A GENETIC ANALYSIS OF TELOMERASE TRANSLOCATION AND TELOMERE FUNCTION IN *K. LACTIS*

by

DANA ELIZABETH HAGER UNDERWOOD

(Under the direction of Michael J. McEachern)

ABSTRACT

Telomeres are DNA-protein complexes that protect the ends of linear chromosomes. The non-genic DNA is typically composted of repetitive T/G-rich sequences, and maintained by telomerase, a specialized reverse transcriptase. In the yeast *K. lactis*, the telomeric repeats are 25 base-pairs in length and copied from a 30 nucleotide template within the telomerase ribonucleoprotein complex. In order to examine the functional regions contained within the telomere and the telomerase template, strains were constructed such that each contained a single base change in the region of the *TER1* gene encoding the telomerase template. Mutations were made at all 30 template positions, and the effects were initially examined by measuring the lengths of the telomeres in the mutant yeast. Telomeres were also cloned to examine the types of mutant telomeric repeats added by telomerase. Typically, the mutations were incorporated as single base-changes in the newly synthesized telomeric repeat. Mutations leading to telomere elongation, telomere shortening, delayed telomere elongation, and telomeric repeats of aberrant sizes were identified. Mutations causing each phenotype were often confined to discrete regions of the template. Mutations leading to telomere elongation and aberrantly sized telomeric repeats were examined further. Extreme telomere elongation was found to be independent of *RAD52*-dependent recombination. However, the formation of the large amount of

extrachromosomal and single-stranded telomeric DNA observed in these mutants was found to be dependent on *RAD52*. Subtelomeric recombination was also found to be elevated in these mutants. Mutations in the ends of the telomerase template led to the synthesis of telomeric repeats that were not 25 base-pairs in length. By examining the telomeric repeats in these mutants, it was determined that mutations which disrupted the base-pairing between the telomere and the template that occurs prior to the synthesis of new telomeric repeats caused the telomere to align with a different region of the template. Mismatches, therefore, were typically neither extended nor removed by a nucleolytic activity. It was also determined that a region outside of the template might play a role in stabilizing the base-pairing interactions between the telomere and the template prior to the synthesis of new telomeric repeats.

INDEX WORDS: Telomere, Telomerase, Repeat, Reverse transcriptase,
Ribonucleoprotein, *RAD52*

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DEDICATION

I dedicate this dissertation to my husband, Keith Underwood. He encouraged me to pursue this program of study knowing that it would add miles of highway and many additional stresses to our relationship. His support, patience, and understanding were an incredible comfort during my time in graduate school and I look forward to beginning our lives together, in one place for the first time.

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CHAPTER1

INTRODUCTION AND LITERATURE REIVEW

In order to survive, organisms must maintain the integrity of their genomes during DNA replication and cell division. In eukaryotes and certain prokaryotes, this process is complicated by fact that the chromosomes are linear; the chromosome ends must be replicated and protected. During normal DNA replication, however, there is no mechanism to replace the final RNA primer that would be at the tip of the lagging-strand chromosome. Failure to replicate the chromosome ends would lead to shortening of the chromosomes and eventually the loss of important genetic information, while failure to protect the ends would cause them to be identified by the cell as double-strand breaks (DSBs) and thus 'repaired' by the cell. Repair by either homologous recombination or non-homologous end joining could, however, lead to genomic instability (Hackett et al., 2001). It is therefore necessary for cells to have a mechanism for maintaining chromosome ends in such a way that they are fully replicated and also not recognized as DSBs.

Prokaryotes with linear chromosomes utilize mechanisms that are quite different from eukaryotes to maintain their chromosome ends. In *Streptomyces lividans*, a protein is covalently attached to the end of the chromosome, where there are multiple palindromic sequences (reviewed in Hinnebusch and Tilly, 1993). Replication could occur in a manner similar to the replication of linear plasmids in the spirochete *Borrelia*, which uses a fold-back mechanism to form a hairpin (Hinnebusch and Barbour, 1991; Qin and Cohen, 1998). One critical difference, however, is that in *Borrelia* the new chromosome replication would begin from the 3' end of the chromosome, forming a concatemer. In *Streptomyces*, the terminal binding protein would serve as the primer.

Among eukaryotes, the basic mechanism is quite different but fairly conserved, with the exception of a few organisms, including *Drosophila*. In the fly, the chromosome ends are composed of 2 types of transposable elements (Biessmann et al., 1992; Levis et al., 1993; Pardue and DeBaryshe, 1999). Although the total length of the retrotransposon tract is regulated, it is unclear how this is done.

TELOMERE MAINTENANCE BY TELOMERASE

Most eukaryotes maintain their chromosome ends through a complex mechanism that begins with the addition of repetitive DNA sequences, which are subsequently bound by an array of proteins. This protein-DNA complex, known as the telomere, allows the chromosome ends to be fully replicated and then protected. The first part of this process involves adding telomeric DNA to the 3' ends of the chromosome. This is done by telomerase, a specialized reverse transcriptase (Blackburn, 1992). This ribonucleoprotein is comprised of an RNA component containing the template for new telomere synthesis (Greider and Blackburn, 1989; Singer and Gottschling, 1994) and a protein component, which has the reverse transcriptase activity (Lingner et al., 1997). The RNA component, called *Tlc1* in *S. cerevisiae* (Singer and Gottschling, 1994) and *Ter1* in *K. lactis* (McEachern and Blackburn, 1995), contains a template that is A/C-rich in most species, leading to the synthesis of T/G-rich telomeric DNA. The template can vary in length; many species, ranging from *Tetrahymena* (Greider and Blackburn, 1989) to humans (Feng et al., 1995), have 6 nucleotide telomeric repeats reverse transcribed from a 9-11 nucleotide template. A number of yeast, however, have telomeric repeats of up to 26 nucleotides, which are copied from a template with 30 or more nucleotides (McEachern and Blackburn, 1994; McEachern and Hicks, 1993). One example this type of organism is the budding yeast *Kluyveromyces lactis*, the model organism for this study, which will be described in

greater detail below. Mutations introduced into a telomerase template can be copied into the telomere via the process described in the next paragraph. In some instances the mutations can lead to dramatic changes in the length of the telomeres (McEachern and Blackburn, 1995; Prescott and Blackburn, 1997b; Yu et al., 1990); proteins likely to be involved in this process will also be described in subsequent sections. The RNA component of telomerase can vary from the 146 nucleotide gene found in *Tetrahymena paravorax* (McCormick-Graham and Romero, 1995) to the RNA of over 1.3 kb that is found in *S. cerevisiae* (Singer and Gottschling, 1994). While the structure of the small ciliate telomerase RNAs is predicted to contain 3 stem-loop structures that keep the template region unpaired and accessible for interaction with the telomere (Lingner et al., 1994; McCormick-Graham and Romero, 1995), the structure of the larger RNAs is likely to be much more complicated. Through experiments in which deletions in the *TER1* gene of *K. lactis* were made, it has been shown that several short regions of the RNA are dispensable for normal telomerase function (Roy et al., 1998). Not surprisingly, however, certain regions of the RNA are required for telomerase activity. In addition to the critical template region, some mutations affect telomerase assembly, while in others the deletions lead to a conformational change that apparently affects telomerase function although not the ability of the RNA to assemble into an RNP complex (Roy et al., 1998). As with human telomerase, approximately half of the telomerase RNA molecule is not necessary for telomerase function *in vitro* (Autexier et al., 1996).

The template of the telomerase RNA is identified largely by 2 characteristics. First, it contains a sequence that is the reverse complement of the T/G-rich strand of the telomere. Second, it is bordered by terminal repeats at each end. The repeats, which are usually 3-5 nucleotides in length, are critical for aligning the telomerase RNA with the template (Autexier and Greider, 1995; Greider and Blackburn, 1989; Prescott and Blackburn, 1997b) (Fig.1-1). After telomerase forms its initial base-pairing interaction

between the 3' end of the telomere and the 3' end of the RNA template, new telomeric DNA is reverse-transcribed from the telomerase template onto the tip of the telomere. Because the 3' end of the template is a direct repeat of the 5' end of the template, the sequence reverse transcribed from the 5' end of the template is available to base-pair with the 3' end of the template in order to initiate synthesis of the next telomeric repeat (reviewed in Greider, 1995). In *K. lactis,* the terminal repeats are 5 nucleotides in length; the 3' terminal repeat, where telomerase aligns, will be termed TR1-5 (for Terminal Repeat positions 1-5) and the 5' repeat, where telomere synthesis stops, will be called TR26-30. In *vitro* analysis has shown that the *K. lactis* telomerase typically does not copy the entire template (Fulton and Blackburn, 1998). Instead, synthesis usually stops at position 28, the middle nucleotide in TR26-30. It is thought that by copying the first 3 nucleotides of TR26-30, which will only base-pair perfectly with the first 3 nucleotides of TR1-5, *K. lactis* telomerase is able to synthesize perfect 25 nucleotide repeats. An examination of this premise will be described in the third chapter.

The identical telomeric repeats made in *K. lactis* contrast dramatically with those made in *S. cerevisiae*, which are very heterogeneous (Cohn et al., 1998; Shampay et al., 1984). Work published by Forstemann and Lingner states that the heterogeneity is due to 2 different mechanisms (Forstemann et al., 2000; Forstemann and Lingner, 2001). First, multiple binding registers are accessible within the telomerase template, potentially allowing telomere synthesis to start at several different places in the template. Second, telomerase appears to be nonprocessive immediately after binding, leading to abortive telomere synthesis. If a specified amount of the template is copied, however, the core of the 17 nucleotide template is then copied processively (Ray and Runge, 2001).

Structural analysis of *TER1* in *K. lactis* has identified a region involved in a long-range base-pairing interaction just beyond the 5' end of the template (Tzfati et al., 2000). This structure appears to be one of the highly conserved structural elements between a

number of *Kluyveromyces* species. The sequence, which is only 10 nucleotides in length, interacts with a sequence that is 200-350 nucleotides upstream of the template. Disruption of the structure causes the telomerase to read through the end of the template and copy the adjacent sequence into the telomere, indicating that this structure is critical for defining the end of the telomerase template. Interestingly, the telomeres in these mutants are shorter than in wild-type strains, despite the repeats being longer than wild-type. It is likely, however, that it is difficult for telomerase to align with the new sequence at the telomeric tip, which would not be homologous to TR1-5.

Telomerase appears to function as a dimer (Prescott and Blackburn, 1997a; Wenz et al., 2001), although it is not clear that it is processive *in vivo*. In some species, telomerase adds many consecutive repeats to one telomere in a processive manner (reviewed in Greider, 1995), while in others it is nonprocessive, adding single repeats to mutliple telomeres in a distributive manner (Collins and Greider, 1993; Lee et al., 1993). *In vitro*, *S. cerevisiae* and *K. lactis* telomerases are typically non-processive and remain attached to the DNA in a stable association (Fulton and Blackburn, 1998; Prescott and Blackburn, 1997b); other *in vitro* experiments have found that *S. cerevisiae* telomerase is processive and can add up to 250 nucleotides in the presence of ATP (Lue and Wang, 1995). Telomerase in *S. castelli* also appears to be processive *in vitro* (Cohn and Blackburn, 1995). It is unclear which mode of activity occurs *in vivo*, although experiments indicate that *S. cerevisiae* telomerase may be non-processive *in vivo*.

Some confusion surrounds the telomerase assembly pathway in yeast. The human telomerase RNA (hTR) has been shown to contain box H/ACA small nucleolar RNA (snoRNA)-like motifs. These motifs are required for proper accumulation and processing of telomerase RNA, and suggest that there is a nucleolar stage in human telomerase maturation (Lukowiak et al., 2001). Consistent with this, the telomerase reverse transcriptase component (hTERT) has also been shown to localize to the

nucleus (Etheridge et al., 2002). In yeast, however, a binding site for Sm proteins and the presence of a 5'-2,2,7-trimethylguanosine (TMG) cap had suggested that the transcript is matured through the same process as snRNAs (Seto et al., 1999). This process involves transport of the RNA into the cytoplasm, where the Sm proteins bind and remain associated during transport back to the nucleus. Consistent with this, the telomerase RNA can be co-immunoprecipitated with the Sm proteins (Seto et al., 1999). It has also been shown that an ORF inserted into the RNA is translated, indicating that the RNA is likely to enter the cytoplasm (Teixeira et al., 2002). Interestingly, however, overexpressed Est2p, the reverse transcriptase component of telomerase, accumulates in the nucleoli and does not redistribute in the nucleoplasm until the RNA component is overexpressed (Teixeira et al., 2002). Therefore, yeast telomerase assembly might involve both cytoplasmic and nucleolar stages.

DOUBLE-STRAND BREAK (DSB) REPAIR IN YEAST

As mentioned above, one critical role of telomeres is that they prevent the DNA ends from being recognized as DSBs. When DNA damage in the form of DSBs occurs in the cell, there are two main pathways used to repair the breaks: homologous recombination (HR) and non-homologous end joining (NHEJ). Homologous recombination occurs when the break is repaired using a donor molecule that shares sequence homology with the broken molecule (Leung et al., 1997), while NHEJ does not use homologous molecules and instead joins ends that share, at the most, a few base-pairs of homology (reviewed in Tsukamoto and Ikeda, 1998).

Homologous repair of DSBs occurs via multiple mechanisms. The two common ones that will be described here both require that the double-strand break undergo 5'→3' processing such that single-stranded molecules are formed. The single-stranded ends can then invade the homologous donor duplex DNA, leading to the formation of Holliday

junctions. DNA synthesis is then initiated and the gap created by the processing of the double-strand break region can be filled. The heteroduplex DNA can then be resolved, leading to a short region that has undergone gene conversion; the original sequence around the DSB has been 'converted' to the sequence from the intact DNA. This process appears to be dependent on *RAD51*, a homolog of *E. coli* RecA, which is involved in strand-transfer reactions (Benson et al., 1998, and reviewed in Haber, 1995; Kuzminov, 2001; Sonoda et al., 2001). A second process, called break-induced replication (BIR), begins in much the same manner as a gene conversion event (reviewed in Kraus et al., 2001). Once one strand invades, however, a normal replication fork is established and DNA synthesis continues to the end of the molecule. BIR can occur in the absence of *RAD51* (Malkova et al., 1996). It is, however, dependent on *RAD50*, *MRE11*, and *XRS2*, which comprise the MRX complex; its role in NHEJ will be described in more detail and its role at telomeres will be described in a subsequent section.

Although homologous recombination is the primary method of repair in yeast, NHEJ can occur. There are three protein complexes known to be involved in this process-the Ku heterodimer, the MRX complex, and DNA ligase IV (reviewed in Tsukamoto and Ikeda, 1998). Ku is known to bind DNA ends in a sequence-independent manner, and can bind single-stranded DNA at a reduced affinity. Ku shows preferential binding to blunt ends, hairpins, and duplex-single-strand transitions, consistent with its role in repairing DNA damage (Bianchi and de Lange, 1999; Falzon et al., 1993; Torrance et al., 1998). One model for how Ku mediates NHEJ involves the Ku heterodimer binding to DNA ends and then recruiting Sir2p, Sir3p, and Sir4p to the DNA end (Martin et al., 1999). These silencing factors, whose roles at telomeres will be described in a subsequent section, are involved in forming condensed chromatin at the

site of the break. End-joining is then thought to be catalyzed by DNA ligase IV. It is unclear how the MRX complex is involved in NHEJ.

RECOMBINATIONAL TELOMERE MAINTENANCE

In yeast, deletion of genes encoding the telomerase RNA component, the reverse transcriptase component, or Est1p, a telomerase-associated component that will be described in detail below, causes eventual senescence in most of the cells (Lendvay et al., 1996; Lundblad and Blackburn, 1993; Lundblad and Szostak, 1989; McEachern and Blackburn, 1995; McEachern and Blackburn, 1996; Singer and Gottschling, 1994). This is caused by the gradual shortening of the telomeres that occurs when telomerase is not present to add new telomeric repeats. After approximately 100 generations, the cells sense that the telomeres are critically short and stop dividing. It is unclear if the cells senesce because of short telomeres per se or if the shortened telomeres have caused the telomeric end structure to become disrupted. The rare surviving yeast cells that lack telomerase have found another way to maintain sufficient telomere length (Lundblad and Blackburn, 1993). These cells use a recombinational mechanism to elongate their telomeres. In *S. cerevisiae*, this elongation has been shown to occur through either a *RAD50* or a *RAD51* recombination pathway (Chen et al., 2001; Le et al., 1999). Two types of recombinational events have been observed at telomeres. The first, Type I, occurs via a *RAD51* dependent mechanism (Chen et al., 2001). Type I survivors maintain sufficient telomeres through the amplification of subtelomeric DNA,including Y' elements (Lundblad and Blackburn, 1993; Teng and Zakian, 1999). Y' elements are repetitive DNA sequences, approximiately 5-7kb in length, located in the subtelomere of *S. cerevisiae*, where they are interspersed with tracts of telomeric-like sequences (Chan and Tye, 1983; Walmsley et al., 1984). One model for the mechanism of forming Type I survivors states that the telomere is resected back until the Y' element

is at the telomere tip, where it then recombines with other Y' elements. Because some telomeres have multiple Y' elements, this mechanism would lead to an amplification of the Y' elements in the cell. Telomeres composed of expanded tracts of Y' elements retain short tracts of telomeric repeats at their ends.

The formation of Type II survivors requires the presence of *RAD50* (Chen et al., 2001). These survivors maintain their chromosome ends primarily by forming elongated tracts of the TG_{1-3} telomeric sequence, although there can be some amplification of Y' elements (Teng and Zakian, 1999). Type I survivors are at a growth disadvantage as compared to Type II survivors, although both grow more slowly than wild-type cells (Le et al., 1999). One model for the expansion of the TG_{1-3} tract in Type II survivors involves the formation of telomeric loops, the end of which could then prime copying around the entire loop (Chen et al., 2001). The mechanism for the formation of the loops will be described below. Interestingly, the human RAD50p had been proposed to associate with telomeric loops found in human cells (Zhu et al., 2000), which is consistent with the idea that copying telomeric DNA loops could be a step in the *RAD50*-dependent formation of survivors. Formation of Type II survivors also requires the presence of *RAD59*, which normally plays a role in mitotic recombination (Chen et al., 2001). Consistent with the requirement for *RAD50* or *RAD51* for survivor formation, deletion of both *RAD50* and *RAD51* in addition to telomerase causes senescence with no survivors in yeast (Le et al., 1999). In addition, cells lacking both *RAD52* and telomerase do not form survivors (Lendvay et al., 1996). This occurs because *RAD52* is required for both forms of homologous recombination in yeast, so *tlc1 rad52* double deletions have neither telomerase nor recombination to maintain their telomeres. For this reason, when examining the effect of recombination on a pathway it is common to delete *RAD52* in order to knock out the function of both homologous recombination pathways in yeast.

Deletion of the *TER1* gene in *K. lactis* has also been shown to cause senescence, with only a small percentage of the cells continuing to grow as survivors (McEachern and Blackburn, 1996; McEachern and Iyer, 2001). Only Type II survivors have been found. The survivors appear to be generated when one telomere acquires additional telomeric repeats, and this elongated telomere is spread to the other chromosome ends via recombination between the homologous subtelomeric regions. Short telomeres may be elongated by a rolling circle mechanism, where a circle of telomeric DNA is copied many times to generate a long telomeric array. It has been shown that circles containing telomeric sequences can be copied in this manner when introduced into the cells (Natarajan and McEachern, 2002).

TELOMERIC CHROMATIN STRUCTURE

Whether recombination or telomerase is used to maintain telomeres, it is apparent that the cell has mechanisms for monitoring and responding to individual telomeres that are not the appropriate length. Telomerase normally acts on the shortest telomeres in the cell, indicating that it is not the average length of the telomeres but the length of each individual telomere that is monitored (Marcand et al., 1999). The average length of the telomeres can vary a great deal between different species, however; telomeres of only a few hundred nucleotides each are found in yeast (Shampay et al., 1984), while in humans the telomeres are many kilobases long (Harley et al., 1990). The telomere is composed of two different DNA components. At the base of the telomere, immediately adjacent to the subtelomeric DNA, is the double-stranded component of the telomere. In most species, the majority of the telomere exists as double-stranded DNA for most of the cell cycle, and this part of the telomere is bound by many of the proteins that will be described below. The second telomeric component, found at the tip of the telomere, consists of single-stranded telomeric DNA. Often called

a G-overhang because the DNA strand involved in this structure is the T/G-rich strand, the overhang is a 3' extension that varies greatly in length between species (Henderson and Blackburn, 1989; Makarov et al., 1997; Wellinger et al., 1993b; Wright et al., 1997). In ciliates, the phyla in which telomeres were discovered and much of the early telomere work was done, the G-overhang length is tightly regulated. Despite having 6-nucleotide T_2G_4 telomeric repeats, the G overhangs in *Tetrahymena thermophila* are typically 14-15 or 20-21 nucleotides in length (Jacob et al., 2001). The length of the overhang, coupled with the fact that the sequence at the end of the telomere is that copied from the middle, not the end, of the template, indicates that the overhang is not merely a function of telomerase addition. It is thought that formation of the overhang may be due to nucleolytic processing, an idea that will be discussed further in conjunction with yeast models. In *Oxytricha nova*, a species with 8 nucleotide T_4G_4 repeats, the overhang is exactly 16 nucleotides (Gottschling and Zakian, 1986). This corresponds with the sequence required for binding by the α/β heterodimeric single-stranded binding proteins found in that species (Gottschling and Zakian, 1986; Gray et al., 1991; Price and Cech, 1987). In *Euplotes aediculatus*, both the double-stranded and single-stranded telomere component lengths are tightly regulated; the double-stranded length is 28 base-pairs, while the overhang is 14 nucleotides long (Klobutcher et al., 1981).

There are at least 2 important differences between the G-overhangs examined in ciliates and those detected in yeast or humans. First, the length of the G-overhang appears to be shorter in ciliates than in higher eukaryotes. The single-stranded component of a human telomere appears be between 30-200 nucleotides (Huffman et al., 2000; McElligott and Wellinger, 1997; Wright et al., 1997); in *S. cerevisiae* the overhang appears to be less than 30 nucleotides throughout most of the cell cycle, although it increases to approximately 150 nucleotides in a cell-cycle dependent manner (Dionne and Wellinger, 1996; Wellinger et al., 1993b). The length of the overhang also

appears to be less tightly regulated in yeast than it is in ciliates, since is length seems to fall within a range rather than being a precise number of nucleotides. Second, the G-overhang length in ciliates does not appear to be cell-cycle dependent; the overhang length remains constant throughout the cell cycle, although it can be lengthened by one unit repeat during starvation conditions (Jacob et al., 2001). In yeast, however, the formation of single-stranded telomeric overhangs occurs in a cell-cycle dependent manner in wild-type cells (Wellinger et al., 1993b). Formation of the G-overhang occurs in late S phase, which is when subtelomeric and telomeric DNA is replicated (Stevenson and Gottschling, 1999; Wellinger et al., 1993a). Although the seemingly obvious explanation is that the overhangs are seen because the lagging strand has not yet synthesized the complement to the DNA recently added by telomerase, this does not appear to be the case. Instead, there appears to be a resection step in which there is enzymatic degradation of the C/A telomeric strand. This degradation, which leads to the formation of single-strand overhangs in both the presence and absence of telomerase, happens in a 5' → 3' manner (Dionne and Wellinger, 1996).

A number of unusual structures that could be formed by the G-rich telomeric overhangs have been proposed. One well known example is the G-quadruplex or G-quartet structure (Balagurumoorthy and Brahmachari, 1995; Balagurumoorthy et al., 1992). This structure, which is known to be formed *in vitro* with G-rich DNA, offers interesting possibilities for a telomeric cap structure. It has been unclear that the *in vitro* model has any biological relevance, but recent data are beginning to address that issue. The G-quadruplexes that form *in vitro* block telomere addition by telomerase. It was thought that a compound that stabilized the G-quadruplexes *in vivo* might decrease telomerase addition to telomeres in the cell. Compounds that specifically stabilize G-quadruplexes were identified via *in vitro* biochemical experiments and then tested and found to inhibit telomerase function *in vitro* (Riou et al., 2002). Promising compounds

were then used to treat human cells in culture. The compounds that showed the best stabilization of the G-quadruplexes were the same ones found to inhibit telomerase activity *in vitro* and cause growth arrest *in vivo*. This indicates that there may be some biological relevance to the G-quadruplex model, at least in human cells. Another model involves the formation of a loop structure at the telomere end. In this model, first proposed by Griffith et al to describe structures found in *in vitro* human cells, the single-stranded DNA forms a 't loop' that is stabilized by the invasion of the single-strand into the duplex DNA (Griffith et al., 1999). Once strand invasion occurs, the single-strand from the chromosome end can displace a region of the duplex and base-pair with the complementary strand. The process appears to involve telomere-binding proteins that will be described below, although the well-known tumor suppressor p53 has been shown to interact specifically with single-stranded telomeric DNA and t-loop junctions *in vitro* (Stansel et al., 2002). A loop structure has also been reported to exist at micronuclear telomeres in the ciliate *Oxytricha fallax* (Murti and Prescott, 1999) and also at trypanosome telomeres (Munoz-Jordan et al., 2001), indicating that t-loops may be a general mechanism of telomere tip protection.

PROTEINS ACTING AT THE TELOMERE

S. cerevisiae proteins that bind the telomeric single-strand

Although the single-stranded part of the telomere accounts for a smaller fraction of the total telomere length than does the duplex section, this region of the telomere is of critical importance because it is through the chromosome tip that telomerase acts. There are a number of proteins that bind to the single-stranded overhang and modulate telomerase access to the telomere (Fig. 1-2). In *S. cerevisiae*, Cdc13p appears to be a critical component of the telomeric end, interacting with a diverse collection of proteins. Cdc13p binds directly to telomeric single-strands (Lin and Zakian, 1996); deletion of

CDC13 is lethal (Weinert and Hartwell, 1993). It is through interactions with Cdc13p that many of the telomeric tip proteins function. Because some Cdc13p interactions are with proteins that increase telomere length, while others are with proteins that negatively regulate telomere length, alleles of *CDC13* have been found that lead to telomere lengthening, while others cause telomere shortening (Chandra et al., 2001). This is consistent with early analysis of the first two *CDC13* alleles examined (Nugent et al., 1996). *cdc13-1*^{ts} was found to be temperature sensitive, and at the restrictive temperature the telomeric C-strand was degraded, leading to an accumulation of single-stranded DNA that triggers a *RAD9*-dependent G2 arrest (Garvik et al., 1995; Lydall and Weinert, 1995). *cdc13-2*^{est} mutants, conversely, had telomeres that shortened progressively and had a senescence phenotype. These different results led to the conclusion that there was more than one role for Cdc13p at the telomere.

Est1p is perhaps the most important protein that interacts with Cdc13p and affects telomerase function. This interaction has been clearly shown by the identification of an allele-specific suppressor of the phenotype caused by one *cdc13* allele (Pennock et al., 2001). Mutations in Est1p lead to gradual telomere shortening and eventual senescence but do not cause the immediate lethality seen in the absence of *CDC13* (Lundblad and Szostak, 1989). The primary role of Est1p appears to be its interaction with Tlc1, the RNA component of telomerase (Zhou et al., 2000). Although Est1p binds free single-stranded telomeric ends, it does so at a much lower affinity than Cdc13p (Nugent et al., 1996; Virta-Pearlman et al., 1996), indicating that the primary role of Cdc13p may be to form a bridge between the telomere and telomere-associated proteins while Est1p links Cdc13p to telomerase. Consistent with this, fusion of Cdc13p to Est2p bypasses the requirement for Est1p in maintenance of telomere length (Evans and Lundblad, 1999). Recent experiments, however, indicate that Est1p may be a cell-cycle specific activator of telomerase (Taggart et al., 2002).

Cdc13p also interacts with a number of other proteins that affect telomere length, including the catalytic subunit of DNA polymerase α, Stn1p, and the Ku recombination complex. The interaction between Cdc13p and Pol1p, the catalytic subunit of DNA polymerase α, was first identified via a two-hybrid assay (Qi and Zakian, 2000). This interaction was not surprising; certain mutations in DNA polymerase α can lead to telomere elongation (Adams and Holm, 1996; Carson and Hartwell, 1985), and DNA primase and polymerases α and δ are required for telomerase-mediated telomere addition (Diede and Gottschling, 1999). Because DNA polymerase α is the only one of the three yeast DNA polymerases to contain a primase activity, it was predicted to have a significant role in synthesizing the C/A-rich telomeric lagging strand. Mutations that affect the interaction between Pol1p and Cdc13p were identified, and these mutations led to telomerase-dependent telomere elongation (Qi and Zakian, 2000). These results are consistent with the expectation that lagging-strand synthesis of the C/A-rich strand and telomerase-mediated synthesis of the G/T-rich strand are coordinated via interactions with Cdc13p. It is unclear, however, whether Cdc13p recruits both telomerase and the polymerase complexes to work coordinately or if there is competition between the complexes. It is possible that other proteins acting at the chromosome tip play a role in coordinating leading and lagging strand synthesis at the tip of the telomere, perhaps through a Cdc13p-dependent mechanism. One candidate protein is Stn1p (Grandin et al., 1997). Delivery of Stn1p to the telomere can suppress a null mutant of *CDC13*, indicating that it is sufficient for end protection (Pennock et al., 2001). Overexpression of Stn1p can suppress the lengthening defect seen in *pol1* mutant strains (Chandra et al., 2001), consistent with a role in coordinating the lagging-strand synthesis with telomerase-mediated telomere synthesis. Stn1p has also been shown both genetically and biochemically to interact with Ten1p *in vivo* (Grandin et al., 2001).

Ten1 mutant alleles, like *stn1* alleles, can lead to significant telomere elongation, suggesting that they function together in telomere capping. *TEN1*, like *STN1* and *CDC13*, is an essential gene (Grandin et al., 2001). Stn1p appears to compete with Est1p for interaction with Cdc13p at an overlapping binding site, providing another layer of regulation to the complex (Grandin et al., 2000). A model has been proposed in which Cdc13p recruits telomerase to the telomere by binding to the single-stranded overhangs present at S-phase. Stn1p displaces telomerase via a competitive interaction, where it is then able to positively regulate C/A strand synthesis. Once the single-stranded overhang is no longer present, Cdc13p would have no binding site and telomerase recruitment would cease (reviewed in Evans and Lundblad, 2000).

A *S. cerevisiae* protein that binds DNA ends

The fact that the Ku heterodimer is present at telomeres has been a source of confusion, since Ku is known to function in the NHEJ pathway (reviewed in Tsukamoto and Ikeda, 1998). It had been thought that it would be necessary to keep Ku sequestered from telomeric ends lest they be acted on by the pathway and fused together. Inactivation of the Ku complex through the use of a temperature-sensitive (ts) allele or deletion of the genes, however, leads to dramatic telomere shortening (Boulton and Jackson, 1996; Boulton and Jackson, 1998) while at the same time the single-stranded overhangs become longer (Gravel et al., 1998; Porter et al., 1996). Deletion of either of the Ku genes has also been shown to cause a disruption to the localization of telomeres within the nucleus (Laroche et al., 1998), giving further evidence that Ku is involved in telomeric structures. Cells deleted for Ku are temperature-sensitive (Barnes and Rio, 1997; Boulton and Jackson, 1996) and arrest when the *RAD9* checkpoint is activated (Barnes and Rio, 1997; Teo and Jackson, 2001), presumably by the increased amount of single-stranded DNA. In Ku mutant strains, the single-strand overhangs are

present throughout the cell cycle, as compared with the cell-cycle regulated fluctuations in the overhangs found in wild-type cells (Gravel et al., 1998; Polotnianka et al., 1998). This had led to the conclusion that the Ku complex is involved in part of the cap structure at the telomere, and is consistent with evidence that Ku interacts with the telomerase RNA and may play a role in telomerase recruitment or activation. Recent evidence, however, suggests that if the telomeres are made artificially long by introduction of a *RIF1Δ* allele then there is an increase in the number of cell divisions before loss of viability in Ku- cells grown at elevated temperatures (Gravel and Wellinger, 2002). This indicates that the overall duplex telomere length is of greater consequence to the cell than the disruption of a cap structure at the chromosome ends.

S. cerevisiae proteins that bind the duplex telomere

Although much of the activity at the telomere occurs at the single-stranded tip, many of the proteins that bind to telomeres and play critical roles in regulating their length function at the double-stranded region that is internal to the telomeric tip. The best characterized of the double-stranded binding proteins regulating telomere length in yeast is Rap1p [Shore, 1994 #419, reviewed in Marcand et al., 1997b). This protein, which is essential (Shore and Nasmyth, 1987), does not function solely in telomere length regulation. Its name, repressor activator protein 1, stems from its role in transcriptional regulation; Rap1p is an activator of some genes while it is involved in silencing others (Kurtz and Shore, 1991; Shore, 1994). The function that Rap1p serves at a given locus appears to depend on the context at that site, indicating that other regulatory proteins also interact with Rap1p. Rap1p binds to specific sequences that are fairly conserved both between yeast species and between its different places of action (regulatory regions of genes and also at telomeres) (Graham and Chambers, 1994; Idrissi et al., 1998; Idrissi and Pina, 1999). The binding of Rap1p to its binding site

occurs through the interaction of two Myb-like domains (Henry et al., 1990; Konig et al., 1996), each of which binds to part of the consensus site. The DNA binding domains are connected by a flexible hinge region, and is has been shown that there is some degree of flexibility to the spacing between the half-sites of the consensus binding sequence (Konig et al., 1996; Wahlin and Cohn, 2000). The DNA binding domain is the only essential part of the protein, although the C-terminal domain of the protein is the site of interaction for the numerous proteins that interact with Rap1p in association with its regulatory functions (Hardy et al., 1992; Hecht et al., 1996; Moretti et al., 1994; Wotton and Shore, 1997). Overexpression of Rap1p has been reported to be toxic to yeast cells (Freeman et al., 1995), and it is proposed that this is either due to high levels of the protein binding the DNA and affecting transcription or to titration of other Rap1p-interacting proteins in the cell. The *K. lactis RAP1* gene has also been identified (Larson et al., 1994) and will be described in more detail in the section discussing the selection of the experimental system; it appears to function in much the same manner as in *S. cerevisiae*.

