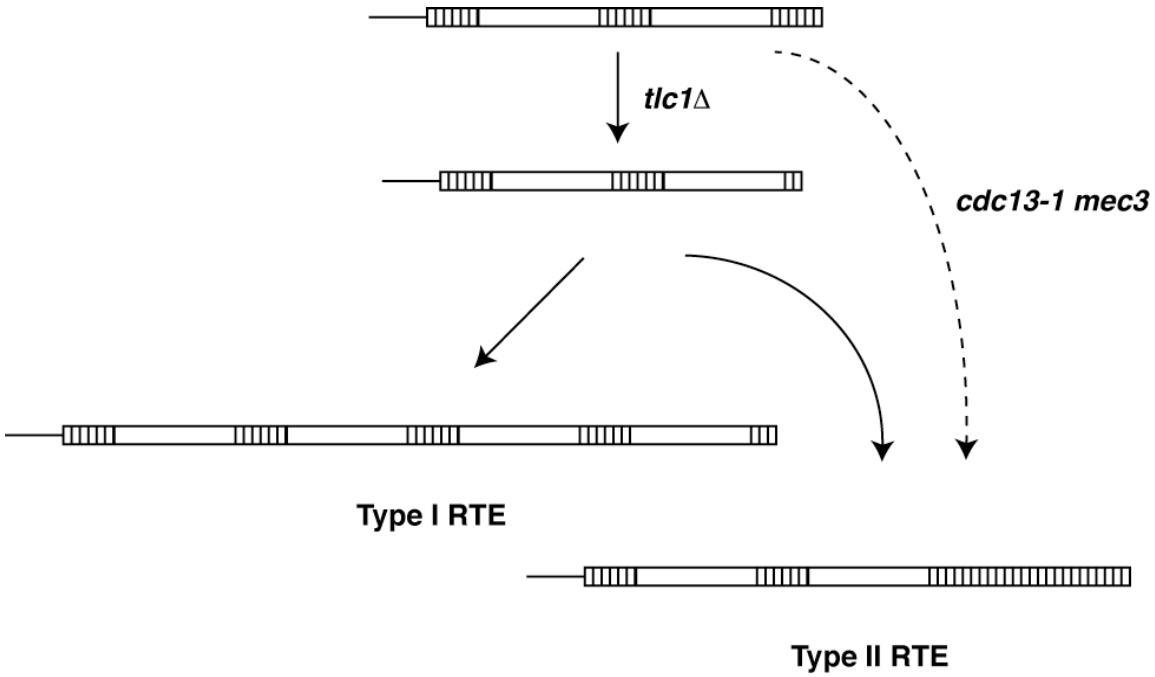

tlc1Δ
cdc13-1 mec3
Type I RTE
Type II RTE

Figure 1.2. Models of double-strand break repair. (A) During synthesis dependent strand annealing (SDSA), a double-strand break initially undergoes 5′ to 3′ resectioning. A 3′ overhang can then strand invade a homologous chromosome. This strand invasion is extended by DNA polymerases and is then dissociated and annealed back to other end of the double strand break to form a continuous DNA strand. The gaps on the strands can then be elongated by DNA polymerases and re-ligated. This type of double-strand break repair causes a localized non-reciprocal gene conversion. (B) During single strand annealing (SSA), there is a double-strand break between direct repeats. After resectioning of the 5′ ends, two direct repeats can anneal together and the remaining pieces of DNA are degraded, causing a deletion event. (C) During break-induced replication (BIR), only one end is available with homology to a non-broken sequence. That end strand invades a homologous sequence and, after an extended delay thought to be due to abortive attempts to do SDSA, a replication fork is created that extensively copies the invaded sequence, often all the way to then end of a chromosome. The other strand copies the invaded strand simultaneously, similarly to the progression of a normal replication fork.

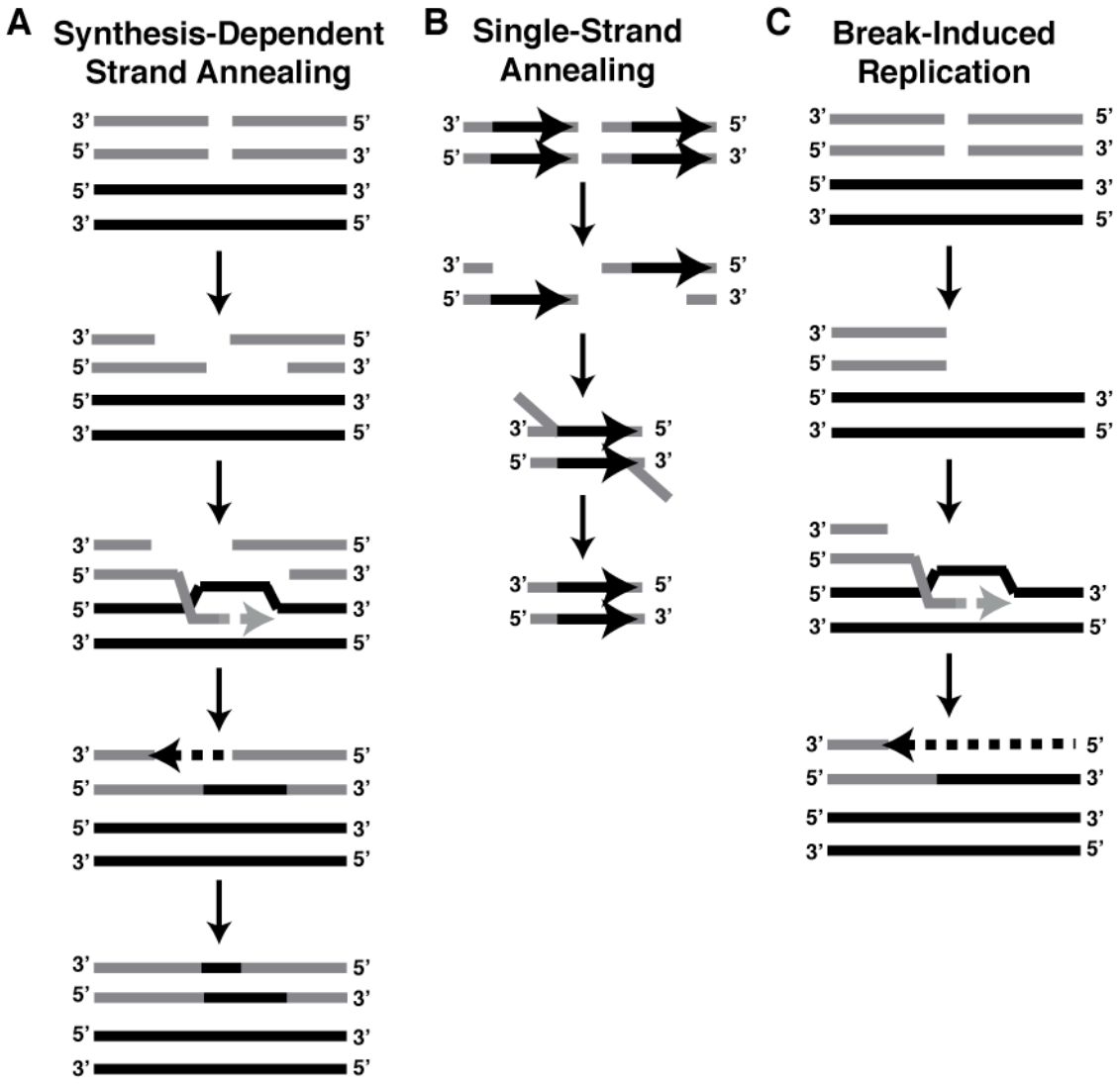

A
Synthesis-Dependent Strand Annealing
B
Single-Strand Annealing
C
Break-Induced Replication
3'
5'

Figure 1.3. The Roll and Spread Model. The model predicts that a telomerase deletion causes telomere shortening. When telomeres reach a critically short length, telomeres appear to be able to recombine and copy other telomeres and occasionally duplicate their subtelomeric sequence. A circle of telomeric repeats can then act as a rolling circle template to elongate a single telomere. This elongated telomere can then serve as a template for other telomeres to copy it in BIR-like events. Subtelomeres are shown with a horizontal line and representative subtelomeric polymorphisms are shown with vertical lines. Small boxes signify telomeric repeats and boxes are shaded to differentiate between repeats originally from a particular telomere. Figure adapted from (133).

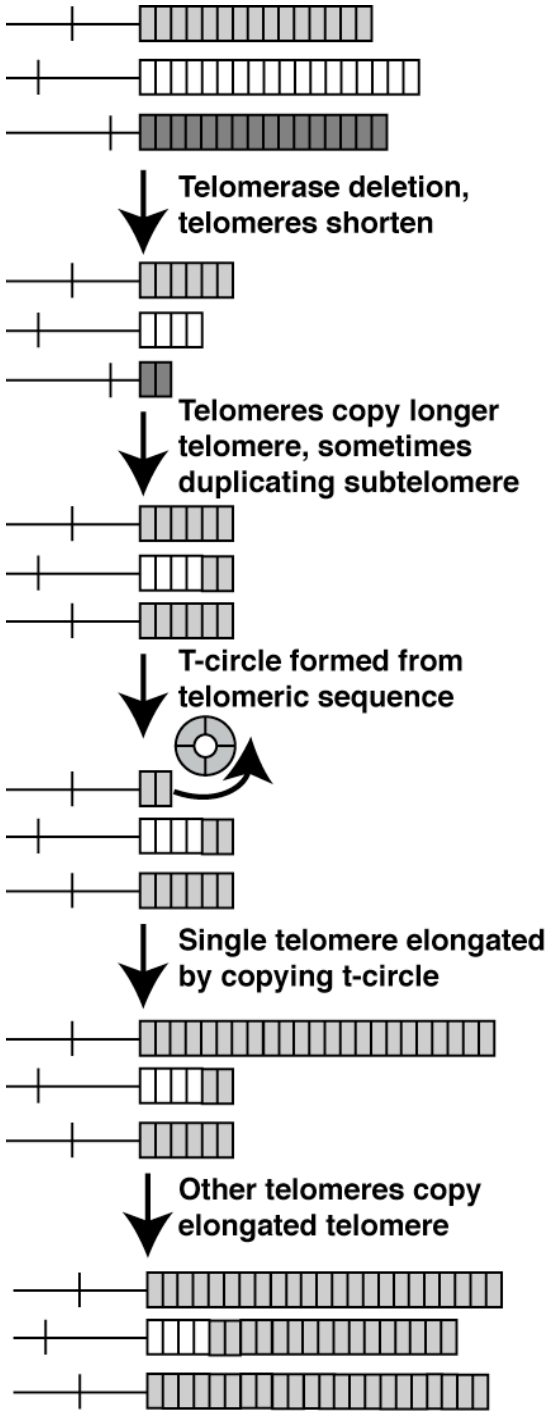

Telomerase deletion,
telomeres shorten
Telomeres copy longer
telomere, sometimes
duplicating subtelomere
T-circle formed from
telomeric sequence
Single telomere elongated
by copying t-circle
Other telomeres copy
elongated telomere

Figure 1.4. Differences between Type II RTE and Type IIR RTE. This diagram shows the differences between telomere uncapping in a telomerase deletion mutant and certain other mutants that undergo RTE. In both cases, telomere recombination is brought about by the loss of protection (uncapping) of telomeres (indicated by the star). In telomerase deletion mutants, uncapping only occurs once telomeres shorten below a critical size (~100 bp in *K. lactis*). Once telomeres are elongated by RTE, telomere capping is restored and RTE is blocked until one or more telomere becomes critically short again. At a telomere containing a protein defect such as in the *stn1-M1* mutant, telomeres are uncapped at all lengths and prone to recombination at all times. In this case, RTE is cannot be shut off and leads to the formation of extremely long telomeres. The horizontal lines signify subtelomeric elements. Figure adapted from (86).

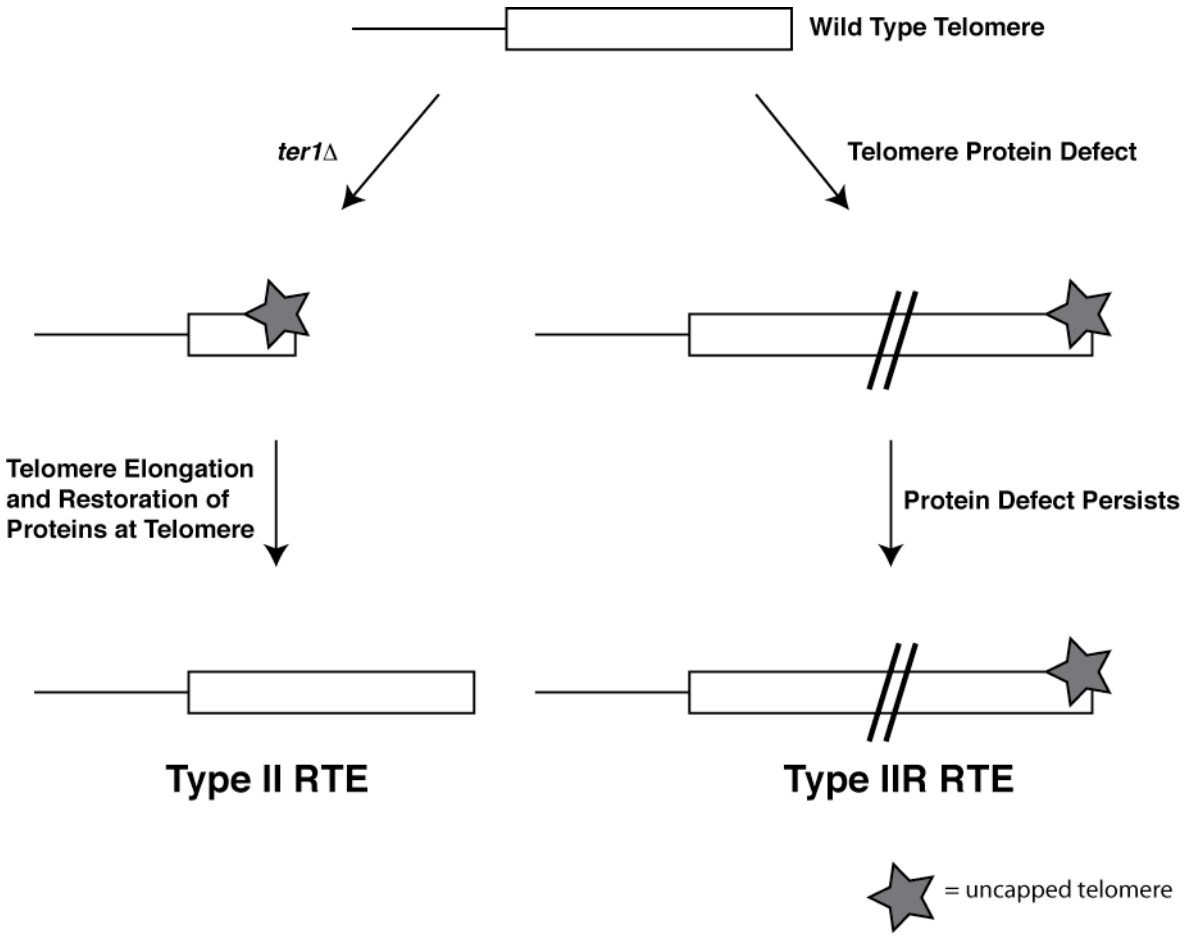
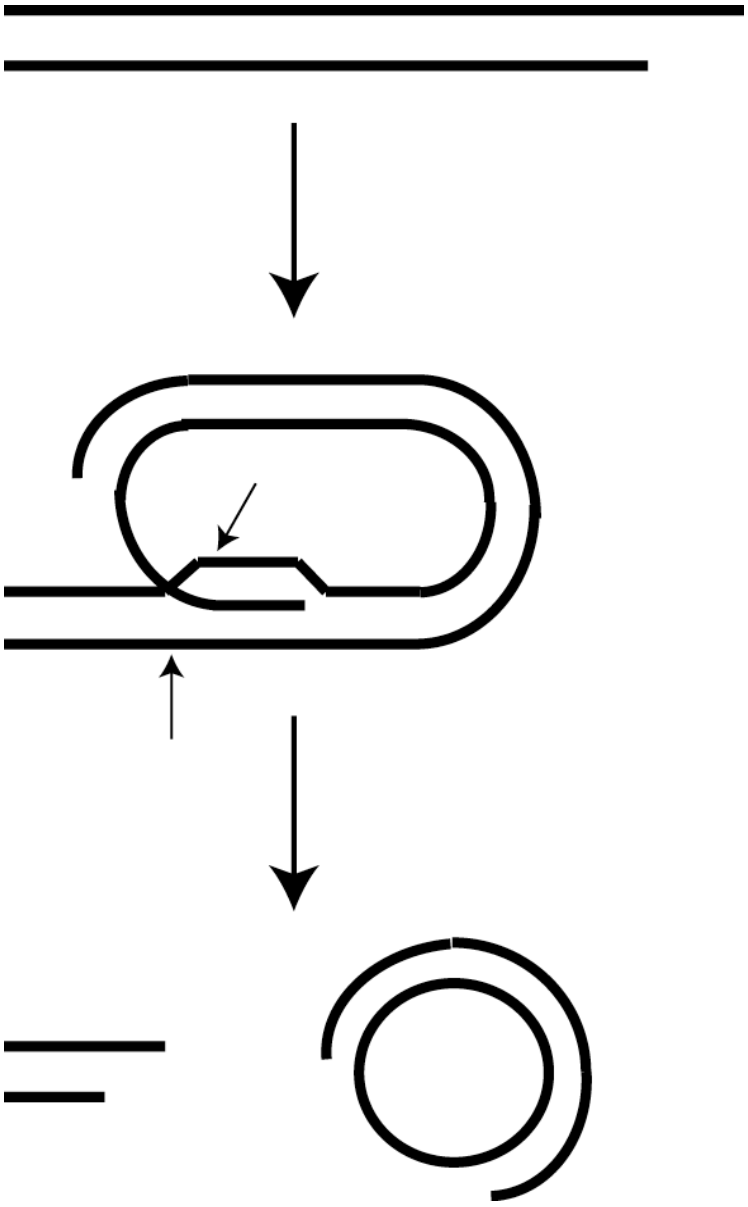

Wild Type Telomere
ter1Δ
Telomere Protein Defect
Telomere Elongation and Restoration of Proteins at Telomere
Protein Defect Persists
Type II RTE
Type IIR RTE
= uncapped telomere

Figure 1.5. Terminal deletion model for TRD that produces a t-circle. In this diagram, an elongated telomere undergoes an intramolecular strand invasion to form a t-loop. If the t-loop is cleaved as indicated by the small arrows, it truncates the telomere and produces a t-circle. Figure adapted from (24).



CHAPTER 2

MUTANT TELOMERIC REPEATS IN YEAST CAN DISRUPT THE NEGATIVE REGULATION OF RECOMBINATION-MEDIATED TELOMERE MAINTENANCE AND CREATE AN ALT-LIKE PHENOTYPE[1]

[1] Bechard LH, Butuner BD, Peterson GJ, McRae W, Topcu Z, and MJ McEachern. 2009. *Molecular and Cellular Biology*. 29(3): 626-639.

Abstract

Some human cancers maintain telomeres using Alternative Lengthening of Telomeres (ALT), a process thought to be due to recombination. In *Kluyveromyces lactis* mutants lacking telomerase, recombinational telomere elongation (RTE) is induced at short telomeres but is suppressed once telomeres are moderately elongated by RTE. Recent work has shown that certain telomere capping defects can trigger a different type of RTE that results in much more extensive telomere elongation that is reminiscent of human ALT cells. In this study, we generated telomeres composed of either of two types of mutant telomeric repeats, Acc and SnaB, that each alter the binding site for the telomeric protein Rap1p. We show here that arrays of both types of mutant repeats present basally on a telomere were defective at negatively regulating telomere length in the presence of telomerase. Similarly, when each type of mutant repeat was spread to all chromosome ends in cells lacking telomerase, they led to the formation of telomeres produced by RTE that were much longer than those seen in cells with only wild type telomeric repeats. The Acc repeats produced the more severe defect in both types of telomere maintenance, consistent with its more severe Rap1 binding defect. Curiously, although telomerase deletion mutants with telomeres composed of Acc repeats invariably showed extreme telomere elongation, they often also initially showed persistent very short telomeres with few or no Acc repeats. We suggest that these result from futile cycles of recombinational elongation and truncation of the Acc repeats from the telomeres. The presence of extensive 3′ overhangs at mutant telomeres suggest that Rap1p may normally be involved in controlling 5′ end degradation.

Introduction

Telomeres are the DNA-protein complexes present at the ends of linear chromosomes (11, 52, 83). Telomeric DNA is composed of short tandem repeats, commonly between 5-26 base pairs in size. Telomeres vary widely in size between organisms, but are generally

maintained at a relatively stable length within an organism. An essential role of telomeres is to protect chromosome ends from the homologous recombination (HR) and non-homologous end-joining (NHEJ) that normally act at broken double-stranded DNA ends (8, 21). However, the inability of replicative polymerases to fully replicate ends causes telomeres to shorten gradually over time, compromising their role in end protection. To prevent this problem, the great majority of eukaryotes use the ribonucleoprotein enzyme telomerase that can add telomeric repeats to the telomere ends (1, 40). Telomerase is recruited to the telomere in large part via telomere binding proteins that bind to a short 3′ overhang in the DNA at the telomere end (71).

