RECOMBINATIONAL TELOMERE ELONGATION IN

*Kluyveromyces lactis*

by

SHOBHANA NATARAJAN

(Under the direction of Dr. Michael J. McEachern)

ABSTRACT

There are at least two pathways of telomere maintenance in yeast and mammalian cells, one being telomerase-mediated and the other being dependent on homologous recombination. Yeast cells normally depend on telomerase to maintain their telomeres. We propose that recombination-dependent rolling circle replication around a circular template may be a mechanism by which telomeres are elongated in a single step. This could lead to the formation of post-senescence survivors among cells that lack telomerase activity. We have found that DNA circles of 1.6 kb, composed of telomeric repeats and a marker gene can be used as templates to form tandem arrays at telomeres. The process is partially dependent on *RAD52*, a gene involved in homologous recombination. The elongated sequence derived from a DNA circle may spread to most or all telomeres in a cell by gene conversion events that initiate at subtelomeric or telomeric positions. We have also analyzed some features of telomere elongation templated by DNA circles to gain better understanding of the process. A host strain that has elevated recombination rates at telomeres can efficiently utilize a circular telomeric template to form and spread tandem arrays, but a host strain with mostly fused chromosomes is not efficient at either process. A circular template is far more efficient at forming telomeric tandem arrays than an equivalent linear insert. Subtelomeric sequences present in the circular template may affect the initial alignment between the template and the host telomeric end, leading to the occurrence of telomeric deletions in the arrays that form. Either strand of a *URA3*-telomere circle can be utilized to form telomeric tandem arrays although the presence of 3’ G-rich tails at chromosome ends suggests that the C-rich strand would be preferentially utilized. Similarly, either strand of 100 nt telomeric circles can promote the formation of telomeric tandem arrays. We have observed putative telomeric circles of this size in a telomerase-template mutant that has very long telomeres. This suggests that uncapped telomeres can promote the formation of very small circles.

INDEX WORDS: telomere, telomerase, homologous recombination, DNA circle, rolling circle replication, *TER1*, *RAD52*
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SHOBHANA NATARAJAN

B.Sc., University of Bombay, India, 1994

M.Sc., Madurai Kamaraj University, India, 1996

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by

SHOBHANA NATARAJAN

Approved:

Major Professor: Michael McEachern

Committee: Sidney Kushner
Claiborne Glover
Marcus Fechheimer
Anna Karls

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To patti, amma, appa, Jo and Harish
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW
Early history of telomeres

Telomeres are protein-DNA complexes present at the ends of linear chromosomes. They protect chromosome ends from degradation, fusion and incomplete replication (8, 32, 35, 67, 74, 80, 117, 139). Telomeres were first described, as the ends of chromosomes that have some essential function, by Muller in 1938 when he discovered that fruit flies treated with X-rays could not survive with terminal chromosomal deletions or inversions (91, 92). Further investigation in maize by Barbara McClintock revealed that chromosomes without telomeres tended to form dicentric chromosomes due to end-to-end fusion of sister chromatids. Resolution of the dicentrics during meiosis by introduction of asymmetric chromosomal breaks led to the loss of chromosomal sequences in some cases and duplication of the end in other cases. The presence of telomeres at chromosomal ends seemed to protect the ends from degradation and fusion (78, 79).

Muller and McClintock saw the significance of telomeres before the chemical nature of genetic material was shown to be DNA. When the semi-conservative replication model for DNA was proposed, it was suggested that there would be an incomplete replication of chromosomal ends (132), leading to loss of unique chromosomal DNA with every round of cell division. It was also suggested that this loss of DNA sequence could lead to cellular senescence (99). Several models were proposed to explain the complete replication of chromosomal ends. It was suggested that DNA replication led to the formation of concatameric DNA, which was later processed to form individual chromosomes (132). It is now known that some viruses adopt this strategy to replicate
their genomes. The presence of palindromic DNA at chromosome ends was suggested as a solution for incomplete DNA replication (18). A model by Bateman suggested that telomeric DNA forms a hairpin loop that connects the two strands of the chromosome (1). It was proposed that during replication, inverted repeats are produced that are resolved by introducing staggered nicks. The nicks then fold back to form hairpin loops. Some viruses like the vaccinia virus do have hairpin loops to solve the end-replication problem.

The structure of telomeric DNA began to be resolved when tandemly repeated sequences at the termini of extrachromosomal rRNA genes were found in the hypotrichous ciliate, *Tetrahymena thermophila* (9). These sequences were GT-rich and formed diffuse bands in Southern blots, which suggested that the termini had variable lengths. It was later demonstrated that DNA molecules in the macronuclei of several ciliates had GT-rich sequences with the general formula \( T_4G_4 \) or \( T_2G_4 \) running 5' to 3' toward the chromosome end (58). Telomeric repeats were also cloned from yeast and shown to be TG\(_{1,3} \) repeats that allowed the maintenance of linear plasmids (120). Subsequently, it has been demonstrated that telomeric DNA is typically composed of short (5-8 bp) TG-rich repeat units, the sequence and number of copies of which are species-specific.

In order to understand the mechanism of telomeric DNA synthesis, it was proposed that telomere expansion involved a terminal transferase activity (113), which was later found in cell extracts of *T. thermophila* (40). *In vitro*, six nucleotide long GT-rich repeats were found to be added onto \( T_2G_4 \) oligos and also onto the TG\(_{1,3} \) ends of
yeast telomeric sequences. This was initially thought to be a non-templated mechanism. It was then shown that an enzyme (telomerase) with an essential RNA component determines the sequence of DNA that is recognized and synthesized at chromosome ends (41, 42).

**Telomerase-mediated telomere maintenance is the primary mode of telomere maintenance in yeast, ciliates and mammals**

The enzyme telomerase adds telomeric repeats to telomeres shortened by multiple rounds of DNA replication (42, 88, 114, Figure 1.1). This is the primary mechanism for telomere replication and expansion in yeast, ciliates and mammals. The telomeric repeat is templated by an RNA component of telomerase and is copied onto single-stranded TG-rich DNA ends by the telomerase reverse transcriptase. The telomerase RNA component has been found in yeast (Tlc1 in *Saccharomyces cerevisiae*, Ter1 in *Kluyveromyces lactis*) and mammals (hTR in humans, mTR in mice). The telomerase reverse transcriptase subunit has also been found in yeast (Est2 in *S. cerevisiae*, Trt in *Schizosaccharomyces pombe*) and mammals (hTERT in humans and mTERT in mice). It is known that in both yeast and mammals, telomerase exists as a dimer (3, 106, 133). This implies that sister chromatid ends could be elongated simultaneously.

All the components of telomerase have not yet been identified. Est1 is a subunit of the telomerase holoenzyme and a single-strand DNA-binding protein that can associate with G-rich 3' telomeric tails in *S. cerevisiae* (71, 74, 130). It was found in a screen designed to detect mutants defective in telomere maintenance. Yeast defective for Est1
gradually lose telomeric repeats and undergo growth senescence (Est = ever shorter telomeres). It has recently been shown that telomere dysfunction in est1-Δ mutants can increase the mutation rate of genes near telomeres and cause terminal deletions and chromosomal fusions (45).

Two other proteins, Est3 and Cdc13, have also been found to be required for yeast telomerase activity in vivo (66). Est3 is a subunit of the telomerase holoenzyme whose function is not known (90). Cdc13 is a single-strand DNA-binding protein that, as described below, plays more than one role at telomeres. Deletion of EST2, EST3 or TLC1 (the telomerase RNA component) leads to the “ever shorter telomere” phenotype initially observed in Est1, but deletion of Cdc13 is lethal.

**Other telomere-binding proteins**

The structure of telomeric DNA and the function of telomerase are regulated by several proteins that interact at chromosome ends. These proteins prevent telomeric DNA from being targeted for degradation, recombination and fusion. Telomere proteins also help regulate the continuous process of telomere synthesis and telomere loss and hence help maintain functional telomeres. Some of these proteins are known to bind double-stranded DNA while others bind single-stranded DNA. Most of the telomere-binding proteins that have been found so far have been studied in S. cerevisiae. Structural and functional homologs in other organisms are being found, and there seem to be several common features in the process of telomere length regulation between lower and higher eukaryotes. Telomeric DNA, the telomerase holoenzyme and telomere-binding proteins
act in concert to preserve telomere function and form a “cap” at chromosome ends. Mutations that affect any of these functions can potentially lead to “uncapping” and hence dysfunctional telomeres. The proteins that act in concert at a telomeric end to preserve its structure and function are shown in Figure 1.2.

Cdc13 of *S. cerevisiae* is a single-strand DNA-binding protein that helps recruit telomerase to its single-stranded DNA substrate through its DNA-binding domain and hence positively regulates telomere length (97). Subsequent to the recruitment of telomerase, Cdc13 negatively regulates telomere length in conjunction with the negative regulator, Stn1 (19, 37). It was shown by two-hybrid analysis that Cdc13 interacts with Stn1 (37). Overexpression of Stn1 suppresses the temperature-sensitive defect of the *cdc13-1* allele (37). *cdc13-1* and *stn1-1* temperature-sensitive mutants accumulate single-strand overhangs in telomeric DNA at the restrictive temperature (34, 37). It has been recently shown that Cdc13 may function in recruiting Stn1 to the telomeric end and that Stn1 is an essential mediator of telomeric end protection (101). Since Cdc13 and Stn1 (via interaction with Cdc13) bind to the single-strand overhangs present at telomeric ends, they may also play a role in regulating the length of the single-strand overhang (53). Cdc13 recruits Stn1 using the same domain that it uses to recruit telomerase (19).

Another regulator of telomere length, *TEN1* was isolated as a suppressor of the *stn1-1* temperature-sensitive mutation (36). The interaction between Stn1 and Ten1 was confirmed by co-immunoprecipitation studies. Ten1 also associates with Cdc13 (36). Other mutations in Ten1 lead to telomere lengthening (36). It has been proposed that the
Cdc13-Stn1-Ten1 complex recruits telomerase and plays a role in telomere capping by protecting the telomere.

Single-strand binding proteins have been found in other organisms as well. Pot1 has been found to bind single-stranded DNA in Schizosaccharomyces pombe and humans (2). Deleting POT1 from S. pombe leads to a rapid loss of telomeric DNA and chromosome circularization. Single-strand binding proteins have also been found in Oxytricha nova, Euplotes crassus and Stylonychia mytilis. In O. nova, the end-binding protein has two subunits: α and β. The β subunit interacts with DNA weakly but induces a conformational change in the α subunit (39), allowing the latter to bind DNA in a sequence-specific manner (30). The two subunits do not bind to each other in the absence of DNA. The structure of the two subunits bound to single-stranded telomeric DNA shows that the DNA component is buried within the structure, indicating that the telomeric end is physically protected by this protein (50). The end-binding proteins of E. crassus (131) and S. mytilis (31) share homology with the end-binding protein from O. nova. The recently determined structure of Cdc13 indicates that it shares structural similarity to the O. nova α protein despite the lack of amino acid homology (85).

Another protein important for telomere function in S. cerevisiae is Rap1 (15, 115). RAP1 is an essential gene that is also involved in silencing at mating type loci and transcriptional activation of many genes (60, 63, 119). ScRap1, which binds double-stranded telomeric DNA, has a C-terminal domain that is important for its role in regulating telomere length (22, 62, 72, 75, 119) and also for its role in promoting transcriptional silencing near telomeres (63, 87). Deletion of the ScRap1 C-terminus
leads to the formation of elongated telomeres. The number of Rap1p molecules bound to telomeres can be sensed by the cell, which leads to a strict regulation of telomere length (77). The Rif1 and Rif2 proteins, described below, are also required for this process. Rap1 has also been identified as a dsDNA-binding protein that binds to telomeres and regulates their length in Kluyveromyces lactis (59, 65). Orthologs of ScRap1 have been found in S. pombe and in human cells, though each of these appear to bind to telomeric DNA indirectly via interactions with other telomere proteins (70, 100).

ScRap1 promotes transcriptional silencing near telomeres by recruiting Sir2, Sir3 and Sir4 (silent information regulator proteins). The Sir proteins are involved in silencing at telomeric and mating type loci (86, 87). Mutations in any of these genes abolish silencing. Rif1 and Rif2 were identified as proteins that are recruited by the C-terminal domain of ScRap1 to telomeres (Rif = Rap1 interacting factor). Mutations in Rif1 and Rif2 lead to a moderate increase in telomere length and improved silencing. Deletion of both Rif1 and Rif2 results in a more dramatic increase in telomere length. Rif1 and Rif2 may be proteins that function as a complex that is involved in telomere length control (135). Thus, Rap1 may exist in two alternative complexes at telomeres, one associated with Rif proteins and another associated with Sir proteins (77).

TRF1 and TRF2 are mammalian proteins which bind ds telomeric DNA (12, 70, 127). TRF1 and TRF2 are present ubiquitously in cells as homodimers, and TRF1 stays bound to telomeres throughout the cell cycle, negatively regulating telomere length. TRF1, TRF2 and ScRap1 have Myb-related DNA-binding domains. The telomere-binding protein Taz1 of the fission yeast Schizosaccharomyces pombe also has a Myb-
like DNA binding domain and shares homology with TRF1 (23). TIN2, which is a protein that interacts with TRF1 and localizes at telomeres, may be the mediator of TRF1 function. TIN2 may be functionally similar to yeast Rif1 (57). Mammalian TRF2 may also be involved in capping at telomeres due to its association with chromosomal termini and with “T-loop” structures. The single-stranded ends of mammalian telomeres have been seen to loop back and invade centromere-proximal double-stranded telomeric repeats to form “T-loops” (43). Deletion of TRF2 leads to chromosome fusions, confirming its role in telomere capping (128). TRF2 also recruits hRAP1 to telomeres.

Another protein that is associated with telomeres in mammals and yeast is KU. In addition to being involved in non-homologous end joining (55), KU plays an important role in telomere capping (52). Deletion of KU in mouse cell lines leads to telomere fusions (51). TRF1 binds mammalian KU and recruits it to telomeres. Yeast Ku also serves as an end-binding protein that has a role other than joining DNA ends (38). Deletion of Ku in yeast cells leads to shortening of telomere length (11), disruption of the subnuclear organization of telomeres and a re-distribution of the Sir protein complex, which eliminates the telomere position effect mediated by the Sir proteins (64). Deletion of Ku also leads to the presence of G-strand overhangs throughout the cell-cycle, indicating that it plays a role in establishing normal telomere structure (38). The exact role of KU at telomeres is not yet known.
Homologous recombination and retrotransposition maintain telomeres in some species lacking telomerase

Unlike the ciliated protozoa, yeast and mammalian cells, some organisms like the mosquito *Anopheles gambiae*, the onion *Allium cepa* and the midge *Chironomus pallividittatus* do not have telomerase activity. *Chironomus* lacks any simple repeated sequence at its chromosomal termini. Instead, it has complex tandem repeats of 340-350 bp that can be classified into a few sub-families (21). The repeat units seem to be derived from a common master unit. Recombination-based mechanisms are thought to generate these repeats in different combinations at the termini. In *Anopheles gambiae* and *Allium cepa*, telomere maintenance is thought to involve unequal crossover events promoted by homologous recombination (5, 104). A recombination-dependent mechanism was also proposed to play a role in the maintenance of telomeric repeats in linear mitochondria within the genus of *Tetrahymena* due to the presence of direct terminal repeats of 31-53 bp (89). Recently, mitochondria with linear chromosomes have been found with varying numbers of copies of direct repeats that are 738 bp long in *Candida parapsilosis* (95). *Candida salamanticensis* and *Pichia philodendra* mitochondrial DNA also have terminal repeats that may function as telomeres (123). The mechanism of maintenance of these telomeric repeats is not yet known although extragenomic circular DNA containing telomeric repeats has been seen under the electron microscope (123). These telomeric circles may be involved in a recombination-dependent mechanism of telomere maintenance.
Yet another mechanism for maintenance of chromosome ends has been found in the fruit fly, *Drosophila melanogaster*. Hybridizations using TTAGG telomeric repeats from other insects did not produce any signal in the genomic DNA of *D. melanogaster* (98). This result suggested that this organism may have unique sequences at chromosomal termini. Moreover, mutant *Drosophila* with viable terminal chromosomal deletions had occasionally been obtained (56, 67), although DNA sequences were steadily being lost from the chromosome ends (6, 67). Terminal sequences of *D. melanogaster* were found to be composed of two classes of non-LTR retrotransposons called Het-A and TART (7, 56, 68, 124, 126). Like other organisms, *Drosophila* has no inherent way of preventing terminal loss from DNA replication but uses transposition of non-LTR sequences to replenish the lost DNA. Het-A sequences are also added onto newly-formed chromosome ends (124), suggesting that retrotransposons may be responsible not only for the maintenance of chromosome ends but also for establishing tandem repeats at chromosome ends that did not previously have retrotransposons.

**Processing and repair of double-strand breaks in yeast**

In the absence of telomerase function, telomeres shorten, which eventually can lead to the formation of “uncapped” telomeres. Uncapped telomeres are subject to high rates of homologous recombination and chromosome fusion (82, 83, 93). A mutation or deletion in the telomerase RNA template can alter the binding of proteins at telomeres leading to deregulation and improper functioning of telomeres. This suggests that short, uncapped telomeres resemble double-strand breaks (DSBs). Therefore, dysfunctional
telomeres may be subject to the same repair processes by which DSBs are repaired. To understand dysfunctional telomeres, it is therefore important to understand how DSBs are sensed and repaired.

DSBs are a form of DNA damage that are known to occur in many, if not all, cells. They can be caused by radiation damage or processing of arrested replication forks (112). If left unrepaired, DSBs can lead to severe consequences including cell cycle arrest and cell death (4). DSBs in yeast are primarily repaired by homologous recombination (44). In order to understand this process, DSBs were introduced into substrate molecules by induction of the HO endonuclease, and their repair was studied in various mutant backgrounds (108, 118, 134). These experiments suggested that there are four important steps in the repair of a DSB: resection of the DSB ends, strand invasion and new DNA synthesis, removal on non-homologous DNA tails and later steps in gene conversion. Resection of the ends and strand invasion by the 3’ ends have been confirmed. It is thought that resolution of intermediates leads to a gene conversion event.

It has recently been shown that a related process called break-induced replication (BIR) can repair certain DNA ends and resume a collapsed replication fork (61, 84, 112). BIR is a non-reciprocal recombination event that starts with strand invasion of an intact homologous chromosome by a broken DNA end and proceeds by copying the sequence templated by the intact chromosome. In yeast, a BIR event can extend all the way to the end of the chromosome. There seem to be at least two pathways by which BIR repairs broken chromosomes in S. cerevisiae, both of which require Rad52 (76, 116). One of these pathways is dependent on Rad51, and the other is dependent on Rad50. rad51-Δ
rad50-Δ double mutants fail to repair broken chromosomes. The difference between the gene conversion that occurs in repair of a DSB and the non-reciprocal recombination event that occurs in BIR is shown in Figure 1.3.

Another process by which DSBs can be repaired is non-homologous end-joining (NHEJ). In NHEJ, two non-homologous ends are ligated together (44, 125). NHEJ appears to be the most common DSB repair method in mammalian cells. Analysis of the junctions formed by end-joining in mammalian cells has shown that a significant proportion of events occur with very small (<5 bp) or no complementary sequences (111). When there are a few complementary bases involved in the process, there may be ligation between the complementary bases and filling in of the gaps by DNA replication (103). The Rad50-Mre11-Xrs2 complex may be involved in the processing of DSBs by non-homologous end-joining in yeast and mammalian cells (17).

**Homologous recombination can maintain yeast telomeres**

Though yeast cells normally utilize a telomerase-dependent mechanism for telomere maintenance, they sometimes can utilize the process of homologous recombination to maintain their telomeres. Studies in *S. cerevisiae* and *K. lactis* showed that telomeres could be maintained in the absence of telomerase and that the process was dependent on the product of the *RAD52* gene (73, 80). Discovery of Est1 as a subunit of telomerase in *S. cerevisiae* and further research in an est1-Δ strain showed that homologous recombination played an important role in telomere maintenance in the absence of telomerase (73, 74). An est1-Δ strain, when followed over several generations,
shows progressive telomere shortening leading to cellular senescence. Although most cells do not survive beyond the point of peak senescence, a few rare survivors that have elongated telomeres appear in the population. Most initial survivors of $est1^{-}\Delta S.\ cerevisiae$ have extensive amplifications in the subtelomeric regions (73, Figure 1.4).