Rap1p appears to serve as a negative regulator of telomere length; mutations in the telomere that disrupt its binding can lead to severe telomere elongation in *S. cerevisiae* (Prescott and Blackburn, 2000) and *K. lactis* (Krauskopf and Blackburn, 1996; Krauskopf and Blackburn, 1998). Interestingly, low-level overexpression of Rap1p in *S. cerevisiae* also leads to modest telomere elongation (Conrad et al., 1990), although this is thought to be due to the titration of other factors that limit telomere length. It has been proposed that telomere length in yeast is determined through a Rap1p counting mechanism (Marcand et al., 1997a). This model was the result of experiments in which Gal4p binding sites were inserted near a yeast telomere in a strain in which the C-terminal domain of Rap1p was fused to the Gal4p DNA binding domain. It was found that telomeres in these strains were shortened, as were telomeres in strains that had

additional telomeric sequences were inserted 30-90 nucleotides internal to the telomere. This led to the conclusion that the cell correlates the length of the telomeric tracts with the number of Rap1p molecules present at the telomere and adds new telomeric repeats as needed based on this number.

Rap1p may serve as a nucleation site for the large protein complexes found at telomeres, as it has been shown to interact with a number of proteins (Hardy et al., 1992; Hecht et al., 1996; Moretti et al., 1994; Wotton and Shore, 1997). Rif1p and Rif2p, named because they are Rap1p interacting factors, are involved in regulating telomere length (Hardy et al., 1992; Wotton and Shore, 1997). Mutation of either *RIF* gene can lead to increases in telomere length, while deletion of both genes causes dramatic telomere length elongation (Wotton and Shore, 1997). It has recently been proposed that it is actually the Rif proteins, not Rap1p, that are used to 'count' telomere length (Levy and Blackburn, personal communication). In an experiment similar to that described for Rap1p, Rif1p or Rif2p were artificially targeted to regions near the telomere, where they were found to cause a decrease in telomere length. If the Rif1p and Rif2p components of the telomere complex do nucleate on a Rap1p molecule, then it is not surprising that the same result would be obtained whether Rap1p or either of the two Rif proteins were tethered near a telomere.

Another factor acting at telomeres is the MRX (Mre11/Rad50/Xrs2) complex. The multifunctional complex is involved in double-strand break repair via NHEJ and recombination, as described above. *rad50* cells show a decrease in the amount of the 5'-3' processing that is responsible for single-strand tail formation, indicating that MRX may be involved in forming the single-strand tails necessary to serve as a substrate from telomerase (Sugawara and Haber, 1992). The complex is phosphorylated and activated by Tel1p (Haber, 1998; Ritchie and Petes, 2000). Tel1p is a homolog of the human ATM protein, which is described below, and leads to telomere shortening when deleted

(Greenwell et al., 1995). A related kinase, Mec1p, is thought to facilitate telomerase access in the absence of Tel1p by phosphorylating histones and decondensing the chromatin (Chan et al., 2001). The kinases are not believed to affect telomerase function, as a telomerase allele leading to long telomeres is still functional in *tel1 mec1* cells (Chan et al., 2001; Tsukamoto et al., 2001).

Telomeric silencing is another phenomena associated with the ends of the chromosome. A form of transcriptional repression thought to be caused by unusual chromatin structures, the silencing of genes near telomeres has been used in a manner similar to telomere length to gauge the function of the telomeres. A number of the genes shown to influence telomere length also affect telomeric silencing (reviewed in Grunstein, 1997). One such group of genes not described above is the SIR (silent information regulator) genes. The SIR proteins are also thought to be recruited by the C-terminal domain of Rap1p (Moazed et al., 1997; Moretti et al., 1994; Strahl-Bolsinger et al., 1997). These genes have the opposite effects of the Rif1p and Rif2p genes (Grunstein, 1997), despite interacting with the telomere through the same nucleating protein; mutations lead to modest telomere shortening and a decrease in telomeric silencing (Palladino et al., 1993). There may be a competitive interaction between the Rif and Sir complexes at the Rap1p C-terminal. Two Sir proteins, Sir3p, and Sir4p, have been shown to interact both with each other and with Rap1p by two-hybrid assays and immunoprecipitation experiments (Cockell et al., 1995; Hecht et al., 1996; Moretti et al., 1994; Strahl-Bolsinger et al., 1997). Sir2p interacts with Sir4p and is thus a part of the complex (Moazed and Johnson, 1996; Strahl-Bolsinger et al., 1997). These proteins also interact with the histones (Hecht et al., 1995; Hecht et al., 1996), where Sir2p acts as a histone deacetylase (Imai et al., 2000), when present at non-telomeric loci. This information has led to a model for a fold-back structure at the telomere, which would allow the proteins bound to the telomere to interact with the Sir complexes associated

with subtelomeric histones to form telomeric heterochromatin (Grunstein, 1997; Luo et al., 2002).

Telomere binding proteins in the fission yeast *S. pombe*

All of the yeast telomere-associated proteins described thus far have been from the model yeast *Saccharomyces cerevisiae*, a budding yeast. Telomere-related work has also been done in the fission yeast *Schizosaccharomyces pombe*. The differences between the mechanisms of telomere length regulation in the different types of yeast will be described briefly. Although *S. pombe* homologs have been found for both Rap1p and Rif1p, they appear to function somewhat differently than in budding yeast (Kanoh and Ishikawa, 2001). The critical determinant of telomere function in *S. pombe* seems to be Taz1p (Cooper et al., 1997), a protein with no real homolog in budding yeast despite similarity with the human TRF1 and TRF2 proteins (described below). Mutations in *taz1*$^+$ lead to telomere elongation, although *taz1*$^+$ is only required under nitrogen starvation conditions (Godhino-Ferreira and Promisel Cooper, 2001). Taz1p also appears to play a critical role in capping the telomeres, preventing them from being acted in by the Ku recombination machinery (Godhino-Ferreira and Promisel Cooper, 2001). Interestingly, Taz1p is thought to be involved in recruiting *S. pombe* Rap1p and Rif1p homologs (spRap1p and spRif1p) to the telomeres, where association of spRif1p is not dependent on spRap1p (Kanoh and Ishikawa, 2001). Taz1p has been shown to associate with both spRap1p and spRif1p by two-hybrid assay, although spRap1p did not associate with spRif1p. This is quite different than in *S. cerevisiae*, where Rif1p is thought to be recruited through an interaction with the C-terminal tail of Rap1p. Although deletion of *rap1*$^+$ and *rif1*$^+$ leads to telomere elongation, neither gene is required in *S. pombe*. A recently discovered *S. pombe* telomere binding protein, Pot1p, also has no known budding yeast homologs (Baumann and Cech, 2001). It has been reported to bind G-

strand telomeric DNA and may be similar to Cdc13p in function; like Taz1p, Pot1p has a close human homolog. A further discussion of this activity is given in the section about human telomere binding proteins.

RELEVENCE TO HUMAN DISEASE

Telomere pathways are implicated in a number of human diseases. This is due to the fact that, unlike yeast and even mice, telomerase is normally not active in most human somatic tissues (Harley et al., 1994; Newbold, 1997; Wright et al., 1996). Because human telomeres are 10-15 kilobases long (Allsopp et al., 1992; de Lange et al., 1990) (as compared to hundreds of nucleotides in yeast) the telomeres do not typically appear to shorten enough to cause problems during a human lifetime. This does, however, cause the cells to have a limited lifespan in culture, as their telomeres will eventually shorten and cause the cells to enter replicative senescence, the point where human cells stop dividing (reviewed in Hayflick, 1997). If the cells are then transformed with a viral oncogene, such as SV40, they will continue to divide until they reach crisis (Hara et al., 1991; Shay et al., 1991). Very few cells survive crisis, but ectopic expression of the telomerase reverse transcriptase component (hTERT) can bypass senescence, prevent crisis, and allow cells to become immortalized (Bodnar et al., 1998; Vaziri and Benchimol, 1998; Wang et al., 1998). Cellular senescence can also be bypassed by ectopic hTERT expression, indicating that the telomere shortening that occurs in its absence may be a cause of both senescence and crisis. The concept of limited cellular lifespan due to telomere loss has been linked to the aging process, although no causality has been shown (Hayflick, 1997). It is also thought to be a barrier to carcinogenesis, as cells would have to divide many times in order to form a cancer. One requirement for cancer formation, therefore, is that the cells must eventually find some way to maintain their DNA ends. Typically this is done through the reactivation of

telomerase (Counter et al., 1992), the expression of which is often upregulated (Kim et al., 1994). Some cells, however, develop other methods for maintaining their telomeres called ALT (for alternative lengthening of telomeres), which is thought to be recombination-mediated (Bryan et al., 1997; Bryan et al., 1995, and reviewed in Henson et al., 2002). Human telomeres are also bound by a number of proteins, which are thought to play a role in signaling telomere length to the cell-cycle machinery and the apoptotic pathways.

TRF2 is a crucial component in mammalian telomere protection (reviewed in (de Lange, 2002). It coats the telomere throughout the cell cycle, interacting directly with the double-stranded component of the telomere (Bilaud et al., 1997; Broccoli et al., 1997). It acts as a negative regulator of telomere length, and also plays a role in stabilizing the single-stranded overhang (van Steensel et al., 1998). It is found, along with TRF1, at the t-loops that occur at the tips of human telomeres (Griffith et al., 1999; Stansel et al., 2001). Interestingly, the tumor suppressor p53 has also been shown to interact with human t-loops *in vitro* (Stansel et al., 2002). Known functions of p53 include its role as a transcriptional activator, its roles at the G1 and G2 checkpoints (reviewed in Cox and Lane, 1995), and its role in responding to telomeric damage (Chin et al., 1999; Karlseder et al., 1999). It is known to have high affinity to single-stranded DNA, and recent work from indicates that it may play a role in the formation of the telomeric t-loop (Stansel et al., 2002). TRF2 also plays a role in recruiting other complexes to the telomere; hRAP1, the human homolog of the yeast Rap1p that was discussed in detail in the preceding section (Li et al., 2000), and the MRE11 complex (Zhu et al., 2000) which, based on data from yeast, is expected to be involved in recombination and play a role at telomeres. DNA-PK, a protein kinase involved in DSB repair, is a heterotrimeric complex that contains the human Ku proteins and is also found to interact with the telomeres, TRF1, and TRF2 (Bailey et al., 1999; d'Adda di'Fagagna et al., 2001; Hsu et al., 1999; Samper

et al., 2000). TRF1 is also a negative regulator of telomere length and appears to be involved in the pairing of telomeric tracts to form a more complex structure (Griffith et al., 1998). It is regulated by at least two different types of proteins (reviewed in Goytisolo and Blasco, 2002). TIN2 interacts directly with TRF1 and is thought to positively regulate telomeric repeat pairing; it is also a negative regulator of telomere length (Kim et al., 1999). The other type of regulation occurs through the homologous proteins TANK1 and TANK2 (Kaminker et al., 2001; Smith et al., 1998). TANK1 poly-ADP-ribosylates TRF1 to inactivate it, while TANK2, which has been shown to interact with TRF1, might affect telomere length through its action on other proteins.

In addition, the protein POT1 has been reported to be present at human telomeres. Unlike telomerase, *POT1* appears to be a housekeeping gene and is transcribed constituitively in a number of tissues (Baumann and Cech, 2001). It binds to the G-rich single-stranded region of the telomere, and is homologous in function and sequence to the Pot1 telomere-binding protein found in the fission yeast *S. pombe*. The authors propose that Pot1p may be involved in recruiting telomerase or simply end protection, as its deletion in *S. pombe* leads to rapid telomere shortening (Baumann and Cech, 2001).

A number of human diseases are attributed to defects in genes involved in telomeres. In patients with premature aging syndromes such as *Ataxia telangiectasia* (AT), Bloom's syndrome, and Werner syndrome, the genes ATM, BLM, and WRN, respectively, are mutated. ATM is involved in the phosphorylation and activation of p53 to signal DNA damage (Banin et al., 1998; Canman et al., 1998; Khanna et al., 1998); p53 activation would be expected to trigger a DNA damage checkpoint. It is also involved in p21-mediated cell cycle arrest (Barlow et al., 1997; Xu et al., 1998). Both BLM and WRN are members of the RecQ family of DNA helicases (reviewed in Wu and Hickson, 2001). Although it is not clear how the helicases are involved in the premature

aging found in patients, three lines of evidence indicate that telomeres and telomere dysfunction may play a role in the diseases. First, BLM has been shown to localize to telomeres (Yankiwski et al., 2000). Second, reintroduction of telomerase prevents the unusually rapid senescence of Werner cells in culture (Wyllie et al., 2000). Third, this class of helicases is required for one type of recombinational telomere elongation in yeast (Cohen and Sinclair, 2001).

One model for how these helicases may be involved in human disease stems from an examination of their interactions with telomeric chromatin. In one experiment, attempts to amplify telomeres through PCR led to the apparent formation of a network of telomeric DNA that did not run into an agarose gel. Treatment with the WRN helicase, however, caused the structure to be resolved so that it could enter the gel (Ohsugi et al., 2000). Although these structures have not been reported *in vivo*, this experiment shows one potential role for helicases at telomeres. In another experiment, cells from $ATM^{-/-}$ mice were examined. Although the telomeres were short, there were extra telomeric spots seen in the cells from $ATM^{-/-}$ mice as compared with wild-type (Hande et al., 2001). This was attributed to the presence of extrachromosomal broken DNA molecules. When the experiment was repeated with human cells taken from AT patients, roughly half of the cells were found to contain extrachromosomal telomeric DNA (Hande et al., 2001). Interestingly, the presence of extrachromosomal circular telomeric DNA has been reported in telomerase-negative immortalized human cells (Ogino et al., 1998). In these cells, the telomeric DNA has been reported to be in both the cytoplasm and the nucleus. Although it is unclear both what the effects of having extrachromosomal telomeric DNA would be and whether it is a cause or an effect of the defect, the findings are not confined to mammals. Extrachromosomal fragments of telomeric DNA have also been reported at certain stages of the developing wheat embryo, and these fragments disappear with further development (Bucholc and Buchowicz, 1995).

Typically, mouse is used as a model of human disease. In issues associated with telomeres, however, there are significant differences between mice and humans. First, mice, like yeast, have constituitively active telomerase (Kipling and Cooke, 1990; Martin-Rivera et al., 1998; Prowse et al., 1993; Prowse and Greider, 1995; Zijlmans et al., 1997). When telomerase knockout mice were made (Blasco et al., 1997), the mice were found to be viable and healthy (Rudolph et al., 1999). After several generations, however, the mice began to develop problems. By the 4^{th} generation the litter sizes were reduced, and by the 6^{th} generation both male and female mice showed significantly reduced fertility; eventually the mice became completely infertile. It was found that their reproductive tissues were not developing properly and never reached full adult size (Lee et al., 1998). Lymphocytes taken from late generation mice and grown in culture showed abnormal chromosome structure and end-to-end chromosome fusions at DNA ends lacking telomeres, and cells from these mice also showed increased levels of aneuploidy (Lee et al., 1998). Because progressive shortening of telomeres in cultured cells had been shown to limit replicative capacity, it was desirable to see if any correlation existed between telomere length and aging at the organismic level. Although certain symptoms associated with aging, such as gray hair and hair loss, were found to occur early in late-generation telomerase-deficient mice, there was no premature aging as determined by tests for the development of age-related disorders such as osteoporosis, cataracts, or arteriosclerosis (Rudolph et al., 1999). The mice did, however, show an increased early mortality of 'natural causes' for which no definite cause of death could be determined. These results indicate that, although the mouse model may mimic certain human conditions associated with telomere defects, it may be difficult to utilize the model organism. In order to duplicate the telomere loss, it is necessary to knockout telomerase from the mice, which will lead to infertile mice within 6 generations.

SELECTION OF EXPERIMENTAL SYSTEM

Kluyveromyces lactis was chosen for this study because it has several advantages for the study of telomeres. First, it has many of the useful features found in the commonly used model *Saccharomyces cerevisiae*: the ability to exist as either a haploid or a diploid for easy genetic manipulation, the ability to mate and be easily transformed for the introduction of new gene alleles, the availability of strains with selectable markers, and even the ability to use *S. cerevisiae* genes to complement *K. lactis* genomic deletions. In the field of telomere research, however, *K. lactis* has several advantages over *S. cerevisiae*. First, *K. lactis* has perfect 25 nucleotide telomeric repeats (McEachern and Blackburn, 1994). Typically, telomeres are composed of perfect repeats but in *S. cerevisiae* the repeats are very heterogeneous (Cohn et al., 1998; Shampay et al., 1984). The heterogeneity appears to be due to incomplete copying of the template (Forstemann et al., 2000; Forstemann and Lingner, 2001), and makes it more difficult to study the effects of mutations made in the template because they will not be copied into every telomeric repeat. Although the 25 nucleotide repeat in *K. lactis* is longer than those of most organisms, this long repeat has advantages for telomere researchers. As described above, a large number of proteins bind to and act on telomeres. With a very short repeat, the proteins must have overlapping binding sites; mutation of a telomeric repeat is probably disrupting a potential binding site for multiple different proteins. In *K. lactis*, however, the chances of a particular change in the telomere disrupting only a single binding site are increased. There is even the possibility that it will be possible to identify discrete functional regions of the telomeric repeat, which would be expected to correspond to specific protein binding sites. In addition, analysis of the Southern blots used to examine telomeres is less complicated in *K. lactis* for 2 reasons. First, *K. lactis* blots have fewer bands since

the cells contain only 6 chromosomes (12 telomeres); in *S. cereviseae* there are 16 chromosomes or 32 telomeres. Second, in *K. lactis* there are no telomeric sequences at subtelomeric locations. This ensures that there is no complexity caused by the presense of non-telomeric bands that hybridize with a telomeric probe. Finally, Rap1p, the critical double-stranded telomere binding protein, has been isolated in *K. lactis* (Larson et al., 1994) and shown to behave similarly to *S. cerevisiae* Rap1p (Krauskopf and Blackburn, 1996; Krauskopf and Blackburn, 1998), indicating that the large telomeric repeats are subject to some similar regulatory mechanisms as the smaller heterogeneous repeats. A *K. lactis* homolog of *STN1* has also been identified (S. Iyer and M.J. McEachern, unpublished data).

Like *S. cerevisiae*, mutations in the *K. lactis* telomerase template can lead to extreme changes in telomere length (McEachern and Blackburn, 1995; McEachern and Iyer, 2001; McEachern et al., 2000). Typically the mutations lead to either immediate telomere elongation or telomere shortening, which may occur more gradually. Three unique telomere-and-telomerase-associated phenotypes had been previously identified, making *K. lactis* an interesting organism for telomere research. First, *K. lactis* has the only phenotypically silent telomerase template mutation described to date (McEachern et al., 2002). This telomerase allele, *ter1-7C*, creates a *Bcl*I restriction site, making it particularly useful for examining telomere dynamics in the wild-type situation without perturbing the system. Second, telomeric fusions, in which the chromosome ends become joined in an end-to-end manner, have been found in mutants severely disrupted for Rap1p binding (McEachern et al., 2000). Third, an unusual delayed elongation phenotype has been identified (McEachern and Blackburn, 1995). The telomeres initially appear to be short, but when the cells are passaged the telomeres suddenly become very long. These mutations are outside of the Rap1p binding site and therefore their primary defect is not predicted to be in Rap1p binding. In addition, initial

characterization of the telomere length defects seen in some of the mutants has been done. It has been shown that, in some of the mutants with very long telomeres, Rap1p binding is reduced 30-100 fold, while in delayed elongation mutants there is no reduction of Rap1p binding (Krauskopf and Blackburn, 1996). Experiments have also shown that, by deleting the C-terminal conserved part of the Rap1p molecule, the rate of telomeric turnover at the telomeric tip can be increased when combined with a delayed elongation *TER1* allele (Krauskopf and Blackburn, 1998). *In vitro* binding of the Rap1p molecule to telomeric DNA is unchanged in the truncation. Interestingly, this is the part of Rap1p thought to nucleate the interactions with Rif1p and Rif2p and thus be involved in telomere tract 'counting' in *S. cerevisiae* (Hardy et al., 1992; Wotton and Shore, 1997).

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FIGURES

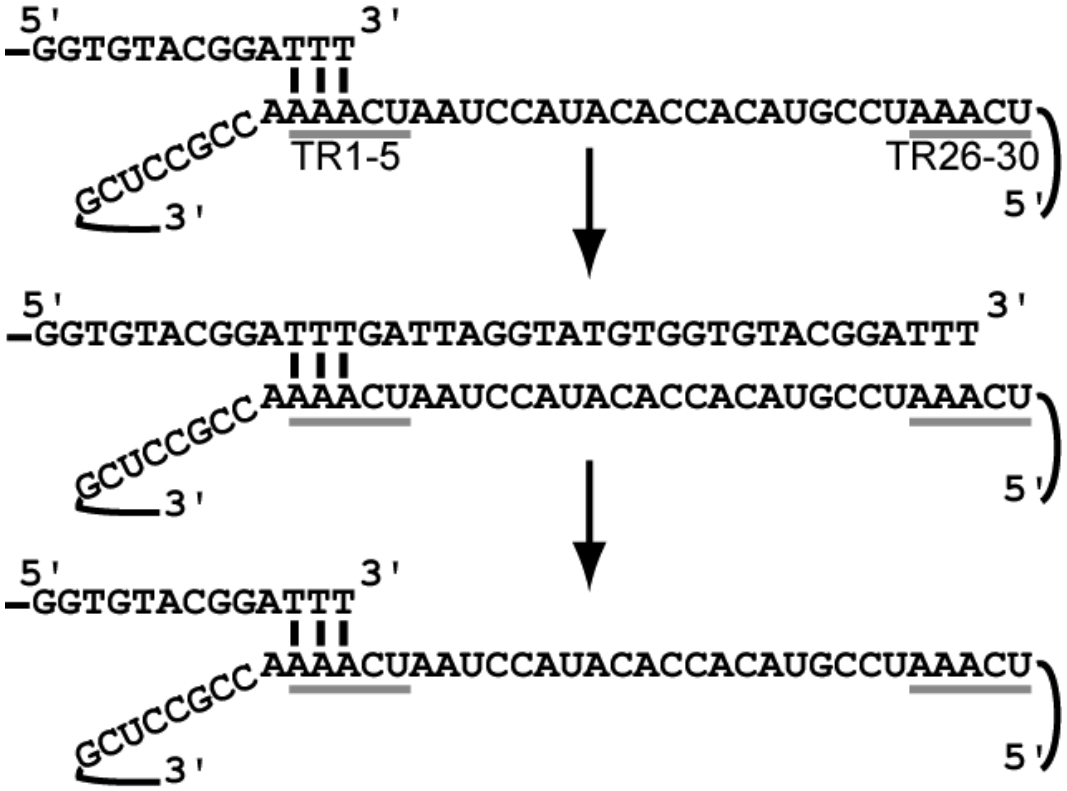

Fig. 1-1. Model for telomerase translocation in *K. lactis*. The top panel shows the telomerase RNA aligning with the telomere to synthesize a new telomeric repeat. The terminal repeats that define the end of the template are underlined and labeled. The center panel shows the synthesis of a new telomeric repeat. Following telomere repeat synthesis, telomerase can dissociate from the telomere and, using the terminal repeats of the template, realign with the 3' terminus of the telomeric repeat.

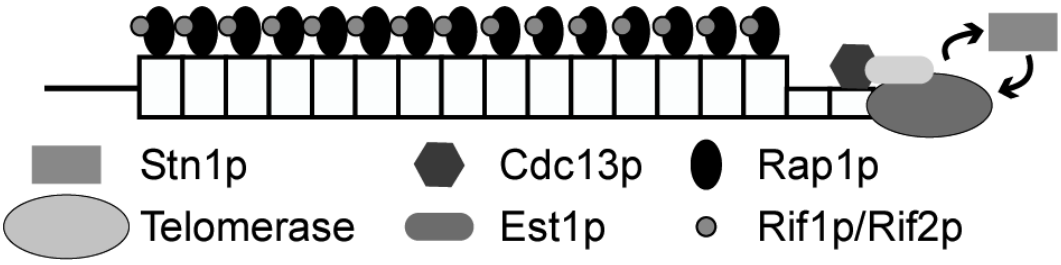

Fig. 1-2. The telomeric repeats are bound by many proteins. Both the double-stranded and single-stranded components of the telomere are bound by proteins. This figure is a simple model which includes some of the important telomere-binding proteins. The double-stranded component (indicated by the large white boxes) are bound by Rap1p, which interacts with Rif1p and Rif2p. The single-stranded region of the telomere (shown with small white boxes) is bound by Cdc13p. Est1p, Stn1p, and telomerase all interact with this part of the telomere.

CHAPTER 2

TOTALLY MUTANT TELOMERES: SINGLE-STEP MUTAGENESIS OF TANDEM REPEAT DNA SEQUENCES[1]

[1] Underwood, D. H., and McEachern, M. J. (2001). Totally mutant telomeres: single-step mutagenesis of tandem repeat DNA sequences., Biotechniques *30*, 934-5, 938.

Telomeres, the tandem repeats that cap the ends of linear eukaryotic chromosomes, are difficult to study molecularly. They are present at the end of each chromosome in the cell, and each telomere is composed of multiple tandem repeats. Also, telomeres are replicated by a combination of two processes (Blackburn, 1994). Most telomeric sequences are replicated, like the rest of the genome, by DNA polymerase. The sequence lost when telomeric tips are not fully replicated is added by telomerase, a reverse transcriptase. Telomerase is a ribonucleoprotein, and its RNA component contains the template for new telomere synthesis. One method for studying telomeres *in vivo* involves mutating the telomerase RNA template, causing altered telomeric repeats to be added to the chromosome ends. Although very powerful because the tips of every telomere in the cell are mutated, this approach has two drawbacks. First, telomerase only acts at the tip of the chromosome, so internal repeats remain wild-type even after long-term growth (McEachern and Blackburn, 1995; McEachern et al., in preparation; Yu and Blackburn, 1991); basal wild-type repeats can have a marked effect on telomere function (Marcand et al., 1997; McEachern and Blackburn, 1995). Second, telomerase template mutants alter both the telomeres and telomerase. Because certain telomerase template mutations can affect telomerase function (Gilley et al., 1995; Yu and Blackburn, 1991) it can be difficult to discern the true cause of a mutant phenotype. In order to help circumvent these problems, we have developed a technique that can create a telomere composed solely of mutant repeats and is not dependent on an altered telomerase template.

Oligonucleotide mutagenesis protocols often involve mutating two sites simultaneously; one site is the desired mutation and the second is a vector mutation that reduces the background of non-mutated plasmids. We investigated whether this approach could be used to simultaneously mutate every telomeric repeat in a cloned yeast telomere. Mutagenesis was done on a pBluescript plasmid containing a cloned

wild-type *K. lactis* telomere, the associated subtelomeric sequence, and a *URA3* gene (Fig. 2-1A). The telomeric insert was comprised of 11.5 repeats, which is within the normal size range for *K. lactis* telomeres (McEachern and Blackburn, 1995); each repeat was composed of an identical 25 base pair sequence.

The first total telomere mutant we attempted to make required a single base change in each repeat. This mutation, referred to as Bcl because it creates a *Bcl*I restriction site, is phenotypically silent when made by telomerase (McEachern et al., 2002; Roy et al., 1998) and is therefore expected to be useful for studying 'wild-type' telomeres. An introduced restriction site facilitates the study of telomeres *in vivo* and is also useful for screening mutagenized plasmids. The sequence to be altered was composed of 25 base pair repeats, so a 25nt oligonucleotide containing the mutation near the center was used for the mutagenesis. Because the first and last repeats of the telomere border non-telomeric sequence, making some mutations would not be possible with only one mutagenic oligonucleotide. Due to the position of the nucleotide altered in the Bcl mutation, however, one oligonucleotide would be expected to be sufficient to mutate every repeat containing the targeted site (Fig. 2-1B).

A modified Kunkel mutagenesis protocol was followed (Kunkel et al., 1987). The plasmid to be mutagenized was made single stranded using a standard helper phage. Approximately 500ng of the single stranded template was annealed with 60ng and 30ng, respectively, of kinased mutagenic oligonucleotide and T7 oligonucleotide in a volume of 12.5µl. The T7 oligonucleotide was used to initiate DNA synthesis from a second site on the plasmid. An approximately 25-fold molar excess of oligonucleotide was added, so that the number of mutagenic oligonucleotides exceeded the number of target sites. The reaction was put in a 65° C heat block and allowed to slow-cool to 30° C. Once the annealing reaction was completed, a 10X reaction mix, composed of 2.5µl of synthesis mix (100mM HEPES, pH 7.8, 10mM DTT, 50mM $MgCl_2$, 2.5mM of each dNTP, and 5mM

ATP), 4.5 Weiss units ligase, and 0.4 units T4 polymerase (as defined by New England Biolabs), was added. The reaction was mixed on ice, incubated at room temperature for 5 minutes, and then the primer extension and ligation reaction was done at 37C° for 3-4 hours.

The synthesis products were transformed into *E. coli* XL1 by a standard heat-shock protocol and plasmids were extracted from the resulting colonies. Because *Bcl*I does not digest dam methlylated DNA from *E. coli*, the plasmids were screened by digestion with *Sau*3AI. Each of four plasmids tested contained novel *Sau*3AI sites. One of these was sequenced, and was found to have the expected base change at each of the eleven sites.

We next attempted to generate a total telomere mutant containing the Kpn mutation, which generates a *Kpn*I restriction site by changing two bases in the telomeric repeat. The Kpn telomerase mutant has an unusual length regulation defect that results in elongation of the telomeres only after hundreds of cell divisions (McEachern and Blackburn, 1995). Lengthening occurs only after a sufficient number of basal wild-type repeats have been replaced with mutant ones. For the Kpn telomere, eleven repeats could be altered using a single oligonucleotide, as was done with the Bcl total telomere mutant. The partial repeat bordering the subtelomere does not contain the positions being altered in the Kpn mutant but may retain a binding site for Rap1p, a negative regulator of telomere length (Krauskopf and Blackburn, 1998). To disrupt Rap1p binding and thus remove the negative regulation possibly conferred by the border repeat, we simultaneously added an oligonucleotide that targeted a base change to the fourth nucleotide of the telomeric repeat adjacent to subtelomere. This mutation, if incorporated, would generate an *Acc*I restriction site (Fig. 2-1C), and is known to cause telomere elongation (McEachern and Blackburn, 1995). The procedure was done as

described above for the Bcl mutant, except that 6ng of kinased oligonucleotide specific to the basalmost repeat was also used.

All 4 plasmid clones recovered contained novel *Acc*I and *Kpn*I sites as judged by restriction digests. These clones were then sequenced and one containing all the expected mutations was identified. Those clones that were not totally mutated had most repeats containing the expected Kpn mutation but they also contained 1-3 wild-type repeats. It is possible that the failure to mutate all repeats was due to an insufficient amount of Kpn oligonucleotide in the reaction.

To introduce the total Bcl telomere into *K. lactis*, an *Eco*RI + *Sac*II digest was first done to cut out the telomere and its associated *URA3*-tagged subtelomeric sequence (see map, Fig. 2-1A). The digest was then transformed into a *ura3 K. lactis* strain, where one telomere was replaced through homologous recombination between the subtelomeric sequences. Figure 2-2 shows restriction digests with *Sma*I and *Bcl*I that confirm the presence of the totally mutated Bcl telomere in the yeast. *Sma*I cleaves the *URA3*-containing telomere into a fragment, which then separates from the other telomeric fragments in the cell. As expected, *Bcl*I digestion cleaved away the *URA3*-Bcl telomere but not a control *URA3*-wild type telomere. In other experiments, by transforming the Bcl total telomere into a *K. lactis* strain containing a mutant telomerase RNA gene (*TER1*) that adds Bcl repeats onto telomeric ends, we have made strains with one telomere completely composed of Bcl repeats (data not shown).

In conclusion, we have shown that oligonucleotide mutagenesis can be used to simultaneously alter every repeat in a tandem array of short repeats. This has allowed us to generate a “totally mutant” telomere in yeast. Although it is not clear if our approach can be used in systems with typical short telomeric repeats, it will be a valuable tool in systems like *K. lactis* that contain long uniform telomeric repeats. In addition, it could be used for mutagenesis of other kinds of repeated sequences. It is

possible that by increasing the molar excess of mutagenic oligonucleotide the procedure can be adapted to simultaneously mutate more repeats or smaller repeats.