Telomeres are protected from degradation and repair by specific proteins. In humans, a complex of six proteins called shelterin caps and protects the telomere (15). In addition, a looped structure called a t-loop, thought to be a strand invasion of the 3′ DNA overhang into internal duplex telomeric DNA, seems to also facilitate end protection in many species (28). In yeast such as *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, the double-stranded telomeric DNA is bound by Rap1p, while the single-stranded 3′ overhang is bound by the trimeric Cdc13p/Stn1p/Ten1p complex (4, 32). Rap1p participates in the negative regulation of telomere length and its loss from the telomere results in telomere end-end fusions (37, 44, 51, 63). Proteins associated with Rap1p at the telomere include Rif1p and Rif2p, which participate in telomere length regulation, and Sir2-4, which participate in telomeric silencing (71). The Cdc13p/Stn1p/Ten1p complex is required to recruit telomerase and lagging strand replication proteins to the telomere. It also protects the DNA end against recombination events (12, 19, 24-27, 64).

In humans, telomerase is present only at low levels in most somatic tissues and telomeres become progressively shorter with each cell division (69, 70). Once telomeres reach a critically short length, they trigger a permanent growth arrest called replicative senescence. Because of this, immortalized cells, including the great majority of cancers, have a telomere maintenance method, most commonly telomerase (34). However, a significant minority of cancers use ALT

(Alternative Lengthening of Telomeres), to maintain telomeres (55). The telomeres in typical cells displaying ALT are highly heterogeneous in length, and while many are much longer than normal human telomeres, others are abnormally short (6, 38, 56, 65). The telomeres of ALT cancers can copy DNA from telomere to telomere and therefore are thought to maintain their lengths by recombination (17). Recombination proteins such as RAD51 and the MRN complex as well as telomere binding proteins are present in ALT cells in subnuclear bodies called APBs (ALT-associated PML Bodies) (89-91). Extrachromosomal telomeric DNA of both linear and circular form is also found abundantly in ALT cells (9, 62, 85).

Yeast mutants lacking telomerase have been important model systems for understanding how recombination can maintain telomeres. Upon deletion of telomerase in both *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, cells display telomere shortening and growth senescence that is followed by the occasional production of better growing post-senescence survivors that arise from recombinational telomere elongation (RTE) (42, 47, 49). RTE in yeast telomerase deletion mutants appears to be triggered by the telomeres becoming too short (50). Once below ~100 bp in length, telomeres from both *S. cerevisiae* and *K. lactis* become able to initiate recombination (73, 77). In *S. cerevisiae*, two distinct types of survivors have been observed that differ in both their telomeric structure and in the genes required for their formation. Type I survivors display amplified subtelomeric Y′ elements and have short terminal tracts of telomeric repeats (42, 75). Their formation requires Rad52 and the canonical HR repair proteins Rad51, Rad55, and Rad57 (39). Type II survivors, in contrast, lack subtelomeric amplification and instead have elongated tracts of telomeric repeats. Their formation requires Rad52 and depends also on Rad50, Rad59, and Sgs1 instead of the Rad51 group of proteins (13, 74). In *K. lactis*, only Type II survivors normally occur (47). A variety of experimental evidence in both *K. lactis* and *S. cerevisiae* suggests that Type II post-senescence survivors arise through a 'roll and spread' mechanism whereby an elongated telomere is first formed by a rolling circle copying of a very small telomeric circle (t-circle)(29, 41, 58, 59). This is followed by additional break-

induced replication (BIR) events that copy the elongated sequence onto other telomeric ends (77).

More recently, it has become clear that RTE in yeast can become induced by certain perturbations in telomeric capping proteins even when telomeres are not abnormally short. For instance, in *S. cerevisiae*, a *cdc13-1 yku70* mutant at the semi-permissive temperature caused Type II survivors to form after a period of senescent-like growth without appreciably shortened telomeres (23). In *K. lactis*, a mutation in the telomere-associated protein Stn1p (*stn1-M1*) led to RTE that produced highly elongated and unstable telomeres and other features that distinguished it from the RTE of telomerase deletion survivors (33). The *stn1-M1* cells had a chronic moderate growth defect but failed to display the large changes in growth rate that characterize senescence and survivor formation. This unusual RTE, termed Type IIR ("runaway"), is thought to be due to a telomere capping defect that renders telomeres prone to initiate HR in a manner largely or entirely independent of their size. Interestingly, in a recent finding, the *stn1-281t* allele of *S. cerevisiae* similarly led to long heterogeneous telomeres as well as inviability in the absence of *RAD52* (66). The close similarity of Type IIR RTE to the ALT phenotypes of certain human cancers and cell lines makes it an especially important phenomenon to understand.

It was recently shown that the sequences from a single telomere engineered to contain only mutationally-tagged telomeric repeats could sometimes be spread to all other telomeres in the cell during the formation of post-senescence survivors in a *K. lactis* mutant lacking a functional *TER1* gene encoding the telomerase RNA (77). Here, we have taken advantage of this technique to test whether either of two telomeric sequence mutations perturbs the manner in which RTE occurs in telomerase-negative cells. These mutations make base changes within each telomeric repeat that fall within the binding site of double strand telomere binding DNA binding protein Rap1. We demonstrate that both of these mutations can in fact lead to recombinational telomere maintenance with characteristics similar to Type IIR RTE.

Materials and Methods

Strains and culturing conditions.

All strains used in this article with the exception of those used in Figure 6C and 6D are derivatives of 7B520 (*ura3*, *his2-2*, and *trp1*) (88). The wild type strain CBS 2359 was used in Figure 6C and 6D and the *ku80*Δ mutant used as a control was in a CBS 2359 background (36). The *ter1-19A(Acc)* and *ter1-24T(SnaB)* single mutants and heteroalleles were made by a plasmid loop-in replacement process using pTER-BX:UA, previously described in (48, 50). All of the transformations of the single mutant telomere were made in 7B520 with a *ter1* deletion mutation (68). *TER1* was reintroduced into the cells with the plasmid pJR31, a derivative of pKL316, which contains a *HIS3* gene (68). The SnaB and Acc mutant telomeres were constructed by performing oligonucleotide-directed mutagenesis on a plasmid (pAK25ΔB) that contained a cloned wild type *K. lactis* telomere as described previously (77, 81). The pAK25ΔB plasmid was derived from pAK25 by filling in the overhangs of the unique *Bgl*II site next to the *URA3* gene inserted into the subtelomeric sequence (50).

In general, cells were grown on YPD rich medium (yeast extract, peptone, dextrose). The selective plates used were SD minimal lacking either histidine, uracil, or both. Transformations were plated on selective medium supplemented with 1M sorbitol. YPD liquid medium was used for growing cells for genomic DNA preparations.

To generate cells with a single mutant telomere, DNA fragments containing a mutant telomere with a subtelomeric *URA3* gene were transformed into *K. lactis* cells as described previously (50). pAK25 derivatives containing only Acc or SnaB repeats were cleaved with *Eco*RI and *Sac*II to release the *URA3*-tagged telomeric fragment. This fragment was then transformed into a *ter1*-Δ mutant containing pJR31 and plated on medium lacking histidine and uracil and supplemented with 1M sorbitol. Fragments used were ones that contained 15 or >28 SnaB repeats and one with 14 Acc telomeric repeats. Transformants were restreaked onto plates

lacking uracil and histidine in order to eliminate any untransformed cells. In individual transformants, the numbers of mutant repeats retained on the mutant telomere after wild type repeats were added to the end was commonly somewhat fewer than the number present on the transforming fragment. Genomic DNA preparations were conducted to confirm that a single telomere had been replaced.

Transformants confirmed to have a single mutant telomere were streaked on YPD medium and patched onto a plate lacking uracil and another lacking histidine. The YPD streaks were observed for the formation of senescing cells, as indicated by partially rough colonies. The rough edges were restreaked onto new YPD medium and also patched to the above mentioned selective plates. The appearance of rough colonies corresponded with loss of the His+ phenotype as expected for loss of the *TER1*-containing pJR31 plasmid. The screening on medium lacking uracil confirmed that the single long telomere was still present. Senescing cells were then serially restreaked on YPD every 3-4 days with each streak representing up to 20-25 cell divisions. The growth on these plates was then scored from 0-4 after loss of pJR31. A score of 0 represented no growth and a score of 4 represented wild type growth. Scores in between were based on size and the degree of roughness of the colonies. After two to three streaks of senescence, when growth scores first leveled or began to improve, scoops of cells from the plates were taken for genomic DNA preparations. At this point, cells were considered to be "survivors" and were found to have telomeres that were lengthened relative to those of cells in a highly senescent state. Typically, multiple serial streaks were done on survivor cell lineages once they were formed.

Gel electrophoresis and Southern blotting.

Restriction digests of yeast genomic DNAs were carried out in the presence of RNase and were run on 0.8% agarose gels in Tris Borate buffer unless otherwise specified in the text. They were visualized by ethidium bromide treatment prior to blotting. Gels were blotted onto a

Hybond N+ (Amersham Biosciences, Piscataway, N.J.) membrane in 0.4M NaOH. They were allowed to transfer for one day and crosslinked using UV light from an electronic crosslinker.

Hybridizations were conducted in 7% SDS, 0.5M EDTA, and 0.5 M Na_2HPO_4 as described (14). For telomeric probes, the G-stranded *Klac*1-25 oligonucleotide (ACGGATTTGATTAGGTATGTGGTGT) was end-labeled with g ^{32}P ATP and allowed to hybridize with the membrane for at least 4 hours at 48° Celsius. The membrane was then washed in 100 mM Na_2HPO_4 and 2% SDS three times at 48° Celsius and visualized using a Molecular Dynamics (Sunnyvale, CA) Storm phosphor imager. For subtelomeric probes, a 615 base pair doubly cleaved *Hin*dIII subtelomeric fragment from plasmid pMya was gel purified using a QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). This fragment contains the *K. lactis* sequence between the *Eco*RI site and the *URA3* gene pictured in Figure 2A. pMya is a derivative of pAK25ΔB made by deleting the telomeric repeats after *Sac*I digestion and religation. The subtelomeric fragment was labeled with a-^{32}P dATP using the large Klenow fragment and a Stratagene (La Jolla, CA) Prime-It II® Random Primer Labeling kit.

In-gel hybridization.

0.7% or 0.8% agarose gels were run using the same protocol to that of the gels for blotting. The *Eco*RI-digested DNA used for these gels was split in half with half being used in each of two agarose gels, one that was subject to Southern blotting (denatured gels) and the other that was used as follows for in-gel hybridization. As described previously (16), gels were soaked in 2X SSC (0.3 M NaCl 0.03 M sodium citrate, pH 7.0) for 30 minutes. They were then blotted to near dryness using Whatman 3 mm chromatography paper for approximately 1.5 hours. The flattened gels were then hybridized in 10X SSC overnight with a C-strand-specific oligonucleotide (ACACCACATACCTAATCAAATCCGT) in order to visualize single stranded telomeric G-strand DNA. The denatured gels were hybridized to either a C-strand-specific probe

or a G-strand specific probe. After hybridization, the gel was washed 4 times in 0.25X SSC for 1.5 hours per wash followed by visualization on a phosphor imager.

Exo I Digestion.

13.3% of the total DNA from a genomic prep from a 1.5 ml overnight culture was incubated in 20 units of *Eco*RI enzyme prepared by New England Biosciences (NEB, Ipswitch, MA) in NEBuffer EcoRI (50 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$, 0.025% Triton X-100 (pH 7.5@25°C)) for 3 hours prior to digestion with Exo I. 20 units of Exo I enzyme prepared by NEB was added after a buffer change from NEBuffer *Eco*RI to NEBuffer Exo I (67 mM Glycine-KOH (pH 9.5), 6. mM $MgCl_2$, 10 mM 2-mercaptoethanol) using Quantum Prep® PCR Kleen Spin Columns (Bio-Rad Laboratories, Hercules, CA). Reactions were incubated for 3 hours at 37°C and run on agarose gels prepared for in-gel hybridization.

Results

SnaB mutant repeats are infrequently incorporated at telomeres when synthesized by telomerase.

The sequence of telomeric repeats in an organism is specified by the template region of the RNA subunit of its telomerase. Mutational analysis of the template region of the *K. lactis* telomerase RNA (*TER1*) revealed that mutations within the left side of the Rap1 binding site, including *ter1-19A(Acc)* ("Acc" in Fig. 2.1A), lead to rapid and often severe telomere elongation that appears to be due to a disruption in Rap1p binding to the affected telomeric repeats (37, 48, 80). However, mutations in the right side of the Rap1p binding site including *ter1-24T(SnaB)* ("SnaB" in Fig. 2.1A), lead to telomeres that stabilize at shorter than normal lengths (50, 80) (Fig. 2.1A, B). It was suggested that this region of the template encodes not only the Rap1p binding site but also another function that is required for the efficient ability of telomerase to add

sequence onto a telomere. As a test of this, *ter1-24T(SnaB)* cells were transformed with an integrative plasmid (pTER-BX-UA) containing the *ter1-19A(Acc)* gene. Transformants were found, as expected for homologous integration, to typically contain both the *ter1-24T(SnaB)* and the *ter1-19A(Acc)* alleles separated by vector sequences. Several such hetero-allelic transformants were then examined for their telomere lengths. Results from this analysis (Fig. 1B) showed that telomeres in these strains invariably displayed a highly elongated smear of telomeric *Eco*RI fragments ranging from ~3 kb to >12 kb (Fig. 2.1B, "S+A" samples). This contrasts with other experiments that showed that introducing *ter1-19A(Acc)* into cells with a wild type *TER1* produced a comparatively slight telomere elongation phenotype (53). These data indicated that the presence of the *ter1-24T(SnaB)* allele, unlike a wild type *TER1* allele, did not substantially interfere with the telomere elongation caused by the *ter1-19A(Acc)* allele. Further passaging of the *ter1-24T(SnaB)*/*ter1-19A(Acc)* hetero-allelic strains for five serial restreaks (~100-125 cell divisions) showed both continued telomere elongation and the appearance of some sharp telomere-hybridizing bands, which, based on previous work with *ter1-19A(Acc)* and other *ter1* template mutations producing rapid telomere elongation, are likely to be telomere-telomere fusions ((51) and data not shown). Control transformants of identical structure except for both *ter1* alleles being *ter1-24T(SnaB)* exhibited a short telomere phenotype similar to the original *ter1-24T(SnaB)* mutant (Fig. 2.1B "S+S" sample). This indicated that the number of copies of *ter1*, by itself, was not leading to telomere elongation.

Digestion of the elongated telomeric fragments from *ter1-24T(SnaB)*/*ter1-19A(Acc)* hetero-allelic strains with restriction enzymes (*Sna*BI and *Acc*I) that specifically cleave each type of mutant repeat but not the wild type repeats produced very different results. Digestion with *Acc*I led to the disappearance of the great majority of the telomeric signal (not counting the residual wild type telomeric repeats remaining at basal positions of the fragments) from each of several hetero-allelic transformants that were examined (Fig. 2.1B and data not shown). In contrast, digestion with *Sna*BI produced a large smear of telomeric signal from ~100 bp to >1 kb

in size with a signal intensity roughly similar to the *Eco*RI-digested control with uncleaved telomeric repeats. These results are consistent with the great majority of the long telomeres in *ter1-24T(SnaB)/ter1-19A(Acc)* hetero-allelic strains being composed of Acc repeats with SnaB repeats only occasionally becoming incorporated. From these experiments, we conclude that either the SnaB telomerase is defective at synthesizing telomeric repeats or that SnaB telomeric repeats are defective at being extended by telomerase.

Both SnaB and Acc repeats are defective at negatively regulating telomere length in the presence of telomerase.

Rap1p binding to telomeric repeats negatively regulates the ability of telomerase to extend yeast chromosome ends (44). Mutant telomeric repeats defective at binding Rap1p should therefore be poorly able to negatively regulate telomere length in the presence of telomerase. To test the Acc and SnaB repeats, cloned *K. lactis* telomeres were first constructed to be composed entirely of either Acc repeats or SnaB repeats. These telomeres, containing a *S. cerevisiae URA3* gene inserted into adjacent subtelomeric sequence and referred to as "STU" (subtelomeric *URA3*) telomeres, were then transformed into *K. lactis* cells. This led to the transforming fragment integrating via subtelomeric homology and replacing one native telomere in each transformant (50) (Diagrammed in Fig. 2.2A). The length of a STU telomere could be readily assessed by digestion with *Xho*I, which cleaves next to the *URA3* gene and allows separation of the STU telomere from all other telomeres in the cell (Fig. 2.2B, C). When the STU telomere was composed of either wild type repeats or of the phenotypically silent Bcl mutant repeats, it was found, as expected, to be of wild type length (53, 81) (Fig. 2.2C). In contrast, both Acc and SnaB repeats showed defects in telomere length regulation with STU telomeres composed of them maintaining lengths substantially longer than wild type telomeres (Fig. 2.2B, C). Acc repeats appeared to be completely "uncounted" as the array of wild type repeats present at the end of a STU telomere with 13 Acc repeats was the same size as wild type telomeres (compare

*Xho*I + *Acc*I digests (X+A) of Acc13 clones in Fig. 2.2C with *Bsr*BI digests (B) of wild type STU clones). This is consistent with the past observation that Acc repeats have a severe defect in binding Rap1p (37). The length regulation defect of SnaB repeats was less severe. A SnaB telomere estimated to have ~10.2 SnaB repeats had a terminal wild type repeat tract (X+S lane of SnaB 10) that was shorter than wild type telomeres and a telomere having ~27 SnaB repeats had a terminal wild type repeat tract that was shorter still (faint signal at ~0.3 kb in X+S lanes of SnaB 27 samples). We conclude that the SnaB mutant repeats are partially defective at regulating telomere length. Using the amount of wild type addition to the end of the mutant repeats, we established that SnaB repeats in the constructs tested retain roughly 40% of their ability to regulate telomere length. For example, a SnaB clone with ~10.2 SnaB repeats was estimated to have ~12.3 terminal wild type repeats. Because our control wild type telomeres averaged 15.9 repeats, the 10.2 SnaB repeats had the same length regulation ability as 3.6 wild type repeats (15.9 minus 12.3).

Constructing telomerase deletion mutants with telomeres composed of Acc or SnaB mutant repeats.

We next examined the effect of the mutant telomeric repeats on recombinational telomere maintenance. We hypothesized that telomeres composed of mutant telomeric repeats might result in chronic telomere capping defects that in turn promoted the formation of very long telomeres by recombination ('runaway' Type IIR RTE, outcomes 4-6 of Fig. 2.3A). To test this, we took advantage of a technique that we had previously shown to result in the spread of sequence from a single telomere to the eleven other telomeres in a *K. lactis* cell (77). Cells with a genomic *ter1*-Δ allele but having a wild type copy of *TER1* on a plasmid were first constructed to contain a single STU telomere containing SnaB or Acc repeats (Fig. 2.3A). As described above, these mutant STU telomeres were extended by the resident wild type telomerase and had a total size (mutant plus wild type repeats) that was longer than other telomeres in the cell.

These transformants were then streaked on rich medium and clones were identified (by their rough colony phenotype and loss of the *HIS3* marker) that had lost the *TER1* plasmid.

In the absence of telomerase, growth senescence ensued and after two to three serial streaks it was possible to identify post-senescence survivors that showed improved growth and contained telomeres that had been elongated by recombination. As observed previously when a single abnormally long, but functionally wild type, telomere was present, the presence of the Acc and SnaB STU telomere led to a detectable partial suppression in the senescence of a *ter1*-Δ mutant ((77) and data not shown). Topcu *et al*. also showed that the sequence of the single abnormally long telomere is spread to all eleven other *K. lactis* telomeres during post-senescence survivor formation at a frequency exceeding 90% (77). We therefore expected that the mutant telomeric repeats from the abnormally long Acc and SnaB STU telomeres would commonly be spread to all telomeres in the ensuing survivors. The efficiency of spreading could be judged by the frequency by which telomeric restriction fragments could be shortened by the restriction enzyme able to specifically cleave the Acc or SnaB telomeric repeats.

Fig. 2.3A diagrams several conceptual outcomes of the fate of the telomeres after the mutant repeats have spread to all of the telomeres. Among "limited RTE" scenarios, telomeres never become more elongated than has been observed in Type II RTE in *K. lactis* regardless of whether the mutant repeats constitute none, some, or all (outcomes 1-3) of the newly acquired telomeric sequences. Alternatively, Type IIR "runaway" RTE might occur as a result of the presence of mutant repeats. This might involve the extreme elongation of all telomeres with uniform tracts of the mutant telomeric repeat (outcome 4). Two alternative possibilities are that some telomeres remain short (perhaps because of sufficient numbers of residual wild type repeats; outcome 5) or that elongated telomeres are not all homogeneous in sequence with some or all containing interspersed wild type repeats (outcome 6).

We observed that, in two separate experiments, 30% (7/23) of survivors and 58% (7/12) of survivors derived from cells with an Acc telomere, had spread mutant repeats to all telomeres

(Figures 2.3, 2.4, and data not shown). Amongst survivors derived from cells with a SnaB telomere, our results varied depending upon the length of the mutant telomere. Only 8% (1/12) of survivors derived from a cell starting with a STU telomere estimated to have 10 SnaB repeats were found to have spread SnaB repeats. However, 62% (8/13) of survivors derived from a cell starting with a STU telomere estimated to have ~27 SnaB repeats were found to have spread SnaB repeats. The low frequency of spreading in survivors derived from the shorter SnaB telomere is similar to that previously observed with a STU telomere carrying a wild type length of phenotypically normal Bcl repeats (77). The survivors derived from cells with Acc or the longer SnaB STU telomeres displayed a somewhat lower spreading frequency compared to the 94% observed with an elongated Bcl telomere. This could reflect an increased instability of the mutant SnaB and Acc telomeres once gradual sequence loss had eliminated the terminal wild type repeats.