Subtelomeric regions of $S.\ cerevisiae$ contain Y' elements. Y' elements are subtelomeric repeats that are highly conserved and fall into two major size categories (5.2 and 6.7 kb). They are usually bracketed by short stretches of telomeric repeats. Other repeat families like the X-elements are also present in $S.\ cerevisiae$ subtelomeric regions, but these are much less conserved and are centromere-proximal as compared to Y' elements. The X-elements are mosaic elements that are more variable than the Y' element. They range from 30-150 bp in length and are well conserved. Their mosaic nature arises due to differences in copy-number and arrangement. In $est1^{-}\Delta$ survivors, the Y' elements and the 50-100 bp of telomeric repeats interspersed in between them increase by as much as 200-fold. Chromosome ends that did not previously have Y' elements acquire the amplified subtelomeric sequence from other telomeres. The expansion of Y' elements and telomeric repeats in subtelomeric regions probably provides a reservoir of telomeric repeats at the chromosome ends and hence, prevents telomere loss in the absence of telomerase. It was proposed that the expansion of Y' elements and telomeric repeats in subtelomeric regions may occur by non-reciprocal cross-over (73). The terminal G-rich tract of telomeric repeats remains constant in size in this class of survivors. Mutants in other components of yeast telomerase also senesce and form post-senescence survivors like the $est1^{-}\Delta$ strain (66, 117, 122).
Deletion of TER1, which is the telomerase RNA template in *Kluyveromyces lactis*, leads to the occurrence of a class of survivors that have elongated telomeres (81). Unlike the subtelomeric expansion seen in *S. cerevisiae*, survivors in *K. lactis* have elongated their telomeric repeat tracts. *K. lactis* lacks the repetitive Y' and telomeric sequences in subtelomeric regions and ter1-∆ survivors do not appear to have amplified subtelomeric regions. rad52-∆ter1-∆ double mutants of *K. lactis* have a dramatically reduced occurrence of post-senescent survivors (80), which establishes a role for homologous recombination in telomere maintenance in the absence of telomerase. It has been shown that subtelomeric recombination in *K. lactis* is enhanced by as much as 1000-fold in ter1-∆ strain (82). In a ter1-∆ strain, recombination between telomeric repeats is also enhanced (Topcu and McEachern, unpublished data). It has been proposed that recombination near telomeres can lead to the spread of telomeric sequences among chromosome ends by a mechanism like break-induced replication.

Further analysis of est1-∆ survivors revealed that there are two types of survivors that lead to telomere maintenance in *S. cerevisiae* (122, Figure 1.4). The survivors that were seen initially with subtelomeric expansions were called Type I survivors. Type II survivors do not have subtelomeric expansions but have elongated telomeric tracts, like the survivors seen in ter1-∆ *K. lactis*. Type II survivors tend to have a growth advantage over Type I survivors. Thus, although Type I survivors are the first to emerge in a given population, Type II survivors outgrow them in liquid-grown populations of survivors.

The two classes of survivors are formed by different types of recombination events (defined by *RAD50*- and *RAD51*-dependent pathways, respectively). The genetic
requirements for the two pathways of recombinational telomere maintenance are similar to the genetic requirements of the two pathways for BIR as discussed in the previous section indicating that BIR may be a mechanism by which telomeric DNA is elongated by recombination. The subtelomeric expansions in the case of Type I survivors are dependent on RAD51, RAD54 and RAD57 (20, 121). The formation of Type II survivors is dependent on RAD59, the RAD50-MRE11-XRS2 complex and also on Sgs1 helicase, but is inhibited by Rif1 and Rif2 (20, 121). Type II survivors can undergo gradual or abrupt changes at telomeres. The telomeric repeats are lost gradually due to DNA replication, or they may go through rapid truncations by intra-molecular recombination (16, 69). Type II survivors can originate very quickly from very short telomeres. It has been proposed that extrachromosomal telomeric circles may be responsible for abrupt, large and variable increases in telomere length (121).

Telomeres and telomerase in mammalian cells

Normal human somatic cells grown in vitro proliferate until they reach a discrete point at which population growth ceases, but cells remain alive and karyotypically normal (49). This period is termed the M1 stage of aging or “replicative senescence”. This block can be overcome by activation of viral oncogenes or suppression of p53 and pRB (tumor suppressor genes) by mutation. After a period of proliferation, human somatic cells reach a second block called a “crisis point” or the M2 stage at which there is an enormous increase in cell death and the occurrence of chromosomal abnormalities. Both replicative senescence and crisis appear to be consequences of shortened telomeres.
Human cells possess telomeric repeat tracts at the end of each chromosome. In embryonic cells, the tracts are typically >10 kb in size. Most normal human somatic cells lack telomerase activity, and telomeres become shorter as they continue to replicate both *in vitro* (47) and *in vivo* (47, 48). As telomeres progressively shorten, the protective structure formed by the DNA and proteins gets disturbed and triggers DNA damage responses that lead to cell-cycle arrest. The continuous loss of telomeric sequences from chromosome ends has been demonstrated to be a major cause of replicative senescence (10). Replicative senescence is a mechanism that is thought to help prevent the formation of tumors in human tissues. Cancer cells and immortal cell lines usually express telomerase and hence, prevent loss of unique sequences (24, 25, 88). Overexpression of hTERT, the telomerase reverse transcriptase, leads to the proliferation of cells beyond the point of replicative senescence in almost all cell types that have been studied so far (10, 28, 107, 129). Conversely, repression of hTERT in telomerase-positive cells leads to gradual telomere shortening, chromosome fusions and eventual cell death (46, 140). Past the point of replicative senescence, telomeres continue to shorten until crisis. Short telomeres lead to genomic instability due to an increased occurrence of telomere fusions and karyotypic abnormalities. Thus, telomere erosion is thought to be a preventive measure against the generation of malignant cells. Continued expression of telomerase is sufficient to achieve cellular immortality in many human cell types (26). Alternate pathways for telomere maintenance other than telomerase activation can also lead to immortalization.
**Alternate lengthening of telomeres (ALT) in human cells**

Research on mechanisms of recombinational telomere maintenance has gained particular significance since a telomerase-independent pathway for telomere maintenance and elongation has been found in ~10% of human cancers and about 50% of immortalized human cell lines. The telomerase-independent pathway in mammalian cells has been called “alternate lengthening of telomeres” or ALT (13). ALT appears to be more prevalent in tumors derived from mesenchymal tissues (109). This may be because mesenchymal cells commonly have slower cell turnover and may lose telomeric repeats at a slower rate as compared to epithelial cells (13). Some cell lines may have both an active telomerase and an ALT pathway. It is not yet clear if both mechanisms co-exist in the same cell or are present in different sub-populations. Whereas telomerase\(^+\) cell lines have telomeres that are usually less than 12 kb, ALT\(^+\) cells have a mean telomere length of \(>20\) kb, but the lengths vary between 3 kb and 50 kb (13, 14). Correspondingly, telomere lengths in individual ALT\(^+\) cells, as seen by fluorescence in situ hybridization, are extremely heterogeneous (138).

ALT\(^+\) cells also have aggregates of proteins that form APBs (ALT-associated PML bodies). PML bodies are nuclear protein bodies that are normally present in mammalian cells. The numbers of PML bodies are greatly increased in ALT\(^+\) cells. APBs have been found to contain several replication-, recombination- and telomere-associated proteins like RAD52, RAD51, RPA, MRE11/RAD50/NBS1 complex, WRN (human homolog of yeast SGS1), BLM, TRF1 and TRF2 (54, 136-138, 141).
The mechanism of ALT may involve events mediated by homologous recombination as in yeast. Experimental support for involvement of homologous recombination in ALT comes from the fact that a DNA tag placed within the repeats of one telomere expanded to ten different telomeres within 63 population doublings in human cells (29). Circular DNA composed of telomeric repeats, which may act as a substrate for telomere elongation promoted by rolling circle replication, has been found in some human cells (110). The formation of tandem arrays of telomeric and non-telomeric sequences has also been reported (94), which may indicate that certain ALT\(^+\) cells may have similarities with the yeast Type I survivors.

Studying the characteristics and mechanisms of telomerase-independent telomere maintenance may prove important for the diagnosis, prognosis and treatment of cancer. Currently, telomerase activity is used as an indicator for malignancy and as the target for designing anti-cancer therapeutics (27). Tumors that employ ALT as the mechanism for maintaining telomeres are predicted to be resistant to drugs that target telomerase. Also, ALT\(^+\) cells resistant to anti-telomerase drugs would likely arise because of strong selective pressure. It is known that if the ALT pathway is repressed by fusing an ALT\(^+\) cell to a normal cell, cell lines that lack an active telomerase senesce and eventually die (102). This indicates that normal cells have an active mechanism to repress ALT and to rapidly shorten their telomeres. It was also shown in this study that ALT and telomerase could co-exist in the same cells. Other reports have suggested that telomerase leads to repression of ALT (33). It will be useful to design combination therapies that target both the telomerase-dependent and ALT pathways.
Current Experiments

We have used *Kluyveromyces lactis* as a model organism to study the mechanism of recombinational telomere elongation. *K. lactis* is closely related to *S. cerevisiae* and the genomes of the two species show appreciable synteny. However, despite the similarities between the two yeast species, they have several differences, which has made it easier to employ *K. lactis* in several of our experiments. For example, *K. lactis* has 6 chromosomes and does not have complex subtelomeric regions. In contrast, *S. cerevisiae* has 16 chromosomes and telomeric repeats and Y' elements in subtelomeric regions. The telomeric repeat in *K. lactis* is 25 bp long and uniform as compared to *S. cerevisiae*, which has telomeric repeats that are heterogeneous in sequence and length.

In this study, we provide evidence suggesting that rolling circle replication may be the mechanism of recombinational telomere elongation in *K. lactis* cells. It is known that rolling circle replication is used as a means of increasing the copy number of some phage genomes and also for the replication of some plasmids (96). It results in the generation of tandem copies derived from the sequence of a circular template. The formation of tandem arrays by rolling circle replication is depicted in Figure 1.5. A 3' end, which can be provided by a unit of RNA, DNA or protein is required to prime the replication. Telomeric circles that may act as templates for rolling circle replication may be generated spontaneously at chromosome ends due to the fact that telomeric DNA is composed of direct repeats. It is known that direct repeats can easily loop-out in the form of circles. A transposon-mediated mechanism has been proposed for the formation of single-stranded circles by resolution of a figure-eight molecule (105).
We propose that there are two steps involved in the formation of long telomeres bearing tandem copies of sequence derived from a telomeric circle: 1) a process capable of lengthening a telomere and 2) a process capable of spreading the sequence of the elongated telomere to other chromosome ends. One or both processes are dependent in part on the product of the \textit{RAD52} gene. We have also analyzed various features of the process of circle utilization to gain better understanding of the process.

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Figure 1.1 Telomerase mediated telomere extension.

The telomerase holoenzyme (shown in pink) catalyzes the synthesis of new telomeric repeats at chromosome ends. The telomerase RNA component aligns with the 3' end of a telomeric repeat, and a single telomeric repeat is synthesized by the reverse transcriptase activity of telomerase. Newly synthesized DNA is shown in blue. Translocation of the telomerase occurs followed by re-alignment of the template RNA with the 3' end of the newly synthesized repeat. This re-alignment allows for another round of synthesis. Sequences shown represent the telomeric repeat and telomerase RNA from the yeast *Kluyveromyces lactis*. Telomeres from other organisms differ from *K. lactis* in having shorter telomeric repeats and a correspondingly shorter telomerase RNA template. This figure is taken from D. Underwood.
A 25 nt repeat is copied in WT

new DNA synthesis

binder again for next synthesis
Figure 1.2 Proteins bound at a telomeric end of *S. cerevisiae*.

Proteins that bind to telomeric repeats at a chromosome end of *S. cerevisiae* are shown. The G-rich telomeric repeats are purple rectangles and the C-rich telomeric repeats are blue rectangles. Telomeric ends typically have a G-rich single-stranded tail. The components of the telomerase holoenzyme that have been identified so far (Est1, Est2, Est3 and Tlc1 RNA) add telomeric repeats to the single-stranded tail. Cdc13 is an end-binding protein that regulates the length of the single-strand overhang at the telomeric end. Cdc13 aids the interaction of the telomerase holoenzyme and the Stn1 protein with the telomeric single-stranded region. Stn1 may also regulate the length of the single-strand overhang. Ten1 is known to associate with Stn1. Rap1 binds double-stranded telomeric repeats and recruits Rif1, Rif2 and the Sir complex to telomeres. Rif1 and Rif2 regulate telomere length, like Rap1. Sir2, Sir3 and Sir4 are involved in mediating silencing at telomeres. The Rad50-Mre11-Xrs2 complex is involved non-homologous end-joining. Ku probably protects chromosome ends.
Figure 1.3 Gene conversion versus break-induced replication

The steps in the repair of double-strand breaks by gene conversion and break-induced replication (BIR) are shown. The black lines represent the donor molecule and the red lines represent the damaged molecule. Once a double-strand break (DSB) is detected, there is resection of 3' ends followed by strand-invasion of the overhangs into a homologous chromosome. When new DNA synthesis occurs, the gap created due to resection is filled (gene conversion) or DNA synthesis proceeds until the end of the chromosome, replacing all sequences that are telomere-proximal (break-induced replication).
Resection of 3' ends

Strand invasion

Synthesis of new DNA

Resolution

Two homologous chromosomes

DSB formation

Resection of 3' ends

Strand invasion

Synthesis of new DNA

Resolution

Gene conversion

Break-induced replication
Figure 1.4 Type I and Type II survivors of *S. cerevisiae*

The structure of subtelomeric and telomeric sequences in Type I and Type II survivors of *est1-Δ S. cerevisiae* as compared to wild-type and *est1-Δ* senescent strains is shown. The blue boxes represent Y' elements and the orange boxes represent telomeric repeats. Y' elements are repeat structures present in the subtelomeric regions of *S. cerevisiae* chromosomes. Type I survivors have short terminal tracts of telomeric repeats and have amplifications of subtelomeric Y' elements and internal tract telomeric repeats. Type II survivors have elongated terminal tracts of telomeric repeats.
Figure 1.5 Rolling circle replication at a chromosome end

A model for the formation of elongated telomeres by rolling circle replication is shown. The G-rich telomeric strand is shown in purple and the C-rich telomeric strand is shown in blue. A double-stranded telomeric circle can template the synthesis of telomeric repeats primed by the 3' end of a chromosome. Strand-invasion of the circular telomeric template by a free 3' G-rich telomeric end followed by DNA synthesis around the C-rich strand of the circle leads to elongation of the telomeric G-strand. Multiple rounds of DNA synthesis around the circular template leads to the formation of a very long G-rich telomeric strand. Lagging strand synthesis leads to the formation of the C-rich strand. The red line represents RNA primer for lagging strand synthesis.
Strand-invasion of a circular template by a chromosome end

First round of extension of 3’ end of chromosome

Second round of replication by displacement of originally synthesized strand

Third round of replication

Multiple rounds of strand displacement and DNA replication

Lagging strand synthesis

Elongated telomeric end
CHAPTER 2

RECOMBINATIONAL TELOMERE ELONGATION PROMOTED BY DNA CIRCLES

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Abstract

Yeast mutants lacking telomerase are capable of maintaining telomeres by an alternate mechanism that depends on homologous recombination. We show here, by using *K. lactis* cells containing two types of telomeric repeats, that recombinational telomere elongation generates a repeating pattern common in most or all telomeres in survivors that retain both repeat types. We propose that these patterns arise from small circles of telomeric DNA being used as templates for rolling circle gene conversion and that the sequence from the lengthened telomere is spread to other telomeres by additional, more typical gene conversion events. Consistent with this, artificially constructed circles of DNA containing telomeric repeats form long tandem arrays at telomeres when transformed into *K. lactis* cells. Mixing experiments done with two species of telomeric circles indicated that all of the integrated copies of the transforming sequence arise from a single original circular molecule.

Introduction

Telomeres are the protective DNA-protein complexes at the ends of chromosomes (Reviewed in 35, 57). The DNA of telomeres in most eukaryotic organisms is composed of tandem arrays of 5-8 bp direct repeats. These repeats in part serve as binding sites for specific proteins that “cap” the telomeres and prevents them from eliciting the repair responses that are normally activated by broken DNA ends. Loss of this function can lead to cell cycle arrest, telomere fusions, and high rates of recombination near telomeres (3, 6, 14, 29, 33, 34, 38, 39, 48). The functioning of telomeres is thus critical for proper cell growth and chromosome stability. Because DNA polymerases are unable to fully
replicate ends, the DNA of even properly capped telomeres are intrinsically unstable, prone to progressive shortening with every cell division (26, 42). Most organisms avoid this problem because of the enzyme telomerase, which adds new telomeric repeats de novo onto telomeric ends (18, 36). Most human somatic cells have little or no telomerase activity and their telomeres are subject to gradual shortening (19, 22, 49). Sufficient telomere shortening in human cells triggers the permanent growth arrest of replicative senescence (7, 27). This is thought to be an adaptation to reduce the rate of cancer formation as almost all human cancers are found to have an active telomere maintenance pathway, most typically due to the presence of telomerase (22).

Although sequence addition by telomerase is the major mechanism of telomere elongation in eukaryotic cells, it is not the only mechanism. The ends of chromosomes in the fruit fly *Drosophila melanogaster* are maintained by occasional transposition of retrotransposons (4, 24, 53). Other organisms, including the midge *Chironomus*, the mosquito *Anopheles gambiae*, and the plant *Alium Cepa* have telomeres composed of complex repeat families that are thought to be maintained by recombination (5, 13, 43, 47).

Recombinational telomere elongation can also occur in some cells that normally utilize telomerase. Yeast cells without telomerase undergo gradual telomere shortening and growth senescence within 50-100 cell divisions (26, 32, 50). Cells that survive beyond the point of senescence have restored longer tracts of telomeric repeats to their chromosomal ends through recombination. In *S. cerevisiae*, there are two genetically distinguishable pathways of recombinational telomere maintenance, one which amplifies subtelomeric sequences while maintaining short telomeres (type 1) and the other that
lengthens telomeric repeat arrays (type 2) (23, 25, 51). In *K. lactis*, which lacks the subtelomeric blocks of telomeric repeats found in *S. cerevisiae*, only the equivalent of type 2 survivors are seen (31).

Some human cells are capable of maintaining telomeric repeat arrays at their chromosome ends in the absence of telomerase. This telomerase-independent pathway of telomere maintenance is called alternate lengthening of telomeres (ALT). ALT was first observed in certain in vitro immortalized cell lines (10, 37, 44). Subsequently, it has been found that 5-10% of human cancers also appear to maintain long telomeres despite an absence of telomerase (9). Recently, direct evidence for telomeric recombination in ALT cells has been found, suggesting that these cells maintain telomeres through homologous recombination (15). These data indicate that recombinational telomere maintenance can occur in human cells and is likely of significance to a number of human cancers.

How cells can utilize recombination to generate longer telomeric repeat tracts has remained perplexing. In *K. lactis* cells, short telomeres are subject to large increases in the rates of subtelomeric gene conversion and can lead to the frequent spreading of a marker gene from one telomere to most or all other telomeres in the cell (33). We show here, by marking *K. lactis* telomeres with two types of telomeric repeat, that recombinational telomere elongation generates a common repeating pattern within most telomeres in a given survivor. We further show that long repeating arrays are formed at chromosome ends when cells are transformed with DNA circles containing telomeric repeats. These results suggest that rolling circle gene conversion may be a mechanism for recombinational telomere elongation.
Materials and Methods

Strains

The *K. lactis* ter1-Δ strain was derived from haploid, wild type *TER1, K. lactis* 7B520 (*Ura3*, *His3*, *Trp*) (56). *TER1* was deleted using the plasmid loop-in, loop-out procedure (32). The ter1-Δ strain with wild-type telomeric repeats at the base and Bcl telomeric repeats at the tips was derived by transforming a senescing ter1-Δ strain with an integrative plasmid (pTER-BX:UA *TER1*-*Bcl*) containing *TER1*-Bcl, forming a ter1-Δ/TER1-*Bcl* heteroallele. Plating on 5-fluoro orotic acid selected for cells that had “looped out” either the *TER1*-Bcl allele or the ter1-Δ allele. Clones containing only the ter1-Δ allele were identified by their early senescence rough colony phenotype. *K. lactis* transformation was done by electroporation as described for *S. cerevisiae* (2).

