STUDIES OF THE MECHANISM OF RECOMBINATIONAL TELOMERE ELONGATION IN AN ALTERNATIVE LENGTHENING OF TELOMERE (ALT)-LIKE *KLUYVEROMYCES LACTIS STN1-M1* MUTANT

by

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(Under the Direction of Michael J. McEachern)

ABSTRACT

The generation and progression of most human cancers requires a telomere maintenance mechanism. While most cancers use telomerase to maintain telomeres, 5-10% maintain telomeres by a recombination-dependent mechanism termed alternative lengthening of telomeres (ALT). The telomeres in ALT cells are highly dynamic and display heterogeneous sizes ranging from barely detectable to highly elongated. However, the details of how telomeres change and are maintained in ALT cells are unknown. Here, we have studied the ALT-like telomere dynamics in the *stn1-M1* mutant of the budding yeast *Kluyveromyces lactis*. The first study showed that telomeric circles (t-circles) could promote telomere elongation during both the establishment and the maintenance stages of the *stn1-M1* state. It also characterized the dynamics of those telomeres. We found that they displayed constant changes of the size and the structure, as well as sometimes drastic loss or gain of telomeric sequences. Our data further suggest that the turnover of sequences involves a concerted amplification mechanism, an outcome consistent with copying t-circles. . In the second study, we examined telomere elongation during the establishment stage of the *stn1-M1* state by using mutationally-tagged telomeric repeats. Our

results suggested that before net telomere elongation occurred, telomeres first undergo a sudden shortening to lose the outermost telomeric repeats. Our results also showed that, as predicted by the roll-and-spread model, all elongated telomeres were commonly derived from sequence coming from a single telomere. Meanwhile, we also found that mismatch repair and telomerase had large effects on spreading of sequences from one mutationally-tagged telomere to others. Our study expands the knowledge of the mechanism of runaway recombinational telomere elongation in budding yeasts and provides clues to understand the mechanism of telomere generation and maintenance in ALT cells.

INDEX WORDS: telomere, recombination, alternative lengthening of telomeres (ALT), *stn1-M1*, *Kluyveromyces lactis*.

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DEDICATION

I would like to dedicate this thesis to my parents, who provide me the great freedom to choose my own territory to explore, the constant attention to make me feel important to this world and the unyielding support to enable me to go forward when facing all kinds of difficulties. Meanwhile, I would like to dedicate this thesis to my most-trusted and supportive friend and partner, Qiaozhi, whom I will walk with to the end of my life. I also would like to dedicate this thesis to my son, Yale (Chengye), who brings me endless joy and excitement, and lets me understand what my parents have been through to make me a man.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

A BRIEF HISTORY OF THE DISCOVERY OF TELOMERES

Telomere, a word derived from greek noun telos “end” and meros “part”, is named for the region at the ends of linear eukaryotic chromosomes, which protects the inner chromosomes from damage. As early as the 1930s, the linear eukaryotic chromosomes were believed to contain a specialized structure to protect ends when Hermann J. Müller found that the ends of irradiated chromosomes in the fruit fly *Drosophila melanogaster* were different from the other parts and free from alterations (154). Two years later, when Barbara McClintock studied dicentric chromosomes while performing corn genetics she proposed that a telomeric structure was required for maintaining the chromosome integrity and to prevent the end-to-end fusion formed by broken ends, which would result in a “breakage-fusion-bridge” cycle that was deleterious to cellular survival (139). Thirty years later, in the 1970s, both James D. Watson and Alexei M. Olovnikov independently recognized that the incomplete synthesis of the lagging strand during DNA replication would lead to the continual loss of chromosomal sequencse. This is because, during the lagging strand synthesis when RNA primers are removed from the Okazaki fragments, a gap will be left at the 3’ end of the DNA (164, 226). Considering Leonard Hayflick’s hypothesis of limited divisions in human somatic cells (83), Alexei M. Olovnikov suggested that

chromosomal sequences would be gradually lost during cell division until reaching a critical level, when cell divisions stopped (164). James D. Watson proposed that some protective mechanism was needed to prevent chromosomal shortening (226).