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FIGURES

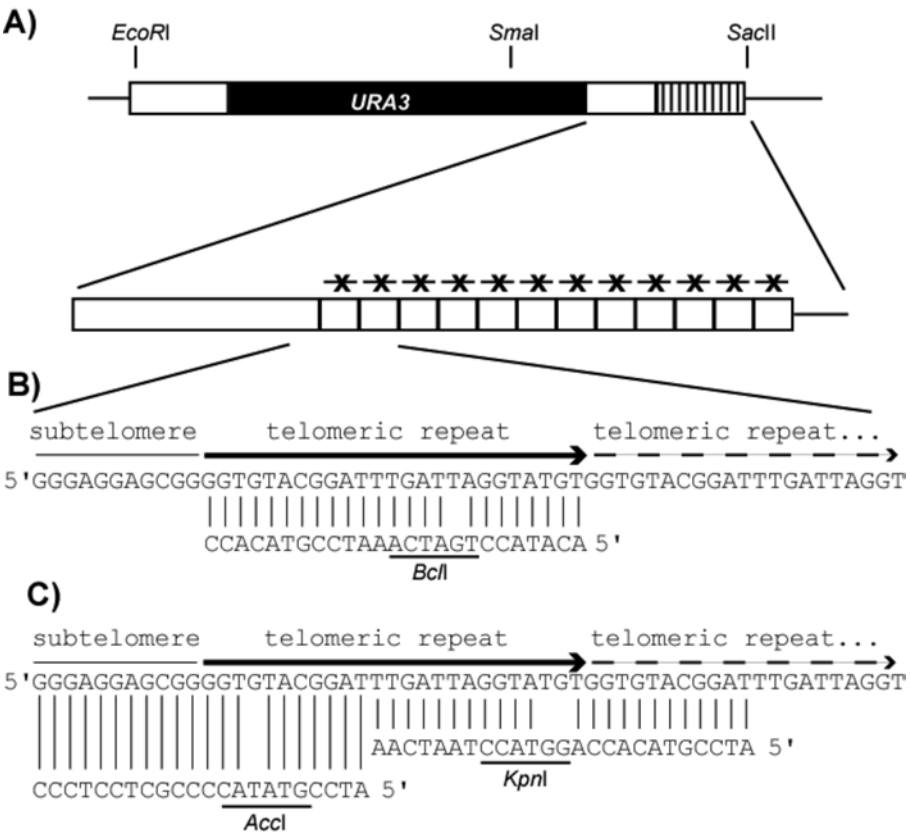

Figure 2-1. Mutagenesis procedure. A) The part of the plasmid being mutagenized and transformed into yeast is shown. White boxes indicate the *K. lactis* subtelomeric region. Unlabelled small boxes indicate telomeric repeats; mutagenic oligonucleotides are indicated by lines marked with X. B) The oligonucleotide used to make the Bcl mutation is shown base-paired to the single stranded template. The dashed lines indicate that there are additional telomeric repeats that bind the oligonucleotide as shown for the first repeat. C) Two oligonucleotides were used to make the Kpn mutatation. One oligonucleotide was used to mutate 11 of the repeats, while the other altered the repeat adjoining the subtelomere. The Acc oligo specific to the basal repeat was complementary to part of the subtelomeric sequence. The dashed lines indicate that there are additional telomeric repeats that bind the oligonucleotide as shown for the second repeat.

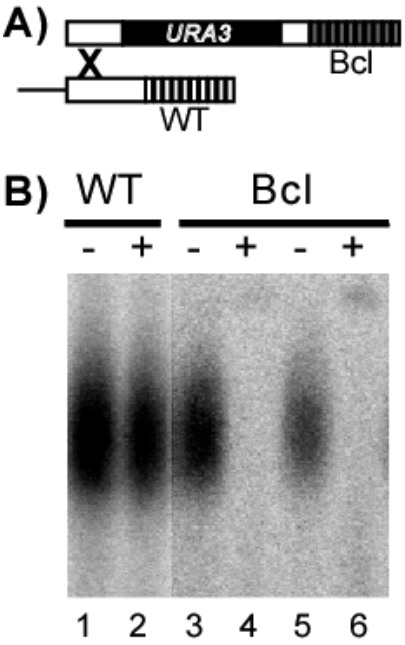

Figure 2-2. Transformation of totally mutated telomeres into yeast. A) The recombination event required for integration of a totally mutated telomere into a yeast chromosome is shown. The white boxes indicate subtelomeric sequences, the black box is the *URA3* gene, the gray boxes represent the Bcl telomere, and the small white boxes are the WT telomeric repeats. Because there is selection for the *URA3* gene, recombination must occur with the centromere-proximal subtelomeric sequence. Eleven of the 12 *K. lactis* subtelomers contain homologous sequence, so by using one of those subtelomeres it is possible to integrate the totally mutated telomere at any one of those 11 telomeres. B) DNA from *K. lactis* cells transformed with a fragment containing the totally mutated telomere is cut in the *URA3* gene with *Sma*I, which separates the *URA3*-tagged telomere from the native telomeres on a gel. Lanes 1-2 are of a wild-type *URA3*-tagged telomere, while lanes 3-6 show a Bcl *URA3*-tagged telomere. The clones are digested with *Sma*I alone (lanes marked -) or *Sma*I + *Bcl*I (lanes marked +).

CHAPTER 3

GENETIC DISSECTION OF THE *K. LACTIS* TELOMERE AND EVIDENCE FOR TELOMERE CAPPING DEFECTS IN *TER1* MUTANTS WITH LONG TELOMERES[1]

[1] Underwood, D.H., Natarajan, S., Carroll, C., and McEachern, M.J. To be submitted to Genes and Development.

ABSTRACT

A genetic dissection of the *K. lactis* telomere was performed by using mutant telomerase RNA (*TER1*) alleles to incorporate mutated telomeric repeats. This analysis has shown that each telomeric repeat contains several functional regions, some of which may physically overlap. Mutations in the right side of the Rap1p binding site and in the terminal repeats of the template typically lead to telomere shortening, while mutations in the region adjacent to the 3' terminal repeat exhibit elevated recombination despite having telomeres of nearly wild-type length. Mutations in the left half of the Rap1p binding site lead to the formation of long telomeres that are suppressed in the presence of high-copy *RAP1*. In contrast, mutations outside but immediately adjacent to the left side of the Rap1p binding site cause a delayed telomere elongation phenotype, and mutated telomeric repeats from one delayed elongation mutant are shown to be defective at regulating telomere length in cells with wild-type telomerase. Mutants with highly elongated telomeres also exhibit signs of telomere capping defects, including elevated levels of subtelomeric recombination and the largely *RAD52*-dependent formation of extrachromosomal and single-stranded telomeric DNA.

INTRODUCTION

Telomeres are complexes composed of proteins and repetitive DNA sequences that cap the ends of linear chromosomes, protecting them from DNA loss during chromosomal replication and from nucleolytic attack. Although the typical telomeric repeat is 5-8 nucleotides long, some yeast species have telomeric repeats that are much longer (McEachern and Blackburn, 1994). In the budding yeast *K. lactis* the telomeres are composed of perfect copies of a 25 nucleotide repeat. The long, homogeneous repeats make this species ideal for genetic dissection of the functions occurring at the telomere. Telomeres are synthesized by telomerase, a ribonucleoprotein containing the

template for new telomere synthesis in its RNA component (reviewed in (Weilbaecher and Lundblad, 1999). In order to synthesize *K. lactis'* 25 nucleotide repeat, a 30 nucleotide template is utilized; the first five nucleotides (terminal repeat positions 1-5, or TR1-5) are identical to the last five nucleotides (TR26-30), which provides telomerase with the ability to base-pair with the telomere for new DNA synthesis (Fulton and Blackburn, 1998). A mutation made in the telomerase template will typically be copied into the telomere during DNA synthesis (McEachern and Blackburn, 1995; Prescott and Blackburn, 1997; Singer and Gottschling, 1994; Yu et al., 1990). It is thus possible to examine the effects of mutating specific nucleotides of the telomeric repeat. This type of analysis is complicated by the fact that both the telomerase template RNA and the telomere are altered; determining which is the cause of any defects seen can be difficult.

Yeast telomeres are composed of tracts of duplex telomeric repeats, with a region of single-stranded DNA at the telomeric tip that is seen in a cell-cycle specific manner (Wellinger et al., 1993). The length of the duplex telomeric tract is regulated via the effects of several proteins (reviewed in Greider, 1996), the most important of which appears to be the Rap1p, an essential gene product that is also involved in transcriptional regulation (reviewed in Shore, 1994). Rap1p binds directly to double-stranded telomeric repeats in both *S. cerevisiae* and *K. lactis* (Krauskopf and Blackburn, 1996; Longtine et al., 1989), where it recruits other proteins such as Rif1p and Rif2p (Hardy et al., 1992; Wotton and Shore, 1997). The number of Rap1p molecules bound to the telomere also appear to be 'counted', such that new telomeric repeats are not added when the appropriate number of Rap1p molecules are bound at the telomere (Marcand et al., 1997a). In this manner Rap1p acts as a negative regulator of telomere length, and may be involved in bending the telomeric DNA (Gilson et al., 1993; Muller et al.; Vignais and Sentenac, 1989). Cdc13p, on the other hand, is a single-strand telomere binding protein that appears to interact with other proteins that act on telomeric

ends (Chandra et al., 2001; Evans and Lundblad, 1999; Lin and Zakian, 1996; Nugent et al., 1996; Pennock et al., 2001). The telomerase complex interacts with Cdc13p through Est1p, while Stn1p appears to bring a negative regulatory function to the telomeric tip through its interactions with Cdc13p and Ten1p (Grandin et al., 2001; Grandin et al., 1997). *CDC13* and *EST1* are both essential in *S. cerevisiae*, where Cdc13p and Est1p play crucial l roles in providing the protective capping function at telomeres.

Experiments in *Saccharomyces cerevisiae* (Prescott and Blackburn, 2000), as well as in *K. lactis*, have shown that altering the telomeric sequence can have dramatic consequences to the cell in terms of telomeric length regulation (McEachern and Blackburn, 1995; McEachern et al., 2000). In *K. lactis*, point mutations in the *TER1* template that produce telomeric repeats with the corresponding change have been shown to cause several different telomere length phenotypes. Mutations in the left half of the Rap1p cause telomere elongation, and in some instances the uncapped telomeres fuse. Other mutations lead to telomere shortening, which also appears to signal telomere uncapping to the cell; these mutants have elevated levels of subtelomeric recombination (McEachern and Iyer, 2001). Two classes of mutations have been reported only in *K. lactis*: delayed telomere elongation (McEachern and Blackburn, 1995), in which telomeres in the mutant cells initially shorten but eventually become very long and phenotypically silent mutations. Both the immediate and delayed telomere elongation phenotypes were recessive, indicating that the elongation is due to telomeric defects; elongation due to an increase in telomerase activity would be expected to be dominant. Although several telomere-length phenotypes have been reported, the extent of the domains has not yet been determined. In this report, the telomeric domains have been more completely examined and one group of telomerase template mutations leading to long, uncapped telomeres, has been examined in more detail. Some of these mutants are known to have telomeres that are defective at binding to Rap1p, while

others are outside the Rap1p binding site. The effects of these long telomeres on the chromosomes will be further characterized.

MATERIALS AND METHODS

Mutagenesis of the *TER1* template

The plasmid pTER-BX-UA (McEachern and Blackburn, 1995) was mutated via one of two methods. Some mutations were constructed using a single-stranded template and a mutagenic oligonucleotide (Kunkel et al., 1987). The majority of the mutations were made using the Quik-change mutagenesis kit (Stratagene, LaJolla, CA). Oligonucleotides of approximately 25 nucleotides were used.

Strains

Strains used in this study are derivatives of haploid *K. lactis* 7B520 (Wray et al., 1987). The strain is *ura3*, *his3*, and *trp1*. A His^+ revertant was used to construct many of the *ter1* template mutant strains. Wild-type refers to the 7B520 parental strain, and the control strain used in the experiments is the strain Bcl+His, a His^+ revertant strain containing the phenotypically silent *TER1-Bcl* allele. Previously characterized *ter1* template mutants used in this study (McEachern and Blackburn, 1995; McEachern and Blackburn, 1996; McEachern and Iyer, 2001; McEachern et al., 2000; McEachern et al., 2002) were constructed in the 7B520 strain. Mutants not previously reported were constructed primarily in the Bcl+His strain, although some (*ter1-13G, ter1-21C, ter1-28A)* were constructed in the 7B520 strain using the plasmid loop-in/loop out method as described previously (McEachern and Blackburn, 1995). In order to be confident that any phenotypes observed were due to the introduction of the intended mutation, the experimental design included redundancy at two steps. For the majority of the mutations discussed in this work, two independent plasmids containing the desired mutation were

utilized. Two independently derived strains containing the mutation were then isolated from each plasmid. Thus, four strains containing each mutation were isolated.

Typically, two of the four clones were then used for long-term passaging. The long-term passaging of the strains was carried out by serial restreaking on rich media (YPD plates). The strains were streaked to single colonies every 3-4 days. Each streak is estimated to represent 20-25 cell divisions. Transformation was carried out using a modified procedure similar to established protocols used for *S. cerevisiae*. A 1 ml overnight culture was grown to saturation and the next day 0.5 ml YPD liquid was added to the culture to return the cells to the growth phase. The cells were grown for 1-2 hours, and then 1.5 ml cells were pelleted at 3000 x g for 5 minutes. The supernatant was removed and the cells were resuspended in 500 μl cold H_2O and repelleted. The cells were resuspended in 80 μl cold H_2O. 10 μl each of 10X TE and 1M LiAc was added and the mixture was incubated at 30° C for 45 minutes. The cells were incubated for an additional 15 minutes after the addition of 2.5 μl 1M DTT. 400 μl cold H_2O was added and the cells were repelleted. The cells were serially resuspended and pelleted in 250 μl cold H_2O, 200 μl cold 1 M sorbitol, and 30 μl cold 1 M sorbitol. The cells were incubated on ice 2 minutes after the addition of DNA. The cells were then electroporated at 1500 V, resuspended in 1 M sorbitol, and plated on appropriated selective plates.

*ter1 rad52*Δ double mutants were constructed by mating the *ter1-14T, ter1-16T,* and *ter1-17T* mutants with TAQ-STU-19 (*URA3, his 3, rad52*Δ). Diploids were selected on media lacking histidine and uracil and passaged on YPD media for 14 serial restreaks to allow the telomeres to shorten. The diploids were then streaked on sporulation plates and the resulting tetrads dissected. DNA was prepared from cells restreaked onto YPD after tetrad dissection.

Construction of Bcl and Kpn STU telomeres

The Bcl and Kpn STU (subtelomeric *URA3*) telomeres were made by mutating a derivative of the pAK25 plasmid (McEachern and Iyer, 2001) as described in (Underwood and McEachern, 2001). Briefly, using a variation on the mutagenesis procedure described by Kunkel *et al* , one to three oligonucleotides were used to mutate each of the eleven telomeric repeats in a cloned telomere. An *Eco*RI-*Sac*II fragment plasmid that contains a *URA3* gene inserted into the subtelomeric region and 11.5 of mutated or wild-type telomeric repeats was transformed into yeast strains as described above. Genomic DNA was prepared and digested with *Xho*I to release the STU telomere; *Bcl*I and *Kpn*I digests were done as appropriate. Fragments were visualized on four-percent gels.

Subtelomeric recombination assay

An *Eco*RI-*Sac*II fragment from the Bcl-STU plasmid that contains a *URA3* gene inserted into the subtelomeric region and 11.5 Bcl telomeric repeats was transformed into *K. lactis* cells. In some cases (as described in Results) it was transformed directly into the *ter1* strains to be assayed. In other cases (as described in Results) the fragment was transformed into the UHA strain (*ura3, his3, ade2, rad52Δ*) and mated with the *ter1* strain to be assayed; the diploids were sporulated, and the tetrads were dissected. The resulting strains were screened for the presence of *RAD52*, the *URA3*-marked telomere, and the presence of the mutated telomerase. In the Bc-STU strain used for mating, there appears to be a subtelomeric duplication at the Bcl-STU telomere. As this might affect levels of subtelomeric recombination, *TER1* strains resulting from the same diploids were used as controls. Rates of *URA3* loss by gene conversion were determined using plates containing 5-flouro-orotic acid (5-FOA) as previously described

(McEachern and Iyer, 2001). Rates of gene conversion on a per cell basis were estimated by the method of the median (Snedecor and Cochran, 1980).

Hybridizations and quantification

Restriction enzyme digested yeast genomic DNA was run on 0.8% or 1% agarose gels, stained with EtBr, and Southern blotted onto Hybond N+ membranes (Amersham Biosciences, Piscataway, NJ). Hybridizations were carried out in Na_2HPO_4 and SDS (Church and Gilbert, 1984). Telomeric oligonucleotides used were the G-strand oligonucleotide Klac1-25 (ACGGATTTGATTAGGTATGTGGTGT) and its reverse complement C-oligo (ACACCACATACCTAATCAAATCCGT). Telomeric hybridizations and washes were performed at 45° or 55° C. Washes were done using 200mM Na_2HPO_4 and 2% SDS. The subtelomeric probe was a 400bp *Eco*RI-*Sac*I fragment from the plasmid pAK25. Hybridizations and washes were performed at 60° or 65° C; washes were done using using 100mM Na_2HPO_4 and 2% SDS.

The quantification of broken DNA was determined by PhosphorImager analysis. The assumption was made that there are 12 telomeres per cell, each with a maximum of 20 telomeric repeats, in the *TER1-7C(BcI)* strain. Each telomere would therefore be less than 500bp long. The total telomeric signal in the uncut *TER1-7C(BcI)* sample was then compared with the telomeric signal seen in the broken DNA running at 450-550 bp in the mutant strains. Comparable amounts of DNA were used in the samples; relative intensities of telomeric signal between the uncut *TER1-7C(BcI)* DNA and the broken pieces of mutant DNA running at approximately 450-550 bp indicated the relative numbers of molecules in each strain. The numbers calculated were likely to be an underestimate of the number of broken telomeric molecules in the mutant cells. Because the probe was to a wild-type sequence, there might have been somewhat weaker hybridization intensity in the mutant telomeric repeats.

In-gel hybridization

In-gel hybridizations were performed using a variation on a previously described method (Dionne and Wellinger, 1996). Thin (5-7mm thick) low-percentage agarose gels (0.7%) were run at 30 volts for 16 hours. The gel was stained with ethidium bromide for photographing and then soaked in 2X SSC for at least 30 minutes. The gel was then dried for 4-5 minutes per side until it was thicker than plastic wrap but thinner than Whatman paper. The gels were then placed in 10X SSC and hybridized with the desired probe at 23°C overnight. The gels were washed 2-3 times for at least 1.5 hours per wash in 0.25X SSC.

Transformation and growth of high-copy *RAP1* strains

Mutant *ter1* strains were transformed with either the plasmid pCXJ3 (Chen, 1996) or pCXJ3+RAP, which contains the *K. lactis RAP1* gene (a gift from A. Krauskopf). The transformants were selected for on media lacking uracil. The strains were subsequently grown on plates containing 125 μg/ml G418 in order to select for higher plasmid copy number. Previous results indicated that, at this concentration, there are more than 100 copies of the plasmid per cell (Chen, 1996). In order to maintain selection for the plasmid, these strains were not grown in overnight culture before DNA extraction. Instead, scoops of cells were taken from the plates and prepared by the normal protocol.

Two-dimensional gel analysis

Two-dimensional gel analysis was performed as described by S.N. and M.J.M. (manuscript in preparation). 15 cm x 15 cm 4% NuSieve 3:1 (ISC Bioexpress, Kaysville UT) agarose gels containing chloroquine were used to run a sample of ~20 μg of genomic DNA in two dimensions. The first dimension was run in 1.6 L of 0.5 X TBE

containing 0.6 μg/ml chloroquine at 5 V/cm for 6 hours. The gel was then stained in a solution of 3 μg/ml chloroquine for 5 hours. For the second dimension run, the gel was rotated by 90˚ and run in 1.6 l of 0.5 X TBE containing 3 μg/ml chloroquine at 5 V/cm for 6 hours. The gel was blotted onto Hybond N+ membranes (Amersham, Piscataway NJ) for at least 24 hours prior to hybridization.

Exonuclease sensitivity of potential single-stranded circles was determined by treating ~20 μg of genomic DNA with 0.1 units of *Exo*I for 1 hour in the presence of 7 ng of a 66 nt non-telomeric linear oligo control. The reaction mixture was run on a two-dimensional gel and the DNA was transferred to a nylon membrane as described above. A control reaction that was untreated with *Exo*I was also run in two dimensions and transferred to a nylon membrane. The presence or absence of the 66 nt linear control was detected by hybridization to a complementary oligo at 45º C for 4 hours. The presence or absence of telomeric DNA resistant to *Exo*I was detected by hybridization to the telomeric oligonucleotides described above *K. lactis* telomeric DNA at 45º C for 4 hours.

Cloning of long telomeres

In order to obtain telomeric fragments that were short enough to be stable in a plasmid, a strategy was devised to clone the broken pieces of telomeric DNA found in the strains with long telomeres; some of the fragments are known to be around 500 base-pairs in length. Total yeast genomic DNA was treated with T4 DNA polymerase and dNTPs to fill in any overhangs and make the ends blunt. A *Sac*I linker was ligated onto the blunt ends and the construct was cut with *Sac*I. The T4 DNA polymerase-treated genomic DNA was then ligated into a pBluescript that had been linearized by *Sac*I digestion. Plasmids extracted from the *E. coli* transformants were screened by

hybridizing Southern blots of digested plasmid DNA with telomeric oligonucleotides. Those hybridizing with the telomeric probe were sequenced.

RESULTS

Mutational analysis of the *K. lactis TER1* template

The long telomeric repeat in *K. lactis* provides an excellent system for genetic dissection of the telomere. By making point mutations in the template of the *K. lactis* telomerase RNA (*TER1*), it was possible to generate yeast cells having specific sequence changes in the newly added telomeric repeats of every chromosome end in the cell. At least one of the three possible point mutations was made at each of the 30 positions in the telomerase template. The mutants were named according to the position of the base change within the template, such that the change that would cause position 1 of the newly synthesized *telomeric strand* to be changed from a T to a G is called *ter1-1G*. Previously published mutations were renamed to be more consistent with the nomenclature used in this paper and will be described by both names, such as *ter1-18C(Bsi).*

Two clones containing each mutation, typically independently constructed (see Materials and Methods), were isolated and their telomere lengths examined every ten serial restreaks (200-250 generations) for sixty restreaks. Fig. 3-1 shows a Southern blot of telomeric restriction fragments from a representative mutation at each template position. Two timepoints-the first (Fig. 3-1A) and fiftieth (Fig. 3-1B) restreaks-are shown; the results from the mutants constructed are summarized in Table 3-1. Telomere lengths in the mutants varied widely, and in many mutants the telomere length changed significantly over time. In addition, some mutants exhibited elevated levels of subtelomeric recombination as judged from loss of one or more telomeric *Eco*RI restriction fragments. The disappearance of telomeric restriction fragments from

Southern blots was previously shown to occur in several *ter1* mutant backgrounds that had short telomeres (McEachern and Iyer, 2001), and was found to involve subtelomeric gene conversion. The subtelomeric sequences of at least 11 of the 12 telomeres in *K. lactis* 7B520 share substantial, but imperfect homology. Subtelomeric recombination, therefore, commonly led to the loss of subtelomeric polymorphisms. Increased levels of subtelomeric recombination is thus a good indicator of compromised telomeric function. Abnormal colony phenotypes, which arise from abnormal cellular morphologies and cell division defects (McEachern and Blackburn, 1995; Smith and Blackburn, 1999), were observed only in strains scored as having very long telomeres. In these strains, the colonies had a rough appearance and irregular edges when compared to the smooth colonies observed in wild-type strains.

Interestingly, only five of the 38 mutations examined showed no evidence of telomere dysfunction; these five mutants had telomeres of wild-type length and exhibited no evidence of subtelomeric recombination as judged by loss of telomeric restriction fragments (Table 3-1). Mutations at three of those positions (16, 20, and 28), however, had wild-type length telomeres when one point mutation was made but long telomeres when a different mutation was made at the same site. This suggests that, at most, two positions in the telomere have no base-specific function. It remains possible that mutations those two positions (positions 7 and 13) might cause telomeric defects if different base substitutions were examined. One of the phenotypically silent strains, *TER1-7C(BcI)*, has been used extensively to examine non-template *TER1* mutations and telomeric turnover (McEachern et al., 2002; Roy et al., 1998; Tzfati et al., 2000).

Mutations in the 5-nucleotide terminal repeats of the *TER1* template often led to telomere shortening and subtelomeric recombination. At early timepoints, nine of the thirteen strains with mutations in these regions had short telomeres; seven of these had also lost *Eco*RI telomeric fragments by the 50th streak. In work that will be described

elsewhere, many mutations in this region have been shown to cause aberrant telomerase translocation events, leading to the synthesis of telomeric repeats that were sizes other than the normal 25 bp in length (Underwood et al, manuscript to be submitted to Molecular Cell). The inability of telomerase to align properly with the telomere during new telomeric repeat synthesis may, at least in part, underlie the telomere length defects seen in these mutants. As discussed below, however, it is possible that the 5 bp telomeric sequence encoded by the terminal repeats of the *TER1* template have additional telomeric functions.

Mutations in template positions 4-9 led to telomeres that were initially wild-type in length or slightly short (Fig. 3-1A, Table 3-1). However, after 50 streaks the telomeric regions in five of the six mutants had changed significantly. These mutants had lost many of their subtelomeric restriction fragments. In the *ter1-4C* and *ter1-9T* mutants the major group of telomeric *Eco*RI fragments, which is normally 1 kb in length and comprised of seven of the twelve telomeres in the cell, was lost. The telomeres in the *ter1-5C* and *ter1-6G* mutants had elongated significantly, and, along with the *ter1-8T* mutant, had a smeary appearance. Although it is unclear whether this region encodes a discrete telomeric function, these results clearly show that it does play a role.

It was previously shown that some mutations within the Rap1p binding site found in the *K. lactis* telomeric repeat led to immediately elongated telomeres and a significant reduction in Rap1p binding (Krauskopf and Blackburn, 1996; McEachern and Blackburn, 1995; McEachern et al., 2000). However, two other mutations in the Rap1p binding site led to telomere shortening. The more extensive mutational analysis presented here confirmed that mutations in the left and right halves of the Rap1p binding site have opposite effects on telomere length. At least one of the mutations tested at each position in the left side of the consensus Rap1p binding site (positions 16-20) led to immediate or gradual telomere elongation (Table 3-1 and Figure 3-1), consistent with

Rap1p's role as a negative regulator of telomere length (reviewed in Marcand et al., 1997b). Although mutations in the right side of the putative Rap1p binding site (positions 21-25) were also expected to cause defective Rap1p binding, four of the six strains with point mutations in this region had telomeres that remained short throughout the entire 50 restreak timecourse examined. None displayed any signs of the telomere elongation that characterized mutations in the left side of the Rap1p binding site (McEachern et al., 2000 and this work, Fig. 3-1 and Table 3-1). This data clearly shows that template positions 21-25 encodes a positive telomeric function.

Mutants containing *ter1-17T*, *ter1-19C*, and *ter1-19A(Acc)* showed immediate severe telomere elongation, in which many of the telomeres ran at limit mobility in cells from the first streak, the earliest time point that could be examined (McEachern and Blackburn, 1995; McEachern et al., 2000, and this work, Fig. 3-1A). The telomeric hybridization patterns of these mutants also had a smeary appearance that extended the length of the lane; this point will be discussed in more detail below. These mutants will henceforth be described as 'immediate' elongation mutants. In contrast, *ter1-16T*, *ter1-16A*, *ter1-18C(Bsi),* and *ter1-20G* initially had a more modest lengthening phenotype (McEachern et al., 2002 and this work, Fig, 3-1A); by the 5^{th} or 10^{th} streak, however, their telomeres lengths approached or equaled those exhibited by the immediate elongation mutants (Fig. 3-1B and data not shown). These mutants will be referred to as 'gradual' elongation mutants. It is likely that the immediate and gradual elongation mutants represent the same defect, such as a decrease in Rap1p binding, and vary only by the severity of the resulting phenotype (Krauskopf and Blackburn, 1996). Telomeric fragments from the *ter1-16T* and *ter1-17T* mutants were cloned and shown to contain only the expected point mutation (data not shown). Also, the telomeric repeats templated by *ter1-18C(Bsi)*, and *ter1-19A(Acc)* contained the restriction site introduced into the template by the point mutations (McEachern and Blackburn, 1995), indicating

that the telomeres might contain of 25 bp repeats with the point mutation as the only change. These results suggested that aberrant telomerase translocation does not typically occur in these mutants.

In cases where heteroallelic *K. lactis* cells containing both a wild-type *TER1* allele and a mutated *ter1* allele were examined, the mutated allele was typically recessive (McEachern and Blackburn, 1995 and data not shown). This would generally be expected, since repeats added by the wild-type telomerase could presumably provide the necessary telomeric functions. It was found, however, that mutations causing immediate telomere elongation can lead to gradual telomere elongation even in the presence of wild-type *TER1*. In heteroallelic strains containing both *ter1-19A(Acc)* and *TER1,* it was found that the telomeres gradually lengthened (Fig. 3-2). After the cells were passaged for 50 streaks, the telomeres were typically several times longer than the 250-500 bp telomeres seen in wild-type cells. Elongation was very slow, however, when compared to the nearly instant elongation typically observed in strains containing only the mutant allele (Fig. 3-1A). The *ter1-19A(Acc)*/*TER1* heteroallele lacked the long smear of telomeric DNA and the abnormal colony phenotype characteristic of the *ter1-19A(Acc)* mutant. It can therefore be concluded that telomeric termini containing mixtures of wild-type and *ter1-19A(Acc)*-templated telomeric repeats are not fully competent at telomere length regulation.

Several of the mutants with changes in the positions adjacent to the left side of the Rap1p binding site- *ter1-11G, ter1-14T*, *ter1-15G, ter1-10A/12C(Bgl)* and *ter1-13C/14C(Kpn)*- led to delayed telomere elongation (McEachern and Blackburn, 1995 and this work). This unusual phenotype was characterized by telomeres that initially shortened but eventually became extremely long (Fig. 3-1A and B). Some of the mutations led to elongation within 150 generations (5 streaks) while others took more than 1000 generations (50 streaks) to elongate. Although the telomeres became long

only after repeated passaging, the elongation itself was often rather abrupt, often occurring within only a few streaks. In the *ter1-14T* mutant, elongation occurred between the 5th and 10th streaks, similar to the timing of elongation previously observed in the *ter1-13C/14C(Kpn)* mutant (McEachern and Blackburn, 1995). The *ter1-11G* and *ter1-15T* mutants, on the other hand, exhibited a smeary hybridization pattern after 30 streaks but did not become extremely long until after 50 streaks. Similarly, the previously reported *ter1-10A/12C(Bgl)* mutant also elongated only after extensive passaging (McEachern and Blackburn, 1995). Once the telomeres did elongate, however, they remained very long through repeated passaging. The smeary hybridization pattern and slightly elongated telomeres in the *ter1-12C* mutant, which initially had short telomeres, indicate that it may have also undergone delayed telomere elongation if it had been passaged further. These results confirm that, in addition to the Rap1p binding site, there is another discrete region of the *K. lacits* telomeric repeat that acts in a distinct manner to serve a crucial role in proper telomere length regulation.

Rap1p overexpression suppresses some *ter1* lengthening alleles

In previously published work, Krauskopf and Blackburn showed that telomeric repeats containing the *ter1-19A(Acc)* or *ter1-18C(Bsi)* mutations had weakened Rap1p binding *in vitro* (Krauskopf and Blackburn, 1996). The repeat templated by *ter1-19A(Acc)* had the most severe defect of the repeats examined, while *ter1-18C(Bsi)* led to a less severe binding defect. In stark contrast, the telomeric repeats made by two delayed elongation mutants *ter1-10A/12C(Bgl)* and *ter1-13C/14C(Kpn)*, which have mutations outside the Rap1p binding site, appeared to bind Rap1p at least as well as wild-type repeats (Krauskopf and Blackburn, 1996). This indicated that the inability to bind Rap1p was not the primary problem in the delayed elongation mutants. Based on this work, it was predicted that telomere elongation caused by weakened Rap1p binding

could be suppressed by the presence of more Rap1p in the cells. Therefore *K. lactis RAP1* was introduced on a high-copy plasmid into cells of the three types of telomere elongation mutants. In addition, cells containing either *TER1-7C(Bcl)* or *ter1-24C(SnaB)* were also transformed. *TER1-7C(Bcl)* has no telomere length defect and thus served as a control, while the *ter1-24C(SnaB)*-templated repeats contain a mutation on the right side of the Rap1p binding site and lead to telomere shortening. The plasmids used contained both *URA3* and G418 resistance as selectable markers. Transformants were initially plated on plates lacking uracil. They were subsequently streaked on plates containing 125 μg/ml G418 to select for higher plasmid copy number; this drug concentration was shown to select for *K. lactis* cells with more than 100 copies of the parental plasmid per cell (Chen, 1996).

Telomere length was examined by Southern blots at the first, third, and tenth streaks; the tenth streak is shown (Fig. 3-3). The results indicated that, in mutants in which the telomeres began to elongate immediately, there was in inverse correlation between lengthening in the original mutant strain and the level of suppression by high-copy *RAP1*. In strains containing *ter1-16T* and *ter1-18C(Bsi)*, which had a gradual lengthening phenotype, the telomere elongation was strongly suppressed by the introduction of the *RAP1*-containing plasmid. In the *ter1-18C(Bsi)* mutant, the telomeres became essentially wild-type in length within ten streaks, while in the *ter1-16T* mutant, which had initially elongated more rapidly, the telomeres shortened significantly though they remained somewhat longer than wild-type telomeres. In both mutants, the smear of telomeric hybridization signal was virtually absent once the telomeres had shortened (data not shown). Conversely, the *ter1-17T* and *ter1-19A(Acc)* immediate elongation mutants showed little suppression of telomere lengthening when in the presence of the high-copy *RAP1* plasmid. Although the amount of telomeric hybridization signal seen at limit mobility in the Southern blots was reduced, the vast majority of the telomeres

remained very long. The *RAP1* plasmid had little or no effect on the telomere length in the *ter1-24C(SnaB)* mutant, which had short telomeres, or the *TER1-7C(BcI)* strain, which had wild-type length telomeres. There was also little effect seen in the delayed elongation mutant *ter1-14T*, which had a mutation outside the putative Rap1p binding site, consistent with idea that the primary defect in delayed elongation mutants was not the inability of the telomeric repeats to bind to Rap1p. Interestingly, however, the delayed elongation mutant showed a moderate improvement in colony phenotype after introduction of the high-copy *RAP1* plasmid. A similar improvement in colony phenotype was seen in the gradual elongation mutants containing high-copy *RAP1* (data not shown). These results support the idea that the long telomeres produced by mutations in the left side of the Rap1p binding site are caused by a defect in Rap1p binding. Therefore, the disruption of a function other than Rap1p binding is responsible for the short telomeres caused by mutations on the right side of the Rap1p binding site.