Telomere cloning

Telomeres were cloned by plasmid rescue as follows. A pRS423-derived plasmid, with a *HIS3* marker gene, containing a ~590 bp *EcoRI*-*XbaI* fragment of *K. lactis* subtelomeric sequence was transformed into the ter1-Δ/TER1-*Bcl* heteroallele strain where it integrated ('looped-in') next to a telomere. Transformants were selected on plates lacking histidine and then plated on 5-FOA to select for ter1-Δ cells and passaged by serial streaking on solid medium to produce senescent cells and later, the formation of post-senescence survivors. Genomic DNA from His+ survivors was cleaved with *XhoI*, to detach the plasmid-telomere fragment from chromosomal DNA, treated with T4 polymerase to blunt ends, and ligated to circularize the fragment. After
transformation into *E. coli*, plasmids containing cloned telomeres could be recovered. As selection for a subtelomeric marker leads to the spread of that marker to multiple telomeres in senescing *ter1-Δ* cells (33), different cloned telomeres from a given survivor likely represent different chromosome ends.

**Southern hybridizations and quantitation of *URA3* copy number**

Southern blotting was done using Hybond N+ membrane. All hybridizations were done in 500mM Na$_2$HPO$_4$ and 7% SDS. The telomeric probe used in our hybridizations is the Klac1-25 oligonucleotide (32). This probe was used at 50°C. The subtelomeric probe was generated from the ~590 bp *EcoRI*-*XbaI*. The *URA3* probe used was a *HindIII* fragment from pMH3 containing the *S. cerevisiae* *URA3* gene (20). The subtelomeric and *URA3* probes were hybridized at 65°C. The number of copies of *URA3* was determined by analysis with a PhosphorImager.

**Isolation of circle S and circle P**

The two circles (“circle S” and “circle P”) differ at a single restriction enzyme site (*S* = *SalI*, *P* = *PvuI*). Circle S was constructed by circularizing a *BamHI*-*BglII* fragment from plasmid pMH3-1Tel and circle P was constructed in a like manner after first filling in the *SalI* site to create a *PvuI* site. pMH3-1Tel was derived by integrating a *DpnI* fragment from pAK25 (33) containing ~11.5 telomeric repeats and ~120 bp of subtelomeric sequence into pMH3.
Results

Repeating patterns are produced by recombinational telomere elongation.

To investigate the mechanism by which recombination generates elongated telomeres, we constructed yeast cells with a disrupted telomerase RNA gene (*ter1-Δ*) and containing telomeres composed of basal wild type repeats and terminal mutant repeats (Bcl repeats) (Figure 2.1A, Materials and Methods). We reasoned that the pattern of the two repeat species present in the telomeres that underwent recombinational elongation would provide clues about the mechanism that produced the elongation. The Bcl mutation present in the terminal repeats does not perturb telomere function and produces a *Bcl*I restriction site by changing one position within the 25 bp telomeric repeat (30, 33, 54).

Digestion of genomic DNA from most post-senescent survivors indicated that elongated telomeres in these cells either lacked Bcl repeats entirely (outcome 1, Figure 2.1B) or had them confined to the basal regions of one or more telomeres (outcome 2, Figure 2.1B) (data not shown). These telomere structures are inconsistent with Bcl repeats having become amplified during the recombinational telomere elongation and survivors containing them and were not studied further. Other survivors (34 of 231), however, were more informative. *Bcl*I digestion of genomic DNA from these survivors, but not from pre-survivor cells, excised very small fragments that intensely hybridized to a telomeric probe (Figure 2.1C). These were predicted to be blocks of wild type repeats bordered on each side by half Bcl repeats. For most such survivors, all telomeres in the cell were cleaved by *Bcl*I and hence contained Bcl repeats. When the *Bcl*I-cleaved DNA was run on high percentage agarose gels (lower panel of Figure 2.1C and data not
shown), it was found that the small telomeric fragments were the sizes expected for multiples of the *K. lactis* 25 bp telomeric repeat, the great majority between 50 and 150 bp (1-5 wild type repeats, correcting for the presence of a 1/2 Bcl repeat on each end). Within a given survivor clone, the excised small fragments were mostly a single size (most notably clones 1, 5, 6, 7 and 8). Different clones, however, displayed different sized BclI fragments. These data suggested that a common pattern of wild type and Bcl repeats were often present in most or all elongated telomeres generated in a post-senescence survivor. The presence of a single predominant size class for blocks of wild type repeats further suggested the possibility that the elongated telomeres often had a simple repeating pattern of wild type and Bcl repeats. To address this, we cloned telomeres from three independent survivors containing interspersed wild type and Bcl repeats. Sequencing telomeric DNA from these survivors confirmed the frequent presence of repeating patterns (outcome 3, Figure 2.1B). The telomeres from survivors we examined commonly contained a repeating unit composed of four repeats, either 3 wild type and 1 Bcl or 2 wild type and 2 Bcl repeats. The blocks of wild type repeats observed in the sequenced clones were in agreement with our restriction digestion data (Figure 2.1C and data not shown). Our results confirmed that recombinational telomere elongation in *K. lactis* commonly involved: 1) a process capable of generating repeating patterns within telomeres, and 2) a process capable of spreading a common repeating pattern to multiple telomeres.
DNA circles containing telomeric repeats can greatly promote telomere elongation.

A possible way for a short telomere in a senescing telomerase deletion mutant to both become elongated and acquire a repeating pattern would be for it to initiate a gene conversion event utilizing a small circle of telomeric repeats as a template. A telomere extended by rolling circle gene conversion would acquire a repeating pattern that matched the size and pattern of the circle that was copied.

A rolling circle model predicts that an exogenously provided DNA circle containing telomeric repeats could become incorporated at telomeres as a long tandem array. To test this, a DNA circle was constructed by ligating the ends of a restriction fragment containing \textit{URA3} and a \textit{K. lactis} telomere. The resulting 1.6 kb monomeric circle was purified by isolation from a gel, and then transformed into \textit{K. lactis} \textit{ter1-}\textcopyright \textit{∆} cells as well as cells containing a functional telomerase (\textit{TER1}). As the circle lacked a replication origin, maintenance of sequences derived from it required incorporation into a chromosome. We analyzed some of the transformants that grew on uracil-lacking plates by Southern blotting and hybridization to determine their telomere structure. The terminal \textit{EcoRI} fragments of the chromosomes were visualized through use of a subtelomeric probe that hybridizes to 11 of the 12 such fragments in the haploid \textit{K. lactis} genome (Figure 2.2A). Some \textit{TER1} transformants had structures consistent with integration of a single copy of the transforming circle at a telomere, or at non-telomeric positions (Figure 2.2A,B, lane 4, and data not shown). However, many \textit{TER1} transformants (32 of 48) and all \textit{ter1-}\textcopyright \textit{∆} transformants (45 of 45) appeared to have one or more telomeric \textit{EcoRI} fragments that were greatly elongated, running at or near limit
mobility in the gel (>15 kb Fig. 2A and B, lanes 3, 5 and 6). Untransformed controls, in contrast, contained no telomeric fragments larger than 3.5 kb (including the single telomere not hybridizing to the subtelomeric probe). The elongated telomeres hybridized very intensely to *URA3* (Figure 2.2B) and telomeric probes (data not shown and Figure 2.3). These data indicated that telomeres in the transformants had become elongated, and often greatly elongated, by incorporation of sequence from the transforming 1.6 kb circle. The extent of subtelomeric sequence present as elongated bands (Figure 2.2A) is indicative of how many telomeres were elongated by addition of sequence derived from the transforming DNA circle and differed between *TER1* and *ter1-Δ* transformants. *TER1* transformants had one or sometimes two lengthened telomeres while *ter1-Δ* transformants had many and often most of their twelve telomeres lengthened. Control experiments using linear DNA containing *URA3*, subtelomeric sequence, and telomeric repeats, but with ends incompatible with self ligation, readily integrated by replacing one or more native telomere but never formed tandem arrays (33, 55 and data not shown), indicating that formation of the highly elongated telomeres depended upon the transforming DNA having a circular structure.

Cutting DNA from transformants with long telomeres (both *TER1* and *ter1-Δ*) with restriction enzymes that cleaved the transforming circle at a single position generated a 1.6 kb fragment that hybridized intensely to *URA3* (Figure 2.2D) and telomeric probes and was often plainly visible on ethidium bromide stained gels (data not shown). Such digests also produced one or more faint bands (visible with *URA3* and subtelomeric probes) of sizes consistent with centromere-proximal junction fragments.
(“J” in Figure 2.2E), and short diffuse terminal fragments (visible with URA3 and telomeric probes; “T” in Figure 2.2E, data not shown). These results are fully consistent with the sequence from the circle having integrated at telomeres as long tandem arrays. The number of copies of the 1.6 kb unit present in transformants was estimated by measuring URA3 hybridization signal on a Southern blot using a strain with a single integrated copy of URA3 as a control. The copy number of the URA3-telomere insert was estimated to be 6-20 in TER1 transformants and 30-180 in ter1-Δ transformants (data not shown). The highest copy numbers are the equivalent of about 2% of total genomic DNA.

Unlike the elongated telomeres of TER1 transformants, those of ter1-Δ transformants ran as complex ladders of bands (Figure 2.2B and see also 2.3B). Most of these bands hybridized with a subtelomeric probe and likely represent telomeres containing different numbers of the integrated 1.6 kb sequence (1-10+ copies). However, not all bands in lanes from the ter1-Δ transformants that hybridized to the URA3 probe also hybridized to the subtelomeric probe (see for example, the lowest band visible in lanes 5 and 6 of Figure 2.2B). This suggested that DNA present in these bands was not associated with telomeres. When uncut genomic DNAs from the ter1-Δ transformants were run on gels, several bands that hybridized to telomeric and URA3 probes were present that migrated well ahead of the bulk chromosomal DNA band (Figure 2.2C and data not shown). These bands were not detected in wild type transformants or in an untransformed ter1-Δ control. We conclude that extrachromosomal derivatives of the 1.6 kb sequence were present in these cells. Although we have not characterized these DNA
species, their positions in gels suggest that they could be circular and linear monomers and multimers of the 1.6 kb *URA3*-telomere sequence. Lacking an ARS element, such structures could not be stably maintained in cells but might instead be frequently produced by the high level of recombination experienced near telomeres in *ter1-Δ* cells (33). Our experiments have shown that none of the sequences of the 1.6 kb *URA3*-telomere circle are capable of driving autonomous replication in *K. lactis* (data not shown).

In addition to having one or two telomeres extended by tandem arrays from the transformed 1.6 kb circles, all other telomeres in *TER1* transformants were slightly elongated, apparently by the addition of extra telomeric repeats (Figure 2.2A and see also Figure 2.3B). This may reflect an ability of the extra telomeric repeat arrays on the greatly extended telomere to bind and titrate a protein such as Rap1 that negatively regulates telomere length. A similar lengthening phenomenon has been observed in *S. cerevisiae* when extra copies of telomeric repeats were present on a multi-copy plasmid (46).

**All integrated copies of the 1.6 kb sequence are derived from a single transforming circular DNA molecule.**

To test whether the tandem arrays that formed at telomeres in *TER1* and *ter1-Δ* transformants were derived from a single original transforming DNA circle or from independent integrations of multiple circles, we performed a mixing experiment. A second form of the 1.6 kb circle was constructed by first filling in a unique *SalI* fragment
to create a unique \textit{Pvu}I fragment in the precursor plasmid. The two forms of 1.6 kb circle (\textit{Sal}I “circle S” or \textit{Pvu}I “circle P”; Figure 2.3A) were then generated, purified, and introduced singly or together (in similar amounts) into \textit{ter1-}\Delta and \textit{TER1} strains and the telomeres from the resulting transformants were then examined (Figure 2.3B, C). Figure 2.3A shows the expected general structure if sequence from circle S and/or circle P form tandem arrays at chromosome ends. The composition of these arrays was expected to differ if derived from a single transforming circle than if derived from many circles. If a single circular molecule was the ultimate source of all the tandem arrays in a transformant, then the arrays should be cleaved completely down to 1.6 kb units by the restriction enzyme specific to one form of circle and completely resistant to digestion with the enzyme specific for the other form of circle. In contrast, if arrays form from integration of multiple transforming circles, then telomeric DNA from any given transformant should contain both ‘S’ and ‘P’ versions of the 1.6 kb sequence and should be cleavable with both \textit{Sal}I and \textit{Pvu}I. Transformation rates of the two circles, transformed singly or together, were similar indicating that transformation was not biased for one molecule or the other.

Figure 2.3B shows hybridization data from representative control clones of \textit{TER1-WT} (top) and \textit{ter1-}\Delta (bottom) that had been \textit{individually} transformed with either circle S or circle P. Filters of genomic DNA cleaved with \textit{EcoRI} (-), \textit{EcoRI-Sal}I (S) and \textit{EcoRI-Pvu}I (P) (as indicated in row labeled ‘secondary digest’) were sequentially probed with subtelomeric, \textit{URA3}, and telomeric probes. As seen earlier with circle S alone (Figure 2.2), tandem telomeric arrays were formed and these were cleaved down to 1.6 kb units that hybridized to telomeric and \textit{URA3} probes when digested with the restriction enzyme 62
specific for the transforming circle (SalI for circle S and PvuI for circle P). Arrays remained at limit mobility and no 1.6 kb URA3-hybridizing bands were observed when samples were digested with the enzyme specific for the other form of circle.

Subtelomeric junction fragments (indicated with J in panel A) that hybridized to subtelomeric and URA3 probes are indicated with arrows in the panels showing the URA3 hybridization. Very short and diffuse terminal fragments (indicated with T in panel A) were also observed with the telomeric probe (data not shown).

Figure 2.3C shows hybridization data from representative clones of TER1-WT (top) and ter1-Δ (bottom) that had been transformed with a mixture of comparable amounts of circle S and circle P. Digests and hybrizations shown are arranged as for Figure 2.3B. The data show that transforming K. lactis cells with mixtures of S and P circles produced tandem telomeric arrays that, within a given transformant, contained sequences derived from only one form of circle. 22 of 22 TER1 and 28 of 28 ter1-Δ contained tandem arrays of either the P version or the S version of the 1.6 kb sequence, but not both. 12 of the 22 TER1 transformants had utilized circle S and the other 10 had utilized circle P to form tandem arrays. 8 of 28 ter1-Δ transformants had utilized circle S and the other 20 had utilized circle P. These data indicate that even in cells with >100 integrated total copies spread among multiple telomeres, all copies can ultimately be derived from a single transforming molecule. The absence of clones containing sequences from both forms of circle may indicate that, in our experiments, only one circular molecule normally entered a cell about to be transformed.
Formation of telomeric tandem arrays is reduced but not eliminated in a *rad52* mutant.

We next tested whether the formation of the long *URA3*-telomere arrays was dependent upon *RAD52*, a gene required for most forms of homologous recombination in *S. cerevisiae*. Because *rad52* *ter1*-Δ mutants have extremely poor viability (31), this experiment was necessarily limited to a *rad52 TER1* strain. When transformed with the 1.6 kb ‘P’ circle, a *rad52 TER1* strain showed a reduced ability to form Ura3⁺ transformants when compared to a *RAD52 TER1* strain (Figure 2.4). A control ARS plasmid transformed both strains equally well. The precise degree to which the *rad52* mutation reduced circle P transformation was difficult to determine because of the heterogeneous colony size observed on transformation plates (Figure 2.4). However, we estimate that the degree of reduction is 75-95%. The colonies that did form in a *rad52* background were examined to determine whether long telomeric arrays had formed. While the tiniest colonies of both *rad52* and *RAD52* strains did not contain detectable *URA3* sequences (and were presumably abortive transformants), the small to medium size transformant colonies did. As shown in Figure 2.5, these transformants routinely (23 of 43) had the same characteristics as *RAD52 TER1* circle P transformants; a novel telomere running at limit mobility in *EcoRI* digests which was cut down to a 1.6 kb fragment by *PvuI* and which hybridized intensely to *URA3* and telomeric probes. The transformants were confirmed as being derived from the original *rad52* deletion mutants through hybridization to a *RAD52* gene probe. Our data indicate that absence of *RAD52* function significantly reduces, but does not eliminate, the ability of a transforming circle to generate a long tandem array at a telomere. This result is consistent with either a single
mechanism that is partially $RAD52$-dependent or with two mechanisms, the major of which is $RAD52$-dependent, for forming tandem arrays by copying the 1.6 kb circle.

**Discussion**

To explain recombinational telomere elongation in $ter1-\Delta$ survivors, we propose the “roll and spread” model shown in Figure 2.6. It proposes that formation of long telomeres by recombinational telomere elongation is brought about by two distinct types of events. In one, a rolling circle gene conversion utilizes a tiny circle of telomeric DNA as a template to generate a long telomere in a single step. If the circle contained two species of telomeric repeat, the resulting elongated telomere would have a repeating pattern based upon the unit structure of the circle being copied. In the second, more common type of event, other gene conversions using the elongated telomere as a template spread its sequence onto other telomeres in the cell.

It was previously shown that short telomeres, even in the absence of growth senescence, caused gene conversion rates at subtelomeric positions to be highly elevated (33). In a $ter1-\Delta$ strain, the degree of increase was measured as $\sim 800$ times higher than seen in a $TER1$ control. This enormously elevated gene conversion frequency was shown to be readily capable of copying a marker gene initially next to a single telomere onto most or all other chromosome ends in the cell. Recent data indicates that recombination between telomeres themselves is also greatly enhanced in a strain lacking telomerase (Z. Topcu and M. McEachern, unpublished data).

Our findings reported here show that recombinationally elongated telomeres within a given post-senescence survivor typically contain a common pattern of telomeric
repeats. This strongly supports the hypothesis that the generation of post-senescence survivors in \textit{ter1-\Delta} mutants is dependent upon spreading of a sequence from one elongated telomere to many, and sometimes all, other telomeres in the cell. As even a single short or missing telomere is likely to be enough to prevent normal growth (3, 48), there would be strong selective pressure on \textit{ter1-\Delta} cells to spread a long telomeric sequence to all other telomeres. The spreading of an elongated telomeric repeat array between different chromosome ends may not be a completely random process. Conceivably, by virtue of having many telomeric repeats, a long telomere would preferentially be a target of strand invasion by short recombinogenic telomeres and therefore preferentially be a donor of sequence information.

The results with transforming circular DNA molecules provides strong circumstantial support for rolling circle gene conversion being a process that \textit{K. lactis} cells can carry out. Our data clearly show that a single circular molecule is responsible for generating the long tandem arrays in \textit{TER1} and \textit{ter1-\Delta} transformants. The typical structure of \textit{TER1} transformants, where a single telomere has acquired all the copies of 1.6 kb sequence argues that integration occurs through a concerted process. In principle, the 1.6 kb circle could integrate singly at a telomere and then expand into a tandem array through multiple unequal crossovers. This would have to be an extremely efficient process to account for long arrays being present as soon as transformants can be examined. At least two additional things argue against unequal recombination accounting for array formation. First, the expected intermediate structures (telomeres with only a few tandem copies of the 1.6 kb sequence) are not observed in \textit{TER1}
transformants, even in *rad52* cells which have a greatly reduced rate of homologous recombination. Second, we have shown directly (Figure 2.2) that a *TER1* transformant carrying a single copy insert (the transformant shown in lane 4 of Figure 2.2A-D) will exist stably for at least 100 cell divisions without expanding into a long tandem array (data not shown).

As seen in Figure 4, transformants of the 1.6 kb circle that grew on plates lacking uracil were heterogeneous in size compared to ARS plasmid controls. Some colony size heterogeneity is characteristic of integrative transformation in general for *K. lactis*. In some organisms, telomeres are known to be able to transcriptionally silence nearby genes (1, 17, 41) and we cannot rule out the possibly that some degree of telomeric silencing might occur in our 1.6 kb circle transformants. However, the 1.6 kb circle transformants remain *Ura*⁺ over at least 100 generations of non-selective growth (data not shown), suggesting that silencing is either uncommon or insufficient to render cells *Ura*⁻. Also, telomeric silencing failed to be observed when a single copy of the same *URA3*-gene fragment used in the 1.6 kb circles was placed within ~120 bp of a *K. lactis* telomere (33).