SnaB repeats can promote the formation of longer telomeres through RTE than can wild type repeats.

Nine *ter1*-Δ survivor clones that had acquired SnaB repeats at most or all telomeres (henceforth called SnaB survivors) were followed by serial restreaking on YPD plates. The telomeres from each streak were then examined by Southern blotting. Fig. 2.3B shows genomic DNA from four representative SnaB survivors and a control survivor that retained only wild type telomeric repeats, each digested with *Eco*RI alone and also with *Eco*RI + *Sna*BI. The double digestion cleaved all SnaB mutant telomeric repeats and typically left behind much shorter fragments containing subtelomeric sequence and a small number of wild type repeats. Results from this analysis (Figure 2.3B) showed that SnaB survivors showed a more variable range of telomere lengths than has been seen in ordinary *ter1*-Δ survivors containing only wild type telomeric repeats.

Three of the nine SnaB survivors, including survivor 16 (Fig. 2.3B), displayed telomeres that never became more than moderately elongated (with estimated sizes of telomeric repeat arrays remaining typically not more than several hundred base pairs). This result is similar to the limited Type II RTE (outcomes 1-3, Fig. 2.3A) that occurs in survivors of telomerase deletion with wild type repeats (47) and the control survivor in Fig. 2.3B. These three SnaB survivors all displayed quite similar outcomes. In streaks 1-2, telomeres remained very short but typically were cut slightly shorter still upon digestion with *Sna*BI (see streak 2 of survivor 16 in Fig. 2.3B) consistent with many or all telomeres having acquired one or more SnaB repeats. In subsequent streaks however, moderate telomere elongation (telomeric *Eco*RI fragments all < ~5 kb) was present that was invariably accompanied by a fragment less than ~0.2 kb in size in *Eco*RI+*Sna*BI double digests that hybridized intensely to a telomeric probe (see streaks 3-5 of survivor 16, Fig. 2.3B). This fragment almost certainly represents short blocks of wild type repeats that are interspersed among SnaB repeats in many or all of the telomeres of these survivors (Type II RTE outcome 2 of Fig. 2.3A). Similar interspersion of wild type repeats was observed in many survivors derived from *ter1*-Δ cells with basal wild type repeats and terminal Bcl repeats where it was thought to be a consequence of roll and spread amplification (59). Telomeric circles with both wild type and SnaB repeats most likely arise from a single telomere containing both repeat types, and would be predicted by the roll and spread model to produce repeating patterns of the two repeat types if copied by a rolling circle gene conversion. We suggest that sufficient concentrations of interspersed wild type repeats in SnaB survivors render those telomeres relatively resistant to further telomeric recombination.

The most common outcome for SnaB survivors, including survivors 3, 4, and 7 in Fig. 2.3B, was that by streak two, they displayed telomeres of unusually large sizes, which frequently migrated at positions above 5 kb in the gel. Characteristically, telomeric hybridization signal in these survivors extended to limit mobility (>20 kb) in gels and also showed up in the wells of the gel. These features have not been seen in *K. lactis* telomerase deletion survivors with wild type

repeats, and argue that SnaB repeats can lead to the formation of much longer telomeres from RTE than can wild type repeats (47). We conclude that these SnaB survivors display a telomere length phenotype that is intermediate between limited Type II RTE and runaway Type IIR (outcomes 1-3 and 4-6 of Fig. 2.3A, respectively).

The elongated telomeres in SnaB survivors appear to have variable degrees of stability. In some cases, such as survivor 3 of Fig. 2.3B, telomeres appeared relatively stable over many cell divisions, with changes in the telomeric fragment pattern largely limited to the gradual shortening expected for cells lacking telomerase. Many SnaB survivors with long telomeres showed little if any sign of wild type repeats except those few that are adjacent to subtelomeric sequences. This may imply that telomeres composed solely of SnaB repeats can often resist uncapping and engaging in recombination for many consecutive cell divisions. In other cases, however, long telomeres in SnaB survivors can be highly unstable. A particularly dramatic example of this can be observed between the third and fourth streaks of survivor 4 of Fig. 2.3B. Here, very long and heterogeneous telomeric signal, commonly reaching to limit mobility, changed abruptly into a pattern of much shorter telomeric fragments, migrating at positions below 4 kb in the gel. This indicates that telomeres composed of SnaB repeats can be subject to high rates of becoming truncated. Interestingly, the shortened telomeres of survivor 4 at streaks 4-5 appeared to contain interspersed wild type repeats as indicated by the short fragment hybridizing to a telomeric probe in the *Eco*RI+*Sna*BI digest (Fig. 2.3B). This adds further support to the idea that interspersed blocks of wild type repeats can prevent further elongation by recombinational processes of telomeres containing SnaB repeats.

ter1-Δ SnaB survivors with long telomeres displayed growth characteristics that were different than those of telomerase deletion survivors with wild type repeats. Whereas *ter1*-Δ survivors with WT repeats vary widely from streak to streak, from highly senescent very slow growth to growth indistinguishable from wild type cells (47), SnaB survivors with long telomeres showed more constant growth characteristics, from slightly senescent to normal (data

not shown). SnaB survivors with short telomeres and interspersed WT repeats instead appeared to more closely resemble telomerase deletion survivors with wild type repeats in having more variable growth characteristics.

Acc repeats promote Type IIR RTE.

Seven independent *ter1*-Δ survivor clones that had acquired Acc repeats at all telomeres (henceforth called Acc survivors) were followed by serial restreaking and Southern blotting. The results from this analysis showed that Acc survivors showed a highly elongated and heterogeneous pattern beginning from the first streak examined when hybridized with a telomeric probe (Fig. 2.4A). Cleavage of DNA from these survivors with *Acc*I eliminated almost all telomeric signal, consistent with the long telomeric sequences being composed almost entirely of Acc repeats. The bands remaining after *Acc*I cleavage correspond to shortened telomeric fragments that retained small numbers of wild type telomeric repeats adjacent to subtelomeric sequence. The smear of telomeric signal in the *Eco*RI digests visible below ~0.7 kb in cells with Acc telomeric repeats is too small to have intact subtelomeric sequences and has been shown to be at least largely circular in nature (E. Basenko and M. McEachern, unpublished data). Past work has shown that small extrachromosomal telomeric sequence present in long telomere mutants is primarily double and single stranded circles (29). In 6 out of the 7 Acc survivors, while the telomeric signal sometimes varied in intensity between streaks, the general pattern of long and heterogeneous telomeric signal did not vary greatly either between survivors or between streaks 1-5 of the same survivor. After 10 streaks, however, most Acc survivors showed a reduced amount of low molecular weight telomeric signal and the high molecular weight telomeric signal was more frequently in sharp bands (Figure 2.4B). These results suggest that the telomere phenotype of the Acc survivors might gradually change over continued passaging, most likely toward a state favoring a more stable telomere function. The colony phenotypes of Acc survivors always showed a slight to moderate senescence phenotype (rough

colonies and slightly slower growth). This supports the idea that their telomeres were never completely wild type in their function.

In one Acc survivor (survivor 7 in Fig. 2.4A), substantial telomere shortening was seen between the first and third streak. The reason for this change is not clear. One possibility is that, as appears to occur in some SnaB survivors, the mutant phenotype is suppressed by the presence of a sufficient number of wild type repeats interspersed with the mutant repeats.

DNA samples from the Acc survivors were next probed with a subtelomeric probe. Unlike the telomeric probe, this probe does not exaggerate the abundance of long telomeric fragments nor was it expected to detect extrachromosomal telomeric DNA. Results from this (Fig. 2.4C) were striking. As expected, smears of signal extending to high molecular weights were observed, consistent with the presence of long telomeric fragments. However, in most time points of most Acc survivors examined, a substantial amount of signal in *Eco*RI digests was seen to run at short sizes that were nearly identical to the bands that had been digested with *Eco*RI+*Acc*I. This indicated that many of the telomeres in Acc survivors were often very short and contained few if any Acc repeats. Those Acc survivor samples that showed little or no short *Eco*RI fragments (most notably Acc survivor 6, streak 5 and Acc survivor 12, streak 5 in Fig. 2.4C) instead showed another still shorter fragment in *Eco*RI+*Acc*I digests (asterisk in Fig. 2.4C). This additional band was never present in digests with *Eco*RI alone and was not detectable with a telomeric probe. It therefore likely represents chromosome ends that contain no basal wild type repeats. Our results suggest that telomeres in Acc survivors tend to exist either in a heterogeneously long state or, if some wild type repeats remain basally, they remain very short and essentially without Acc repeats. The results also suggests that the presence of even small numbers of basal wild type repeats can stabilize telomeres to persist at short lengths for long enough periods of time to permit their detection as a significant fraction of the total telomere population in Acc survivors. SnaB survivors were also probed with a subtelomeric

probe and were not seen to contain telomeres that were very short among the long telomeres, which is consistent with the lesser recombination defect in SnaB survivors (data not shown).

Wild type repeats are variably present within the long tracts of Acc repeats in Acc survivors.

To further examine the structure of telomeres in Acc survivors, *Rsa*I digests were performed. This restriction enzyme cleaves wild type *K. lactis* telomeric repeats but not Acc repeats. Figure 2.5 shows *Bsr*BI digests of DNA from a number of Acc survivors alone or in combination with *Rsa*I digests. As expected, the telomeric signal from a wild type control was completely eliminated by *Rsa*I digestion (Fig. 2.5, leftmost lanes). Also as expected, telomeric signal from each Acc survivor was found to be substantially resistant to *Rsa*I digestion. With Acc survivor 2, for example, there was little or no sign of *Rsa*I cleavage of telomeric arrays at any of three streaks examined, consistent with the long telomeres containing Acc repeat tracts uninterrupted by wild type repeats. Acc survivor 10 similarly shows little evidence of telomere cleavage by *Rsa*I. However, with the other four Acc survivors examined (4, 9, 12, & 6), there was a pronounced shift of high molecular weight signal to smaller sizes after cleavage with *Rsa*I that was often accompanied by the appearance of some sharp bands below 5 kb. These results indicate that at least small numbers of wild type repeats can often be interspersed within the long Acc telomeres. Clearly the wild type repeats in these clones are unable to provide proper telomere function. Presumably they are not present in sufficient concentrations or at the correct positions (the ends) to be able to correct the defects caused by the more abundant Acc repeats. The more severe defect of Acc repeats may also act to render interspersed wild type repeats less able to provide telomere function than is the case with SnaB survivors.

Very small bands produced by *Rsa*I digestion (most prominent in Acc survivor 12 and streaks 3 and 5 of Acc survivor 6) conceivably could represent small tandemly repeating units containing both wild type and Acc repeats similar to the repeating arrays observed in some

telomerase deletion survivors with wild type repeats generated from cells with two types of telomeric repeats (59). The changes in the *Rsa*I digestion profile of Acc survivor 6 over a five streak growth course showed dramatic changes in the telomeric signal <0.5 kb. These data indicate that there can be rapid and substantial turnover of telomeric sequences in Acc survivors.

Abundant single-stranded DNA is seen at telomeres in Acc and SnaB survivors.

Previously, it was shown that *ter1* long telomere mutants of two distinct classes (immediate (e.g., *ter1-19A(Acc)*) and delayed elongation) have an abundance of single-stranded telomeric DNA, specifically of the G-rich strand, at their telomeres (80). To test for the presence of single-stranded DNA in Acc and SnaB survivors, in-gel hybridizations were performed using a telomeric oligonucleotide probe complementary to the sequence of the G-rich telomeric strand. The results obtained from Acc survivors and *ter1-19A(Acc)* mutants are shown in Figure 2.6A. The ethidium bromide gel picture is provided as a loading control. *Eco*RI digestions were prepared and half of each was run on a gel for standard Southern blotting (denatured gel) and the other half was run on a gel for the in-gel hybridization. The wild type control showed telomeric signal in the denatured gel but little or none detectable in the in-gel hybridization. However, the in-gel hybridization showed that appreciable single-stranded telomeric DNA was present both in the *ter1-19A(Acc)* strains as well as in the Acc survivors. Some variability in the extent of the single-stranded telomeric DNA was evident and likely reflects variations in the length or other features of the telomeres in these cells. Figure 2.6B illustrates the presence of abundant single-stranded DNA in a SnaB survivor (Survivor 4 of Fig. 2.3B) followed for 5 streaks. As can be seen, telomeres in these SnaB survivors display abundant single-stranded telomeric DNA, particularly when the telomeres are very long. Our results demonstrate that elevated levels of single-stranded telomeric DNA are present in Acc and SnaB survivors and that it forms in a telomerase-independent manner.

We next tested whether the single stranded telomeric DNA we detected in Acc survivors existed as 3′ overhangs by digesting with Exo I. Figure 2.6C shows digestion of Acc2, Acc9, and Acc10 with *Eco*RI and with *Eco*RI+Exo I. The denatured and in-gel hybridizations to a C-strand telomeric probe as well as the ethidium bromide-stained control are shown. Digestion with Exo I produced a 36-55% decrease in the signal present in the in-gel hybridizations. These results are consistent with at least a sizable fraction of the single stranded telomeric DNA in Acc survivors existing as long 3′ single stranded overhangs. Partial resistance of telomeric single-stranded DNA to Exo I digestion was also observed in *stn1-t* mutants with elongated telomere in *S. cerevisiae* (66). Conceivably, the resistant fraction in both cases might represent single-stranded gaps.

We also examined the senescing *ter1*-Δ precursors to Acc survivors that contained a single mutant telomere for the presence of long 3′ overhangs. We found that the telomere with the Acc repeats produced a prominent in-gel hybridization signal to a C-strand telomeric probe that was sensitive to Exo I digestion. This signal was not seen in the same cells at the earliest stages of senescence (when the telomere would still be capped with many wild type repeats) nor was it seen in the short telomeres with only wild type repeats that were present in the same cell (Fig. 6C and data not shown). Similar results were found in senescing cells with the single SnaB telomere (data not shown). On the other hand, neither *ter1-24T(SnaB)* cells, which have very few SnaB repeats at the ends of each of the twelve telomeres, nor *ter1*-Δ cells lacking any mutant telomere, show increased 3′ overhang signal relative to wild type controls (Fig, 2.6E). We conclude that telomeres terminating in extended tandem arrays of Acc or SnaB repeats are not able to protect telomeres from extensive degradation of 5′ ends.

Discussion

Length regulation defects of Acc and SnaB repeats in the presence of telomerase are consistent with Rap1 binding defects.

Our results here show that both SnaB and Acc mutant telomeric repeats are defective at regulating telomere length in cells expressing telomerase. While SnaB repeats retain a partial ability to negatively regulate telomere length when present basally at a telomere, Acc repeats appear to be completely defective in this function. Telomeres with an array of Acc repeats thus acquire a terminal array of wild type telomeric repeats that is the full size of normal telomeres.

The defect of the SnaB and Acc repeats in regulating telomere length in the presence of telomerase is very likely the result of defects in their ability to bind the Rap1p protein. Rap1p is well known to be a key negative regulator of telomere length in *S. cerevisiae* and *K. lactis* through its ability to bind double-stranded telomeric repeats (37, 44). Also, the base changes of both telomeric mutations fall within the Rap1p binding site and disrupt Rap1p binding *in vitro* with the extent of disruption of binding greater for the Acc mutation than for the SnaB mutation ((37) and A. Krauskopf and E. H. Blackburn, personal communication). Although a basal array of Acc repeats is completely "uncounted" with respect to regulating telomere length, this may not mean that there is a complete absence of Rap1p bound to them *in vivo*. Not all sequences able to bind Rap1p appear able to regulate telomere length (30, 37, 80). In *K. lactis*, for example, a basal array of 'Kpn' mutant repeats, each with two base changes near but not in the Rap1p binding site, are strongly defective at being "counted", yet bind Rap1p with at least normal affinity as individual repeats *in vitro* (30, 37, 80). This lack of counting could result from cooperative interactions between Rap1p molecules or Rap1p's known ability to bend DNA (22, 54, 84).

What is the defect of SnaB repeats that interferes with telomerase-mediated telomere maintenance?

A number of lines of evidence argue that SnaB mutant telomeric repeats have a second defect that inhibits telomerase's ability to add sequence onto the mutant telomeric ends. The *ter1-24T(SnaB)* mutant produces telomeres that are substantially shorter than wild type, a characteristic shared with several other *ter1* template mutations that alter the right side of the Rap1p binding site (50, 80). In addition, combining the *ter1-24T(SnaB)* base change in *cis* with the *ter1-19A(Acc)* mutation almost completely blocks the extreme elongation normally caused by the latter allele (51). Also, our data here shows that cells containing separate *ter1-24T(SnaB)* and *ter1-19A(Acc)* alleles exhibit extensive telomere elongation through incorporation of Acc repeats but little incorporation of SnaB repeats. The very poor accumulation of SnaB repeats in a *ter1-24T(SnaB)* mutant presumably accounts for why telomeres can remain short in this mutant despite the mutant repeats being defective in the negative regulation of both telomerase and RTE.

The defect, or defects, in SnaB repeats that blocks sequence addition by telomerase is not fully understood. One defect is likely to be that the *ter1-24T(SnaB)* mutation interferes with the base pairing between the telomerase RNA and the 3′ end of the telomeric DNA. Recent evidence has indicated that telomeric DNA copied from positions 22-24 of the template base pair with positions adjacent to the template and that this partially explains the short telomere phenotypes of mutations at positions 22-24 ((82), Z. Wang and M. McEachern, unpublished data)(dotted lines in Figure 2.1A show alignment regions). An additional possibility is that the SnaB mutation also interferes with the binding of Cdc13p or Est1p, proteins that interact with the 3′ single-stranded tail of telomeres and are required for recruitment or activation of telomerase (19, 20, 64, 72). The binding site of Cdc13p within *S. cerevisiae* telomeric sequences does appear to overlap the Rap1p binding site (18). Whether this is true in *K. lactis* has not been determined.

Both SnaB and Acc telomeres are defective at regulating RTE.

SnaB and Acc repeats can promote the formation of telomeres by RTE that are much longer than those seen in comparable telomerase deletion mutants containing only wild type telomeric repeats. This elongation, particularly in the case of Acc survivors, appears to be similar to that of the Type IIR RTE originally described in the *stn1-M1* mutant of *K. lactis* (33). Type IIR RTE was defined as RTE that produces very long telomeric repeat tracts due to a capping defect that makes telomeres prone to inducing recombination in a manner independent of their length. This contrasts with 'ordinary' Type II RTE where the telomeric recombination is initiated by a capping defect brought on by critically short telomeres and appears to be suppressed by even modestly elongated telomeres. It could be noted though that standard Type II RTE could potentially produce a long telomere, but only in a single step such as perhaps copying a circular template or copying another telomere that was already long.

Other facts in addition to the abnormally long telomeres of SnaB and Acc survivors suggest that both types of mutant repeats produce Type IIR RTE. Both SnaB and Acc survivors that have amplified mutant but not wild type telomeric repeats normally lack the irregular cycles of growth senescence and recovery typical of telomerase deletion mutants with only wild type repeats. Instead, they have no obvious growth defects or else modest chronic growth defects that do not appreciably change with passaging. This is similar to the *stn1-M1* mutant, which had continuous moderate growth and cellular defects presumably stemming from the continual presence of telomeric ends that triggered a DNA damage response.

Another key feature predicted for Type IIR RTE is extreme and constitutive telomere instability. Even relatively long telomeres are expected to be highly unstable in length and not able to shorten gradually over multiple cell divisions as occurs in ordinary *K. lactis* Type II survivors. This certainly appears to be the case with Acc survivors. Although the long smeared telomeric signal of Acc survivors in Southern blots remained relatively constant over time, we interpret this not as telomeric stability but rather as a steady state of high instability. The

presence of telomeric DNA in highly smeared signal indicates that telomeres in Acc survivors (at least those composed of primarily Acc repeats) are typically too unstable to exist at or near a discreet size for the entire 20-25 cell divisions of time needed to generate enough cells for Southern analysis. However, with the extreme size and continuous presence of long telomeres in these cells, it is not currently possible to fully exclude the possibility that a minority of relatively stable telomeres could be present in Acc survivors. The abundance of extrachromosomal telomeric DNA in Acc survivors serves as further evidence of frequent telomeric recombination. SnaB survivors, though clearly less extreme than Acc survivors, also showed instances of sudden large changes in the sizes of telomeres that in survivors with wild type repeats would have been long enough to be relatively resistant to such changes.

Our results here with Acc survivors raise the question of the relative contributions of telomerase and HR to the long telomere phenotype of the *ter1-19A(Acc)* mutant. As another member of the "immediate elongation" class of *ter1* template mutations that have mutations in the Rap1 binding site have been shown to produce very long telomeres independently of the critical recombination gene *RAD52*, the extreme telomere elongation in this class of mutants very likely can arise independently by two completely different mechanisms, Type IIR RTE and unregulated telomerase addition. The abundant extrachromosomal telomeric circles in these mutants, in contrast, are present in *RAD52* cells but absent in *rad52* mutants. The high rate of telomeric recombination in *ter1-19A(Acc)* mutants suggests that it is highly likely that the Type IIR RTE is actively occurring in the presence of telomerase, as is the case for the Type IIR RTE in the *stn1-M1* mutant (33).

