T-CIRCLES AND T-LOOPS PLAY A ROLE IN *KLUVEROMYCES LACTIS* RECOMBINATIONAL TELOMERE ELONGATION

by

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(Under the Direction of Michael McEachern)

ABSTRACT

Recombinational telomere elongation (RTE) known as ALT (alternate lengthening of telomeres) is the mechanism of telomere maintenance in up to 5-10% of human cancers. The telomeres of yeast mutants lacking telomerase can also be maintained by recombination. The roll-and-spread model has been proposed to explain this elongation in the yeast *Kluveromyces lactis*. Our model suggests that a very small (~100 bp) circular molecule of telomeric DNA is copied by a rolling circle event to generate a single long telomere. The sequence of this primary elongated telomere is then spread by recombination to all remaining telomeres. Data presented here will show that *K. lactis* cells can efficiently utilize synthetic single-stranded telomeric circles of 100 nt to elongate there telomeres. It is also shown here, by 2D gel analysis and electron microscopy, that small circles of single and double-stranded telomeric DNA (t-circles) are commonly made by recombination in a *K. lactis* mutant with long telomeres. To our knowledge this is the first report of single-stranded t-circles as a product of telomere dysfunction. We also present evidence that t-loop structures like those originally isolated from human cells are present on some *K. lactis* telomeres. Our data shows phenotypic similarities between the telomeres found in *ter1-16T* and those previously analyzed in human ALT cells.

INDEX WORDS: t-loops, t-circles, Recombinational Telomere Elongation (RTE), Alternate Lengthening of Telomeres (ALT), The Roll-And Spread model

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DEDICATION

This work is dedicated to the people who are most responsible for its creation, my family, To my parents, Neil and Maureen thank you for teaching me to value my mind and to appreciate the gift of learning. Your constant support and encouragement has been a harbor to me during many storms. To my loving husband Eugene thank for pushing me to achieve my goals. And finally to Maxwell, my pumpkin, thank you for the joy you give me every night when I walk through the door.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Telomere Structure and Function

Telomeres are nucleoprotein structures at the ends of linear chromosomes. Most telomeres consist of 5-8 bp G-rich repetitive sequences, which are bound directly and indirectly by telomeric proteins. There are several components of telomeric structure that seem to be crucial players in normal telomeric function. These include the double stranded telomeric repeats, the single stranded 3' G-rich overhang of telomeric repeats, the protein complexes which bind specifically either the double- or single-stranded telomeric regions, and the higher order structure of DNA and proteins called the telomeric cap. In concert these components act to perform multiple functions, which have implications not only for telomeric structure but on cell cycle control, and genomic stability.

One vital role of the telomere complex is to protect the linear chromosome from sequence loss. While the majority of telomeric DNA is copied through the action of normal DNA replication, some telomeric sequence is lost each cell division due to the incomplete replication of linear chromosomes. Because telomeric DNA does not contain coding DNA they serve as a physical buffer to the loss of coding DNA. Telomeres have also evolved a specialized system for their own extension that counteracts sequence loss incurred by incomplete linear replication and nucleolytic processing of the chromosome

end. The addition of telomeric repeats is carried out in most species by the reverse transcriptase telomerase (Blackburn 1992). Telomerase consists of multiple protein subunits and an RNA subunit that contains the template for the organism-specific telomeric repeat. The multiple subunits of telomerase have been best characterized in the yeast *S. cerevisiae*. These include the protein components encoded by *EST1*, *2*, and *3* and the RNA subunit encoded by *TLC1* (Lundblad and Szostak 1998; Singer and Gottschling 1994). Telomerase is recruited to the telomere through the association of the single-stranded telomeric binding protein Cdc13p and the telomerase subunit Est1p (Lundblad and Szostak 1989; Hughes et al. 1997; Evans and Lundblad 1999; Evans and Lundblad 2000). Once at the telomere the telomerase RNA aligns with the single-stranded overhang and the template region is copied by the catalytic component of telomerase, Est2p (Counter et al. 1997; Evans and Lundblad 2000). The single-stranded overhang which telomerase associates with is created post-replication through nucleolytic activity (Dionne and Wellinger 1998). Unlike the processing of double-stranded breaks the 5' telomeric ends are only modestly degraded leading to a short 3' overhang. In yeast this short (12-14 bp) overhang persists throughout the cell cycle, is elongated during S phase, and is dependent upon Mrellp (Larrivee et al. 2004).

The sequence of telomeric repeats seems to vary amongst yeast species but is completely conserved within vertebrates (Meyne et al. 1989; McEachern and Blackburn 1994; Cohn et al. 1998). Yeast telomeric repeats differ in their sequence, length and the fidelity of that sequence along the telomeric tract. However, most yeast telomeric sequences share a common T+G motif which is common not only to yeast telomeric repeats but related to the repeat of most other eukaryotes (McEachern and Blackburn

1994). Much of what is known about telomere biology has been worked out in the yeast *Saccharomyces cerevisiae*. The telomeric repeat of this organism (5'$TG_{(1-3)}$3') is not homogeneous. The yeast *Kluveromyces lactis* has a 25 bp repeat (5' TTTGATTAGGTATGTG GTGTA CGGA3') which is maintained with fidelity at the telomeric ends (McEachern and Blackburn 1994). This has lead to the development of *K. lactis* as one of a handful of genetic models that are commonly used to study telomere biology.

The study of higher order structure found at telomeric ends has led to the discovery of what is now known as a t-loop. The t-loop structure consists of a D-loop formed by the invasion of terminal single stranded telomeric DNA into more internal duplex telomeric repeats. These structures have been isolated and visualized at the telomeres of humans, mice, chickens, pea plants, and the macronuclei but not the micronuclei of ciliated protozoans (Griffith et al. 1999; Murti and Prescott 1999; Cesare et al. 2003; Nikitina and Woodcock 2004), indicating that the ability of telomeres to enter into a t-loop structure may be widely but probably not universally evolutionarily conserved. It is currently not known what roll t-loop structures plays in telomere biology of any organism. In humans the existence of a t-loop structure seems to be promoted by associations of TRF2 at the loop stem structure (Griffith et al. 1999) (Stansel et al. 2001). TRF2 association with telomeres is required for their protection from end to end fusions (van Steensel et al. 1998). T-loop maintenance through TRF2 may be an important part of maintaining a "capped" telomeric state. It is also known that a mutant of TRF2, $TRF2^{\Delta B}$, exhibits loss of t-loop structure and results in the creation of extrachromosomal telomeric circles (Wang et al. 2004), indicating that destabilizing this structure could lead to losses

of telomeric DNA. While it seems likely that some higher order structure results from the interaction of telomeric binding proteins at the telomere in yeast the exact role of such a structure has yet to be determined.

Along with telomeric length maintenance the telomeric cap protects the chromosome ends from being recognized as double-stranded DNA breaks. If the telomeric cap fails, telomeres become accessible targets for DNA repair proteins. Inappropriate processing of the telomeres can lead to structures known to induce genomic instability. For instance, telomeres may be fused together through the processes of non-homologous end joining (NHEJ) creating dicentric chromosomes and leading to breakage-fusion-bridge cycles as originally described by McClintock (McClintock 1941). Alternatively, a longer region of 3' single-stranded DNA produced by end resection of the 5' telomeric strand could make telomeres more prone to inter- or intra-telomeric recombination events (Hackett and Greider 2003).

Yeast Telomere Structure and Binding Proteins

Much of what is known about telomeric structure and function has come out of work done on the budding yeast species *S. cerevisiae*. This section will discuss the structure and function of the most well known protein components of the yeast telomere. Most of the proteins included have also been found in the yeast *K. lactis* which likely has similar telomeric cap components.

The major double stranded telomeric binding protein in yeast is Rap1p (Repressor Activator Protein 1). Rap1p is an essential protein (Shore and Nasmyth 1987) and is known to act in a context specific manner as both a repressor and activator of

transcription (Shore 1994). As an activator, Rap1p is involved in the transcription of most ribosomal protein genes, glycolytic enzyme genes, and at the *MAT*α mating type genes; as a repressor Rap1p acts at both mating type loci as well as near the telomeres (Shore 1994).

Rap1p directly binds telomeric repeats through interaction with its two Myb domains. The most well studied telomeric role of *RAP1* is its activity as a negative regulator of telomere length. Yeast telomere length is tightly controlled through a feedback mechanism which counts the number of Rap1p molecules that are bound to an individual telomere (Marcand et al. 1997). It has been proposed that the mechanism by which Rap1p molecules are counted may involve a higher order telomeric structure. Possibly, binding of a specific number of Rap1p molecules creates a telomeric conformation that is inaccessible to the reverse transcriptase telomerase. The loss of Rap1p binding sites through telomeric shortening may force a change in this conformation, allowing telomerase access to the telomere (Marcand et al. 1997).

Rap1p's ability to regulate telomere length requires its association with Rap1p-interacting factors 1 and 2 (Rif1p and Rif2p) (Hardy et al. 1992; Levy and Blackburn 2004; Wang RC. 2004). A lack of *RIF1* and *RIF2* can be compensated for by fusing a PDZ protein oligomerization domain from the rat gene GRIP1 protein to the Rap1ΔCp, a mutant of Rap1p lacking the Rif protein binding site, protein indicating that one functionally important role of the Rif proteins at the telomere is to cause associations between Rap1p molecules which are tethered to telomeric repeats (Levy and Blackburn 2004). This data supports the theory that a conformational change controlled by the binding of Rap1p is an important player in yeast telomere length maintenance.

Rap1p association with DNA promotes a bend in the DNA thought to be greater than 50° (Gilson et al. 1993; Muller et al. 1994). Taken together, the important role of Rap1p oligomerization and Rap1p's ability to bend DNA suggest that the creation of a higher order loop-back structure promoted by the Rap1p/Rif1p/Raif2p protein complex may be a significant factor in the negative regulation of yeast telomeres. A structure like this has previously been suggested as a player in telomere length control and transcriptional repression (Evans and Lundblad 2000; Zaman et al. 2002).

The *K. lactis* telomeric repeat offers an excellent model for studying the role of the telomeric repeat sequence in length regulation processes. Mutations in the template region of the telomerase RNA subunit of *K. lactis,* encoded by *TER1*, cause mutant repeats to be exclusively added to the telomeric end. Mutant *ter1* templates containing changes on the left side of the Rap1p binding site caused elongation of telomeres (McEachern and Blackburn 1995; Krauskopf and Blackburn 1996; Krauskopf and Blackburn 1998). An extensive study in which each position of the 25 bp repeat was altered uncovered several functional domains of the *K. lactis* 25 bp repeat (Underwood et al. 2004) (Fig.1.1). The Rap1p binding site encompasses positions 15-26. Underwood et. al. demonstrated that the Rap1p binding site could be divided into two functional domains. Mutations in the left side of the site including positions 16-20 caused immediate telomere elongation while mutations in the right side of the site, positions 22-24, caused telomere shortening. Mutations within the left side of the Rap1p binding site also seem to be coincident with elevated levels of recombination near the telomeres and the production of a large amount of ECTR, and extension of the single-stranded 3' overhang (Underwood et al. 2004).

In addition to functioning as a negative regulator, Rap1p also serves as a tethering point for molecules involved in heterochromatin formation and gene repression at and near the telomere, a process called the telomere position effect (TPE). The Ku70p-/Ku80p complex competes with Rif1p and Rif2p for binding with Rap1p. This interaction allows for the recruitment of Sir2p Sir3p and Sir4p (Silent Information Regulator) to the telomere (Mishra and Shore 1999).

Rap1p is a major player in telomere length regulation and structure and mediates the protein-DNA interaction within the double-stranded region of the telomere. However, the more distal and single stranded portion of the telomere is controlled through the action of another protein, Cdc13p, which interacts dynamically with a set of proteins to both protect the single-stranded region from degradation and control the access of telomerase to the telomeric end.

Cdc13p binds the single stranded G-rich telomeric overhang and in coordination with a number of binding partners regulates the addition of telomeric repeats both through the action of telomerase and DNA polymerase alpha (Qi and Zakian 2000). *CDC13* is an essential gene and loss of its function causes cells to enter G2 cell cycle arrest (Weinert and Hartwell 1993; Garvik et al. 1995). In addition to this function, Cdc13p also protects the single-stranded 3' end from inter-telomeric recombination events (Grandin et al. 2001a). Extension of the 3' strand of telomeric DNA is accomplished through recruitment of the Est1p telomerase subunit by Cdc13p (Qi and Zakian 2000). Cdc13p negatively regulates telomere length and protects telomeric ends through its association with two other essential proteins Stn1p and Ten1p. Stn1p binds Cdc13p in a region overlapping the Est1p binding site and competes with telomerase for

binding of Cdc13p (Grandin et al. 1997). The negative regulation mediated by Stn1p also requires Ten1p. Ten1p has been shown to associate in vivo with both Stn1p and Cdc13p (Grandin et al. 2001b). Cdc13p's interaction with Stn1p is also involved in coordinating extension of the G and C-rich strand of the telomere (Chandra et al. 2001). Cdc13p, Stn1p, and Ten1p, integral components in the dynamic telomere-lengthening machine, also perform a major capping function, protecting yeast telomeres from the recombinational pathways, which normally act on single-stranded DNA overhangs.

Yeast Cell Cycle Checkpoint and Repair Proteins Involved in Telomere Maintenance

Checkpoint pathways monitor the genome for DNA damage and regulate cell cycle progression in response to DNA damage signals. Persistence of either DSBs or single-stranded DNA signals a damage response, which can elicit a cell cycle arrest (Lee et al. 1998; Usui et al. 2001). A number of proteins which play key roles in triggering check point pathways or in DNA repair are also involved in normal maintenance of yeast telomeres including, the yeast ATM-like kinases Tel1p and Mec1p, the MRX complex, and the Ku70p/Ku80p hetero-dimer (Tsukamoto et al. 2001; Larrivee et al. 2004; Fisher and Zakian 2005).

Tel1p and Mec1p seem to be involved in redundant pathways of telomere maintenance. Deletion of one of the ATM-like kinases leads to stable shortening of telomeres but does not cause loss of cell viability. The degree of shortening is much greater in a *tel1* mutant than in *mec1* mutants. Double mutants of *tel1* and *mec1* illicit a telomerase deletion-like phenotype characterized by telomere shortening that leads to

replicative senescence (Ritchie et al. 1999). Tel1p also seems to be involved in providing a capping function which, along with the telomerase RNA subunit Tlc1, prevents telomere fusions to double-stranded breaks (Ritchie et al. 1999; Chan and Blackburn 2003). Double mutants of *tel1* and any of the three components of the MRX complex exhibit the same shortened telomere phenotype and are therefore believed to act in the same pathway of telomere protection (Ritchie and Petes 2000). These same partners are involved in a specific cell-cycle checkpoint pathway called the Tel1p- and Mre11p-dependent checkpoint or TM-checkpoint. The TM-checkpoint acts independently of Mec1p and recognizes the persistence of unrepaired double-stranded breaks (Usui et al. 2001). Loss of functional human ATM leads to the onset of a premature aging disease known as ataxia telangiectasia (AT) (Metcalfe et al. 1996). Similar to *tel1Δ mec1Δ* yeast mutants, AT cells have shortened telomeres. AT cells also exhibit high levels of genomic instability which leads to an increased frequency of tumorigenesis (Wong et al. 2003).

The Ku70p/Ku80p heterodimer is involved in the process of non-homologous end joining. Ku70p/Ku80p binds DSBs and in humans recruits the catalytic subunit DNA-PKcs to form the DNA-dependent protein kinase Additionally, Ku70p/Ku80p bring together broken ends and prepare DSBs for ligation (reviewed in Featherstone and Jackson 1999). The Ku70p/80p heterodimer also binds double-stranded telomeric DNA. In *S. cerevisiae* Ku70p/80p is required for telomeric length maintenance as well as in the control of single-stranded overhang length (Boulton and Jackson 1996; Porter et al. 1996). Ku70p/Ku80p also binds a loop structure found on the *S. cerevisiae* telomerase RNA, *Tlc1*, and this interaction is required for efficient recruitment of telomerase to the *S. cerevisiae* telomeres (Fisher and Zakian 2005). In the absence of Ku70p/Ku80p the

telomeric end is vulnerable to degradation by the 5'→3' exonuclease Exo1p (Gravel et al. 1998). Deletions in Ku70p and Exo1p causes the accumulation of longer than normal 3' telomeric overhangs and eventually extension of the single-stranded region can elicit a DNA damage response through the *MEC1* checkpoint pathway (Chen et al. 2001; Maringele and Lydall 2002). Interestingly, a *ku80Δ* mutant in *K. lactis* has no change in overall telomere length but does experience elongation of the 3' single-stranded overhang and an increased rate of recombination near the telomere indicating that *KU80* is not required for length maintenance, it does contribute to the maintenance of the telomere "cap" in *K. lactis* cells (Iyer et. al., unpublished data).

Another set of proteins with overlapping functions in DNA repair and telomere maintenance are components of the MRX complex. The MRX complex (Mre11p, RAD50p, Xrs2p) is involved in the maintenance of both overall telomere length and the single-stranded telomeric overhang. Mutations in all three of the MRX components leads to a decrease in telomere length in the yeast *S. cerevisiae* (Boulton and Jackson 1998). Similarly, in *K. lactis, mre11Δ* and *ras50Δ* mutants each lead to a decrease in telomere length (Iyer et. al. unpublished data). The MRX complex has been identified as an important player in many recombination pathways including meiotic homologous recombination, non-homologous end joining, and homologous repair of IR-induced DSBs (reviewed in Krogh and Symington 2004). The Mre11 protein possesses both a 3'→5' exonuclease activity as well as a single-stranded endonuclease activity (Furuse et al. 1998; Paull and Gellert 1998; Trujillo and Sung 2001). Yeast telomeres maintain single-stranded G-rich 3' overhangs throughout the cell cycle, which are elongated during S-phase. Mutations in *MRE11* decrease the length of the constitutive overhang but do not

alter the S-phase extension of 3' overhangs. This indicates that the MRX complex is important for maintenance of normal length 3' overhangs but not in the extension of those overhangs during S-phase (Larrivee et al. 2004).

The MRX complex has also been found to be important for the recruitment of telomerase to Cdc13p (Tsukamoto et al. 2001). Additionally, it has been shown that the MRX complex has a high affinity for G4-DNA binding and that it is specifically an efficient endonuclease of G-rich telomeric sequences (Ghosal and Muniyappa 2005). G4-DNA contains non-Watson-Crick base interactions known as G-quartets and it is thought that this type of structure may form on a telomeric G-rich 3' overhang (Sen and Gilbert 1992). It has been suggested that MRX may function through the cleavage of G-quartet DNA from the 3' tip allowing for the association of Cdc13p and subsequently telomerase (Ghosal and Muniyappa 2005).

While not all of the pathways that connect telomere uncapping to DNA damage responses and cell cycle arrest are understood, it is clear that many proteins associated with the telomere are also players in cell cycle control. Understanding the pathways that connect telomere biology to cell cycle control is a central goal in understanding the mechanism by which human cells bypass cellular senescence during oncogenesis.

Human Telomere Dysfunction in Cancer

As in other organisms, normal human telomere structure performs roles, in length regulation and telomere capping. A complex tethered to the telomere through TRF1 controls human telomere length. TRF1 is active as a homodimer and contacts telomeric DNA through the single Myb-type DNA binding domain of each TRF1 molecule

(Bianchi et al. 1999). The binding of TRF1 to the telomeric DNA also creates a bend in the duplex DNA similar to the bend created by Rap1p on yeast telomeres (Bianchi et al. 1999). Tankyrase interacts with and negatively regulates TRF1 by poly(ADP-ribosyl)ation (Smith et al. 1998). TRF1 also complexes with TIN2 and TPP1, proteins thought to connect TRF1 with the two other proteins which bind human telomeric DNA, TRF2 and POT1. Mutants of POT1 lacking an intact DNA binding domain exhibit immediate and extensive telomere elongation (Loayza and De Lange 2003). It has been proposed that the interaction of TRF1 with POT1 controls telomerase's access to the chromosome end and that through this activity they are negative regulators of telomere length (Loayza and De Lange 2003; de Lange 2005). The second function of the human telomere structure is to provide a protective cap. TRF2 is the main player in providing this function and loss of TRF2 leads to rapid impairment of end protection resulting in chromosome end fusions (van Steensel et al. 1998). Human RAP1 associates with TRF2 and is also a negative regulator of telomere length. However, it does not directly bind human telomeric repeats like scRap1p (Larrivee et al. 2004). An integral part of telomeric integrity seems to be the formation of a t-loop. The D-loop within the t-loop structure is stabilized by TRF2 association with the duplex telomeric DNA created at the point of single stranded invasion (Griffith et al. 1999; Wang RC. 2004).

Mortal human cells, which include most human somatic cells, do not contain active telomerase and therefore have limited replicative lifespans (Counter et al. 1992). This limited lifespan of human cells was initially identified by Leonard Hayflick in 1965 (Hayflick 1965; Shay and Wright 2000). This lifespan was subsequently linked to the shortening of telomeres caused by incomplete replication in the absence of telomerase

(Counter et al. 1992). This shortening occurs at a variable rate depending on cell type and growth conditions (Counter et al. 1992). Human cells that reach their replicative limit are triggered through a p53-mediated pathway to enter an arrest called replicative senescence, or mortality phase 1 (M1), which occurs just before the G1/S transition. If there is a defect in the signaling which allows the cell to bypass M1 replicative senescence the cell then continues to divide until entering a stage known as crisis (Wright et al. 1989). Crisis is a period of mass cell death characterized by the existence of uncapped chromosomes, leading to fusion of telomeric ends which may drive the genomic instability that could contribute to tumorigenesis (Fig.1.2) (DePinho and Polyak 2004).

Over half of all human cancers harbor mutations in the p53 gene (Greenblatt et al. 1994) and in two separate studies with mice 100% of animals lacking p53 gene developed cancers (Donehower et al. 1992; Jacks et al. 1994; Artandi and Attardi 2005). These data indicate a powerful link between p53 dysfunction and the inability of cells to enter replicative senescence. The exact mechanism by which telomeres elicit the p53-dependent cell cycle arrest in response to telomere shortening is not understood. However, several interesting pieces of information linking p53 to the telomeric role in triggering senescence have recently been uncovered. The shortest telomeres seem to associate with p53 when the cells are nearing senescence (Zou et al. 2004). Further, these same short telomeres seem to be involved most prominently in telomere-telomere association near senescence. Shay and Wright have proposed that it is not just the shortest telomeres but also the telomere-telomere associations that short telomeres tend to be partners in that trigger the beginning of senescence (Zou et al. 2004). p53 is known to

associate with both single-stranded telomeric overhangs and with t-loop junctions (Stansel et al. 2002). It is also known that the p53 is phosphorylated by ATM in the presence of double-stranded breaks (Appella 2001) linking p53 to the ATM DNA damage response pathway. The p53 pathway remains an attractive target for research and development of anti-cancer therapies.

Once cells enter the M2 crisis stage a telomere maintenance program must take effect for the cells to gain immortalization (Shay and Wright 2004; Colgin and Reddel 1999). Two possibilities for this exist. First and most frequently, cancer cells reactivate telomerase (Shay and Bacchetti 1997). Alternatively, a recombinational pathway of telomere maintenance called Alternate Lengthening of Telomeres (ALT) can be initiated (Colgin and Reddel 1999; Dunham et al. 2000). ALT cancers utilize non-reciprocal inter-telomeric recombination events to elongate their telomeric ends (Dunham et al. 2000). These cells also display a number of distinctive phenotypes, which separate them from telomerase-positive cancers. ALT cancer telomeres are extremely long and heterogeneous in length (Colgin and Reddel 1999). They display types of genomic instability not seen in telomerase positive cancers including both destabilization of some minisattelites and loss of telomeric DNA from chromosomal ends (Cesare and Griffith 2004; Jeyapalan et al. 2005). Another phenotype indicative of ALT cancers is the presence of nuclear bodies named ALT-associated promyelocytic leukemia bodies (APBs) (Yeager et al. 1999). APBs appear coincident with the onset of ALT and contain a number of proteins involved in both replication and recombination (Nabetani et al. 2004). These bodies also associate with extra-chromosomal telomeric repeats (ECTR) and have been shown to be sites of DNA synthesis (Yeager et al. 1999; Nabetani et al. 2004).