Although both *TER1* and *ter1-Δ* cells have the ability to greatly extend their telomeres when transformed with a DNA circle containing telomeric repeats, there are notable differences in the behavior of the two strains that likely reflect the differences between normal telomeres and dysfunctional short telomeres. While *TER1* circle transformants have only one or two telomeres with tandem arrays of the *URA3*-telomere sequence, *ter1-Δ* transformants typically have multiple telomeres with tandem arrays. This is almost certainly due to the highly recombinogenic nature of short telomeres and
their propensity to spread a sequence from one telomere to other telomeres. Consistent with this, atypical ter1-Δ transformants, initially containing few telomeres with tandem arrays, have a much greater proportion of their telomeres with arrays after an additional passaging of ~125 cell divisions, even in the absence of selection for URA3 (data not shown). A second difference between TER1 and ter1-Δ circle transformants is that only the latter contain detectable extrachromosomal species derived from the URA3-telomere sequence. The mechanism(s) by which these form is not known and could potentially be different than the formation of tiny telomeric circles. A third difference concerns the size heterogeneity of the tandem arrays at telomeres. While arrays in TER1 cells typically exist as a single band running at limit mobility in our gels, arrays in ter1-Δ are highly heterogeneous (1->10 copies of the 1.6 kb sequence). This, coupled with the abundance of extrachromosomal species derived from the 1.6 kb sequence in their cells, suggests that the tandem arrays of ter1-Δ transformants are prone to recombining at high rates. This would not be surprising as the terminal group of telomeric repeats in an array, which is likely responsible for all telomere function, remain both relatively short (data not shown) and are still subject to gradual sequence loss. We suggest that the telomeres of our circle transformants reach an equilibrium where recombination events that lengthen and spread the arrays are balanced by recombination events and perhaps other processes that shorten or delete them.

Rolling circle gene conversion could readily account for the patterns of repeats we observed in the telomeres of ter1-Δ survivors. If a small circle of DNA containing both wild type and Bcl repeats were used as a template for rolling circle gene conversion, it
would produce a repeating pattern of those two repeat types in the resulting elongated telomere, as observed in most of those survivors that retained any Bcl repeats. Although the major class of survivors we observed lacked Bcl repeats, this does not suggest the existence of a second mechanism of recombinational elongation. Rather, these survivors likely arose from copying circles of DNA composed solely of wild type repeats. As the senescing cells contained only wild type repeats in the more basal part of the telomere (as diagrammed in Figure 2.1A), it is not surprising that Bcl repeats could be completely lost in most cells prior to the formation of survivors with elongated telomeres.

The simplest form of our hypothesis would be for a short telomere to undergo elongation by direct strand invasion into a small circle of telomeric DNA followed by rolling circle DNA synthesis (Figure 2.6). However, it is conceivable that rolling circle synthesis and integration at a telomere could occur as separate steps. Rolling circle replication might be initiated extrachromosomally on a telomeric circle if the 3' end from a gap or broken telomeric fragment were available as a primer. Once an extrachromosomal tandem array was formed, strand invasion by a telomere followed by gene conversion could lead to the array becoming incorporated at a chromosome end.

Our data would suggest that circles as small as 100 bp can serve as templates for rolling circle DNA synthesis. Although we have thus far been unable to identify small telomeric circles in K. lactis ter1-Δ cells (unpublished data), there is precedent for DNA circles composed of telomeric repeats being present in some mammalian cells (45) and circles as small as ~100 bp are formed in vivo from the unusual mitochondrial telomeres of the yeast Candida salmanticensis (52). There is also precedent in vitro for circles as small as 34 nucleotides serving as templates for rolling circle DNA synthesis (16). How
tiny telomeric circles might form in senescing *ter1*-Δ cells is not clear but they could easily be imagined to be an occasional byproduct of the very high rates of subtelomeric and telomeric recombination that occur when telomeres become very short (33). Recombination between repeats of a single telomere or annealing between broken single stranded fragments of telomeric DNA would each, in principle, be able to form circles containing whole numbers of telomeric repeats. Shortening of telomeres through intratelomeric recombination has been documented to sometimes occur in *S. cerevisiae* (11). If a small circle of telomeric repeats was produced by this process in a *ter1*-Δ strain, it conceivably could immediately be utilized by that telomere for rolling circle gene conversion. We suggest that a limiting factor for recombinational telomere elongation may be the formation of telomeric circles and not the ability to utilize circles for telomeric elongation. Our results with transforming artificially created circles indicate that both *TER1* and *ter1*-Δ cells can effectively utilize DNA circles to lengthen their telomeres.

We cannot rule out the possibility that the telomere elongation in some or all *ter1*-Δ post-senescence survivors arises through unequal recombination between telomeres rather than rolling circle gene conversion. However, it is more difficult to imagine how unequal recombination could generate the long telomere sizes or the repeating patterns common to most telomeres that are seen in survivors. Irregularities in the patterns of some recombinationally elongated telomeres (Figure 2.1B, clones 1b, 3c, 12a) indicate that telomeric repeat arrays at chromosome ends can be altered by recombination events other than those that copy DNA circles.
A notable difference between the URA3-telomere circle transformants and ter1-Δ post-senescence survivors is the much lower degree of telomere elongation in the latter (typically hundreds of base pairs and not more than a few thousand bp). This might reflect a markedly lower degree of processivity for a DNA polymerase copying a very small circle. It could easily be imagined that a 100 bp circle would not be large enough for the proper assembly of a fully processive DNA polymerase holoenzyme.

Post-senescence survivors in S. cerevisiae are of two types. Type 2 survivors have elongated telomeric arrays very similar to the survivors in K. lactis (51). Type I survivors, in contrast, have long alternating arrays of telomeric repeats and subtelomeric Y' elements (26), a pattern very reminiscent of the arrays of URA3 and telomeric repeats seen in K. lactis cells transformed with the 1.6 kb circle. The different structures of Type I and Type II survivors, combined with differences in the genes required for them (12, 23) indicate that there are mechanistic differences in how they arise. It will be of interest to determine whether the K. lactis circle transformants resemble Type I transformants in other ways.

DNA ends lacking telomeric repeats in yeast are processed to generate 3' overhangs which can then strand invade homologous duplex DNA to initiate a localized gene conversion or establish a replication fork that can sometimes copy sequence all the way to the end of the donor chromosome (8). The latter process, termed break-induced replication (BIR), could be the mechanism by which our 1.6 kb circles are copied to produce highly elongated telomeres. In S. cerevisiae, BIR appears to be highly RAD52-dependent (28). It is noteworthy then that telomere elongation promoted by DNA circles in K. lactis is partially RAD52-independent. This could mean it occurs by a different
mechanism or it may simply be due to differences in the experimental systems. Disruption of RAD52 function is known to greatly reduce but not completely eliminate telomerase-independent telomere elongation in K. lactis ter1-Δ cells (31).

Our experiments have shown that DNA circles containing telomeric repeats are potent triggers of recombinational telomere elongation. The use of endogenously formed circular DNA molecules as templates to extend telomeric ends could potentially explain other examples of telomerase-independent telomere elongation. As mentioned, Type II survivors of S. cerevisiae cells lacking telomerase elongate telomeres in a manner essentially identical to that seen in K. lactis ter1-Δ survivors and Type I survivors amplify alternating Y' elements and telomeric sequences. DNA circles composed of Y' and telomeric repeats have been reported to exist in S. cerevisiae (21). Mammalian cells that are ALT+ (and believed to maintain telomeres by recombination) often build very long telomeres in the absence of telomerase (10). In one reported example, the amplified DNA appeared to be a tandem array of telomeric and non-telomeric sequences (40). It will be of great interest to determine whether such telomeric circles underlie the ALT phenomenon of mammalian cells.
Acknowledgements

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References


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Figure 2.1. Repeating structure within telomeres of ter1-Δ survivors. (A) Strategy for construction of ter1-Δ strains with two types of telomeric repeats.

The ter1-Δ strain with wild-type telomeric repeats at the base and Bcl telomeric repeats at the tips was derived by forming a ter1-Δ/TER1-Bcl heteroallele. Plating on 5-fluoro orotic acid selected for cells that had “looped out” either the TER1-Bcl allele or the ter1-Δ allele. Clones containing only the ter1-Δ allele were identified by their senescent rough colony phenotype. (B) Classes of outcomes observed in post-senescence survivors derived from ter1-Δ strains with wild-type and Bcl repeats. Light gray boxes indicate wild type repeats; dark gray boxes indicate Bcl repeats and white boxes indicate partial repeats not distinguishable as either wild type or Bcl. Thin lines represent subtelomeric sequence. Telomere structures shown for outcomes 1 and 2 are approximate; based upon BcI restriction digestion and not sequencing. Telomere structures for outcome 3 are cloned and sequenced examples of recombinationally elongated telomeres from three independent post-senescence survivors. There is no sequence variation from wild-type telomeric repeats in the cloned telomeres except for the position that is expected to create a BcI restriction site. (C) Southern blot, hybridized with a telomeric probe, of a senescent ter1-Δ strain (Δ) and eleven post-senescence survivors derived from it that retained Bcl repeats (1-11). Pairs of lanes show DNA from individual survivors digested with EcoRI and EcoRI - BcI. Among the twelve telomeres, subtelomeric EcoRI sites are present ~1-3.5 kb from telomeric ends. The double digest yields blocks of wild-type repeats. Lower panel shows these blocks resolved on a 4% NuSieve agarose gel. Size markers for both panels are indicated. Cloned telomeres in panel B (1a,1b,1c and 3a, 3b, 3c) came from
clones #1 and #3 respectively of panel C. Cloned telomere 12a of panel B was isolated from a cloned not shown in panel C.
**A**

- senescing ter1-∆
- ter1-∆ + TERT-Bcl
- loss of Bcl repeats
- senescing ter1-∆
- ter1-∆ survivors

**B**

**Classes of ter1-∆ survivors**

1. No retention of Bcl repeats
2. Only basal Bcl repeats
3. Repeating patterns

**C**

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kb

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Figure 2.2. Long tandem arrays formed at telomeres after transformation with a DNA circle containing *URA3* and telomeric repeats.

(A) Southern blot, hybridized with a subtelomeric probe, of EcoRI-digested DNA from wild type *TER1* (WT) and *ter1-*Δ*(Δ)* strains untransformed (lanes 1-2) and after transformation (lanes 3-6) with a 1.6 kb *URA3*-telomere circle. (B) Same filter as ‘A’ after stripping and reprobing with a *URA3* probe. (C) Southern blot, hybridized to a telomeric probe, of uncut genomic DNA from *TER1* (WT) and *ter1-*Δ*(Δ)* strains transformed with the 1.6 kb *URA3*-telomere circle. Transformants shown are the same as those shown in ‘A’ and ‘B’. (D) Southern blot, hybridized with a *URA3* probe, of EcoRV-digested from wild type *TER1* (WT) and *ter1-*Δ*(Δ)* strains untransformed (lanes 1-2) and after transformation (lanes 3-6) with a 1.6 kb *URA3*-telomere circle. (E) Diagram of 1.6 kb *URA3* - telomere circle transformation and structures of single and multiple tandem inserts and a telomere. Gray boxes indicate blocks of telomeric repeats, black boxes indicate *URA3*, white boxes indicate a short subtelomeric sequence present on DNA circles and stippled boxes indicate subtelomeric sequence used as probe in panel A and not present on the circles. Subtelomeric EcoRI sites are 1-3.5 kb from telomeric ends in untransformed *K. lactis* cells. Positions of EcoRI sites(RI) and EcoRV (RV) sites are indicated. Abbreviations: J, centromere-proximal junction fragment; T, telomeric end fragment.
Figure 2.3. Transformations with two species of circle produces arrays derived from only one species.

(A) Diagram of telomere structures before and after introduction of a mixture of two species of 1.6 kb *URA3* - telomere circles that differ only by a single restriction site. Grey and black boxes are blocks of telomeric repeats and *URA3*, respectively. White boxes are subtelomeric sequence present on DNA circles and stippled boxes are subtelomeric sequence not present on the circles. R, S, and P indicate sites for *Eco*RI, *Sal*I, and *Pvu*I, respectively. S/P indicates sites that will either be *Sal*I or *Pvu*I depending upon which circle the site is derived from. J and T indicate the junction fragment with subtelomeric sequences and the terminal telomeric fragment, respectively. (B) Southern blots of representative clones of *TER1* (top) and *ter1-Δ* (bottom) transformed with either circle S or circle P are shown hybridized with subtelomere, *URA3*, or telomeric probe, as indicated. Untransformed control is shown digested with *Eco*RI and transformants are shown digested with *Eco*RI (-), *Eco*RI - *Sal*I (S) or *Eco*RI - *Pvu*I (P). The type of transforming circle used is indicated on top. Faint band at 2.2 kb in *URA3*-probed lanes containing the dark 1.6 kb fragment are trace partials left over from cleaving the tandem arrays. Positions of molecular weight markers (in kilobase-pairs) are indicated. (C) Southern blots of two representative clones each of *TER1* (top) and *ter1-Δ* (bottom) transformed with both circle S or circle P are shown hybridized with subtelomere, *URA3*, or telomeric probe, as indicated. The two clones represent one example each of clones exhibiting tandem arrays of either a S version or a P version. Digests were done as for B. Junction fragments (J) are marked with arrows in the *URA3* panels of 2B and 2C.
Figure 2.4. *URA3*-telomere circle transformation into *rad52* and *RAD52* strains.

Photographs show sections of plates with Ura\textsuperscript{+} transformants. Equal amounts of both the *URA3*-telomere circle and the autonomously replicating ARS plasmid control were used for both strains.
No DNA  ARS plasmid  URA3+Tel. circle

RAD52

rad52

2mm
Figure 2.5. Long tandem arrays at telomeres can be formed in a \textit{TER1 rad52} strain transformed with a \textit{URA3}-telomere circle.

Southern blot of untransformed (C) and three \textit{rad52} clones transformed with a \textit{URA3}-telomere circle. The untransformed control is shown digested with \textit{EcoRI} and the transformed clones are shown digested with \textit{EcoRI} and \textit{EcoRI - PvuI}, as indicated. The same filter is shown hybridized with subtelomeric, \textit{URA3} and telomeric probes. Faint bands at 1.6 kb in \textit{EcoRI - PvuI} digested samples hybridized with the subtelomeric sequence are residual signal from prior \textit{URA3} hybridization. Size markers (in kb) are shown at left.
**Figure 2.6. The “roll and spread” model.**

The formation of one long telomere is postulated to occur via a rolling circle gene conversion, copying either the 1.6 kb circle (circle transformants) or a very small telomeric circle (ter1-Δ survivors). Inset box depicts telomeric end processed to have a 3' single strand overhang (shaded thin boxes) that strand invade a telomeric circle. In ter1-Δ cells, the very high rate of telomeric gene conversion can spread sequence from one long telomere onto many or all other telomeres of the cell under selective pressure for post-senescence survivors. The net result is that a common pattern is present in most or all of the elongated telomeres.
ter1Δ mutant with 2 repeat types

Gene conversion using telomeric circle as template

Genes conversion using elongated telomere as template
CHAPTER 3

FACTORS INFLUENCING THE RECOMBINATIONAL EXPANSION AND SPREAD OF TELOMERIC TANDEM ARRAYS IN *Kluyveromyces lactis* ²

²Natarajan, S. and M.J. McEachern. To be submitted to *Molecular and Cellular Biology.*
Abstract

We have previously shown that DNA circles containing telomeric repeats and a marker gene can promote the recombinational elongation of telomeres in *K. lactis* by a mechanism proposed to involve rolling circle DNA synthesis. Wild-type cells acquire a long tandem array at a single telomere, while telomerase deletion (*ter1-Δ*) cells, acquire an array and also spread it to multiple telomeres. In this study, we further examine the factors that affect formation and spread of telomeric tandem arrays. We show that a telomerase+ strain with short telomeres can efficiently form and spread arrays, while a telomere fusion mutant is not efficient at either process. Surprisingly, telomeric repeats are frequently deleted from a transforming *URA3*-telomere circle at or prior to the time of array formation by a mechanism dependent upon the presence of subtelomeric DNA in the circle. We further show that in a *ter1-Δ* strain, long tandem arrays can arise from telomeres initially containing a single-copy insert of the *URA3*-telomere sequence. However, the reduced rate of array formation in such strains suggests that single-copy inserts are not typical intermediates in arrays formed from *URA3*-telomere circles. Using heteroduplex circles, we have demonstrated that either strand of a *URA3*-telomere circle can be utilized to form telomeric tandem arrays. Consistent with this, we demonstrate that 100-nucleotide single-stranded telomeric circles of either strand can promote recombinational telomere elongation. Putative single-stranded telomeric circles of about this size were identified in *ter1-16T*, a long telomere mutant that exhibits telomere-capping defects similar to *ter1-Δ*. 
Introduction

Research on mechanisms of recombinational telomere maintenance has gained increasing significance since telomerase-independent telomere maintenance has been recognized in ~10% of cancers. The telomerase-independent pathway in mammalian cells has been called “alternate lengthening of telomeres” or ALT (1). The mechanism of ALT appears to involve events mediated by homologous recombination as in yeast (4, 17, 21). Studies in yeast have shown that there are two types of survivors that form by different pathways when the telomerase-mediated pathway for telomere maintenance is not functional (3, 12, 25, 26). Type I survivors have subtelomeric expansions and require RAD51; and Type II survivors have telomeric expansions and require RAD50 and SGS1 (3, 13, 25, 26). Type II survivors of *S. cerevisiae* sometimes produce abrupt changes in telomere length that seem to occur in a very short span of time and may occur by a single-step process (11, 25). The single-step process may involve the formation and utilization of extrachromosomal telomeric circles by a mechanism like rolling circle replication. Invasion of internal telomeric tracts by single-stranded telomeric tails has been proposed as a mechanism for rapid telomeric deletions in *S. cerevisiae* (2). Extrachromosomal telomeric circles may form during the process of rapid telomeric deletions. Circular DNA composed of telomeric repeats that may act as substrates for telomere elongation by rolling replication have been found in some human cells (22). The formation of tandem arrays of telomeric and non-telomeric sequences in mammalian cells has also been reported (19). In *Kluyveromyces lactis*, subtelomeric recombination is enhanced in strains that lack a fully functional telomerase by as much as 1000-fold (15).
It has been proposed that subtelomeric recombination can lead to the spread of telomeric sequences among chromosome ends by a mechanism like break-induced replication.

We have previously shown that *K. lactis* cells provided with a circle containing a marker gene and telomeric repeats will generate telomeres with long tandem arrays of DNA derived from the transforming circle (18). The formation or utilization of the circle may be dependent on homologous recombination. We proposed that once a telomere has expanded by rolling circle gene conversion, the expanded telomeric sequence can be spread to other chromosomal ends by other gene conversion events. In this work, we have analyzed the process of circle utilization to gain better understanding of the process. We have shown that the telomeric structure of the host and the circular template have certain requirements to efficiently promote the formation of tandem telomeric arrays.

**Materials and Methods**

**Strains**

The ter1-Taq, ter1-AccSna, msh2-Δ and ter1-Δ msh2-Δ strains are derivatives of haploid *K. lactis* 7B520 (Ura−, His−, Trp−) (27). The ter1-Taq, ter1-Δ and ter1-AccSna strains have been described previously (14, 15). The TER1/ter1-Δ msh2-Δ heteroallele strain was obtained from the lab of V. Lundblad (23). The ter1-Δ msh2-Δ and TER1 msh2-Δ strains were constructed by the loop-out of TER1 or ter1-Δ respectively from the TER/ter1-Δ msh2-Δ heteroallele strain as described before (14). ter1-16T was generated by the loop-in/loop-out procedure. The ter1 allele used for the loop-in had a point
mutation in the Rap1 binding site of the template region (D. Underwood and M. McEachern, unpublished).

*IUT-WT1* was generated when a single copy of circle S was incorporated at a telomere in a *TER1* strain that was transformed with circle S (18). To generate *IUT-Taq*, we mated *ter1-Taq* to *IUT-WT1*. However, the *TER1* and *ter1-Taq* haploid spores that were produced from this mating had single circle S inserts at 2 or 3 telomeres (data not shown), apparently because the insert had spread to other telomeres. This spreading occurred prior to the formation of ascospores as the *URA3* gene was detectable in 50-100% of spores instead of the expected 50%. The initial presence of short telomeres in the diploid that came from the *ter1-Taq* parent may have led to increased recombination events at the telomeres. We then used a *IUT-WT* haploid strain that had single copy circle S inserts at two telomeres to mate with a *ter1-Δ* strain to generate *IUT-Del*. Tetrad dissection of *TER1/ter1-Δ* diploids produced haploid spores of *ter1-Δ* and *TER1* that had single copy circle S inserts at 1, 2 or 3 telomeres (data not shown). Examples of these strains were then used to test whether the 1.6 kb circle S inserts could expand into long tandem arrays. As a control, the single copy circle S insert in the *TER1* strain was introduced into another *TER1* strain by mating to generate additional *IUT-WT* strains. Serial re-streaks of the strains generated was done by passaging a mixture of colonies on YPD agar.