The unusual persistence of short wild type telomeres in early Acc survivors.

Many Acc survivors exhibit persistent short telomeric bands in the first several streaks after they are generated. These short telomeres are composed largely or entirely of wild type repeats and their behavior is therefore not likely to be representative of telomeres containing

appreciable numbers of Acc repeats. The persistence of these short wild type telomeres remains difficult to fully explain. Almost certainly, the presence of a small number of wild type repeats provides a degree of telomere function that allows the short telomere to be somewhat resistant to recombinational processes. Consistent with this, disappearance of the persistent short telomeres is correlated with loss of the wild type repeats from those telomeres.

What is more perplexing is how the persistently short telomeric bands can remain a constant size over several streaks despite the absence of telomerase. We have previously seen an example of short telomeres in *K. lactis* persisting for a number of streaks after the introduction of a particular *ter1* template mutation that produced mutant telomeric repeats and elongated telomeres (51). However, in this case, the persistent short telomeres clearly displayed gradual shortening prior to the point where they became elongated. This was interpreted as indicating that some telomeres did not acquire mutant telomeric repeats for many cell divisions (perhaps because of inefficiency of the mutant telomerase template) and instead underwent gradual replicative sequence loss until either telomerase or recombination finally made them much longer. This explanation cannot account for the persistent short telomeres in Acc survivors because those telomeres do not undergo gradual shortening over the multiple streaks where they persist (Fig. 2.4C). This forces us to the conclusion that the persistent short telomeres are being actively elongated by recombination during the time period where they appear to be persisting at very short sizes.

One possible explanation is that the same set of short telomeres is repeatedly elongated by recombination to only very small extents so as to maintain their short sizes. This seems implausible given that telomerase is absent and other telomeres in the same cells undergo RTE that routinely adds kilobases to their lengths. An alternative possibility is that the persistently short telomeric bands represent a semi-stable intermediate state of telomeres that are otherwise regularly undergoing recombination events that may either greatly lengthen or greatly truncate them (Fig 2.4D). The persistent short telomeric bands would therefore represent a percentage of

all telomeres that retained a minimal number of basal wild type repeats. Given the sizable percentage of total subtelomeric signal present in the persistently short telomeric bands (Fig. 2.4C), this model would seem to require that telomeric truncations could routinely remove essentially all but the basal-most wild type repeats from a telomere with terminal Acc repeats. Supporting the possibility of such large deletions is the observation that "immediate elongation" *ter1* template mutations including *ter1-19A(Acc)* undergo turnover of basal wild type repeats in spite of having highly elongated telomeres (53). That wild type repeats in Acc survivors might be particularly resistant to loss would not be entirely surprising given that protecting chromosome ends from degradation and recombination is their normal function. Precedents are known in both *K. lactis* and *S. pombe* where defects in telomere binding proteins can give rise to very rapid and dramatic shortening of all long telomeres in the cell (2, 3, 33, 57).

Disruption of Rap1 binding is the most likely cause of the Type IIR RTE of SnaB and Acc survivors.

The simplest possible explanation for the enhanced tendency of the Acc and SnaB repeats to recombine is a defect in Rap1p binding. Both mutations fall within the Rap1 binding site and both interfere with Rap1p binding *in vitro*. Moreover, the more modest RTE phenotype in SnaB survivors relative to Acc survivors correlates with the SnaB mutant having a lesser Rap1p binding defect as judged by both telomere length defects in the presence of telomerase and *in vitro* binding studies. In *S. cerevisiae*, the Rif1p and Rif2p proteins bind to the Rap1p C-terminus and play crucial roles in mediating Rap1p's role in the negative regulation of telomere length in the presence of telomerase (31, 74, 87). In the *K. lactis* genome, *RIF1* but not *RIF2,* has been identified. We have found that deletion of *RIF1* in *ter1*-Δ mutants does not produce an obvious Type IIR RTE phenotype (O. Sprusansky and M. McEachern, unpublished data). Thus, we conclude that the Type IIR RTE phenotypes of SnaB and Acc repeats act independently, or at least not primarily, through affects on Rif1p interactions at the telomere. It also seems unlikely

that the additional defects of SnaB repeats that interfere with telomere elongation by telomerase in *ter1-24T(SnaB)* cells could be solely responsible for the weaker RTE phenotype of SnaB survivors relative to Acc survivors. If disrupted Cdc13p binding leads to both the short telomeres of *ter1-24T(SnaB)* cells and a Type IIR phenotype, it would predict, contrary to our observations, that SnaB survivors would have a more extreme RTE phenotype than Acc survivors. We cannot rule out, however, that defects in Cdc13p binding make contributions to the RTE phenotypes of Acc or SnaB survivors. Finally, the anticipated base-pairing defect of SnaB repeats with the region next to the Ter1 template (Fig. 2.1A) would be expected to be specific to telomerase-mediated telomere elongation and not affect telomeric recombination.

How might Rap1 negatively regulate telomeric recombination?

An important conclusion from our results is that the SnaB and Acc telomeric repeat mutations disrupt the negative regulation of both telomerase- and recombination-mediated telomere elongation. This suggests that there is some overlap in the negative regulation of telomere elongation by telomerase and by recombination. Previously reported data would also seem to support this idea. The 'Kpn' mutation of the *K. lactis* telomeric repeat (a double base change that does not affect Rap1p binding *in vitro* (37)) also disrupts both elongation processes (48, 77). In *S. cerevisiae*, the MRX complex and the Tel1p and Mec1p kinases, contribute to both telomerase- and recombination-mediated telomere maintenance (5, 35, 43, 61, 67, 78, 79) while the Rif1p and Rif2p proteins act to inhibit both processes (31, 74, 87).

A known overlap between sequence addition by telomerase and recombination is that both require the formation of 3′ overhangs. Normal telomeres of *S. cerevisiae* are exonucleolytically processed to acquire 3′ overhangs of >25 nucleotides during S phase (10). Recent data has suggested that formation of these short overhangs may be a key regulated step in telomerase mediated telomere elongation (60). These 3′ overhangs are thought to be good substrates for binding and sequence addition by telomerase but too short to be efficient substrates

for recombination. In contrast, non-telomeric broken DNA ends in yeast (at least outside of G1 phase) are well known to be degraded at their 5′ ends to produce long 3′ overhangs that can serve as substrates for Rad51p binding and strand invasion (86). Thus, the size of the 3′ telomeric overhang is likely to be critical for determining whether it can be elongated by telomerase or recombination.

Our results support the possibility that Rap1p acts to prevent recombination from initiating at telomeres by helping block the action of one or more exonucleases that degrade 5′ strand ends. By interfering with Rap1p binding, SnaB and Acc mutant repeats present at telomeric ends would allow the formation of 3′ overhangs long enough to provoke the telomeres to initiate homologous recombination. In the case of at least the Acc repeats, the long overhangs may also stimulate sequence addition by telomerase. Precedent exists for a protein that binds the double-stranded part of telomeric DNA to protect against formation of 3′ overhangs. Absence of the Taz1 protein in the fission yeast *S. pombe* leads to longer 3′ overhangs than are present at telomeres of wild type cells (76). One possibility for an exonuclease that might be blocked by Rap1p is ExoIp. ExoIp is known to be an important contributor to the 3′ overhangs that are generated at broken ends and certain dysfunctional telomeres, though it seems not to be required for the short overhangs of normal yeast telomeres (45, 46, 76).

The different roles that Rap1p plays at yeast telomeres likely depend on different regions of the telomeric repeat tract. The ability to negatively regulate telomere length in the presence of telomerase can clearly be carried out by Rap1 binding sites located within the most basal part of the telomere. In contrast, the ability of Rap1 to block telomere-telomere fusions from non-homologous end joining (NHEJ) appears to be played by only the most terminal telomeric repeats (7, 51, 63). It would seem highly likely that the proposed ability of Rap1p to regulate the nucleolytic degradation of the 5′ end of the telomere also is localized to the more terminal part of the telomere. This may explain why the *ter1-19A(Acc)* mutant displays massive telomere elongation in spite of having near-normal numbers of wild type repeats basally at telomeres (48).

It remains to be determined whether Type IIR RTE occurs through a roll and spread mechanism as has been suggested for Type II RTE (59). Although telomeric circles may be abundant in *K. lactis* mutants undergoing Type IIR RTE, the continuous presence of long telomeres in these cells would seem to provide suitable alternative templates for short telomeres to copy to become long. Perhaps an important place for rolling circle synthesis to contribute to Type IIR RTE is at the very early stages after a mutant's creation during the formation of the first long telomere in the cell.

Human ALT cells utilize recombination to maintain their telomeres in the manner that is often highly reminiscent of Type IIR RTE in yeast. The ALT phenotype does not occur in most human cells that undergo severe telomere shortening, suggesting that it may require one or more mutations in order to occur. Although ALT cells are unlikely to have mutant telomeric repeat sequences, our results here strengthen the idea that mutations that cause chronic telomere capping defects that promote telomeric recombination and are not suppressed by telomere elongation are likely required. Future studies with yeast Type IIR may therefore be of considerable importance in gaining insight to telomere maintenance in human ALT cancers.

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Figure 2.1. The long telomere phenotype of *ter1-19A(Acc)* mutation is dominant to the *ter1-24T(SnaB)* mutation in *trans*. (A) Diagram of the *K. lactis* telomerase RNA template region. The strand shown is the complement of that present in the RNA. The numbers shown signify the coordinates used for base positions in and around the template. Rap1p binds at the sequence shown overlined. Base substitutions making *Acc*I and *Sna*BI restriction sites are indicated. The underlined sequences are involved in accurate alignment of the template with the telomeric DNA during telomerase translocation. (B) Southern blot of telomeric hybridization to DNA from *ter1-24T(SnaB)* and *ter1-19A(Acc)* cells created by integration of a *ter1-19A(Acc)*-containing plasmid into haploid *ter1-24T(SnaB)* cells. Two independent heteroallelic strains are shown (S+A). DNA from a wild type (WT) and a matching control containing two *ter1-24T(SnaB)* alleles are also shown. Each DNA is shown digested with *EcoR*I (R), *EcoR*I and *Acc*I (R+A) and *EcoR*I and *SnaB*I (R+S). Markers (M) are shown in kb.

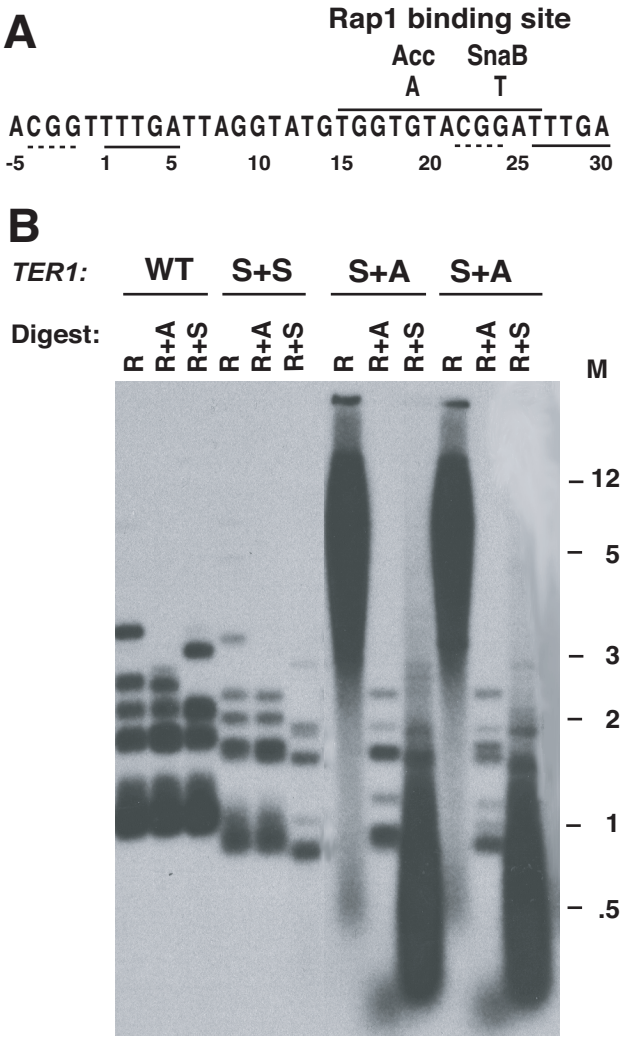
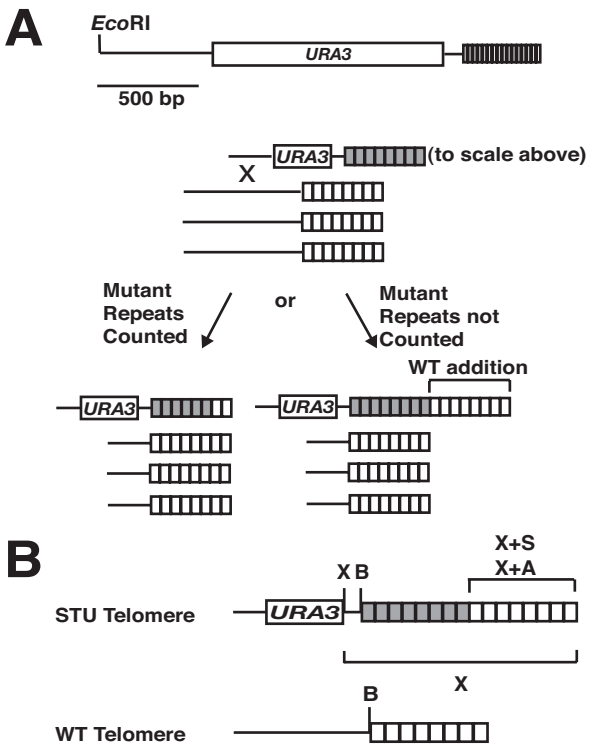

A
Rap1 binding site
Acc I
SnaB I
ACGGTTTTGATTAGGTATGTGGTGTACGGATTTGA
-5
1
5
10
15
20
25
30
B
TER1:
WT
S+S
S+A
S+A
Digest:
R
R+A
R+S
R
R+A
R+S
R
R+A
R+S
R
R+A
R+S
M
12
5
3
2
1
.5

Figure 2.2. SnaB and Acc telomeric repeats are defective at regulating telomerase addition to their ends. (A) Diagram of experimental method for replacing a native telomere with a mutant telomere (grey boxes represent mutant repeats and white boxes represent wild type repeats). Restriction fragments containing mutant telomeres were transformed into wild type cells where they each replaced a single native telomere by recombination between common subtelomeric sequences. Upon integration, the mutated telomeres acquire some number of terminal wild type repeats (white boxes) from the resident wild type telomerase. See text for details. A scale diagram of a STU telomeric fragment is shown at the top. (B) The STU telomeres have a unique *Xho*I (X) site at the end of the *URA3* fragment. A *Bsr*BI (B) site is located 3 bp upstream of the telomeric repeats that is present at 10 of 12 telomeres. The tagged repeats each have Acc or SnaB (A or S) restriction site so that the wild type addition onto them can be measured. The brackets represent fragments generated by particular digests. (C) Southern blot of SnaB and Acc telomeres. The leftmost four lanes show two independent wild type STU telomere transformants cut by *Xho*I (X) or *Bsr*BI (B). The band between 0.5 and 0.9 kb is the STU telomere in *Xho*I digests. The slightly smaller band in the *Bsr*BI digests represents 10 of the 12 telomeres. The central six lanes show transformants that received a SnaB STU telomere with ~10 SnaB repeats (SnaB 10) or ~27 SnaB repeats (SnaB 27) cut by *Xho*I or by a double digest with *Xho*I and *Sna*BI (X+S). The rightmost lanes show *Xho*I and *Xho*I + *Acc*I digest (X+A) of two transformants that received an Acc STU telomere with 13 Acc repeats. The wild type addition in the different double digest lanes can be seen as a light smear near the bottom of the gel. The bracket marks the range of positions of the STU telomere fragments. Markers (M) are shown in kb.


A
EcoRI
URA3
500 bp
URA3
(to scale above)
X
Mutant
Repeats
Counted
or
Mutant
Repeats not
Counted
WT addition
URA3
URA3
B
X+S
X+A
X B
STU Telomere
URA3
X
B
WT Telomere

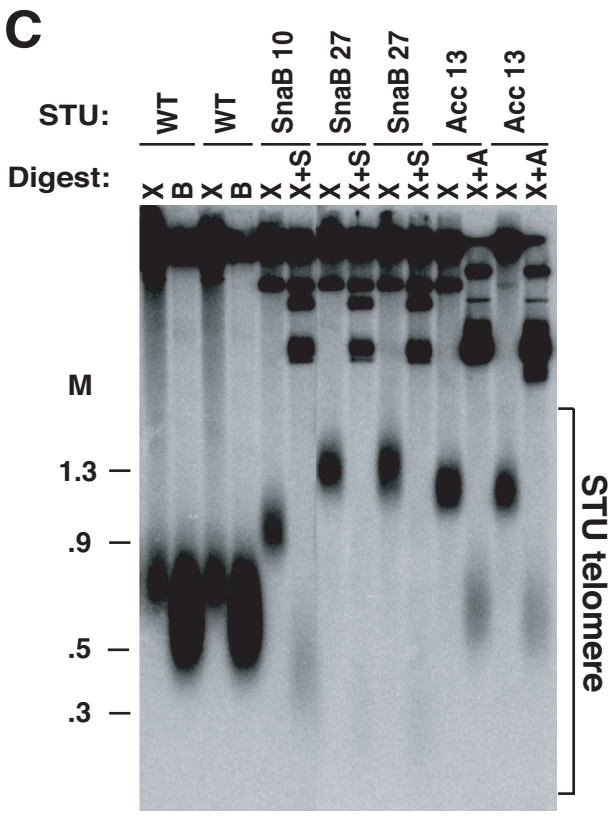

C
STU:
WT
WT
SnaB 10
SnaB 27
SnaB 27
Acc 13
Acc 13
Digest:
X B X B X X+S X X+S X X+S X X+A X X+A
M
1.3
.9
.5
.3
STU telomere

Figure 2.3. SnaB telomeric repeats can promote the formation of long, unstable telomeres through RTE. (A) Scheme for generating *ter1*-Δ cells containing mutant repeats. Cells containing a single STU telomere with mutant telomeric repeats (left drawing) were deleted for telomerase and allowed to senesce (middle drawing). The long size of the mutant telomere greatly enhances the likelihood that the mutant repeats will spread to all other chromosome ends during RTE. Drawings at right show several possible outcomes for the telomere structures. Outcomes 1-3 depict typical moderate telomere lengthening seen in Type II survivors shown with only wild type repeats (outcome 1), interspersed wild type and mutant repeats (outcome 2), or only amplification of mutant repeats (outcome 3). The asterisk depicts a different possibility that defective mutant repeats could be effectively "capped" by wild type repeats. Outcomes 4-6 depict potential results with Type IIR RTE generating very long telomeres. Outcome 4; all telomeres mutant and long. Outcome 5; mix of mutant long telomeres and shortened telomeres. Outcome 6; Long mutant telomeres with some interspersed wild type repeats. Gray and white boxes are mutant repeats and wild type repeats, respectively. (B) Southern blot hybridized to a telomeric probe of *ter1*-Δ survivors with telomeres containing SnaB repeats. Each gel shows a separate SnaB survivor followed for five serial restreaks after senescence. The first gel is a control survivor that retained only wild type repeats, while the other gels show examples of some of the spreading patterns. DNA from each sample is shown digested with *EcoR*I (-) and with *EcoR*I + *SnaB*I (+). Underneath the gels is indicated the type of repeat primarily amplified. Markers (M) are shown in kb.