In late 1970s, Elizabeth H. Blackburn and Joseph G. Gall discovered that the ciliated protozoan *Tetrahymena thermophila*, in addition to the micronucleus with normal chromosomes, contained a macronucleus with fragmented chromosomes, the ends of which were composed of a tandemly repeated hexanucleotide sequence 5'CCCCAA3' (16). Later on, Jack W. Szostak and Elizabeth H. Blackburn demonstrated the telomere function of the terminal repetitive sequences from *Tetrahymena*, by joining the terminal fragments from *Tetrahymena* to a yeast vector to construct a linear plasmid, which could be stabilized in yeast. Their work also suggested that an unknown enzyme synthesized the telomeres (205). This enzyme, later called telomerase, was first identified by Carol W. Greider and Elizabeth H. Blackburn in *Tetrahymena* cell- free extracts. They showed that telomerase had the activity to add 5'TTGGGG3' repeats onto a synthesized primer (72). The identification of human telomeres (153) and human telomerase (151) sparked research in both the cancer and cellular senescence fields, which will be described in more detail below.

TELOMERIC STRUCTURES AND FUNCTIONS

Telomeres in budding yeast

Telomeres are composed of telomeric DNA and its associated proteins (144, 237). Telomeric DNA in most eukaryotic species are composed of tandem arrays of short repeats (usually 5-26 bp), typically with G-clustered sequence on the strand with 5' to 3' orientation toward chromosomal ends. Some species, including mammals (51), the budding yeast

Kluyveromyces lactis (142), the ciliated protozoan *Tetrahymena thermophila* (16) and the plant *Arabidopsis thaliana* (177) (Table 1.1) have telomeric repeats that are uniform in size and sequence. However, in other species, the repeats are variable. One well known example of this is the budding yeast *Sacchromyces cerevisiae* (189), where the highly variable telomeric repeats are generated by the misalignment of the telomerase RNA template and the newly synthesized telomeric strands (173) (Table 1.1).

Over 90% of the telomeric DNA is double-stranded (47) and is associated with double-stranded DNA-binding proteins (Figure 1.1). In *S. cerevisiae*, Rap1p binds to the double-stranded telomeric DNA as a monomer via its DNA-binding domain (DBD), which contains two Myb/homeodomain-like motifs (65, 108). Rap1p negatively regulates telomere lengths (110), which involves a counting mechanism (182) that is carried out in association with the telomere length regulators Rif1p and Rif2p (80, 110, 229). Actually, counting Rap1p is indeed counting Rif proteins because tethering either Rif protein to telomeres is sufficient to shorten telomeres proportionally to the number of Rif molecules tethered (121). Genetic studies suggest that the negative regulation of telomere length by Rap1p/Rif1p/Rif2p works through inhibiting MRX/Tel1p function (30, 46, 176) that would otherwise promote telomerase recruitment and telomere elongation. This result is consistent with the fact that the telomere elongation in Rif mutants is telomerase-dependent and *RAD52*- independent (208).

Rap1p also mediates silencing at the silent mating-type loci (HML and HMR) (204) as well as near telomeres (111) by recruiting a silencing information regulator protein complex (including Sir2p, Sir3p, and Sir4p) through its C-terminal domain (171). The C terminus of Rap1p is thought to directly interact with Sir3p and Sir4p, Sir4p would then recruit Sir2p, a histone deacetylase, to deacetylate histone tail lysines, specifically H4K16. This deactivation in

turn creates a high-affinity binding site for the silencing information regulator complex to interact and modify adjacent nucleosomes and spread the heterochromatin state further along the chromosome (91, 150, 171, 181, 183, 207).