**Mating and sporulation**

Matings were performed on malt extract agar for two days and diploids were selected on SD plates lacking uracil and histidine. Diploids were allowed to sporulate on
minimal sporulation medium (10% potassium acetate) for two days. Individual spores from tetrad dissection were allowed to grow on YPD for three days before further analyses.

**Southern hybridization and quantitation of URA3 copy number**

Hybridizations to telomeric, subtelomeric and URA3 probes and quantitation of URA3 copy numbers were done as described previously (18). A second subtelomeric fragment of 113 bp was used to determine if there were any subtelomeric deletions of the tandem arrays formed in circle N transformants. This 113 bp fragment was obtained as a BsrBI-HindIII fragment from the plasmid Stumaker II, which is a derivative of pBluescript containing telomeric and subtelomeric sequence from a chromosome end of K. lactis (15). The 113 bp subtelomeric fragment was hybridized at 50°C. Hybridization washes were done in 200 mM Na$_2$HPO$_4$ and 2% SDS for a total of 15 min. URA3-RAD52 hybridizations were done using a 2.2 kb DraI-BglII fragment from the plasmid p52ΔC:UB, a derivative of pBluescript containing the K. lactis RAD52 gene disrupted by URA3 from S. cerevisiae. Hybridization was done at 65°C. Hybridization washes were done in 100 mM Na$_2$HPO$_4$ and 2% SDS for a total of 45 min. URA3 copy numbers were determined using the PhosphorImager.

**Construction and isolation of the circle with no subtelomeric region (circle N), heteroduplex circles (circle H) and the 100 nt circle**

Circle with no subtelomeric sequence, Circle N, was isolated as described previously for circle S and circle P (18) by ligation of a gel purified BamHI-BglII
fragment. The plasmid construct used for generating circle N was pMH3-Tel-NoST, which lacked subtelomeric sequence. pMH3-Tel-NoST was created by introducing an \textit{XhoI-BsrBI} telomeric fragment from pMH3-Tel (18) into pMH3 (\textit{XhoI-NruI} site), which resulted in a telomeric insert present in the opposite orientation as compared to what was originally present in pMH3-Tel. All plasmids were maintained in \textit{E. coli} XL1.

Heteroduplex circle, Circle H, was made using a construct derived from pMH3-2UraTel that had two tandem copies of the \textit{BamHI-BglII} 1.6 kb \textit{URA3}-telomere sequence (Figure 3.7A). This was constructed by introducing a second copy of the \textit{BamHI-BglII} fragment containing the \textit{URA3}-telomere sequence into the \textit{BglII} site of pMH3-Tel. Two fragments were gel purified from this plasmid; a 1.6 kb \textit{SalI} fragment that had the entire length of the \textit{URA3}-telomere sequence and an \textit{XmaI-SacI} fragment contained all of the 1.6 kb \textit{URA3}-telomere sequence except for a 70 bp region at the end of the \textit{URA3} gene that contained a \textit{BsiEI} site. Two oligomers that spanned the 70 bp region were made as complementary strands that could fill the gap in the \textit{XmaI-SacI} fragment. One of the two oligomers, which corresponded to the strand that contained the G-rich telomeric sequence, had two mismatched bases that created a \textit{SnaBI} site and eliminated the \textit{BsiEI} site. The other oligomer, which corresponded to the strand that contained the C-rich telomeric sequence, had two mismatched bases that created an \textit{AatII} site and destroyed the \textit{BsiEI} site. The \textit{SalI} and \textit{XmaI-SacI} fragments were mixed in equal proportions, boiled for 5 min and allowed to anneal by slow cooling (about 1 hr) to 25°C in the presence of the \textit{SnaBI} and \textit{AatII} containing oligomers to form two linear (the original \textit{SalI} and \textit{XmaI-SacI} fragments) and two nicked circular structures (one containing a \textit{SalI}-derived Watson strand and an \textit{XmaI-SacI}-derived Crick strand bridged by one oligomer and the other
containing a SalI-derived Crick strand and an XmaI-Sacl-derived Watson strand bridged by the other oligomer). The mixture was run on 1% agarose without ethidium bromide at 6 V/cm for 4 hr to separate the nicked circular forms from the linear DNA. The nicked circles were gel-purified and ligated in the presence of an excess of the two oligos that bridged the gap created by the XmaI-Sacl fragment to generate circle H. Thus, the circle H preparation contained two types of 1.6 kb circles with two-base mismatched heteroduplexes each, one that had a BsiEI-SnaBI mismatch (BsiEI on telomeric G-strand and SnaBI on the C-strand) and the other that had a BsiEI-AatII mismatch (BsiEI on telomeric C-strand and AatII on the G-strand).

A 100 nt oligomer composed of three 25 bp telomeric repeats flanked on either side by non-telomeric sequence ("A" on the 5' side and "B" on the 3' side) was synthesized. This oligomer was circularized by bringing the ends together with another 25 nt oligomer composed of sequence complementary to the non-telomeric sequence of the 100 nt oligomer (B'-A') in the presence of T4 DNA ligase (Also see Figure 3.8A). One hundred nt circles composed of either the C- or the G-rich telomeric strand were made in the same way. The G-strand 100 nt telomeric circle transformants were derived from circles composed of telomeric repeats that had a BclI restriction site. 250-300 ng of the 100 nt circle along with 0.5-1 µg of p1B3, an ARS-containing plasmid (27), was used for each transformation.

Two-dimensional gels to look for circular DNA of ~100 nt

15 cm x 15 cm 4% NuSieve 3:1 (ISC Bioexpress, Kaysville UT) agarose gels containing 0.6 µg/ml 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 hr. The gel was then stained in a solution of 3 µg/ml chloroquine for 5 hr. 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 hr. The gel was blotted onto Hybond N+ membranes (Amersham, Piscataway NJ) for at least 24 hr prior to hybridization.

Exonuclease sensitivity of potential single-stranded circles was determined by treating ~20 µg of genomic DNA with 0.1 units of \textit{E.coli} ExoI for 1 hr in the presence of 7 ng of a 66 nt non-telomeric linear oligo control. \textit{E.coli} ExoI digests single-stranded DNA and single-stranded regions of double-stranded DNA into single nucleotides. 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 ExoI 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 45°C for 4 hr. The presence or absence of telomeric DNA resistant to ExoI was detected by hybridization to a 25 nt C-rich sequence of \textit{K. lactis} telomeric DNA at 45°C for 4 hr.
Results and Discussion

Formation and spread of long tandem arrays in a telomerase$^+$ strain transformed with a URA3-telomere circle.

We previously reported that single molecules of a DNA circle containing URA3 and telomeric repeats could promote formation of tandem arrays at telomeres in both TER1 and ter1-∆ strains (18). Transformants of the TER1 strain typically had a tandem array at only a single telomere whereas transformants of the ter1-∆ strain invariably had tandem arrays at multiple telomeres. These results suggested that while both strains could form a tandem array from a URA3-telomere circle, only the ter1-∆ strain could spread the sequence from the initial array to other telomeres. We wanted to test if the URA3-telomere DNA circle could lead to both the formation and spread of tandem arrays in a telomerase$^+$ strain that is not prone to recombinational telomere elongation. To do this, we transformed circular DNA molecules containing telomeric repeats and URA3 into ter1-Taq, a strain which has been shown to have stably short telomeres, high rates of subtelomeric recombination, but no detectable tendency to elongate telomeres via recombination (15). The short telomere phenotype of ter1-Taq appears to be due to an aberrant translocation step that leads to telomerase synthesizing abnormally long 31 bp telomeric repeats (D. Underwood and M. McEachern, unpublished results). We introduced two types of 1.6 kb URA3-telomere circles (shown in Figure 3.1A) that differed at one restriction site (circle S, which had a SalI site and circle P, which had a PvuI site) either singly or as a mixture. Transformants that grew on medium lacking uracil were analyzed by Southern blotting. The top panel of Figure 4.1B shows one each of ter1-Taq transformants derived from circle S and circle P. The use of a subtelomeric
probe that hybridizes to 11 of the 12 telomeric EcoRI fragments showed that some telomeres in 32 of 32 examined transformants had become greatly elongated. When cleaved with either SalI or PvuI (2° digest), these extended telomeres were largely cleaved down to units near 1.6 kb in size that hybridized intensely to URA3 and telomeric probes. These data indicated that sequence from the URA3-telomere circles integrated at telomeres in ter1-Taq cells as long tandem arrays. When ter1-Taq was transformed with a mixture of circle S and circle P, almost all of the resulting transformants had arrays that were composed entirely of sequence derived from a single type of circle: 63 out of 64 ter1-Taq transformants had arrays that could be cleaved by either SalI or PvuI, not both (Figure 3.1B, bottom and data not shown). The single transformant that had arrays that could be cleaved by both SalI and PvuI is shown in the bottom panel of Figure 3.1 B (clone 1). This exceptional transformant aside, these data are consistent with our past results and indicate that all copies of the integrated 1.6 kb sequence present in a transformant are ultimately derived from a single circular URA3-telomere molecule.

Overall, the URA3-telomere circle transformants of ter1-Taq bore more resemblance to circle transformants of ter1-Δ than to those of TER1 cells. Most notably, ter1-Taq and ter1-Δ transformants had multiple telomeres that had acquired arrays. We estimated the number of telomeres that had elongated to form tandem arrays in a given transformant by quantitation of the percentage of subtelomeric hybridization signal above 3 kb in EcoRI-digested genomic DNA (Figure 3.2). 3 kb is larger than the size of the largest EcoRI telomeric fragment detected by the subtelomeric probe in DNA from untransformed cells and any subtelomeric signal above this size must be due to incorporation of sequence from the URA3-telomere circle. In TER1 transformants, only
one or at most two of the twelve telomeres had tandem arrays whereas in ter1-Taq and ter1-Δ transformants, typically, many of the twelve telomeres had arrays. The total number of integrated copies of the 1.6 kb sequence derived from the circle was measured by determining the hybridization intensities of URA3 relative to a single-copy gene control, as described previously (18). Among the 17 ter1-Taq transformants that were analyzed, we estimated that the range of URA3 copy number varied between 16-145. This is similar to the 30-180 copies seen earlier in ter1-Δ transformants. Another similarity of ter1-Taq circle transformants to those of ter1-Δ is the presence of extrachromosomal ladders of bands that may correspond to linear and circular forms of the URA3-telomere sequence that have excised out of the array (data not shown). Such extrachromosomal species were not detected in TER1 transformants (18).

Thus, we have shown that transformation with URA3-telomere circles can lead to both formation of long tandem arrays and the spreading of the arrays to multiple telomeres in ter1-Taq, a telomerase+ strain with stably short telomeres. We conclude that the efficient spread of tandem arrays to multiple telomeres requires neither senescence nor the tendency to undergo recombinational telomere elongation in the absence of exogenously added telomeric circles. However, spreading does appear to require elevated recombination rates near telomeres.

The URA3-telomere circle frequently deletes telomeric repeats prior to formation of tandem arrays.
When either circle S or circle P was transformed into *K. lactis* cells (*TER1*, *ter1-Taq* or *ter1-Δ*) the resulting tandem arrays were often seen to be composed of unit copies that were slightly smaller than 1.6 kb when digested with *SalI* or *PvuI*, respectively, and probed with *URA3* (data not shown). We analyzed circle S and circle P transformants further to determine the location of the deleted sequence. To do this, DNA from randomly selected circle S and circle P transformants was digested with *XhoI* and *PstI*, which cleaved the *URA3*-telomere unit into three fragments: two *URA3* fragments (of expected sizes of ~1.0 kb and 0.2 kb) and one telomeric fragment (of expected size ~0.4 kb). The top panels of Figure 3.3A show hybridization to a telomeric probe of *XhoI*-*PstI*-digested circle S and circle P transformants of *TER1*, *ter1-Taq* and *ter1-Δ* strains and a control digestion of the plasmids from which circle S and P were derived. The bottom panels of Figure 3.3A show hybridizations to a *URA3* probe of the same filters as in the top panels. The sizes of the two *URA3* fragments are constant (~1.0 kb and 0.2 kb) in all examined transformants from each of the three strains indicating that there are no deletions in the *URA3* regions of the arrays. In contrast, the telomeric fragment (~0.4 kb) was observed to be variable in size between transformants, particularly in *ter1-Taq* and *ter1-Δ* strains. This showed that the deletions in the *URA3*-telomere units were in the region of the circle derived from the cloned *K. lactis* telomere. This telomeric region contained 113 bp of subtelomeric DNA in addition to the tract of telomeric repeats. The restriction enzyme *BsrBI* recognizes a site just 3 bp from the border of subtelomeric and telomeric sequences and separation of the two regions by *BsrBI* cleavage showed that all deletions mapped within the telomeric repeats.
The presence of subtelomeric sequence in circle S and circle P might influence strand invasion by a native telomeric sequence into either circle. We therefore determined that this short subtelomeric sequence was required for the deletions to occur. This was done by first constructing a circle with no subtelomeric homology (circle N). To first confirm that circle N could promote the formation of tandem arrays, it was introduced into \textit{TER1}, \textit{ter1-Taq} and \textit{ter1-Δ} strains and DNA from the transformants was examined. As shown in Figure 3.4, the lack of subtelomeric sequences did not hamper the formation of tandem arrays in any strain. The arrays that formed had largely the same features as the ones transformed from circle S and circle P in \textit{TER1}, \textit{ter1-Δ} and \textit{ter1-Taq} strains (Figure 3.1 and 18). Intense 1.6 kb bands were observed in \textit{URA3} and telomeric hybridizations when the arrays were cut with a restriction enzyme that cuts once in each unit of the array. Additionally, multiple telomeres had arrays in \textit{ter1-Taq} and \textit{ter1-Δ} transformants while \textit{TER1} transformants had arrays at only one or two telomeres.

In circle N transformants, deletions within the \(~1.6\) kb band were greatly reduced. Among seventeen clones each of \textit{TER1}, \textit{ter1-Taq} and \textit{ter1-Δ}, Southern analysis showed that only one clone of each had a deletion in the telomeric part of the array (\textit{SmaI-PstI} digests and hybridizations shown in Figure 3.3B). These results suggest that the presence of subtelomeric sequence in circle S/P actively promotes telomeric repeat deletions within the circle prior to or at the time of formation of a tandem array at a telomere. Although we cannot rule out the possibility that the subtelomeric sequence is a hotspot for recombination, we suggest that it acts by complicating the possibilities for strand invasion by a native \textit{K. lactis} telomere. Since the telomeres are composed of perfect
tandem repeats, strand invasion of a short telomere at a native chromosome end could occur in multiple different registers into a DNA circle containing multiple telomeric repeats (Figure 3.4C). If the DNA circle also contained adjacent subtelomeric sequence, continued strand annealing extended into that sequence could in principle result in a single stranded loop of telomeric repeats being extruded from the middle of the annealed DNA. If this loop was excised and the break resealed by ligation, the resulting DNA circle would contain fewer telomeric repeats. Incorporation of the sequence from the deleted circle by a rolling circle mechanism would then result in a tandem array with all units containing the deletion.

**The formation of long tandem arrays is greatly reduced in a mutant with fused telomeres.**

We predicted that the absence of free telomeric ends in a *ter1* mutant that had circular chromosomes due to telomere fusions might prevent *URA3*-telomere circles from forming tandem arrays. The *ter1-AccSna* mutant has two substitutions in the template region of the telomerase RNA gene that result in telomeric repeats with a disrupted Rap1 binding site. Early passages of this mutant have short telomeres, but within about 20 restreaks (400-500 cell generations), most or all of the telomeres have undergone fusions, resulting in the apparent circularization of the six chromosomes (16). These fusions retain telomeric repeats that could serve as targets for homologous recombination with a *URA3*-telomere circle. Circle S and an ARS plasmid control were introduced into *ter1-AccSna* cells by transformation. By normalizing transformation frequencies using the ARS plasmid, we found that transformation of circle S into *ter1-AccSna* was reduced by about
20-fold relative to a *TER1* strain (Table 3.1). It was concluded that fused telomeres cannot efficiently integrate sequence from the *URA3*-telomere circle.

We then analyzed the telomeric structure of the *ter1-AccSna* transformants by Southern analysis (Figure 3.5B). Six of ten transformants had hybridization patterns inconsistent with either tandem arrays or multi-copy integration and were not studied further. One of these is shown as clone 2 of Figure 3.5. The other four transformants (including clone 1 of Figure 3.5) had patterns consistent with telomeric tandem arrays. *EcoRI* digestion produced a band running at limit mobility that hybridized to subtelomeric, *URA3* and telomeric probes, while *SalI* digestion cut this band down to 1.6 kb units. In each of these four transformants, we found one of the six original chromosomal fusion bands to be shifted up to limit mobility indicating that the occasional tandem array that forms does not spread to other chromosome ends. Tandem arrays may be present at one or both ends of the chromosome. We did not determine how many telomeres were elongated or if the elongated telomeres formed chromosome fusions.

How tandem arrays can sometimes form in *ter1-AccSna* cells is not clear. Some free telomeric ends may be present in populations of *ter1-AccSna* cells. Although the telomere fusions in *ter1-AccSna* cells are fairly stable, they may be subject to occasional resolution to free telomeres, as is thought to occur in other *ter1* template mutants that lead to fusions (16). The formation of a tandem array may therefore occur due to the occasional presence of these free telomeric ends. Alternatively, the process of integration of circle S at the telomeric repeats within a fusion may have led to DNA strand breaks that resolved the fusion and thereby permit array formation. Even if tandem arrays initially formed at a non-fused telomere, the presence of the Ter1-*AccSna* telomerase
would lead to dysfunctional telomeric repeats being added on to the ends, which in turn would be expected to lead to a new telomere fusion.

**Testing the ability of a single 1.6 kb URA3-telomere insert to expand into a long tandem array.**

A prediction of the rolling circle replication model is that a long tandem array can arise in a single step. In principle however, long tandem arrays could arise from multiple unequal recombination events (Figure 3.6B) starting from just a single-copy insert of the URA3-telomere sequence. We therefore wanted to see if a single-copy URA3-telomere insert present at one or more telomeres could lead to the formation of tandem arrays in host cells carrying different ter1 alleles. Among the circle S TER1 transformants that we initially obtained (18), we had one transformant that had a single-copy of circle S integrated at a telomere. The existence of this clone indicated that expansion of a single insert into a long tandem arrays was not an automatic event in a TER1 strain.

To more thoroughly examine the potential ability of the single-copy URA3-telomere insert to expand into a long tandem array, we generated haploid TER1, ter1-Taq and ter1-Δ strains (called IUT-WT, IUT-Taq and IUT-Del respectively), carrying one or more telomeres with the single insert and followed them over multiple streaks of growth (see Materials and Methods for details of strain construction). Results from following the IUT-WT, IUT-Taq and IUT-Del strains over five consecutive streaks (100-125 cell divisions) are shown in Figure 3.6A. The top panel shows hybridization to a URA3 probe of two clones each of IUT-Taq and IUT-Del as well as a IUT-WT control. The bottom panel shows hybridization of the same filters to a telomeric probe. As seen previously,
the *IUT-WT* strain maintained the fragment with the single-copy circle S insert at its original size (2.6 kb) with no sign of hybridization signal at higher positions in the gel. Moreover, the intensity of the 2.6 kb *URA3* band did not change over serial restreaks, which indicated that there was no obvious duplication of the *URA3* band through spreading to other telomeres. In contrast, *IUT-Taq* and *IUT-Del* strains were able to expand the single circle S inserts into long tandem arrays. In 14 out of 19 *IUT-Taq* clones and each of 6 *IUT-Del* clones that we analyzed, tandem arrays derived from *URA3*-telomere sequence formed by the 4th re-streak. In the 2nd and 3rd re-streaks of the strains shown, although long arrays are often detectable, most of the *URA3* signal is at ~2.6 kb, the position of the single-copy band.