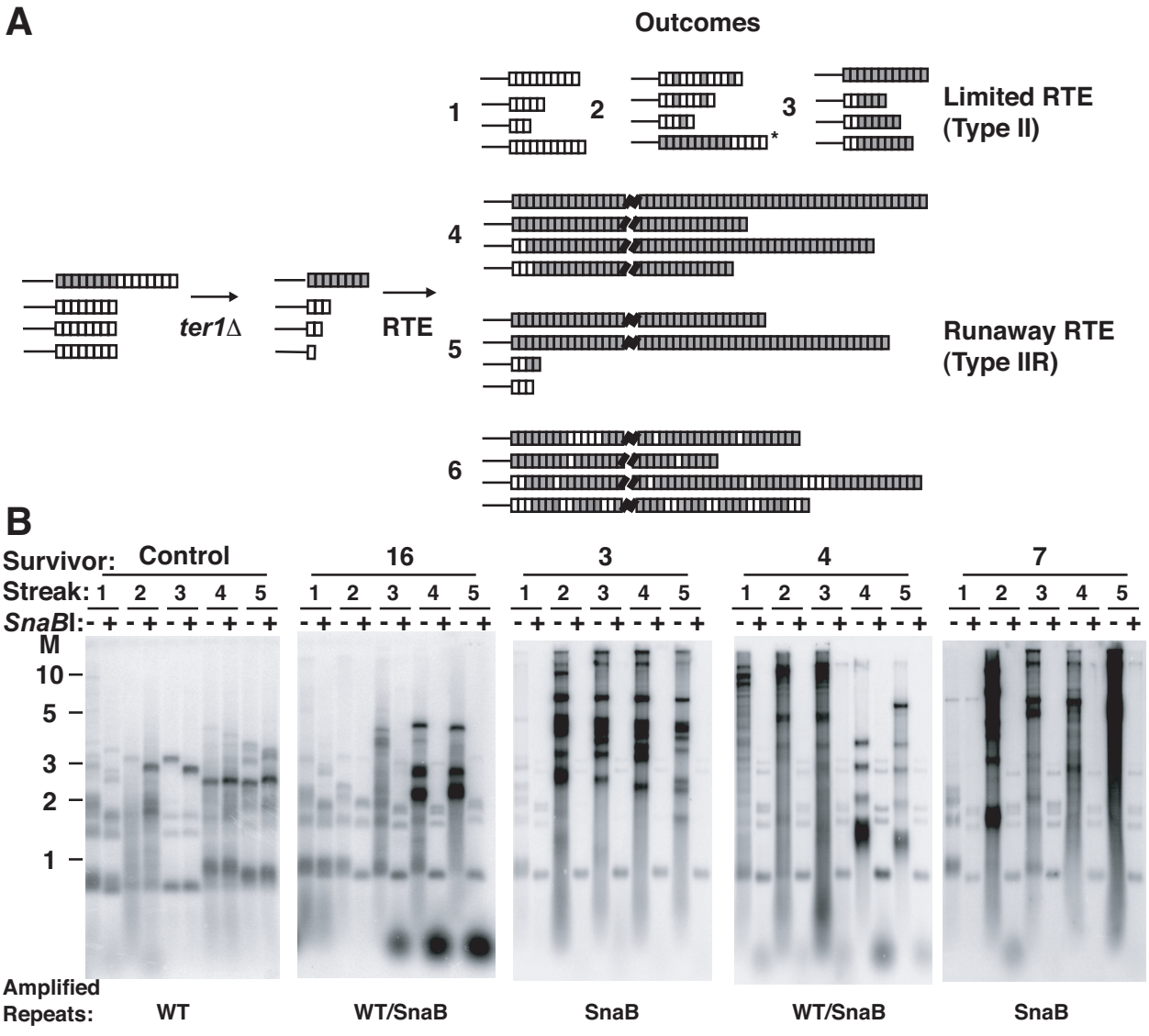

A
Outcomes
1
2
3
*
Limited RTE
(Type II)
4
5
Runaway RTE
(Type IIR)
6
ter1Δ
RTE
B
Survivor:
Control
16
3
4
7
Streak:
1 2 3 4 5
SnaBI:
- + - + - + - + - +
M
10
5
3
2
1
Amplified
Repeats:
WT
WT/SnaB
SnaB
WT/SnaB
SnaB

Figure 2.4. Acc survivors exhibit Type IIR RTE after spreading but can have persistant short telomeres. (A) Southern blots of six independent Acc survivors. Each gel shows DNA from an independent survivor that was serially restreaked 5 times after survivor formation. Samples are shown digested with *Eco*RI (-) and *Eco*RI + *Acc*I (+). A telomeric probe was used for hybridization. (B) Southern blot of telomeres of Acc survivors after ten streaks. The digests and probe are the same as above. (C) Persistent short telomeres in Acc survivors. Southern blots of DNA from wild type cells, *ter1*-Δ cells and Acc survivors are shown hybridized to a subtelomeric sequence common to 11 of 12 telomeres. The dot indicates the position of a group of telomeric fragments when they contain only a small number of telomeric repeats. The signal in this band in the *Eco*RI +*Acc*I digests represent the total amount of this group of telomeres and the signal in the *Eco*RI digests represents the fraction of the telomeres that are very short even without cleavage of the Acc repeats. The asterisk marks the position of subtelomeric fragments in *Eco*RI + *Acc*I digests that have lost all detectable wild type repeats and that consequently are not detectable with the telomeric probe. Molecular weight markers (M) are shown in kilobases. (D) Model for persistent short telomeres in early Acc survivors. In the case on the left, a telomere is shown with a basal region of wild type repeats (white box) and a long terminal region of Acc repeats (gray region) that is unstable and, as a consequence, highly heterogeneous in length in a population of cells. Truncated forms of the telomere that retain only the basal wild-type repeats may confer a semi-stable state that is relatively resistant to being re-elongated by recombination. However, in the situation on the right, an unstable long telomere with no basal wild type repeats could be subject to similarly high rate of truncation events but be unable to stabilize any particular short-sized telomere.

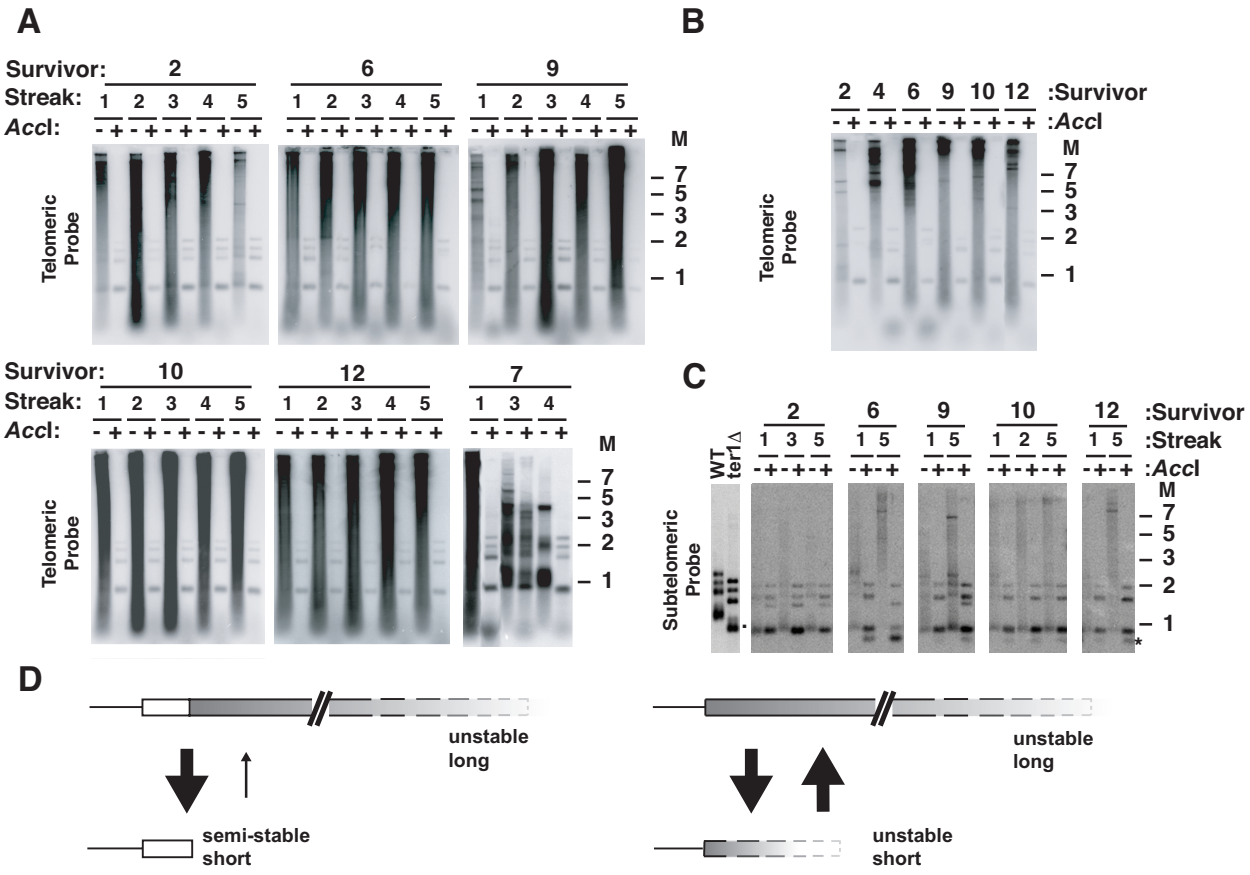

A
Survivor:
Streak:
AccI:
Telomeric Probe
M
7
5
3
2
1
B
:Survivor
:AccI
C
WT
tert1Δ
:Streak
Subtelomeric Probe
D
unstable long
semi-stable short
unstable short

Figure 2.5. Acc survivors can contain WT repeats within their long tracts of Acc repeats. Southern blots show Acc survivors digested with *Bsr*BI (-) or *Bsr*BI + *Rsa*I (+) and hybridized to a telomeric probe. All survivor numbers correspond to those in Figure 4. At left is a wild type control where the telomeres are completely cut away by *Rsa*I. The central and right gels show results from serial restreaks of Acc survivors 2 and 6. Asterisks on the survivor numbers indicates that the DNAs shown are from different subclones of the Acc survivors than are shown in Fig. 4. Markers (M) are shown in kb.

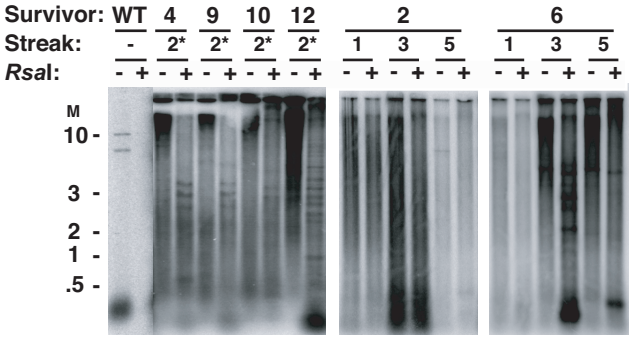
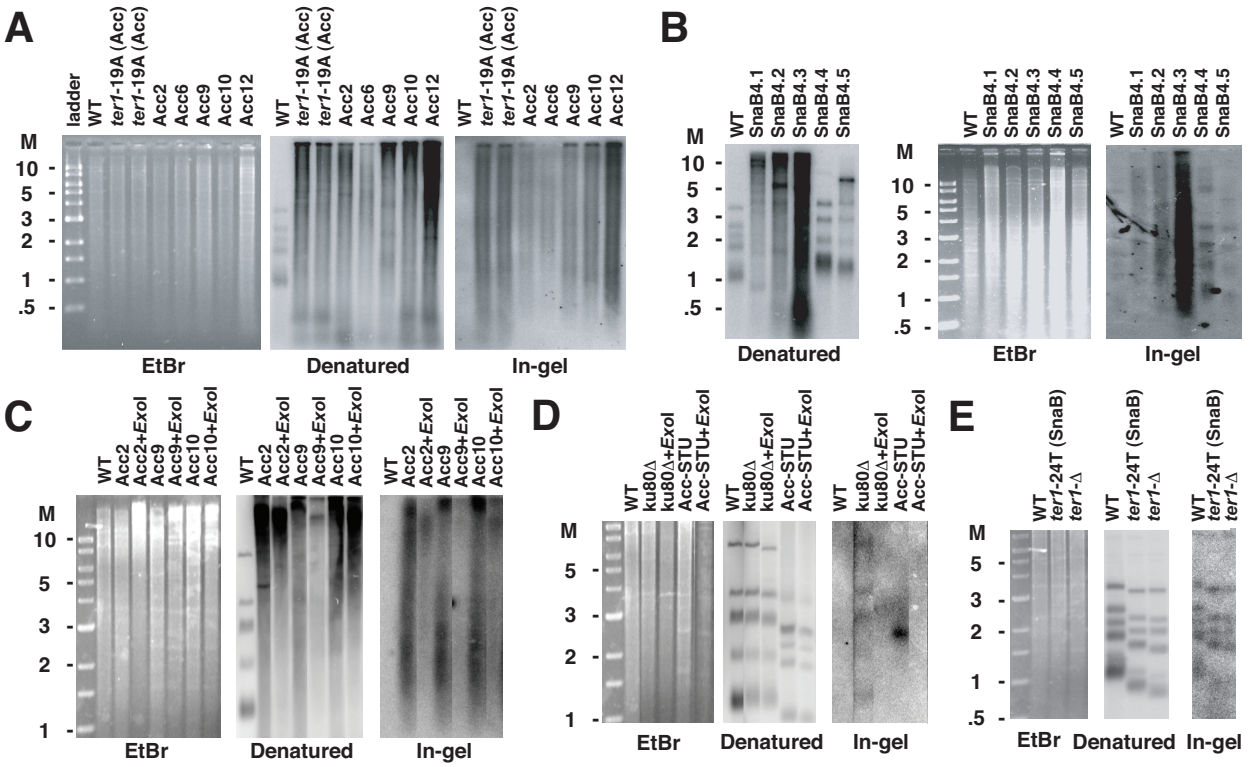

Survivor: WT 4 9 10 12 2 6
Streak: - 2* 2* 2* 2* 1 3 5 1 3 5
RsaI: - + - + - + - + - + - + - + - + - + - +
M
10
3
2
1
.5

Figure 2.6. Telomeres in Acc and SnaB survivors have substantial amounts of single-stranded DNA. (A) Ethidium-stained gel (EtBr), Southern blot, and in-gel hybridization of DNA from Acc survivors. The first lane in each is a wild type control. The second and third lanes are two independent samples of *ter1-19A(Acc)*. The remaining lanes are the same survivors as shown in Figure 4. (B) EtBr-stained gel, Southern blot, and in-gel hybridization of *Eco*RI-digested DNA from each of five streaks of SnaB survivor 4. The streak numbers are notated after the survivor number above the gel. Also shown is the wild type strain 7B520. Please note that the Southern blot and in-gel only in Figure 6B ran differently and therefore have different size markers. (C) EtBr-stained gel, Southern blot, and in-gel hybridization of *Eco*RI-digested and *Eco*RI + Exo I digested DNA of Acc survivors number 2, 9, 10, and 12 along with that of a wild type control from the wild type strain CBS. Both the Southern blot and in-gel hybridization were probed with a C-stranded telomeric oligonucleotide. (D) EtBr-stained gel, Southern blot, and in-gel hybridization of *Eco*RI-digested cells of the wild type strain CBS 2359, and the *Eco*RI and *Eco*RI + Exo I-digested DNA of *ku80*Δ cells and senescent *ter1*-Δ cells containing an Acc-STU telomere that has not yet spread to other telomeres. (E) EtBr-stained gel, Southern blot, and in-gel hybridization of *Eco*RI-digested DNA of the wild type strain 7B520, a *ter1-24T(SnaB)* strain, and a *ter1*-Δ strain. Molecular weight markers (M) are shown in kb.


A
ladder
WT
ter1-19A (Acc)
ter1-19A (Acc)
Acc2
Acc6
Acc9
Acc10
Acc12
M
10
5
3
2
1
.5
EtBr
Denatured
In-gel
B
WT
SnaB4.1
SnaB4.2
SnaB4.3
SnaB4.4
SnaB4.5
Denatured
EtBr
In-gel
C
WT
Acc2
Acc2+ExoI
Acc9
Acc9+ExoI
Acc10
Acc10+ExoI
EtBr
Denatured
In-gel
D
WT
ku80Δ
ku80Δ+ExoI
Acc-STU
Acc-STU+ExoI
EtBr
Denatured
In-gel
E
WT
ter1-24T (SnaB)
ter1-Δ
EtBr
Denatured
In-gel

CHAPTER 3

AN ABNORMALLY LONG TELOMERE IN THE YEAST *KLUYVEROMYCES LACTIS* IS SUBJECT TO BOTH TRUNCATION AND ELONGATION[2]

[2] Bechard LH, Jamieson N, and MJ McEachern. To be submitted to *Molecular and Cellular Biology*.

Abstract

While the major mechanisms of telomere length changes are thought to be replicative sequence loss and sequence addition by telomerase, other processes have been shown to be involved. Recombination has been extensively studied as a method to lengthen telomeres, but it has also been shown to shorten telomeres in a process called Telomere Rapid Deletion (TRD). In this study, we observe size changes that occur in the yeast *Kluyveromyces lactis* at an artificially long telomere tagged with repeats containing a phenotypically silent *Bcl*I restriction site. As observed in *S. cerevisiae*, we found that TRD was common (4×10^{-3}/cell division) and preferentially truncated the long telomere to near wild type size. However, we did not detect an increase in the rate in meiosis. We also found that about half of mitotic TRD events were associated with deep turnover of telomeric repeats, suggesting that telomeres were often cleaved to well below normal length prior to being re-extended by telomerase. Despite undergoing a high rate of TRD, the long Bcl telomere showed no increase in the rate of subtelomeric gene conversion, a highly sensitive test of telomere dysfunction. Our results suggest that telomere truncation is not confined to abnormal telomeres but also occurs in normal telomeres where it likely accounts for deep turnover within telomeres that has been previously shown to occur. Finally, we report that in addition to undergoing TRD events, the long Bcl telomere could, at a somewhat lower frequency, become substantially longer. This elongation occurred through the addition of more Bcl repeats, indicating that it was caused by recombination.

Introduction

Telomeres are the complex of DNA and proteins that protect the ends of chromosomes from being recognized as double-stranded breaks (11, 43, 78). Due to the inability of the

replicative polymerases to fully replicate ends, telomeres gradually shorten over time in the absence of a specialized means of maintaining them (58, 83). In the majority of eukaryotes, the ribonucleoprotein telomerase acts to extend telomere ends by adding nucleotides to a short 3′ overhang at telomeres (1, 32). In what is thought to be an anti-cancer adaptation, most human cells have little or no telomerase, which leads to replicative senescence when telomeres become too short (70). Furthermore, telomeres are normally "capped" by a complex of proteins, which protect them from non-homologous end-joining (NHEJ) and homologous recombination (9, 14). In some organisms, including humans, telomere capping may also involve telomeric loops (t-loops), the structures resulting from the intramolecular strand invasion of the 3′ overhang at telomeres into the internal telomeric repeats (18).

While gradual loss and extension by telomerase are the most common modes of telomere length changes, other processes, most notably recombination, have been shown to affect telomere length under certain circumstances. Some DNAs, including the chromosomes of the mosquito *Anopheles gambia* and the linear mitochondrial DNA of certain yeasts and ciliates, appear to use recombination as their normal method of telomere maintenance (46, 57, 66, 74). Telomerase-deficient mutants of a number of yeast species are able to maintain their telomeres by recombination (36, 39, 51, 71). In *K. lactis* and *S. cerevisiae*, the species that have been most studied, telomere elongation appears to occur by a "Roll and Spread" model, in which a small circle of telomeric repeats (t-circle) first acts as a template for rolling circle DNA synthesis to elongate a single telomere. The sequence from the first long telomere can then be copied in a Break-Induced-Replication (BIR)-like event to elongate the other telomeres in the cell (19, 35, 52, 53, 75). In both species, this recombinational telomere elongation is dependent on the major recombination protein *RAD52* and is initiated by the loss of telomere capping brought on by very

short telomere lengths (36, 39, 42). More recent work has demonstrated that recombinational telomere elongation can also occur as a consequence of certain mutations directly affecting telomere capping (4, 16, 17, 24, 29, 60, 86). Interestingly, these mutations frequently create telomeres that are longer and more heterogeneous than telomeres undergoing recombination caused by the loss of telomerase.

Recombination has also been shown to be an important telomere maintenance mechanism in some cancer cells. Telomerase activity is very low or absent in most human somatic cells (26). While the majority of human cancers reactivate telomerase to maintain telomeres and immortalize cells, a significant minority use a recombinational mechanism called Alternative Lengthening of Telomeres (ALT) (5, 26, 65). Telomeres become highly heterogeneous in ALT cancer cells, with some telomeres being very long and others having few or no telomeric repeats. Extrachromosomal telomeric DNA in both linear and circular forms is commonly found in these cells (10, 50, 82). Various types of telomeric recombination have been demonstrated to be common in ALT cells. Experiments using a sequence tag introduced on one telomere have shown that recombination events can transfer the sequence to other telomeres as well as duplicate it at the same telomere (13, 47). Also, reciprocal sister chromatid exchanges have been shown to occur at greatly elevated rates in ALT cells (2).

Telomeres can also be subject to dramatic shortening events under a variety of different circumstances including normal growth and development in some organisms. Examples include the abrupt shortening of all telomeres in newly developed macronuclei of the ciliate *Euplotes crassus* (79), the rapid trimming back to normal size of telomeres greatly lengthened by continuous mitotic growth in *Tetrahymena thermophila* (30), and the large truncations and elongations occurring during antigenic variation in *Trypanosoma brucei* (49). Even the

comparatively large telomere attrition per cell division of relatively long telomeres in human fibroblasts and in telomerase mutants of *Caenorhabditis elegans* have been proposed to involve additional mechanisms other than gradual loss due to the end-replication problem (12, 23). Considerable evidence now suggests that oxidative damage to telomeres can accelerate telomere attrition in human cells (64, 69, 80, 81).

Sudden shortening events are commonly associated with telomeres that have capping defects. Early studies in immortalized human cells showed dramatic shortenings, sometimes losing all telomeric signal, at a single telomere that had become long (48). Human cells containing mutations of the double-stranded telomere binding protein TRF2 show a depletion of 3′ overhangs and telomere deletions that are the size of t-loops (77, 82). A *K. lactis* mutant with very long telomeres occasionally displayed better-growing colonies with much shorter telomeres (40). Even in situations where recombinational maintenance is occurring, there can also be concurrent large shortening events. Evidence from a *K. lactis* mutant lacking telomerase and having telomeric repeats defective for binding Rap1 suggested that rapid truncations to very short sizes occurred regularly in cells with very long telomeres maintained by recombination (4).