Delayed elongation mutant repeats fail to regulate telomere length in *TER1* cells

The unusual delayed elongation phenotype seen in strains with certain *ter1* alleles was thought to be due the inability of the mutant telomeric repeats to perform the function(s) required of repeats at the base of the telomere (McEachern and Blackburn, 1995). If this was the case, introducing the mutant repeats at the base of the telomere would be expected to cause elongation of that telomere even in the presence of wild-type *TER1*. It is possible to mutate every repeat of a telomere *in vitro* and then replace one telomere in a yeast strain with the totally mutated telomere (Underwood and McEachern, 2001), and this experiment was performed using two types of total telomere mutants. Telomeres composed entirely of either Bcl repeats or Kpn repeats were created. Their names refer to the one-or-two base substitutions in the repeats; Bcl repeats created a *Bcl*I restriction site and were the type of repeat templated by the

phenotypically silent allele *TER1-7C(Bcl)*, while Kpn repeats created a *Kpn*I restriction site and were of the type predicted to be synthesized by the delayed elongation mutant *ter1-13C/14C(Kpn).* These constructs will henceforth be referred to as Bcl-STU or Kpn-STU (subtelomeric *URA3*) telomeres. Digestion with *Xho*I cleaves a site next to the *URA3* gene in the marked telomere to yield a telomeric restriction fragment that can be separated from all other *Xho*I telomeric restriction fragments from the cell. This allowed the lengths of the marked telomeres to be readily examined. While a typical wild-type *K. lactis* telomere was composed of 10-20 telomeric repeats, the STU construct contained only 11.5 telomeric repeats (Fig. 3-4A, input fragment). Therefore, when a wild-type STU or Bcl-STU is introduced into strains with either wild-type or *ter1-7C(Bcl)* telomerase alleles, it was not unexpected that a small number of telomeric repeats were added by the endogenous telomerase. This yielded a telomeric fragment of the size expected for a wild-type telomere, which was somewhat larger than the input fragment (Fig. 3-4A, compare lanes 1, 2, 8, and 10 to the input fragment length). Introduction of a wild-type STU telomere into a *TER1-7C(Bcl)* strain followed by cleavage with *Xho*I + *Bcl*I released the wild-type STU telomere that was introduced. As seen in Fig. 3-4A, lane 9, the fragment released was somewhat shorter than the input fragment, likely due to replacement of the wild-type repeats with Bcl repeats via the normal process of telomeric turnover. Conversely, when a Bcl-STU was introduced into a wild-type *TER1* strain, digestion with *Bcl*I cleaved the STU telomere and released a small telomeric fragment composed of the wild-type telomeric repeats added to the tip of the Bcl-STU (Fig. 3-4A, lane 3). In contrast to the results observed when the Bcl-STU and wild-type STU were used, when a telomere composed entirely of Kpn repeats was introduced into either a wild-type strain or the *ter1-7C(Bcl)* strain, a full array of 10-20 wild type repeats was added on to the Kpn-STU fragment. The telomeric fragment became significantly longer than the input fragment (Fig. 3-4A, lanes 4, 6, 11, and 14, compare with input fragment),

and was elongated to a greater extent than either the wild-type or Bcl-STU telomere constructs (Fig. 3-4A, compare lanes 4, 6, 11, and 14 with lanes 1, 2, 8, and 10). When the length of wild-type *TER1* or *TER1-7C(Bcl)*-templated telomeric tracts added to the Kpn-STU telomere were examined by *Kpn*I digestion, they were found to be almost as long as the input fragment (Fig. 3-4A, lanes 5, 7, 13, and 16). Because the input fragment also contains some subtelomeric sequence it longer than just 11.5 telomeric repeats, indicating that the telomeric tracts added to the Kpn-STU telomere contained at least twelve telomeric repeats. The size of the Kpn-repeat-containing region of the Kpn-STU telomere was examined by *Xho*I + *Bcl*I digestion. In one clone it had become longer than the input fragment (Fig. 3-4A, lane 12), while the fragment from the other clone was the same size as the original input fragment (Fig. 3-4A, lane 15). Possible reasons for the size increase seen in one clone are addressed in the Discussion. These results indicate that the mutant repeats do not perform the basal functions required for normal telomere length regulation, despite their ability to bind Rap1p very well.

Subtelomeric recombination is elevated in mutants with highly elongated telomeres

Elevated levels of subtelomeric recombination were shown to occur in *K. lactis* mutants with short telomeres in a *RAD52*-dependent manner (McEachern and Iyer, 2001). This recombination could be monitored by two methods. First, as described above, Southern blots could be examined for loss of telomeric restriction fragments. Second, a telomere tagged with a subtelomeric *URA3* insert (a STU telomere) could be introduced into the mutants and the rates of *URA3* loss via subtelomeric gene conversion determined by plating the cells on media containing 5-FOA (McEachern and Iyer, 2001). The long telomeres in these mutants often ran at limit mobility and were very smeary in appearance, making it impossible to see a loss of telomeric bands in a Southern blot. Because subtelomeric recombination rates appeared to correlate with

telomere dysfunction, assays to measure subtelomeric recombination were done using *ter1-14T*, *ter1-18C(Bsi)*, and *ter1-19A(Acc)* cells. These mutants represented the three types of severe telomere elongation mutants (delayed, gradual, and immediate, respectively). A strain containing the phenotypically silent *TER1-7C(Bcl)* was used as a control. A Bcl-STU telomere was first transformed into the *TER1-Bcl, ter1-18C(Bsi),* and *ter1-19A(Acc)* strains; transformants were then screened and those in which a Bcl-STU telomere had replaced a single native telomere were chosen for further study. In other experiments, the Bcl-STU telomere was transformed into a strain of opposite mating type and mated into the *ter1-14T* and *ter1-19A(Acc)* strains (see Materials and Methods), and the spores resulting from the diploid were screened to find the progeny that contained the *URA3* gene and the mutated telomerase allele.

Rates of subtelomeric recombination were assayed as previously described (McEachern and Iyer, 2001). The mutant strains with long telomeres were observed to have rates of subtelomeric recombination that were 8-1900 fold higher than the control (Table 3-2). In order to determine the baseline recombination levels in the Bcl-STU strains resulting from mating, wild-type *TER1* progeny from the mating were also analyzed. The rate of 3.3×10^{-4} observed in the wild-type *TER1* strain, which is approximately four-fold higher than the rate of 7.6×10^{-5} observed for *TER1-7C(Bcl)*, may have been elevated due to the presence of long telomeres containing mutated repeats that were acquired from the *TER1/ter1-14T* or *TER1/ter1-19A(Acc)* diploids. *TER1-7C(Bcl)* cells were previously shown to have the same subtelomeric recombination rate as *TER1* cells (McEachern and Iyer, 2001). One unexpected finding was that the rates of gene conversion in the *ter1-19A(Acc)* strain were quite different in the two assays performed. Introduction of the telomere by transformation directly into the *ter1-19A(Acc)* strain resulted in a gene conversion rate of 1.4×10^{-1}, compared with a rate of marker loss of 2.6×10^{-3} when the marked telomere was introduced by mating

ter1-19A(Acc) with a strain containing a STU-marked telomere. The second result was very similar to the rate of 2.5 $x10^{-3}$ seen when the Bcl-STU was transformed into the *ter1-18C(Bsi)* strain. There are several possible explanations for the different results seen in the assays, including slight structural differences in the STU telomere (see Materials and Methods), the method of introduction of the marked telomere into the cell, the particular telomere containing the *URA3* marker, and the strain background.

The strain exhibiting delayed elongation, *ter1-14T,* was particularly interesting. Telomeres in the mutant initially shortened and then became extremely long; the mutant was assayed under both conditions. Only a two-fold difference in recombination rates was observed between strains from early and late timepoints (2.6 $x10^{-3}$, compared with 5.6 $x10^{-3}$). This suggests that a capping defect caused by mutant telomeric repeats, rather than telomere length *per se*, led to elevated levels of subtelomeric recombination. One caveat to the experiment, however, is that a subset of the telomeres in the cells used at the early timepoint were likely to be long by the time that the assay was completed, and the telomeres were therefore likely to exist as a heterogeneous mixture of lengths. Despite the complications described, it is clear that severe telomere elongation mutants of each type examined have defects in telomere protection that led to elevated levels of subtelomeric recombination.

RAD52 is not required for the formation of very long telomeres

In the absence of telomerase, yeast cells can maintain telomeres by recombinational mechanisms (Chen et al., 2001; Lundblad and Blackburn, 1993; McEachern and Blackburn, 1996; Teng et al., 2000; Teng and Zakian, 1999). Therefore, it was of interest to know whether recombination played a role in the formation of the very long telomeres seen in strains containing certain *TER1* template mutations. Strains from all three types of elongation mutants were mated with a *RAD52* deletion strain in

order to make *ter1* strains that were deleted for *RAD52*. For this experiment, *ter1-14T* represented the delayed lengthening class, *ter1-16T* was an example of the gradual lengthening phenotype, and *ter1-17T* was used to represent the immediate lengthening phenotype. Diploids were made, which contained both normal length and elongated telomeres from the two parental strains. The cells were passaged for 14 streaks to allow the telomeres to shorten. Once the long telomeres shortened and all of the telomeres were of comparable lengths, the cells were sporulated. The telomeres in the diploid strains did not shorten quickly, although the smear of DNA seen in the mutant strains was significantly reduced (data not shown). This was consistent with the idea that the telomeres in the diploid cells were capped and generally shortened gradually over time by normal telomeric turnover as previously described (Krauskopf and Blackburn, 1998; Smith and Blackburn, 1999). The progeny haploid strains were examined for the presence of long telomeres using Southern blots and probed to determine whether they had wild-type *RAD52* or the *rad52Δ* allele (Fig. 3-5). For each of the three mutations examined, strains were isolated that contained long telomeres and lacked *RAD52*; both *RAD52* and the telomere elongation phenotype segregated meiotically 2:2, as expected for single genes. Because the telomeres in the diploid were not extremely long and lacked the smeary appearance of the mutants, the long telomeres seen in the mutants were clearly formed after sporulation. One minor difference was seen in the *ter1-14T* mutant. Although the telomeres had lengthened considerably, those in the *rad52Δ ter1-14T* strain were not quite as long as those in the *RAD52 ter1-14T* strain (Fig. 3-5). Because only one *rad52Δ ter1-14T* clone was examined, it is possible that the difference is due to strain variability and that given sufficient time it would lengthen to the same extent as the *RAD52* strain. These results indicated that, while *RAD52* could play a minor role in the formation of long telomeres in the delayed elongation mutant, it was not required for the extreme telomere elongation seen in any of the mutants examined.

Extrachromosomal DNA is produced in strains with extreme telomere elongation in a *RAD52*-dependent manner

One unusual phenotype of *K. lactis ter1* mutants with very long telomeres was that all of them contained a smear of telomeric DNA extending from the top of the blot (greater than 20 kb) to the bottom (less than 200 bp) (Fig. 3-1). To further characterize this DNA, uncut yeast genomic DNA was run on an agarose gel and serially probed with a subtelomeric probe and probes specific to each telomeric strand (Fig. 3-6). Because the DNA was uncut, chromosomal DNA was expected to run at limit mobility. In samples from both wild-type and mutant cells, essentially all of the visible subtelomeric signal was seen at limit mobility. This indicates that the subtelomeric DNA was not generally degraded or broken during preparation. When the gel was subsequently reprobed with the telomeric probes, a different pattern was seen. With either strand-specific telomeric probe, the majority of the signal was also seen at limit mobility. However, in the mutants with long telomeres 10-25% of the telomeric signal was seen a long smear comprised of molecules that were less than 3 kb in length, the size below which it is clear that very little of the telomeric DNA could be attached to subtelomeric DNA (Table 3-3 and unpublished data). Included in this is the large peak of hybridization signal migrating at positions smaller than approximately 0.5 kb; there were estimated to be approximately 2-10 molecules of this size per mutant cell (see Materials and Methods). In contrast, in the phenotypically silent *TER1-Bcl* control only 3% of the telomeric DNA was below 3 kb in length. These results indicate that, in the mutants examined, a significant percentage of the telomeric DNA is extrachromosomal; it was not possible to determine how much of the signal above 3 kb in the mutants was also extrachromosomal telomeric DNA. Two differences between the signal seen with G-strand and C-strand telomeric probes were observed. When the C-oligonucleotide probe was used, the area between approximately 0.5 kb and 1 kb displayed a very significant decrease in the telomeric

hybridization intensity, which was not apparent when the G-strand probe was used. In addition, the large peak of hybridization signal migrating at positions smaller than approximately 0.5 kb ran slightly lower when visualized with the C-strand telomeric probe.

Although cells with mutated *TER1* alleles could elongate in the absence of *RAD52*, the character of the telomeric DNA present in the cell was different in *ter1 rad52Δ* double mutants. The long broken smear of telomeric DNA seen in the long telomere mutants was significantly reduced in the *rad52Δ* strains, and the material below 3 kb in length was essentially eliminated (Fig. 3-6 and Table 3-3). These data indicate that recombination was involved in the formation of most of the broken DNA.

Long telomere mutants have increased amounts of single-stranded telomeric DNA

In *S. cerevisiae,* extensive 3' single-stranded overhangs can be generated at DSBs (double strand breaks) and uncapped telomeres (Garvik et al., 1995; Lydall and Weinert, 1995; Sugawara and Haber, 1992). In order to see if the broken DNA in *ter1* mutants with long telomeres contained single stranded telomeric DNA, nondenaturing in-gel hybridizations were done (Dionne and Wellinger, 1996) (Fig. 3-7). When probed with the G-strand oligonucleotide, which hybridizes with the C-rich telomeric DNA, little signal was seen. In contrast, when the C-strand telomeric oligonucleotide was used the lane showed a smear of strong signal. Much of the signal was in the lower half of the gel, where the smallest fragments migrated at approximately 1 kb. In the wild-type *TER1* control, only a trace amount of hybridization signal was visible at the top of the gel. Because the DNA examined was uncut, only the very large pieces running at limit mobility were likely to be G-strand overhangs attached to the chromosome. Therefore, a majority of the single-stranded telomeric DNA in the long telomere mutants was extrachromosomal. The lack of telomeric hybridization signal seen with the G-strand

telomeric oligonucleotide in the in-gel hybridization indicates that the signal seen with this probe under denaturing conditions (as shown in Fig. 3-6) was double stranded. The telomeric signal in the in-gel hybridization migrated between limit mobility and the double-stranded 1 kb size marker. This may suggest that the relatively weak hybridization signal seen between approximately 1 kb and 0.5 kb in DNA from long telomere mutants probed with the C-strand telomeric probe (Fig. 3-6) may, in part, be due to the absence of single-stranded DNA. When the in-gel hybridization was done with an approximately 400 bp subtelomeric probe derived from sequence within approximately 600 bp of the base of the telomeres, no signal was seen (data not shown), indicating that the single-stranded character did not appreciably extend into the subtelomeric region of the chromosome. The smear of single-stranded DNA was considerably diminished in mutants deleted for *RAD52* (data not shown). Because *RAD52* would not be expected to be required for the formation of 3' tails, the reduced level of single-stranded telomeric DNA could be explained by the decrease in the amount of extrachromosomal molecules in the cells.

In subtelomeric recombination assays and Rap1p overexpression experiments in which *ter1-18C(Bsi)* and *ter1-19A(Acc)* were included (this work and data not shown), they behaved in a manner similar to *ter1-16T* and *ter1-17T*, respectively. The telomeric repeats in these mutants, however, contained *Bsi*WI and *Acc*I restriction sites, respectively. These sites were utilized in an effort to quantify the length of the single-stranded overhang predicted to be present in both these strains; cutting the double-stranded component of the telomere with *Bsi*WI and *Acc*I would be expected to release the single-stranded component. Digestion of genomic DNA with the appropriate enzyme (*Bsi*WI or *Acc*I) did not yield a detectable band or smear that hybridized only to the C-strand telomeric probe (Fig. 3-8). This suggests that no single-stranded overhangs greater than 50 nt in length remain after cleavage with either of those enzymes. Very

small molecules, however, would not attach to the membrane so extremely short overhangs would not be easily detected using a procedure that involved blotting the DNA onto a membrane. It is also possible that long single-stranded overhangs were present but cleaved by low-efficiency single-strand digestion activity in the enzymes. However, the data was more consistent with the possibility that short single-stranded telomeric sequence existed that was less that 50 nucleotides in length.

Arrays of small, possibly circular, double-stranded and single-stranded telomeric molecules are present in mutants with extreme telomere elongation

Two-dimensional gel electrophoresis was used to examine the population of small (less than 500 bp) extrachromosomal DNA seen in the *ter1-16T* mutant. The two dimensions differed by the concentration of chloroquine used (see Materials and Methods). Using this scheme, double-stranded linear DNA was expected to form a straight diagonal line, while non-linear DNA may run ahead of or behind the diagonal. When DNA from the *ter1-16T* mutant was examined, non-linear fragments that did not run on the diagonal were seen when the blots were probed with the C-strand oligo (Fig. 3-9A, marked with arrows) but not when the G-strand oligo was used (Fig. 3-9B). These migrated at positions in the gel corresponding to double-stranded linear DNA of approximately 50 to 200 bp or more in length. This indicated that the molecules in these spots were largely or entirely single-stranded. A second series of distinct spots that ran on the diagonal were seen with both the C-strand and G-strand probes, indicating the presence of double-stranded species of molecules (Fig. 3-9A and 3-9B, and data not shown). These molecules ran in the gel in a size range similar to that of the single-stranded molecules. When a *ter1-16T rad52Δ* double mutant was examined (Fig. 3-9C and 3-9D), no telomeric hybridization was seen at small DNA sizes, consistent with previous data (Fig. 3-6), indicating that all of the small telomeric molecules observed in

the two-dimensional gels were products of homologous recombination. This result, combined with the discrete sizes of the telomeric hybridization, suggests that the spots differ in size by multiples of 25 bp or 25 nt, the size of the *K. lactis* telomeric repeat. If the single-stranded molecules seen in Fig. 3-9A were circular, it would be predicted that they would be resistant to cleavage by *Exo*I, an exonuclease specific for single-stranded DNA. To examine this, samples of DNA were treated with *Exo*I. As a control, an excess of a 66 nt oligonucleotide (oligo A) was included in the *Exo*I treatment and run with the sample. When the blot of *Exo*I-treated DNA was probed with an oligonucleotide complementary to oligo A (oligo A'), no signal was seen (compare Fig. 3-9G with Fig. 3-9E), indicating that the control oligo was completely digested. When the same blots were probed with the C-strand telomeric probe, the spots that were not on the diagonal were still visible, indicating that they were resistant to *Exo*I digestion (Fig. 3-9H, compare to Fig. 3-9F). This strongly supports the idea that the small single-stranded telomeric DNA molecules were circular.

DISCUSSION

The *K. lactis* telomeric repeat contains of several functional domains

Mutagenesis of the entire *K. lactis* telomerase template was used to incorporate mutations into the telomeric repeats. This analysis has revealed that the telomeric repeat contains multiple functional regions. One is a 5 bp region of the repeat that is templated by two regions in the *TER1* template, the terminal repeats, which are 5 nucleotides in length and mark the ends of the template. They are critical for proper translocation, the process by which telomerase dissociates and realigns with the template to make new telomeric repeats. In work presented elsewhere, many mutations in this region were shown to cause aberrant translocation events (Underwood et al, manuscript to be submitted to Molecular Cell), leading to the synthesis of telomeric

repeats that are longer or shorter than the 25 bp repeat synthesized by wild-type telomerase (Underwood et al, manuscript to be submitted to Molecular Cell). The elimination of specific base-pairing interactions between the telomere and the telomerase template, which disrupts normal translocation, is likely to be largely responsible for the telomere shortening often seen in when mutations are made in the *TER1* terminal repeats. However, some data suggests that telomeric nucleotides copied from the terminal repeats of the *TER1* template may serve another function. The short-telomere phenotype seen in both the *ter1-3C* and *ter1-28C* point mutants could have been entirely due to the inability of telomerase to translocate properly, as both alleles lead to the synthesis of abnormally sized telomeric repeats. However, a double point mutant that was altered at positions 3 and 28, *ter1-3C/28C,* contained identical mutations in both *TER1* template terminal repeats and synthesized 25 bp repeats with the expected single-base substitution. The telomere length, however, remained quite short (data not shown), indicating that the sequence copied into the telomere from the terminal repeats may play an important role in telomere length regulation. Additional experiments will be needed to separate the effects of altered telomerase function from the effects of disrupted telomere function in these mutants.

The Rap1p binding site, the most highly conserved feature of the telomeric repeat among many related yeasts (McEachern and Blackburn, 1994), was a predicted functional domain within the telomeric repeat, but the analysis of this region was not as simple as might have been expected. The exact protein contacts between Rap1p and its telomeric binding site have not been determined, but alignment of the telomeric sequence with the *S. cerevisiae* Rap1p sites used to determine the crystal structure (Konig et al., 1996; Taylor et al., 2000) suggests that the *K. lactis* Rap1p site as depicted in Figure 3-1 and Table 3-4 (positions 16-25) is probably correct. However, it is also possible to get a close alignment of the canonical Rap1p site with the *K. lactis* telomeric

sequence if the alignment begins at position 14, instead of the usual position 16. This alignment is not as consistent with the phenotypes resulting from mutations in the region, but it can not be completely excluded as an alternative Rap1p binding site.

Mutations leading to immediate and gradual telomere elongation in *K. lactis* disrupt nucleotide-specific contacts identified in the Rap1p crystal structure (Table 3-4). At position 17, the *ter1-17T* allele leads to telomere elongation, whereas *ter1-17A* does not. It is possible that changing position 17 to a thymine disrupts a specific contact between Rap1p and its site, while a change to adenine does not. Interestingly, the *ter1-16A/17A* double mutant had normal length telomeres, even after passaging for 130 streaks, when introduced into cells by plasmid loop-in/loop-out but causeed telomere elongation when introduced into a *ter1-Δ* strain with short telomeres (McEachern and Blackburn, 1995). In the latter protocol, basal wild-type repeats that could influence telomere length are largely gone before the mutant telomerase is expressed. As all of the mutants described in this study were made by plasmid loop-in/loop-out, it is possible that some of the mutants with telomeres of wild-type length, such as *ter1-17A*, could also have a 'cryptic' telomere elongation phenotype that would only be detectable if few or no wild-type repeats remained in the telomeres containing the mutant repeats.

In *S. cerevisiae*, Rap1p has been shown to be a negative regulator of telomere length (Gilson et al., 1993; Marcand et al., 1997a). The results presented here provide further evidence that *K. lactis* Rap1p is also a negative regulator of telomere length. Mutations in the left side of the Rap1p binding site lead to telomere elongation. The presence of *RAP1* on a high-copy plasmid was able to suppress telomere elongation caused by some of these mutations. This strongly argues that the immediate and gradual telomere elongation phenotypes were due to defects in Rap1p binding, and also indicates that the presence of additional copies of Rap1p leads to an increase in cellular Rap1p levels. An increased level of Rap1p might suppress the long telomere phenotype

by forcing the mutant repeats with reduced Rap1p binding affinity to bind Rap1p. The lack of effect on telomere length in wild-type cells may indicate that the *K. lactis* telomeres are normally fully saturated with Rap1p. The presence of high-copy *RAP1* in *S. cerevisiae* can actually lead to modest telomere elongation through a mechanism that is unclear (Conrad et al., 1990). The extent of suppression of telomere lengthening by high-copy *RAP1* is consistent with the degree of disruption of Rap1p binding observed *in vitro* (Krauskopf and Blackburn, 1996). This may allow the effect of Rap1p overexpression to be used to predict the relative degree of disruption to the Rap1p binding site for mutations not analyzed *in vitro*. It is also interesting to note that the presence of high-copy *RAP1* in wild-type cells did lead to an obvious growth defect. This is in contrast to the results seen in *S. cerevisiae*, where high levels of Rap1p are toxic (Chambers, 1996; Freeman et al., 1995). Whether this difference is due to differences in Rap1p overexpression levels in the two species or some other reason is unknown.

Mutations in the right side of the Rap1p binding site were typically found to cause telomere shortening; none caused the extreme telomere elongation seen when the left side of the site was mutated. This surprising result could be explained by proposing that this region encodes two functional domains: a Rap1p binding site and also a site, which partially overlaps with the Rap1p site, that positively modulates telomere length. A likely explanation is that this region is a binding site for either Cdc13p or Est1p, proteins that bind single-stranded telomeric DNA and recruit telomerase to the telomeric tip (reviewed in Evans and Lundblad, 2000). Although some mutations in the right side of the Rap1p binding site would also be expected to disrupt Rap1p binding, the inability to recruit telomerase to the telomere would limit or prevent the extensive telomere lengthening typically seen in mutations in the Rap1p binding site. Therefore, the identification of *K.*

lactis CDC13 and *EST1*, and the examination of their DNA binding specificity, will be of great interest.

Another functional domain of the *K. lactis* telomeric repeat is the region adjacent to the left side of the Rap1p binding site. Mutations in at least four different positions cause the telomeres to become short initially, but eventually lead to severe delayed telomere elongation. The initial shortening of the telomeres in these mutants strongly suggests that this region of the telomere serves a positive function in telomere length regulation, which is disrupted by the mutations. It is not clear if this effect is due to a defect separate from that leading to telomere elongation, or if the two phenotypes are different manifestations of the same defect. The delay in formation of the long telomeres appears to be due to the requirement for telomeric turnover to replace some percentage of the telomere with mutant repeats before telomere length regulation is critically disrupted (McEachern and Blackburn, 1995). For this reason, the role that the internal telomeric repeats play in length regulation was examined. Mutated telomeric repeats, containing the mutation specified by *ter1-13C/14C(Kpn)* were introduced into the base of a single telomere in a *TER1* background. This led to the addition of a full array of 10-20 wild-type repeats onto the 11.5 mutant repeats. This indicates that the Kpn telomeric repeats are not capable of fulfilling the length regulation functions of basal telomeric repeats. It remains unclear whether telomeric repeats at different positions within the basal part of the telomere have equal effects on length regulation. Interestingly, in three of the six Kpn-STU mutants in a *TER1-7C(Bcl)* background examined (Fig. 3-4 and data not shown), the size of the Kpn-STU fragment recovered after *Bcl*I cleavage was longer than the size of the input fragment. This elongation could not be telomerase mediated, as any repeats added by *TER1-7C(Bcl)* would contain the *Bcl*I restriction site and thus be cleaved. It is therefore likely that the three Kpn-STU telomeres acquired wild-type repeats by recombining with the wild-type base of another telomere in the cell. Although

only a small number of clones were examined, these results suggest that, in addition to a defect in telomere length regulation, the mutant repeats of the Kpn-STU may also have a capping defect that makes them prone to recombination.

The presence of the high-copy *RAP1* plasmid had little or no effect on the delayed elongation mutant examined, indicating that the primary defect of this mutant is not in Rap1p binding. There are, however, several possible explanations for the telomere elongation phenotype seen in the delayed elongation mutants. First, it is possible that the mutations increase the strength of binding for a positive regulatory protein. A second possibility is that the mutations may directly alter interactions between Rap1p and the telomeric DNA. Rap1p is known to cause a bend in the telomeric DNA when bound. The nucleotides adjacent to the Rap1p binding site may provide additional contacts with Rap1p that affect DNA bending, and thus the telomeric chromatin structure. Third, mutations in the delayed elongation domain of the telomere could cause a defect in binding a protein that interacts with the telomereic DNA, perhaps only in the presence of Rap1p, which also serves a negative regulatory function. Possible candidates include Rif1p and Rif2p, which interact with the telomere via Rap1p and may be part of the mechanism by which telomeric repeats are 'counted' by the cell (Marcand et al., 1997a, D. Levy and E.H. Blackburn, personal communication).

A final region of the template was defined by mutations in template positions 4-9. Mutations in this region led to modest changes in telomere length and unexpectedly high levels of subtelomeric recombination, as determined by loss of subtelomeric restriction fragments in Southern blots. In several of the mutants, the telomeric hybridizations developed a smeary appearance over time. Mutations leading to delayed elongation often developed a smeary appearance before elongating, indicating that these mutants might also elongate if give sufficient time. This region might therefore be a less critical

component of the delayed elongation domain of the telomeric repeat. Alternatively, it could delineate another domain that contributes to proper telomere function.

Mutants with severe telomere elongation exhibit telomere capping defects

One of the main roles of telomeres is to prevent the chromosome ends from being recognized as DSBs. Mutations made in the telomeric repeats would thus be expected to cause the telomeres to be treated similarly to double-strand breaks. *TER1* template mutants with long telomeres exhibit defects in both colony and cellular morphology, reminiscent of yeast cells arrested with unrepaired DSBs (Smith and Blackburn, 1999; Weinert and Hartwell, 1993). They are also likely to be subject to the same 'repair' by homologous recombination and/or non-homologous end joining (NHEJ) that occurs at DSBs. Short telomeres in *K. lactis* were previously shown to have elevated levels of subtelomeric recombination (McEachern and Iyer, 2001). In this work, *K. lactis* delayed, gradual, and immediate telomere elongation mutants were examined by recombination assays, and all three were shown to have elevated levels of subtelomeric recombination. The frequency of recombination did not correlate directly with the length of the telomeres. Indicative of this, the *ter1-14T* delayed elongation mutant displayed elevated rates of subtelomeric recombination at both timepoints examined, despite the expectation that the telomeres were very different in length. This is consistent with the idea that high rates of subtelomeric recombination result from compromised telomere capping and can occur in the presence of long or short telomeres with capping defects. Although the STU recombination assays indicate that subtelomeric recombination is elevated in the mutants, they do not address whether recombination within the telomeric repeat tracts is also elevated.

TER1 template mutants with very long telomeres also exhibit a change in the composition of telomeric DNA in the cell. Not only are the telomeres very long, but a

large amount of the telomeric DNA is extrachromosomal, existing as broken fragments that run from the top of the gel to sizes of less than 100 bp. When wild-type *TER1* is introduced into a *ter1* mutant with long telomeres, the telomeres remain much longer than wild-type. However, the telomere lengths are more sharply defined, and the smear of telomeric fragments is not present (Krauskopf and Blackburn, 1996; Maddar et al., 2001; Smith and Blackburn, 1999, and data not shown). A similar response is seen in the *TER1/ter1-19A(Acc)* heteroallelic strain (Fig. 3-2). These data indicate that the extrachromosomal telomeric DNA is an effect of telomere uncapping that can be reversed by the presence of wild-type repeats near the telomeric termini. Interestingly, although telomere elongation does not depend on *RAD52,* formation of the broken DNA in the *ter1-14T, ter1-16T, ter1-17T*, and *ter1-19A(Acc)* (data not shown) mutants is largely *RAD52*-dependent. This result which strongly suggests that recombination within tracts of telomeric repeats is highly elevated in the long telomere mutants. Recombination between or within the long tracts of telomeric DNA could thus result in the formation of extrachromosomal telomeric DNA molecules that may potentially be subject to further telomerase-mediated telomere addition.

Highly elevated rates of recombination within the telomeric repeat tracts have been shown to occur in *ter1-Δ* mutants (Z. Topcu and M. J. McEachern, unpublished data). Telomeric recombination may also explain why, when the long telomeres in the mutants shortened upon introduction of wild-type *TER1* or high-copy *RAP1*, shortening was more rapid than the 5 bp per end per cell division would be expected from replicative sequence loss (McEachern and Blackburn, 1995). The rapid rate of telomere shortening could be related to the telomere rapid deletion (TRD) that has been observed in *S. cerevisiae* (Bucholc et al., 2001; Li and Lustig, 1996), when. When significant telomere shortening occurred in some of the high-copy *RAP1* experiments described in this work, the telomeres initially shortened by as much as 4.5 kb in only three restreaks

(60-75 generations) (data not shown); this is more than ten times the telomere shortening rate in *ter1-Δ* cells (McEachern and Blackburn, 1995). Turnover of telomeric repeats close to the base of the telomere has previously been shown to occur in *TER1* template mutants with highly elongated telomeres (McEachern and Blackburn, 1995; McEachern et al., 2002). This further supports the idea that very long telomeres in the *TER1* template mutants examined are highly dynamic and prone to high rates of telomeric deletion, recombination, and unregulated telomeric repeat addition by telomerase.

Telomeric recombination is clearly involved in the formation of the small, possibly circular telomeric molecules seen as discreet spots when DNA from the *ter1-16T* mutant is run on two-dimensional gels. Surprisingly, one class of small molecules appeared to be composed largely or entirely of circular single-stranded G-rich telomeric DNA. It is possible that the formation of telomeric circles could be initiated by single-stranded telomeric overhangs forming loops by strand-invading into a portion of the duplex telomeric DNA on the same telomere. The D-loop structures formed could subsequently undergo further processing to excise circular molecules composed entirely of telomeric repeats. Although it is unclear whether the circles would contribute to the maintenance of long telomeres in cells of the *ter1-16T* mutant, telomeric circles can be utilized to lengthen telomeres in the absence of *TER1* (Natarajan and McEachern, 2002 and S. Natarajan, in preparation).