This indicates that the *URA3*-telomere signal remained at its original size in most cells in the examined population. Only at the ~4th streak did arrays appear to be present in the bulk of the cells of the population. In many *IUT-Taq* clones the additional *URA3*-hybridizing bands acquired after passaging the clones were of sizes that were consistent with being dimers or trimers of the 1.6 kb insert. This suggests that unequal crossing-over as diagrammed in Figure 3.6B may be occurring. In some *IUT-Taq* and most *IUT-Del* clones, many additional *URA3*-hybridizing bands migrating at high molecular weights were acquired after serial passaging. These probably represent telomeres with multiple *URA3*-telomere inserts and excised ladders of extrachromosomal *URA3*-telomere sequence. Such species have previously shown to be abundant in *ter1-A* cells containing long tandem *URA3*-telomere arrays (18).

Our results indicate that a single *URA3*-telomere insert at a telomere is not capable of expanding into a long tandem array in *TER1* cells. This rules out unequal
crossover (Figure 3.6B) as a possible mechanism for the formation of long tandem arrays by \textit{URA3}-telomere circles in a strain with normal telomeres. Although long tandem arrays can form from single \textit{URA3}-telomere inserts in \textit{ter1-Taq} and \textit{ter1-\Delta} cells, the kinetics of their formation appear inadequate to account for the near immediate formation and spread of long tandem arrays in cells of the same mutant strains that have been transformed with a \textit{URA3}-telomere circle. We conclude that single \textit{URA3}-telomere inserts are unlikely to be common intermediates to long tandem array formation in \textit{URA3}-telomere circle transformants of \textit{ter1-Taq} and \textit{ter1-\Delta} cells.

Multiple unequal crossover events could be one way by which telomeres could expand a single copy insert of circle S to tandem copies. Alternatively, the expansion at telomeres could also be triggered by circular \textit{URA3}-telomere DNA molecules that “loop out” from the single-insert molecule (Figure 3.6B). We suggest that a combination of unequal crossover and rolling circle gene conversion might contribute to the formation of tandem arrays in strains with highly recombinogenic telomeres that initially have only single-copy \textit{URA3}-telomere inserts.

\textbf{Either strand of a \textit{URA3}-telomere circle can be used for the formation of tandem arrays.}

An important question about the long tandem array formation promoted by the \textit{URA3}-telomere circle is whether there is a strand-bias in the utilization of a circular template to form tandem arrays at telomeres. The presence of single-stranded G-rich telomeric tails at chromosomal 3’ ends (10) suggested that the C-rich strand of a circular telomeric template might be exclusively used to template the formation of tandem arrays.
In order to differentiate between the use of the C-rich and the G-rich telomeric strands of a URA3-telomere circle, we assembled two heteroduplex circles (circle H1 and H2; collectively referred to as circle H), as shown in Figure 3.7A. The heteroduplex circles had the same sequence as circle S except for two-base mismatches that eliminate a BsiEI site and create unique restriction enzyme sites (SnaBI on the telomeric C-strand of circle H1 and AatII on the telomeric G-strand of circle H2). The preparation of a mixture of these two heteroduplex circles was introduced by transformation into ter1-Δ msh2-Δ cells and TER1 msh2-Δ cells. Strains deficient in mismatch repair were used so that the mismatches in the heteroduplex circles would not be repaired.

Heteroduplex DNA could in principle produce sectored transformants with different sectors having sequence information derived from different strands. Care was therefore taken to maximize the chances of detecting sectored transformants by using entire colonies from the transformation plates to inoculate cultures for DNA preparations. As before, genomic DNA from transformants was examined by Southern blotting. Digestion with EcoRI was used to visualize telomeric fragments containing intact tandem arrays. Secondary digests that included BsiEI, SnaBI or AatII were used to see which enzyme could cut the telomeric arrays down to units of 1.6 kb. Southern analysis of DNA from circle H transformants showed that, as expected, the great majority produced hybridization patterns consistent with formation of tandem arrays. Three examples of ter1-Δ msh2-Δ transformants are shown in Figure 3.7B.

Table 3.2 shows the distribution of the restriction sites present in the arrays of the ter1-Δ msh2-Δ and TER1 msh2-Δ transformants that were analyzed. Greater than half of
all transformants in both strains (39/67 *ter1-Δ msh2-Δ* and 28/48 *TER1 msh2-Δ*) were found to have arrays containing only a single type of the three restriction sites. This indicates that utilization of sequence from only one strand of circle H was the most common means of generating the telomeric tandem arrays. Many transformants, however, had arrays that contained more than one of the three types of restriction sites (one example is shown in Fig. 3.7B). In some cases *SnaBI* and *AatII* sites were present together (13/67 *ter1-Δ msh2-Δ* and 3/48 *TER1 msh2-Δ* transformants). This indicated that arrays had been derived from at least two transforming molecules. In other cases, when *BsiEI* sites were present together with one other type of site, arrays may have been derived either from two different molecules or from two strands of the same molecule. Multiple subclones of five *ter1-Δ msh2-Δ* transformants of this type contained the same restriction site profiles as the parental clone. This suggests that the original transformants were not mixtures of two kinds of cells that each contained a single type of restriction site (as might have occurred from segregation of the *URA3*-telomere heteroduplex). Arrays in a few transformants were not cleaved by any of the three enzymes. These may have resulted from errors in the oligonucleotides that were used in the construction of circle H.

The most striking result from our data was that *SnaBI* sites and *AatII* sites were present in arrays with equal frequencies. This was true in both *ter1-Δ* and *TER1* strains. Combining results from both recipient strains, 36 of 115 transformants had arrays with at least some *SnaBI* sites and 37 of 115 had arrays with at least some *AatII* sites. If the C-rich telomeric strand of *URA3*-telomere circles was the only strand used as the template to generate the arrays, *AatII* sites would not be present at all in the transformants. We
conclude that array formation can arise with roughly equal frequency from copying either
the C-rich or the G-rich strand of a transforming URA3-telomere circle. With that
assumption, it would be expected that transformants would occur in a 2:1:1 ratio
containing arrays with BsiEI, SnaBI, and AatII sites, respectively. However,
transformants containing arrays with only BsiEI sites were distinctly more abundant than
those containing only one of the other enzyme sites. This may have arisen from lingering
discontinuities in the strands of circle H that included annealed oligonucleotides.

The relatively high percentage of transformants with arrays containing more than
one type of restriction site was somewhat surprising. We suspect that this was largely or
entirely due to cells that took up more than one heteroduplex molecule. Although
experiments with circle S and circle P (this work and 18) indicated that incorporation of
sequence from two molecules was rare, the circle H transformations were carried out
using a greater amount of DNA. That clearly would have increased the likelihood of
cells taking up more than one URA3-telomere circle.

How the 1.6 kb URA3-telomere circle led to the generation of tandem arrays at
telomeres is unclear. The fact that introduction of the URA3-telomere circle into TER1
cells typically produces a tandem array at only a single telomere suggests that array
formation occurs in a single step. Conceivably, an additional step might be used to
integrate the array at a telomere. Array formation has been proposed to be due to some
type of rolling circle replication (18), a possibility that raises many interesting questions.
One such question is, what primes the replication? Our data suggests that a mechanism
exists for priming DNA synthesis on either strand of circle H. Strand invasion of a
telomeric 3’ end into a URA3-telomere circle is expected to be able to prime replication of
the telomeric C-strand. Processing of recombination intermediates could also routinely generate means of priming replication of the telomeric G-strand. Alternatively, the displaced telomeric sequence of G-strand of the *URA3*-telomere circle might be able to recruit a DNA polymerase. G-strand telomeric DNA provides binding sites for the telomere binding protein Cdc13, which is known to be able to bind to DNA polymerase α (20). Another question concerns whether the DNA synthesis that generates tandem arrays resembles break-induced replication (BIR). In BIR events, a replication fork is established after invasion of a 3’ end, leading to replication that can proceed all the way to a telomere (7, 8). Tandem array formation by a rolling circle synthesis, by its nature, involves displacement DNA synthesis. This would seem to preclude a typical replication fork.

100 nucleotide telomeric circles of either strand can generate tandem arrays at telomeres.

Sequencing data suggested that the elongated telomeric arrays in *ter1-Δ* survivors containing two kinds of telomeric repeats can be composed of repeating units of 100 bp (18). We therefore wanted to test if circles of that size and composed mainly of telomeric repeats could promote the formation of tandem arrays at telomeres. We created single-stranded 100 nt circles composed of three telomeric repeats (75 nt of C-rich strand) and 25 nt of non-telomeric sequence (including a *ClaI* restriction site) by annealing the two ends of a linear 100 nt oligo with a 25 nt bridge oligo that was complementary to the non-telomeric sequence in the presence of DNA ligase(Materials and Methods; Figure 3.9A). We introduced the 100 nt circle into *ter1-Taq* cells by co-transforming it with p1B3, an
ARS-containing plasmid that had a *URA3* marker gene. The *ter1-Taq* strain was chosen because it had short recombinogenic telomeres but did not display the growth senescence and unstable telomere lengths of *ter1-Δ*.

We initially screened pools of ~10 transformants each for the presence of an ~100 bp *ClaI* fragment that hybridized to a telomeric probe. This size fragment is expected if the 100 nt circle integrates in the form of tandem arrays (Figure 3.8A). From screening 30 such transformant pools, we found four that released a small fragment when cut with *ClaI*. We then attempted to isolate the individual transformants containing the 100 bp telomeric *ClaI* fragment from all four pools by re-streaking individual colonies from each pool, isolating DNA from each, and testing for the presence of bands that hybridized to a probe made with the bridge oligo. Two transformants that had bands that hybridized to the bridge oligo were successfully isolated. One of these (C1) had multiple hybridizing bands while the other (C2) had only a prominent pair of closely spaced bands (Figure 3.8, left panels). The DNA fragments that hybridized to the bridge oligo also appeared to hybridize to telomeric and subtelomeric probes (Figure 3.8B, C and data not shown). As observed with the pooled transformants, cleavage of C1 and C2 with *ClaI* also produced telomere-hybridizing bands of 100 bp. We conclude from these data that sequence from the 100 nt circle had become incorporated as tandem arrays at one or more telomeres.

To examine the stability of the integrated telomere-bridge tandem arrays, the two *ter1-Taq* transformants were grown for several serial restreaks on YPD plates. Shown in Figure 3.8C is a Southern blot of *XbaI*-cleaved DNA from these cells after hybridization to telomeric, subtelomeric and bridge oligo probes. The results from this analysis showed that the integrated arrays were highly unstable. The C1 transformant lost all but one band
that hybridized to the bridge oligo by the second streak and had lost all bands by the third streak. The C2 transformant lost its bands that hybridized to the bridge oligo completely by the second streak. The reason for this instability is unclear. It could be due to non-telomeric DNA (the bridge sequence) being present at multiple positions throughout the elongated telomeres.

We next tested whether 100 nt circles composed of the G-rich telomeric strand could also lead to the formation of tandem arrays. As before, 100 nt “G-strand” circles were generated \textit{in vitro} using a 100 nt oligo containing three telomeric repeats and a 25 nt bridge oligo. As a control, 100 nt “C-strand” circles were generated again. Each type of circle was then transformed into \textit{ter1-Taq} cells along with p1B3. From each co-transformation, DNAs from 35 pools (each composed of \textasciitilde{}10 \text{Ura}^{+} transformants) were then isolated and examined by Southern blotting. Consistent with our initial results, at least 6 pools derived from the “C-strand” circle were found to exhibit \textit{Eco}RI fragments that hybridized to a bridge oligo probe as well as 100 bp \textit{Cla}I fragments that hybridized to a telomeric probe. Results with the “G-strand” circle were similar. At least 9 of the 35 pools exhibited \textit{Eco}RI fragments that hybridized to a bridge oligo as well as 100 bp \textit{Cla}I fragments that hybridized to a telomeric probe. Three of these pools (PG1-3) are shown in Figure 3.9B (right panels). The \textit{Cla}I telomeric fragments in clones C1 and C2 and in pools PG1, PG2 and PG3 were confirmed to be 100 bp on a 4\% agarose gel in all the clones/pools shown (data not shown). We conclude that 100 nt circles of either strand can lead to recombinalational telomere elongation through the formation of telomeric tandem arrays.
The simplest model for telomere elongation would be for the 3’ single-stranded end of a telomere to strand-intrude a telomeric circle (annealing to the C-rich strand) and act directly as the primer for DNA synthesis around the circle (Figure 3.9 C). Our results with both 100 nt circles and the 1.6 kb heteroduplex circles argue strongly against this model being the only mechanism for copying the sequence of a circle. Instead, there must be equally efficient mechanisms for circles of either strand to serve as templates for generating elongated arrays of telomeric repeats. One possibility is that rolling circle replication often occurs extrachromosomally. Once a telomeric array is generated, it could readily be incorporated at a chromosome end (Figure 3.9 A, B). How extrachromosomal rolling circle replication would be primed is not clear. Conceivably, small single-stranded telomeric pieces can be generated that may anneal to the circle (Figure 3.9A). Alternatively, the mechanism that primes telomeric Okazaki fragment synthesis might be able to prime rolling circle replication (Figure 3.9B). The yeast single-strand protein-binding complex, which includes Cdc13, is thought to help recruit DNA polymerase α to a telomeric end and bring about synthesis of the second strand (20). It is possible that a single-stranded “G-strand” telomeric circle could recruit a DNA polymerase in the same manner.

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

**Extrachromosomal single-stranded DNA of a long telomere mutant includes species forming discrete spots on a two-dimensional gel.**

We have examined preparations of genomic DNA from *ter1-Δ* cells for small telomeric circles by running the DNA on two-dimensional gels. Thus far, we have been unable to detect any extrachromosomal DNA that hybridizes to a telomeric probe in *ter1-Δ* cells. This may not be surprising since telomeric circles or extrachromosomal linear DNA may be very rare in occurrence and hence, hard to detect. However, abundant extrachromosomal telomeric DNA is observed in some *ter1* template mutants that produced very long telomeres (D. Underwood and M. McEachern, manuscript in preparation). Despite their very different telomere length phenotype, such mutants appear to have telomere-capping defects that make them resemble *ter1-Δ* mutants in certain respects. For one, they contain substantial amounts of single-stranded telomeric DNA, specifically of the G-rich strand. Resection from the 5' end to produce 3' tails (G-strand tails at telomeres) is characteristic of broken DNA ends and telomeres in cells with defects in the essential capping protein Cdc13 (5). Also, the long telomere template mutants, like *ter1-Δ* mutants, display greatly enhanced rates of subtelomeric recombination (D. Underwood and M. McEachern, manuscript in preparation). Finally,
the cellular and colony morphologies of the long telomere mutants is also often abnormal and bear resemblance to those of a ter1-∆ strain (16, 24). Therefore, it is likely that telomere-processing reactions would often be similar in ter1-∆ and the long telomere ter1 template mutants.

We ran uncut genomic DNA of one of the long telomere mutants, ter1-16T on a two-dimensional gel where the gel running conditions for the two dimensions utilized different concentrations of chloroquine (See Materials and Methods). Linear double-stranded DNA forms a linear diagonal in a two-dimensional gel. Figure 3.10 panels A and B show two-dimensional gels of ter1-16T genomic DNA probed with C and G telomeric strand oligos, respectively. In addition to the extrachromosomal DNA that forms a linear diagonal, discrete spots (marked with arrows in Figure 3.10) that run off the diagonal can be seen that hybridize only to the C strand oligo. We estimate that these spots migrate near positions of double-stranded linear fragments between fifty and two-hundred base pairs. Discrete spots were also observed in the linear diagonal that hybridized to both the G and C strand telomeric oligos. These may be double-stranded extrachromosomal telomeric DNA. The formation of these spots and the other extrachromosomal DNA visible on this gel is dependent upon RAD52 (Figure 3.10 C, D), which indicates that a recombination-dependent mechanism is involved.

The shift in mobility of the discrete spots from the diagonal of linear double-stranded DNA of ter1-16T, visible in the C-oligo hybridization (Figure 3.10 A) suggested that these spots may be single-stranded telomeric DNA circles. In order to test the exonuclease sensitivity of the putative circles, we ran ExoI-treated genomic DNA from ter1-16T and a 66 nt linear DNA control in two dimensions. While the linear control was
completely digested by ExoI, we found that the discrete spots of telomeric DNA were resistant (Figure 3.10 E-H). This suggests that the spots may be single-stranded circular telomeric DNA.

Some models for how small G-strand telomeric circles might form in ter1-16T cells are shown in Figure 3.11. Each postulates the initial presence of a 3' overhang at the dysfunctional telomere. 3' overhangs are normally produced at yeast telomeres during S phase (10). 3' overhangs are also produced at broken DNA ends during DNA repair processes. In one model (Figure 3.11A), a 3' telomeric end strand-invades a more internal region of the same telomere to form a t-loop. Nicking of the G-strand followed by ligation of the 5' end to the strand-invaded 3' telomeric end would produce a G-strand circle. Although t-loops are thought to be protective structures at mammalian telomeres (6), in this model the t-loop is likely to be an abnormal byproduct of an uncapped telomere. In a variation of this model (Figure 3.11B) the break in the G-strand tail is shown occurring prior to t-loop formation. An alternate mechanism for forming G-strand circles could be through annealing of single-stranded telomeric pieces. Breaks occurring in the 3' overhang of a telomere would generate single-stranded linear pieces. If occasional C-strand pieces containing telomeric repeats were also available, annealing between the two strands followed by ligation could produce largely single-stranded circles (Figure 3.11C). This would be directly analogous to the mechanism we used to form 100 nt circles \textit{in vitro}. A partially double-stranded fragment from a telomeric end could also potentially lead to circle formation in a similar fashion (Figure 3.11D).
References


Table 3.1. The number of circle S transformants is greatly reduced in a strain with fused telomeres.

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<th>Strain</th>
<th>TER1</th>
<th>ter1-AccSna</th>
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<tbody>
<tr>
<td>No. of circle S transformants</td>
<td>267</td>
<td>4</td>
</tr>
<tr>
<td>No. of ARS plasmid transformants</td>
<td>16,000</td>
<td>5,000</td>
</tr>
<tr>
<td>Relative circle S transformation frequency</td>
<td>1.00</td>
<td>0.05</td>
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</table>

The number of circle S transformants obtained in TER1 and the fused telomere strain, ter1-AccSna is shown as compared to the number of ARS plasmid transformants obtained.
Table 3.2. Restriction sites derived from individual strands of a heteroduplex circle mix in telomeric tandem arrays of transformants

<table>
<thead>
<tr>
<th>Restriction sites present in arrays</th>
<th>ter1-Δ msh2-Δ</th>
<th>TER1, msh2-Δ</th>
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<tr>
<td>SnaBI only</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>AatII only</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>BsiEI only</td>
<td>29</td>
<td>20</td>
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<tr>
<td>BsiEI and SnaBI</td>
<td>6</td>
<td>8</td>
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<tr>
<td>BsiEI and AatII</td>
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</tr>
<tr>
<td>SnaBI and AatII</td>
<td>4</td>
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</tr>
<tr>
<td>BsiEI, SnaBI and AatII</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>No. of transformants with arrays not cleaved by BsiEI, SnaBI or AatII</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>No. of transformants with inserts but no tandem arrays*</td>
<td>0</td>
<td>4</td>
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<tr>
<td>Total no. of transformants analyzed</td>
<td>67</td>
<td>48</td>
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* These transformants seem to have single copy inserts of sequence derived from the circle.

The number of transformants that had tandem arrays with BsiEI, SnaBI and/or AatII are shown. Numbers of transformants are shown for two strains: TER1 msh2-Δ and ter1-Δ msh2-Δ.
Figure 3.1. Long tandem arrays form in ter1-Taq when transformed with circle S or circle P.

A. The structure of circle S and circle P and the expected structure of a tandem array formed by circle S and/or circle P at a telomere are shown. The black boxes are the URA3 gene, the gray boxes represent telomeric repeats and the small white boxes represent subtelomeric sequence present in circles S and P. The stippled box represents internal subtelomeric sequence not present in the circles. Restrictions sites SalI, PvuI, EcoRI and XbaI are indicated as “S”, “P”, “R” and “X”, respectively. B. Southern hybridizations of one circle S and one circle P transformant of ter1-Taq are shown in the top panel. Southern hybridizations of two circle S+P transformants are shown in the bottom panel. An untransformed control is shown in each case for comparison. Every transformant is represented by three lanes for three types of restriction digests: EcoRI, EcoRI+SalI and EcoRI+PvuI. The primary digest (-) is always EcoRI, which cuts at a subtelomeric position that can vary among telomeres. The secondary digests are either SalI or PvuI, represented by “S” or “P” on the top of each lane. The type of circle (S or P) used for the transformation is represented above the panels for each transformant. Size markers in kb are also indicated. The same membrane has been probed sequentially with three probes: XbaI-EcoRI subtelomeric fragment, URA3 and telomeric probes as indicated at the bottom of each panel.
Figure 3.2. Multiple telomeres acquire arrays in circle S and circle P transformants of ter1-Taq.