The best-studied example of dramatic telomere shortening is Telomere Rapid Deletion (TRD), which has been shown to truncate abnormally long telomeres in *S. cerevisiae* (34). These truncations predominantly shorten telomeres down to the size of resident wild type telomeres and were proposed to represent a trimming mechanism that shortened telomeres that had become too long. *S. cerevisiae* TRD is partially dependent on the major recombination protein Rad52 and also shows a dependency on the Mre11p-Rad50p-Xrs2p complex and the Ku70/80 heterodimer (6, 34). The retention of introduced *Hae*III sites at relatively more basal positions in telomeres that had undergone TRD led to the proposal that TRD occurred though a

terminal deletion initiated by a t-loop intermediate (6). TRD showed a 30-70-fold increase in meiosis, which was dependent on the meiotic bouquet protein Ndj1p (25). TRD has also been reported at abnormally long telomeres in *Arabidopsis thaliana*, but does not appear to be dependent on paralogs of RAD51 or on the MRE11-RAD50-NBS1 complex (84). Interestingly, in human cells overexpressing telomerase RNA, telomeres become long and heterogeneous in length and t-circles become abundant, presumably because of trimming by a TRD-like mechanism (61).

In order to determine the fate of a long telomere in *K. lactis* cells, we have introduced an abnormally long telomere made up of phenotypically silent repeats containing a *Bcl*I restriction site into cells with otherwise wild type telomeres. Like *S. cerevisiae*, we observed abundant TRD that shortened telomeres to apparent wild type size. However, about half of these events were associated with deep turnover of telomeric repeats. We also report a lower frequency of further elongation of the Bcl telomere brought about by recombination.

Materials and Methods

Strains and Culturing Conditions.

The *K. lactis* strain ZT-LBT1 containing the long Bcl telomere in this study was a derivative of 7B520 (*ura3 his2-2 trp1*) (85). It contained a deletion of the *K. lactis* telomerase RNA gene *TER1* (67). *TER1* was replaced in these cells using the plasmid pJR31, which is a derivative of pKL316 (67). This plasmid contains a *S. cerevisiae HIS3* gene, which complements the *his2* mutation in 7B520, for selection. The long Bcl telomere with *URA3* adjacent to it was constructed and transformed into *K. lactis* cells to replace a single telomere as

described previously (75). To create a *rad52Δ* strain with a long telomere, cells containing the long telomere were mated to SI-E4 (*ade2-202 rad52Δ*), a strain of the opposite mating type (Shilpa Iyer, unpublished data).

For the mitotic TRD studies, clones of the strain containing the long Bcl telomere were plated on SD minimal medium lacking histidine in order to retain pJR31 and were grown for 3-4 days to allow adequate growth. The *rad52Δ* and post-meiosis strains were plated on YPD (yeast extract-peptone-dextrose) after ascertaining they had obtained the wild type *TER1* allele after mating.

Matings were conducted on malt extract and diploids were grown on YPD after initial selection on an SD plate lacking tryptophan. Sporulation of the diploids took place on minimal sporulation medium and spore dissections took place on YPD with 1 M sorbitol. DNAs from the resulting spores were subjected to Southern blotting in order to determine which of them had obtained both the *rad52Δ* allele and the long telomere. Membranes were probed with the sequence of the *RAD52* gene from the plasmid pSK(KlRAD52) to determine which had received the *rad52Δ* allele (45).

TRD was observed in meiosis by mating the ZT-LBT1 strain containing the long telomere to the *K. lactis* strain GG1958 (*ade2-202*). After tetrad dissection, DNA from all four spores was examined by Southern blotting to determine whether TRD had occurred.

Detecting and quantifying length changes in the long Bcl telomere.

ZT-LBT1 cells from a freezer stock were plated onto SD plates lacking histidine. After one additional streak on SD lacking histidine, several parent colonies from this plate were serially diluted and replated onto fresh SD plates lacking histidine to obtain independent

colonies. Colony subclones from these plates were chosen for genomic DNA preparations and were subjected to *Xho*I digest for visualization by Southern blot.

To calculate TRD rates, we obtained 518 subclones from 15 different parent colonies. For our analysis, we considered any subclone containing a Bcl telomere that was at least 200 bp shorter than the long Bcl telomere precursor as having undergone TRD. Then, we obtained a frequency of colonies that had undergone TRD in from each parent colony. In order to determine the rate of TRD, we used the method of the median (31). In the parent colonies 2 and 3, there was a small amount of degraded DNA in the lanes running below ~0.5 kb which obscured our ability to see partial TRD events. Therefore, the number of partial TRD events is likely to be higher than the number listed.

Gel electrophoresis and Southern blotting.

Restriction digests of yeast genomic DNA preps were conducted in the presence of RNase and run on 0.8% agarose gels in Tris Borate buffer unless otherwise specified in the text. The gels were subsequently blotted on Hybond N+ Membranes in 0.4 M NaOH. They transferred for approximately one day and the membranes were then cross-linked using UV light from an electronic crosslinker.

The membranes were probed using the telomeric G-stranded telomeric probe *Klac*1-25 (ACGGATTTGATTAGGTATGTGGTGT), which was labeled with [γ-^{32}P]ATP. They were hybridized for at least 4 hours at 48 ° C in 500 mM Na_2HPO_4 and 7% sodium dodecyl sulfate and washed three times for 5 minutes in 100 mM Na_2HPO_4 and 2% sodium dodecyl sulfate. Filters were visualized using a General Electric (Sunnyvale, CA) Storm phosphorimager.

Subtelomeric Gene Conversion Assay.

In order to measure the subtelomeric gene conversion rate near the long Bcl telomere, we used an assay described previously (42). Briefly, we used the loss of the subtelomeric *URA3* marker beside the long Bcl telomere as a measure of telomere stability. *K. lactis* cells were serially diluted and spots of these dilutions were plated onto SD plates lacking histidine, SD plates lacking both uracil and histidine, and SD plates lacking histidine but containing the drug 5′-FOA, which selects for cells lacking *URA3*. Colony counts from 5′-FOA plates lacking histidine and SD plates simply lacking histidine were made to determine the frequency of *ura3* cells. 5′-FOA resistant clones derived from cells containing a *URA3*-tagged telomere were shown to have replaced the tagged telomere and *URA3* gene with sequence from another telomere (42, 54). The method of the median was used to determine the subtelomeric gene conversion rate (31).

Results

A long telomere undergoes frequent rapid deletion in *K. lactis*.

In order to look for the occurrence of TRD at telomeres in *K. lactis*, we took advantage of a previously constructed strain that contained a single ~1.5 kb telomere in cells that otherwise have telomeres of the normal 400-600 bp length (75). The long telomere has a *URA3* gene located next to it and each of its repeats contain the phenotypically silent Bcl mutation that creates a *Bcl*I restriction site (Fig. 3.1A) (44). Although the long Bcl telomere would gradually shorten to normal length upon long term passaging of cells containing it, short term passaging permitted testing for TRD events. To do this, we prepared genomic DNA from 518 subclones of

15 parent colonies containing the long Bcl telomere and digested them with *Xho*I, which cleaves the Bcl telomere next to the *URA3* gene and allows separation of it from all other telomeric fragments in gels. As shown in Fig. 3.1B, most subclones retained a Bcl telomeric fragment of ~1.5 kb, the original size of the telomere. However, some subclones were found to have part or all of their Bcl telomere shortened by large increments, including many that were reduced to being near the normal ~500 bp length. That the altered size of the fragment was due to shortening of the Bcl telomere was indicated by the decrease in intensity of hybridization to the telomeric probe (Fig. 3.1B) as well as by mapping with other restriction enzymes (data not shown). The clones containing a mixture of a long and a shortened Bcl telomere (Fig. 3.1B, lanes 11 and 20) represent cases where the shortening event occurred in a cell division occurring shortly after plating for single cells and consequently resulted in only a fraction of the Bcl telomere being short in the cell population examined. For the sake of our calculations, we considered Bcl telomeres that were >200 bp shorter than the initial telomere as having undergone TRD events. In total, 62 out of 518 colonies (12%) had undergone complete TRD (Table 3.1) and at least 6 colonies had undergone partial TRD. Due to a particularly high TRD frequency from precursor colony 3 and because not all TRD events from each precursor are necessarily independent, the median frequency of 5.2% likely represents a more accurate measure of TRD frequency. This corresponds to a TRD rate of ~4 X 10^{-3} per cell division.

Figure 3.2 shows a summary of the lengths of a representative group of 33 of the TRD events that were observed. Twenty of these events produced telomeres that had shortened to lengths between 0.6-0.8 kb, which was within 200 bp of the wild type control *URA3*-tagged telomere shown in Fig. 3.1A. The remaining 13 TRD events produced shortening to intermediate lengths. Because we used a conservative cutoff of 200 bp shorter than the precursor

telomere to define a TRD event, it remains possible that the number of intermediate events may actually be somewhat underestimated. Nonetheless, our results suggest that TRD in *K. lactis*, like that reported in *S. cerevisiae* (34), preferentially shortens long telomeres to near wild type length.

TRD events are frequently associated with turnover deep into the telomere.

While the majority of the TRD events we observed shortened the long Bcl telomere to approximately wild type size, a question that remained was whether the telomere was actually first shortened to below wild type size and then re-extended to normal size by the resident telomerase. Because any addition to a shortened Bcl telomere by telomerase would add on wild type repeats, we were able to address this question. Figure 3.3A shows two potential outcomes after TRD shortens a telomere to approximately wild type size. The first, (Fig, 3.3A, left), is that the telomere is shortened to near wild type size. In this case, cleavage with *Xho*I + *Bcl*I will result in cleavage of the telomere into monomeric repeats that will not show up in a Southern blot. In the second possible outcome (Fig. 3.3A, right), TRD shortens the telomere to well below wild type size, which then becomes re-elongated to normal length by the resident telomerase. In this alternative, *Xho*I + *Bcl*I digestion will cleave any basal Bcl repeats but will leave a visible smeared band representing the terminal array of wild type repeats.

We cleaved DNA with *Xho*I and *Xho*I + *Bcl*I from15 subclones that had undergone TRD events to near wild type telomere length in order to test for the presence of wild type repeats in the shortened telomere. In nine of the subclones (two of which are shown in Fig. 3.3B), *Bcl*I digestion eliminated all hybridization signal produced by the Bcl telomere, consistent with the shortened telomeres in these subclones being composed entirely, or almost entirely, of Bcl

repeats. Notably, in these clones, the length of the shortened telomere was slightly longer than wild type. However, in 7 of the 16 subclones, those where the shortened telomere was closest to wild type in length (including cases 1 and 4 in Fig. 3.3B), we found that wild type repeat arrays estimated to be ~100-300 bp were present on the end of the shortened Bcl telomere. By taking the length of the telomere in the *Xho*I digest and subtracting the size of both the 133 bp subtelomeric region and the wild type repeat array left over after BclI cleavage, we estimated that subclone 4 had ~7 Bcl repeats remaining while subclone 1 had just 3-4 Bcl repeats remaining. Out of the other 5 events, 4 were estimated to have only ~7 Bcl repeats remaining after TRD and 1 had ~9 repeats (data not shown). Our results suggest that some TRD events of the long Bcl telomere may shorten the telomere to well below normal length prior to a re-extension of the telomere to normal size, presumably mediated by telomerase.

The Long Bcl Telomere is susceptible to further lengthening by the addition of more Bcl repeats.

Although an abnormally long telomere should be resistant to elongation by both telomerase and recombination, we observed 24 events, mostly partial events (Table 3.3), where the long Bcl telomere was observed to have become further elongated. Four of these events are indicated with the black arrowheads in Fig. 3.4A. In subclone 1, the lengthened telomere is a partial event, visible as a faint band present above the original long telomere band. In subclone 2, the lengthening event appears to be complete, but two partial TRD events can also be seen. In subclone 4, two lengthened telomeric bands are present as partial events. The largest appears to be about twice the size of the original long telomere, or ~3 kb. In subclone 6, the lengthening

event added ~750 bp to the length of the original Bcl telomere and appears to be present in the entire sample.

DNA from 20 of the samples showing lengthening events was cleaved with *Bsr*BI, which cleaves 3 bp away from the telomeric repeats and/or at restriction sites within the *URA3* gene (Fig. 3.4B and data not shown). Each of the *Bsr*BI digests shifted the elongated telomere to a position ~130 bp shorter than a digest by *Xho*I. This was consistent with each of the longer fragments being longer telomeres. Out of the six complete lengthening events we observed (3 from the original TRD analysis and 3 later purified from clones containing partial lengthening events), two that had increased their sizes more than 0.5 kb were seen to have a clearly increased signal intensity in a Southern blot (Fig. 3.4A and B). Since the *Klac*1-25 probe hybridizes to each telomeric repeat, this signal increase represents an increase in the number of telomeric repeats present on the telomere. These data taken together argue that the elongation events of the Bcl telomere are in fact due to the acquisition of additional telomeric repeats. A summary of the sizes of 21 of the lengthening events we observed is shown in Fig. 3.5. While the majority of these lengthening events appear to add less than 1 kb of telomere repeats to the long Bcl telomere, four of the lengthening events approximately double the length of the original long telomere. None of the partial lengthening events observed contained a corresponding partial shortening event of the same size (Fig. 3.4A and data not shown). We conclude from this that the mechanism of lengthening did not involve reciprocal sister chromatid exchanges.

DNAs from four samples representing the largest complete elongation events (with estimated additions of 650 bp, 750 bp, 1100 bp and 1500 bp) were also cleaved with *Bcl*I in order to observe whether the elongated telomeres were composed of the Bcl repeats or of wild type repeats. Additions composed of wild type repeats as would be expected by either sequence

addition by telomerase or recombinational copying of sequence from another telomere would produce sizable bands of predicted sizes that were resistant to *Bcl*I digestion. With all four samples, including the two clones shown in Fig. 3.4B, the addition of the *Bcl*I enzyme resulted in the apparent complete digestion of the telomere (Fig. 3.4B and data not shown). We conclude that the lengthening in these clones occurred by a mechanism that largely or entirely added on additional Bcl repeats. This indicates that the mechanism involved recombination of the Bcl telomere either with itself or with a sister Bcl telomere.

The rate of subtelomeric gene conversion is similar near both long and normal-length Bcl telomeres.

The high rates of both TRD events and lengthening events at the long Bcl telomere suggest that long length might destabilize telomere function. To address this possibility, we took advantage of an existing assay that can measure rates of subtelomeric gene conversion in *K. lactis*. Eleven of the twelve *K. lactis* telomeres share subtelomeric homology immediately adjacent to the telomeric repeats (55). These sequences can undergo highly elevated rates of homologous recombination when telomere function is compromised (7, 24, 42, 76). This recombination can be measured by quantifying the loss, through gene conversion, of a *URA3* gene inserted next to a single telomere (42). These gene conversions were found to replace both telomeric and subtelomeric sequence from one telomere with sequence from another in what mechanistically are likely to be break-induced replication (BIR) events (54).

Serial dilutions of cell suspensions made from freshly grown colonies of the long Bcl telomere strain and a control subclone that had previously undergone TRD to produce a Bcl telomere of wild type length were spotted onto 5′-FOA medium, which selects for *ura3* cells, as

well as onto control medium without 5′-FOA. The results from our analysis show that both long and normal length Bcl telomeres exhibit similar levels of subtelomeric gene conversion (Table 3.2). Thus, by this measure at least, the long telomere is not inherently more unstable than a wild type length telomere. The rate of subtelomeric gene conversion in both telomeres was a slightly higher than that originally reported for a wild type *K. lactis* telomere (42). This is not likely to be due to the mutant Bcl repeats, as a Bcl telomere was previously shown to display a similar rate as a wild type telomere (44). A more likely explanation may that the strains used in this study have the telomerase RNA gene carried on a plasmid rather than at its normal chromosomal locus.

The long Bcl telomere can still undergo TRD in a *rad52Δ* strain.

Rad52 is the protein that is most broadly involved in various types of homologous recombination in yeast and TRD in *S. cerevisiae* has been shown to be partially dependent on its presence (34). Because telomeric repeats in *K. lactis* are much more homogeneous in size and sequence than those of *S. cerevisiae*, it might be expected that telomeric recombination would be highly Rad52-dependent. To test this, we monitored the stability of the long Bcl telomere in strains constructed to lack *RAD52* that were generated through mating and sporulation. As shown in Fig. 3.6 and Table 3.3, TRD was found to be common in the *rad52Δ* strains, having occurred in 9 of the 178 (5.1%) subclones examined. Because of our relatively small sample, we cannot exclude that there may be a partial dependency of *RAD52* for TRD of the long Bcl telomere. However, our results clearly demonstrate the occurrence of a high rate of *RAD52*-independent TRD. Our data suggest that TRD in *rad52*Δ cells is similar to TRD in *RAD52* cells in primarily producing shortened telomeres of wild type or near wild type length. This was the case in six of

the nine TRD events that were identified in *rad52* strains. While we did not see any elongation events that lengthened telomeres more than ~100-300 bp longer than the precursor strain in the *rad52Δ* strains, we did observe two apparent slight elongation events, one of which is shown in lane 8 of Fig. 3.6B.

We next investigated whether the TRD events that shortened the Bcl telomere to wild type size produced deep turnover within the telomere. Out of the three TRD events that had shortened telomeres to close to wild type size that we tested in *Xho*I and *Xho*I + *Bcl*I digests, two were found to be associated with turnover events that had left only ~8 and ~6 Bcl repeats (Fig. 3.6B). The other subclone was completely cleaved by *Bcl*I indicating that it had not undergone detectable turnover (data not shown).

TRD does not exhibit a large increase in frequency during meiosis in *K. lactis.*

Studies in *S. cerevisiae* showed a 30-70 fold increase of TRD in meiosis, which was dependent on the meiotic bouquet formation protein Ndj1p (25). In order to determine if the TRD frequency is similarly increased in *K. lactis*, we mated the strain containing the long telomere with a wild type strain of the opposite mating type, sporulated the diploids, and dissected tetrads. Because the long Bcl telomere was present in one out of the two parent cells, we observed that the *URA3*-tagged Bcl telomere segregated 2:2, as expected. After examining this telomere in spores from 42 tetrads, (84 spore cells), we observed only 1 TRD event (data not shown). The other spore that had obtained the long telomere in this tetrad had not shortened, indicating that the TRD event had occurred post-replication. Although the 1.2% TRD frequency we observed in meiosis is slightly higher than the per cell division rate of TRD in mitotic cells, it is much lower than the meiotic TRD frequency of 11-23% per meiosis previously reported in *S.*

cerevisiae (25). We conclude that TRD does not have a comparatively large increase in meiosis in *K. lactis*.

Discussion

It is increasingly clear that telomere maintenance can involve processes in addition to elongation mediated by telomerase and gradual shortening from incomplete replication. Homologous recombination is now well established as a mechanism for maintaining telomeres in certain circumstances where telomeres are dysfunctional (41). Even telomeres without obvious functional defects can undergo truncations of sizes too large to be accounted for by gradual sequence loss. Examples of this include the accelerated telomere shortening from oxidative damage in cultured human cells (80), the truncations of ciliate macronuclear telomeres by an unknown mechanism (30) and the TRD of artificially elongated telomeres in *S. cerevisiae* (34). In our studies here, we used a single long telomere in *K. lactis* to look for sudden changes in its size.

TRD has been best characterized in *S. cerevisiae* where it is primarily a phenomenon that shortens abnormally long telomeres that exist in the presence of normal length telomeres (34). The TRD we observed occurring at a long Bcl telomere in *K. lactis* has several similarities to TRD in *S. cerevisiae*. First, TRD in both organisms was very abundant. We estimated that it occurred at a frequency of 4×10^{-3} per cell division in mitotically growing *K. lactis*, quite close to the frequency of 1.2×10^{-3} reported for mitotically growing *S. cerevisiae*. Second, like *S. cerevisiae*, TRD in *K. lactis* appears to mostly shorten telomeres to within 200 base pairs of wild type size. This occurs in spite of the Bcl telomere initially being ~1000 bp longer than wild type. Thirdly, in both species, a significant fraction of TRD events can occur in cells lacking the major

recombination gene *RAD52*. In *S. cerevisiae*, an estimated one third of TRD events occurred in the absence of *RAD52*. In our work, which required more labor-intensive screening using Southern blots, we found that TRD occurred in a *rad52* mutant at a frequency that at least approached that seen in *RAD52* cells. However, our experiments were not sensitive enough to tell whether some, or perhaps most, of the TRD events we saw were *RAD52*-dependent. Although *RAD52* is required for the bulk of homologous recombination in yeast, some recombination, including some telomeric recombination, can be observed in its absence (39, 53, 62, 63). One significant difference that was found between TRD in *K. lactis* and *S. cerevisiae* was its frequency in meiosis. In *S. cerevisiae*, TRD increases 30-70 fold in meiosis relative to its rate in mitosis (25). However, we did not see a corresponding increase in the meiotic rate of TRD in *K. lactis*. The basis of this difference is not known.

The mechanism by which TRD occurs also remains unclear. It is entirely possible, and perhaps likely, that more than one mechanism is involved. DNA damage or nucleolytic cleavage could potentially explain some *RAD52*-independent events. Lustig and coworkers proposed that most TRD in *S. cerevisiae* occurs through the formation of an intramolecular strand invasion of a telomeric end into its more internal repeats followed by cleavage of the t-loop structure (Fig. 3.7A) (6). In support of this, they observed that TRD events involved deletion of the more terminal parts of the telomere and were not associated with reciprocal exchanges. Our experiments similarly did not show evidence for reciprocal sister chromatid exchange (Fig. 3.1 and data not shown). Such events would have led to events of sudden lengthening of the Bcl telomere being equal in number to the TRD events that shortened it. While some lengthening events were observed in our experiments, these were much less commonly observed than the TRD events. Although the t-loop deletion model (Fig. 3.7A) remains attractive, we would point

out that telomeric end strand invasions could also happen in trans, into another telomere (Fig. 3.7B). This conceivably could provide a mechanism for how the presence of normal length telomeres in a cell could promote TRD at abnormally long telomeres (34). Whether normal length telomeres promote TRD in *K. lactis* was not addressed in our study.