Recently, it has been shown that ALT cancers also produce abundant t-circles (Cesare and Griffith 2004). These structures are proposed substrates for rolling-circle-mediated telomere elongation in both human cancers and in yeast telomerase deletion survivors (Natarajan and McEachern 2002; Cesare and Griffith 2004). This phenotype is shared with a specific mutant of the TRF2 basic domain ($TRF2^{\Delta B}$). In this latter case, the production of t-circles seems to be related to a loss of TRF2 capping and protection at the t-loop junction (Wang RC. 2004). Two genes involved in homologous recombination, XRCC3 and NBS1, are required for the production of t-circles in a $TRF2^{\Delta B}$ mutant. This suggests that the resolution of t-loops through homologous recombination is responsible for the production of t-circles.

Telomere Dysfunction and Genomic Instability: A Link to Tumorigenesis

The disruption of capping at the telomere can be defined as the disruption of the mechanism by which telomeres are distinguished from double-stranded breaks. This outcome is common to several situations in which the telomeric cap is compromised including dyskeratosis congenita, Bloom's syndrome, Werner's syndrome and in ALT cancers (Mohaghegh and Hickson 2002; Chang et al. 2003). Several pieces of information tie telomere dysfunction to a loss of global genomic integrity. Mice deficient in the telomerase RNA gene (mTR) have an increased rate of chromosomal end-to-end fusions and an increased incidence of cancer (Blasco et al. 1997; Reddel et al. 2001). Further, yeast cells lacking telomerase have up to a 10 fold increase in the mutation rate (Hackett et al. 2001) and this genomic instability is known to be fueled by Exo1p 5' end resection and the increased formation of single stranded DNA at chromosome arms

(Hackett and Greider 2003). Another piece of work specifically aimed at investigating genomic instability in ALT cells showed an increase of minisattelite instability at one locus (Jeyapalan et al. 2005).

The link between telomere uncapping in ALT cells and increased genomic instability has yet to be elucidated. Mounting evidence suggests that the phenotype of ALT cancers may fuel genomic instability (Chang et al. 2003; Reddel and Bryan 2003). This genomic instability may lead to increased cell death in some cases decreasing the malignant potential of tumors (Qi et al. 2005). Conversely, the ALT genomic instability may also allow ALT tumors to gain mutations in regions known to be cancer "hot spots" and which are directly correlated with tumor malignancy (O'Hagan et al. 2002). A large fraction of ALT positive tumors show loss of heterozygosity (LOH) within the same regions of chromosome 8. It may be significant that this LOH is 50% at a locus adjacent to the gene encoding the WRN helicase, is a protein important to maintaining a normal telomeric cap (Shigeeda et al. 2003). It is likely that general genomic instability can, at some frequency, give rise to rearrangements that favor the progression into malignancy. It has also been hypothesized that ALT cancer cells could be an intermediate stage in the formation of telomerase-positive malignancies which are capable of metastasis (Chang et al. 2003; Reddel and Bryan 2003).

Recombinational Telomere Elongation (RTE) in Yeast

Yeast cells normally constitutively express telomerase and therefore do not go through a telomere shortening induced senescence which human cells experience. However, if telomerase is deleted from yeast cells, a growth senescence occurs as a result

of gradual telomere shortening and rare *RAD52*-dependent survivors arise (Lundblad and Blackburn 1993; McEachern and Blackburn 1996). These survivors have established a telomerase-independent mechanism of telomere maintenance. This pathway is dependent upon a series of recombinational events and is called recombinational telomere elongation (RTE). In *Saccharomyces cerevisiae* two distinct pathways of RTE exist resulting in what are known as Type I and Type II survivors. Each type of survivor is distinct in the resulting structure of their telomeres as well as in their gene requirements. Type I survivors amplify the Y' subtelomeric repeats and maintain short distal telomeric repeat tracts. Type I survivor formation in *S. cerevisiae* requires *RAD52*, *RAD51*, *RAD54*, and *RAD57*, the canonical genes required for mitotic DSB repair. Type II survivor formation, in which just the distal telomeric repeats are amplified, requires *RAD52*, *RAD50*, *RAD59* and the RecQ helicase *SGS1* (Chen et al. 2001; Cohen and Sinclair 2001) (Fig. 1.3). Each of these sets of genes defines a subset of the *RAD52* epistasis group and the requirements for a distinct type of recombinational event occurring at the telomeric end. Additionally, it is now known that the *RAD51*-dependent pathway of recombination requires a much larger region of homology (approximately 100 bp) than the *RAD50*-dependent pathway (as little as 33 bp) during non-telomeric break-induced replication events (Ira and Haber 2002).

The yeast *K. lactis* does not contain telomeric repeats within its subtelomeric region and therefore does not produce Type I elongated telomeres. *K. lactis* cells with a deletion of the telomerase RNA gene (*ter1*Δ) produce *RAD52*-dependent survivors which contain elongated tracts of telomeric repeats (McEachern and Blackburn 1996). *K. lactis* cells containing two types of phenotypically wild type repeats which then experienced

deletion of *TER1* gave rise to survivors containing both types of repeats. The telomeres of survivors derived from an individual clone all contained multiple copies of the same short (of less than 100 bp) pattern of the two repeat types. Between separate clones the pattern of repeats differed.

These results led to the development of the roll-and-spread model of RTE, which consist of three basic steps (Natarajan and McEachern 2002) (Fig.1.4). The first step is the creation of a small t-circle from a telomeric end. In the second step the t-circle serves as a template for the extension of a single telomeric tract through rolling circle DNA synthesis. Evidence has shown that a 1.6 kb circles containing both telomeric and non-telomeric sequence can be utilized very efficiently as a template for telomere extension (Natarajan and McEachern 2002). The third and final step yields elongation at all or most telomeric ends by spreading the newly formed long telomeric sequence through non-reciprocal recombination events. It has also been shown that a single elongated telomere is efficiently spread to all chromosome ends in 90% of *ter1*Δ survivors (Topcu et al. 2005) (Fig.1.5).

K. lactis ter1Δ survivors undergo episodes of elongation followed by gradual shortening. Presumably, this pattern of elongation and shortening represents the cell's ability to cap telomeres that are above a certain critical length and the loss of this cap when telomeres shorten below this length. Short telomeres participate in more recombination than normal length telomeres (McEachern and Iyer 2001). Strikingly, the point at which telomere length becomes sufficiently short to trigger recombination with another telomere occurs largely within a narrow window of telomere length, at between ≈75 and 100 bp long (Topcu et al. 2005). This suggests that *K. lactis* telomeres require at

least four full repeats to maintain a capped structure that is resistant to initiating recombination. The ability for *K. lactis* survivors to recap telomeres elongated through RTE suggests that the processes of telomere capping and telomere length maintenance are distinct from each other.

It seems likely that the rate limiting step in survivor formation and what may make it relatively rare is the creation of a t-circle. The uncapping of *K. lactis* telomeres that allows the creation of a t-circle is likely the spark which initiates the progression into RTE. The initial evidence that supported the roll and spread model indicated a t-circle containing as little as 100 bp was the likely template for elongation. Interestingly, 100 bp is near the length at which telomeres become prone to instability recombination. One attractive interpretation of these two pieces of data is that circle creation can only happen when the telomeres become "uncapped" and that this typically restricts the size of t-circle made by senescing *ter1Δ* cells to 100 bp or less. Alternatively, the short length of senescing *ter1Δ* telomeres may inhibit the production of t-circles. In such a case a rare longer telomere experiencing uncapping may produce the t-circle. In any case the production of t-circles is likely the rate-limiting-step that *ter1Δ* cells must overcome to become survivors.

Telomere Rapid Deletion

Recently, the telomeres of several organisms have been found to exist in higher order structures known as t-loops. The proposed structure of a t-loop resembles a recombination intermediate and presumably needs to be protected from being "resolved" by DNA polymerases and nucleases that would normally act at such a structure. In

humans t-loops are stabilized by TRF2. A mutant of the TRF2 basic domain, $TRF2^{\Delta B}$, can not provide t-loop stabilization and this results in telomere length reduction and the creation of t-loop size t-circles (Wang et al. 2004).

A very interesting parallel to RTE involves a mechanism of telomere length control in the yeast *S. cerevisiae* known as telomere rapid deletion (TRD). This process provokes a rapid loss of telomere length at one telomere at a time and has been proposed to be a means of keeping telomere lengths within a narrow size range (Li and Lustig 1996). The hypothesized mechanism of TRD occurs through the resolution of a t-loop like structure (Bucholc et al. 2001b). TRD requires *RAD50* (a member of the MRX complex), a feature it has in common with the Type II RTE. In fact all members of the MRX complex are required for TRD. However, the nuclease activity of Mre11p is not (Bucholc et al. 2001a; Bucholc et al. 2001b; Williams et al. 2005). The C-terminus of Rap1p, which is required for its ability as a negative regulator of telomere length, is not required for TRD. However, in a Rap1p C-terminus mutant, TRD becomes much less precise (Bucholc et al. 2001b). TRD frequency is greatly increased during meiosis and serves as a means of precisely resetting telomeres to wild type length (Joseph et al. 2005). TRD occurs preferentially on telomeres that are significantly larger than the other telomeres within the cell. (Joseph et al. 2005).

Other forms of rapid telomere loss have been observed in both mice and in human cells. One piece of evidence indicating that TRD may occur in other eukaryotes, discussed above, involves the resolution of t-loop structures in the $TRF2^{\Delta B}$ human cells. This evidence strongly suggests that a TRD-like mechanism can result in both loss of telomeric sequence and in the creation of a t-circle equivalent to the deletion size (Wang

RC. 2004). The NBS1 gene, a partner in the human homologue of the MRX complex, is required for t-circle formation in TRF2$^{\Delta B}$ cells. It is possible that the MRX complex is responsible for the formation of a single-stranded overhang which inserts into the more internal duplex DNA forming a t-loop structure which is a proposed intermediate stage in both TRD and t-circle creation (Bucholc et al. 2001a; Bucholc et al. 2001b; Wang RC. 2004).

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Figure 1.1 The *K. lactis* telomeric repeat contains 25 bp. The Rap1p binding site is located along positions 16 to 25 as indicated by the blue line. Mutants within the Rap1p binding site can be divided into two subsets. Mutations within the left side of the Rap1p site (positions16-20) lead to long telomere phenotypes as indicated by the red bracket. Mutations within the right side of the Rap1p binding site (positions 21-25) lead to short telomere phenotypes as indicated by the green bracket

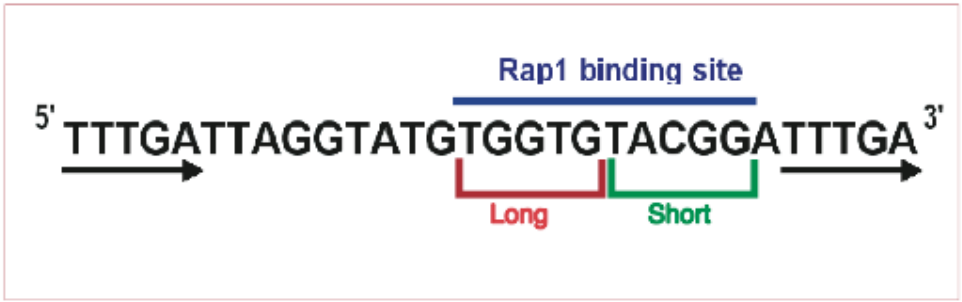

Rap1 binding site
5'
TTTGATTAGGTATGTGGTGTACGGATTTGA
3'
Long
Short

Figure 1.2 Telomeres and cellular immortalization in human cell cultures. Telomeres gradually shorten in the absence of telomerase activity until reaching a critically short length that triggers an M1 senescence. Bypassing this senescence occurs in cells with a mutation in genes controlling the p53 mediated cell cycle checkpoint pathway. Cells continuing to divide beyond senescence eventually enter crisis a stage marked by massive cell death and major genomic instability. In order to survive crisis the cell must either reactivate telomerase or develop a telomerase-independent manner of elongating their telomeres.

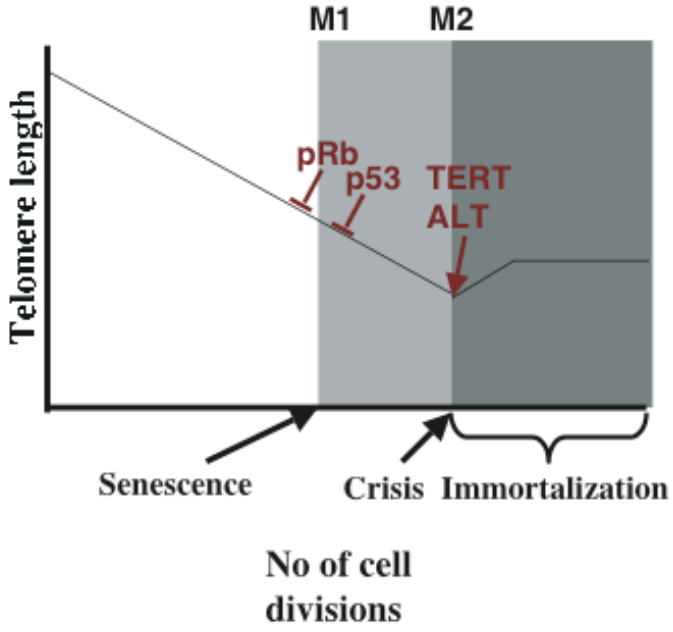

M1
M2
Telomere length
pRb
p53
TERT
ALT
Senescence
Crisis
Immortalization
No of cell divisions

Figure 1.3 Two types of survivors arise after telomerase deletion in *S. cerevisiae*. Both types of survivors depend upon *RAD52*. The yellow boxes represent the subtelomeric Y' elements of *S. cerevisiae*. The black dashes represent $TG_{(1-3)}$ telomeric repeats. Type I survivor telomeres contain amplifications of the Y' elements and a few distal telomeric repeats. Type II survivors telomeres have extended regions of telomeric repeats.

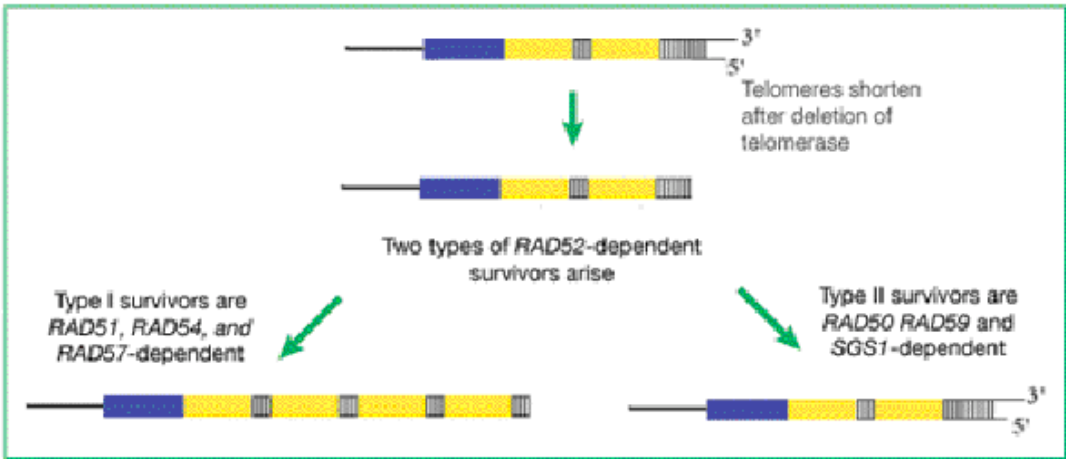

3'
5'
Telomeres shorten after deletion of telomerase
Two types of RAD52-dependent survivors arise
Type I survivors are RAD51, RAD54, and RAD57-dependent
Type II survivors are RAD50 RAD59 and SGS1-dependent
3'
5'

Figure 1.4 The roll-and-spread model of recombinational telomere elongation consists of three steps. (Natarajan and McEachern 2002) This model depicts a three-stage mechanism for recombinational telomere elongation (RTE). Stage one is the formation of a circle from an existing telomere. Stage two is a rolling circle replication event resulting in one elongated telomere. Stage three consists of gene conversion events that spread the original elongated telomeric sequence to several telomeric ends.

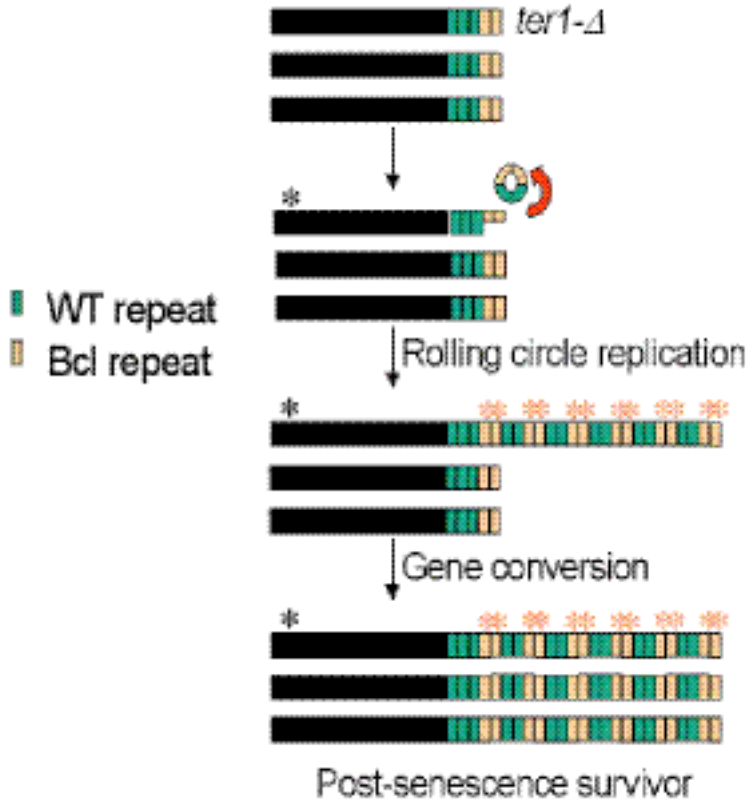
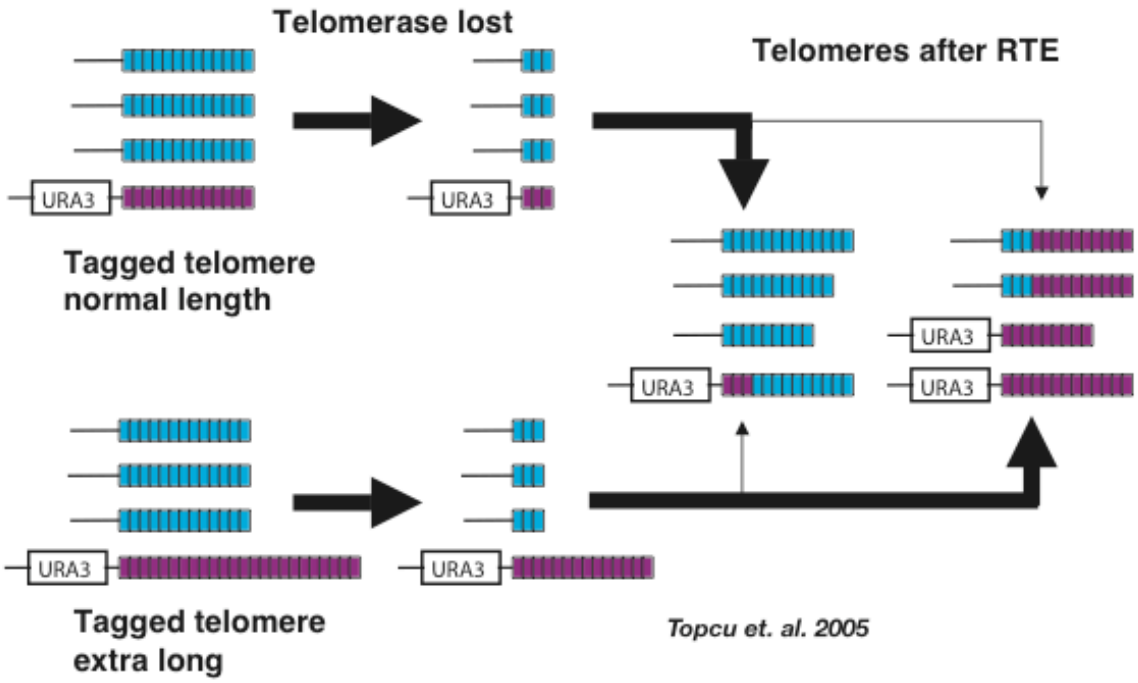

ter1-Δ
WT repeat
Bcl repeat
Rolling circle replication
Gene conversion
Post-senescence survivor

Figure 1.5 A long marked telomere is preferentially spread to all the telomeres in a cell. Topcu et. al. demonstrated that a single long telomere is spread to all the telomeres in a senescing *ter1Δ* cell 90% of the time. Marked telomeres of normal length are spread to all telomeres about 10% of the time indicating that they are not preferred over telomeres of similar lengths.


Telomerase lost
Telomeres after RTE
URA3
URA3
Tagged telomere
normal length
URA3
URA3
URA3
URA3
URA3
Tagged telomere
extra long
Topcu et. al. 2005

CHAPTER 2

SMALL T-CIRCLES ARE UTILIZED AND PRODUCED BY *KLUVEROMYCES LACTIS* TELOMERES[12]

INTRODUCTION

Telomeres are the nucleoprotein structures found at the ends of linear chromosomes (Bailey and Goodwin 2004; Cech 2004; Chan and Blackburn 2004). Telomeric DNA consists of tandem arrays of short repeats that have G-rich and C-rich strands. The telomeric DNA is divided into a proximal double-stranded region and a more distal single-stranded 3' overhang (Fletcher 2003; Neidle and Parkinson 2003). This overhang is comprised solely of the G-rich strand and is elongated through the activity of the reverse transcriptase, telomerase (Wei and Price 2003). Telomerase minimally consists of a template RNA that is reverse transcribed onto telomeric ends by the catalytic protein subunit.

Telomeric DNA also associates with a large assemblage of proteins (Vega et al. 2003; Smogorzewska and de Lange 2004). The telomere protein/DNA complex acts as a cap that is dynamic in composition and arrangement. It is the dynamic protein/DNA telomere complex that both controls telomere metabolism and protects telomeres from

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[1] Figure 2.1 appears in Natarajan, S., C. Groff-Vindman, and M.J. McEachern. 2003. Factors influencing the recombinational expansion and spread of telomeric tandem arrays in *Kluyveromyces lactis*. *Eukaryotic Cell* **2**: 1115-27.