The percentage of elongated telomeres was estimated as the amount of subtelomeric signal found above 3 kb relative to the total subtelomeric signal in a lane of EcoRI-digested DNA for URA3-telomere circle transformants of TER1 (9 transformants), ter1-Taq (24 transformants) and ter1-Δ (24 transformants) strains. Eleven of the twelve telomeres hybridize to the subtelomeric probe.
Strain

Subtelomeric signal > 3 kb

TER1  ter1-Taq  ter1-Δ

Strain

10  20  30  40  50  60  70  80  90

0  0.5  1  1.5  2  2.5  3  3.5  4  4.5  5  5.5  6  6.5  7  7.5  8  8.5  9  9.5  10
Figure 3.3. The presence of subtelomeric sequence in a *URA3*-telomere circle promotes deletion of telomeric repeats.

A. Southern blots of 17 circle S transformants each of *TER1, ter1-Taq* and *ter1-Δ* strains are shown. DNA from the transformants was digested with *Xho*I and *Pst*I and probed with a telomeric probe (top panel) and a *URA3/RAD52* probe (bottom panel). The *RAD52* signal was used as a single-copy control. The telomeric and *URA3* fragments that are formed are detected with the two probes. The *Xho*I+*Pst*I fragment containing the telomeric repeats is apparent in the top panel at ~0.2-0.4 kb. The plasmids from which circles S and P were derived have also been digested with *XhoI*-*PstI*-*BglII* to release the equivalent telomeric fragment and probed with the telomeric probe (top panel) to show that telomeric deletions are not present in the original transforming molecule (see dark band at 0.4 kb; the slightly bigger and much fainter band is signal from vector sequences). The expected map of a telomeric array formed by circle S is shown below the Southern blots. B. Southern blots of 17 circle N (no subtelomeric region) transformants each of *TER1, ter1-Taq* and *ter1-Δ* strains are shown. The transformants have been digested with *Sma*I and *Pst*I and probed with a telomeric probe (top panel) and a *URA3/RAD52* probe (bottom panel). The expected map of a telomeric array formed by circle N is shown below the Southern blots. Fragments containing telomeric repeats are visible near ~0.3 kb (top panel). “P” stands for *Pst*I and “S” stands for *Sma*I. C. A model for how subtelomeric DNA present in a transforming circle may lead to deletions within the block of telomeric repeats is shown. Thinner boxes represent single-stranded DNA. In short telomere strains, different alignments of the subtelomeric and telomeric regions of
the circle and a strand-invading chromosome end could occur (e.g., alignments 1 and 2 as shown). Alignment 1 can lead to a single-stranded loop between two regions of paired duplex DNA if base pairing extends into the subtelomeric sequence present on the circle (white box). Excision of this loop followed by ligation of the ends would then lead to deletion of some telomeric repeats from the circle (which originally has 11.5 telomeric repeats). Copying the resultant circle would lead to arrays composed of units with fewer repeats.
A

Strain: TER1  ter1-Taq  ter1-∆  plas SP

Telomeric

\begin{align*}
12.0 & \quad 4.0 & \quad 1.0 & \quad 0.5 & \quad 0.2 \\
\end{align*}

RAD52

\begin{align*}
12.0 & \quad 4.0 & \quad 1.0 & \quad 0.5 & \quad 0.2 \\
\end{align*}

~0.4 kb

B

Strain: TER1  ter1-Taq  ter1-∆

Telomeric

\begin{align*}
12.0 & \quad 4.0 & \quad 1.0 & \quad 0.5 & \quad 0.2 \\
\end{align*}

RAD52

\begin{align*}
12.0 & \quad 4.0 & \quad 1.0 & \quad 0.5 & \quad 0.2 \\
\end{align*}

~0.3 kb

C

Alignment 1

Excision of telomeric repeats from circle

Circle with fewer telomeric repeats

Array with fewer telomeric repeats

Alignment 2

Circle with full length telomeric repeats

Array with full length telomeric repeats

135
Figure 3.4. Long tandem arrays form after transformation of a *URA3*-telomere circle lacking subtelomeric sequences.

A. The structure of a circle with no subtelomeric sequence (circle N) and the expected structure of a tandem array templated by circle N is shown. The black box is the *URA3* gene, the gray box represents telomeric repeats and the white and the stippled box represent subtelomeric sequences. “X” stands for *XbaI*, “R” stands for *EcoRI* and “S” stands for *Sall*. B. Southern hybridizations of one transformant each of *TER1*, *ter1-Taq* and *ter1-Δ* circle N are shown as well as untransformed controls. Every transformant is represented by two lanes for two types of restriction digests: *EcoRI* and *EcoRI*+*Sall*. Size markers in kb are also indicated. The same membrane has been probed sequentially with three probes: subtelomeric, *URA3* and telomeric probes as indicated at the bottom of each panel. The subtelomeric “stump” released can be seen with the subtelomeric probe at a position below 1 kb in the *TER1* transformant. Hybridization to the telomeric probe does not reveal the unelongated telomeres well, especially in the lanes representing the *ter1-Δ* transformant.
**A**

Circle N

---

**B**

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**TER1**

- N N - N N - N N

**ter1-Taq**

- - + - - + - - +

**ter1-Δ**

- - + - - + - - +

---

**SalI**

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---

**1.48kb**

---
Figure 3.5. Some circle S transformants of ter1-AccSna form tandem arrays.

Southern blot of untransformed (U) and two ter1-AccSna clones transformed with a URA3-telomere circle (circle S) is shown. The untransformed control is shown digested with EcoRI and the transformed clones are shown digested with EcoRI and EcoRI +SalI, as indicated. The same filter is shown hybridized with subtelomeric, URA3 and telomeric probes. Size markers (in kb) are shown at left.
URA3

--- + - +

Telomeric

--- + - +

Probe:

1.0

1.6

3.0

12.0

SalI

U 1 2 1 2 1 2 U U

139
Figure 3.6. A single copy of circle S integrated at a telomere can expand to form tandem arrays in short telomere mutants but not in a wild-type strain.

A. Shown are Southern hybridizations of serial re-streaks of one TER1, two ter1-Taq and two ter1-Δ clones that initially had one or more telomeres containing a single copy of the URA3-telomere insert. The top panel shows hybridization of EcoRI-digested genomic DNA to URA3 and the bottom panel shows the same filters hybridized to a telomeric probe. B. Models for expansion of a single copy URA3-telomere insert into tandem arrays. Multiple unequal recombination events could generate long tandem arrays. Alternatively, excision of a circle followed by rolling circle gene conversion might generate arrays. The black box represents the URA3 gene, the gray box represents telomeric repeats and white and stippled boxes represent subtelomeric sequences.
A

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</table>

URA3

Telomeric

12.0

3.0

1.0

B

Unequal recombination

Additional unequal recombination

Loop-out of circle

Rolling circle replication

1.6 kb

(n)
Figure 3.7. Absence of strand bias in utilization of a URA3-telomere circle during formation of telomeric long tandem arrays.

A. An outline of how heteroduplex circles (circle H) were generated and the expected structure of a tandem array at a telomere derived by transforming a circle H mixture is shown. Plasmid pMH3-2UraTel was digested with SalI and XmaI-SacI to generate two linear fragments, which were boiled together, annealed and gel purified as gapped circles. The gaps were filled with oligos that contained mismatches that created SnaBI and AatII restriction sites on different strands of the circle as shown. “R” stands for EcoRI, “B” stands for BsiEI, “S” stands for SnaBI and “A” stands for AatII. The sequence of the XmaI-SacI fragment that was absent in the gapped circles and the sequence of the oligos used to bridge the gaps are also shown. B. Southern blots of three transformants obtained in a ter1-Δmsh2-Δ double mutant are shown. Every transformant is represented by four digests (indicated above every lane) and three hybridizations (indicated below). An untransformed control (U) digested is also shown with each hybridization. For all four digests of each transformant, the primary digest is EcoRI, which generates telomeric fragments and the three secondary digests are either BsiEI, SnaBI or AatII. Note that SnaBI cleaves one of the EcoRI bands to a slightly smaller size.
**XmaI-SalI fragment with the BsiEI site**

\[ \text{XmaI} \quad \text{CCCGGGAATCTCGTGTA... (50 bp)...CGAGC} \]
\[ \text{SalI} \quad \text{CTTAGAGCCAGCAT... (50 bp)...GC SalI} \]

**SnaBI containing oligo (76 nt)**

\[ \text{SnaBI} \quad \text{CCCGGGAATCTCGTGTA... (50 bp)...CGAGC} \]

**AarII containing oligo (66 nt)**

\[ \text{AarII} \quad \text{CTTAGAGCCAGCAT... (50 bp)...GC} \]
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**Probe:**

**Subtelomeric**

![Subtelomeric](image)

**URA3**

![URA3](image)

**Telomeric**

![Telomeric](image)
Figure 3.8. Sequence from a 100 nucleotide telomeric circle can form tandem arrays at telomeres.

A. *In vitro* generation of a 100 nt circle and the expected structure of a tandem array formed by the integration of its sequence at a telomere is shown. The partially single-stranded circle was generated by ligating the ends of a 100 nt oligo brought together by annealing to a bridging oligo that is complementary to a 25 nt non-telomeric region of the 100 nt oligo. Gray boxes represent telomeric repeats, the white boxes represent non-telomeric sequence that forms the bridge between the telomeric repeats in the circle and the black box represents subtelomeric sequence. B. Southern blots of two C-strand 100 nt circle ter1-Taq transformants (C1 and C2) and three pools of G-strand 100 nt circle ter1-Taq transformants (PG1, PG2, PG3) that have a telomeric array formed with sequence derived from the circles are shown. The probes used are the telomeric oligo and the bridging oligo. Each transformant is represented by two lanes for two digests (*Eco*RI and *Eco*RI+*Cla*I). *Eco*RI generates the telomeric fragments and *Cla*I cuts once within each unit of the array leading to the formation of a 100 nt band. The 100 nt band is not very clearly visible in PG1, PG2 and PG3 because they are pools of ~10 transformants each. C. Southern blots of serial re-streaks of the same two C-strand 100 nt circle ter1-Taq transformants (C1 and C2) shown in B, digested with *Xba*I. *Xba*I generates smaller telomeric fragments for 9 of the 12 telomeres in TER1 K. lactis, making it easier to detect the telomere(s) that has been elongated by formation of array(s) derived from the 100nt circle. “Immediate subtelomeric” and telomeric hybridizations as well as hybridization to the 25 nt bridge oligo shown in part A of this figure are shown.
A

\[
\begin{align*}
GATACGAAGTCC & \quad (TCCGTACACATAATCAA)_3 \quad GTAGATCTGCAT C \\
+ \\
GGACTTCGTATCGATGCAGATCTAC
\end{align*}
\]

\[
\text{Mostly single-stranded 100 nt circle}
\]

\[
\text{Tandem array templated by the circle}
\]

B

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| U 1 2 3 4 | 1 2 3 4 | 1 2 3 4 |

| Telomeric |
| Bridge oligo |
| Immediate subtelomeric |
Figure 3.9. Formation of telomeric tracts promoted by telomeric circles of either strand.

A. A model for the formation of long telomeric tracts by rolling circle replication initiated by extrachromosomal telomeric fragments is shown. Linear fragments of telomeric DNA can then initiate rolling replication around a circle bearing complementary sequences leading to the elongation of the linear fragment. The complementary strand can then be synthesized and the double-stranded telomeric fragment can be integrated at a chromosome end leading to telomere elongation. B. The formation of telomeric arrays templated by a C-rich telomeric circle may also be primed by the 3’ end of an Okazaki fragment. C. The telomeric G-rich strand can invade a telomeric C-stranded circle to eventually generate long telomeres by rolling replication of the G-strand around the circle and lagging strand synthesis of the C-strand. Black boxes represent G-strand telomeric repeats, gray boxes represent C-stranded telomeric repeats and white and stippled boxes represent subtelomeric sequences.
Formation of telomeric circles

Extension of chromosomal G-rich telomeric end and lagging strand synthesis

Formation of long tracts of extrachromosomal ss telomeric DNA

Template switching of DNA replication primed by an Okazaki fragment

Synthesis of second strand

Formation of long tracts of extrachromosomal ds telomeric DNA

Integration of extrachromosomal telomeric repeats at a chromosome end
Figure 3.10. Small extrachromosomal telomeric DNA in a long telomere mutant.

The panels shown in this figure are two-dimensional agarose gel electrophoreses. The numbered arrows indicate the directions of electrophoresis of the first and second dimensions. The extrachromosomal DNA largely forms a linear diagonal in these gels. Panel A shows uncut genomic DNA of ter1-16T RAD52 probed with the telomeric C-strand oligo. At least four discrete spots that have been marked with arrows can be seen to have run off the linear diagonal. Panel B shows the same filter shown in panel A probed with the telomeric G-strand oligo. Panel C shows uncut genomic DNA of ter1-16T rad52-∆ probed with the telomeric C-strand oligo. Panel D shows the same filter shown in panel C probed with the telomeric G-strand oligo. Panel E shows uncut genomic DNA of ter1-16T RAD52 + linear 66 nt non-telomeric oligo (called oligo A) probed with the complementary oligo A'. Panel F shows the same filter as in panel E probed with the telomeric C-strand oligo. The signal from the non-telomeric oligo A has not been stripped off. Panel G shows genomic DNA of ter1-16T RAD52 + linear 66 nt non-telomeric oligo A treated with ExoI exonuclease probed with the complementary oligo A'. Panel H shows the same filter as in panel G probed with the telomeric C-strand oligo. The signal from the non-telomeric oligo A has not been stripped off. The positions of the size markers in bp shown in panels F and H may not be precise because they have been determined by running a 25 bp ladder on a separate gel that was stained with ethidium bromide.
Figure 3.11. Formation of extrachromosomal telomeric circles in a strain with long telomeres.

Models for the formation of “G-strand” telomeric circles from chromosomal telomeric tracts are shown. Black boxes represent G-strand telomeric repeats and gray boxes represent C-stranded telomeric repeats. A. A t-loop structure at a telomeric end may form by looping back of the G-rich telomeric strand. A telomeric circle may be generated if a nick is produced at the point where the telomeric strand folds back. B. This is a variation of the model shown in part A. An extrachromosomal G-strand telomeric fragment may form a circular structure via ligation of the ends annealing with the complementary sequence present at the chromosome end. C. An extrachromosomal G-strand telomeric fragment as shown in Part B may circularize via ligation of the ends annealing to a complementary extrachromosomal fragment. D. Recessed 5' ends lead to the formation of sticky ends on a double-tranded telomeric fragment, which may ligate to form partially or completely double-stranded telomeric circles.
CHAPTER 4

MITOTIC TELOMERIC RECOMBINATION IN WILD-TYPE

*Kluyveromyces lactis*
Introduction

A recombinational pathway of telomere maintenance has been implicated as the cause for cellular immortalization in certain types of human tumors and immortalized cell lines (1, 2, 5). The mechanisms by which recombination acts to maintain long telomeres have not been very well studied. It has been proposed that recombination events that occur at chromosome ends may depend on a process called break-induced replication (BIR), which leads to non-reciprocal change (i.e., gene conversion) of all DNA elements from the point of cross-over to the chromosome end (6, 7). Telomeres in yeast and mammals are usually maintained by telomerase. The absence of a functional telomerase leads to cellular senescence in yeast. When EST1 or other genes encoding components of the S. cerevisiae telomerase are deleted, the cells undergo senescence but a few survivors emerge that have elongated their telomeres (9, 10). Such survivors in S. cerevisiae are known to arise from two pathways that are dependent on homologous recombination, one of which leads to subtelomeric expansions (involving Y' elements and internal blocks of telomeric repeats), and the other to telomeric expansions (3, 9, 15, 16). The two pathways also differ in their requirements for Rad50, Rad51 and other recombination-related proteins (3, 15). Deletion of telomerase in the related yeast Kluyveromyces lactis also leads to telomeres shortening, growth senescence and eventually to the formation of post-senescence survivors. These survivors appear to be the type with elongated telomeres (11). It is known that K. lactis cells that lack a telomerase or that have stably shortened telomeres have highly elevated rates of subtelomeric recombination (13). This elevated recombination rate is thought to be crucial for the production of post-senescence survivors. In this work, we have devised an assay that can detect recombination between
telomeric repeat arrays and determine whether gene conversion events that replace subtelomeric sequence also replace the telomeric sequence.

**Materials and Methods**

**Strains**

The strain used in this study is a derivative of *K. lactis* 7B520 (*ura3*, *his3*, *Trp*; 18), which is considered the wild-type strain in all our experiments. The mutationally-tagged telomere strains used in this study were constructed by transforming 7B520 with an ~1.5 kb *EcoRI*-SacII linear DNA fragment from the plasmid Bcl-Stumaker II. The plasmid Bcl-Stumaker II was derived from Stumaker II (a derivative of pBluescript containing a cloned wild-type telomere of *K. lactis*) by oligonucleotide mutagenesis that mutated each of the 11 telomeric repeats present in the plasmid into Bcl repeats (17). Plasmids were propagated in *E. coli* XL1.

**Southern hybridization**

Southern blotting was done using Hybond N+ membrane (Amersham Pharmacia, Piscataway, NJ). All hybridizations were done in 500 mM Na$_2$HPO$_4$ and 7% SDS (4). The telomeric probes used in our hybridizations were the 25 nucleotide (nt) Klac 1-25 oligomer (12) and a 12 nt Bcl oligomer matching the part of the 25 nt telomeric repeat with the single base change (underlined) leading to the formation of the *Bcl*I restriction site (TGATCAGGTATG). This probe was used at 26°C. Hybridization washes were done for a total of five minutes with wash buffer containing 200 mM Na$_2$HPO$_4$ and 2% SDS. The subtelomeric probe was generated from the ~590 bp *EcoRI*-*XbaI* from the
plasmid KL-11B (13). The subtelomeric probe was hybridized at 65°C. Hybridization washes were done for a total of 45 minutes with wash buffer containing 100 mM Na₂HPO₄ and 2% SDS.

**Yeast transformation**

*K. lactis* transformation was done using a protocol similar to the one used for *S. cerevisiae*. The protocol used for the transformation required 20 ml of yeast culture grown to O.D._600_ of 0.5-1.5 for each DNA sample that was introduced. The cells were pelleted and resuspended in 5 ml of sterile water. 1 M TE (Tris Hcl + EDTA) and 0.1 M lithium acetate was added to the cells, and they were incubated at 30°C for 45 min. The cells were then incubated in 2.5 mM DTT at 30°C for 15 min. After two washes with ice cold sterile water and two washes with ice cold 1 M sorbitol, the cells were concentrated to a final volume of 40 µl, which was used for the electroporation of DNA. Electroporation was done using a mixture of yeast cells and DNA at 1500 V in an Eppendorf Electroporator.

**Results**

**Constructing strains with mutationally-tagged telomeres**

In order to determine the frequency of recombination near telomeres in yeast cells with a fully functional telomerase, we used *K. lactis TER1* strains engineered to have one out of twelve telomeres composed largely of mutationally-tagged telomeric repeats. The mutationally-tagged telomere strains were generated by transforming a *TER1* strain with an ~2.2 kb linear piece of DNA bearing a *URA3* gene, subtelomeric homology and 11.5
mutant telomeric repeats, which integrated at and replaced one of the twelve chromosome ends (Figure 4.1A). The mutation present in each of the 11.5 telomeric repeats was a single base change that led to the formation of a BclI restriction site in each telomeric repeat. Telomeres composed of Bel repeats are phenotypically normal by several criteria including telomere length and subtelomeric recombination rates (13, 14, 17). In this experiment, we generated nine mutant strains that each had one native telomere replaced by the URA3-Bcl telomere construct. Six different telomeric ends were replaced among the nine strains that we generated (data not shown). The telomeric ends that were replaced by the URA3-Bcl telomere construct could be visualized by Southern blotting as a shift up of the original telomeric band by ~1.2 kb as shown in Figure 4.1A. When the Bel telomere-tagged strains are passaged, wild-type telomeric repeats are added onto the termini because of the presence of a wild-type TER1 gene.