Normal telomere function in most eukaryotes involves the formation of small single-stranded 3' tails at telomeric termini. In *S. cerevisiae,* the short single-stranded G-overhangs have been shown to form in a cell-cycle dependent manner, independently of telomerase (Dionne and Wellinger, 1996; Wellinger et al., 1993). One consequence of telomere uncapping can be the formation of much larger 3' G-strand tails at the telomeric tip through the resection of the telomeric C-rich strand. For example, single-

stranded overhangs that can extend thousands of base pairs from the telomere are found throughout the cell cycle in some *cdc13* mutants (Garvik et al., 1995; Lydall and Weinert, 1995). In the *TER1* template mutants with long telomeres examined in this work, there is a substantial amount of single stranded telomeric DNA that is composed almost entirely of the telomeric G-rich strand. No evidence was found to indicate that the single-stranded overhangs in these mutants are extremely long, nor were the single-stranded regions found to extend into the subtelomere. The fact that no long single-stranded 3' tails were observed after cleavage of the double-stranded telomeric DNA with restriction enzymes could indicate that there are relatively short single-stranded 3' tails at telomeric ends. The large amount of single-strand hybridization signal seen in mutants with long telomeres would therefore be due to the presence of short 3' tails on both the chromosomal and extrachromosomal telomeric ends. Despite the extreme length of the duplex telomeres in the mutants examined, the single-stranded component at the telomeric tip may be somewhat regulated. The single-stranded component of the telomere may form a structure, such as a T-loop (Griffith et al., 1999; Munoz-Jordan et al., 2001) or G-quartet structure (Balagurumoorthy and Brahmachari, 1995; Balagurumoorthy et al., 1992), that limits its size. Alternatively, if the slow rate of 5' end resectioning seen at DSBs (Sugawara and Haber, 1992) also occurs at uncapped telomeres, relatively short telomeric 3' tails may initiate recombination events before the tails become large. However, extensive regions of single-strandedness form in the presence of specific *cdc13* mutations prior to cell cycle arrest (Lydall and Weinert, 1995). Whatever the length of the single-stranded overhang, however, it is clear that the formation of single-stranded DNA occurs at a high level in mutants with long, uncapped telomeres.

Summary

Taken together, the results presented here show that alterations in the sequence of the telomeric repeat typically lead to changes in telomere length that may have a number of causes, including disruption of the binding site for Rap1p. The effects on telomere length may be specific to whether the disruption occurred at the telomeric tip or the base of the telomere. Certain mutations can result in partial telomere uncapping, which can cause telomeres to be seen as DSBs. Recombination within or near the telomere is a frequent consequence. This recombination, however, may be the cause of further problems because it can cause the excision of large amounts of telomeric DNA from some mutated telomeres, forming many telomeric repeat-containing molecules that are not attached to the chromosome. Putative circular telomeric molecules, which could be copied to add more telomeric repeats to the already long telomeres, could conceivably further exacerbate the telomere length defects. Linear extrachromosomal molecules composed of mutant telomeric repeats, on the other hand, may be acted on by the same cellular machinery that acts at the true telomeres and at DSBs. They might therefore titrate the proteins involved in telomere regulation while also further activiating recombination and other DNA repair processes.

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FIGURES

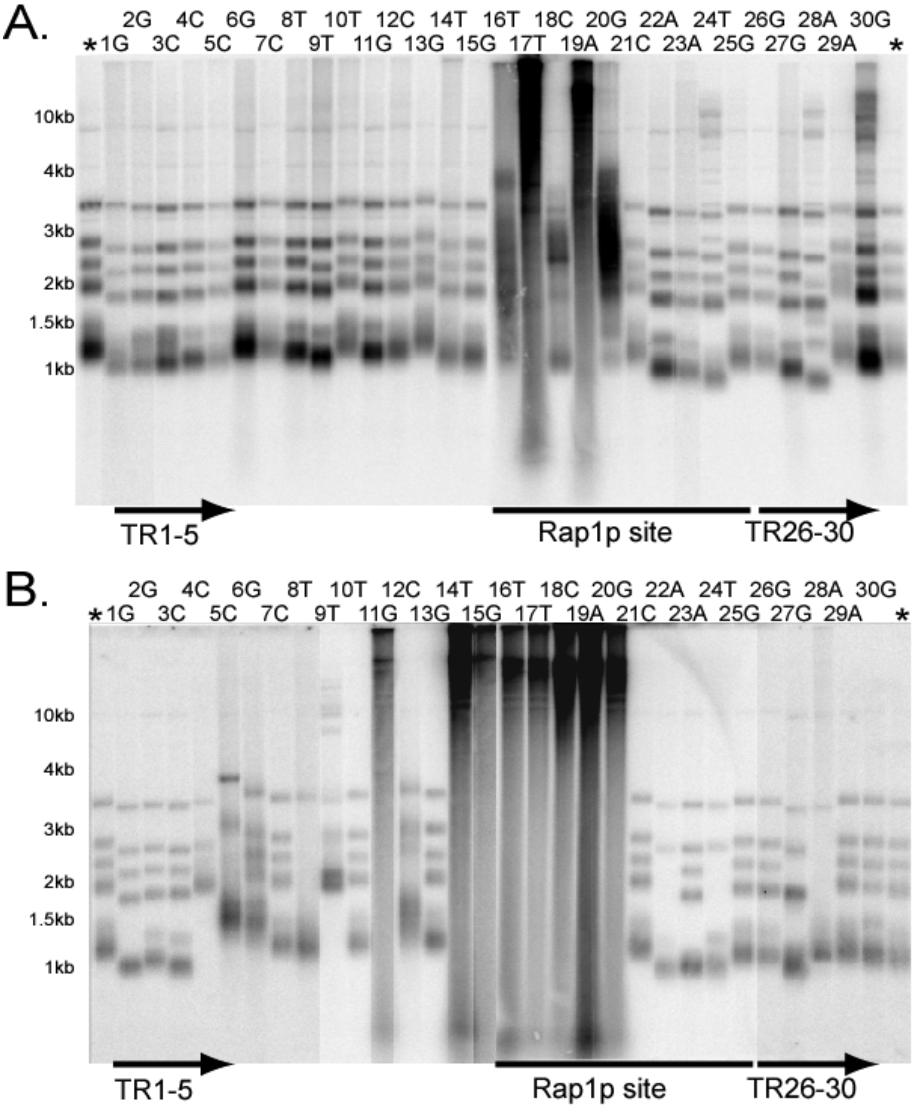

Fig. 3-1. Most point mutations in the *TER1* template cause changes in telomere length that worsen after passaging. A) Each of the 30 positions in the *K. lactis* telomerase template was mutated independently to create a collection of point mutants. A Southern blot of *Eco*RI digested genomic DNA from newly created mutants is shown, probed with a telomeric oligonucleotide. The symbol * indicates the parental *TER1* control. Labeled lanes indicate the template position that is mutated in the strain shown in that lane. Strains are shown in order, with the leftmost lane being position 1 and the rightmost lane being position 30. The Rap1p binding site is underlined; the terminal repeats are underlined with arrows. Size markers, in kb, are shown. **B)** Southern blot of the telomere length at the 50th streak is shown for all strains except the mutant at position 3, where the 29th streak was shown. Note the delayed elongation at positions 11, 14 and 15, adjacent to the left side of the Rap1p binding site.

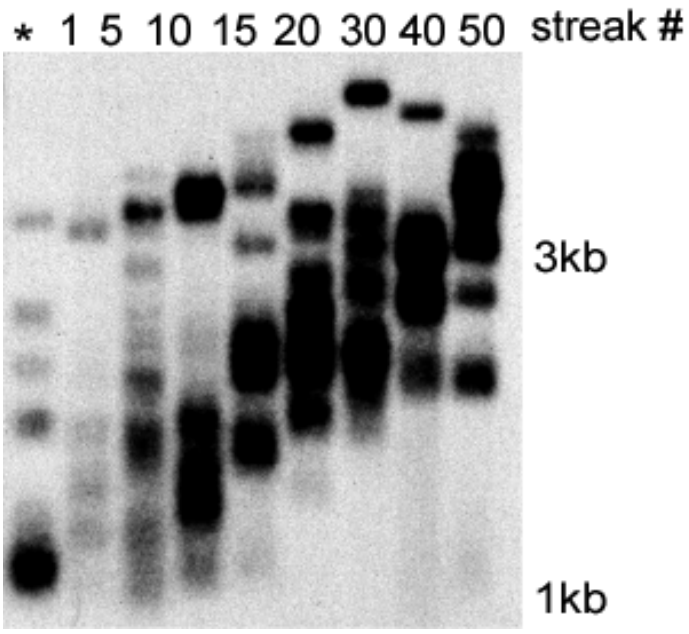

Fig. 3-2. The telomere elongation phenotype of the *ter1-19A(Acc)* mutant is not completely recessive. A heteroallelic strain containing both *TER1* and *ter1-19A(Acc)* was grown for 50 restreaks and DNA samples were periodically isolated. Shown is a Southern blot of *Eco*RI-digested genomic DNA from several time points. DNA from *TER1-7C(Bcl)* was used as a control (lane marked *). Size markers (in kb) are shown.

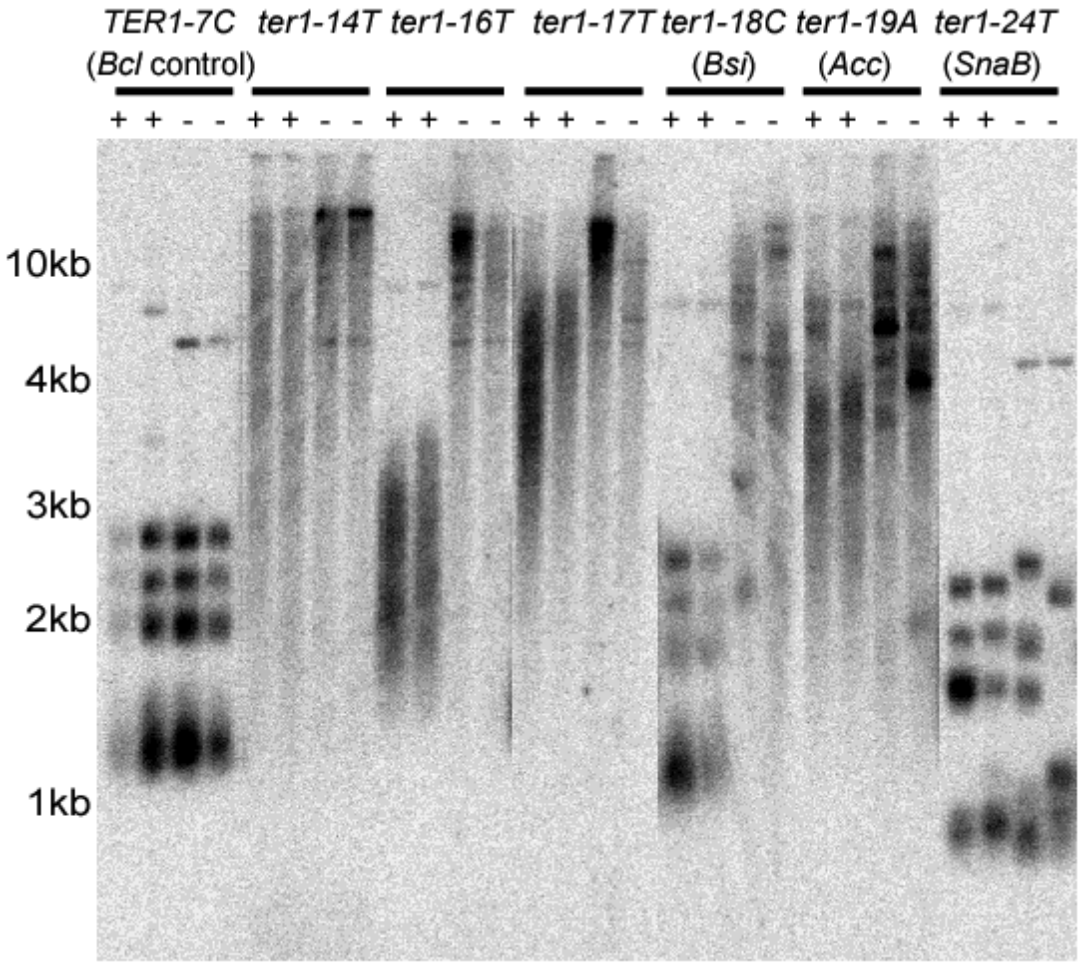

Fig. 3-3. High-copy *RAP1* suppresses telomere elongation in some *ter1* template mutants. Strains with mutated *TER1* alleles were transformed with plasmids +/- the gene encoding Rap1p. DNA was prepared from the strains after 10 serial streaks on 125μg/ml G418. The blot is shown, probed sequentially with a subtelomeric probe and a telomeric probe. For each mutant line, two independent transformants are shown for the high-copy *RAP1* (+) and vector-only control (-).

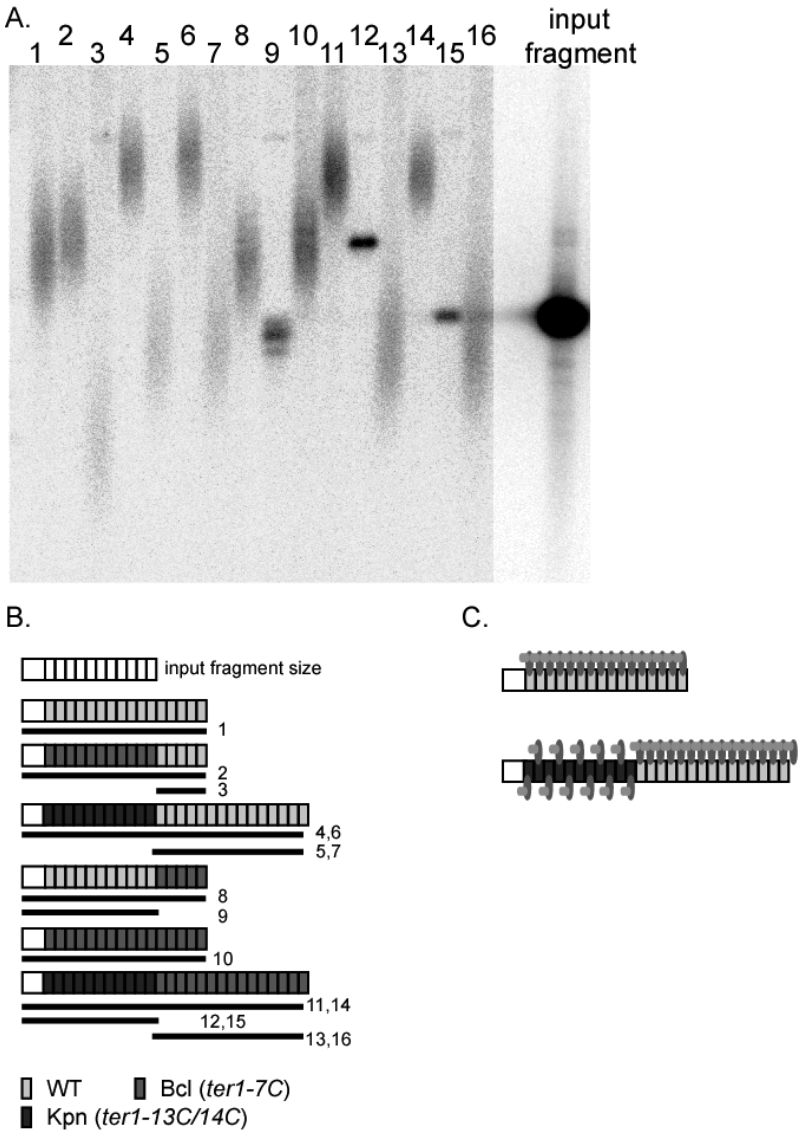

Fig. 3-4. Basal telomeric repeats play a role in telomere length regulation. A) Wild-type, Kpn, and Bcl-STU constructs the length of the input fragment were transformed into *TER1* and *TER1-7C(Bcl)* strains. The STU telomere was separated from the cellular telomeres by digestion with *Xho*I. Lane 3 shows digestion with *Xho*I + *Bcl*I to release the newly-added wild-type repeats. Lanes 5, 7, 13, and 16 show digestion with *Xho*I + *Kpn*I to release the newly-added wild-type or Bcl repeats. Lane 9 shows digestion with *Xho*I + *Bcl*I to release the wild-type-STU input fragment. Lanes 12 and 15 show digestion with *Xho*I + *Bcl*I to release the Kpn-STU. **B)** A schematic diagram of the fragments examined in (A) is shown. The input fragment is shown in white. In all other telomeres, the white box indicates the subtelomeric region. Light gray indicates wild-type repeats, medium gray indicates Bcl repeats, and dark gray indicates Kpn repeats. **C)** A model of the defect seen in Kpn repeats shown. Wild-type repeats bind Rap1p (elongated oval) and interact properly with another telomeric protein, such as Rif1p or Rif2p (light gray circles). Kpn repeats bind Rap1p in an abnormal manner, leading to a disruption of the interaction with the other telomeric factor.

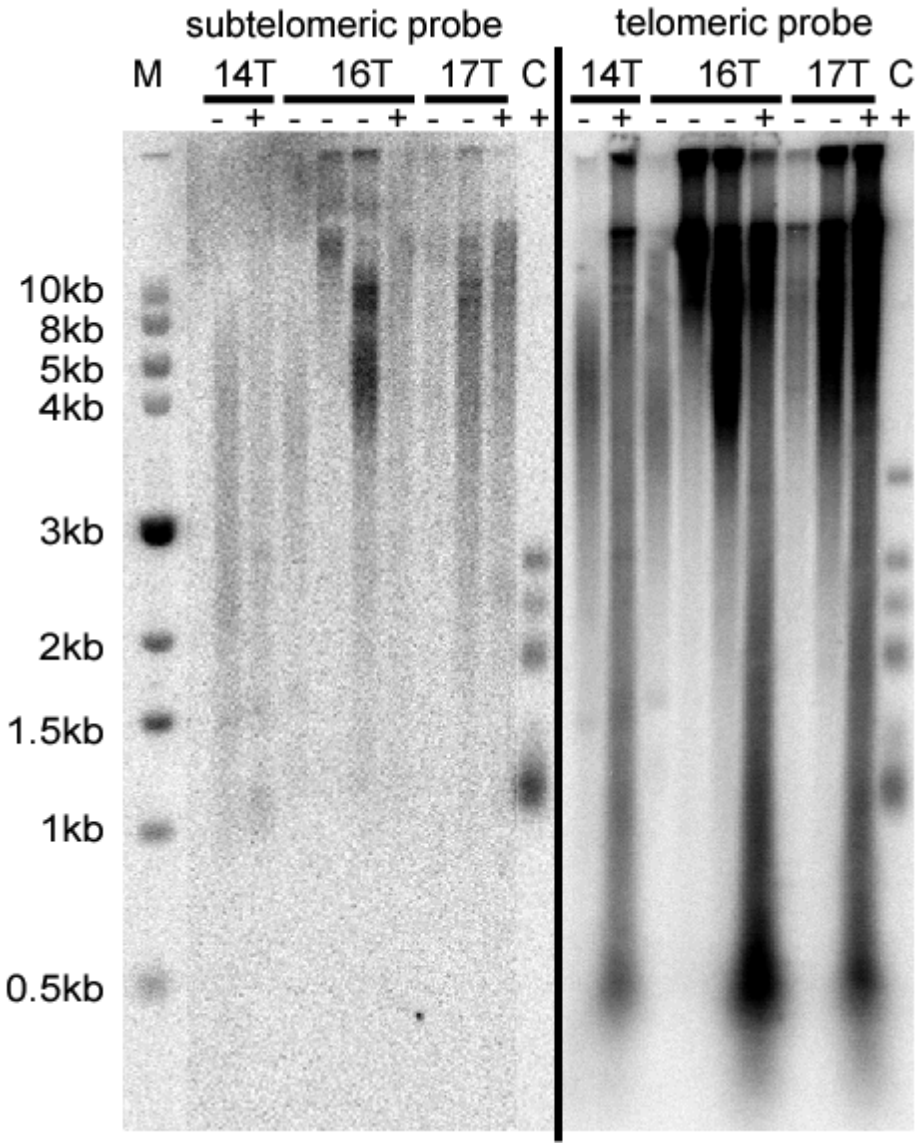

Fig. 3-5. *RAD52* is not required for extreme telomere elongation. *Eco*RI-digested genomic DNAs from *ter1-14T, ter1-16T,* and *ter1-17T* mutants and a wild-type control (lane C) was hybridized with a subtelomeric probe (left panel). After stripping the membrane to remove the subtelomeric probe the filter was rehybridized to the the telomeric probe. The – indicates *rad52* strains and the + indicates *RAD52* strains. Telomere lengths are most clearly visualized with the subtelomeric probe. Size markers (in kb) are shown.

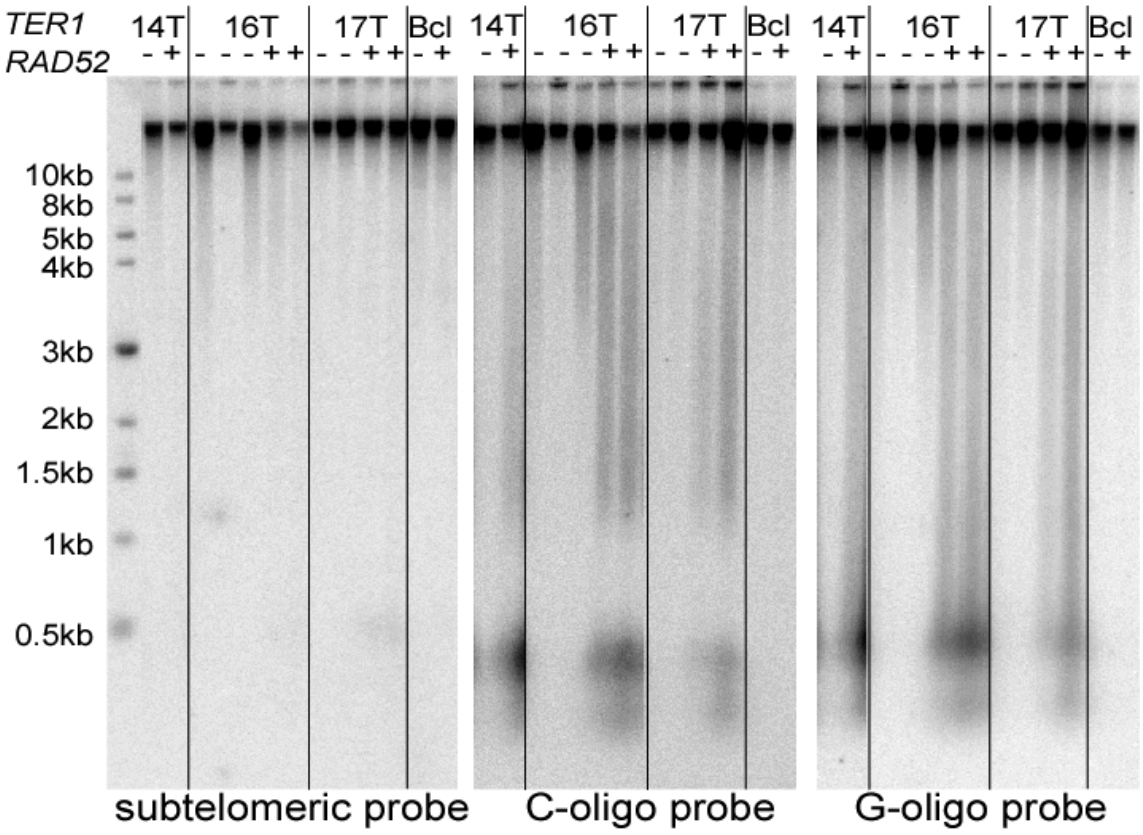

Fig. 3-6. A significant amount of the telomeric DNA is present as extrachromosomal pieces in strains with very long telomeres. Uncut genomic DNAs from *ter1-14T, ter1-16T,* and *ter1-17T* mutants and the phenotyically wild-type *TER1-7C(Bcl)* control were blotted and serially probed with a subtelomeric probe, a C-strand telomeric oligonucleotide probe, and a G-strand telomeric oligonucleotide probe. Lanes are marked with – or +, indicating the absence or presence of *RAD52* in the strain. Size markers (in kb) are shown.

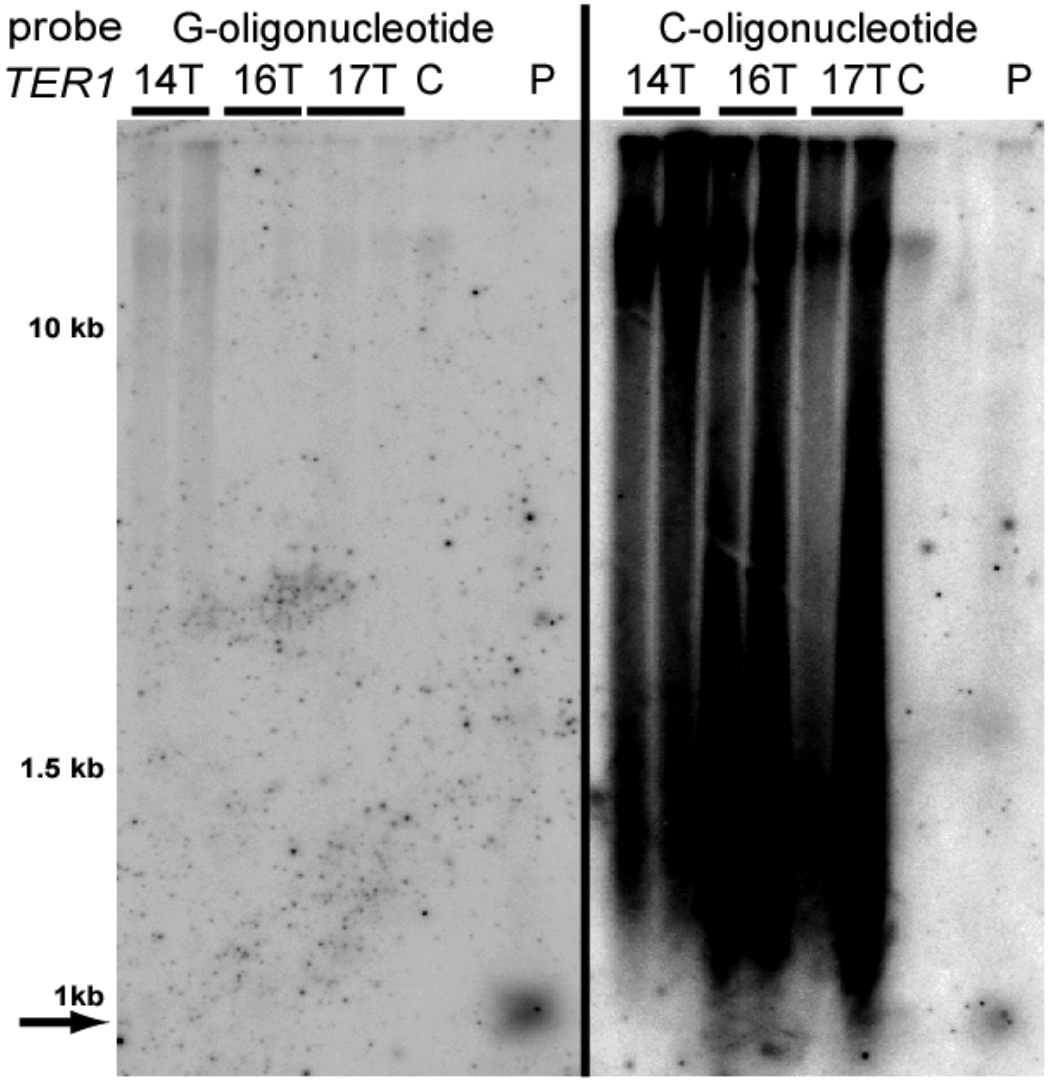

Fig. 3-7. Mutants with long telomeres show large amounts of single-stranded telomeric G-strand DNA. Uncut genomic DNAs from *ter1-14T, ter1-16T,* and *ter1-17T* mutants and the phenotyically wild-type *TER1-7C(Bcl)* control (lane C) were run on a low-percentage gel. Two samples from each mutant were examined. An in-gel hybridization was used to visualize the single-stranded DNA. The lane marked P contains an approximately 500 bp fragment from a plasmid that has telomeric sequence (marked with an arrow). Denaturation by boiling prior to loading onto the gel caused the fragment to run aberrantly and thus it is not indicative of molecule size. The positions of double-stranded DNA size markers (in kb) are shown.

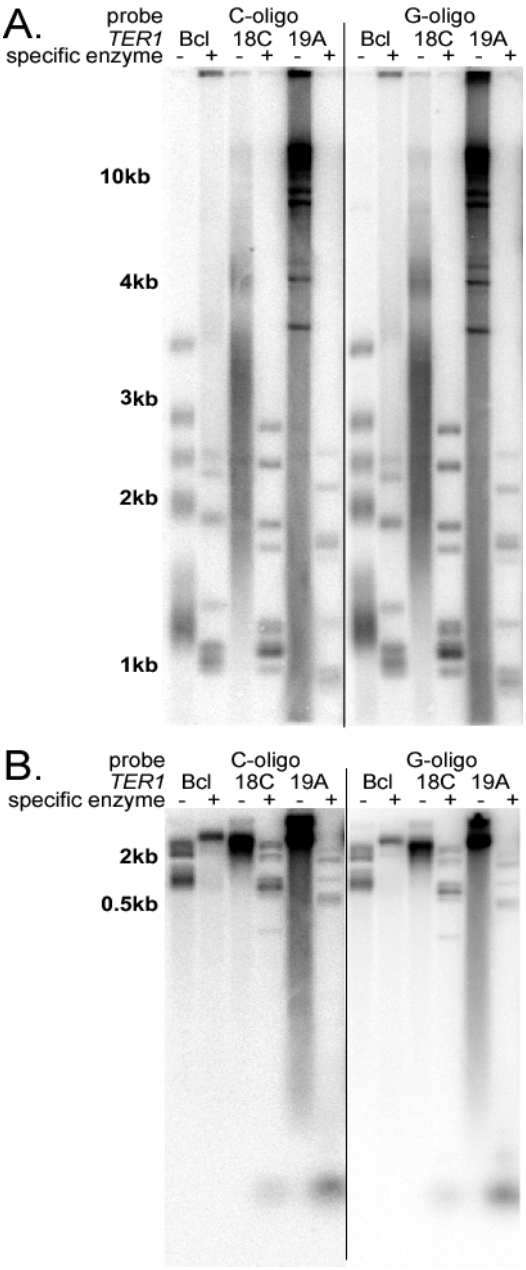

Fig. 3-8. Single-stranded overhangs in *ter1-18C(Bsi),* and *ter1-19A(Acc)* may be short. DNAs from *TER1-7C(Bcl), ter1-18C(Bsi),* and *ter1-19A(Acc)* were digested with *Eco*RI -/+ the specific restriction enzyme that cuts the mutant repeats in the strain (*Bcl*I, *Bsi*WI, and *Acc*I, respectively). Blots were sequentially probed with a C-strand telomeric oligo (left panel) and the G-strand telomeric oligo (right panel). **A)** To look for long overhangs, the gel was run such that the smallest fragments seen are approximately 1kb. **B)** To ascertain that no small fragments missed in the analysis in described in **(A)**, a high-percentage gel was used to separate smaller fragments. Again, no overhang was detected with the C-oligo. The very small fragments seen in both hybridizations are the small duplex molecules generated when each mutant telomeric repeat is cut by the specific restriction enzyme.