[2] Figures 2.2, through 2.8 appear in Groff-Vindman, C., A.J. Cesare, S. Natarajan, J.D. Griffith, and M.J. McEachern. 2005. Recombination at long mutant telomeres produces tiny single- and double-stranded telomeric circles. *Mol. Cell. Biol.* **25**: 4406-12.

being recognized as double-stranded breaks. One proposed mechanism for telomere protection is the creation of telomeric secondary structures such as t-loops which are stabilized through the action of telomeric DNA binding proteins (Griffith et al. 1999; Tomaska et al. 2004b). The maintenance of telomere length is important to the normal functioning of cells. In most human somatic cells telomerase activity is very low or absent so that telomeres gradually shorten, eventually reaching a state which triggers a growth arrest called replicative senescence (Wright and Shay 1992; Cristofalo et al. 2004). Mutations or other conditions that bypass senescence produce further telomere shortening and take cells into crisis, a state of high genetic instability and cell death caused by widespread telomere dysfunction (Shay and Wright 2004). In order to survive crisis and become immortal a means of telomere elongation must arise (Cong et al. 2002; Scheel and Poremba 2002). Most cancer cells are immortal due to the presence of telomerase (Shay and Bacchetti 1997). A subset of cells that emerge from crisis maintain their telomeres through ALT, a process that involves inter-telomeric recombination (Bryan et al. 1997; Dunham et al. 2000; Reddel and Bryan 2003). Studies have shown that telomeres of ALT cells are both highly heterogeneous in size and frequently much longer than telomeres in normal human cells and telomerase positive cancer cells (Bryan et al. 1995; Bryan et al. 1997). ALT cells also commonly contain intranuclear structures known as ALT associated PML bodies or APBs. APBs contain recombination proteins, telomeric DNA, and replication factor A, and have therefore been suggested to play a role in the mechanism of ALT telomere elongation (Yeager et al. 1999; Nabetani et al. 2004). While the structure of telomeric DNA associated with APBs is unknown,

extrachromosomal telomeric repeats (ECTR) have recently been extracted from ALT cell lines and have been shown to at least partially exist in circular conformations (Cesare and Griffith 2004; Wang RC. 2004).

Yeast cells have constitutively active telomerase and normally maintain their telomeres within a fixed size range (Evans and Lundblad 2000). However, deletion of telomerase in yeast cells leads to telomere shortening and coincident growth senescence (Lundblad and Szostak 1989; Singer and Gottschling 1994; McEachern and Blackburn 1995). Although most cells eventually die from this senescence some post-senescence survivors emerge. These survivors, which are dependent upon the recombination gene *RAD52* (Lundblad and Blackburn 1993; McEachern and Blackburn 1996), elongate their telomeres through recombinational telomere elongation (RTE) a process analogous to human ALT. In Saccharomyces cerevisiae, there are two distinct pathways of RTE, each of which produces a distinctly different elongation pattern. Type I survivors have amplification of the long Y' subtelomeric elements and short extensions of the telomeric $TG_{(1\text{-}3)}$ repeats (Lundblad and Blackburn 1993; Teng and Zakian 1999; Chen et al. 2001a). Type II survivors lack subtelomeric amplification and are characterized by long telomeric $TG_{(1\text{-}3)}$ extensions which are heterogeneous in length (Teng and Zakian 1999; Chen et al. 2001a). Both type I and type II survivors of Saccharomyces require *RAD52* as well as type specific sets of recombination genes. Type I survivors require *RAD51*, RAD54, and RAD57 while type II survivors require *RAD50*, *RAD59*, and the helicase SGS1 (Le et al. 1999; Teng et al. 2000a; Chen et al. 2001b; Cohen and Sinclair 2001; Huang P 2001).

Only type II survivors arise when the telomerase RNA gene (*TER1*) is deleted from Kluveromyces lactis cells (McEachern and Blackburn 1996). Inspection of telomeric sequence from *K. lactis ter1*-Δ survivors derived from cells with telomeres composed of basal wild type and terminal phenotypically silent mutant repeats revealed repeating patterns containing both types of repeats (Natarajan and McEachern 2002a). Most or all telomeres within a clone shared a single pattern but patterns varied between survivor clones. These data lead to the roll-and-spread model which proposed that a three step process involving the creation and utilization of small telomeric circle (t-circle) (≈100 bp) was the mechanism by which *K. lactis* survivors were creating survivors. First, in this model a t-circle is created from a single telomeric end which is experiencing some level of uncapping. This first step is then followed by a rolling circle type synthesis around the newly created t-circle yielding a linear array of the t-circle sequence. Finally, in the third step of this model the newly formed array of telomeric sequence is spread through gene conversion to most or all other telomeres (Natarajan and McEachern 2002a).

It has been previously shown that a 1.6 kb circle containing telomeric repeats, a marker gene, and subtelomeric sequence can play a role in telomeric elongation. After introduction of such a circle into a *ter1*-Δ cell, tandem arrays of the circular sequence can be found at multiple telomeric ends (Natarajan and McEachern 2002a). However, it is possible that the large portion of non-telomeric DNA found on these circles as well as their large size makes them less realistic templates for the elongation seen in naturally occurring *K. lactis* RTE .

In this work we provide support for two of the three steps of the roll-and-spread model of RTE. First, we show that a small mostly single-stranded t-circle of telomeric DNA can be used as a donor of telomeric DNA in a *K. lactis* telomerase mutant that has stably short telomeres. Second, we show that a long telomere mutant *ter1-16T* efficiently creates both single and double-stranded t-circles some as small 100 nt/bp. These results demonstrate that *K. lactis* telomeres are capable of making and utilizing small t-circles much like those proposed to be the instigators for RTE in *K. lactis ter1*-Δ cells.

MATERIALS AND METHODS

Yeast Strains

The strain 7B520 (ura3-1 his2-2 trp1) originally described by Wray et al. (Wray et al. 1987a) was used as wild type in this study. The telomerase RNA gene mutant *ter1-16T* and *ter1*-Taq are mutants of 7B520 originally described by Underwood et al. (Underwood et al. 2004). The double mutant *ter1-16T RAD52*Δ was created by mating of the single mutant *ter1-16T* with TAQ-STU-19 (ura3 his2-2 *RAD52*Δ) also containing one chromosome with a subtelomeric insert of the *S. cerevisiae* URA3 gene (M.J. McEachern and S. Iyer unpublished data). Diploids were selected for on plates lacking both histidine and uracil and sporulated. Tetrad dissection was performed and each spore was screened for *RAD52* and telomere length phenotype by Southern blot analysis (Underwood et al. 2004).

Southern Blotting

Yeast genomic DNA (cut or uncut) was run on 1% Sea Kem LE agarose gel (Cambrex Bio Science Rockland Inc., Rockland, ME) or 4% Gene Pure 3:1 agarose gel (ISC Bioexpress, Kaysville, UT) and then transferred onto Hybond N+ membrane (Amersham Biosciences, Piscataway, NJ). Southern blots were hybridized and washed at 47°C or 50°C with γ-P^{32} labeled probes. Probes were either Klac1-25 G-strand telomeric probe (5'-ACGGATTTGATTAGGTATGTGGTGT-3') or the KC25-1 C-stranded telomeric probe (3'-ACACCACATACCTAATCAAATCCGT-5'). All hybridizations were carried out in the presence of 500mM Na_2HPO_4 and 7% sodium dodecyl sulfate (SDS) (Church and Gilbert 1984). All washes were done in 100 mM Na_2HPO_4 and 2% SDS.

Creation of small single-stranded telomeric circles

A 100 nt oligomer composed of three 25 bp telomeric repeats flanked on either side by non-telomeric sequence ("A" on the 5' side and "B" on the 3' side) was synthesized. This oligomer was circularized by bringing the ends together with another 25 nt oligomer composed of sequence complementary to the non-telomeric sequence of the 100 nt oligomer (B'-A') in the presence of T4 DNA ligase (Also see Fig. 1.1A). 100 nt circles composed of either the C- or the G-rich telomeric strand were made in the same way. The G-strand 100 nt telomeric circle transformants were derived from circles composed of telomeric repeats that had a *Bcl*I restriction site. 250-300 ng of the 100 nt circle along with 0.5-1 ng of p1B3, an ARS-containing plasmid (Wray et al. 1987b), was used for each transformation.

2-Dimensional Gel Analysis

Genomic DNA from *ter1-16T* (uncut or Exonuclease I treated) were run at 75V for 6 hours on 4% non-denaturing Gene Pure 3:1 agarose gel containing 0.6 μg/ml chloroquine. These gels were then soaked in 0.5 X TBE containing 3 μg/ml chloroquine. The gels were then rotated 90° and run in the second dimension for 6 hours at 75V. Both dimensions were run in 0.5 X TBE running buffer with chloroquine concentrations equal to that of the gel.

Isolation of Extrachromosomal telomeric DNA

Uncut genomic DNA from *ter1-16T* was run on 0.8% agarose gels at 30 V for 90 min. DNA migrating below a 500 bp linear marker was cut from the gel. This DNA was electro-eluted from the gel fragments at 90V for 1 hour while enclosed by 12-14,000 MWCO Spectra/Por dialysis tubing (Spectrum Laboratories Incorporated, Ranch Dominguez, CA). Solutions containing eluted DNA were concentrated by using microcon model YM-10 as directed by manufacturer (Amicon Bioseparations, Raleigh, NC).

Electron Microscopy

Gel isolated low molecular weight *K. lactis* DNA was incubated with 20 μg/ml T4 gene product 32 (gift of Nancy Nossal, NIH, Bethesda MD) for 5 min. in a buffer containing 10 mM HEPES pH 7.5 and 1 mM EDTA. The samples were treated with 0.6% glutaraldehyde on ice for 10 min and chromatographed over a 2.5 ml BioGel A-1.5M column (Bio-Rad, Hercules, CA). Fractions containing DNA and DNA-protein

complexes were prepared for electron microscopy(Griffith and Christiansen 1978) and examined on an FEI Tecnai 12 instrument (Eindhoven, The Netherlands). Images were captured using a Gatan Ultrascan US4000SP digital camera (Gatan, Pleasanton, CA) and molecule dimensions determined using Gatan Digital Micrograph 3.0 software. Images for publication were captured on sheet film, digitized using ACT-1 (Nikon, Tokyo, Japan) software and Nikon SMZ1000 steroscope. Brightness and contrast were adjusted using Adobe Photoshop (Adobe Systems, San Jose, CA).

RESULTS

One hundred-nucleotide telomeric circles of either strand can generate tandem arrays at telomeres.

Sequencing data suggested that the elongated telomeric arrays in *ter1-Δ* survivors containing two kinds of telomeric repeats can be composed of repeating units of 100 bp (Natarajan and McEachern 2002b). We therefore wanted to test if circles of that size and composed mainly of telomeric repeats could promote the formation of tandem arrays at telomeres. We first created single-stranded 100 nt circles composed of three telomeric repeats (75 nt of C-rich strand) and 25 nt of non-telomeric sequence (including a *Cla*I restriction site) by annealing, in the presence of DNA ligase, the two ends of a linear 100 nt oligo with a 25 nt bridge oligo that was complementary to the non-telomeric sequence in the presence of DNA ligase (Materials and Methods; Fig. 1.1A). Formation of circles was confirmed by showing that denatured ligation reactions could be hybridized to a labeled bridge oligo in a Southern blot while identical samples that had not been ligated could not. We introduced the 100 nt circle into *ter1-Taq* cells by co-transforming it with

p1B3, an ARS-containing plasmid that had a URA3 marker gene. The *ter1-Taq* strain was chosen because it had short recombinogenic telomeres but did not display the growth senescence and unstable telomere lengths of *ter1*-Δ.

We initially screened pools of ~10 transformants each for the presence of an ~100 bp *Cla*I fragment that hybridized to a telomeric probe. This size fragment is expected if the 100 nt circle integrates in the form of tandem arrays (Fig. 1.1A). From screening 30 such transformant pools, we found four that released a small fragment when cut with *Cla*I. We then attempted to isolate the individual transformants containing the 100 bp telomeric *Cla*I fragment from all four pools by re-streaking individual colonies from each pool, isolating DNA from each, and testing for the presence of bands that hybridized to a probe made with the bridge oligo. Two transformants that had bands that hybridized to the bridge oligo were successfully isolated. One of these (C1) had multiple hybridizing bands while the other (C2) had only a prominent pair of closely spaced bands (Fig. 1.1, left panels). The DNA fragments that hybridized to the bridge oligo also appeared to hybridize to telomeric and subtelomeric probes (Fig. 1.1B and data not shown). As observed with the pooled transformants, cleavage of C1 and C2 with *Cla*I also produced telomere-hybridizing bands of 100 bp. We conclude from these data that sequence from the 100 nt circle had become incorporated as tandem arrays at one or more telomeres.

We next tested whether 100 nt circles composed of the G-rich telomeric strand could also lead to the formation of tandem arrays. As before, 100 nt "G-strand" circles were generated in vitro using a 100 nt oligo containing three telomeric repeats and a 25 nt bridge oligo. The telomeric repeats in the G-strand circle were each constructed to have a single base pair change that makes a *Bcl*I restriction site. The *Bcl* mutant repeats are

functionally normal but serve as tags that can be readily identified by digestion with *Bcl*I (McEachern et al. 2002). As a control, 100 nt "C-strand" circles were generated again. Each type of circle was then transformed into *ter1-Taq* cells along with p1B3. From each co-transformation, DNAs from 35 pools (each composed of ~10 Ura^+ transformants) were then isolated and examined by Southern blotting. Consistent with our initial results, at least 6 pools derived from the "C-strand" circle were found to exhibit *Eco*RI fragments that hybridized to a bridge oligo probe as well as 100 bp *Cla*I fragments that hybridized to a telomeric probe. Results with the "G-strand" circle were similar. At least 9 of the 35 pools exhibited *Eco*RI fragments that hybridized to a bridge oligo as well as 100 bp *Cla*I fragments that hybridized to a telomeric probe. Four clones positive for hybridization to the bridge oligo were isolated from G-strand circle transformant pools. DNA from two of these clones (G1 and G2) that are hybridized with subtelomeric, telomeric, and bridge probes are shown in Fig. 1.1B. These clones again displayed characteristics expected of having telomeres that had been extended by tandem copies of the transforming circular sequence. Each produced *Cla*I fragments of ~100 bp that hybridized to a telomeric probe and *Bcl*I fragments of still smaller size that hybridized to a bridge oligo. The G2 clone was particularly notable. As judged from the subtelomeric hybridization, the majority of the 12 telomeres in these cells had acquired arrays derived from the G-strand circle. Judging by the sizes of restriction fragments in a gel run a longer distance (not shown), we estimate that the extended telomeres in the G2 clone were shortened by an average of ~430 bp from *Bcl*I cleavage and ~380 bp from *Cla*I cleavage. These studies show that 100 nt circles of either strand can lead to recombinational telomere elongation through the formation of telomeric tandem arrays.

To examine the stability of the integrated telomere-bridge tandem arrays, the two original C-strand circle *ter1*-Taq transformants were grown for several serial restreaks on YPD plates. Shown in Fig. 1.1C is a Southern blot of *Xba*I-cleaved DNA from these cells after hybridization to telomeric, subtelomeric and bridge oligo probes. The results from this analysis showed that the integrated arrays were highly unstable. The C1 transformant lost all but one band that hybridized to the bridge oligo by the second streak and had lost all bands by the third streak. The C2 transformant lost its bands that hybridized to the bridge oligo completely by the second streak. Telomeric tandem arrays from other clones, including the G1 and G2 clones shown in Fig. 1.1B, seemed less unstable (data not shown). Because the *ter1-Taq* telomerase normally maintains telomeres at a short size, gradual loss of the elongated telomeres was to be expected. However, the reason for the high rate of instability in some clones is not clear. Conceivably, the presence of non-telomeric DNA (the bridge sequence) at multiple positions throughout an elongated telomere can destabilize it. In *S. cerevisiae*, it has been shown that even normal telomeric sequences can be subject to rapid large deletions (Bucholc et al. 2001a).

2D gel analysis of extrachromosomal telomeric DNA from ter-16T cells

The long telomere mutant *ter1-16T* has extremely elongated telomeres relative to wild type (Fig. 2.2A compare lanes 2 and 3 with 5 and 6) (Underwood et al. 2004). Also apparent in the *ter1-16T* sample is the ECTR, which can be seen running under 1 kb in lane 1 of Fig. 2.2A. This ECTR is especially abundant between the 100 and 500 bp linear markers (Fig. 2.2, A, lane 4). Based on the unit size of repeating patterns in the telomeres of post-senescence survivors (Natarajan and McEachern 2002a), we predicted that circles

at least as small as 100 bp can sometimes be formed in vivo by a senescent *ter1*-Δ cell. However, *ter1*-Δ cells at various stages of senescence do not produce amounts of extrachromosomal telomeric repeats (ECTR) that are detectable by Southern blot (data not shown) (McEachern and Blackburn 1996). This is not surprising as they are presumably produced only rarely in this mutant.

Previous work done on telomerase RNA template gene (*TER1*) mutants has shown that mutants within and near the Rap1p binding domain produce extremely long telomeres and abundant ECTR (McEachern and Blackburn 1995; Krauskopf and Blackburn 1996; Krauskopf and Blackburn 1998; Underwood et al. 2004). However the nature of the ECTR in these mutants was not previously investigated. We therefore selected a representative long telomere mutant that produces abundant ECTR, *ter1-16T* (Fig. 2.2A), for detailed analysis. This mutant contains a 1 bp change in the Rap1p binding region of the telomerase RNA template (Underwood et al. 2004). Consistent with the hypothesis that decreased Rap1p binding at *ter1-16T* telomeres causes its telomeric phenotype, both the long telomere phenotype and presence of abundant ECTR are partially suppressed when Rap1p is over expressed in *ter1-16T* (Underwood et al. 2004).

2D gel analysis was employed to investigate the structure of ECTR made by *ter1-16T*. Two ladders of telomeric DNA migrating faster than 500 bp are seen on 2D Southerns hybridized to a C strand telomeric probe (Fig. 2.2C and E). One of these ladders migrates faster than the other in high concentrations of the intercalating agent chloroquine. This faster moving ladder hybridizes only to the C stranded telomeric probe (Fig. 2.2C and E). In contrast, the slower moving ladder hybridized to both telomeric strand probes (compare Fig. 2.2D and E). We therefore conclude that one ladder is

double-stranded ECTR and the other ladder is single-stranded ECTR specifically composed of the G-rich strand of *K. lactis* telomeric DNA. The single-stranded ladder is resistant to the single-strand specific Exonuclease I in conditions where a single-stranded control oligonucleotide is completely digested away (Fig. 2.2E and data not shown).This indicated that the molecules in the single-stranded ladder of ECTR do not contain nuclease-accessible ends (Fig. 2.2E).

Neither ladder of spots was visible in a (*ter1-16T RAD52*Δ) double mutant (Fig. 2.2B) in spite of the fact that long telomeres persist (Underwood et al. 2004). This finding indicates that the creation of both the ss and ds small ECTR species is a recombination driven process. Because homologous recombination is a precise process, we further conclude that the discreet spots in each ladder represent forms differing by integral numbers of the 25 bp/nt telomeric repeat.

Electron Microscopy of ECTR from *ter1-16T*

We next examined the small ECTR from *ter1-16T* by electron microscopy. Uncut genomic DNA from *ter1-16T* was run a short distance on a 0.8% agarose gel. Material running ahead of a 500 bp linear marker was extracted and purified. The extracted ECTR was treated with the single-stranded binding protein T4 gene product 32 and visualized by electron microscopy (Fig. 2.3). Greater than 75% of the *ter1-16T* ECTR molecules visualized were circular. Of the circular DNA molecules, approximately 60% were double-stranded and 40% were single-stranded. 51 single-stranded circles estimated sizes ranging from 125 nt to 400 nt and 106 double-stranded circles ranging in size from 100 bp to 1600 bp were measured as shown in Fig. 2.4. Previously telomeric circles have

been referred to as t-circles (Cesare and Griffith 2004; Tomaska et al. 2004a). The number of small t-circles counted may have been underestimated due to the difficulty involved in visualizing DNA circles near or below 100 bp/nt in size. 90% of all circles measured were under 400 bp/nt in size, consistent with the migration patterns of this DNA as seen on both 1D and 2D gels. Amongst the circles examined we found a few surprisingly large circles. It is not clear why these larger circles migrated faster than the 500 bp linear marker. Limitations of digital molecular measurement did not allow precise sizing of circles. Both the double and single-stranded circles are expected to be multiples of the 25 bp increment of the *K. lactis* telomeric repeat as indicated by the incremental nature of the telomeric ladders visualized on 2D Southerns and by their dependence on homologous recombination. Our results demonstrate that tiny telomeric circles can readily be generated by telomeric recombination in at least one Class of mutant with long dysfunctional telomeres.

In the course of examining the telomeric DNA sample containing circles we also visualized several double-stranded circles with double-stranded tails (Fig. 2.5). These structures were not abundant in the sample, representing less than 1% of the total number of molecules visualized. Because this sample was not subjected to any crosslinking most noncovalent structures were not preserved. The small ECTR from *ter1-16T* may therefore have contained many more circle with tail type structures that were lost in sample preparation. There are several possible explanations for these structures. They could represent protective t-loops (Griffith et al. 1999). Alternatively, they could be intra-molecular strand invasions that occurred as a result of telomere uncapping. If this happened on extrachromosomal linear pieces, either end of the fragment might be

involved in the strand invasion. Finally, the structures could be rolling circle replication (RCR) intermediates. Larger structures of this sort have been shown to be intermediates of RCR in a variety of systems (Chen et al. 2001a; Backert 2002).

DISCUSSION

We have demonstrated here that a small mostly single-stranded telomeric circle can be utilized to create arrays of its sequence at the telomeres of the mutant *ter1-Taq*. It is possible that *ter1-Taq* cells have telomeres which inefficiently produce t-circles and that this leads to its inability to undergo RTE. The simplest model for recombinational telomere elongation using a circular DNA template would be for the 3' single-stranded end of a telomere to strand-invade a telomeric circle (annealing to the C-rich strand) and act directly as the primer for DNA synthesis around the circle. Our results with both 100 nt circles and the 1.6 kb heteroduplex circles argue strongly against this model being the only mechanism for a telomere to acquire the sequence from copying a circle. Instead, there must be efficient mechanisms for circles of either strand to serve as templates for generating elongated arrays of telomeric repeats. One possibility is that rolling-circle-replication often occurs extra-chromosomally. Once a telomeric array is generated, it could readily be incorporated at a chromosome end. How extrachromosomal rolling-circle-replication would be primed is not clear. Conceivably, small single-stranded telomeric pieces are generated that can anneal to the circle. Another possibility for the priming of G-strand telomeric circles might be to use a mechanism related to that which primes telomeric Okazaki fragment synthesis. The yeast single-strand protein-binding complex, which includes Cdc13p, is thought to help recruit DNA polymerase to a

telomeric end and bring about synthesis of the second strand (Qi and Zakian 2000). It is conceivable that a single-stranded "G-strand" telomeric circle could recruit a DNA polymerase in the same manner.

The recombinational telomere elongation that occurs in *ter1 K. lactis* mutants in the absence of exogenously added DNA circles results in telomeres that have been extended by only moderate amounts, typically hundreds to low thousands of base-pairs. Based on this, we had postulated that if rolling circle replication of ~100 nt/bp telomeric circles was responsible for the generation of elongated telomeres in *ter1* post-senescence survivors, then the extent of telomere elongation produced by 100 nt circles should be equally moderate. Our data here are consistent with that prediction. While transformation of *ter1-Taq* with the 1.6 kb *URA3*-telomere circle leads to telomeric bands that routinely extend to limit mobility in agarose gels, transformation of the same strain with 100 nt circles typically produces much less extensive telomere elongation.

A major prediction of the roll-and-spread model is that senescing *K. lactis* cells lacking telomerase will occasionally be able to generate small circles of telomeric DNA that can trigger RTE (Natarajan and McEachern 2002a). T-circles at least as small as 100 bp/nt would need to be produced by such cells to account for the repeating patterns in the elongated telomeres of post-senescence survivors (Natarajan and McEachern 2002a). Our results here demonstrate that at least some *K. lactis* cells with dysfunctional telomeres can readily produce small telomeric circles.