The extreme tips of telomeric DNAs are single-stranded with protruding 3' overhangs (84, 228). Despite its relatively small size compared to the double-stranded telomeric DNA, the single-stranded G tail carries out important functions as reflected by the existence of a conserved mechanism to generate it from blunt ends (47). The single-stranded G tails in budding yeast remain short (10-15 nt) for most of the cell cycle, but are lengthened transiently in the late S phase (47). Single-stranded tail formation is independent of telomerase which suggests that it may be generated from exonucleolytic end processing (135, 227). The MRX(N) (*MRE11-RAD50-XRS1* in budding yeast; *MRE11-RAD50-NBS1* in humans) complex works in concert with Sae2p, a protein with endonuclease activity (104), to contribute to the resection of C-rich strand of telomeres (29, 113). Recent data showed that the MRX complex appears only at leading-strand telomere (58). At the same time, additional activities that contribute to end processing are supplied by Exo1p, a 5'-3' double strand specific exonuclease and probably the combination of Sgs1p, a member of RecQ helicase family with 3'-5'helicase activity, along with the Dna2p nuclease (212). In summary, one model suggests that MRX-Sae2p may specialize in leading-strand telomere end processing while Exo1p, Sgs1p and Dna2p may extend the single-stranded overhangs produced by removal of RNA primers at lagging-strand telomeres (130).

The newly generated single-stranded G tails lead to the increased association of Cdc13p, a single-stranded DNA-binding protein with specificity for TG rich telomeric repeats. The peak of binding being in late S phase (162) (Figure 1.1). Cdc13p could positively regulate telomere length via recruiting telomerase (31), in which MRX and Tel1p/Mec1p also play important roles.

In late S phase, the increased association of the MRX complex to telomeres leads to the recruitment of the Tel1p protein kinase [ortholog of mammalian ATM, which belongs to phosphatidylinositol 3' kinase-like kinase (PIKK) family] through an interatction with Xrs2p (179, 192). Tel1p then phosphorylates Cdc13p at its phosphorylation sites (SQ motif) within the "recruitment domain" (215), which subsequently will recruit telomerase through its interaction with its Est1p subunit (162, 166). Est1p interacts with telomerase RNA (*TLC1*) and is important for telomerase activity *in vivo*. Mec1p (another PIKK kinase, ortholog of mammalian ATR) also plays an important role in telomerase recruitment (178); however, its role appears less central than Tel1p, because the *tel1* mutant has a more severe effect on telomere length than a *mec1* mutant. In addition to its role in recruiting telomerase, Cdc13p could prevent the formation of extended single-stranded G tails which would otherwise lead to growth arrest in a *RAD9*-dependent manner (60) and contribute to telomere capping (60, 162).

One possible mechanism by which Cdc13p prevents the single-stranded G tails from being extended further is through coordinating C-strand synthesis by interacting with Pol1p, a subunit Polα-primase complex. Consistent with this hypothesis, point mutations in either *CDC13* or *POL1* that reduces the interaction between Cdc13p and Pol1p leads to telomerase-mediated telomere lengthening (175). The understanding of telomeric capping function carried out by Cdc13p has been enhanced by the discovery of *STN1* and *TEN1*, which suppress the thermosentivitity of *cdc13-1* cells and are required for regulating the single-stranded G tails (68-69, 232). Cdc13p, Stn1p, and Ten1p form a heterotrimeric complex called the CST complex (Figure 1.1). Stn1p is proposed to negatively regulate telomere length by competing with Est1p for binding with Cdc13p as well as contributing to the capping function (31, 146). Consistent with this idea, *stn1* mutants in both *S. cerevisiae* and *K. lactis* have extended single-stranded

telomeric DNAs (69, 93). The capping function of CST is contributed by Stn1p associated with Ten1p, because co-overexpression of Ten1p and the N terminus of Stn1p could bypass Cdc13p to carry out the telomere capping function (168). Studies have shown that Stn1p interacts with Ten1p through its N terminus and with Cdc13p and Pol12p, the latter being another subunit of the Polα-primase, through its C terminus (76, 169, 174). When overexpressed, Stn1p is redirected to nontelomeric chromosome sites via its interactions with Pol12p (61). Given Cdc13p interacting with Pol1p and Stn1p interacting with Pol12p, CST may be tightly coupled with the prevention of extended single-stranded G tails by helping promote C strand synthesis. Recently, the *STN1* and *TEN1* homologs have been found in plants, fission yeast and mammalian species (136, 146, 199, 224), suggesting their conserved roles in telomere capping and regulation during evolution. Interestingly, CST is structurally related to the eukaryotic single-strand binding protein replication protein A (RPA; which is a heterotrimer of RPA70, RPA32, and RPA14 subunits in humans; and Rpa1p, Rpa2p, and Rpa3p in yeast) (63, 92, 203). These results suggest that the gene encoding CST could have evolved from RPA ancestor genes to perform specialized functions at telomeres.