**Screening for telomeric and subtelomeric recombination in a strain with normally functioning telomeres**

We followed twelve colonies each of the nine URA3-Bcl-tagged telomere strains over 15 re-streaks (300-360 cell generations) each. Southern analysis was done to determine if there was any duplication, deletion or translocation of either the URA3 gene or the Bel telomeric repeats from their original position to any other telomere due to subtelomeric or telomeric recombination. Genomic DNA from the passaged clones was digested with EcoRI and EcoRI-BclI and sequentially probed with a subtelomeric probe, a Bel-specific telomeric oligo and the URA3 probe. Figure 4.1C diagrams five possible events that might be expected to occur and the alterations in Southern hybridization...
patterns that they would produce. We found only one clone that had a duplication of the
URA3-Bcl telomere among the 108 clones (12 colonies from each of 9 strains) that we
analyzed. Because a duplication of the URA3 gene may present itself as only an increase
in intensity of a band, we cannot be certain that other duplications were not overlooked.
The single extra URA3 band suggested that duplication of the subtelomeric DNA that
includes the URA3 insert occurred at one telomere out of 32,400 (1/108 colonies x 1
telomere x 300 cell generations) per cell generation, equivalent to a frequency of 3x10^{-5}
per telomere per cell generation. In this experiment, we found no clones that had lost the
URA3 gene, nor did we find clones that had lost all Bcl repeats but retained the URA3
gene from the original marked telomeres. There was also no evidence for Bcl repeats
having spread to additional telomeres. Figure 4.2 shows the number of events that were
observed among five possible outcomes of recombination near telomeres. We conclude
that recombinational spreading of telomeric repeats from one telomere to other telomeres
is rare in normal K. lactis cells.

**Loss of subtelomeric URA3 is accompanied by loss of all Bcl repeats**

One way by which recombinational spread near telomeres may occur is by a gene
conversion event that leads to non-reciprocal exchange of DNA sequences within a short
stretch of the chromosome. Another mechanism for the spread of subtelomeric and
telomeric sequences is a non-reciprocal exchange of DNA sequence that initiates as a
replication fork at a subtelomeric position and proceeds all the way to the end of the
chromosome. This latter mechanism, called break-induced replication (BIR) has been
proposed to occur at double-strand breaks (DSBs) under some circumstances (8). The
difference between gene conversion of a short stretch of DNA and BIR has been depicted in Figure 4.2.

We wanted to determine if short patch gene conversion or BIR was responsible for recombination events near chromosome ends. In order to do this, we selected for the loss of the subtelomeric $URA3$ gene on media containing 5-fluorouracil (5-FOA) in the nine $URA3$-Bcl-tagged telomere strains. We picked twelve 5-FOA-resistant colonies from each of the nine strains and analyzed them by Southern blotting to determine the fate of the Bcl repeats. Genomic DNA was digested with $EcoRI$ and $EcoRI$-$BclI$ and sequentially probed with a subtelomeric probe, a Bcl-specific telomeric oligo and the $URA3$ probe. Among all the 108 (9 x 12) clones that we analyzed, we found co-loss of $URA3$ and Bcl telomeric repeats. We also did not detect the spread of any Bcl telomeric repeats to other telomeres that might have arisen before or during the recombination event that eliminated $URA3$. Our results are consistent with the theory that recombinational exchanges near $URA3$-tagged telomeres that result in the loss of $URA3$ proceed by a mechanism like BIR.

**Discussion**

It has been previously shown that recombination rates at subtelomeric regions are elevated in strains with short telomeres but not in strains that have a functional telomerase and normal length telomeres (13). In this study, we wanted to estimate the frequency of recombination within the telomeres themselves under normal conditions. The assay that we designed allowed us to potentially detect both subtelomeric and telomeric recombination events in a strain with normal telomeres. Our evidence suggests
that, in wild-type *K. lactis* cells, the frequency of both subtelomeric gene conversion and telomeric recombination occurs at frequencies lower than $10^{-5}$ per telomere per cell generation. The observation of a single subtelomeric-telomeric duplication among 108 clones in ~300 cell generations is not inconsistent with the rate of subtelomeric recombination that was previously observed ($6.7 \times 10^{-6}$ per telomere per cell generation; 13). The failure to observe evidence for recombination within telomeric repeat arrays argues that telomere-telomere recombination is also normally a rare event. However, the quick turnover of the most distal telomeric repeats due to telomerase-mediated sequence addition means that we cannot rule out that recombination may be more common specifically at telomeric termini.

We have also examined whether recombination events selected to result in the loss of the subtelomeric *URA3* gene also results in the loss of Bcl repeats. In all cases, we observed co-loss of *URA3* and the Bcl telomeric repeats, consistent with BIR events that originated at subtelomeric positions and proceeded all the way to the end of the telomere, hence, replacing all sequences to the telomeric end. In this experiment there was also no evidence for recombination events between telomeric repeats. Since we have followed only one out of twelve telomeres, we may have overlooked telomeric recombination events that occurred between the other eleven telomeres. In any case, we have seen no evidence for increased rates of recombination between telomeric repeats in a *TER1* strain.

Other experiments done in our laboratory suggest that recombination between telomeric repeats is highly enhanced during the formation of *ter1-Δ* survivors (Topcu and McEachern, unpublished). Mutationally-tagged Bcl telomeres, similar to those described here, were generated in a *ter1-Δ* strain. The telomere tagged *ter1-Δ* strains were followed
over several re-streaks to look for duplication, deletion or translocation of \textit{URA3} or the Bcl repeats. A sizeable percentage of survivors were observed to have lost \textit{URA3} and the Bcl repeats. Up to 10\% of survivors were found to have spread the Bcl repeats to most or all other telomeres in the cell, often with retention of basal wild-type repeats. This indicates that recombination between telomeres can be greatly enhanced in a \textit{ter1-\Delta} strain.

In yeast cells, recombination near telomeres seems to be an adaptive mechanism to ensure survival by replenishing telomeric repeats in the absence of telomerase. The formation of post-senescence survivors, which arise after cells lacking a functional telomerase undergo senescence, almost certainly requires elevated recombination rates near telomeres. As some human cancers appear to utilize recombination to maintain telomeres, it will be important to study the mechanisms by which it occurs.

References


**Figure 4.1. Assay for telomeric and subtelomeric recombination and possible outcomes.**

A, left side) The integration of a linear fragment bearing *URA3* (black box), subtelomeric sequences (stippled boxes) and Bcl telomeric repeats (light gray boxes) occurs at a telomere. Other telomeres are composed entirely of wild-type repeats (darker gray boxes). A “totally-mutant telomere strain” is generated, which has one telomeric end replaced with the mutant, in this case Bcl, telomeric repeats. A, right side) Southern blots of DNA from three different clones, each digested with *Eco*RI, that have the *URA3*-Bcl telomere insert replacing one of three different telomeres, probed with telomeric and *URA3* probes. The white dots in the telomeric probe shows the position to which the three different telomeres are shifted due to the *URA3*-Bcl telomere insert. Either new bands may appear (clone # 1) or an existing band may become a doublet and hence more intense (clones #2 and 3) due to the *URA3*-Bcl telomere insert. Size markers in kb are shown on the right. The position of telomeric *Eco*RI fragments of an untransformed clone are shown as black circles/oval to the left. Multiple bands are produced in an *Eco*RI digest due to the different positions of the *Eco*RI site in the subtelomeric region of the 12 telomeres (indicated as R1-R12 bands on the left). B) Initial structure and C) five different outcomes of subtelomeric or telomeric recombination are shown. C1) Duplication of both *URA3* and the Bcl telomere. C2) Duplication of the Bcl telomere without duplication of *URA3*. C3) Spread of some Bcl repeats to another telomere. C4) Loss of *URA3* and the Bcl telomere. C5) Loss of *URA3* with retention of the Bcl telomere. The patterns on the left are schematic representations of Southern blots digested with *Eco*RI and probed with the subtelomeric probe, Bcl oligo and the *URA3*
probe of what might be observed from each type of recombination event. The black boxes represent telomeric bands that light up and the white boxes represent telomeric bands that do not light up with the respective probe. The gray box in outcome 3 represents a slightly shorter band that hybridizes with lower intensity. On the right are three telomeric ends representing each outcome. The dark gray boxes represent wild-type telomeric repeats and the light gray boxes represent Bcl telomeric repeats. The stippled boxes are subtelomeric regions and the black boxes are \textit{URA3}. The subtelomeric probe used in our hybridizations does not hybridize to the R12 band.
A

Clone: 1 2 3 1 2 3

kb

1.0

2.0

3.0

βclI

B

Initial URA3-Bcl telomere with wild-type repeats at terminus

Probe: Subtel Bcl URA3 oligo

Duplication of URA3 and Bcl telomere

Duplication of Bcl telomere only

Spread of Bcl telomeric repeats only

Loss of URA3 and Bcl telomere

Loss of URA3 with retention of Bcl telomere

C

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Figure 4.2. Results of assaying for telomeric and subtelomeric recombination.

Five possible outcomes due to recombination in or near telomeres in a “total Bcl telomere” strain and the number of events that we observed of each type are shown. a) Duplication of $URA3$ and the Bcl telomere caused by subtelomeric gene conversion. b) Duplication of the Bcl telomere without duplication of $URA3$ caused by telomeric gene conversion. c) Spread of some Bcl repeats to another telomere. d) Loss of $URA3$ and the Bcl telomere caused by subtelomeric gene conversion (previously shown to be $6.7 \times 10^{-6}$ (13). e) Loss of $URA3$ with retention of the Bcl telomere. The dark gray boxes represent wild-type telomeric repeats and the light gray boxes represent Bcl telomeric repeats. The stippled boxes are subtelomeric regions and the black boxes are $URA3$. 
Outcomes after recombination

- a. Duplication of URA3 and Bcl telomere
  - Number of clones observed out of 108 clones followed over 300 cell generations: 1
  - Estimated frequency: $3 \times 10^{-5}$

- b. Duplication of Bcl telomere
  - Number of clones observed: 0
  - Estimated frequency: $<3 \times 10^{-5}$

- c. Spread of Bcl telomeric repeats only
  - Number of clones observed: 0
  - Estimated frequency: $<3 \times 10^{-5}$

- d. Loss of URA3 and Bcl telomere
  - Not determined
  - Estimated frequency: $6.7 \times 10^{-6}$ (previously determined)

- e. Loss of URA3 with retention of Bcl telomere
  - Number of clones observed: 0
  - Estimated frequency: $\leq 7 \times 10^{-8}$
CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS
In this study, we have presented data that support the hypothesis that recombinational telomere elongation can be promoted by rolling circle replication around a circular telomeric template. Homologous recombination plays a role in the maintenance of yeast telomeres when telomerase mediated telomere maintenance is dysfunctional or absent due to a mutant or absent telomerase, respectively (3, 4). In order to study the process of recombinational telomere elongation, we designed an experiment to follow two types of telomeric repeats through the process of formation of post-senescence survivors in *K. lactis* cells lacking the telomerase RNA template (*TER1*). Telomeres in survivors retaining both repeat types produced small blocks of wild-type telomeric repeats when digested with a restriction enzyme that cut within the other type of telomeric repeat. This restriction analysis coupled with cloning and sequencing of telomeres from these survivors showed that telomeres in a given clone were composed of uniform alternating blocks of the two types of repeat. This suggested the possibility that DNA circles may be responsible for the formation of the uniform blocks of repeats in the elongated telomeres by a mechanism involving rolling circle replication.

To test the rolling circle replication hypothesis, we introduced 1.6 kb circles containing telomeric repeats and a *URA3* marker gene into three different strains of *K. lactis*: wild-type *TER1*, which had normal length telomeres, and *ter1-Taq* and *ter1*-*Δ*, which had short, recombinogenic telomeres (5). Southern analysis showed that most of the transformants had utilized the input circle to generate long tandem arrays at telomeric ends. The units of these tandem arrays had the sequence of the input circle, which supported the idea that a circular template could be utilized to form elongated telomeres. Typically, the majority of telomeres in *ter1-Taq* and *ter1*-*Δ* strains had acquired the
tandem arrays. On the other hand, *TER1 K. lactis* had tandem arrays at one or two telomeres. Another difference between transformants of *TER1 K. lactis* and that of *ter1-Taq* and *ter1-Δ* *K. lactis* was the copy number of sequence derived from the circle in the tandem arrays at telomeres; the former had 10-20 copies and the latter had 30-180 copies. This and other data suggested that a tandem array initially formed at a single telomere. Subtelomeric or telomeric gene conversions could then spread the elongated sequence to most or all telomeres in strains that had a dysfunctional telomerase and high recombination rates at or near telomeres. *TER1* cells did not have enhanced recombination rates, unlike *ter1-Taq* and *ter1-Δ* and hence did not spread the sequence acquired from the circle to other telomeres, but retained it at the original one or two telomeres.

Although the data so far supported the rolling circle replication hypothesis, it was possible that multiple circles were getting into the transformants, integrating at telomeres independently of one another and forming tandem arrays at telomeres. In order to confirm that telomeric tandem arrays could be derived from a single transforming molecule, we transformed *TER1*, *ter1-taq* and *ter1-Δ* cells with two types of *URA3*-telomere circles, that differed at a single restriction enzyme site. We expected sequence derived from both circles to be present in the telomeric tandem arrays of a given transformant if multiple circles were responsible for the formation of the telomeric arrays. We found that a given transformant had tandem arrays derived from one type of circle or the other, not both. This indicated that the telomeric tandem arrays were derived from one circular molecule, which was consistent with the rolling circle replication hypothesis.
URA3-telomere circle transformants obtained in a TER1 rad52-∆ strain showed that the utilization of the circle to form arrays at one or two telomeres was mostly but not completely dependent on Rad52. Other data indicated that the spread of the sequence to other telomeres is strongly Rad52-dependent (5). We propose that the formation of the tandem arrays at telomeres from a circular molecule occurs by rolling circle replication of the telomeric end around the circular template. The tandem arrays then spread to other telomeres by gene conversion at subtelomeric or telomeric positions. We refer to this as the “roll and spread model”. A similar mechanism may be responsible for the formation of survivors that appear in a population of ter1-∆ K. lactis and est1-∆ S. cerevisiae cells that are senescent. There are two types of survivors that form in an est1-∆ strain of S. cerevisiae (2, 4, 7). Type I survivors have amplification in their subtelomeric regions involving Y' elements and internal blocks of telomeric repeat while Type II survivors have elongated telomeres. The tandem arrays that form in our circle transformants in K. lactis are similar in structure to Type I survivors. Both have alternating stretches of telomeric and non-telomeric sequences. It is known that Type I and Type II survivors also differ in the requirement for Rad50 and Rad51, which are proteins that are involved in two different pathways of homologous recombination (1, 6). Type I requires Rad51, while Type II requires Rad50. It will be useful to determine the requirement of Rad50 and Rad51 for the process of formation of tandem arrays by utilization of a circle with telomeric repeats and a marker gene.

We have analyzed some of our TER1 circle transformants over 100 generations of unselected growth and found the arrays to be reasonably stable. Other experiments can be done to more sensitively determine the stability of the tandem arrays that form from a
circular template. The rate of loss of the \textit{URA3} marker gene in our circle transformants can be determined. It will be useful to compare the rate of loss of the arrays, and hence their stability, between different strains. The requirement for elevated rates of recombination for the spread of the tandem arrays can be tested by mating a \textit{TER1 URA3}-telomere circle transformant with arrays at one or two telomeres with a \textit{ter1-\Delta} strain that has high recombination rates at telomeres. The \textit{ter1-\Delta} haploid spores obtained from such a mating will probably display very high rates of both loss and duplication of the tandem array.

In this study, we have also presented some details of the features of telomere elongation promoted by DNA circles. We have found that subtelomeric homology present in a circle is not required for the formation of arrays, but its presence leads to the formation of telomeric deletions in the resulting arrays. A prediction of the rolling circle replication hypothesis is the requirement of some type of telomeric end to prime replication around a circular template. As expected, a strain with mostly circular chromosomes cannot efficiently utilize a circular template to form or spread tandem arrays. We have also shown that while a circular \textit{URA3}-telomere template can promote the formation of telomeric tandem arrays, a linear \textit{URA3}-telomere does not. A single copy of the 1.6 kb \textit{URA3}-telomere sequence inserted at a telomere cannot expand to form arrays in a \textit{TER1} strain, but can do so in \textit{ter1-Taq} and \textit{ter1-\Delta} strains, presumably by unequal recombination or by the formation of a \textit{URA3}-telomere circle. The process of formation of tandem arrays from a single-copy insert in short telomere strains commonly requires 80-100 cell generations. Circle transformants usually produce arrays in less than 20 cell generations.
We have also tried to determine if there is any strand-bias in circle utilization. Since telomeric ends possess single-stranded 3' G-rich tails, a prediction might be that the C-strand of the circle would be a more efficient template for rolling replication. Our data are inconsistent with this prediction. We have used circular *URA3*-telomere templates with unique restriction sites to mark the two strands. We have found that in transformants of both *TER1* and *ter1-*Δ, sequence derived from either of the two strands can be present in the tandem arrays. Since the formation of survivors may require utilization of a circle that may be close to 100 nt in size, we tried to determine if a single-stranded 100 nt telomeric circle (composed of three C-rich telomeric repeats) can prime telomere elongation. Since this circle lacks a marker gene, it has not been easy to identify transformants that have taken up the 100 nt circle. We screened pools of transformants to look for clones that had formed telomeric tandem arrays and isolated two *ter1-Taq* transformants so far that have produced telomere elongation with sequence derived from the 100 nt circle. We also used a 100 nt circle composed of three G-rich telomeric repeats to transform *ter1-Taq* cells. We screened pools of transformants to look for clones that had telomeric tandem arrays and found nine positive pools. We are still in the process of identifying the individual clones that have the arrays. Thus, small single-stranded telomeric circles of either strand can prime telomere elongation. It will be useful to find more such transformants. It is possible that the utilization of a 100 nt circle and the structure of a telomere elongated by such a circle may be very different from that of a larger 1.6 kb circle that we have used in most of our experiments. It will be important to determine these differences.
We have found putative circles of ~75-200 nt in a telomerase template mutant that has long telomeres. The putative circles, which are resistant to ExoI exonuclease can be seen as discrete spots that hybridize to the G-rich telomeric strand on a two-dimensional gel. Electron microscopy can be done to visualize and hence confirm the presence of telomeric circles.

We have provided circumstantial evidence for the hypothesis that rolling circle replication around a telomeric template can lead to telomere elongation. We have also shown that the utilization of a circular template for telomere elongation can occur rapidly and hence, can explain the abrupt telomere elongation observed in survivors of yeast that lack a functional telomerase. The rate-limiting step in the rolling circle replication process may be the formation of telomeric circles. We have proposed several models for the formation of telomeric circles. Once an elongated telomere is formed, the elongated sequence seems to readily spread to most or all chromosome ends in the cell. Recombination rates are elevated when telomeres are short and can lead to the spread of elongated telomeres among chromosome ends. The formation and utilization of a circular template may also be dependent on homologous recombination, which may be a reason for the dependence of survivor formation on homologous recombination. Rolling circle replication seems to be a very efficient mechanism for telomere elongation as compared to unequal recombination although both processes may be responsible for the formation of long telomeres. Since we have not observed any strand-bias in circle utilization, we propose that telomere elongation by rolling circle replication may occur either at the chromosome end or extrachromosomally. Rolling circle replication-mediated telomere elongation may also be responsible for the elongation of telomeres in some mammalian
cancers and immortalized cell lines. In the absence of telomerase, although most human somatic cells undergo senescence due to telomere shortening, some cells survive and maintain their telomeres via an unidentified mechanism that has been abbreviated to “ALT” (Alternate Lengthening of Telomeres). Some recent data suggest that homologous recombination may be involved in the maintenance of human telomeres in the absence of telomerase.

Our research has provided more insight into the process of recombinational telomere elongation in yeast. Recombination-mediated telomere elongation is gaining more importance since some tumors seem to depend on a recombination-mediated pathway to maintain elongated telomeres. Yeast is a useful model organism because it provides more insight into mechanisms that may be common in most or all eukaryotic systems. So far, therapeutics designed to combat cancers by inhibiting telomere elongation are mainly targeting telomerase. In order to attack the problem from multiple fronts, it will be necessary to gain better understanding of all processes that can lead to telomere maintenance.

References