The production of t-circles by recombination can likely be caused by a variety of telomere capping defects. All *K. lactis* mutants with extremely long telomeres produce abundant ECTR (Underwood et al. 2004). These include mutations such as *ter1-16T* that

produce immediate telomere elongation as a result of Rap1p binding defects, other *TER1* template mutations that cause delayed elongation and do not disrupt Rap1p binding, and a mutation in the gene encoding the telomeric cap protein Stn1p (Krauskopf and Blackburn 1996; Underwood et al. 2004; Iyer et al. 2005). Evidence that these different mutants have telomere capping defects includes highly elevated subtelomeric gene conversion rates and the presence of extensive single-stranded DNA specifically of the G-rich strand (Underwood et al. 2004; Iyer et al. 2005). Preliminary evidence from 2D gel analysis shows that ECTR from representatives of each of these classes of mutants contain telomeric ladders similar to those from the *ter1-16T* mutant (Iyer et al. 2005) (data not shown). Taken together the presence of ECTR ladders in all classes of long telomere mutants suggests that t-circle formation is a general consequence of telomere uncapping.

As senescing *ter1-Δ* mutants also experience very high rates of telomeric and subtelomeric recombination (McEachern and Iyer 2001; Topcu et al. 2005; Topcu Submitted), they are also likely to form t-circles, at least occasionally. However, several factors may act to drastically limit t-circle formation in senescing *ter1-Δ* cells relative to the levels seen in *ter1-16T*. The most obvious is telomere length, which is very short in senescing *ter1-Δ* cells, and very long in *ter1-16T* and other mutants known to have abundant ECTR. Additionally, the relative amounts of single-stranded 3' overhangs might also be very different. Another difference is the apparent nature of the respective capping defects. The telomeres of *ter1-Δ* cells are prone to initiating recombination only when they drop below ~100 bp in length (Topcu et al. 2005). It may therefore be the case that most telomeres in senescing *ter1-Δ* cells become recombination prone at sizes too short to form t-circles via intratelomeric recombination. If *ter1-Δ* cells only rarely make

circles, their formation may be the rate-limiting step of RTE. In marked contrast, the mutants that produce abundant ECTR presumably have perpetual capping defects and are therefore likely to continuously be prone to circle formation and other recombinational processes.

We suggest that all the ECTR structures seen and described above may form from a common recombination product. The model in figure 2.6 proposes that a 3' telomeric overhang strand-invades a more internal region of the telomeric repeat array. Subsequent nucleolytic processing of this structure could give rise to a partially single-stranded t-circle. Degradation of the partial C strand or helicase activity could lead to the creation of a totally single-stranded circle comprised of the G-rich telomeric strand. Extension of the partial duplex strand by a DNA polymerase could lead to a double-stranded circle or, with continued DNA synthesis, to a rolling circle replication intermediate. This model for ECTR production is similar to a model proposed by Lustig and colleagues for telomere rapid deletion (TRD), a process which can lead to the sudden loss of up to thousands of base pairs from a telomere in yeast or human cells (Bucholc et al. 2001b). TRD is largely *RAD52*-dependent in *S. cerevisiae* where it was suggested to arise from resolution of an equivalent intramolecular strand-invasion structure into a shortened telomere and a t-circle (Li and Lustig 1996). TRD and t-circle production may therefore be one and the same.

Intramolecular strand invasion to form a t-loop structure has been proposed to be a protective mechanism that helps provide the capping function of telomeres (Griffith et al. 1999). T-loops have been observed at telomeres of mammals, plants, protozoans, and the yeast Schizosaccharomyces pombe (Griffith et al. 1999; Munoz-Jordan et al. 2001;

Cesare et al. 2003; Tomaska et al. 2004b). Their formation appears to be promoted by certain telomere binding proteins (Stansel et al. 2001; Tomaska et al. 2004b). There are two possibilities for the relationship between a protective t-loop that contributes to telomere capping and an intramolecular strand invasion of the telomeric end that results from defective telomere capping and which may be the precursor to t-circles. The first is that the two structures are fundamentally the same at the DNA level. In this scenario, a t-loop is kept from being processed by the nucleases and polymerases that would normally accompany a recombination event by the complex of telomere proteins. Presumably, this would include the double-stranded telomeric binding protein Rap1p in *K. lactis*. Disruption of this protective complex could therefore directly lead to a t-loop behaving as a recombination intermediate. The alternative possibility is that t-loops are not identical to a recombination intermediate at the DNA level. Conceivably, for example, t-loops could form through non-Watson-Crick base interactions. In this case, the t-loop DNA structure could be intrinsically incompatible with recombination and might need to be disrupted before a telomeric end could initiate a recombination event. Whether the telomeres of *K. lactis* form t-loops is not currently known. The somewhat surprising characteristic of the *ter1-16T* mutant with its very long telomeres to preferentially accumulate t-circles of very small sizes might indicate that normal *K. lactis* telomeres preferentially form tightly folded loop-back or t-loop structures within their normal length of 350-600 bp. Failure to form a properly folded structure has been suggested to be responsible for the relatively abrupt loss of protection against recombination that occurs when *K. lactis* telomeres drop to below ~100 bp in length (Topcu et al. 2005).

What role, if any, the t-circles play in telomere function in *ter1-16T* cells is unknown. The presence of long telomeres and the absence of ECTR in *RAD52 ter1-16T* cells clearly indicate that small t-circles are not required for the telomere elongation in this mutant (Underwood et al. 2004) (Fig. 2.2). However, it remains possible that recombination, possibly including the copying of t-circles, does contribute to the formation of long telomeres in *ter1-16T* cells. The presence of telomeres composed of other mutant telomeric repeats leads to pronounced telomere elongation even in the absence of telomerase (Topcu et al. 2005) (Harris, L., Topcu, Z. and McEachern, M. unpublished data).

Recent evidence indicates that t-circles contribute to RTE in other organisms in addition to *K. lactis*. Telomeric "nanocircles" as small 36 nt can be utilized in vitro for RCR by a variety of DNA polymerases (Hartig and Kool 2004). The sudden emergence of elongated telomeric repeat tracts in Type II survivors of *S. cerevisiae* has been suggested to be triggered by rolling circle copying of t-circles (Teng et al. 2000b). Double-stranded t-circles are abundantly produced from the mitochondrial telomeres of several yeast species including *Candida parapsilosis* and have been proposed to be required for normal mitochondrial telomere maintenance (Tomaska et al. 2000). Most recently, t-circles have been isolated from human ALT cells (Cesare and Griffith 2004; Wang RC. 2004). *Xenopus laevis* has also been found to produce t-circles during part of its normal embryonic development (Cohen and Mechali 2002). The presence of t-circles in this broad group of organisms opens up the possibility that RTE based on rolling circle replication is a phylogenetically diverse phenomenon.

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Figure 2.1 Sequence from a 100 nucleotide telomeric circle can form tandem arrays at telomeres. A) In vitro generation of a 100 nt circle and the expected structure of a tandem array formed by the integration of its sequence at a telomere is shown. The partially single-stranded circle was generated by ligating the ends of a 100 nt oligo brought together by annealing to a bridging oligo that is complementary to a 25 nt non-telomeric region of the 100 nt oligo. Light gray boxes represent telomeric repeats, and the dark gray boxes represent non-telomeric sequence that forms the bridge between the telomeric repeats in the circle. The stippled box represents the region of subtelomeric DNA used as a probe. **B)** Southern blots of two C-strand 100 nt circle *ter1-Taq* transformants (C1 and C2) and two G-strand 100 nt circle *ter1-Taq* transformants (G1 and G2) that have a telomeric array formed with sequence derived from the circles are shown. The probes used are indicated beneath the panels. Each C-strand circle transformant is shown as two digests (*Eco*RI and *Eco*RI+*Cla*I). *Eco*RI generates the telomeric fragments and *Cla*I cuts once within each unit of the array leading to the formation of a 100 nt band. The G-strand circle transformants are shown as thee digests; *Eco*RI, *Eco*RI+ *Cla*I and *Eco*RI+*Bcl*I. *Bcl*I cleaves telomeric repeats derived from the G-strand circle. **C)** Southern blots of serial re-streaks of the same two C-strand 100 nt circle *ter1-Taq* transformants (C1 and C2) shown in B, digested with *Xba*I. *Xba*I generates smaller telomeric fragments for 9 of the 12 telomeres in *TER1 K. lactis*, making it easier to detect the telomere(s) that has been elongated by formation of array(s) derived from the 100 nt circle. Hybridizations to telomeric and bridge probes are shown.

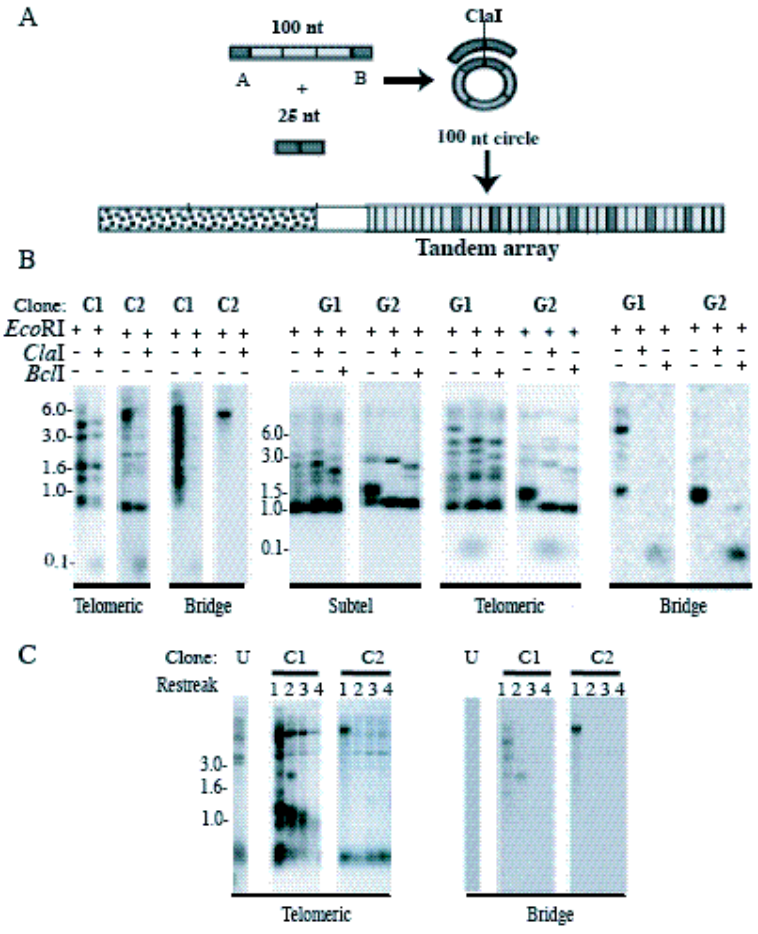

A
100 nt
ClaI
A
B
25 nt
100 nt circle
Tandem array
B
Clone: C1 C2 C1 C2 G1 G2 G1 G2 G1 G2
EcoRI
ClaI
BclI
6.0-
3.0-
1.6-
1.0-
0.1-
Telomeric
Bridge
1.5-
Subtel
Telomeric
Bridge
C
Clone: U C1 C2 U C1 C2
Restreak 1 2 3 4 1 2 3 4 1 2 3 4
3.0-
1.6-
1.0-
Telomeric
Bridge

Figure 2.2 Gel analysis of ECTR from *ter1-16T* A) Southern blot of 1% 1D agarose gels hybridized to C strand telomere probe. Cut and uncut genomic DNA from *TER1*, lanes 1-3 and the *ter1-16T* mutant lanes 4-6, show abundance of small telomeric material running under 500 bp. **B-E)** Southern blots of 2D 4% agarose gels hybridized to either the G-strand or C-strand telomeric probes. **B)** Uncut DNA from *RAD52 ter1-16T* hybridized to the C-strand probe shows that the double mutant produces no ECTR. The spot seen represents chromosomal telomeric signal. Similar results were obtained with the G-strand probe (data not shown). **C)** Uncut *ter1-16T* DNA run with 66 nt control oligo (arrow), hybridized with the C-strand telomeric probe. Two ladders of partially separated spots are visible. **D)** ExoI treated *ter1-16T* genomic DNA hybridized to the G-strand telomeric probe. **E)** Same membrane shown in panel D hybridized to the C-strand telomeric probe. Molecular weights of ds linear control fragments are shown in bp.

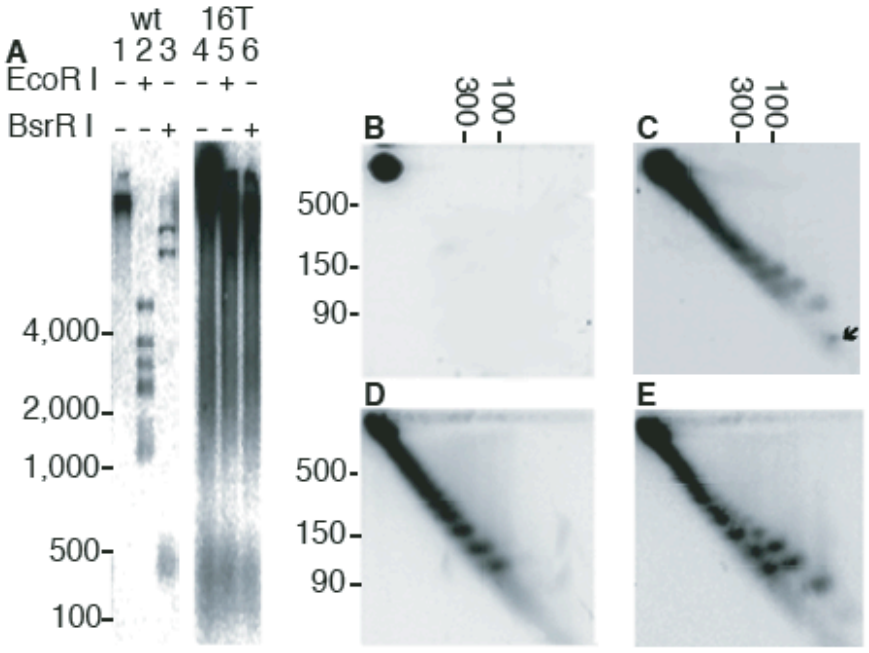

A
wt
16T
1 2 3 4 5 6
EcoR I - + - - + -
BsrR I - - + - - +
4,000
2,000
1,000
500
100
B
C
D
E
300
100
500
150
90

Figure 2.3 Visualization of DNA circles isolated from *K. lactis* survivors. A-F) Electron micrographs of double strand DNA circles. Circle sizes are 1580, 1285, 155, 230, 265 and 190 bp respectively for A-F. **G-J)** Electron micrographs of single strand DNA circles bound by T4 gene 32 single strand DNA binding protein. Circle sizes are 360, 365, 400 and 330 nt for G-J respectively. Samples were directly mounted onto thin carbon coated foils and rotary shadowcast with tungsten (Materials and Methods). Shown in negative contrast. Solid bar is equivalent to 200 bp (A-F) or 235 nt (G-I).

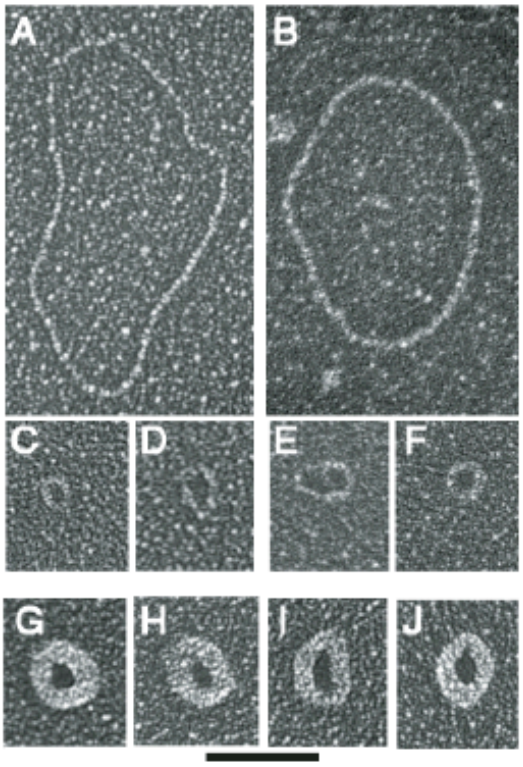

A
B
C
D
E
F
G
H
I
J

Figure 2.4 Size distribution of t-circles. A) double stranded t-circle sizes **B)** single stranded t-circle sizes. For both panels bars represent t-circles ranging from 12.5 bp/nt below and above size indicated. 90% of the circles measured fell below 400 bp/nt.

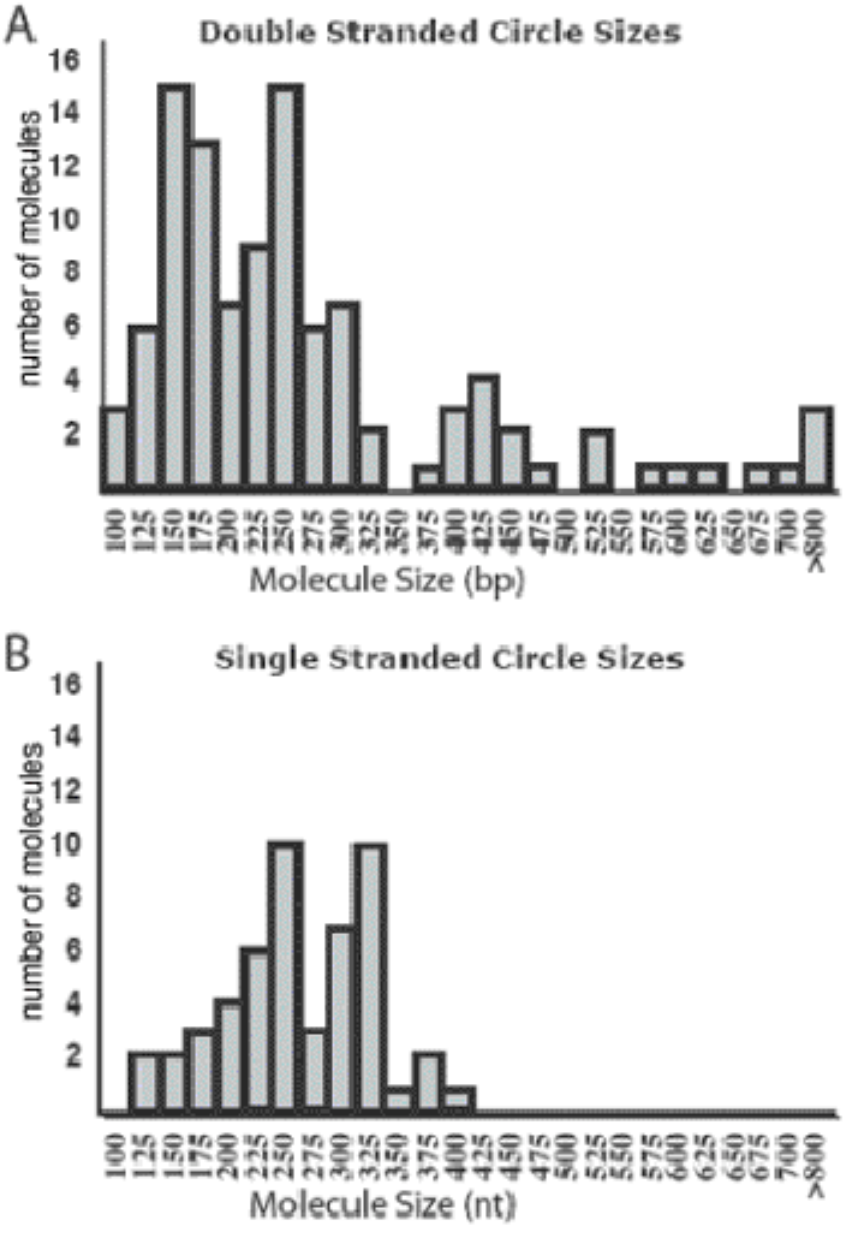

A
Double Stranded Circle Sizes
number of molecules
16
14
12
10
8
6
4
2
100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700 >800
Molecule Size (bp)
B
Single Stranded Circle Sizes
number of molecules
16
14
12
10
8
6
4
2
100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700 >800
Molecule Size (nt)

Figure 2.5 Visualization of circle-with-tail structures present in *K. lactis ter1-16T* ECTR. A-D) Electron micrographs of molecules resembling rolling circle replication intermediates. Estimated sizes of circular and tail portions of molecules shown in A-D are: 230 and 570; 430 and 730; 140 and 240; and 300 and 210 bp respectively. Molecules were prepared as in Figure 2. Bar is equivalent to 200 bp.

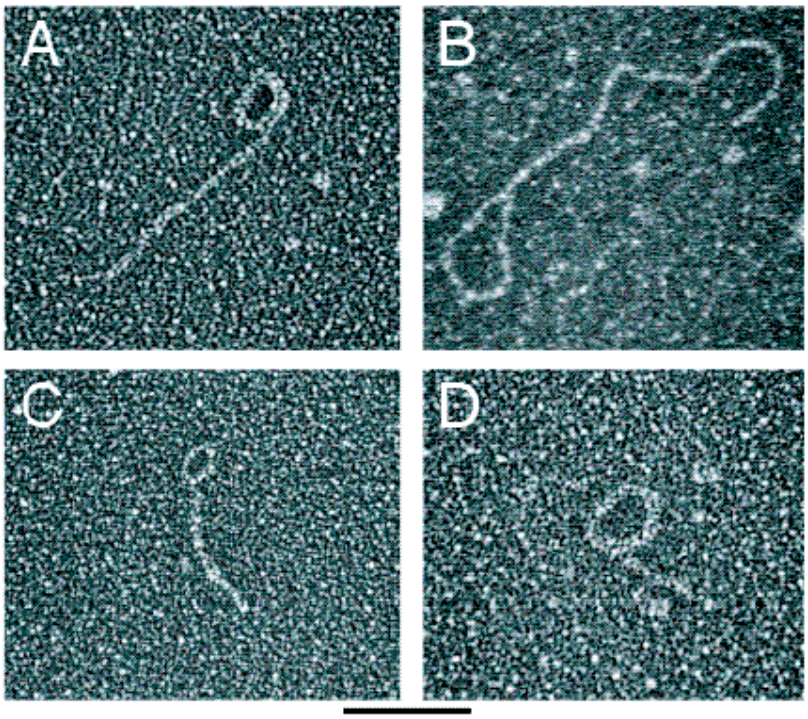
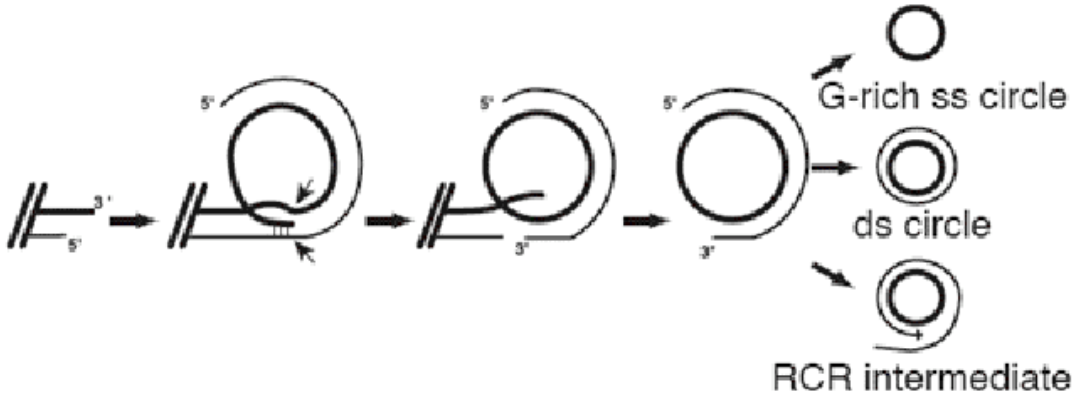

A
B
C
D

Figure2.6 Model of t-circle creation. Intramolecular strand invasion of the G-rich strand 3' telomeric tip and internal telomeric C-rich strand. Cutting of both telomeric strands and ligation of the G-rich strand creates a partially double stranded circular intermediate. This initial product could be degraded to leave a single G-rich stranded circle, filled in to create a double stranded circle, or used as a template for rolling circle replication (RCR).