A particularly notable result was that the long Bcl telomere could sometimes undergo sudden elongation events. Our results suggest that further lengthening of the long Bcl telomere occurs by copying more Bcl repeats. This indicates that elongation occurs by recombination and not by telomerase, which in our experiments could synthesize only wild type repeats. There are a number of possibilities for how elongation that added Bcl repeats could occur. We believe that unequal reciprocal exchange between sister chromatids is unlikely to be the explanation. This model would predict that in those subclones where elongation by, for example 300 bp, had occurred in only a fraction of the Bcl telomere, there would be a corresponding equal fraction that was shortened by 300 bp. Such simultaneous reciprocal losses were not observed (Fig. 3.4 and unpublished data). Another potential model is that the long telomere was generated by copying a telomeric circle. This model cannot be ruled out, but seems unlikely given that it requires a second, earlier recombination event (to create a t-circle) and that the elongation events we observed were never more than double the size of Bcl telomeres and mostly produced lengthening of less than 900 bp. This latter observation suggests instead that lengthening may occur through a BIR event that follows strand invasion of the end of a Bcl telomere either into its own more internal repeats or into repeats of its sister chromatid (Fig. 3.7A-B). Extension of the invaded 3′ end by a DNA polymerase would then allow copying out to the end of that telomeric sequence. Such a mechanism is predicted to not more than double the size of the original telomere.

A central question regarding the telomeric recombination that we observed is what is its significance to the cell. TRD occurring via a t-loop structure (Fig. 3.7A) has been postulated to be the mechanism by which t-circles form (6, 19, 82). TRD may thus be critical to recombinational telomere elongation, which in yeast telomerase deletion mutants at least, appears to maintain telomeres through a process dependent upon rolling circle copying of a t-circle (35, 52, 53). Although mitochondrial DNA in certain yeast species such as *Candida parapsilosis* might normally utilize this process (57), no information to date has suggested that t-circle formation is of importance to normal yeast chromosomal telomere maintenance.

TRD has been proposed to function as a mechanism that trims abnormally long telomeres to normal size (34). Consistent with this, our data show that TRD in *K. lactis* preferentially shortens telomeres to wild type length. This demonstrates that this feature of TRD is not limited to *S. cerevisiae* and may be more generally conserved. Other data of ours, however, suggest that recombination involving the long Bcl telomere may be more stochastic than orderly. The appreciable incidence of telomere elongation strongly suggests that telomeric recombination occurring at an abnormally long telomere can work in both directions, either shortening or lengthening. Furthermore, the deep turnover into the telomere that is associated with a significant fraction of TRD events suggests that many shortening events initially reduce the Bcl telomere to much shorter than wild type length.

While we cannot completely rule out that the deep turnover occurred slightly after, and independently of, the TRD events, we consider this possibility unlikely. Instead, we favor the idea that TRD in *K. lactis* frequently shortens telomeres to sizes substantially shorter than normal length and that either telomerase or perhaps in some cases, break-induced replication events, lengthens the telomere back to normal size. Whether the events that shorten telomeres to near

normal size and those that shorten telomeres to below normal size are caused by the same mechanism is not known. Our results indicate that deep turnover, like TRD in general, still occurs at an appreciable frequency even in the absence of *RAD52*.

If TRD in *K. lactis* is not strictly a mechanism for trimming long telomeres, it is likely that it also occurs at completely wild type telomeres. Conceivably, recombination at telomeres may be difficult to completely prevent. Both their simple repetitive structure and their 3′ overhangs might serve as features that could greatly promote the likelihood of recombination even if multiple other features of telomeres act to repress it. Evidence suggestive that wild type telomeres undergo TRD comes from past experiments with *TER1-7C(Bcl)* cells, which have a telomerase RNA template mutation that generates the phenotypically silent base change in telomeric repeats. Serial passaging of these cells over the course of hundreds of cell divisions found that Bcl repeats eventually replace all but the innermost 1-4 repeats of the telomeres (44). This deep turnover cannot be readily explained by gradual replicative sequence loss and was postulated to occur via terminal truncations of the telomeres that would typically be repaired by the resident *ter1-7C*(Bcl) telomerase. *K. lactis* telomeres in a telomerase deletion mutant that are below ~100 bp (4 repeats) in size have been shown to be capable of recombining with other telomeres (75). This suggests that TRD events that shorten telomeres to that degree might also initiate homologous recombination. Conceivably, such truncations deep into the telomere might be a cause of the subtelomeric BIR events that can eliminate or duplicate a *URA3* gene placed in subtelomeric sequence next to a telomere (Table 3.2 and (42)). The fact that normal length telomeres are no more resistant than the long Bcl telomere to undergoing these BIR events may add further support to the possibility that normal length telomeres also engage in TRD.

Most models for recombinational repair of DNA suggest that a strand-invaded 3′ end will be used as a primer for at least limited DNA synthesis. This might seem to suggest that strand invasion of a telomeric end into either itself (Fig. 3.7A) or another telomere (Fig. 3.7B) would lead to telomere elongation being more frequent than telomere shortening. Yet the reverse is observed in both *K. lactis* and *S. cerevisiae*. We suggest that it may be more important for telomeric capping function to block recombinational elongation than recombinational shortening. One reason for this is that in most circumstances, shortening of a normal length telomere is likely to be easily and rapidly corrected by sequence addition by telomerase, which is known to be favored at short telomeres (72). Furthermore, recombinational elongation by self or sister copying (models favored by our data) has the potential to cause telomeres to exponentially increase in length if the process ever became even moderately frequent.

Subtelomeric recombination has been postulated to be an adaptive mechanism that can permit rapid evolution of contingency genes located near chromosome ends (38). It is therefore interesting to speculate that TRD, by cleaving deep into telomeres and occasionally triggering them to further recombine, could act as an enhancer of subtelomeric evolution whose rate might be regulated and vary depending upon environmental circumstances. In a similar vein, events that suddenly shorten or lengthen telomeres might be adaptive through their ability to epigenetically alter the expression of nearby genes. In a number of organisms, including *K. lactis*, genes near telomeres can be subject to silencing (3, 8, 15, 20, 22, 27, 33, 37, 56, 68, 73) (59). In *S. cerevisiae* at least, it has been shown that this silencing can be influenced by telomere length (28). Such an effect, in fact, served as the basis of the assay used to measure TRD frequencies in that organism (34).

How significant TRD might be to other organisms remains largely unknown. We would suggest that TRD may be particularly important to human cells. In part, this would stem simply from the long size (relative to yeast) of human telomeres, which would make them vulnerable to losing much more sequence in a single event. More significantly, though, the very low telomerase levels in most human somatic tissues would leave TRD-shortened telomeres without a means of becoming re-extended. Because human cells arrest their growth when a small number of telomeres become too short (21), a small number of TRD events could potentially have dramatic effects on the replicative capacity of a cell. Gaining a better understanding of TRD is therefore clearly a goal of considerable significance.

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Table 3.1. Summary of the shortening and lengthening events associated with the long Bcl telomere. We examined 518 subclones of 15 different parent colonies of the same long Bcl telomere transformant. The categories for complete TRD and complete elongation are the result of shortening or lengthening events that had occurred in the whole colony examined. Alternatively, the number of partial TRD events and partial elongation events represent the number of events we see that occur in only a percentage of the colony with the original long Bcl telomere still visible. The total represents the total number of events in each category.

Parent	Complete TRD	Partial TRD	Complete Elongation	Partial Elongation	Total number of subclones examined
1	3	0	0	1	57
2	0	0	0	0	40
3	38	1	0	1	98
4	12	3	1	12	99
5	4	1	1	4	125
6	0	0	0	0	10
7	0	1	1	0	10
8	1	0	0	0	10
9	1	0	0	0	10
10	1	0	0	0	10
11	1	0	0	2	10
12	0	0	0	1	10
13	0	0	0	0	10
14	1	0	0	0	9
15	0	0	0	0	10
Total	62	6	3	21	518

Table 3.2. Rate of loss, through gene conversion or break-induced replication events, of a subtelomeric *URA3* gene next to the long Bcl telomere compared to a wild type telomere. The number in parentheses represents the number of times the assay was performed.

Strain	***URA3* loss rate [mutation rate ± SE (*n*)]**
Wild Type Bcl	$2.7 \times 10^{-5} \pm 5.1 \times 10^{-6}$ (19)
Long Bcl	$2.8 \times 10^{-5} \pm 1.3 \times 10^{-5}$ (19)

Table 3.3. Summary of the shortening and lengthening events associated with the long Bcl telomere in a *rad52Δ* strain. We examined 178 subclones of 18 different *rad52Δ* parent colonies containing the long Bcl telomere. The 19 parent colonies come from three different spores (1.1-1.6, 2.1-2.3, 3.1-3.9) that had been found to contain the long Bcl telomere and the *rad52* deletion. The number of complete TRD or complete elongation events result from the number of subclones which shortened or lengthened the long Bcl telomere in the entire colony. The number of partial TRD or partial elongation events represent events which had occurred in only a percentage of the colony with the long Bcl telomere still visible. The total represents the total number of events in each category.

Parent	Complete TRD	Partial TRD	Complete Elongation	Partial Elongation	Total Number of Subclones Examined
1.1	0	0	0	0	10
1.2	0	0	0	0	10
1.3	0	0	0	0	10
1.4	0	0	0	0	10
1.5	0	0	0	0	10
1.6	1	0	1	0	10
2.1	0	0	0	0	10
2.2	1	0	0	0	10
2.3	0	0	0	0	10
3.1	2	0	0	0	10
3.2	0	0	1	0	10
3.3	1	0	0	0	10
3.4	1	0	0	0	9
3.5	0	0	0	0	10
3.6	0	0	0	0	10
3.7	1	0	0	0	10
3.8	0	0	0	0	9
3.9	2	0	0	1	10
Totals	9	0	1	1	178

Figure 3.1. Frequent deletions occur at a long telomere in *K. lactis*. (A) Diagram of the single long Bcl telomere introduced into *K. lactis* cells (shown to scale). This telomere contains a *URA3* selectable marker gene (white box) inserted into subtelomeric sequence. The unique *Xho*I site allows the Bcl telomere to be separated from other telomeres in gels. A native *Bsr*BI site 3 bp internal to the telomere is present on 10 out of the 12 wild type telomeres in the cell. The long telomere is made up completely of Bcl repeats containing a single base pair change that makes a *Bcl*I restriction site (gray blocks). After transformation into *K. lactis* cells with wild type telomeric repeats (white blocks), the long Bcl telomere recombines via subtelomeric homology and replaces a single native telomere. (B) This Southern blot, hybridized to a telomeric probe, shows an *Xho*I digest of genomic DNA from subclones of cells containing the long Bcl telomere. The wild type (WT) control is an equivalent telomeric fragment containing a subtelomeric *URA3* gene, but of wild type length and composed of wild type telomeric repeats. After introduction into *K. lactis* cells, the *Xho*I fragment containing the long Bcl telomere measures ~1.5 kb, containing ~55 telomeric repeats, indicating that it is ~3 times longer than a wild type telomere. The position of the introduced telomere is indicated by the bracket. Markers are shown in kilobases (kb).

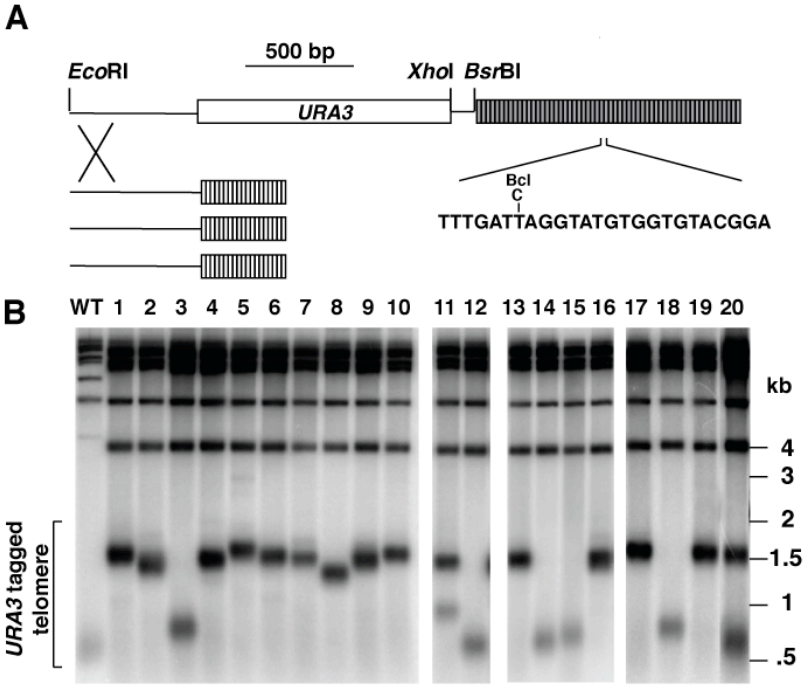

A
500 bp
EcoRI
XhoI
BsrBI
URA3
BclC
TTTGATTAGGTATGTGGTGTACGGA
B
WT 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
kb
4
3
2
1.5
1
.5
URA3 tagged telomere

Figure 3.2. Summary of telomere lengths after TRD. This bar graph shows the length distribution of 33 of the TRD events observed. The telomere length is measured as the size of the *Xho*I Bcl telomeric fragment after TRD and the vertical axis shows the number of samples which have been shortened to within a given size range.

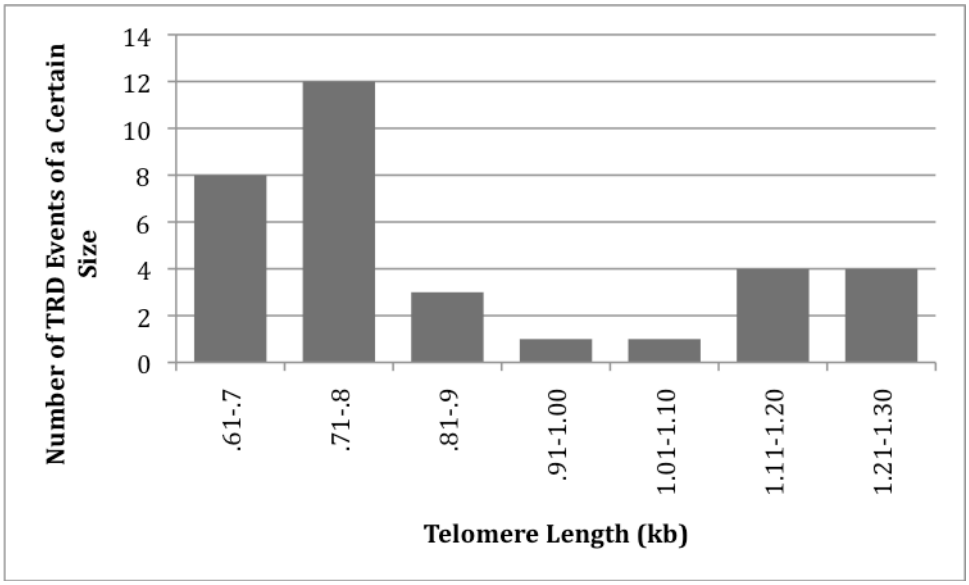

Number of TRD Events of a Certain Size
14
12
10
8
6
4
2
0
.61-.7
.71-.8
.81-.9
.91-1.00
1.01-1.10
1.11-1.20
1.21-1.30
Telomere Length (kb)

Figure 3.3. TRD events can result in turnover deep into the shortened telomere. (A) Diagrammed are two possible outcomes for the long Bcl telomere after undergoing TRD. In the first, on the left, the long telomere has been shortened to wild type size and all of the remaining repeats are Bcl repeats. After cleavage with *Bcl*I, the telomeric repeats are cleaved into individual repeats and a small subtelomeric segment is liberated. In the outcome on the right, the long telomere is shortened to well below wild type size and then re-extended by the wild type telomerase. After cleavage with *Bcl*I, a block of wild type repeats will be left over, the Bcl repeats will be cleaved into individual repeats, and a small subtelomeric fragment will again be liberated. (B) These Southern blots of 2% agarose gels, hybridized to a telomeric probe, shows an *Xho*I and an *Xho*I + *Bcl*I digest of four subclones that have undergone TRD. The same WT control described in Fig. 3.1 is shown on the left.

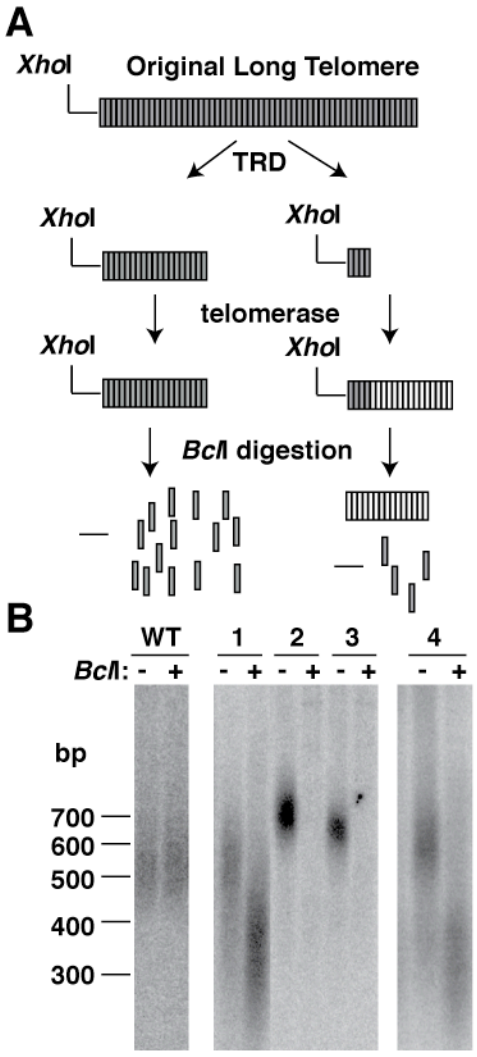

A
XhoI
Original Long Telomere
TRD
XhoI
XhoI
telomerase
XhoI
XhoI
BclI digestion
B
WT
1
2
3
4
BclI: - + - + - + - + - +
bp
700
600
500
400
300

Figure 3.4. Further elongation of the long Bcl telomere can occur and these elongations are made up of Bcl repeats. (A) This Southern blot shows an *Xho*I digest of several of the long Bcl telomeres that have undergone elongation events. A wild type control, described in Fig. 3.1, is shown on the left. Black arrowheads show the position of elongated telomeres in the sample. Signal intensities across the lanes from subclones 5 and 6 are shown on the right. (B) This Southern blot shows cleavages of DNA from two subclones that had undergone elongation events with *Xho*I, *Xho*I+*Bcl*I, and *Bsr*BI, as indicated. A wild type control is shown on the left and C represents a control long telomere that has not undergone TRD or elongation. Positions of elongated telomeres in samples 1 and 2 are shown with slanted black arrowheads and positions of the elongated telomere in samples 1 and 2 after cleavage with *Bsr*BI are shown with white arrowheads. Signal intensities through the lanes of the *Xho*I digest of the control (C) and the *Xho*I digest from subclone 1 are shown on the right. Note that subclones 1 and 2 are not the same subclones as those shown in panel B. Markers for both panels are shown in kilobases (kb).