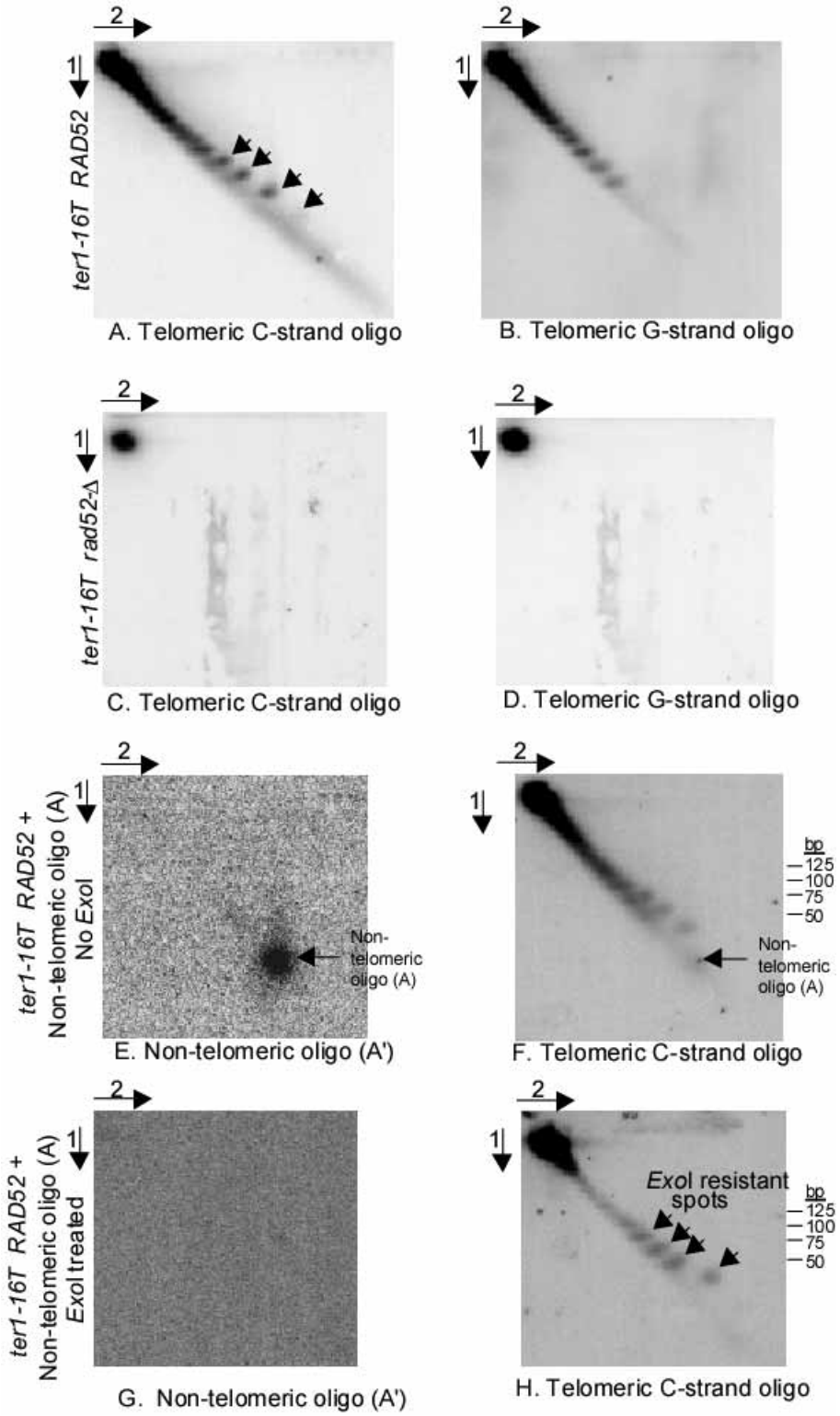
2
1
ter1-16T RAD52
A. Telomeric C-strand oligo
B. Telomeric G-strand oligo
ter1-16T rad52-Δ
C. Telomeric C-strand oligo
D. Telomeric G-strand oligo
ter1-16T RAD52 +
Non-telomeric oligo (A)
No ExoI
Non-
telomeric
oligo (A)
E. Non-telomeric oligo (A')
bp
125
100
75
50
Non-
telomeric
oligo (A)
F. Telomeric C-strand oligo
ter1-16T RAD52 +
Non-telomeric oligo (A)
ExoI treated
G. Non-telomeric oligo (A')
ExoI resistant
spots
bp
125
100
75
50
H. Telomeric C-strand oligo

Fig. 3-9. Two classes of discreetly-sized small telomeric molecules are present in the *ter1-16T* immediate elongation mutant. 2-dimensional gel electrophoresis was used to look for the presence of telomeric circles in the strain containing *ter1-16T.* The blots are presented in pairs, with the probe used for each blot written under each image. **A)** Arrows mark the putative circular molecules seen when the C-oligo is used to look for G-strand molecules. These molecules do not run on the diagonal with the linear DNA. **B)** When the G-oligo is used, no molecules are seen off the diagonal. Interestingly, molecules (expected to be linear duplex molecules) of discrete sizes are seen with this probe. **C)** and **D)** In the absence of *RAD52*, no broken molecules are seen with either probe. **E)** An arrow labeled A marks the location of a 66nt oligonucleotide that was run with the sample as an exonuclease control. The oligonucleotide is clearly visible when A', its complementary oligonucleotide, is used as a probe. **F)** A telomeric oligonucleotide is added to the filter shown in (E), which was not stripped. The signal from A is still visible and runs on the diagonal. **G)** When duplicates of the samples used in (E) and (F) are treated with Exonuclease I, A is completely digested and is not present to hybridize with A'. **H)** Although A was completely digested, the putative non-linear spots seen with the C-oligo probe are still visible, indicating that they are not ExoI-sensitive.

TABLES

			length		recombination	
position	wild-type	mutation	early	late	band loss	STU assay
1	T	G	short	short	+	
2	T	G	short	short	nd	
3	T	C	short	short	nd	
4	G	C	slightly short	wild-type	++	
5	A	C	slightly short	slightly long	++	
6	T	G	wild-type	long	nd	
7	T	C	wild-type	wild-type	nd	wild-type
8	A	T	wild-type	slightly long	++	
9	G	T	short	wild-type	++	
10	G	T	slightly long	wild-type	nd	
11	T	G	wild-type	very long	++	
12	A	C	wild-type	slightly long	+	
13	T	G	wild-type	wild-type	nd	
14	G	T	short	very long	?	elevated
15	T	G	short	very long	++	
16	G	T	long	very long	?	
		A	long	very long	?	
17	G	T	very long	very long	?	
		A	wild-type	wild-type	nd	
18	T	C	long	very long	?	elevated
19	G	A	very long	very long	?	elevated
		C	very long	very long	?	
20	T	G	very long	very long	?	
		C	wild-type	slightly short	nd	
21	A	C	slightly long	slightly long	nd	
22	C	A	short	short	+	
23	G	A	short	slightly short	+	
24	G	T	short	short	++	
		C	short	short	++	
25	A	C	slightly long	slightly long	nd	
26	T	G	wild-type	wild-type	nd	
27	T	G	short	short	++	
		A	short	slightly long	+	
28	T	A	short	short	++	
		G	wild-type	wild-type	nd	
		C	short	short	++	elevated
29	G	A	slightly long	slightly long	nd	
30	A	G	wild-type	slightly long	nd	

Table 3-1. Specific *TER1* template mutations lead to telomeric length and capping defects. The specific base changes made at each position are shown. The changes are shown in relation to the wild-type sequence of the telomeric G-strand. The telomere length phenotypes caused by each group of mutations are shown at two timepoints. Early indicates phenotypes seen in the first 30 restreaks; late indicates phenotypes seen in the restreaks 40-60. Recombination defects are shown when known. Loss of telomeric restriction fragments is indicated in the column labeled 'Band Loss'. + indicates loss of 1-2 restriction fragments, ++ indicates loss of 3 or more restriction fragments, nd indicates that band loss was not detected, and ? indicates that, due to extreme telomere length, it was not possible to determine whether telomeric bands were lost. When subtelomeric recombination assays have been done, results are shown.

strain background	method of Stu introduction	frequency of 5FOA ^{r}	gene conversion rate	relative rate (A)	relative rate (B)
TER1-7C(Bcl)	transformation	1/68889	7.6×10^{-5}	1	2×10^{-1}
ter1-18C(Bsi)	transformation	1/3200	2.5×10^{-3}	32	74
ter1-19A(Acc)	transformation	1/78	1.4×10^{-1}	1928	440
ter1-19A(Acc)	mating	1/3032	2.6×10^{-3}	34	8
ter1-14T early	mating	1/3059	2.6×10^{-3}	34	8
ter1-14T late	mating	1/1535	5.6×10^{-3}	73	17

Table 3-2. Subtelomeric recombination is elevated in strains with long telomeres. Recombination rates were measured in three strains with *ter1* template mutations causing telomere elongation. When describing the *ter1-14T* mutant, the second streak is described as early, while the fourth streak is described as late. In this delayed elongation mutant, the telomeres are elongated by the fourth streak. The frequency with which 5-FOA-resistant colonies arose is given. The gene conversion rate, which indicates the frequency of gene conversion per cell, is calculated from the 5-FOA resistance frequency using the method of the median (Snedecor and Cochran, 1980). The relative rates labeled (A) were compared with those seen in *ter1-7C(Bcl)*, which has wild-type rates. Relative rates labeled (B) were compared to the rates of wild-type *TER1* clones with somewhat elongated telomeres made by mating (see Results).

TER1 allele	*RAD52* +/-	peak	molecule size	% in fraction subtelomeric probe	% in fraction C-oligo probe	% in fraction G-oligo probe
ter1-7C(Bcl)	-	1	> 3 kb	95.6	96.5	96.5
		2	0.6 kb - 3 kb	2.6	1.7	1.7
		3	< 0.6 kb	1.7	1.8	1.8
	+	1	> 3 kb	94.7	96.8	96.8
		2	0.6 kb - 3 kb	3.5	2.0	2.0
		3	< 0.6 kb	1.8	1.2	1.2
ter1-17T	-	1	> 3 kb	91.52	93.3	94.1
		2	0.6 kb - 3 kb	5.529	3.2	2.5
		3	< 0.6 kb	2.951	3.5	3.3
	+	1	> 3 kb	90.666	82.4	86.3
		2	0.6 kb - 3 kb	4.975	11.0	6.0
		3	< 0.6 kb	4.359	6.5	7.8

Table 3-3. Levels of extrachromosomal telomeric DNA are decreased in *rad52Δ* strains. Levels of extrachromosomal DNA were determined in stains with wild-type length (*ter1-7C(Bcl*) or long (*ter1-17T)* telomeres with and without *RAD52*. Similar results are seen for all mutant strains examined. Peak 1 represents DNA greater than 3kb in size, peak 2 encompasses DNA between approximately 500nt and 3kb, and peak 3 represents molecules of less than 500nt. These numbers represent the quantitation data from the *TER1-7C(Bcl)* clones and the first and third lanes of the *ter1-17T* sections from Fig. 3-3.

Telo-A sequence from crystal structure	G	G	T	G	T	A	T	G	G	G	T	G	T
specific residues contacted-G-rich strand	G	G	T						G	G			
	N7	N7							N7	N7			
	O6	O6	O4						O6				
specific residues contacted-C-rich strand				C	A			C	C	C			A
				N4	N3			N4	N4	N4			N3
K. lactis telomeric sequence	G	G	T	G	T	A	C	G	G	A	T	T	T
Telo-A contacts not found in *K. lactis*	+	+	+	+	+			+	+	N4			+
(contacts maintained if +)													
mutation	16T	17T	18C	19A	20C	21C	22A	23A	24T	25C	26G	27G	28C
disruption	N7	N7	O4	N4	-			N4	N7	N7			-
phenotype (WT, short (S), long (L), very long (vL)	L	vL	L	vL	sIS	WT	S	S	S	WT	WT	S	S
mutation	16A	17A		19C	20G				24C			27A	28A
disruption	O6	O6		N4	N3				O6, N7, N4				N3
phenotype (WT, short (S), long (L), very long (vL)	L	WT		vL	vL				S			S	S
mutation													28G
disruption													N3
phenotype (WT, short (S), long (L), very long (vL)													S

Table 3-4. **Different mutations in the Rap1p binding site may disrupt Rap1p binding to different extents**. Comparison of the telomeric sequence used to determine the crystal structure of Rap1p in *S. cerevisiae* (Konig *et al*, 1996) with the putative Rap1p binding site in *K. lactis* indicates that, despite some divergence between the sequences, every specific contact with a residue on a nucleotide is maintained except one. When the phenotypes from the mutants are compared to the predicted contacts, it appears that the mutations with severe effects often cause a disruption of the contact between Rap1p and the telomere. Mutations with mild or wild-type phenotype (*ter1-20C, ter1-21C)*, however, do not disrupt the predicted contacts between Rap1p and the telomere.

CHAPTER 4

TEMPLATE REQUIREMENTS FOR TELOMERASE TRANSLOCATION IN THE YEAST *K. LACTIS*[1]

[1] Underwood, D.H., Zinzen, R., and McEachern, M.J. To be submitted to Molecular Cell.

ABSTRACT

Telomeres are synthesized by telomerase, a specialized reverse transcriptase, which contains a template in its intrinsic RNA component. In *K. lactis*, the repeats synthesized by the wild-type telomerase are 25 nucleotides in length and uniform in sequence. By making mutations in regions in the template thought to direct the synthesis of new telomeric repeats and the observing the types of repeats that are made, it has been possible to determine some of the requirements for telomerase translocation. These mutations typically result in telomeres that are shorter than those of wild-type cells. The mismatches between the telomerase template and the telomere that occur after incorporation of the mutations are normally neither removed nor extended by telomerase. Instead, the mutations lead to the synthesis of aberrant repeats that range in size from 31 bp to 13 bp. Therefore, the specificity by which the telomeric tip aligns with the telomere is critical for the production of the uniform repeats seen in *K. lactis*. In addition, the region immediately 3' of the template may play an important role in translocation of the enzyme.

INTRODUCTION

Telomeres, the DNA-protein complexes found at the ends of linear eukaryotic chromosomes, contain DNA composed of short, repetitive sequences. The sequences are maintained by telomerase, a specialized reverse transcriptase. The enzyme is a ribonucleoprotein, with the template for telomerase synthesis contained within the RNA moiety (Greider and Blackburn, 1989; Singer and Gottschling, 1994). The RNA is bound by the reverse transcriptase component, called Est2p in *S. cerevisiae* (Lingner et al., 1997) and TERT (telomerase reverse transcriptase) in humans (Nakamura et al., 1997), which contains conserved features found in reverse transcriptases (Cech et al., 1997).

At the telomere there is a duplex region that is involved in regulating the length of the telomere, and also a single-stranded region (reviewed in Zakian, 1996). In yeast the single-stranded overhang forms in a cell-cycle specific manner and is not dependent on telomerase for its formation (Dionne and Wellinger, 1996; Wellinger et al., 1993). It is at this part of the telomere that the access of telomerase to the telomere occurs. Cdc13p binds the single-stranded region (Lin and Zakian, 1996), where it plays a critical role in mediating access to the telomere (Chandra et al., 2001; Evans and Lundblad, 1999; Pennock et al., 2001). Stn1p, a negative regulator of telomere length, also interacts with Cdc13p (Grandin et al., 1997); competition between the negative activity of Stn1p and telomerase-mediated telomere addition is thought to be a component of telomere length regulation. Telomerase is conveyed to the telomere by a number of proteins, including Est1p (Evans and Lundblad, 1999) and the Ku complex (Grandin et al., 2000; Peterson et al., 2001), which interact with the RNA component of telomerase. Est1p interacts with the single-stranded region of the telomere, both through direct DNA binding and via interactions with Cdc13p (Evans and Lundblad, 1999; Qi and Zakian, 2000). The role of Ku, a protein involved in non-homologous recombination, is unclear.

The telomerase template, in most organisms examined to date, has terminal repeats of 3-5 nucleotides (nt) at both ends (Feng et al., 1995; McEachern and Blackburn, 1995; Yu et al., 1990). These repeats are thought to be involved in aligning the template with the telomere prior to the synthesis of a new telomeric repeat. The sequence copied from the end of the template would perfectly base-pair with the sequence at the start of the template, providing 3-5 nt of complementarity between the telomere and the template. Because the telomeric repeats are usually very similar within a species, the alignment of the telomere with the template is probably guided by other mechanisms as well. One notable exception to the trend toward uniform telomeric repeats is found in *S. cerevisiae*, which has telomeric repeats that are highly

heterogeneous despite the fact that there is only one allele of the telomerase RNA gene, *TLC1*, per cell (Prescott and Blackburn, 1997b; Singer and Gottschling, 1994). Work explaining the mechanisms by which a single template gives rise to heterogeneous telomeric repeats has led to two important conclusions (Forstemann et al., 2000; Forstemann and Lingner, 2001). First, *S. cerevisiae* telomerase does not always copy the entire length of the template, which would thus preclude it from always using the terminal repeat to align properly. Second, there are multiple binding sites within the template that have very similar sequences, such that alignment could occur at any of them and lead to the synthesis of variant repeats. The telomerase template has also been shown to play a role in the active site function of telomerase. In *Tetrahymena*, mutations in the telomerase RNA template lead to a decrease in enzyme fidelity and premature synthesis termination (Gilley et al., 1995).

The translocation step, in which telomerase dissociates from the newly synthesized repeat and realigns to synthesize a new repeat, offers the potential for variations in telomere synthesis. Despite this, telomeres in the budding yeast *Kluyveromyces lactis* are composed of uniform copies of a 25 base-pair (bp) repeat . The repeat is synthesized from a 30 nt template, which contains 5 nt terminal repeats at its ends (Fulton and Blackburn, 1998; McEachern and Blackburn, 1995). The terminal repeats of the RNA, which are separated by the 20 nt 'core' of the template, will be referred to as TR1-5 (terminal repeat, positions 1-5) and TR26-30 (terminal repeat, positions 26-30) for the remainder of this paper. The term 'positions' in the names refer to the 30 nucleotides in the *K. lactis* telomere; numbered 1-30; 1 is at the 3′ end of the template and 30 is the last possible nucleotide that could be copied from the 5′ end of the template. Just 5' of the template, only three nucleotides from the end of the template, is a base-paired structure. The eight nucleotide region of base-pairing involves a long-range interaction between the RNA sequence at the end of the template

and a region more than 300 nt away. Elimination of the base-pairing in this region *in vivo* causes telomerase to read through the template and incorporate sequences the non-template region adjacent to the template into the telomere (Tzfati et al., 2000). The telomeres are also short in mutants that lack base-pairing in this region. The base-paired structure therefore defines a stop signal beyond the end of the template, but previous studies have shown that, *in vitro*, *K. lactis* telomerase normally stops copying before reaching the end of the template (Fulton and Blackburn, 1998). While the template is 30 nt in length, *in vitro* telomerase typically stopped copying at position 28 of the template. The model proposed from the *in vitro* study was that telomerase normally copies to position 28 of the template and then aligns using only the first 3 nucleotides of TR1-5 (Fulton and Blackburn, 1998). Therefore, in this model positions 4 and 5 would typically be copied from the template, while positions 29 and 30 would not. Because the sequences of TR1-5 and TR26-30 are identical, it is impossible to determine which one is the template for a particular nucleotide in the *in vivo* wild-type system.

In order to test the requirements for normal alignment of the telomerase template with the telomeric tip *in vivo*, a collection of yeast strains containing point mutations in the telomerase template was examined. By cloning telomeres templated by various *TER1* mutants, it was possible to determine whether an aberrant translocation event had occurred. These experiments were also used to address the differences between *in vitro* and *in vivo* telomerase activity and determine the minimum base-pairing interactions required for translocation *in vivo*.

MATERIALS AND METHODS

Mutagenesis of the *TER1* template

The plasmid pTER-BX-UA (McEachern and Blackburn, 1995) was mutated via one of 2 methods. Some mutations were constructed using a single-stranded template and a mutagenic oligonucleotide (Kunkel et al., 1987). The majority of the mutations were made using the Quik-change mutagenesis kit (Stratagene, LaJolla, CA). Oligonucleotides of approximately 25 nucleotides were used.

Strains

Strains used in this study are derivatives of haploid *K. lactis* 7B520 (Wray et al., 1987). The strain is *ura3*, *his3*, and *trp1*. A His^+ revertant was used to construct many of the *ter1* template mutant strains. Wild-type refers to the 7B520 parental strain, and the control strain used in the experiments is the strain Bcl+His, a His^+ revertant strain containing the phenotypically silent *ter1-Bcl* allele. Previously characterized *ter1* template mutants (McEachern and Blackburn, 1995; McEachern and Blackburn, 1996; McEachern and Iyer, 2001; McEachern et al., 2000; McEachern et al., 2002) were constructed in the 7B520 strain. Mutants not previously reported were constructed primarily in the Bcl+His strain, although some were constructed in the 7B520 strain, using the plasmid loop-in/loop out method as described previously (McEachern and Blackburn, 1995). When two telomerase molecules were present in the same cell they existed as heteroalleles and were the product of a plasmid loop-in.

The long-term passaging of the strains was carried out by serial restreaking on rich media (YPD plates). The strains were streaked to single colonies every 3-4 days. Each streak is estimated to represent 20-25 cell divisions. Transformation was carried out using a modified procedure identical to that used for *S. cerevisiae*. The procedure was scaled down proportionately so that for each transformation a 1 ml overnight culture

was grown to saturation. The next day 0.5 ml YPD liquid was added to the culture to return the cells to the growth phase. The cells were grown for 1-2 hours, and then 1.5 ml cells were pelleted at 3000 x g for 5 minutes. The supernatant was removed and the cells were resuspended in 500 μl cold H_2O and repelleted. The cells were resuspended in 80 μl cold H_2O. 10 μl each of 10X TE and 1M LiAc was added and the mixture was incubated at 30° C for 45 minutes. The cells were incubated for an additional 15 minutes after the addition of 2.5 μl 1M DTT. 400 μl cold H_2O was added and the cells were repelleted. The cells were serially resuspended and pelleted in 250 μl cold H_2O, 200 μl cold 1 M sorbitol, and 30 μl cold 1 M sorbitol. The cells were incubated on ice 2 minutes after the addition of DNA. The cells were then electroporated at 1500 V, resuspended in 1 M sorbitol, and plated on appropriated selective plates.

Hybridizations

Restriction enzyme digested yeast genomic DNA was run on 0.8% or 1% agarose gels, stained with EtBr, and Southern blotted onto Hybond N+ membranes (Amersham Biosciences, Piscataway, NJ). Hybridizations were carried out in Na_2HPO_4 and SDS (Church and Gilbert, 1984). The telomeric oligonucleotide used was the G-strand oligonucleotide Klac1-25 (ACGGATTTGATTAGGTATGTGGTGT).

Telomere cloning

The types of telomeric repeats synthesized *in vivo* by the mutated *TER1* alleles was determined by direct cloning of telomeres from yeast cells. Telomeres were cloned from the longest-streaked strains available at the time of the experiment. In some cases, telomeres were cloned after as few as 3 serial restreaks, while in other cases as

many as 40 serial restreaks had occurred. Telomeres have been cloned using three different methods. All three will be described below.

Using a linear plasmid

The plasmid pHISLIN1 (McEachern et al., 2002) contains yeast *CEN* and *ARS* sequences and has two telomeric tracts in opposite orientations, separated by the *URA3* gene. The plasmid was cut with *Xho*I and *Bam*H1 to excise the *URA3* gene to yield a linear plasmid with *K. lactis* telomeres at both ends. The linear DNA was introduced into *ter1* template mutants and transformants were selected for the presence of the plasmid by growth on plates lacking histidine. The DNA prepared from the transformed yeast cells was digested with *Sma*I, which cleaved one telomere from the plasmid. The remaining plasmid was then ligated into a circle and transformed into *E. coli*. The resulting plasmids were sequenced.

Using an integrative plasmid

The majority of the telomeres cloned in thus study were recovered using one of two integrative plasmids used to clone telomeres from yeast cells. The first, pSTH (subtelomeric *HIS3*) was made by inserting a 400 bp *Eco*RI-*Sal*I fragment of subtelomeric DNA from pAK25 (McEachern and Iyer, 2001) into pRS423 (Sikorski and Hieter, 1989). The plasmid was transformed into *his3* yeast and transformants were selected by growth on media lacking histidine. The plasmid integrated at telomeres by subtelomeric homology. In some instances, transformants were pooled in the cultures used to prepare the genomic DNA. Telomeres were recovered by digesting the genomic DNA with either *Sna*BI or *Xho*I, filling in the overhangs with T4 DNA polymerase, and ligating the blunt ends into a circle. After transformation into *E. coli*, the plasmids were prepared and sequenced.

The second plasmid, pMya, is a derivative of the pAK25 plasmid (McEachern and Iyer, 2001) in which the telomeric repeats were deleted by releasing a *Sac*I fragment and then ligating the cohesive ends together. Transformants were selected on media lacking uracil; the plasmid integrated into the subtelomeric sequences via homology with the plasmid. Once genomic DNA was prepared from the transformed cells and treated with T4 DNA polymersae and dNTPs to make the ends blunt, a linker oligonucleotide containing a *Sac*I restriction site was ligated onto the DNA ends. The DNA was then cut with *Sac*I and ligated to form circles. After transformation into *E. coli*, plasmids were screened by Southern blotting and hybridization with a telomeric probe.

Blunt-end ligation of broken DNA

In strains with very long telomeres, it was expected that it would difficult to clone intact telomeres, as they would often be greater than 10kb in length. In order to obtain telomeric fragments that were short enough to be stable in a plasmid, a strategy was devised to clone the extrachromosomal pieces of telomeric DNA known to be present in the strains with long telomeres (D.H. Underwood et al, manuscript to be submitted to Genes and Development, and McEachern and Blackburn, 1995). Total yeast genomic DNA was treated with T4 DNA polymerase and dNTPs to fill in any overhangs and make the ends blunt. A *Sac*I linker was ligated onto the blunt ends and the construct was cut with *Sac*I. The DNA was then ligated into a pBluescript that had been linearized by *Sac*I digestion. The telomeric inserts recovered were between 150-250bp in length. Sequencing was carried out using either the Sequenase kit (Amersham Biosciences, Piscataway, NJ) or by automated flourescent dideoxy sequencing.

RESULTS

Mutations in the terminal repeats of the *TER1* template compromise telomere function

In all known telomerase RNAs, the template region is flanked by short terminal repeats. These terminal repeats (TR) are thought to be important for allowing telomerase to align with the telomere prior to the synthesis of a new telomeric repeat. The model for the synthesis of new telomeric repeats suggests that alignment occurs between the 3' end of the telomere and the 3' TR of the telomerase template. The telomeric repeat would then be reverse-transcribed from the template, and the telomeric sequence copied from the 5' TR would subsequently be used to realign the 3' TR of the RNA with the telomeric tip in order to initiate the synthesis of another telomeric repeat. Figure 4-1A shows *K. lactis* telomerase translocation as described by this model, which will be examined *in vivo* in this work. In *K. lactis*, the telomerase RNA (Ter1) has a 30 nt template region that contains 5 nt termial repeats, referred to as TR1-5 and TR26-30. The terms TR1-5 are TR26-30 will be used to refer to both the 5 nt terminal repeats in the Ter1 template and the sequences in the gene which encodes them, but not the telomeric nucleotides that they template. Telomeres in *K. lactis* are composed of perfect 25 bp repeats, indicating that *K. lactis* normally aligns with the telomere using TR1-5.

To examine the role of the Ter1 terminal repeats, mutations were made such that either TR1-5 or TR26-30 of the telomerase template was deleted. RNAs containing each deletion also contained different point mutations that are phenotypically silent, allowing the activity of telomerase molecules lacking the template terminal repeats to be examined *in vivo*. In mutants containing either deletion allele as the sole source of telomerase, the colonies were rough in appearance and exhibited somewhat slower growth than wild-type *K. lactis*. Although the growth phenotype was similar to that of an early-stage *ter1* null mutant (McEachern and Blackburn, 1996), neither deletion mutant displayed the growth senescence and eventual cell death that is characteristic of *ter1*

null mutants. The cells maintained the ability to grow indefinitely, albeit at the somewhat slower rate. Examination of the lengths of the telomeres by Southern blotting indicated that they had became very short (data not shown). When the *ter1-Δ1-5* and *ter1-Δ26-30* alleles were present in the same cell, telomeres were, at most, slightly longer than in either single mutant (data not shown). Cloning of the telomeres, however, revealed that both of the phenotypically silent point mutations could be incorporated into a single telomeric repeat (Fig. 4-2, indicated with gray nucleotides), indicating that telomerase molecules containing either TR deletion could function. Incorporation of both base-changes was only seen in one of the two cloned telomeres. In the other cloned telomere, only one mutation was incorporated. Both of the cloned telomeres were extremely short (less than 125 bp), consistent with short telomeres seen on the Southern blot. To minimize the possibility of artifacts, all telomeres described in this study were cloned using plasmid rescue techniques instead of PCR-based protocols (see Materials and Methods). Thus, when *ter1-ΔTR1-5* and *ter1-ΔTR26-30* were both present in the same cell, it became possible for chromosome ends to acquire more than one newly-synthesized telomeric repeat. These results show that, although the terminal repeats of the telomerase template were important for proper telomerase function, their deletion did not abolish telomerase activity.

It has been shown that base substitutions in the 20 nt 'core' of the Ter1 template found between TR1-5 and TR26-30 were typically incorporated into the 25 bp telomeric repeat as the expected base changes (Fig., 4-1A and 4-1B). First, telomeres from a strain containing the phenotypically silent allele *TER1-7C(Bcl)* (previously published as *TER1-Bcl*) were cloned and contained only the expected single base-change in each newly synthesized telomeric repeat (Fig. 4-1B) (McEachern et al., 2002; Tzfati et al., 2000). Telomeres were also recovered from three mutants that contained telomerase alleles that led to the formation of very long telomeres, and the telomeres from all three

strains contained only the expected change (unpublished data and McEachern and Blackburn, 1995). Therefore, mutations made in the 'core' of the template were typically copied faithfully into the telomere by telomerase and do not routinely affect the process of translocation.

In order to confirm the role the *K. lactis* telomerase terminal repeats in telomerase translocation, a series of point mutations were made in TR1-5 and TR26-30. The mutations are named in terms of the position that was mutated, such that *ter1-2G* indicates that position 2 of the template was altered and the sequence that would be *copied into the telomere* from that position is a G. *K. lactis* strains containing *TER1* alleles with template point mutations as their only source of the telomerase RNA were constructed. The mutants described in this work were part of a larger project involving mutagenesis of each position in the template (D.H. Underwood et al, manuscript to be submitted to Genes and Development). Telomere length in the terminal repeat mutants was typically found to be shorter than that of wild-type cells. A Southern blot of many of the terminal repeat mutants after forty serial restreaks is shown in Fig. 4-3. In most cases, the telomere lengths after an extended period of growth were similar to the telomere lengths shortly after construction of the mutants (D.H. Underwood et al, manuscript to be submitted to Genes and Development). Mutations in the first 3 positions of TR1-5 led to significant telomere shortening, while mutations in positions 4 and 5 of TR1-5 initially exhibited mild telomere shortening that persisted over an extended growth period (Fig. 4-3). At the latest timepoints examined (50 and 60 serial restreaks, 1000-1250 generations), the telomeres in *ter1-4T* elongated slightly and became nearly wild-type in length (D.H. Underwood et al, manuscript to be submitted to Genes and Development, and data not shown). The telomeres in *ter1-5T*, on the other hand, sometimes became elongated by hundreds of bases (D.H. Underwood et al, manuscript to be submitted to Genes and Development, and data not shown). Mutations

at two positions of TR26-30 led to telomere shortening (Fig. 4-3). The alleles *ter1-27A, ter1-27G, ter1-28A*, and *ter1-28C* led to telomere shortening. However, *ter1-26G, ter1-30G*, and *ter1-28G* had no apparent effect on telomere length and the mutation at position 29 (*ter1-29A*) led to a very mild telomere elongation (Fig. 4-3 and data not shown).

In addition to changes in telomere length, long-term passaging of the *TER1* template mutants revealed that many had elevated levels of subtelomeric recombination. This was indicated by loss of *Eco*RI restriction fragments in Southern blots (Fig. 4-3, compare lanes 2, 4, 5, 6, 9, 13, 15, 18, and 19 with lanes 7 and 8, which showed a banding pattern identical to wild-type, and D.H. Underwood et al, manuscript to be submitted to Genes and Development). Such band loss has been shown to be due to gene conversion events that homogenize subtelomeric polymorphisms (McEachern and Iyer, 2001). This phenomenon is associated with many of the *ter1* mutants with short telomeres and is thought to indicate a compromised telomere capping function. In the cases of *ter1-4T, ter1-5T,* and *ter1-8T*, however, telomeric bands were lost despite the telomeres being close to wild-type in length (Fig. 4-3, lanes 4 and 10, and data not shown). This suggests that these mutants might also have disrupted telomere capping, possibly caused by mutations incorporated into the telomeric repeats.

Three point mutations in TR1-5 and TR26-30 lead to minor shifts in alignment

In order to study the effects of the *TER1* template mutations on telomerase translocation, telomeres were cloned from the mutants. The sequencing of telomeres allowed models to be made that explained the translocation events that would have occurred in order for those particular sequences to be copied by telomerase. Telomeres were cloned from ten terminal repeat mutants; three mutations, *ter1-3C*, *ter1-28G*, and *ter1-2G,* led to one or two nucleotide shifts in the normal alignment between telomerase

and the telomere. When telomeres were cloned from the *ter1-3C* strain, a 26 bp repeat was seen near the terminus of two of the three telomeres cloned, instead of the 25 bp length of wild-type repeats. The small number of mutant repeats recovered may be because the mutants had only been grown for 90 cell divisions prior to cloning the telomeres and therefore contained very few newly synthesized telomeric repeats. Synthesis of the 26 bp repeat appeared to be due to a shift of the template, such that instead of the alignment being between the telomeric tip and positions 1-3 of the template it instead involved the position immediately 3' of the template (position –1) and positions 1 and 2 (Fig. 4-4A). This assumes that the telomeric tip terminates in TTT, copied from positions 26-28. Previous work had indicated that position 28 was the predominant stop site for *K. lactis* telomerase *in vitro* (Fulton and Blackburn, 1998). The mutation at position 3 would disrupt base-pairing of the 3 nt complementary region normally used for alignment. Therefore, the region of telomerase used for alignment was shifted by 1 nt in order to restore perfect base-pairing between the 3' end of the telomere and the template.

Similarly, the *ter1-28G* mutation caused a 1 nt shift into the template where the base-pairing interaction occurred with positions 2-4 of the template (Fig. 4-4B). This led to the synthesis of a 24 bp repeat, which was observed three times in a single telomere. Finally, a mutation at position 2, made by the allele *ter1-2G*, sometimes caused a 27 bp repeat to by synthesized. This long repeat was observed a total of four times in the two telomeres examined (Fig. 4-4C). For this to occur, the TT sequence copied from positions 27 and 28 was predicted to base-pair with positions –1 and 1 at the start of the template. When this occurred, the mutation at position 2 could be copied into the telomere. The shifts in the region of telomerase that was aligned with the telomere in the *ter1-3C, ter1-28G,* and *ter1-2C* mutants indicate that mismatches between telomerase

and the telomeric tip are not typically removed by a proofreading activity; instead, they lead to the synthesis of aberrantly sized telomeric repeats.