5'
3'
5'
5'
3'
5'
3'
G-rich ss circle
ds circle
RCR intermediate

CHAPTER 3

IDENTIFICATION OF T-LOOPS ASSOCIATED WITH A TELOMERE CAPPING DEFECT IN *KLUVEROMYCES LACTIS*

INTRODUCTION

Telomeres are the repetitive sequences at the ends of linear chromosomes and are composed of short (5-8 bp) G-rich repeats. Telomeres employ specialized replication mechanisms that compensate for the incomplete nature of linear semi-conservative replication (Blackburn 1992). Telomeric DNA and its associated proteins cap chromosomal ends, hiding them from DNA repair pathways (Bailey and Goodwin 2004). Loss of a functional cap is associated with an increased rate of telomeric recombination, chromosome fusions and replicative senescence.

It has recently been shown that human single-stranded telomeric DNA is protected in a t-loop structure promoted by the protein TRF2 (Griffith et al. 1999a; Stansel et al. 2002). In t-loops the 3' overhang loops back and invades the more internal telomeric duplex. This action forms a local D-loop structure possibly stabilized by the binding of TRF2 (Stansel et al. 2001b). It is not clear if t-loop structure is the obligate form of a capped human telomere. However, evidence shows a link between loss of TRF2 association and increased chromosomal fusions (van Steensel et al. 1998b). This indicates that the ability at least to stabilize t-loops is an important part of human telomeric capping.

Telomerase is a specialized reverse transcriptase that extends the ends of telomeres in most eukaryotes. In humans somatic cells telomerase activity is very low or non-existent. This means that telomeres shorten with each replicative cycle. The shortening of telomeres acts a replicative clock, limiting the lifespan of human cells (Shay and Wright 2000). In yeast, telomerase is constitutively active and telomeres are maintained within narrow length margins despite replicative telomeric shortening. Deletion of telomerase or its accessory factors in yeast results in telomeric DNA shortening over successive generations, leading to senescence and cell death (Lundblad and Szostak 1989; McEachern and Blackburn 1996b; Lingner et al. 1997). Survival depends on maintaining the telomeric DNA through an alternative, homologous recombination (HR) based mechanism, here referred to as recombinational telomere elongation (RTE) (Lundblad 2002). In *Saccharomyces cerevisiae*, two types of survivors are characterized. Type I survivors have short telomeres and exhibit amplification of the subtelomeric Y' elements, while Type II survivors have long heterogeneous telomeric DNA (Lundblad and Blackburn 1993b; Teng and Zakian 1999b; Teng et al. 2000b; Chen et al. 2001b). Survivors in the related species, *Kluveromyces lactis* are exclusively Type II (McEachern and Blackburn 1996b).

Data coming from the yeast *K. lactis* led to the development of the roll-and-spread model of recombinational telomere elongation (Natarajan and McEachern 2002a). This model depicts a process with three major steps. First, the model predicts that a t-circle is created from a telomeric end and secondly, that this t-circle acts as template for rolling-circle-replication that lengthens a telomere. Finally, this initially elongated tract of telomeric repeats is then spread to all the telomeric ends through non-reciprocal

recombination events. In support of this model, telomeric circles (t-circles) have been shown to strongly promote RTE (Natarajan and McEachern 2002a; Natarajan et al. 2003a) and t-circles as small as 100 bp/nt can readily be formed by dysfunctional telomeres (Groff-Vindman et al. 2005). Additionally, spreading of sequence from one telomere to all other telomeres has been demonstrated to occur during RTE (Topcu et al. 2005). A roll-and-spread mechanism has also been proposed to explain post-senescence survivor formation in *S. cerevisiae* (Lin et al. 2005). The precursor to the creation of a t-circle is thought to be a structure known as a t-loop. T-loop creation involves a loop back of the telomeric 3' overhang that invades the more proximal telomeric duplex creating a D-loop-like structure at the point of 3' invasion.

A subset of human tumors and immortalized cells, termed alternative lengthening of telomeres (ALT), also utilize RTE to maintain their telomeric DNA in the absence of telomerase (Bryan et al. 1995b; Bryan et al. 1997b). The heterogeneous telomeric DNA length in ALT cells suggests a Type II-like phenotype. While complete chromosome uncapping leading to telomere fusions does not occur in human ALT cells, the telomeric DNA appears to be in a very plastic state. ALT telomeres are subject to telomere rapid deletion (TRD) and hyper recombination, despite no increase in overall genomic recombination (Murnane et al. 1994; Dunham et al. 2000b; Bechter et al. 2003; Bechter et al. 2004a; Bechter et al. 2004b; Londono-Vallejo 2004). ALT cell telomeres elongate through non-reciprocal homologous recombination events (Dunham et al. 2000a). ALT cell lines also produce large amounts of extrachromosomal telomeric repeats (ECTR) (Ogino et al. 1998; Tokutake et al. 1998). This DNA is sometimes associated with an ALT specific nuclear body called an APB body (Yeager et al. 1999b). APBs appear

coincident with the onset of ALT and are sites of DNA replication as well as harbors to multiple proteins involved in recombinational processes (Wu et al. 2003; Nabetani et al. 2004). Much of the extrachromosomal telomeric repeat (ECTR) DNA molecules in ALT exists as t-circles. (Cesare and Griffith 2004b; Wang et al. 2004). Exogenous expression in non-ALT human cells of a truncated TRF2 allele lacking the N-terminal basic domain ($TRF2^{\Delta B}$) results in telomere shortening dependent on functional HR pathways and t-circle formation (Wang et al. 2004). These data suggest that t-circles result from a resolution event at the t-loop junction, termed t-loop HR (Wang et al. 2004). Indirect evidence suggests this is also the mechanism of circle formation in human ALT cells, and is consistent with the general telomere instability in the RTE phenotype (Cesare and Griffith 2004b).

In *S. cerevisiae*, the telomeres form a fold back structure mediated by protein-protein interactions functioning to establish the telomere position effect (TPE) (de Bruin et al. 2000). It is unknown if strand invasion of the overhang into the duplex telomeric DNA occurs, thereby generating a t-loop. However, a *RAD52*-dependent telomere rapid deletion (TRD) mechanism in *S. cerevisiae* functioning to shorten overly-elongated telomeres is proposed to result from resolution of a t-loop, or t-loop like intermediate (Lustig 2003). This is very similar to $TRF2^{\Delta B}$ induced t-loop HR (Wang et al. 2004) and suggests that t-loops are able to form in *S. cerevisiae*, even if only transiently.

Mutant strains of *K. lactis* exhibiting the phenotypic hallmarks of telomere uncapping are generated by mutating the template region of the telomerase RNA subunit (*TER1*) corresponding to the telomeric Rap1p binding site (McEachern and Blackburn 1995; McEachern et al. 2000; Underwood et al. 2004b; Groff-Vindman et al. 2005)

(chapter 2, this dissertation). Specifically, many of these mutants contain telomeres with long single-stranded G-rich telomeric DNA believed due to an increased telomeric overhang length as well as large quantities of extrachromosomal telomeric repeats (ECTR) (Underwood et al. 2004b).

K. lactis provides an excellent model for an investigation of telomere structure in a budding yeast species. Previous work identified the *ter1-16T* mutant, which exhibits the phenotypic characteristics of telomere uncapping, including long heterogeneous telomeres, abundant single-stranded G-rich telomeric DNA, high rates of telomere recombination and t-circles (Underwood et al. 2004b; Groff-Vindman et al. 2005), (chapter 2, this dissertation). The *ter1-16T* phenotype appears due to diminished Rap1p binding at the telomere caused by a mutation of the telomeric DNA within the Rap1p binding site. Over-expression of *RAP1* in *ter1-16T* cells significantly shorten telomeres, presumably due to abundant Rap1p overcoming the decreased affinity for the mutant telomere sequence (Underwood et al. 2004b). Doubly mutated *ter1-16T rad52Δ* cells retain long telomeres, although ECTR DNA formation is inhibited (Underwood et al. 2004b; Groff-Vindman et al. 2005) (chapter 2, this dissertation).

Reported here is the first ever visualization of native yeast telomeres. By examining *ter1-16T* with and without exogenous *RAP1* over-expression, we have examined the native telomeres from *K. lactis* in an uncapped state and with the telomere phenotype repressed. Examination of telomeres from *ter1-16T rad52Δ* will allow us to determine what contribution HR plays in telomere structure, specifically in the hyper-recombinagenic uncapped state. In *ter1-16T*, we observe looped molecules of telomeric DNA with physical characteristics similar to previously described t-loops from

mammalian and plant cells. We also observe that *ter1-16T* cells contain t-circles ranging up to the size of an entire telomere, suggestive of t-loop HR. Telomeric DNA from *ter1-16T* in the presence of *RAP1* over-expression (hereon referred to as *ter1-16T RAP1*^{OE}) and *ter1-16T rad52Δ* contained significantly less t-loops and t-circles. These results suggest that t-loops in *ter1-16T* are largely or entirely the result of telomere capping dysfunction and that *RAP1* plays a regulatory role in maintaining proper telomere structure.

MATERIALS AND METHODS

Yeast strains

The telomerase mutant *ter1-16T* and the double mutant *ter1-16T rad52Δ,* previously described in Underwood et. al. 2004, were constructed in a His+ revertant of the 7B520 strain (Wray et al. 1987b).

Induction of *RAP1* over-expression

The telomerase mutant *ter1-16T,* was transformed with either pCXJ3 or pCXJ3+*RAP1*, which contains the *K. lactis RAP1* gene (a gift from Anat Krauskopf). The transformants were selected for on media lacking uracil. The strains were subsequently grown in liquid culture containing 125 μg G418 /ml YPD. It has previously been reported that this level of G418 results in greater than 100 copies of plasmid per cell (Chen 1996).

RNA Extraction and Northern Blot Analysis

RNA was extracted from a portion of the cells harvested for DNA isolation which received the same G418 treatments described above. RNA was extracted using the Qiagen RNA midi-prep kit (Qiagen, Valencia, CA). RNA was run on 1% agarose gels in 1X BPTE. Downward capillary transfer of the RNA onto Hybond N+ membrane was done in the presence of 6X SSC for 16 hours.

Psoralen crosslinking, isolation of genomic DNA, and size fractionation of telomere restriction fragments.

K. lactis cells were grown to OD_{600} of 15 (post-exponential phase). One litter of cells were spheroplasted and isolation of nuclei was performed as described previously (Kim et al. 2004) with the following modification, the lytic enzyme used was 100 μg/ml Zymolyase 100T (Seikagaku). Isolated nuclei were psoralen cross-linked as described previously (Griffith et al. 1999a). After crosslinking, nuclei were re-suspended in lysis buffer containing 0.5% SDS and 1mg/ml Proteinase K. The DNA was extracted by phenol/chloroform and precipitated with ethanol. Genomic DNA was digested with *Alu*I, *Hpa*II and *Nla*III (New England Biolabs, Beverly MA) at enzyme concentrations of 1U/μg for 2 hours, and supplemented with an equal amount of enzyme for an additional 2 hours. During the final hour of restriction enzyme digestion, RNaseA was added to 20 μg/ml for 1 hr at 37^{o}C. Digestions were checked on a 1% agarose gel, and if required more enzyme was added. The DNA was treated with 200 μg/ml of proteinase K for 1 hr at 55^{o}C in the presence of 0.5% SDS and 10 mM EDTA, then extracted twice with phenol and once with chloroform:isoamyl alcohol (24:1). Following ethanol

precipitation, the DNA was suspended in 0.5 mL of 10 mM Tris pH 7.6, 0.1 mM EDTA, 0.1% SDS for size fractionation.

Telomere restriction fragments were separated by gel filtration chromatography using the same apparatus and protocol as described previously (chapter 2, materials and methods). Slot blot analysis of column elution fractions was done as described previously (chapter 2, materials and methods) using a [γ-^{32}P] radiolabeled *K. lactis* C-strand telomere probe (chapter 3, materials and methods).

Electron microscopy

Samples were prepared for EM by surface spreading on a denatured protein film. A 50 μl aliquot of DNA in EM TE was mixed with ammonium acetate (pH 7.9) to a final concentration of 0.25 M. Cytochrome C (Sigma Inc.) was added to 4 μg/ml and the drop placed on Parafilm for 90 s. A parlodion-covered EM grid was touched to the drop and then dehydrated through washes of 75 and 90% ethanol followed by air drying and rotary shadowcasting with platinum–paladium (80:20). Analysis was carried out on a Philips CM12 instrument. Images were captured on sheet film and a Gatan multiscan 794 digital camera (Pleasanton, CA). Molecule dimensions were determined using Gatan Digital Micrograph software. Images for publication were captured by ACT-1 software (Nikon, Tokyo, Japan) utilizing a Nikon SMZ1000 stereoscope. Image contrast was adjusted by ADOBE PHOTOSHOP (San Jose, CA). In the experiments where DNA from the elution column was subsequently digested with *Rsa*I or *Mse*I (NEB), the sample was brought to 0.1 μg/ml of SSB in 1x NEB buffer 1 and incubated on ice for 30 min, then treated with 0.5 units of either restriction enzyme for 15 min at 37°C. The reaction was quenched with

EDTA to a final concentration of 5 mM, passed through a G50 sephadex (Pharmacia, New York, NY) spin column, and prepared for EM by surface spreading.

In the SSB staining experiments, DNA from the eluted fractions was incubated with 0.04 μg/ml of E. coli SSB on ice for 30 min in a reaction containing 20 mM Hepes pH 7.6, 50 mM NaCl. The DNA was prepared for EM by direct mounting onto thin carbon coated grids as described previously (chapter 2, materials and methods)(Groff-Vindman et al. 2005).

Pulsed field gel electrophoresis

Telomere restriction fragments were separated by standard PFGE using a Bio-Rad CHEF DR-III apparatus (Bio-Rad, Hercules, CA) on a 1.0% Pulsed Field Certified (BioRad) agarose gel in 0.5X TBE at 4°C with the following settings; 1.0 s initial switch time, 6.0 s final switch time, 6.0 volts/cm, 120° included angle, 9 hour duration. Following electrophoresis the gel was stained with ethidium bromide, de-stained and visualized with UV light.

Two dimensional gel electrophoresis

Telomere restriction fragments were separated in the first dimension in a 0.6% Gold agarose (ISC Bio Express, Kaysville, UT) gel in 0.5X TBE (44.5 mM Tris Base, 44.5 mM boric acid, 1 mM EDTA) at 1V/cm for 13.5 hours at RT. The gel was stained with ethidium bromide in 0.5X TBE, de-stained in 0.5X TBE, visualized by UV light and the appropriate lanes excised. The excised slab was arranged in a gel casting tray such that it will be perpendicular to the electrical current. A 1.1% agarose (Invitrogen, Carlsbad, CA)

gel in 0.5X TBE containing 300 ng/ml ethidium bromide was poured around the excised slab. Second dimension electrophoresis was carried out at RT in 0.5X TBE containing 300 ng/ml ethidium bromide at 6V/cm for 3 hours. Total DNA was visualized using UV light.

In-gel hybridization

Both standard and 2D PFGE agarose gels were denatured for 45 min (soaked in 1.5M NaCl, 0.5 N NaOH), neutralized for 25 min (Soaked in 3M NaCl, 0.63M Tris pH 7.4), dried and pre-hybridized in Church's Mix (0.5M Na_2HPO_4 pH 7.2, 1 mM EDTA, 7% SDS, 1%Bovine Serum Albumin) at 55°C for one hour. The gels were hybridized with a [γ-^{32}P] labeled *K. lactis* C-strand telomeric probe (chapter 2, materials and methods) (Groff-Vindman et al. 2005). Gels were subsequently washed three times, for 30 min at room temp in 4x SSC (1x SSC is 0.15M NaCl, 0.015 M sodium citrate), then once in 4X SSC, 0.1% SDS for 30 min at 55°C. The signal was visualized using a Storm 840 Phosphorimager (Molecular Dynamics, Piscataway, NJ). In the case of figure 3.1 gels were hybridized to [γ-^{32}P] labeled *K. lactis* C-strand telomere probe in the presence of 10X SSC at 30° for 16 hours. The gels were washed twice for 1.5 hours at 30° in 0.25 X SSC.

RESULTS

Telomeres of *ter1-16T* have a capping defect that is independent of *RAD52,* and that is rescued by over-expression of *RAP1*

Genomic DNA was harvested from *ter1-16T, ter1-16T rad52Δ,* and *ter1-16T* $RAP1^{OE}$ cells using a procedure previously shown to efficiently retain DNA of both large and small molecular weights (Underwood et al. 2004a; Groff-Vindman et al. 2005). The uncut genomic DNA from was tested for the presence an extended G-rich overhang using in-gel hybridization techniques previously described in Underwood et. al.. The resulting Southern blot showed the presence of single-stranded G-rich telomeric DNA ranging from limit mobility to below 500 bp in length in the *ter1-16T* mutant consistent with earlier results (Fig. 2.2).

The double mutant *ter1-16T rad52Δ* also contains the large single-stranded signal indicative of elongated 3' telomeric overhangs but largely lacks small single-stranded material (Fig. 3.1). This is consistent with the almost total loss of small t-circle production in this background that has been previously published (Groff-Vindman et al. 2005) (chapter 2 of this dissertation). These data support the previously reported requirement for *RAD52* in the appearance of *ter1-Δ* survivors (Natarajan and McEachern 2002).

A plasmid, pCXJ13+*RAP1*, containing the *RAP1* gene as well as a G418 selectable marker was transformed into *ter1-16T* cells. It was previously shown that over-expression of pCXJ13+*RAP1* decreases overall telomere length in *ter1-16T* (Underwood et al. 2004a). This plasmid is maintained at 100+ copies per cell when in the presence of high levels of G418. Over-expression of the *RAP1* gene was confirmed by northern blot

analysis (data not shown). In the presence of *RAP1* over-expression the *ter1-16T* mutant has an almost complete reduction in large single-stranded G-rich signal (Fig. 3.1). The over-expression of *RAP1* also abolishes the single-stranded signal running at low molecular weight (Fig. 3.1) as well as reducing overall telomere length (Underwood et al. 2004a). These data suggest that the over-expression of *RAP1* results in a more capped state at the telomeres which both reduces total telomere length, single-stranded overhang length, and the production of t-circles.

ter1-16T cells contain telomeres long enough to be separated from genomic DNA by gel filtration

Previous efforts to visualize yeast telomeres have been complicated by their short length. In the past, enrichment of telomere restriction fragments (TRFs) by gel filtration chromatography proved useful in mammalian and plant cells (Griffith et al. 1999a; Cesare et al. 2003b; Cesare and Griffith 2004b). Wild type *K. lactis* cells possess telomeres of 300-600 bp, which are not long enough to separate from the genomic restriction fragments by gel filtration. As mentioned above, the mutant *ter1-16T* belongs to a class of telomerase mutants, which exhibits extreme telomere elongation. To assure that the telomeres in *ter1-16T* and related mutants were of sufficient length for gel filtration, we measured the telomeres by separating uncut and *Alu*I/*Hpa*II/*Nla*III (A/H/N) digested DNA by pulsed field gel electrophoresis (PFGE) and hybridizing in-gel with a telomere specific probe (Fig. 3.2A).

In the A/H/N digested *ter1-16T* sample, the bulk of telomere signal migrated from approximately 30 kb to 2.5 kb (Fig. 3.2A, left panel, lane 2). This lane also contained

telomeric smears extending to appreciably larger and smaller size. The *ter1-16T* mutant is also known to produce abundant small extrachromosomal telomeric DNA (ECTR) much of which exists in the form of t-circles (Underwood et al. 2004a; Groff-Vindman et al. 2005). However, due to rigorous processes of DNA extraction and purification much of the ECTR signal is lost from the *ter1-16T* sample. Therefore it is unclear how much of the signal at lower molecular weights represents telomeric ends and how much represent ECTR. The double mutant *ter1-16T rad52Δ* also had similarly long telomeres (Fig. 3.3A, left panel, lane 4). In the case of *ter1-16T RAP1*^{OE}, telomeric fragments were substantially shorter with the bulk of signal falling between 5 kb and 1.75 kb (Fig. 3.3A, left panel, lane 6). This shortening was not as great as that observed previously for the same cells (Underwood et al. 2004a) because the cells were grown under *RAP1* over-expression conditions for fewer cell divisions. It should be noted that the A/H/N digestion cuts within the subtelomeric regions leaving an average of 370 bp (with an estimated range of 145-450 bp) of subtelomeric DNA on the telomere restriction fragments (TRFs). The telomere lengths in *ter1-16T* , *ter1-16T rad52Δ* and *ter1-16T RAP*^{OE} were similar in size to the telomeres from human cells successfully used in previous telomere enrichments by gel filtration (Griffith et al. 1999a) and therefore they were judged to be suitable for EM analysis.

Efficient observation of t-loop structures by EM is dependent upon first psoralen photo-crosslinking the nuclei in situ to establish covalent interaction between the invading overhang and the duplex telomeric DNA (Griffith et al. 1999b). Crude nuclei from *K. lactis* were isolated and psoralen photo-crosslinked as in previous experiments using mammalian and plant cells (Materials and Methods) (Griffith et al. 1999b; Cesare

et al. 2003b; Cesare and Griffith 2004b). Following crosslinking the DNA was isolated and reduced to TRFs by treatment with A/H/N. We selected these enzymes specifically due to their lack of non-specific ss nuclease activity that may cleave ss DNA at the t-loop junction (personal communication, New England Biolabs, technical support). Greater than 0.5 mg of A/H/N digested DNA from psoralen crosslinked, or non-crosslinked samples from each of the *K. lactis* strains were passed through a long gel-filtration chromatography column. Elution profiles of DNA concentration and specific telomere content indicated that for all preparations, the telomeric DNA preceded the bulk genomic DNA, evidence of sufficient telomere enrichment (Fig. 3.2B).

Visualization of telomere restriction fragments

DNA from the high molecular weight (HMW), telomere enriched fractions were prepared for electron microscopy by surface spreading on a denatured protein film (Kleinschmidt preparation). In this method, the DNA is coated with denatured cytochrome c thickening the nucleic acid molecule approximately 10 fold and aiding in inhibiting false positive structures. For consistency, we quantitated fractions up to and including the peak of the telomere signal. Higher numbered fractions contained many small DNA molecules that are likely not of telomeric origin.

Sizes of DNA molecules in the highest molecular weight fractions from *ter1-16T* range, up to 30 kb, with an average estimated size of between 5 and 10 kb. Fractions used for EM analysis were just before and up to the peak of telomeric signal. These fractions, numbers 29 and 30, contained an average estimated DNA size near 4 kb. In the *ter1-16T* $RAP1^{OE}$ samples, the average overall DNA size was shorter, consistent with telomere

length. As with previous experiments (Griffith et al. 1999a; Cesare et al. 2003a; Cesare and Griffith 2004a), the most common structural form observed in the HMW preparations from all three strains were long linear DNA molecules (Table 1). Linear DNA molecules are consistent with telomeres that were either not arranged into a t-loop, or telomeres where the strand invasions were not maintained during the enrichment procedure (Griffith et al. 1999a).