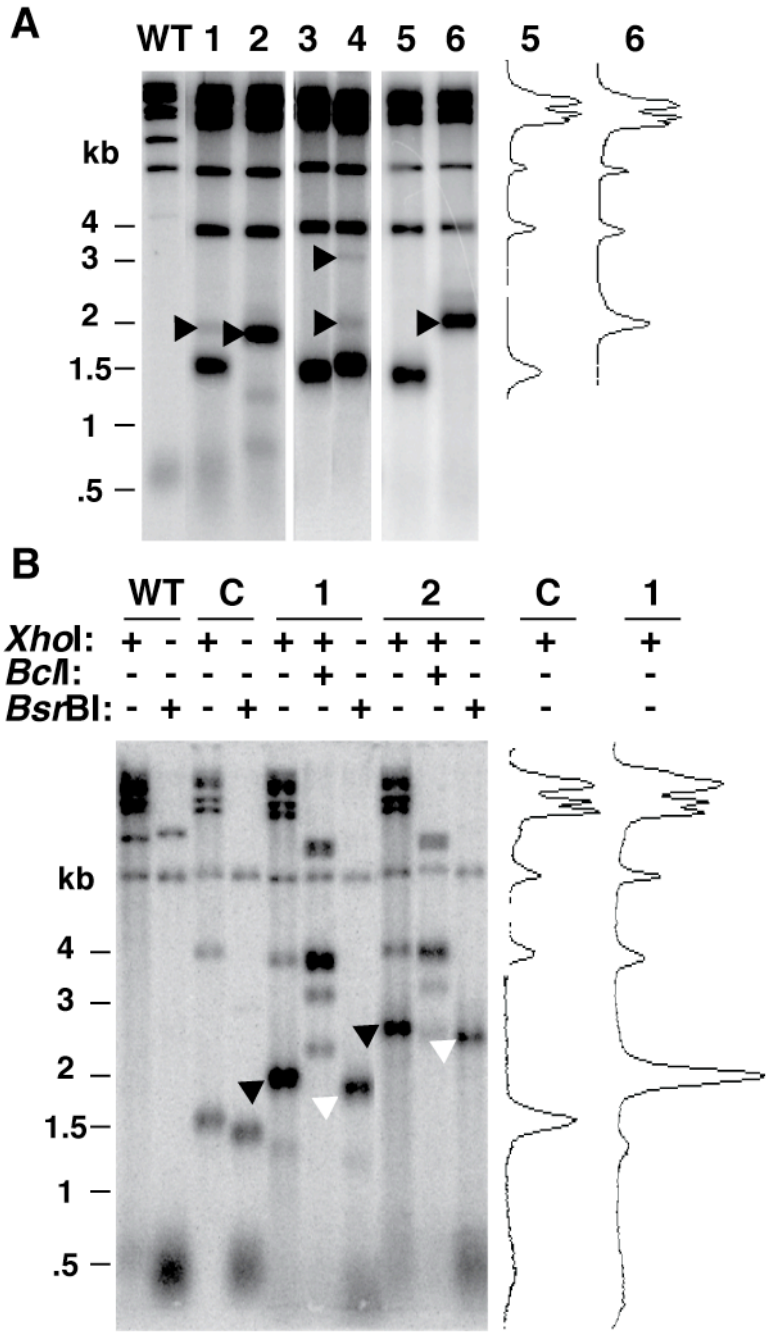

A
WT 1 2 3 4 5 6
5
6
kb
4
3
2
1.5
1
.5
B
WT C 1 2 C 1
XhoI: + - + - + + - + + - + +
BclI: - - - - - + - - + - - -
BsrBI: - + - + - - + - - + - -
kb
4
3
2
1.5
1
.5

Figure 3.5. Summary of lengths of the Bcl telomere after further elongation. This bar graph shows the length distribution of 21 elongation events, including both partial and complete events. The telomere length is measured as the size of the *Xho*I Bcl telomeric fragment after elongation and the vertical axis shows the number of samples which have been lengthened to within a given size range.

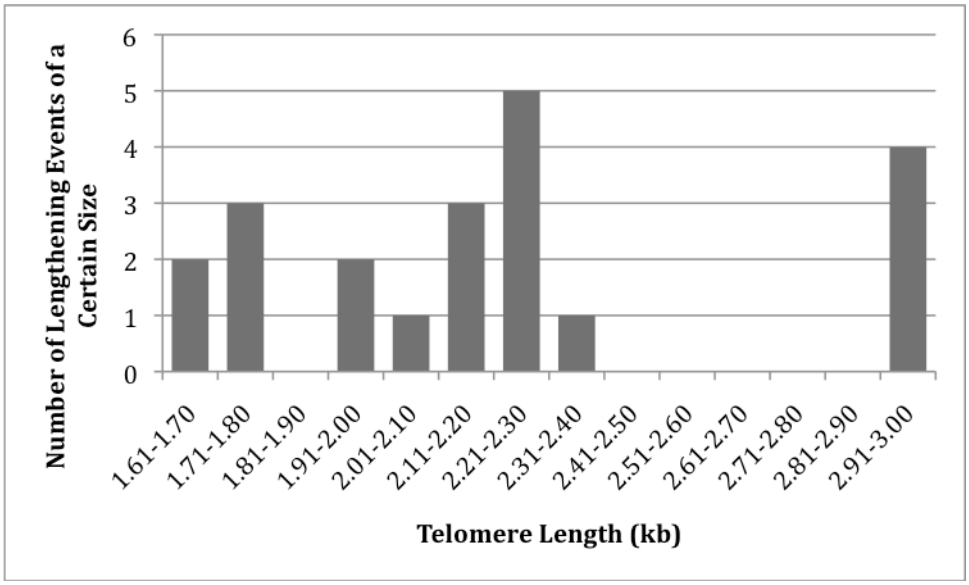

Number of Lengthening Events of a Certain Size
0
1
2
3
4
5
6
1.61-1.70
1.71-1.80
1.81-1.90
1.91-2.00
2.01-2.10
2.11-2.20
2.21-2.30
2.31-2.40
2.41-2.50
2.51-2.60
2.61-2.70
2.71-2.80
2.81-2.90
2.91-3.00
Telomere Length (kb)

Figure 3.6. *rad52Δ* cells still undergo TRD that often has appreciable turnover associated with it. (A) These Southern blots show an *Xho*I digest of two TRD events in a *rad52Δ* strain containing the long Bcl telomere. The lane marked WT contains a *URA3*-tagged telomere that is wild type in length and sequence. The lane marked P contains the precursor *URA3*-tagged long Bcl from immediately prior to isolation of the subclones in the other lanes. (B) This Southern blot shows an *Xho*I digest of an apparent slight elongation event in a *rad52Δ* strain. Lanes marked WT and P indicate wild type and precursor Bcl telomeres, as in (A). (C) This Southern blot of a 2.5% agarose gel shows an *Xho*I and *Xho*I+*Bcl*I digest of two subclones that had undergone TRD to near wild type size in a *rad52Δ* strain. The position of the leftover wild type block of repeats is shown with an arrow. Markers in all panels are shown in kilobases (kb).

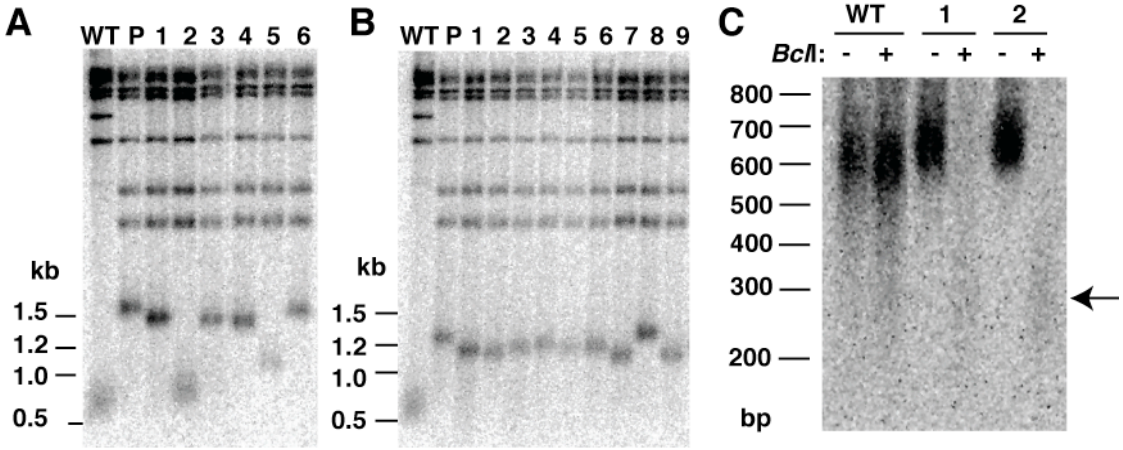
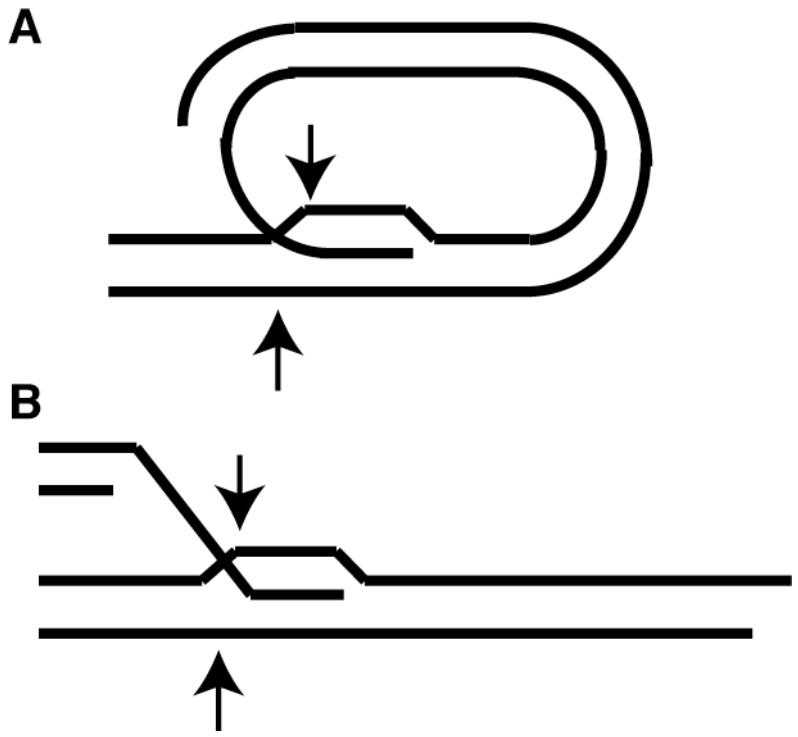

A
WT P 1 2 3 4 5 6
kb
1.5
1.2
1.0
0.5
B
WT P 1 2 3 4 5 6 7 8 9
kb
1.5
1.2
1.0
0.5
C
WT
1
2
BclI: - + - + - +
800
700
600
500
400
300
200
bp

Figure 3.7. Two potential mechanisms of TRD. (A) This model shows an intramolecular strand invasion of a telomeric end into its own telomeric repeats, forming a t-loop. (B) This model shows a strand invasion of a wild type telomere into an abnormally long telomere. Nucleolytic cleavage positions that might produce a TRD event are shown with arrows in each model.


A
B

CHAPTER 4

PERSPECTIVES

The data presented here expand our knowledge of telomere recombination in yeast. Recombinational Telomere Elongation (RTE) is an important mechanism in both yeast cells deleted for telomerase and in human ALT cancers (11, 18, 20). In the yeast *Kluyveromyces lactis*, where RTE has been most extensively characterized, I was able to show that cells containing telomeric repeats that are defective at binding the double-stranded telomeric binding protein Rap1p cause extensive recombination at telomeres and was able to create a system that resembled the telomeres in ALT cancer cells. I was also able to show that Telomere Rapid Deletion (TRD) in *K. lactis* displays generally similar characteristics to TRD in *S. cerevisiae* and is therefore likely to be a related process (17). However, I have also and have proposed new ideas for how TRD could act.

I have shown that cells with telomeric repeats displaying a defect in Rap1p binding have long and heterogeneous telomeres, produced by recombination, traits shared with telomeres in human ALT cancers (30). This phenotype also resembles the recombination present in cells containing a mutation in the single-stranded telomeric DNA capping protein Stn1p, called *stn1-M1* (15). Since an ALT-like phenotype can be created by defects in either of two telomeric capping proteins at telomeres, the question arises as to whether the ALT phenotype is created by protein capping defects. When the human double-stranded binding protein TRF2 is dislodged from telomeres, those telomeres undergo

large deletions (40). This shows that protein binding defects at human telomeres do in fact destabilize telomeres. If other mutations stopped senescence from occurring in these cells, there is a question of whether an ALT-like phenotype would form.

In general, survivors using Type II RTE to maintain their telomeres in *S. cerevisiae* have much longer telomeres (>10 kb) than survivors using Type II RTE (<2 kb) in *K. lactis* (20, 34). Because the two organisms have similar telomere lengths in telomerase positive cells, this difference seems unusual. Based on the data presented here, a potential reason for the length differences between Type II survivors in these yeasts is based on the irregular repeats in *S. cerevisiae*. If some small neighboring groups of telomeric repeats in *S. cerevisiae* are somewhat dysfunctional compared to others, then they would be more likely to become uncapped at a longer length once they were present at the end of a telomere in a senescing telomerase deletion mutant. This is turn would lead to them acquiring a long 3′ overhang and initiating recombination sooner than telomeric sequences that displayed stronger capping ability. Thus, preferential amplification of slightly dysfunctional telomeric sequences naturally present in *S. cerevisiae* could result in a somewhat similar phenotype to the Type IIR RTE phenotype I observed in my studies. The basis of the dysfunction caused by the *S. cerevisiae* repeats might lie in a Rap1p binding defect or potential in a binding defect of another protein, such as Cdc13p. One interesting speculation from this hypothesis would be that the long and heterogeneous telomeres in ALT cancers might become enriched with dysfunctional mutant telomere repeats. Data has shown that the proximal telomeric repeats in normal human cells can differ from the regular telomeric TTAGGG repeat (2, 3, 10). However, telomeres of ALT cancer cells do appear to have interspersions of variant telomere repeats in the distal portion of the telomere (38). The effect of these low numbers of variant repeats on the ALT phenotype is not known.

One interesting hypothesis made from the long 3′ overhangs at telomeres undergoing Type IIR RTE in *K. lactis* is that one role of telomere capping proteins is to protect the telomeres from excessive resectioning. These data suggest that Rap1p could form an important block to the resectioning nucleases and therefore prevent recombination. It would be interesting to determine whether this block requires other proteins to bind the telomeres, such as Rif1p, Rif2p, or the single-strand telomere binding proteins. Data in *S. pombe* suggest that the telomeric double-stranded binding protein Taz1 plays a similar role in preventing long 3′ overhangs from forming at telomeres (23). Recent data has also implicated Rap1p in the repression of recombination at telomeres in mice, which could potentially use a similar mechanism (32).

Data has indicated that a mutation in telomeric repeats just outside of the Rap1 binding site, called the Kpn mutation, can create a similar Type IIR RTE phenotype (36). The question arises as to whether this mutation, while not affecting Rap1 binding, could affect telomere structure in a similar way as a loss of Rap1p binding. Because Rap1p is predicted to bend DNA and because the number of Rap1p molecules appear to be counted at telomeres, I hypothesize that mutations just outside the Rap1p binding site may be able to affect the secondary structure of telomeres and that, while able to bind Rap1p, the Rap1p-dependent counting mechanism may be affected (12, 19). In support of this idea, data has shown that telomerase extends a telomere containing only Kpn repeats past the length of a wild type telomere, indicating that they do not regulate telomerase addition normally (37).

I have shown that a telomere composed of Acc repeats has a long 3′ overhang before its sequence is spread to other telomeres by RTE (5). When telomerase is still active in these cells, a telomere composed of Acc repeats acquires a full wild type length array of wild type telomeric repeats onto its end, thereby creating a total telomere length that is abnormally long. This lack of

regulation of telomerase addition leads to the hypothesis that the long overhang can preferentially recruit telomerase. Other data indicates that short telomeres are preferentially elongated by telomerase (33). There is also existing data showing that overhang size is increased at short telomeres compared to longer telomeres (27). However, increased binding of the telomerase recruitment protein Cdc13p was not shown at short telomeres and the role of the increased overhang length in telomerase recruitment remains unknown (6).

I have shown the presence of telomeric circles (t-circles) in *ter1*-Δ cells that have Acc telomeric repeats (5). Considerable evidence now supports the belief that t-circles serve as templates for generating lengthened telomeric sequences in "normal" *ter1*-Δ cells that have only wild type telomeric repeats and undergo Type II RTE (13, 25, 26). Accumulating evidence also suggests that t-circles can be copied during the Type IIR RTE that creates the very long and heterogenous telomeres of *stn1-M1* cells [(4); J. Xu and M. McEachern, unpublished data]. I predict that t-circles are also able to elongate telomeres in the Acc mutant. However, more studies, such as testing whether repeating patterns of repeats can form in *ter1*-Δ Acc survivors, would be needed to test this. T-circles are also produced by the mitochondrial telomeres of *Candida parapsilosis* and recombination has been proposed as a normal telomere maintenance pathway in the organism *Anopheles gambiae* (28, 31, 35). It will be interesting to see if these organisms follow a similar recombination pathway found in the absence of telomerase in organisms like *K. lactis*, *S. cerevisiae*, and humans.

The studies on Telomere Rapid Deletion described in this manuscript indicate that TRD in *K. lactis* is quite similar to TRD in *S. cerevisiae* (17). These similarities show that the TRD in *K. lactis* and *S. cerevisiae* are likely to have similar mechanistic pathways. However, TRD in *K. lactis* does not appear to simply shorten abnormally long telomeres to wild type size, which was

the proposed role of TRD in *S. cerevisiae* (17). Instead, my results suggest that, while many TRD events do in fact shorten telomeres to near wild type size, many others appear to shorten abnormally long telomeres to well below wild type size. Although TRD events were shown to shorten *Arabidopsis thaliana* telomeres to below wild type size in cells deleted for telomerase components, my data was the first to show a similar truncation in wild type cells (41). If TRD were simply a mechanism to shorten abnormally long telomeres back to normal size, we would expect that truncations would be more precise and perhaps also more common.

Along with an undergraduate, Nathan Jamieson, I have also shown that a long telomere does not perturb subtelomeric recombination rates relative to a wild type telomere. Since many *K. lactis* mutations that perturb telomere capping, sometimes even without perturbing telomere length, also substantially raise subtelomeric recombination rates, this result was unexpected (8, 15, 21, 37). One possible explanation for this result is that TRD shortens a long telomere without the event ever perturbing subtelomeric regions. However, the TRD events that cause deep turnover of telomeric repeats appear to at least be capable of shortening telomeres to below the ~100 bp threshold that causes uncapping and permits the initiation of recombination. Another possible explanation for the equivalent subtelomeric recombination rates at long and normal length telomeres is that wild type telomeres also undergo similar rates of deep turnover by TRD.

In *Arabidopsis thaliana* and human cells overexpressing hTR, however, there is an increased rate of telomere shortening when telomeres are long (29, 41). I have also seen some evidence in my studies that longer telomeres appear to be more prone to TRD than shorter telomeres (data not shown). It is possible that TRD is seen more frequently at a longer telomere than at a shorter telomere while not perturbing the subtelomeric recombination rates because a

wild type telomere that shortens only has the ability to form deep turnover events, while a long telomere can trim to near wild type size or above. Data from *K. lactis* has demonstrated that wild type telomeres have repeat turnover further into the telomere than would be expected by gradual loss and telomerase re-extention (22). However, more work needs to be done to show that the purportive TRD presumably responsible for this turnover at wild type telomeres and the TRD at long telomeres is mechanistically similar (22).

In my studies, I cannot rule out that TRD occurs by a terminal deletion involving a t-loop, which was supported by evidence obtained in *S. cerevisiae* (7). This t-loop cleavage might actually produce a t-circle, which could contribute to RTE by the Roll and Spread Model in cells lacking telomerase or otherwise prone to RTE. However, I also propose a variant of this model in which a wild type telomere can strand invade a long telomere and that the long telomere can be cleaved at the site of the strand invasion. This model would predict similar nucleolytic cleavage at the strand invasion or at a t-loop because the structures of the strand invasions in both cases are equivalent. However, this model might better account for the fact that long telomeres are preferentially shortened to wild type size, which is seen in both *K. lactis* and *S. cerevisiae* (17). It is possible that TRD could occur by several different mechanisms with the truncations to wild type size caused by strand invasion of wild type telomeres into long telomeres and the less precise cleavages occurring by t-loop deletion or simple nuclease cleavages not involving recombination. In potential support of this latter possibility, not all TRD in either *S. cerevisiae* or *K. lactis* was dependent on the major recombination protein *RAD52* (17).

Telomeres in humans and in *Caenorhabditis elegans* undergo a substantially larger telomere attrition per cell division than is predicted from gradual telomere loss due to the end-

replication problem (9, 14). These organisms have telomeres that are multiple kilobases in length, while the telomeres in the yeasts *S. cerevisiae* and *K. lactis* are only hundreds of base pairs long. If TRD were causing large deletions at telomeres in humans at a rate similar to the 4.0×10^{-3} events per telomere per cell division that I have seen in *K. lactis*, large deletions, sometimes of several kilobases, would be frequent. These large deletions would be expected to have a substantial effect on the per cell division rate of telomere attrition in organisms with longer telomeres. However, at shorter yeast telomeres, even a deletion event removing most of the telomere would not have a huge effect on the per cell division telomere attrition rate. In agreement with this idea, the rate of *K. lactis* telomere attrition appears to be ~3-5 base pairs per cell division. While oxidative damage has been shown to be one possible mechanism of accelerated telomere attrition in human cell culture, there is a strong possibility that other mechanisms are also at work (39). It is even possible that the largest cause of telomere attrition in organisms with longer telomeres are TRD events. It will be important to understand the reasons for telomere attrition in human cells to better understand the process of senescence.

While a small number of lengthening events were reported within studies of TRD in both *S. cerevisiae* cells and in *Arabidopsis* cells, they were not studied in detail (17, 41). The analysis in this study finds that lengthening events occur using sequence from the abnormally long telomere itself. The two likely models for lengthening are break-induced replication (BIR)-like events copying telomeric sequence from a sister chromatid or a similar event occurring from the end of a long telomere strand invading itself. The current methods available are unable to differentiate between these models. Recent data in human ALT cells have confirmed that a plasmid tag integrated into a telomere can be duplicated within the same telomere, supporting

that lengthening events can occur via intramolecular or sister chromatid copying of telomeric sequences (24).

Overall, I have contributed two important points to the field of telomere recombination. First, I have shown that Rap1p is an important inhibitor of telomere-telomere recombination in *K. lactis* cells. My studies of TRD in *K. lactis* have also indicated that telomere recombination is potentially at work at fully capped telomeres. The current paradigm in the telomere field is that capped telomeres are not prone to recombination. These studies are important to our understanding of the telomeres in human cells, which normally lack telomerase (16). ALT cancers show frequent recombination and are also prone to large deletions (1, 11). I predict that disruptions to the proper functioning of telomere binding proteins might play an important role in ALT cancers and that a major role of telomeres in human cells might actually be to prevent the occurrence of recombination at normal telomeres.

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