Translocation can occur to sites within the 'core' of the template

A double point mutant made at positions 28 and 30 of the template, *ter1-28A/30G*, caused the sequence of TR26-30 to no longer be identical to TR1-5; instead, it is identical to the 5 nucleotides at template positions 6-10 (Fig. 4-5A). This allele was thus predicted to lead to the synthesis of 20 bp repeats that lacked the sequence from TR1-5. When telomeres were cloned from a strain containing *ter1-28A/30G* as its only telomerase RNA allele, 20 bp repeats of the predicted sequence were found at the tips of the telomeres (Fig. 4-5A). The telomeres were also short, consistent with their appearance on a Southern blot (Fig. 4-5A and data not shown). This indicated that the sequence added at the tip of the telomere by the mutated *TER1* allele could base-pair with a sequence within the 'core' of the template for the next round of telomere synthesis.

If template position 30 is not normally copied by telomerase, as suggested by *in vitro* data (Fulton and Blackburn, 1998), it would be expected that the single mutant *ter1-28A* might lead to the synthesis of the same 20 bp repeats synthesized by *ter1-28A/30G*. To examine this, the allele *ter1-28A*, was made. Telomeres in the strains containing *ter1-28A* were initially short, similar to those in the *ter1-28A/30G* mutant (Fig. 4-3 and data not shown). When *ter1-28A* mutant telomeres were cloned, it was found that the same 20 bp repeat found in the double point mutant was often copied (Fig. 4-5B). This could occur whether telomerase typically stopped copying at either template position 28, where the single mutation could direct the synthesis of the 20 bp repeat, or template position 29, which already matched the sequence needed to base-pair with position 9 and would thus be expected to have no effect. In two of the five clones, however,

aberrant translocation events occurred. In these instances, 4 or 6 bp of additional sequence was found between the incorporation of the mutation at the end of the repeat and the start of the next newly synthesized repeat. In the example where four extra bases were seen (Fig. 4-5B, tip repeat of fourth telomere), a single misalignment with the region near TR1-5 could have led to their incorporation. In the second case (Fig. 4-5B, tip repeat of fifth telomere), however, no single misalignment could account for the extra four nucleotides. One possible explanation is that the additional sequence was incorporated when telomerase 'stuttered' as it tried different alignments in the TR1-5 region before finding an appropriate one and synthesizing a full repeat. The fact that the repeats made by *ter1-28A*, unlike those made by *ter1-28A/30G,* were not all 20 bp in length may indicate that position 30 is copied at low frequency. The nucleotide copied from position 30 might, when incorporated, also be involved in base-pairing during translocation.

The most dramatic example of aberrant translocation leading to altered telomeric repeat size was observed in a strain with the *ter1-27G* allele, a mutant which also had short telomeres (Fig. 4-3). In this case, the sequence copied from positions 26-28 would be TGT. As with the previously described allele, cloned telomeres indicate that more than one type of repeat was synthesized by *ter1-27G.* The most easily explained was a 13 bp repeat that apparently occurred when the telomeric tip base-paired with positions 13-15 of the template (Fig 4-5C). This repeat was observed a combined total of three times in two of the four telomeres cloned (Fig. 4-5C, first and second telomeres). No repeat shorter than 13 bp was found to be synthesized by any *K. lactis TER1* allele to date. In three other repeats, additional sequences were found (Fig. 4-5C, third and fourth telomeres, shown in italics) between the incorporation of the mutation at the end of one repeat and the start of the next newly synthesized repeat. Although the five nucleotides at the beginning of the terminal repeat of telomere three matched TR1-5, it

was unclear what base-pairing interactions led to their incorporation into the telomere. For the small additions found at the end of the terminal repeat in telomere three and the start of the tip repeat in telomere four, it was again unclear what base-pairing interactions caused their incorporation, although they could have been copied from copying TR1-5 or the mutated TR26-30.

Mutations within the 'core' of the template can cause aberrant translocation

The mutations described thus far have all been within either TR1-5 or TR26-30. In order to test whether mutations within the 'core' of the template could also cause aberrant translocation events, the mutation *ter1-8T* was created. This mutation could potentially function in a manner similar to *ter1-28A* in that the sequence copied from positions 26-29 would be a perfect complement to template positions 6-9. The unusual characteristic of this mutant, however, was that template positions 1-4 and 6-9 are identical. Therefore, it was expected that telomerase might be able to base-pair at either location.

It was found that two types of repeats were copied by *ter1-8T* (Fig. 4-6). One type was 25 bp long and contained a single base-change at position 8, consistent with incorporation of the mutant nucleotide into the telomere following normal alignment between the telomere and TR1-5. The other repeats were 20 bp in length, consistent with the telomeric tip base-pairing with telomerase position 6-9. In the six cloned telomeres examined, there was a significant bias towards the 25 bp repeat containing the point mutation; 44 such repeats were found, as compared with 8 of the 20 bp repeats.

Unexpectedly, there was evidence of telomeric recombination in four of the six telomeres cloned from the *ter1-8T* mutant. This *ter1* mutant, like most in this study (see Materials and Methods), was made in a background that initially contained the

phenotypically silent *TER1-7C(Bcl).* Therefore, its telomeres contained wild-type repeats basally and Bcl repeats (templated by the *TER1-7C(Bcl)* telomerase) distally prior to introduction of the *ter1-8T* mutant allele. For a brief time, *TER1-7C(Bcl)* and the *ter1-8T* allele were in the cell simultaneously and thus the telomere might contain both types of mutant repeats intermingled. In this strain, however, wild-type, *ter1-7C(Bcl)*-templated, and *ter1-8T*-templated repeats were interspersed at the telomere (Fig. 4-6, wild-type sequences marked with *). The presence of wild-type repeats nearer to the telomeric tip than repeats templated by *ter1-8T* is not an arrangement that could have been generated by the action of telomerase, as the wild-type *TER1* allele was deleted in the strain prior to the introduction of *ter1-8T*. A likely explanation is the occurrence of recombination between the arrays of telomeric repeats. Long-term passaged *ter1-8T* cells display the loss of one or more of the *Eco*R1 restriction fragments seen in a Southern blot (D.H. Underwood et al, manuscript to be submitted to Genes and Development) that characterizes high rates of subtelomeric recombination (McEachern and Iyer, 2001). This further supports the possibility that telomeric recombination may be enhanced in this mutant.

Template positions 29 and 30 are sometimes copied

Analysis of telomeres cloned from the *ter1-28A* mutant support the hypothesis that, *in vivo*, *K. lactis* telomerase normally copies position 28 of the template. Telomeres cloned from strains with *TER1* mutations at positions 4, 5, 29, and 30 provided a way to examine whether telomerase also copied positions 29 and 30 *in vivo*. If a position was copied *in vivo*, then the presence of a *TER1* allele mutated at that position would lead to incorporation of a mutated nucleotide into the newly synthesized telomeric repeats and could potentially cause aberrant translocation and thus abnormally sized telomeric repeats. Telomeres cloned from a strain containing *ter1-5C* indicated that position 5 was

usually copied by telomerase. If the number of repeats that had incorporated the mutation at position were counted, starting from the first repeat that was known to be synthesized by *ter1-5C*, position 5 was incorporated 18 out of 24 times in the two telomeres examined. When the same calculation was made telomeres cloned from the *ter1-4C* mutant, position 4 was found to be incorporated 17 out of 25 times in the four telomeres examined. In both mutants, the mutation was incorporated into 25 bp repeats as the single expected base-change.

The results obtained from strains containing *ter1-4C* and *ter1-5C* were expected to have an inverse relationship with the numbers obtained when strains mutated at positions 29 or 30 were used. The model shown in Fig. 4-1 predicted that, for each repeat synthesized, the telomeric sequence was copied from either position 4 or 29 in a mutually exclusive manner. The same would be true for positions 5 and 30. When six telomeres from *ter1-29A* were cloned, the repeats were observed to be completely wild-type. Possible reasons for the complete lack of mutant repeats will be described in the Discussion. The telomeres cloned from this strain were slightly longer than average for *K. lactis* (data not shown), consistent with the slightly long appearance of the telomeres on a Southern blot (Fig. 4-2). Only one telomere was recovered from the strain containing *ter1-30G*, but the telomere contained three repeats that showed incorporation of the mutation at position 30. Interestingly, this mutation led to an aberrant translocation event that produced a 13 bp repeat; this apparently occurred through base-pairing between the telomere and positions 15-17 of the template (Fig. 4-7). Although additional base-pairing interactions are not shown in Fig 4-7, several nucleotides within the telomeric repeat could potentially be involved in base-pairing with the template. These include the nucleotides at positions 7, 8, 10, 12, and 13. This repeat is the same 13 bp copied by *ter1-27G*, although they differ by 2 nucleotides due to the sites of the mutations. Although the telomeres in the strain containing *ter1-27G* were short and

recombinogenic (as judged in long-term passaged cells by the loss of *Eco*RI restriction fragments) (Fig. 4-2 and data not shown), telomeres in the strain containing *ter1-30G* were wild-type in length and showed no evidence of the telomeric band loss characteristic of elevated subtelomeric recombination (Fig. 4-2). The results presented here indicate that, although telomerase can copy the 5' end of the template up to and including position 30, copying of positions 4 and 5 is more common.

The region immediately 3' of the template is available for base-pairing and is important *in vivo*

The third *TER1* allele made with a mutation at position 28 was *ter1-28C*. Like *ter1-28A*, *ter1-28C* led to shortened telomeres (McEachern and Iyer, 2001) and translocation defects. When telomeres were cloned from a strain containing *ter1-28C*, telomeric repeats that were 31 bp long were found (Fig. 4-8A). In order for these repeats to be made, the telomere must have base-paired with nucleotides 3' of template position 1 (referred to as –3 and –4, respectively). This indicates that this region of the telomerase RNA was accessible for base-pairing with the telomere. Interestingly, the sequence found at template positions -4, -3, and -2 was identical to that found at template positions 22, 23, and 24 (Fig. 4-8B). This sequence identity, coupled with the fact that the region 3' of the template was available for base-pairing, led to the idea that these positions might be involved in stabilizing the initial alignment between telomerase and the telomere in wild-type cells. As an initial test of this, two point mutations were made in the sequence immediately 3' of the telomere. The first, *ter1-(-4A)*, disrupted the potential for base-pairing between that position and the telomere and led to moderate telomere shortening (Fig. 4-8C). The second was *ter1-(-1A)*. This position, which was normally a mismatch for any potential interaction with the telomeric nucleotide copied from position 25, was altered so that base-pairing could occur. This mutation led to mild

telomere elongation. Elongation was only seen in three of the four clones made. There are two likely explanations for this: the strain with wild-type telomere length might not have replaced the wild-type *TER1* with the mutant, or a suppressor could have arisen. These results clearly indicate that the sequence immediately 3' of the template plays an important role in telomerase function.

DISCUSSION

Proper telomerase translocation requires that each of several steps in the process occur correctly. Therefore, in order to examine telomerase translocation *in vivo* it is necessary to address a number of different questions. First, it is necessary to determine what nucleotides in the telomerase template are reverse-transcribed into the telomeric repeat. It is also necessary to determine what positions within both the telomere and the telomerase template contribute to base-pairing during translocation. Another issue is whether *K. lactis* telomerase has a nuclease activity which could remove mismatched bases from the telomere upon alignment with the Ter1 RNA. In some species, a nuclease activity has been found to be associated with telomerase (Greene et al., 1998; Lue and Peng, 1997). A related question involves determining whether mismatches between the tip nucleotides of the telomere and the telomerase RNA inhibit the ability of telomerase to extend the telomeric tip. All of the factors above combine to determine what telomeric repeats are synthesized by telomerase.

In order to examine telomerase translocation, mutations in TR1-5 and TR26-30 were made and telomeres from the resulting mutants were cloned and sequenced. Models were then created to explain the aberrant translocation events observed in the mutants. Several factors complicated the construction of the models. First, it was not possible to determine how all of the factors described above were affecting a given mutant; although incorporation of the mutation could be easily discerned from the

telomere sequences, the exact stop site of telomeric repeat synthesis, for example, might not always be obvious. It was therefore necessary to view the totality of the data before many conclusions could be made. Also, in some cases only a very few mutated repeats were sequenced. Because many of the mutations lead to telomere shortening, it is often impossible to recover long tracts of mutated repeats. However, the plasmid rescue method utilized is unlikely to introduce errors into the sequence, making the sequences recovered likely to be reliable. The aberrant telomeres recovered contrast sharply with wild-type telomeres, in which the only aberrant translocation events found are rare 26 bp repeats that contain an extra T before the three Ts of positions 1-3 (Y. Tzfati, personal communication). Therefore, because each mutation examined caused different translocation events, specific models were created for each mutant. Although the models may describe only a subset of all events that actually occur, they provide valuable insight into how telomerase functions.

Mismatches between the telomeric tip and the template are not typically removed

A nuclease activity has been reported to be associated with telomerase in *Euplotes crassus, Tetrahymena thermophila,* and *Saccharomyces cerevisiae* (Cohn and Blackburn, 1995; Collins and Greider, 1993; Greene et al., 1998; Lue and Peng, 1997; Melek et al., 1996; Niu et al., 2000; Prescott and Blackburn, 1997b). In *E. crassus*, where the activity has been well characterized, the nuclease has been shown to cleave by an endonucleolytic mechanism. It can remove long stretches of non-telomeric DNA and preferentially cleaves the single-stranded DNA at the junctions of mismatches between the telomeric primer and the telomerase template, thus removing the mismatch (Greene et al., 1998; Melek et al., 1996). In *S. cerevisiae,* mismatches between the telomeric sequence and the telomerase template have been shown to stimulate cleavage of the single-stranded telomeric primer by the telomerase-associated nuclease,

which would also lead to the removal of the mismatch (Prescott and Blackburn, 1997a). However, there was also no evidence that a nuclease activity acted on mutated telomeric repeats in *Tetrahymena*, where mutations in the telomerase template led to lead to the synthesis of aberrantly sized telomeric repeats (Yu et al., 1990). In *K. lactis* strains containing telomerase template mutations, the problem of mismatches between telomerase and the template does not typically appear to be resolved via the action of a nuclease. In the majority of mutants described here, the mismatch between telomerase and the telomere could have been eliminated by the removal of one or two nucleotides from the tip of the telomere. Instead, however, aberrant translocation events were the mechanism by which telomerase dealt with the mismatch. When mutations outside the template caused telomerase to copy the Ter1 sequence beyond TR26-30, the additional sequence did not appear to be cleaved (Tzfati et al., 2000 and Y. Tzfati, personal communication). Instead, aberrant translocation events involving perfect base-pairing between the sequence at the telomeric tip and the telomerase template occurred. The telomeres cloned from a strain containing *ter1-29A* might suggest that a nuclease had removed the terminal nucleotide templated by position 29, but in the absence of that type of activity in other strains the data might best be explained by other mechanisms (described below). These results do not rule out the possibility that a proofreading activity involving a nuclease acts during the synthesis of new telomeric repeats.

Telomerase can copy the entire 30 nt template *in vivo*

An important step in examining telomerase translocation *in vivo* is determining where in the template telomerase synthesis stops. *In vitro* evidence indicates that *K. lactis* telomerase normally stops copying at position 28 of the template (Fulton and Blackburn, 1998), and in creating models of aberrant translocation events it is useful to know if this is also the case *in vivo*. In all ten of the telomeres cloned from strains in

which position 28 of the template was mutated, the mutated nucleotide was incorporated into the telomere. No repeats failing to incorporate the mutated nucleotide were found distal to the basalmost mutated repeats. This indicates that position 28 is normally copied into the telomere. Telomeres were cloned from all four template mutants that could be informative in answering the question of whether the last two nucleotides of the template, positions 29 and 30, are copied. Because the sequence of TR26-30 is identical to that of TR1-5, it is not possible to determine whether positions 29 and 30 are copied in wild-type cells; the sequence could also be copied from positions 4 and 5. Based on the results from the cloned telomeres, it appears that positions 4, 5, 29, and 30 all are copied sometimes. The mutation predicted by each specific allele was sometimes incorporated, and in the case of *ter1-30G* it led to the synthesis of aberrantly-sized repeats that were 13 bp in length. Although no clone containing a mutated telomere was recovered from the strain containing *ter1-29A*, it is clear that if position 30 is copied then position 29 must also be copied at some frequency. There are several possible reasons why the *ter1-29A* mutation was not observed in the telomeres examined. First, it is clear that position 30, and possibly position 29, are not copied during every translocation event. When the mutation templated by *ter1-29A* is copied, however, it is not clear what translocation event(s) would be possible to allow another repeat to by synthesized, as no sequence in the template provides an obvious site for base-pairing with the mutated telomeric tip. It is possible that, due to the specific mutation incorporated, telomeric termini containing the mutation templated by *ter1-29A* are not readily extended because they can not base-pair efficiently with any part of the template. If no subsequent repeat is added, the nucleotides at the tip would be lost during the normal turnover at the telomere and the point mutation in the telomere would be lost. It is also possible that the mutation is incorporated and then the resulting mismatch is cleaved by a telomerase-associated nuclease; this seems unlikely in view of

the results seen with other mutations. Finally, it is possible that it is by chance that no mutated repeats were recovered from this strain.

If positions 29 and/or 30 are not always copied, then positions 4 and/or 5 must sometimes be copied. Telomeres cloned from the *ter1-4C* and *ter1-5C* mutants showed that positions 4 and 5 are often copied. However, wild-type repeats were intermingled with the mutant repeats in a manner similar to the telomeres cloned from the *ter1-8T* mutant (see below). One possible explanation for this is that these repeats were incorporated by telomeric recombination. It is also possible that these repeats were incorporated when telomerase copied the wild-type sequence at positions 29 or 29 and 30 and subsequently extended a mismatched base-pair. Mismatch extension did not typically occur in the other mutants examined in this study (see below); in the strain containing *ter1-30G* the mismatch would also be between template position 5 and the telomere, and yet aberrant translocation rather than mismatch extension was observed. Therefore, it seems unlikely that a mismatch at the same position would be extended in the strain containing *ter1-5C*. The results obtained using strains with mutations at template positions 4, 5, 29, and 30 therefore suggest that, although telomerase can copy the entire template, the stop site for telomere synthesis is usually position 28 of the template.

Mismatches at the 3' terminal nucleotides are not typically extended

More than 250 telomeric repeats, synthesized by different telomerase alleles, have been examined and of these only six repeats contain sequence that can not be ascribed to a single translocation event that involves extending a telomere that base-pairs perfectly with the telomerase RNA. This emphasizes the importance of base-pairing interactions in telomerase function. However, extension of mismatched primers by retroviral reverse transcriptase *in vivo* has been reported to occur approximately 20%

of the time (Pulsinelli and Temin, 1994) so it was of interest whether telomerase also extended such mismatches. Telomerase could potentially respond to two types of telomere-telomerase mismatches - those at the terminal nucleotide of the telomere-Ter1 alignment region and those within the alignment region - in different ways, so both types will be examined. Mismatches at the terminal nucleotide of the telomere, which would cause a mismatch to have to be directly extended, will be addressed first. Telomeric repeat synthesis often stops at position 28, and all three mutations at that position would be expected to usually cause a mismatch to be present at the tip of the alignment region. If base-pairing with the wild-type positions of TR1-5 occurred, telomerase would be forced to extend mismatched nucleotides. In the cases of all three mutations at position 28 of the template, aberrant translocation occurs rather than the extension of a mismatch. In the case of *ter1-28G*, the aberrant event is merely a shift of one nucleotide leading to a repeat that is one nucleotide shorter than the 25 bp wild-type repeat. In a strain containing *ter1-28A*, however, a more dramatic 5 nucleotide shift, leading to the synthesis of a 20 bp repeat, is needed to avoid the presence of a mismatch at the tip of the alignment region. The final mutation at position 28, in the allele *ter1-28C*, causes another dramatic shift in the region of alignment. In this instance, the telomere aligns with a region outside of the template. This alignment, which at the most involves only 2 contiguous complementary nucleotides between the telomere and telomerase, maintains perfect base-pairing at the tip of the alignment region. A final example of the preference for perfect base-pairing at the tip of the alignment region is found when telomeres synthesized by *ter1-30G* are examined. When the mutated nucleotide incorporated at position 30 is observed, an aberrant translocation event is also always observed. This event, which leads to the synthesis of a 13 bp repeat, occurs despite the potential for base-pairing between TR1-5 and the wild-type sequence copied from positions 26-29. The base-pairing interaction that does occur involves a region that is only two

nucleotides long. In the last two examples, aberrant translocation occurs despite the fact that there are a minimum of two or four nucleotides, respectively, that could be involved in base-pairing with TR1-5; the potential for additional base-pairing that could stabilize alignment with TR1-5 will be discussed below. In all of the examples, alignment with TR1-5, the site of alignment in wild-type cells, would have required only that a single mismatch at the end of the alignment region be tolerated.

The second class of mismatches involves those that are not at the tip of the region used for base-pairing and would thus not be directly extended by telomerase. The mutations described in this work offer some evidence that mismatches at the penultimate telomeric nucleotide can also affect alignment during translocation. A mutation at position 2 of the template, as found in the allele *ter1-2G*, might be predicted to have no effect, since base-pairing at the tip of the alignment region would be maintained. The telomeres recovered from a strain containing *ter1-2G*, however, show that an alternative base-pairing occurred in a significant percentage of the repeats. This indicates that, in this mutant, a mismatch between telomerase and the telomere, even one that is not predicted to be at the tip of the alignment region, can affect translocation. The alternative alignment did not occur in every repeat, however. This could be due to the specific site where telomere synthesis stopped; if position 29 was copied, alignment at the normal site would still allow two nucleotides of perfect base-pairing beyond the mismatch at position 2. If copying stopped at position 28, on the other hand, there would only be one nucleotide of base-pairing after the mismatch and thus telomerase might shift to avoid the mismatch. The telomeres cloned from a strain containing *ter1-27G* indicate that a similar situation occurs in this strain. Telomeric repeats synthesized by *ter1-27G* are predicted to most often terminate in the sequence TGT; if position 29 is the terminal position copied then the sequence at the telomeric tip would be TGTG. If a telomeric repeat containing the mutation at position 27 base-paired with TR1-5, the

mismatch would be with position 2 of the template. Despite the fact that the alignment region would likely terminate with one or two nucleotides of perfect base-pairing, no 25 bp repeats containing the G mutation templated by position 27 were recovered. Instead, alignment occurred at an alternate site within the 'core' of the template, which allowed the template to maintain perfect base-pairing with the three nucleotides at the telomeric tip.

The results described in this section emphasize two important points. First, mismatches between the telomeric tip and the telomerase RNA are not typically extended. Second, the minimum preferred amount of base-pairing at the telomeric tip appears to be two nucleotides. The vast majority of the telomeric repeats examined were synthesized following translocation events that maintained perfect base-pairing between the terminal two or three nucleotides at the telomeric tip and the telomerase RNA. In all of the strains, with one possible exception, at least two nucleotides were involved in the alignment between the telomere and telomerase. The one possible exception is found with the allele *ter1-28C*. With this allele, only one base-pair is utilized unless position 29 is also copied. Since the entire template can be copied, it is not unreasonable to think that position 29 might have been copied in this instance. If telomerase can align using only one base-pair to stabilize the alignment, then it might be expected that there would have been more variability in the types of aberrant translocation events observed; other sites in the template could also form a single base-pair with the tip nucleotide of the telomere.

A possible new determinant of telomerase translocation in *K. lactis*

The data presented in this work suggests that telomere-template alignment preferentially occurs in the area of the template near TR1-5. In the few abnormal repeats seen in this study that could not be explained by a single translocation event, the

sequences copied during 'stuttering' appeared to be copied from this region. In addition, the wild-type alignment is always with TR1-5 of the template, despite the potential for a few nucleotides of base-pairing elsewhere in the template, and single base-changes in TR1-5 caused only minor shifts in the region of Ter1 involved in base-pairing. Together, these results suggest that TR1-5 is not the sole determinant of telomerase translocation. It is possible that telomere-associated proteins play a role in positioning telomerase correctly. Another possibility is that three nucleotides, located 3' of the template, may be involved in base-pairing with nucleotides 22-24 of the telomeric repeat (Fig. 4-8). This extra base-pairing could stabilize the interaction between the telomeric repeat and the telomerase template. A mutation in this region outside the template that disrupted base-pairing potential led to telomere shortening, while a mutation that added additional base-pairing potential caused mild telomere elongation, indicating that this region may play a role in telomerase-mediated telomere maintenance. However, it is difficult to test the base-pairing model directly. The obvious test would be to make compensatory changes in the region 3' of the template and in the template itself-for example, to change position -4 and also position 22 of the template in order to maintain the base-pairing predicted by the model. If the base-pairing interaction is the reason for the telomere length defect seen in the *ter1-4-A* strain, restoration of the base-pairing should suppress the defect. Mutations in positions 22-24, however, have short telomeres, indicating that they are involved in a positive telomeric function. These telomeric positions are part of the Rap1p binding site and have been proposed to be part of the binding site for a single-strand telomere binding protein, such as a Cdc13p or Est1p homolog. Although it is possible that the telomere shortening is due to defects in translocation, the length phenotype is more severe than that of the *ter-4-A* strain. This is consistent with the idea that the region may have another function and complicates analysis of the base-pairing requirement.

Effects of TR1-5 and TR26-30 mutations on the telomeres

Many mutations in TR1-5 and TR26-30 were shown to cause aberrant translocation events. However, the effect of the mutations on the cell is not limited to translocation defects. The majority of the mutations in TR1-5 and TR26-30 lead to telomere shortening. There are several possible reasons for this. The mutations could alter telomerase activity by disrupting the alignment between telomerase and the telomere. Mutations in Ter1 could also disrupt the active site of telomerase complex. Mutations outside the Ter1 template in *K. lactis* (Roy et al., 1998) and within the *S. cerevisiae* and *Tetrahymena* telomerase RNA templates (Gilley and Blackburn, 1996; Gilley et al., 1995) have previously been shown to affect telomerase function. Alternatively, the mutations might act through the telomere, perhaps affecting the binding of telomeric proteins and disrupting telomere length regulation or telomere capping. This was suggested by telomeres cloned from a strain containing *ter1-3C/28C* (D.H. Underwood et al, manuscript to be submitted to Genes and Development). In this mutant, the same mutation was made in both terminal repeats; the mutation was copied into the telomere as a single base-change in 25 bp repeats, and yet the telomeres remained short. Further evidence that the telomeric sequences are important is seen in the elevated subtelomeric recombination levels seen in some of the mutants. Telomeric recombination was also observed in one, and potentially as many as three, of the mutants. The clearest example of this was seen in the *ter1-8T* mutant, where two of the telomeres examined contained telomeric repeats synthesized by three different telomerase alleles. As with the *ter1-4C* and *ter1-5C* mutants, the telomerase alleles were replaced in the strain such that wild-type *TER1* and the mutant allele were never present in the cell simultaneously (see Materials and Methods). Repeats synthesized by the wild-type *TER1*, however, were observed closer to the telomeric tip than some of the repeats synthesized by the mutant telomerase, indicating that recombination had

occurred between the tip of one telomere and a more basal region of another. Finally, aberrant telomeric repeats might also affect the interaction between telomere proteins and the telomerase complex. The abnormally sized telomeric repeats might alter the spacing between protein binding sites, perhaps for Cdc13p or Est1p, and the end of the telomere, affecting telomerase recruitment.

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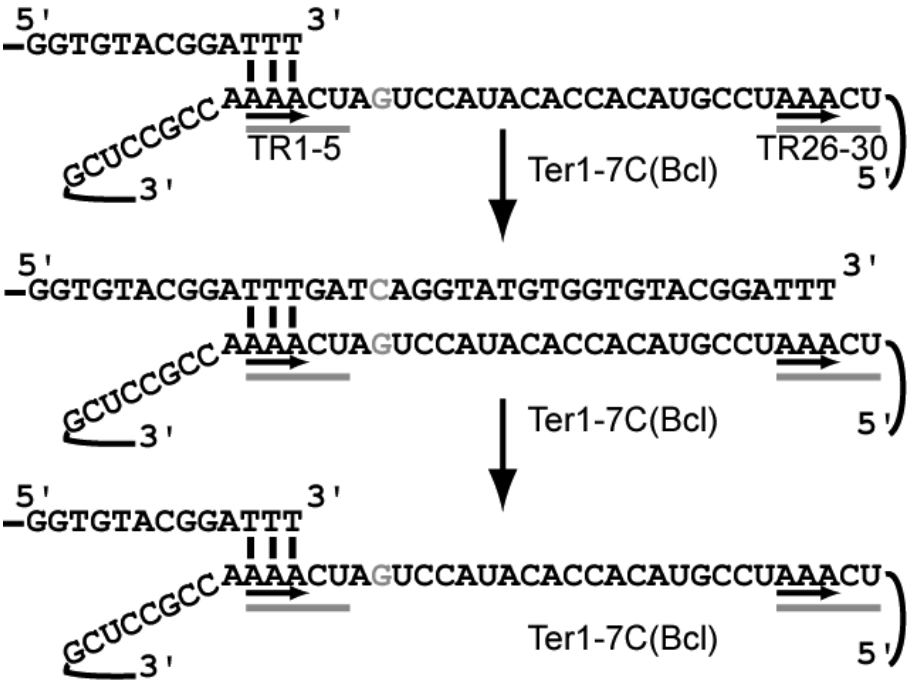
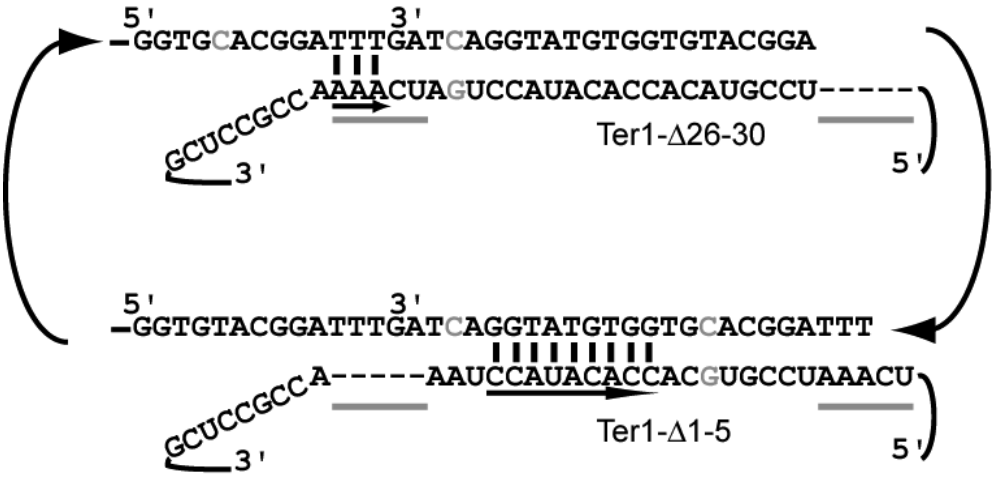
FIGURES



	# of repeats
GGTGTACGGATTT	1
GATTAGGTATGTGGTGTACGGATTT	4
GATCAGGTATGTGGTGTACGGATTT	12
GATCAGGTATGGTGTGTAC	1

Fig. 4-1. Wild-type telomerase translocation. A) A diagram of telomerase translocation in *K. lactis* is shown; the labeling in this diagram will be continued in all subsequent diagrams. The allele *TER1-7C(Bcl)* is shown, with the point mutation shown in gray. The mutation name refers to the point mutation incorporated into the telomeric strand. A short region of the telomeric repeat is shown base-pairing with TR1-5 of the template. TR1-5 and TR26-30 are shown underlined with a gray bar. The region between the underlined segments is the 20 nucleotide 'core' of the template. The nucleotides involved in base-pairing are underlined with a black arrow; in this model, telomerase synthesis is shown to stop at position 28, so there are three nucleotides available to align with the template. Alignment begins at position 1 of the template. Once synthesis is complete, telomerase can dissociate and realign for the next round of telomeric repeat synthesis. **B)** The sequence of a telomere cloned from a strain containing *TER1-7C(Bcl)* is shown. This telomere contains 16 complete telomeric repeats, in addition to the partial repeats at each end. The sequence is shown from the subtelomere-adjacent sequence (top) to the tip of the telomere (bottom). The point mutation is copied into the newly synthesized telomeric repeat. In all figures, the first full line of sequence is the complete 25 bp repeat; all variations can be compared to this line. The point mutations are shown in gray type. The number of each type of repeat is shown at the right of the figure. The repeat at the tip varies from the penultimate repeat only because it is not a complete repeat.



		# of repeats
1)	GGTGTACGGATTT	1
	GATTAGGTATGTGGTGTACGGATTT	2
	GATTAGGTATGTGGTGCACGGATTT	1
	GATCAGGTATGTGGTGCACGGATTT	1
	GA	1
2)	GGTGTACGGATTT	1
	GATTAGGTATGTGGTGTACGGATTT	3
	GATCAGGTATGTGGTGTACGGATTT	1

Fig. 4-2. Two telomerase molecules can work cooperatively to synthesize a telomeric repeat. The model for telomerase translocation in the absence of either terminal repeat is shown. The positions of the deleted nucleotides are marked with dashes, and the regions of the terminal repeats are underlined with gray bars. The telomerase shown on top, from the *ter1-Δ26-30* mutant, also contains a 7C point mutation (shown in gray). This molecule can base-pair using TR1-5, but lacks TR26-30. The second molecule, from the *ter1-Δ1-5* mutant, could base-pair using nucleotides in the template 'core' (underlined with long black arrow) and is marked with the 20C (shown in gray) mutation. Telomeres cloned from the heteroallelic strain are shown below the model. In one telomere, incorporation of both point mutations can be seen (gray nucleotides); in the other only one has been incorporated.