Looped molecules in the psoralen crosslinked, telomere enriched fractions from *ter1-16T*

The HMW crosslinked fractions from *ter1-16T* contained looped molecules consistent with the previously characterized t-loop structure, described as a ds DNA circle with a connected ds DNA tail (Table 1). Preparations from the non-crosslinked *ter1-16T* sample had a 4-fold reduction in the percentage of looped molecules compared to the crosslinked sample, indicating a psoralen contribution to the stabilization of the loop structure (Fig. 3.4A and Table 1). This is consistent with previous examination of telomere structure in mammalian and plant cells (Griffith et al. 1999a; Cesare et al. 2003b). The crosslinked samples from *ter1-16T RAP1*^{OE} and *ter1-16T rad52Δ* had significantly less looped molecules, 3.7 and 7.7 fold respectively, compared to the crosslinked preparation of *ter1-16T* (Fig. 3.4 A, Table 1). In addition, there was no significant difference regarding the abundance of looped molecules when comparing the crosslinked versus non-crosslinked preparations in *ter1-16T RAP1*^{OE} or *ter1-16T rad52Δ* (Fig. 3.4A, Table 1). We note that the total abundance of looped molecules observed here was lower than in previous studies of telomeres from other organisms.

The total size of the looped molecules (measurement of the loop + tail contour) in *ter1-16T* both with and without psoralen crosslinking ranged from 0.7 to 27.2 kb, with a mean size of 6.1 and a median of 3.6 kb. Roughly half of the looped molecules were less than 3 kb (48%, total n = 94) with the other half represented in a distribution of molecules ranging from 3 to 30 kb (52% of molecules) (Fig.3.4B). The distribution of looped molecules, ranging from 3-30 kb in *ter1-16T*, correlates well with the chromosomal telomere signal in the PFGE measurements of telomere length (Fig. 1A). The small loops are consistent with the LMW (low molecular weight) ECTR DNA, or possibly the shortest chromosomal telomeres in *ter1-16T.* A lower percentage of looped molecules were observed in *ter1-16T RAP1*^{OE}. However, analysis of a large total number of molecules gathered enough measurements of looped molecules to analyze the distribution (Fig. 3.4B). Consistent with the shortened telomeres, the vast majority of looped molecules in *ter1-16T RAP1*^{OE} were small (total size loop + tail), with 96% less than 3 kb (total measured = 91, size range 0.5 to 5.6 kb, mean = 1.51 kb, median = 1.08 kb, one outlying molecule measured at 20.1 kb). Not enough looped molecules were measured in *ter1-16T rad52Δ* for accurate size quantitation.

Looped molecules from *ter1-16T* are composed of telomeric DNA and are structurally similar to t-loops isolated from mammalian cells

The 25 bp telomeric repeat in *K. lactis* contains a single *Rsa*I digestion site. Digestion with *Rsa*I therefore reduces *K. lactis* telomeric DNA to 25 bp fragments, below the resolution of EM. *Mse*I cleaves a 4-bp site that is not present in the *K. lactis* telomere sequence. Telomere-enriched HMW, fractions from crosslinked *ter1-16T* were digested

with either *Rsa*I or *Mse*I then examined by EM for the presence of looped molecules (Fig. 3.4C). Incubation of multiple fractions with *Mse*I resulted in an increase in the number of looped molecules observed compared to the initial observation after A/H/N digestion in all cases. In one fraction, digestion with *Mse*I resulted in the number of looped molecules increasing to 14%, close to the number of looped molecules observed in some HMW crosslinked fractions from human ALT experiments. We believe the increase in the number of looped molecules reflects the digestion of non-telomeric DNA in these particular fractions in the presence of *Mse*I.

In contrast, digestion of the same fractions with *Rsa*I resulted in the complete disappearance of looped molecules and the loss of the majority of general DNA content. All EM grids prepared from the *Rsa*I digested telomere enriched fractions had a high background; likely due to an abundance of 25 bp telomeric repeat monomers on the grid surface. A plasmid containing two *Rsa*I digestion sites was digested simultaneously with *Rsa*I. This control resulted in the generation of linear fragments of the expected sizes (data not shown) indicating that the lack of DNA seen in the *Rsa*I digested sample was not due to a non-specific nuclease activity. The resistance of looped molecules to *Mse*I, and their sensitivity to *Rsa*I strongly suggests that the looped molecules are composed of telomeric DNA.

A prediction of t-loop structure indicates that a segment of ss DNA should be present at the t-loop junction, due to strand displacement within the displacement loop (D-loop). Consistent with this, *E. coli* single-stranded DNA binding protein (SSB) localized to the loop junction of 35% of t-loops isolated from human and mouse cells (Griffith et al. 1999a). In a similar experiment we treated the HMW DNA from

crosslinked *ter1-16T* with the T4 gene 32 SSB. This SSB protein localized to greater than 95% of loop junctions of looped molecules from *ter1-16T* (Fig. 3.5A-C). Together with the dependence for psoralen crosslinking to maintain looped structure, the presence of SSB at the loop junction suggests strand invasion mediated looping in *ter1-16T*. The SSB interaction at the loop junction was independent of the size of the looped molecule. However, the amount of the SSB at the t-loop junction varied between a single SSB monomer to large protein amounts, indicating probable variability in the amount of ss overhang invasion (compare Fig. 3.5A,B to C). Unlike previous experiments examining gel purified ECTR DNA in the presence of T4 Gene product 32 (Iyer, S, Cesare, A and McEachern, M. unpublished data), in these experiments SSB binding is exclusively at the junction, not on the tail immediately adjacent to the junction. This difference is significant as it suggests a displacement loop that has single-stranded DNA at the loop junction versus rolling-circle-replication where single-strand DNA is located in the tail.

Greater than 90% of linear molecules also stained with SSB and only on a single end, consistent with SSB binding the 3' telomeric overhang (Fig. 3.5D, E). 1D in-gel hybridization has shown that *ter1-16T* contains an increased amount of single-stranded G-rich telomeric DNA (Fig. 3.1) (Underwood et al. 2004a), likely due to an increased overhang length. Consistent with this, some of the linear molecules were stained extensively at one terminus indicative of a very long overhang (Fig. 3.5E). However, most linear molecules localized minimal amounts of SSB to a single end of the molecule, consistent with the short overhangs believed to occur naturally in budding yeast (Fig. 3.5 D) While we did not observe enough molecules to properly quantitate these experiments,

the significant ss DNA observed on some molecules is consistent with the telomere uncapping phenotype of *ter1-16T*.

Telomere circles in *ter1-16T*

The initial hypothesis that led us to investigate for t-circles in *ter1-16T* was genetic data suggesting that RTE in *ter1Δ* survivors is dependent on rolling-circle-replication initiated on circular telomeric DNA templates as small as 100 bp (Natarajan et al. 2002; Natarajan et al. 2003). Examination of gel-isolated LMW DNA from *ter1-16T* and the *stn1-M1* mutant indicated that t-circles were abundant, and that they were commonly small, as small as 100bp/nt (Groff-Vindman et al. 2005) (chapter 2 this dissertation and S. Iyer and McEachern unpublished data). We therefore expected to see small t-circles and perhaps large t-circles in the experiments described here.

In the HMW telomere enriched fractions from *ter1-16T*, we observed a large number of t-circles ranging in size from 0.5 to 19.3 kb (Fig. 3.7 C and Table 1). The majority of circles were small; with 84% of the total measured circles from the crosslinked and non-crosslinked fractions less than 3 kb in total length (total n = 102). DNA circles in the psoralen crosslinked preparations from *ter1-16T* cells were 6.0 and 8.3 fold more prevalent than in *ter1-16T RAP1* and *ter1-16T rad52Δ* respectively. In the *ter1-16T* $RAP1^{OE}$ sample, 96% of measured circles were smaller than 3 kb (total n = 83, crosslinked and non-crosslinked fractions). We did not measure enough circles from *ter1-16T rad52Δ* for a statistically significant assessment of their size range. Somewhat surprising was that circle abundance in *ter1-16T* as assayed by EM was enriched by psoralen crosslinking (Table 1). This might indicate that some of the circles are not

covalently closed, but instead associated by homologous base pairing that is stabilized by psoralen crosslinking.

To determine if the circular material contained telomeric DNA sequence we employed neutral-neutral standard 2D gel electrophoresis (Fig. 3.8) to separate A/H/N digested TRFs from the *ter1-16T* strains (Fig. 7). In TRFs from *ter1-16T*, a strong arc of telomeric material running consistent with circular DNA was observed in both the crosslinked and non-crosslinked samples, suggesting that the DNA circles observed by EM were t-circles. Equivalent circle arcs were not observed in any of the psoralen crosslinked or non-crosslinked samples from the *ter1-16T RAP1*^{OE} or *ter1-16T rad52Δ*. Resolution of standard 2D gels is limited to circles of ≥ 2 kb. The EM observations indicate that DNA circles in *ter1-16T RAP1*^{OE} were generally below this resolution. The small number of t-circles within the resolution limits of the 2D gels was probably to few to be detected by Southern hybridization. Therefore we separated uncut DNA from *ter1-16T RAP1*^{OE} by 4% chloroquine 2D gels and hybridized with a telomere specific probe, similar to previous experiments identifying small t-circles in *ter1-16T* (chapter 2, this dissertation). In these experiments, a small amount of ECTR DNA consistent with t-circles was observed, though an exposure 3x longer than with *ter1-16T* was required to see even a faint signal (data not shown). The significant decrease in circles in *ter1-16T RAP1*^{OE} is in agreement with the EM results and previous experiments suggesting an effective but not complete suppression of telomere length defect by *RAP1* overexpression in *ter1-16T* cells (Underwood et al. 2004b). 4% 2D agarose gels containing high levels of chloroquine has previously shown that small t-circles were greatly reduced or absent in *ter1-16T rad52Δ* cells.

In the previously described *Mse*I and *Rsa*I digestion experiments, circular molecules were also quantitated (Fig. 3.7B). *Rsa*I digestion of HMW crosslinked fractions resulted in a complete disappearance of circles in one fraction, and an 88% reduction in another, consistent with the circles containing telomeric DNA. Surprisingly, *Mse*I digestion also resulted in a slight reduction in circles. We can not rule out the possibility that some circles are partly or entirely non-telomeric.

We observed small single-stranded DNA circles migrating ahead of a 500 bp linear marker in previous experiments examining gel purified LMW ECTR molecules from *ter1-16T* (Groff-Vindman et al. 2005) chapter 2, this dissertation). In the SSB binding experiments described above, we did not observe any small t-circles (less than 1 kb) in the HMW fractions from *ter1-16T*. Likely, this is because the small single-strand DNA circles are present in the LMW fractions. Consistent with this, a small peak of telomeric material trailing the original telomere peak is present in the gel elution profile of *ter1-16T* (fraction 40, Fig. 3.2B). We incubated DNA from this fraction with SSB and examined it by EM, however, the overwhelming amount of small linear ds DNA molecules prevented identification of small single-stranded circles. We did not observe circles containing both ds and ss DNA in any fractions.

The observation of t-loops and large t-circles in *ter1-16T* is consistent with a mechanism of circle formation by t-loop HR/TRD. Inhibition of HR in *ter1-16T rad52Δ* drastically reduces circle formation, consistent with the requirement for HR in both TRD and t-loop HR (Fig. 3.7A, 3.8 and Table 1). Given the mechanism of t-loop HR, we expect a correlation in size between the loop portion of t-loops, and t-circles. As observed previously in human ALT cells (Cesare and Griffith 2004a), the sizes of t-circles and the

loop portion of t-loops from *ter1-16T* are correlated (Fig. 3.6C). There is some discrepancy at the lowest sizes though this is likely due to experimental design. Enrichment by gel-filtration will result in more HMW DNA molecules. Therefore, the smallest circles would not be expected to appear as often, which is reflected in the distribution.

DISCUSSION

Presented here is the first report to our knowledge describing EM observations of native yeast telomeric DNA. In this study, nuclei from *K. lactis ter1-16T*, *ter1-16T* $RAP1^{OE}$ and *ter1-16T rad52Δ* strains were psoralen crosslinked in situ, the genomic DNA digested, and TRFs enriched for by gel filtration. We observed both t-circles and looped structures in the HWM psoralen crosslinked fractions from the *ter1-16T*. Over-expression of *RAP1,* which suppresses the telomere length defect *ter1-16T* cells, also significantly diminished both t-circle and t-loop occurrence. T-loops and t-circles were also significantly diminished in a *ter1-16T rad52Δ* double mutant, implicating HR in t-loop and t-circle formation in *K. lactis* telomere mutants. The results here support t-loops and t-circles being associated with the RTE phenotype in budding yeast, and also support a regulatory role for *RAP1* in budding yeast telomere structure.

T-loops consist of a 3' strand invasion duplex DNA which appears to be similar to a D-loop structure. This structure, originally described in human cells, seems to represent a structure very similar to a normal recombination intermediate. In order for this structure to be capped, stabilization of the D-loop must occur. While this has not been proven, it has been shown that the $TRF2^{\Delta B}$ mutation leads to production of t-circles which requires

a gene, XRCC2, known to be involved in Holliday junction resolution (Wang RC. 2004). It is possible that TRF2 stabilizes the t-loop after D-loop formation and that this is a structure necessary for capping of human telomeres. In contrast, it may be that in wild type *K. lactis* cells telomeres are prevented from making a t-loop structure through the action of Rap1p. Prevention of telomeric recombination and other aspects of telomeric capping might be independent of t-loop formation in some species.

Four lines of evidence suggest the looped molecules observed here are bona fide t-loops, analogous to the higher order structure observed previously at the telomere in several eukaryotic species (Griffith et al. 1999a; Murti and Prescott 1999a; Munoz-Jordan et al. 2001b; Cesare et al. 2003b; Nikitina and Woodcock 2004b). First, the total length of the looped molecules observed by EM in the HMW fractions correlates with the telomere lengths in the strain of DNA origin. Second, the looped molecules in *ter1-16T* are sensitive to *Rsa*I and resistant to *Mse*I, consistent with the *K. lactis* telomeric DNA sequence. Third, the looped molecules in *ter1-16T* are significantly more abundant in the psoralen crosslinked fractions, accordant with previous observations indicating a requirement for psoralen crosslinking to maintain the t-loop structure (Griffith et al. 1999a). Fourth, SSB localizes to the loop junction of looped molecules in *ter1-16T* accordant with previous observations in mammalian cells, indicating single-stranded DNA at the loop junction due to presence of a D-loop (Griffith et al. 1999a). Several lines of evidence also suggest the circular molecules observed in these studies by EM contain telomeric DNA. Like the looped molecules, circles in *ter1-16T* are sensitive to *Rsa*I and resistant to *Mse*I as expected for *K. lactis* telomere sequence. In addition, 2D gel electrophoresis of TRFs derived from *ter1-16T* revealed an arc of telomeric signal

migrating in a pattern consistent to double-stranded DNA circles. Therefore, we conclude the looped and circular molecules observed here are t-loops and t-circles.

Evidence from human cell culture experiments expressing the TRF2$^{\Delta B}$ allele and analysis of telomeric DNA in human ALT cells supports a mechanism of t-circle formation mediated by improper resolution at the t-loop junction by HR enzymes, termed t-loop HR (Cesare and Griffith 2004b; Wang et al. 2004). This mechanism is similar to the proposed *RAD52*-dependent TRD mechanism that can shorten overly elongated telomeres in *S. cerevisiae* (Lustig 2003). Data presented here further supports the idea that circle formation occurs in a t-loop HR/TRD dependent manner. Specifically, t-circle formation in *ter1-16T* like TRD in *S. cerevisiae* is largely *RAD52* dependent. In addition, the size correlation between t-circles and the loop portion of t-loops in *ter1-16T* is consistent with t-loops being a precursor to t-circles.

Looped molecules in *ter-16T* ranged in total size from < 1 kb to near 30 kb. Previous experiments indicated that the heterogeneous ECTR DNA in *ter1-16T* exists in both circular and linear conformation (Groff-Vindman et al. 2005), (chapter 2 this dissertation). Therefore, we expect that some of the looped molecules observed here are extrachromosomal in origin. This might be especially likely for those looped molecules in *ter1-16T* less than 3 kb, which are smaller than the bulk of the nuclear telomeres as measured by PFGE. While these small looped molecules may not represent telomeric ends removed through digestion, we see no reason why linear ECTR DNA will not be acted on in a similar manner to chromosomal telomeres, and suggest that ECTR t-loops are analogous in structure to chromosomal t-loops.

A linear extrachromosomal piece of telomeric DNA contains both a C-rich 3' end as well as a G-rich 3' end. It is possible that the C-rich 3' end invades the duplex telomeric DNA and these structures are visualized as looped structures. While we can not rule this possibility out, some previously published data argues against it. In the analysis of t-circles created by *ter1-16T* only single-stranded G-rich t-circles are found (Groff-Vindman et al. 2005). These circles are likely created from telomeric ends by resolution of an invasion of G-rich single-stranded DNA. Resolution of this type of t-loop structure would not give rise to C-rich single-stranded t-circles. If it was common for ECTR to form C-rich 3' strand invasions the resolution of those structures should at some frequency result in C-rich single-stranded t-circles. Because C-rich t-circles have not been detected in any analysis of *ter1-16T* DNA, we conclude that C-rich strand invasions are not the structure visualized in this study.

The roll-and-spread model of RTE predicts rolling-circle-replication on a t-circle template leads to the formation of a significantly elongated telomere (Natarajan and McEachern 2002b; Natarajan et al. 2003b; Tomaska et al. 2004b). Due to the similarity of rolling-circle-replication intermediates and t-loops as assayed by EM (Skaliter et al. 1996; Griffith et al. 1999a; Nosek et al. 2005), we cannot exclude that some of the looped molecules here are rolling-circle-replication intermediates. Specifically, looped molecules present in the non-crosslinked samples might represent rolling-circle-replication intermediates as they do not require psoralen for stability. Interestingly, a higher percentage of t-loops in crosslinked versus noncrosslinked *ter1-16T* were larger than 3 kb. This may be a clue that some of these smaller and more infrequent looped structures are indeed rolling-circle-replication intermediates.

In humans, TRF2 functions in chromosome end-capping (van Steensel et al. 1998a; Smogorzewska et al. 2002; Celli and de Lange 2005) and likely plays a role in t-loop formation and maintenance (Griffith et al. 1999a; Stansel et al. 2001a). In budding yeast, no hTRF2 ortholog is present at the telomere (Li et al. 2000; Smogorzewska and de Lange 2004b), indicating that t-loops may not be the wild type budding yeast telomere conformation. Instead, *S. cerevisiae* telomeres appear to terminate in a fold back structure, mediated by Rap1p and its binding partners Sir3p and Sir4p, establishing telomere position effect (TPE) (de Bruin et al. 2000). It is unknown if the 3' overhang invades the duplex telomeric DNA in this structure generating a t-loop.

While our data suggest t-loops are associated with telomere capping defects in budding yeast, we cannot definitively exclude their absence in the wild type state. In wild type *S. cerevisiae*, overhang lengths are 12-14 nt outside of S phase and longer during S-phase (Wellinger et al. 1993; Larrivee et al. 2004). Presumably, wild type *K. lactis* overhang lengths are of similar size. Uncapping in *ter1-16T* due to a lack of Rap1p at the telomere, results in greatly increased single-stranded G-rich telomeric DNA (Underwood et al. 2004b) (Fig.3.1). Overexpression of *RAP1* reduces overhang length to closer to wild type size. Psoralen crosslinking of the strand invasion resulting from a 14 nt overhang will presumably be very inefficient and therefore few loops would be detected experimentally in *ter1-16T RAP1*^{OE}, consistent with our observations. In contrast, the much longer overhangs of *ter1-16T* likely lead to more strand invasion, and presumably greater crosslinking efficiency. Therefore, more looped molecules would be detected in *ter1-16T,* consistent with our observations. Accordant with increased overhang lengths and greater strand invasion, we did see looped molecules with variable amounts of SSB

at the loop junction in *ter1-16T*. Increased strand invasion likely renders the telomeres more susceptible to HR and t-circle generation, suggesting an unstable t-loop structure in *ter1-16T*. The data presented here may suggest that in budding yeast, the t-loop is not a protective, but rather a recombinogenic structure. In this scenario, t-loops in yeast represent a functionally uncapped state, opposite of their ascribed role in mammalian cells.

It is interesting that a small number of t-loops are found in the absence of functional *RAD52. RAD52* is not required for length maintenance in wild type *K. lactis* or for the extreme telomere elongation seen in *ter1-16T* cells (Underwood et al. 2004a). The t-loop structures seen in *ter1-16T rad52Δ* likely do not represent recombination intermediates. Perhaps these few molecules represent the low level of non-obligate but protective t-loops that normally occur in *K. lactis* cells. It is also possible that t-loops form at wild type *K. lactis* telomeres at some frequency and are blocked from initiating recombination. This would mean that t-loops in wild type *K. lactis* were not required for capping yet still represented a capped structure. It has been shown that human telomeres isolated from HeLa cell lines varied from 15-40%. The same study incubating human telomeric DNA with TRF2 protein showed that less than 20% of the molecules were folded into t-loops (Griffith et al. 1999). This evidence suggests that human telomeres may not always occur in a t-loop arrangement and it is likely that there is some flux between a linear and t-loop conformation (Griffith et al. 1999a; de Lange 2005).

Regardless of whether telomeres are arranged into a t-loop structure in wild type *K. lactis,* the presumably diminished Rap1p binding at the telomere in *ter1-16T* undoubtedly leads to a more recombinogenic telomere state. This is supported by past

observation of increased subtelomeric recombination and increased telomeric turnover in related *ter1* mutants that cause *RAP1* binding defects (McEachern et al. 2002a; Underwood et al. 2004a). It is further supported by observations here of increased *RAD52*-dependent inter-telomeric recombination and t-circle formation in *ter1-16T*. Uncapping of the telomere due to diminished Rap1p binding therefore promotes a telomeric state whereby t-circles are readily generated, a requisite step in the roll-and-spread mechanism (Natarajan and McEachern 2002b; Tomaska et al. 2004b; Topcu et al. 2005). In *K. lactis ter1Δ* survivors, Rap1p is functional, and the telomeres become highly recombinogenic only when they reach very short lengths (McEachern and Iyer 2001b; Topcu et al. 2005). T-circles in senescing *ter1Δ* cells would thus be small, as t-loop HR would only occur at telomeres composed of a small number of repeats. This is consistent with the repeating patterns of telomere sequence in the elongated telomeres of *ter1Δ* survivors being ≈100 bp (Natarajan and McEachern 2002b). In *ter1-16T*, Rap1p is not adequately bound to the telomere leading to a perpetual capping defect and frequent telomere recombination. T-circles of various sizes are thus produced frequently from t-loop HR in the absence of adequate Rap1p binding at the telomere. The similarity of observations here to observations of telomeric DNA from human ALT cells suggests analogous telomere instability in the human RTE phenotype (Cesare and Griffith 2004b). Perhaps the t-loops in ALT cells are indicative of a capping defect in which the t-loop is frequently resolved into t-circles much like what occurs at *ter1-16T* telomeric ends.

RAP1 therefore appears to have a functional role in telomere capping and stability in *K. lactis*. Similar capping defects and a tendency toward RTE occur in *S. cerevisiae* with mutation in *CDC13* (Grandin et al. 2001a) and in *K. lactis* with mutation

in *STN1* (Iyer et al. 2005), two members of the Cdc13p-Stn1p-Ten1p telomere capping complex. RTE in yeast therefore appears to be linked to a type of telomere uncapping that is different from the gross chromosome uncapping in mammalian cells leading to telomere fusions. Exactly how telomere uncapping leading to either telomere fusions or RTE remains to be answered.

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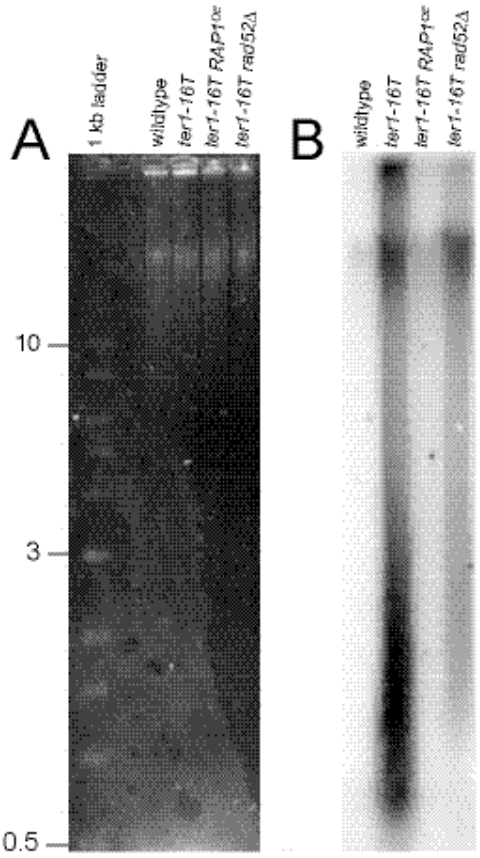
Figure 3.1 In-gel hybridization of undigested genomic DNA from wild type *K. lactis*, *ter1-16T*, *ter1-16T RAP1*^{OE}, and *ter1-16T rad52Δ*. A) A 0.8% gel stained with ethidium bromide shows the quantity of DNA loaded in each lane. DNA size markers (in kilobases) are indicated to the left. **B)** in-gel hybridization of the gel in panel A using a C-rich telomeric strand probe. As the gel was not denatured, material visible represents single-stranded G-rich telomeric DNA.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ter1-16T RAP1oe
ter1-16T rad52Δ

Figure 3.2. PFGE measurement, and gel-filtration chromatography, of telomeric DNA from *K. lactis ter1-16T*, *ter1-16T RAD52*Δ, and *ter1-16T RAP1-OE* strains. A) Telomere length measured by PFGE in *ter1-16T* and related strains. Genomic DNA (2 μg) either uncut (-) or digested with *Alu*I, *Hpa*II and *Nla*III (+), were separated by PFGE (size marker units are kilobases). Total DNA content as visualized by ethidum bromide staining and UV light is shown on the left. Telomeric DNA signal as determined by in-gel hybridization with a [γ-^{32}P] radiolabeled *K. lactis* C-strand telomere probe and visualization by phosphorimager is shown on the right. **B)** Total DNA content, and relative telomeric DNA abundance, in the elution fractions from gel-filtration chromatography of TRFs from *ter1-16T*, *ter1-16T RAD52*Δ, and *ter1-16T RAP1*. DNA content was determined by optical density at 260 nm (OD 260). An equal amount of material from selected fractions was slot blotted and probed with a [γ-^{32}P] radiolabeled *K. lactis* C-strand telomere probe. The slot blot signal was quantitated by phosphorimager and graphed to indicate relative telomeric intensity. Elution profiles from crosslinked samples are shown here. Profiles from non-crosslinked samples of all strains are similar to the profiles shown here (data not shown).

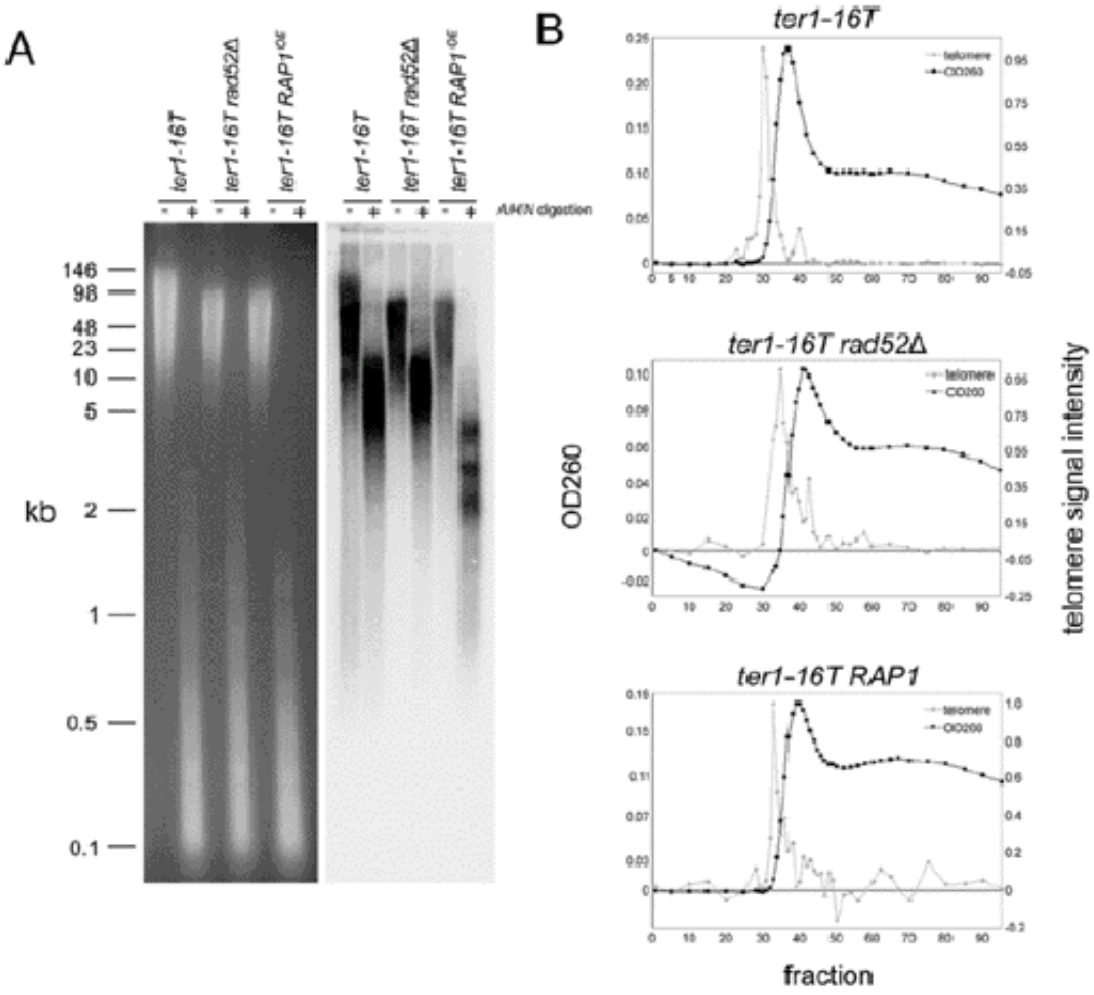
A
ter1-16T
ter1-16T rad52Δ
ter1-16T RAP1OE
- + - + - + - + - + - +
ExoIII digestion
146
98
48
23
10
5
kb
2
1
0.5
0.1
B
ter1-16T
telomere
OD260
ter1-16T rad52Δ
telomere
OD260
ter1-16T RAP1
telomere
OD260
OD260
telomere signal intensity
fraction

Figure 3.3. Looped molecules in the HMW, telomere enriched fractions from *ter1-16T*. A-G) Electron micrographs of looped molecules present in the HMW fractions from *ter1-16T*. DNA was prepared for EM by surface spreading on a denatured protein film and rotary shadowcasting with a platinum-palladium amalgam. Loop and tail sizes are 3.4 and 15.0, 4.2 and 8.7, 0.8 and 6.5, 0.4 and 0.7, 0.5 and 1.0, 0.6 and 1.2, 1.3 and 0.8 kb for panels A through G respectively. Looped molecules in *ter1-16T RAP1-OE* and *ter1-16T RAD52Δ* were structurally similar to the molecules shown here. Images are in negative contrast. Bar is equivalent to 3 kb.

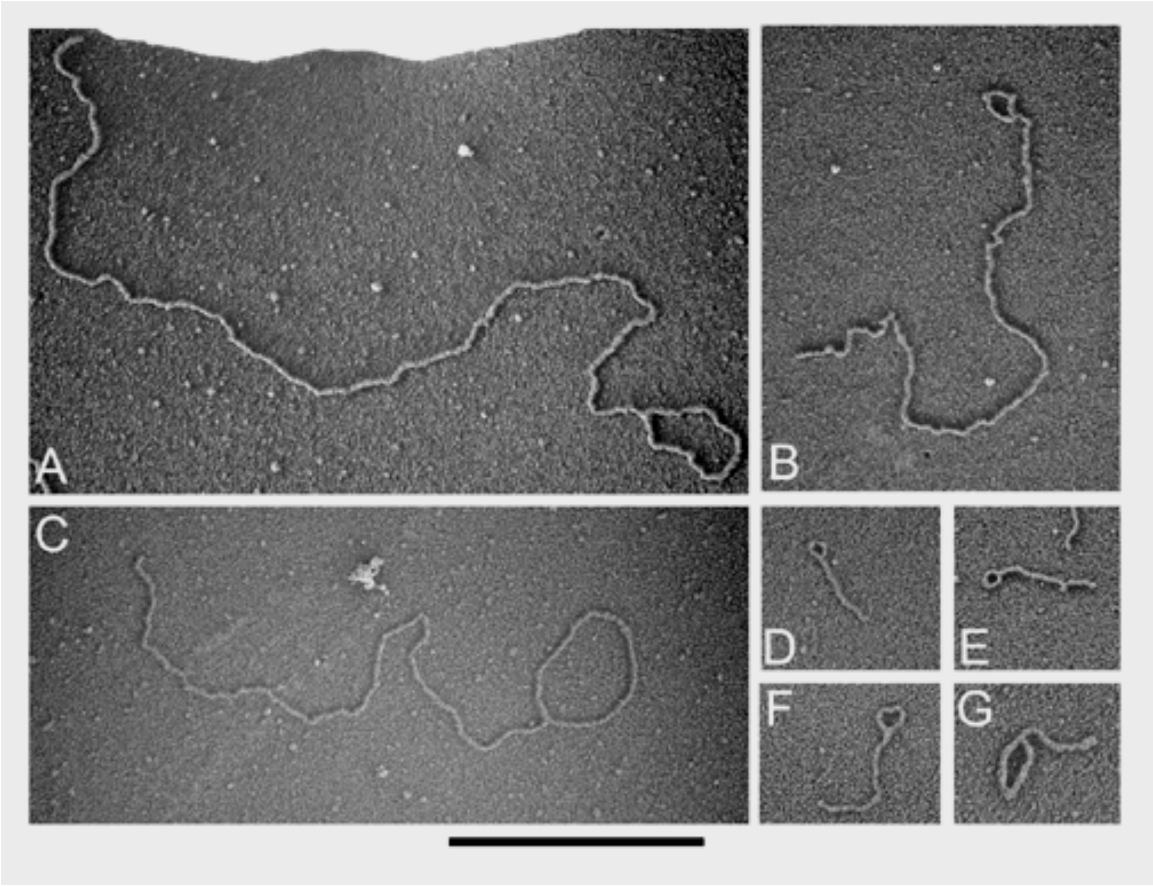
A
B
C
D
E
F
G

Figure 3.4 Looped molecules in *ter1-16T* are consistent with t-loops. A) Distribution of looped molecules observed in the psoralen crosslinked and non-crosslinked samples from *ter1-16T*, *ter1-16T RAP1-OE*, and *ter1-16T RAD52*Δ. **B)** Distribution of the total length (loop + tail) of measured looped molecules in the HMW fractions from *ter1-16T* (n = 94) and *ter1-16T RAP1-OE* (n = 91). **C)** Distribution of looped molecules observed in the indicated HMW, crosslinked fractions from *ter1-16T* after initial digestion with *Alu*I/*Hpa*II/*Nla*III (initial), then subsequent digestions with *Mse*I or *Rsa*I. The *K. lactis ter1-16T telomere* sequence is; 5'-TTTGATTAGGTATGTTGTGTACGGA-3'. The *Rsa*I digestion site is underlined. *Mse*I digests the sequence 5'-TTAA-3'. Fraction 29 *Mse*I digestion sample n=100, fraction 30 *Mse*I digestion sample n=151. Fraction 29 and 30 *Rsa*I digestion sample represent at least 100 molecules examined.

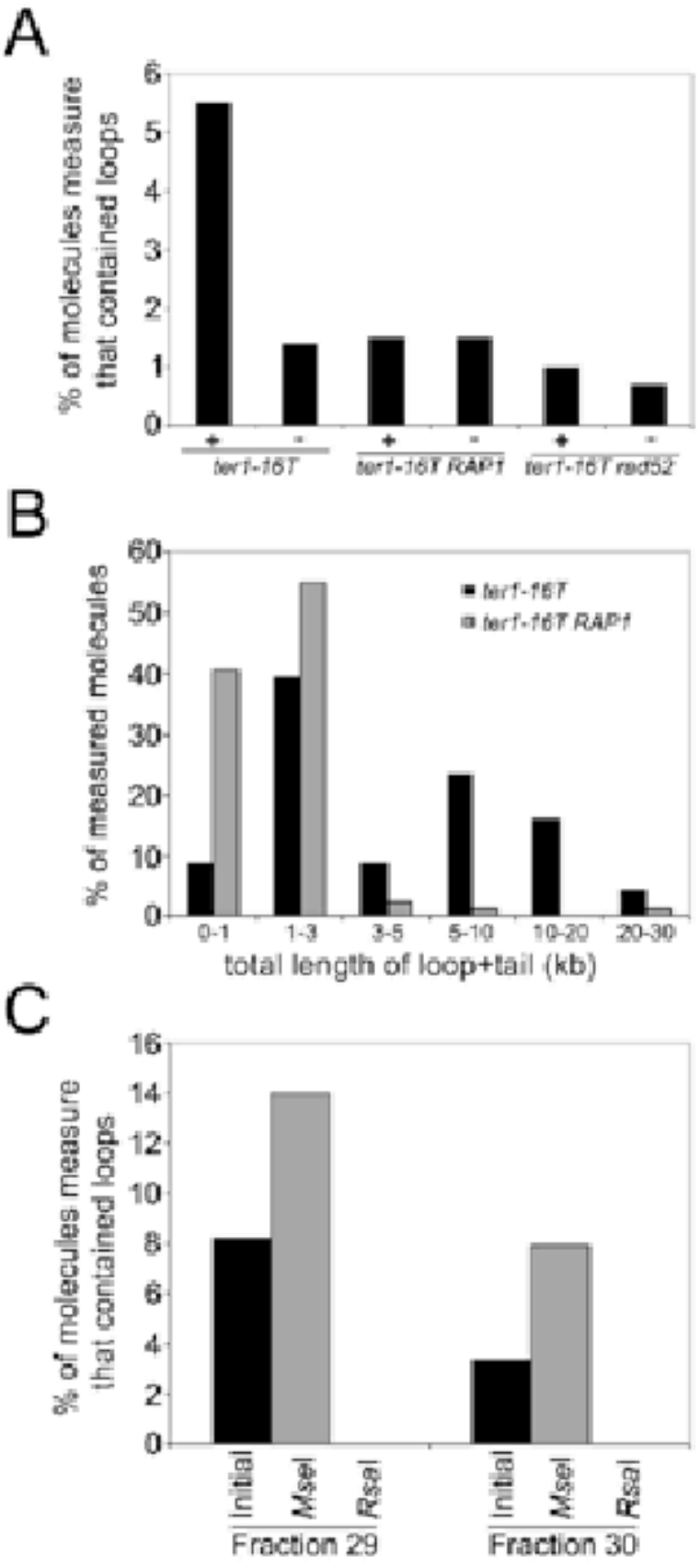
A
% of molecules measure that contained loops
0
1
2
3
4
5
6
+
-
+
-
+
-
ter1-16T
ter1-16T RAP1
ter1-16T rad52
B
% of measured molecules
0
10
20
30
40
50
60
ter1-16T
ter1-16T RAP1
0-1
1-3
3-5
5-10
10-20
20-30
total length of loop+tail (kb)
C
% of molecules measure that contained loops
0
2
4
6
8
10
12
14
16
Initial
MseI
RsaI
Initial
MseI
RsaI
Fraction 29
Fraction 30

Figure 3.5 SSB bound looped and linear molecules from the HMW, crosslinked *ter1-16T* fractions. A-C) Looped molecules with SSB localized to the loop junction. Loop and tail sizes are 0.3 and 5.7, 0.4 and 1.5, 0.7 and 0.6 kb for A through C respectively. **D-E)** Linear molecules with SSB localized to a single molecule end. The length of the ds DNA in D is 8.1 kb. In E, the total length of the ds DNA for the molecule shown is 16.1 kb, with most of ds DNA outside of the image border. Samples were directly mounted onto thin carbon coated foils and rotary shadowcast with tungsten. White arrows indicate the location of SSB protein. Shown in negative contrast. Bar is equivalent to 2 kb.

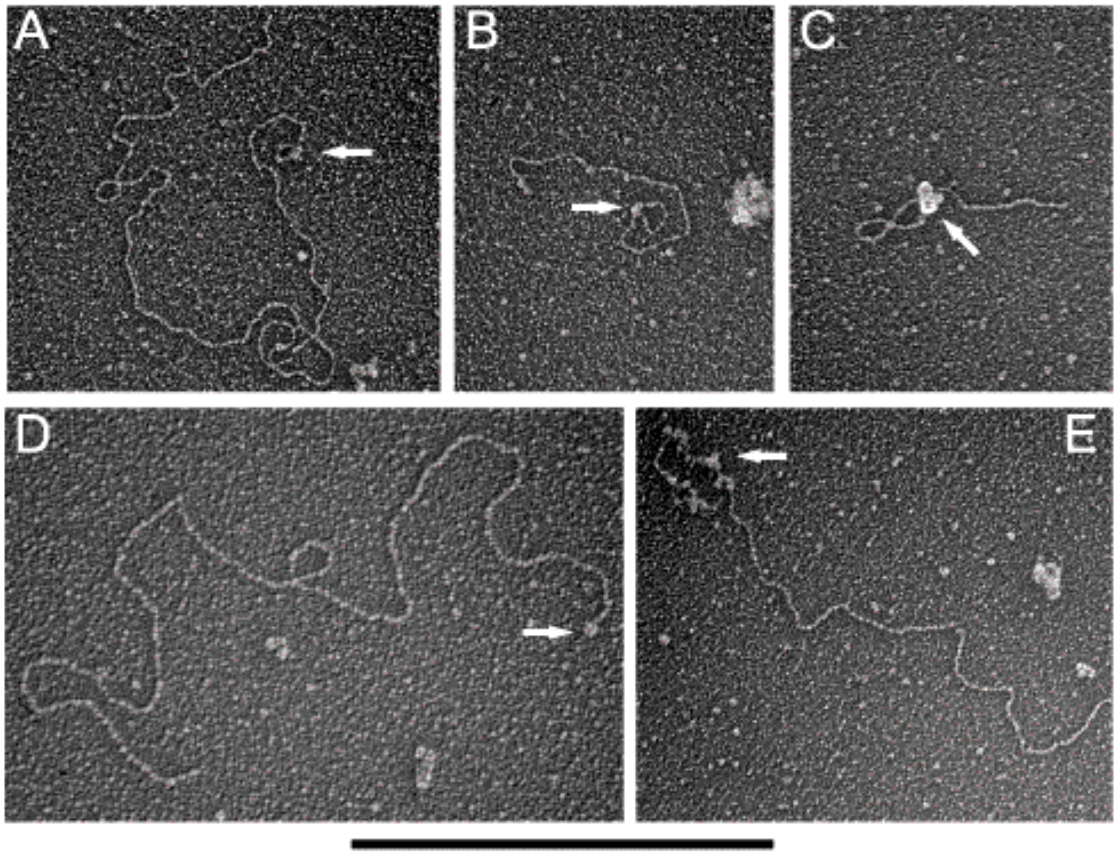
A
B
C
D
E

Figure 3.6 Extrachromosomal DNA circles from *ter1-16T*. A-J) Electron micrographs of circular DNA molecules observed in the telomere enriched fractions from *ter1-16T*. DNA was prepared for EM as in figure 2. Circle lengths are 8.5, 4.6, 4.6, 3.6, 3.3, 0.9, 0.8, 0.7, 1.2, 0.9 kb for A through J respectively. Shown in negative contrast. Bar is equivalent to 2 kb.

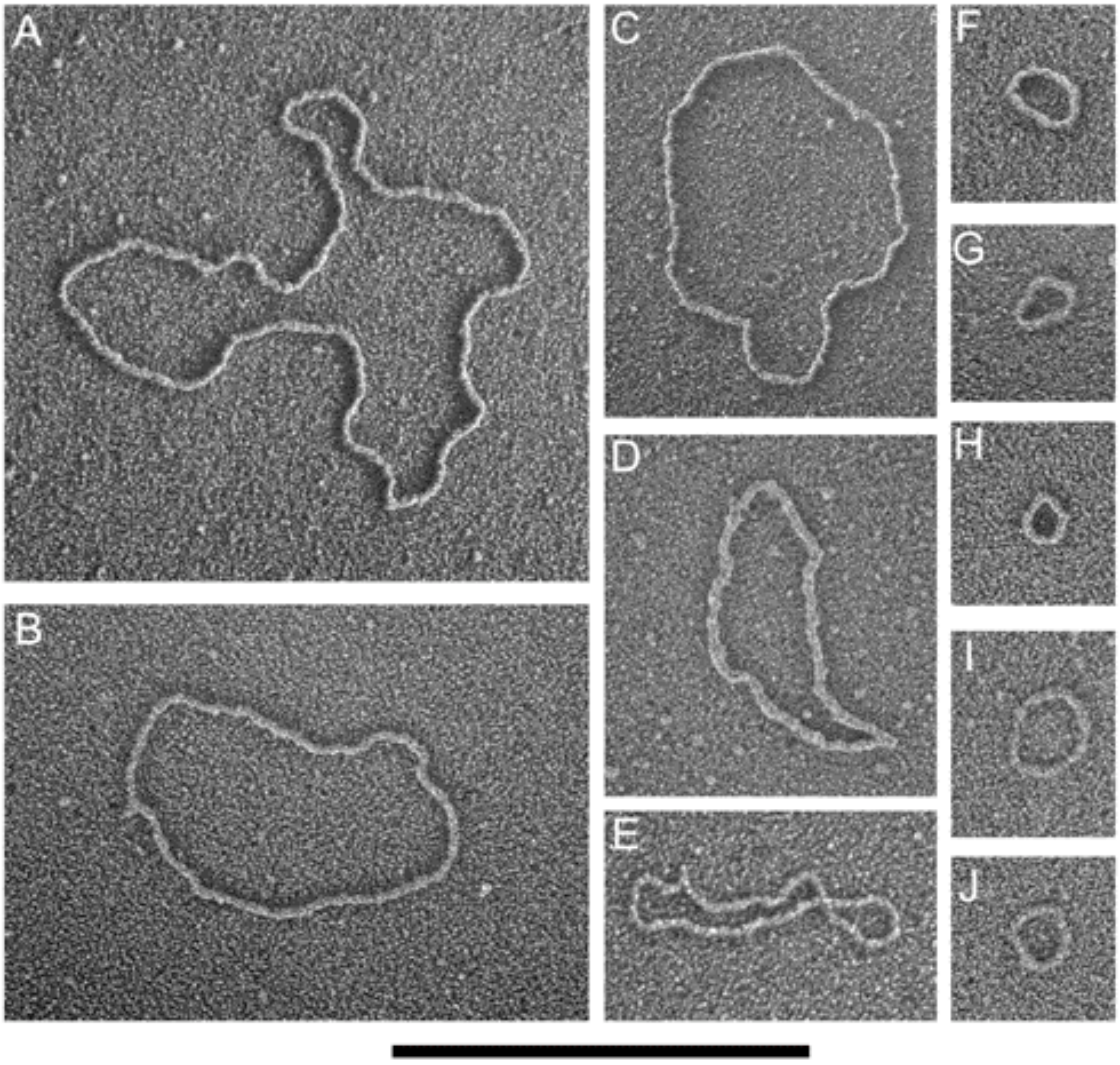
A
B
C
D
E
F
G
H
I
J

Figure 3.7 Circular molecules in *ter1-16T* are consistent with t-circles and t-loop HR. A) Distribution of the abundance of looped molecules observed in the psoralen crosslinked samples from *ter1-16T*, *ter1-16T RAP1-OE*, and *ter1-16T RAD52*Δ. **B)** Distribution of the percentage of circular molecules observed in the indicated HMW crosslinked fractions from *ter1-16T* after initial digestion with *Alu*I/*Hpa*II/*Nla*III (initial), then subsequent digestions with *Mse*I or *Rsa*I (see figure 3.3 for sequence information). **C)** Distribution of the measured loop portion (n = 94) of t-loops and t-circles (n = 102) from *ter1-16T*.