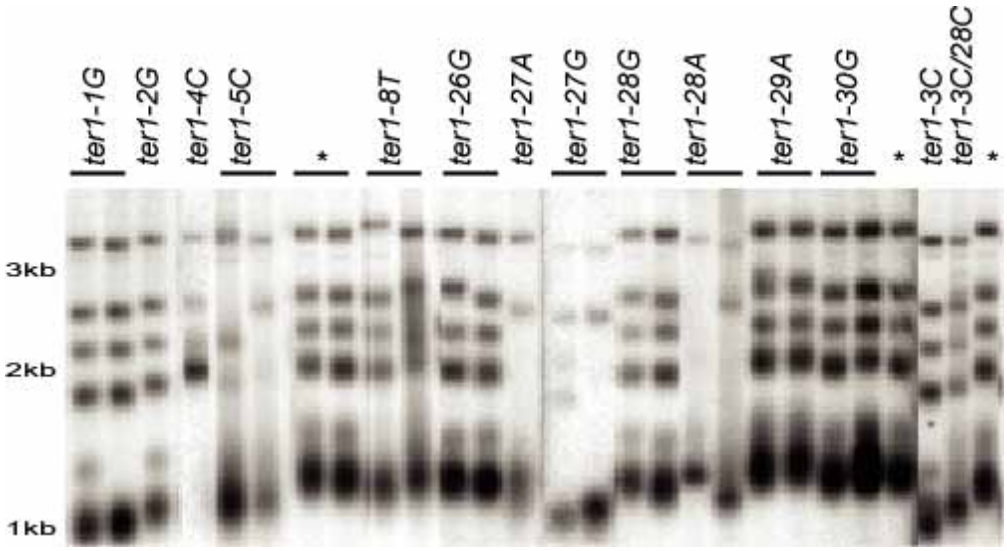
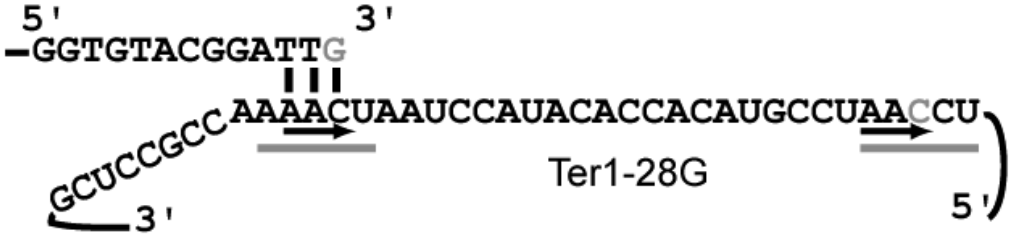


Fig. 4-3. Point mutations in TR1-5 and TR26-30 often lead to telomere shortening. Southern blots of *Eco*RI digested genomic DNA are shown. The names of the *TER1* alleles are shown above the figure. Lines above the lanes indicate that two clones containing the mutation are shown. The fortieth streak was used for all strains except *ter1-3C* and *ter1-3C/28C*, which were shown at the ninth streak. Lanes labeled *TER1-7C(BcI)* show telomeres that were wild-type in length.

A.

	# of repeats
GGTGTACGGATTT	1
GATTAGGTATGTGGTGTACGGATTT	10
CGATTAGGTAT	1

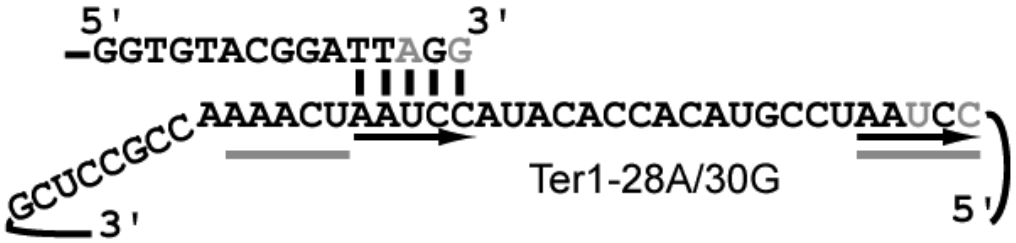
B.



	# of repeats
GGTGTACGGATTT	1
GATTAGGTATGTGGTGTACGGATTT	8
GATTAGGTATGTGGTGTACGGATTG	1
ATTAGGTATGTGGTGTACGGATTG	2
ATTAGGTAT	1

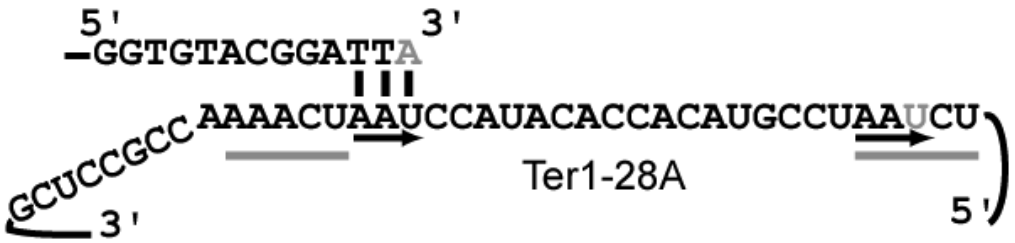
Fig. 4-4. Some point mutations in TR1-5 and TR26-30 lead to minor shifts in alignment with telomerase. A) The telomerase allele *ter1-3C* causes the synthesis of a 26 bp repeat. The presence of the mutation at position 3 of the template causes telomerase alignment to shift by 1 nt, such that alignment begins at a nucleotide 3' of the normal start site. **B) A 24 bp repeat is synthesized in the strain containing *ter1-28G*.** When a G is copied from position 28, the alignment shifts by one nucleotide towards the 5' end of the template. Base-pairing then occurs with positions 2-4 of the template. **C) *ter1-2G* can cause a 27 bp repeat to be synthesized.** When a C is present at position 2 of the template, telomerase alignment is sometimes shifted by 2 nucleotides. In this case, only two nucleotides of base-pairing is apparent between the tip of the telomere and the template.

A.



		# of repeats
1)	GGTGTACGGATTT	1
	GATTAGGTATGTGGTGTACGGATTT	8
	GATTAGGTATGTGGTGTACGGATTA	1
	GGTATGTGGTG	1
2)	GGTGTACGGATTT	1
	GATTAGGTATGTGGTGTACGGATTT	4
	GATTAGGTATGTGGTGTACGGATTA	1
	GGTATGTGGTGTACGGATTA	1
	GGTATGTGGTGTACGG	1

B.



		# of repeats
1)	GGTGTACGGATTT	1
	GATTAGGTATGTGGTGTACGGATTT	5
	GATTAGGTATGTGGTGTACGGATTA	1
	GGTATGTGGTGTACGGATTA	1
	GGTATGTGGTGTA	1
2)	GGTGTACGGATTT	1
	GATTAGGTATGTGGTGTACGGATTT	5
	GATTAGGTATGTGGTGTACGGATTA	1
	GGTA	1
3)	GGTGTACGGATTT	1
	GATTAGGTATGTGGTGTACGGATTT	5
	GATTAGGTATGTGGTGTACGGATTA	1
	GGTAT	1
4)	GGTGTACGGATTT	1
	GATTAGGTATGTGGTGTACGGATTT	6
	GATTAGGTATGTGGTGTACGGATTA	1
	*TTGA*TTAGGTATGTG	1
5)	GGTGTACGGATTT	1
	GATTAGGTATGTGGTGTACGGATTT	7
	GATTAGGTATGTGGTGTACGGATTA	1
	*ATGAGA*TTAGGTATGTGGTGT	1

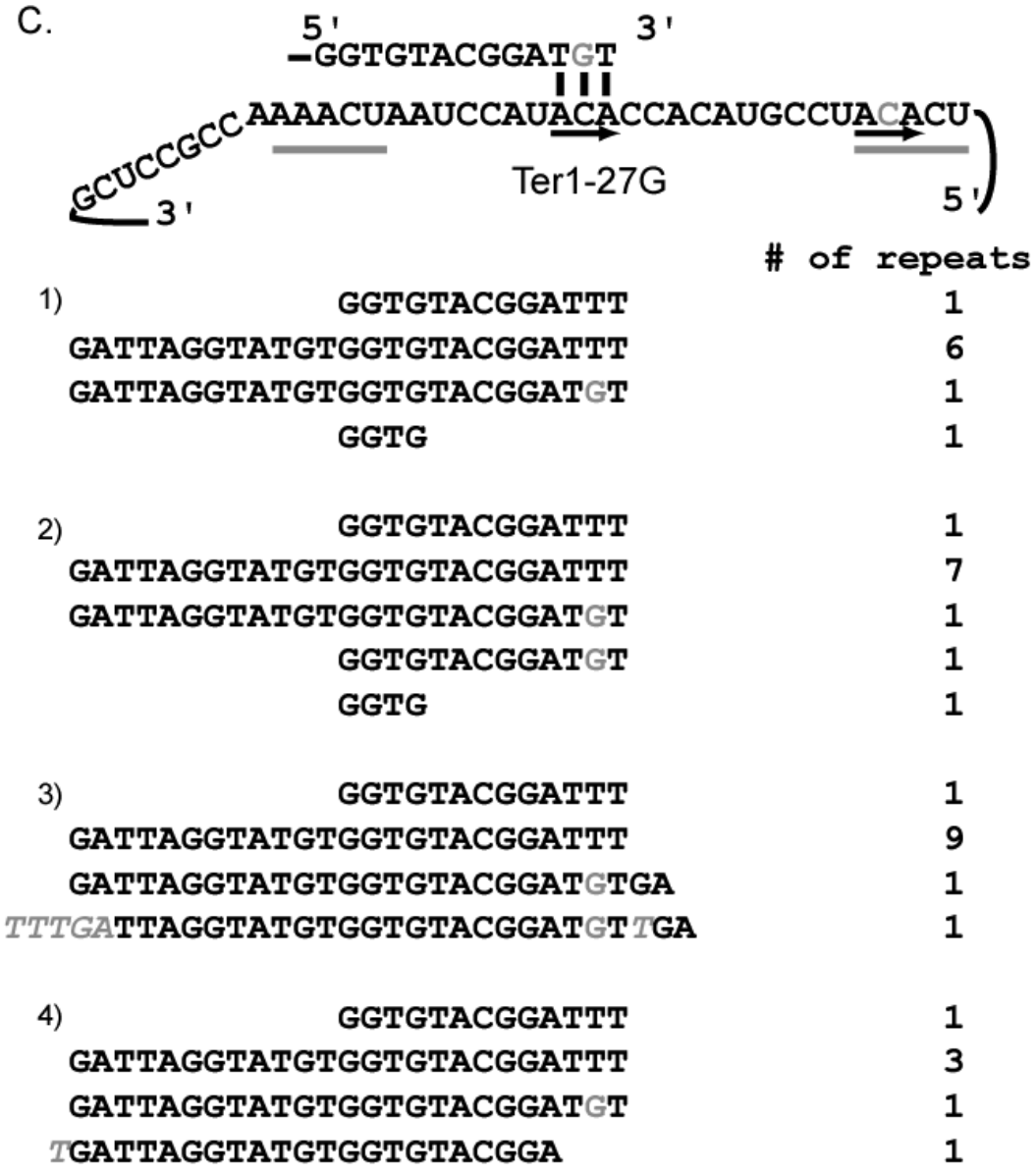
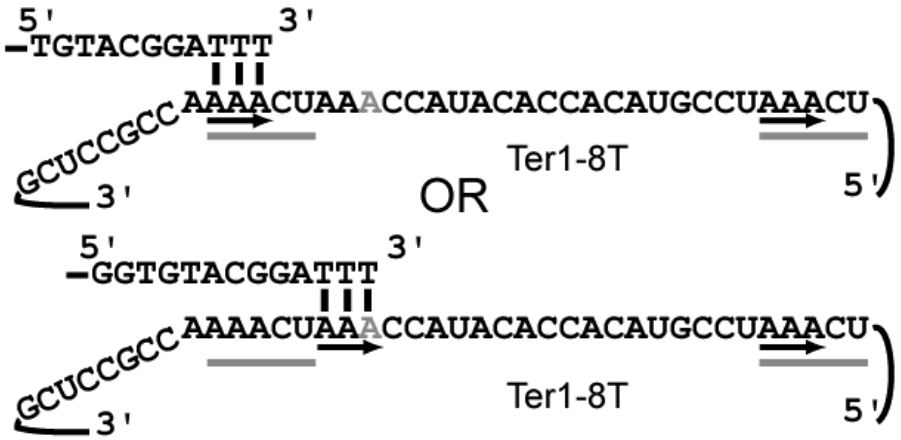
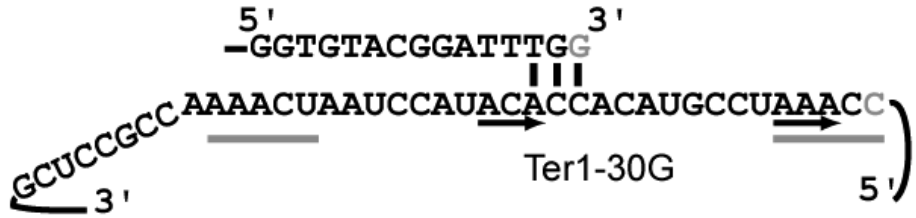


Fig. 4-5. The telomere can align with nucleotides within the 'core' of the template. A) *ter1-28A/30G* copies a 20 bp repeat. The telomeric repeats made in the *ter1-28A/30G* strain are 20 bp long. It is unclear that position 30 is typically copied *in vivo*; however, the mutations cause the sequence in TR26-30 to be identical to the sequence at positions 6-10 (underlined with arrows). The change potentially templated by position 30 is shown in gray, as is the mutation introduced by position 28. **B) *ter1-28A* copies a 20 nt repeat or 'stutters'.** Telomeres cloned from a strain containing *ter1-28A* often contain 20 bp repeats identical to those made in the *ter1-28A/30G* strain. Sometimes, however, sequence that is not easily explained was found (shown in gray italics). **C) *ter1-27G* leads to they synthesis of 13 nt repeats or unusually long repeats.** When a G is copied from position 27, the telomeric tip can base-pair with position 13-15 of the template, leading to the synthesis of a 13 nt repeat. In other cases, aberrant synthesis occurs (extra sequence shown in gray italics).



			# of repeats
1)	GGTGTACGGATTT		1
	GATTAGGTATGTGGTGTACGGATTT		5
	GATTTGGTATGTGGTGTACGGATTT		3
	GAT*T*AGGTATGTGGTGTACGGATTT	*	1
	GATCAGGTATGTGGTGTACGGATTT		1
	GATTTGGTATGTGGTGTACGGATTT		1
	GATCAGGTATGTGGTGTACGGATTT		1
	GATTTGGTATGTGGTGTACGGATTT		2
	GAT		1
2)	GGTGTACGGATTT		1
	GATTAGGTATGTGGTGTACGGATTT		3
	GGTATGTGGTGTACGGATTT		2
	GAT*T*AGGTATGTGGTGTACGGATTT	*	2
	GATTTGGTATGTGGTGTACGGATTT		3
	GGTATGTGGTGTACGGATTT		1
	GATTTGGTATGTGGTGTACGGATTT		1
	GG		1

Fig. 4-6. Mutations in the template 'core' can alter translocation. The mutation *ter1-8T* (shown in gray) can lead to the synthesis of two types of repeats. In the first type, the telomere base-pairs with TR1-5 (shown in upper alignment) and incorporates the mutation as a single-base change (shown in gray in the upper sequence). In the second type (lower alignment), base-pairing occurs within the template and leads to the synthesis of a 20 bp repeat. Sequences from tow telomeres cloned from the *er1-8T* mutants are shown. Wild-type repeats (identified by the italicized nucleotide at position 7 and a * at the end of the repeat) are interspersed with the *ter1-8T* and *ter1-7C* repeats (indicated with the underlined C at position 7) made by telomerase alleles in the cell. These repeats could only have become interspersed via recombination (see text).



	# of repeats
GGTGTACGGATTT	1
GATTAGGTATGTGGTGTACGGATTT	4
GATTAGGTATGTGGTGTACGGATTTGG	1
TGTACGGATTT	1
GATTAGGTATGTGGTGTACGGATTTGG	1
TGTACGGATT	1
GATTAGGTATGTGGTGTACGGATTT	7
GATTAGGTATGTGGTGTACGGATTTGG	1
TG	1

Fig. 4-7. *ter1-30G* can synthesize a 13 bp repeat. A telomere cloned from a strain containing *ter1-30G* contained repeats in which position 30 was copied. In each case, the subsequent repeat synthesized was 13 bp long.

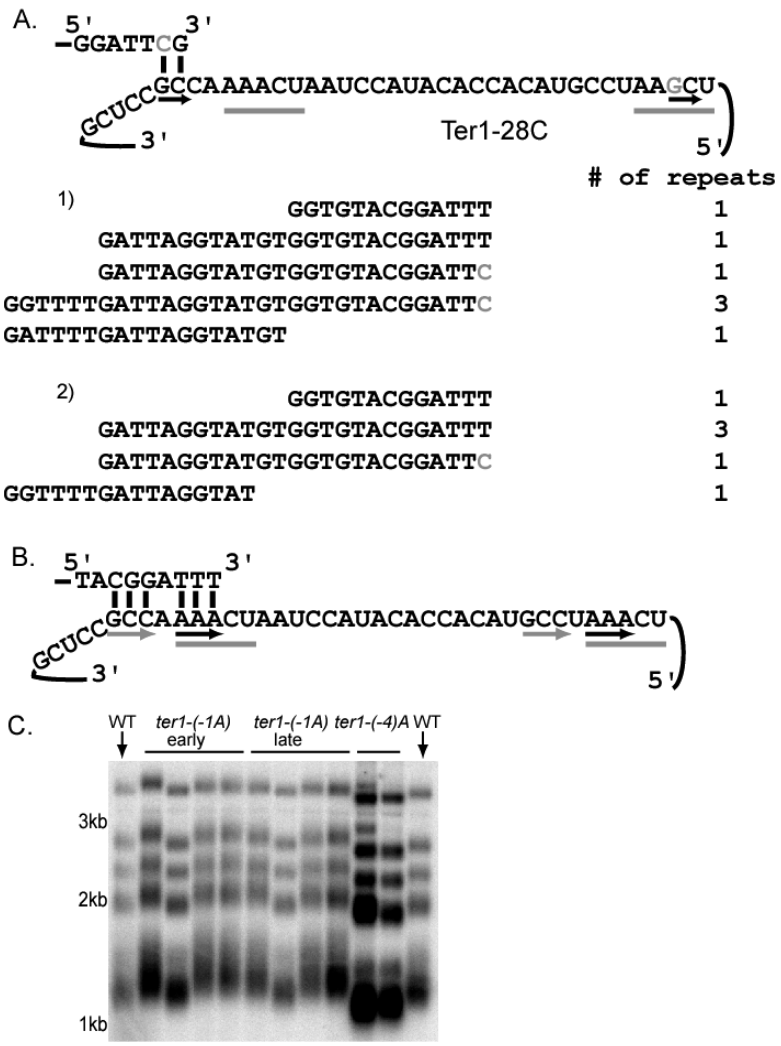


Fig. 4-8. The region 3' of the temeplate is available for base-pairing. A) *ter1-28C* leads to the synthesis of 31 bp repeats. When position 28 and possibly position 29 are copied, the telomeric tip base-pairs with the nucleotides 3' of the template position 1. The positions, referred to as –3 and –4, are underlined with a black arrow at the left of the figure. Multiple repeats were found in one cloned telomere, while a single long repeat was found in the other. **B) The region 3' of the template may stabilize alignment during wild-type translocation.** Alignment of a wild-type telomeric repeat with the telomerase template reveals that additional base-pairing might occur outside TR1-5. The region 3' of the template, at positions –2, -3, and –4, is identical to that at template positions 22, 23, and 24. The telomere copied from these positions might base-pair with the region 3' of the template. Both regions are underlined with gray arrows. **C) Mutations 3' of the template can cause telomere length phenotypes.** Two mutations were made in the region 3' of the template. The first, *ter1-(-1)A*, changes the nucleotide at the –1 position so that it could base-pair with the nucleotide templated by position 25 in the template. Three of the four clones examined exhibit a mild but stable elongation of the telomeres. The second mutation, at the –4 nucleotide (*ter1-(-4)A)*, disrupts its base-pairing potential. This mutation leads to telomere shortening.

CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

A collection of strains was constructed such that each strain contains a point mutation in its *TER1* template. Experiments have been done using some of the strains, and the results described in the preceding chapters have given insight into both telomere and telomerase function. Although some questions have been answered, more have been raised. Some of the possible future directions of the work will be discussed below. The work has been divided into two main sections-one dealing with the telomere and the other dealing with telomerase-although there is obviously a great deal of overlap between them. In the next few pages, a brief conclusion will be given for each section. The conclusions will be followed by a discussion of the future directions of each project.

EXAMINATION OF MUTATIONS AFFECTING TELOMERE FUNCTION

Most mutations made in the telomerase template appear to be copied faithfully into the telomere. Because telomeres in *K. lactis* are composed of uniform 25 bp repeated sequences, each *TER1* template mutation causes each newly synthesized telomeric repeat to have the same single base substitution. This uniformity of the telomeric repeats makes the *K. lactis* system particularly powerful for the study of the telomere. Many of these mutations lead to subsequent changes in telomere length. The mutations leading to a particular change, whether it be telomere elongation, telomere shortening, or delayed telomere elongation, tend to be found in discrete regions of the telomere. This is consistent with the idea that many of the telomere length phenotypes are the result of a change in the ability of a protein to bind the mutated telomeric repeats. The fact that the telomeres can become long or short is consistent with the fact that both

positive and negative regulators of telomere length have been identified in *S. cerevisiae*. Homologs to the *S. cerevisiae* Rap1p and Stn1p have already been identified in *K. lactis*, and it is likely that homologs to other telomere binding proteins also exist. Identification of these homologs is an obvious extension of this project. As there is a *K. lactis* genome sequencing project underway, it may be possible to identify the genes via their homology to *S. cerevisiae* genes and then clone them through a PCR-based approach. Of particular interest the homologs of Cdc13p and Est1p. Mutations on the right side of the putative Rap1p binding site in *K. lactis* lead to telomere shortening instead of the expected telomere elongation. One explanation for this is that the nucleotides on the right side of the site may also comprise a binding site for Cdc13p or Est1p, both of which are single-strand binding proteins that play a role in positively regulating telomere length. Once the genes are cloned in *K. lactis*, it is possible to test this hypothesis by attempting to suppress the telomere shortening phenotype by overexpression of the proteins, an experiment similar to that done with Rap1p. DNA binding assays could also be used to examine the effects of specific telomeric repeat mutations on DNA-protein interactions.

The region of the telomere leading to delayed telomere elongation is of particular interest, as it is possible that this region serves at least two functions. The telomeres initially shorten, but after repeated serial restreaking, they become extremely elongated. There are two possible explanations for this. First, it is possible that mutations in this region disrupt the binding site of a single protein. If this is the case, then the protein would be expected to have a positive affect on the telomeric tip and a negative effect on the more basal region of the telomere. A protein that affects the structure of the telomere could have such an effect. Another possibility is that this region is the binding site for two different proteins, which have opposite effects on telomere length. For example, if a mutation mildly disrupted the binding site for Cdc13p or Est1p at the telomeric tip, then telomere shortening would be expected to occur. Once mutated

telomeric repeats replaced more basal wild-type repeats, however, interactions of a negative regulator of telomere length might be disrupted. The Rif proteins (Rap1p interacting factors, Rif1p and Rif2p) are possible candidates for this function; preliminary evidence suggests that Rif1p and Rif2p are part of the mechanism by which telomeric repeats are 'counted' by the cell (D. Levy and E.H. Blackburn, personal communication). Although Rap1p binding is not disrupted in the delayed elongation mutants examined, it is possible that the binding is altered such that the interactions with the Rif proteins are disrupted and thus not counted, leading to telomeric elongation. These hypotheses can be tested by both *in vivo* overexpression experiments and *in vitro* binding experiments, although it will be necessary to clone the *RIF1* and *RIF2* genes first.

Another interesting question involves the large amount of extrachromosomal DNA seen in strains with very long telomeres. Although the telomere elongation seen in the mutant strains occurred independently of *RAD52*, *rad52 ter1* double mutants showed a significant reduction in extrachromosomal telomeric DNA levels. It is unclear, however, what mechanism(s) are involved in the formation of extrachromosomal telomeric DNA. Although some of the molecules appear to be circular and thus might form by the looping-out of sections of the telomere, it is not clear which *RAD52*-dependent pathway could lead to the formation of what appear to be broken linear telomeric molecules. *RAD52* dependent homologous recombination occurs through two main pathways and is therefore either *RAD50*-dependent or *RAD51*-dependent; deletion of *RAD52* eliminates both pathways. It is possible that, by constructing mutants deleted for either *RAD50* or *RAD51* that some progress could be made in determining the mechanism(s) for the breakage. This is, however, dependent on further determination of the functions of the *RAD50* and *RAD51* genes, as it is not clear that enough is known of their functions to truly determine molecular mechanisms.

Anecdotal evidence indicates that levels of non-homologous recombination are elevated in strains with very long telomeres. Processes such as the looping-in of telomerase-containing plasmids, the knock-out replacement of telomerase constructs, and the replacement of one telomere with a marked cloned telomere are very efficient in wild-type strains; they occur via homologous recombination and the constructs are typically inserted into the expected site. When knock-out replacement of telomerase or telomeric replacement experiments are done in strains with long telomeres, however, it is very difficult to recover the expected construct. It is not yet clear whether recombination levels are different or if the ratio of homologous to non-homologous recombination has changed. These questions can be addressed by performing assays to determine the frequencies of both types of recombination. One method for this assay involves inserting the *S. cerevisiae URA3* gene into the *K. lactis ADE2* gene. The resulting construct can be transformed as a linear fragment into a strain that is *ura3* and *ADE2*; transformants will be selected as by the ability to grow on plates lacking uracil. If the fragment integrates via homologous recombination at the *K. lactis ADE2* locus, the chromosomal *ADE2* gene will be replaced by the disrupted *ADE2* in the construct. Disruption of the *ADE2* gene leads to red colonies, so in this case the transformants would be red. If the construct integrates via non-homologous recombination, however, the chromosomal *ADE2* gene would remain intact, leading to white colonies. Therefore, the number of colonies obtained from the cells would indicate the total level of recombination, while the ratio of red:white colonies would indicate the ratio of homologous:non-homologous recombination. By determining the frequencies of the homologous and non-homologous events in both wild-type and mutant cells, the veracity of the anecdotal results can be determined.

As alluded to in Chapter 3, one problem with the method of mutagenesis is that both the telomere and the telomerase RNA are mutated, making it difficult to elucidate

which structure is functionally defective. One way to examine this is to use mutated STU (subtelomeric *URA3*) constructs. These constructs are composed of a cloned telomere marked with by inserting a *URA3* gene into the associated subtelomeric sequence. The construct is then mutated *in vitro* such that the entire telomere is composed of mutated repeats. By replacing a wild-type telomere with this construct, it is possible to monitor the function of the mutated repeats. If, for example, a *ter1* strain has short telomeres but the STU construct containing the same type of repeats is maintained at wild-type length and does not immediately shorten, then it might be inferred that the short telomeres of the *ter1* mutant are due to a telomerase defect.

In attempting to use a new method for creating the mutated STU constructs, it was found that the Stratagene Quik-Change Multi kit often leads to the expansion or contraction of the size of the telomeric insert. It is possible that this kit could be used to construct STUs of varying lengths but containing the same mutation. These constructs could be particularly useful for dissecting the function of different regions of the telomere. Two applications of these constructs are apparent. First, it will be possible to make phenotypically silent Bcl-STU constructs of varying lengths. If a Bcl-STU that is longer than the length of a wild-type telomere is introduced into the cells, it will be possible to follow the dynamics of the telomere as it shortens. Second, by introducing STUs with very few telomeric repeats into the cells it will be possible to follow the recombination levels of the basalmost telomeric repeats. By examining these levels in mutations with different telomere length phenotypes, it may be possible to determine differences in telomeric recombination levels in different situations.

Another future direction for this work is an examination of the mutations that lead to elevated levels of subtelomeric recombination despite having telomeres at or near wild-type length. No quantitative assays have been done to examine this phenomenon, and it is thus characterized only by the loss of telomeric bands visible in Southern blots

due to loss of subtelomeric polymorphisms by recombination. After performing subtelomeric recombination assays, however, it is somewhat unclear what experiments could help determine an explanation for the events. It is likely that there is some defect in the structure of the telomere that leads to it not being fully protected despite its wild-type length. This defect might be suppressed by overexpression of a component of the telomeric protein complex. There are, however, many proteins that may play a role in the structure at or near telomeres, including histones, the Sir proteins, and the Ku complex; overexpressing all of the proteins involved in a complex in a single cell might not be feasible. DNA binding experiments with specific proteins predicted to interact with this region of the telomere could also be done.

EXAMINATION OF MUTATIONS AFFECTING TELOMERASE FUNCTION

Several of the *TER1* mutations described in this work affect telomerase translocation *in vivo*, causing telomeric repeats of aberrant length to be synthesized. Little is known, however, about other effects that these mutations have on telomerase function. Nothing is known about the stability of the enzyme in the presence of the mutations. Although it is possible to measure *TER1* transcript levels, this information is not fully informative without data on telomerase assembly. For example, if the *TER1* transcript is normally present in excess, a reduction in transcript stability that leads to a reduction in transcript levels might be less detrimental to the telomere function than a mutation that affects the ability of Est2p, the reverse transcriptase component of telomerase, to bind to the RNA. Therefore, in order to develop an understanding of the effects of the mutations on telomerase levels it would be necessary to examine not only RNA stability but the assembly and stability of the telomerase ribonucleoprotein complex. In order to do this, it is necessary to clone the genes for the *K. lactis* homologs of Est1p, Est2p, and Est3p. Est1p is thought to mediate telomerase access to the

telomere through its binding to Ter1 and the telomere. Est2p is the reverse transcriptase component of telomerase, and Est3p is a telomerase component of unknown function. If *EST1, EST2,* and *EST3* are cloned into appropriate vectors, it should be possible to examine *in vitro* assembly and activity of telomerase molecules that contain mutated telomerase RNA components.

Another issue, alluded to above, is the fact that mutations in the telomerase template can affect the translocation of the enzyme. It is unclear whether the telomere shortening often seen in these mutants is due to the mutant telomeric repeats synthesized by the enzyme or is the result of the inability of telomerase to align to synthesize the repeats. If a mutated telomerase has a defect in translocation, it is plausible that the alignment step could occur less efficiently, which could lead to shorter telomeres. Alternatively, the mutant repeats synthesized by the enzyme could be the cause of the telomere shortening. There are at least two ways that these possibilities could be distinguished. First, it is possible to construct mutated STU constructs containing the mutations made by telomerase. If the defect is due to telomerase translocation defects, then the telomere would be maintained at wild-type length. If, however, the telomeric repeats are defective, then one of two things might happen in the presence of a wild-type telomerase. First, if the repeats are completely defective at length regulation, then a full wild-type telomere might be added onto the mutated STU, as occurred with the Kpn-STU. Alternatively, if the mutated repeats are unable to recruit telomerase, then they might be expected to shorten, perhaps and then be extended by recombination. This experiment is somewhat complicated by the fact that the telomere has both single-stranded and double-stranded regions, each of which may have different functions. If a mutated repeat is defective at a single-stranded function (such as binding Cdc13p), for example, then the addition of a few wild-type repeats(which might occur at

low efficiency) would restore this function to the telomere and allow it to be maintained at wild-type length.

A second way to circumvent the problem that aberrant translocation may cause is to make *TER1* alleles that have an altered permutation of the template. This would cause the sequences normally in the terminal repeats that are critical for wild-type translocation to be in the 'core' region of the template. As an example, the template sequence, written as the telomeric T/G rich telomeric strand, is normally TTTGATTAGGTATGTGGTGTACGGATTTGA, with the terminal repeats underlined. An altered permutation might be TTAGGTATGTGGTGTACGGATTTGATTAGG. Although five-nucleotide terminal repeats are still present, the sequence that normally comprises the repeats (underlined) is in the 'core' of the template. Point mutations in the underlined sequence could then be made and would not be expected to affect translocation. Thus, any resulting changes in telomere length would be expected to be caused by a defect in the telomere itself and not telomerase function. A number of the altered permutation *TER1* alleles have been constructed, and all cause slight-to-moderate changes in telomere length. It may therefore be necessary to try to determine the underlying cause of the telomere length phenotype in the altered permutation strains before attempting to make additional template mutations in those mutants.

A final region of the telomerase RNA that has just begun to be examined is the region immediately 3' of the template. The potential for base-pairing between three nucleotides in this region and three telomeric nucleotides copied from part of the telomerase 'core' exists, and this region is separated from the template's 3' terminal repeat by one nucleotide (Fig. 4-8). Two point mutations-one that causes the non-base-paired position to be able to base-pair with the telomere and another that disrupts a potential base-pairing interaction-lead to changes in telomere length. As with the other mutations described, these mutations might have an effect on either translocation of the

enzyme or on telomerase assembly or stability. These possibilities can be tested by using the techniques described above. In order to further characterize this region, additional *TER1* point mutations should also be made. In addition, it would be informative to clone telomeres from these strains. This would allow aberrant translocation events to be identified and the underlying events examined.

SUMMARY

The collection of strains containing mutations in *TER1*, the telomerase RNA template, is an excellent starting point for many experiments examining both telomere and telomerase function. Because only one mutation was made at many of the positions, it may be necessary to make additional mutations in order to fully examine the importance of a region of the template or telomere. The extant strains, however, provide insight into the role(s) of each region. They also allow the telomere to be divided into functional regions, although there is likely to be some overlap of function. Such complications can be resolved through the use of experiments such as those described above.