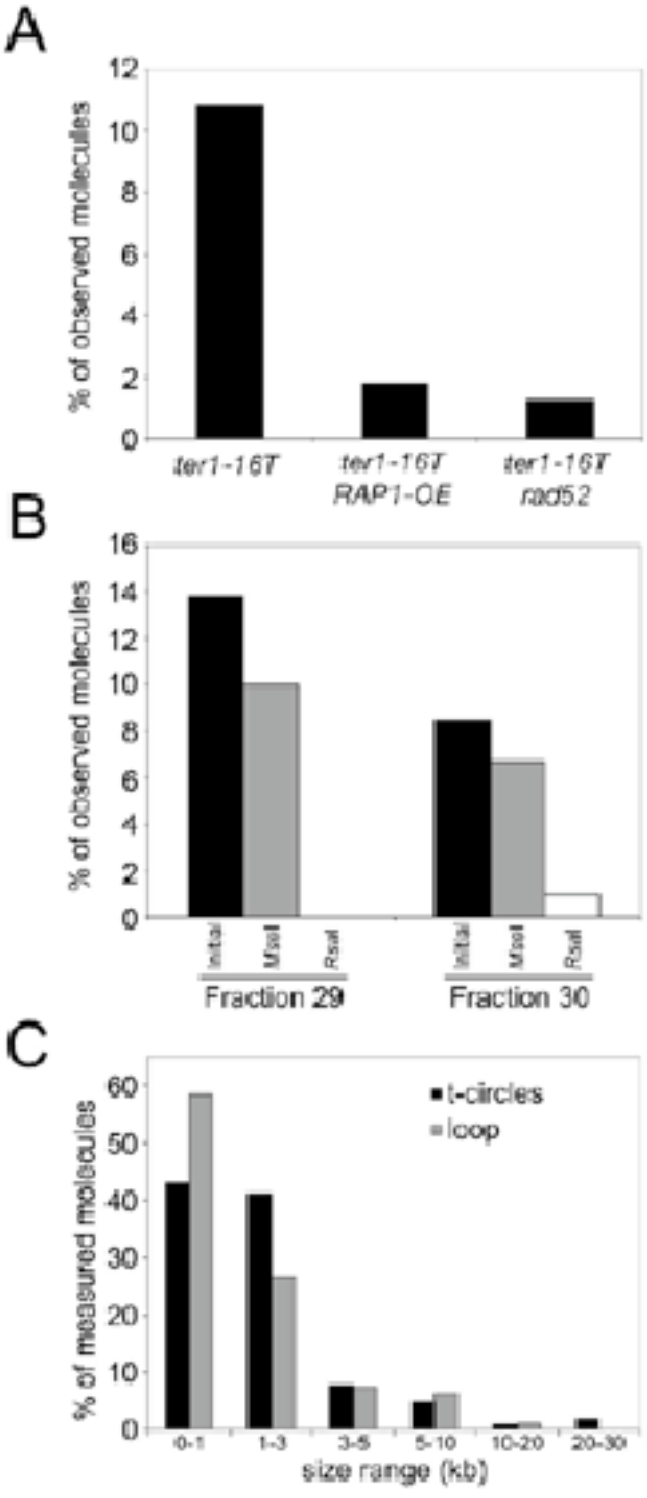
A
% of observed molecules
12
10
8
6
4
2
0
ter1-16T
ter1-16T RAP1-OE
ter1-16T rad52
B
% of observed molecules
16
14
12
10
8
6
4
2
0
Initial
MseI
RsaI
Fraction 29
Fraction 30
C
% of measured molecules
60
50
40
30
20
10
0
t-circles
loop
0-1
1-3
3-5
5-10
10-20
20-30
size range (kb)

Figure 3.8 Standard neutral-neutral 2D gel electrophoresis of TRFs derived from psoralen crosslinked, and non-crosslinked, genomic DNA from *ter1-16T*, *ter1-16T RAP1-OE and ter1-16T RAD52Δ*. Genomic DNA (8 μg) digested with *Alu*I, *Hpa*II and *Nla*III was separated by 2D gel electrophoresis. Total DNA was detected by ethidium bromide staining and UV light (top panels). Telomeric material was detected by in-gel hybridization with a [γ-^{32}P] *K. lactis* C-strand telomere probe and visualized using a phosphorimager (bottom panels). The black and white arrows indicate linear and circular form DNA respectively.

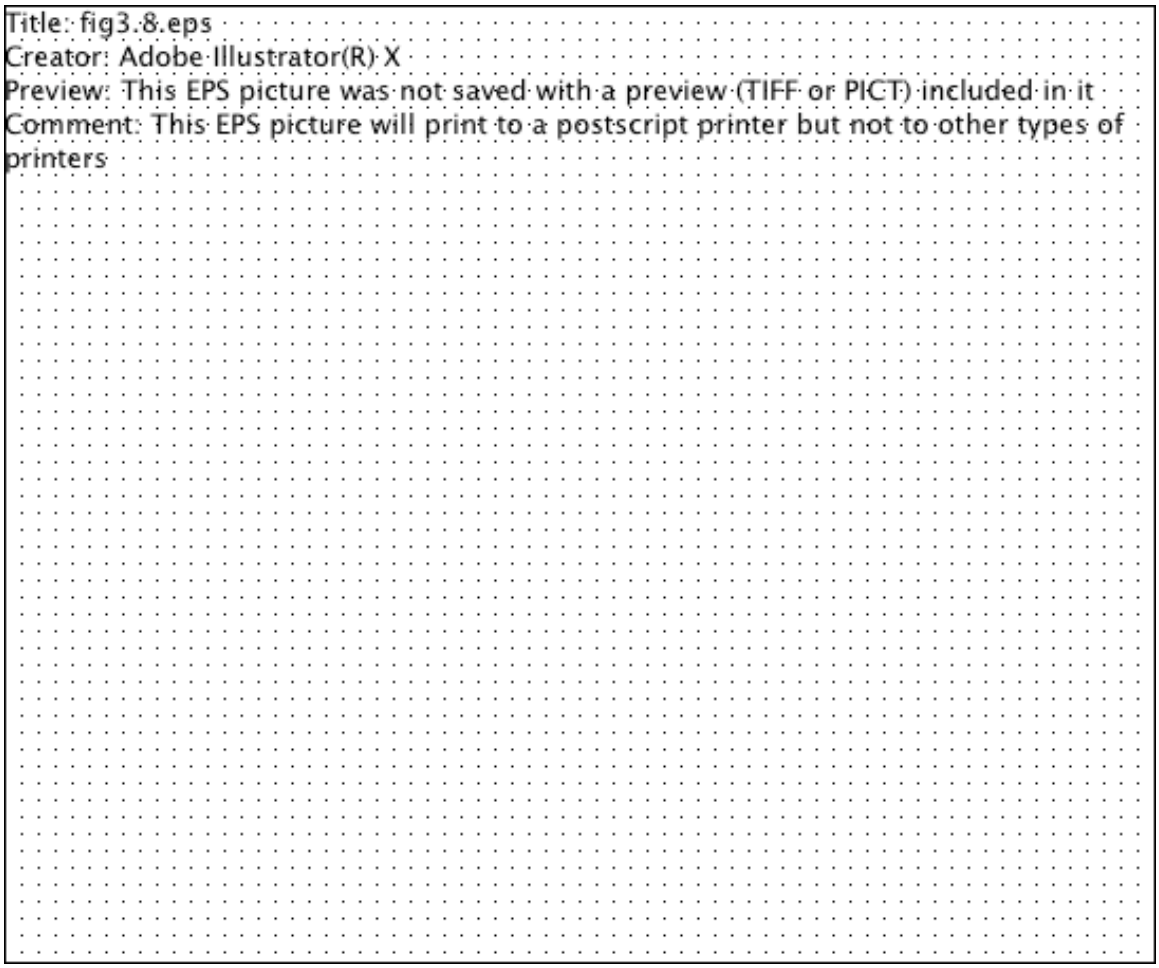
Title: fig3.8.eps
Creator: Adobe Illustrator(R) X
Preview: This EPS picture was not saved with a preview (TIFF or PICT) included in it
Comment: This EPS picture will print to a postscript printer but not to other types of printers

Table 1. Summary of the EM observations from *ter1-16T, ter1-16T RAP1-OE*, and *ter1-16T RAD52Δ*. Chi-square (χ^2) measures the goodness of fit for the crosslinked versus non-crosslinked sample for each strain. The difference is significant in the *ter1-16T* is sample, but not in the *ter1-16T* and *ter1-16T RAD52Δ*.

Sample	Total n	Linear (%)	Loop (%)	Circle (%)	χ^2
ter1-16T					
crosslinked	617	526 (85.3)	34 (5.5)	57 (10.8)	
non-crosslinked	1622	1560 (96.2)	22 (1.4)	40 (2.5)	$p \leq 0.001$
ter1-16T RAP1^{OE}					
crosslinked	2516	2434 (96.7)	38 (1.5)	44 (1.8)	
non-crosslinked	2930	2840 (96.9)	45 (1.5)	45 (1.5)	$p > 0.05$
*ter1-16T rad52*Δ					
crosslinked	1471	1438 (97.8)	14 (1.0)	19 (1.3)	
non-crosslinked	450	444 (98.7)	3 (0.7)	3 (0.7)	$p > 0.05$

Chapter 4

DISCUSSION AND FINAL THOUGHTS

This study has provided the first evidence of a higher order t-loop structure at the ends of yeast telomeres. We have also presented evidence that t-circles are likely made through resolution of a t-loop. This resolution appears to be dependent upon a *RAD52* mediated recombination event and occurs only in the presence of a compromised telomeric cap. Further, we have provided evidence indicating that t-circles are capable templates for rolling circle mediated telomere elongation.

The *ter1-16T* mutant can elongate its telomeres through the action of telomerase. However, it is clear that there is an increase in telomeric recombination in *ter1-16T*. Further, *ter1-16T* produces t-circles, likely competent templates for rolling-circle-replication. These t-circles could promote telomere elongation. Indeed, putative rolling-circle-replication intermediates have been visualized within *ter1-16T* DNA samples (Groff-Vindman et al. 2005). Another telomerase mutant, *ter1-Acc,* that produces a similar but more severe immediate telomere elongation phenotype is also defective in binding Rap1p (McEachern and Blackburn 1995; Krauskopf and Blackburn 1996). Mutants with Acc repeats at their telomeres can achieve extreme elongation via either telomerase or recombination (Harris, L. and McEachern, M. unpublished data). Another mutation, within the gene *STN1,* which encodes a protein involved in capping of the telomeric overhang, also has extremely elongated telomeres that are produced by

recombination (Iyer et al. 2005). Both of these mutants efficiently employ RTE. Telomerase deletion survivors only elongate their telomeres by modest lengths (a few hundred bp) and this is likely due to two things. First, rolling-circle-replication around a small (100 bp) t-circle is not likely to be highly processive. This is supported by the modest elongation seen in *ter1-Taq* cells utilizing a 100 nt synthetic circle (Groff-Vindman et al. 2005); (chapter 2 this dissertation). Secondly, these cells contain wild type telomeric repeats and do not harbor mutations in genes encoding telomeric binding proteins. This is thought to block further recombination once modest telomere elongation has occurred. Extension in the extreme elongation mutants mentioned earlier likely occurs through more processive rolling-circle-replication reactions around larger circles and these large extensions are not acted upon by the normal telomeric cap allowing them to recombine again and be lengthened further. Interestingly, telomerase deletion survivors given large circles (1.6 kb) containing telomeric repeats use these circles to extend their telomeres by many kilobases (Natarajan and McEachern 2002). This indicates that the size of the circle almost certainly plays a role in the extent of the elongation resulting from its replication.

The extreme elongation mutants of *K. lactis* share many phenotypic characteristics with human ALT cells. Human ALT cells have very long heterogeneous telomeres, they produce large amounts of ECTR and recently much of this ECTR was shown to be circular in nature (Cesare and Griffith 2004; Wang RC. 2004). These cells elongate their telomeres through RTE though it has not been shown that the circles play a role in this extension. *K. lactis* extreme telomere elongation mutants may be excellent models for investigating the mechanism by which ALT cells extend their telomeres. ALT

and telomerase-dependent extension of telomeres can coexist in human cell culture (Perrem et al. 2001; Reddel et al. 2001). Perhaps this type of coexistence is similar to the situation in *ter1-16T*.

Data presented here indicates that t-loops exist on the ends of uncapped *ter1-16T* telomeres at some frequency. Over-expression of Rap1p did not increase the number of t-loops observed in *ter1-16T.* Comparison of *ter1-6T* and *ter1-16T RAP1-OE* crosslinked samples instead showed a significant decrease in the number of t-loops after *RAP1* is overexpressed. The telomeric ends of *ter1-16T* cells have greatly elongated telomeric overhangs. These overhangs may promote the formation of t-loops that are stabilized by crosslinking. Perhaps the t-loop structures visualized in this study are not protective but are "uncapped" inter-telomeric recombination intermediates; an idea supported by the major reduction in t-loops found in telomeric samples from *ter1-16T rad52Δ* double mutant. The overexpression of *RAP1* in *ter1-16T* reduces the single-stranded overhangs of *ter1-16T* to near wild type lengths. Therefore, *ter1-16T RAP1-OE* telomeres may not have sufficient overhangs to produce the types of t-loops found in *ter1-16T* samples. Still, a small number of t-loops were observed in all of the samples analyzed.

An alternative explanation of this data is that t-loops do exist at some frequency at the ends of *K. lactis* telomeres but this is not the obligate capped structure. Perhaps, the small number t-loops seen in *ter1-16T RAP1-OE* contain very short regions of single-strand invasions at their D-loops and are not stabilized well by psoralen crosslinking. This "capped" type of t-loop presumably would not require recombination since a similar level of t-loop background is found in *ter1-16T RAD52Δ. RAD52* is notably not required

for the normal maintenance of telomere length. If a t-loop was a normal "capped" structure it is likely that they would form through *RAD52*-independent means.

Knowledge of the genetic requirements of t-loop formation in *ter1-16T* would perhaps shed further light on whether t-loops are capped or uncapped structures. Perhaps, the longer single-stranded overhangs of *ter1-16T* are bound by Rad51p, the eukaryotic RecA equivalent, initiating a recombination event that requires a longer region of homology to occur. If this is true the number of t-loops observed *ter1-16T* DNA after crosslinking should decrease in a *ter1-16T rad51* double mutant. Clearly, a disruption in Rap1p binding, as is seen in *ter1-16T*, increases the frequency at which one type of t-loop forms. Likely, disruptions in other telomeric cap proteins can lead to either increased t-loop formation or increased t-loop resolution. One interesting candidate is the *STN1* gene. Stn1p binds Cdc13p found at the telomeric overhang and these two proteins control the access of telomerase to the telomeric end. A mutant of *STN1, stn1-M1*, has extremely long telomeres produced by RTE as well as elongated single-stranded overhangs (Iyer et al. 2005). This mutant is known to produce abundant t-circles (Iyer, S., Cesare, A., Basenko, E., Griffith, J. and McEachern, M. unpublished data). If t-loops are as frequent or more frequent in *stn1*-M1 than in *ter1-16T RAP1*^{OE} this would indicate that multiple types of capping defects could lead to the formation of "uncapped" t-loops.

If proteins involved in capping structure are required for the presence of t-loops in *ter1-16T* this would be consistent with the existence of "capped" t-loops in *K. lactis* cells. It is known that Nbs1, a component of the MRN complex, the human homologue of the MRX complex, is required for homologous recombination mediated t-loop resolution or t-loop HR (Cesare and Griffith 2004; Wang RC. 2004). The MRX complex is also

required for normal yeast telomere maintenance (Tsukamoto et al. 2001; Larrivee et al. 2004). *RAD50* is a component of the MRX complex in both yeast and the MRN complex in human cells. In both humans and yeast cells the MRX complex is required for normal telomere maintenance and processing of double strand breaks. Perhaps, the MRX complex is responsible for a telomere-processing event, which precedes t-loop formation. Additionally the MRX complex might be involved in t-loop to t-circle resolution. An attractive next experiment is to identify the level of t-loop formation in *ter1-16T rad50* double mutant.

T-circle creation that occurs in senescing *ter1Δ* cells probably also occurs through resolution of a t-loop. However, telomeres in these cells are drastically shorter than in *ter1-16T* cells. Data discussed earlier suggests that t-circles created in *K. lactis ter1Δ* survivors are as small as 100 bp. The Type II pathway of telomerase deletion survival in *S. cerevisiae* is dependent upon the *RAD50 RAD59* recombination pathway, and gives rise to telomere structure similar to that seen in *K. lactis* telomerase deletion survivors. Notably, the *RAD50 RAD59* recombination pathway requires significantly shorter stretches of homology than the *RAD51* mediated pathway. T-circle creation and therefore, survivor formation in *K. lactis* cells may be dependent upon the *RAD50 RAD59* mediated pathway of homologous recombination.

Human t-loops may also exist in both a capped and an uncapped form. Human ALT cells contain t-loops and t-circles. The loop portion of these t-loops and t-circles found in ALT cells have similar sizes. This data suggests that in ALT cells t-loops continue through a process of recombination leading to the excision of a t-circle. Therefore, it seems there is an alteration in the state of the t-loop in ALT cells that causes

them to become “uncapped” t-loops. No matter what the state of wild type telomeres in yeast or human cells it is likely that t-loop resolution into t-circles is an evolutionarily conserved phenomenon of uncapped telomeres.

The roll-and-spread model of RTE suggests that a t-circle is responsible for formation of an elongated telomere in *ter1Δ* cells. It is still unclear whether the circular template acts at the telomere that created it (in cis) or at an alternate telomere (in trans). The resolution of a Holliday junction created at the loop-stem junction of a t-loop can produce either a circle of the G-rich or C-rich strand (Fig.4.1). Either of these circles would still be attached to the telomeric end by its base pairing interactions. If a G circle is created it can easily be displaced through extension of the new 3’ terminus (Fig.4.1). If a C-rich circle is created it could remain attached to the end and become a template for rolling-circle DNA synthesis. Consistent with this model the only single-stranded circles collected from *ter1-16T* cells were composed of G-rich telomeric strand (Fig.2.1). Data indicating that synthetic circles transformed into cells can efficiently elongate telomeric ends argues that circles can act in trans but does not address whether in cis utilization of a t-circle occurs in a *ter1Δ* survivor formation.

RAD52 is required for the efficient formation of t-circles in *ter1-16T*, as well as for the appearance of *ter1Δ* survivors. It is not clear what other genetic requirements for RTE may exist. *S. cerevisiae* telomerase deletion survivors consist of two distinct groups. These two types of survivors are distinguished by the types of telomeric extension which occur at their ends during RTE. Type I survivors require *RAD51*, *RAD54*, and *RAD57* and have amplifications of their Y’ subtelomeric repeats. Type II survivors require *RAD50*, *RAD59*, and *SGS1* and solely extend their telomeric TG(1-3) repeats. *K. lactis*

cells do not have telomeric repeats within their subtelomeric regions and this is likely why a Type I-like pattern is not seen in *ter1Δ* survivors. It is possible that *K. lactis* survivors could also utilize either the Type I and Type II recombinational pathways. If this is true, survivor formation will not be perturbed in cells with mutation in just one of these pathways. An in depth genetic analysis is needed to form a well rounded picture of the mechanism by which RTE occurs in *K. lactis* cells.

Human ALT cells, which extend their telomeres through recombination, exhibit specific types of genomic instability that are not seen in telomerase positive cancers (Jeyapalan et al. 2005). It has been hypothesized that ALT may act as stepping-stone for the development of fully metastatic telomerase-positive tumors. Because telomerase has become a major target in the development of human cancers, the ALT pathway has been suggested as a pathway by which tumors could gain resistance to newly developed treatments. An important task in defining a model of ALT cancer development will be uncovering the connection between RTE and genomic instability. *K. lactis* mutants, such as *ter1-16T* that exhibit high rates of telomeric recombination may be excellent models for investigating this connection. It is possible that elevated levels of recombination at the telomeres result in a decreased ability for the cell to repair every day damage. This could occur because proteins important to recombination and repair processes are sequestered at the telomeric ends.

Here we have provided an analysis of the *ter1-16T* mutant that provides a mechanistic link between telomeric processes in *K. lactis* cells experiencing capping defects and human ALT cells. We have provided details on the structure of telomeres in *ter1-16T* and shown evidence to support a model of t-circle formation through t-loop

resolution much like the t-loop HR proposed to occur in human ALT cells. We have also shown that small t-circles are efficient templates for the extension of telomeres leading to elongation similar to what is seen in telomerase deletion survivors. Continuing this line of investigation will likely lead to a better understanding of the mechanism by which both *K. lactis* telomerase deletion survivors and human ALT cancer cells elongate their telomere.

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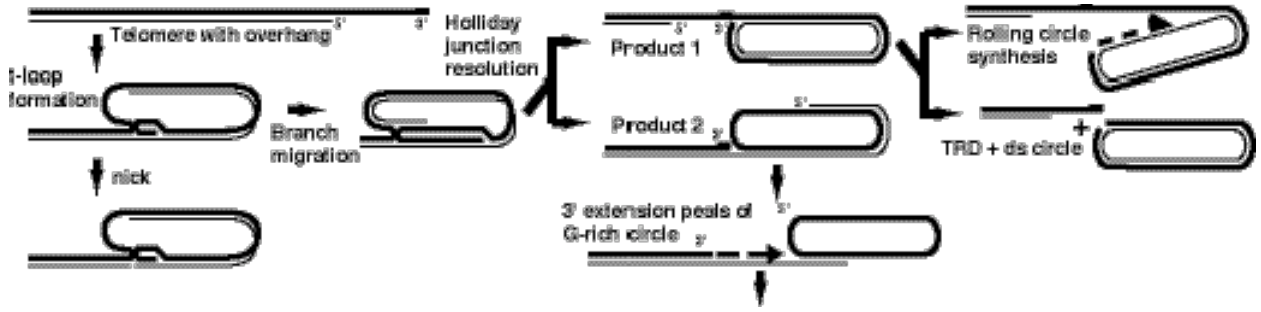
Figure 4.1 T-loop resolution can lead to both circle creation or rolling-circle extension in cis. The invasion of telomeric overhang into the duplex telomeric DNA can produce a holiday junction structure. T-loops can likely be resolved leading to the creation of t-circles (Groff-Vindman et al. 2005)(chapter 2 this dissertation) This holiday junction can be resolved resulting in an intact circular structure of either the G or C-rich strand at the telomere. If a c-rich circular structure is made the G-rich 3' end of the telomere can act as a primer, extending the telomere through rolling-circle-replication around the C-rich circle. If a G-rich circle is created the 3' G-rich strand extension will peal this circle of resulting in a shortened telomere and a free G-rich single-stranded circle.


Telomere with overhang
t-loop
formation
nick
Branch
migration
Holliday
junction
resolution
Product 1
Product 2
3' extension peels of
G-rich circle
Rolling circle
synthesis
TRD + ds circle