Telomeres in mammalian cells

Mammalian telomeres are composed of repetitive 5'-TTAGGG-3'/3'-AATCCC-5' units and are typically 10-15 kilobases in length in humans and 20-50 kb in lab mice (51, 82, 106). The mammalian single-stranded G tail is 50-500 nt, which is thought to strand-invade into the duplex telomeric repeats forming a displacement loop (D-loop) called the t-loop (73). It has been proposed that the t-loop sequesters the chromosome ends and protect them from DNA repair machinery (73).

The telomeric DNA repeats in mammalian cells are associated with the protein complex, shelterin (50), which includes TRF1, TRF2, RAP1, TIN1, TPP1 and POT1. TRF1 and TRF2 bind the double-stranded telomeric DNA through the C-terminal SANT/Myb DNA binding domain (9, 12, 18, 38, 45, 79, 161) as homodimers or as oligomers but do not interact with each other directly (9, 18, 56). RAP1 is the human ortholog of yeast Rap1p, but unlike its yeast counterpart, mammalian RAP1 lacks DNA-binding activity and depends on TRF2 for telomeric localization (122-123). TIN1 is in the central position of shelterin to bridge TRF1, TRF2, and TPP1. TPP1 is recruited by TIN1 and will in turn recruit POT1 (127, 234). This is thought to be the major mechanism of POT1 recruitment to the telomere (89, 127, 231, 235). POT1 was identified based on its similarity to the alpha subunit of the *TEBP*α/β telomere-binding complex in the ciliate *Oxytricha nova* (6) and can bind the single-stranded telomeric DNA in G tails or in the t-loop structure through its N-terminal OB domain (6, 103, 118).

Similar to yeast telomere-binding proteins, which protect the chromosome ends from DNA repair machinery, shelterin protects the mammalian telomeric DNA from being processed as DNA double-strand breaks that would otherwise trigger growth arrest via two PIKK kinases - ATM and ATR (191). Indeed, TRF2 is involved in inhibiting the ATM-dependent pathway. A *mTRF2*-/- mutation in mouse cells, or the expression of a dominant negative TRF2 allele in human cells, generates a DNA damage signal mediated by ATM kinase (25, 52, 100), which in turn leads to the localization of *53BP1*, *MDC1*, and γ*-H2AX* to chromosome ends (48, 53, 206). These foci, named as telomere dysfunction-induced foci (TIFs), are similar to those induced by double-strand DNA breaks in inner parts of chromosomes. POT1 is suggested to be involved in repressing the ATR-dependent pathway, because both TIFs and cell cycle arrest caused by deletion of both POT1s from mouse cells were diminished when ATR but not ATM signaling

was blocked (52). A current model for repressing ATR is that POT, in concert with TPP1 (52, 90, 231), prevents replication protein A (RPA), which has non-specific single-stranded DNA binding activity, from binding to single-stranded telomeric DNA and triggering the DNA damage signal (52).

TELOMERASE AND TELOMERE MAINTENANCE

The ends of linear chromosomes are subjected to shortening because of the end replication problem (164, 226). To solve this problem, most eukaryotes use telomerase, a ribonucleoprotein reverse transcriptase, containing a catalytic protein subunit (*TERT*), a telomerase RNA subunit (*TER*) and species specific accessory proteins (13, 112, 193). The core elements of *TERT* and *TER* are conserved across species, suggesting a common role in telomere replication (33, 35-36, 134, 210). The *TERT* of vertebrates, ciliates, and yeasts contains five core domains: the single-stranded DNA-binding thumb domain; the palm domain which contains the active site for reverse transcription; the finger domain functioning in nucleotide and RNA binding; the TRBD domain, involved in telomerase ribonucleoprotein assembly and repeat addition processivity; and the TEN domain, a novel nucleic acid binding domain, which binds single-stranded DNA weakly and facilitates telomerase processivity by maintaining the association of telomerase with chromosomes during translocation (64, 94, 145, 180). A study showed that *TERT* domains are organized in a ring structure with a right-handed conformation (186), which creates a hole in the interior for holding the RNA template and binding telomeric DNA.

*TER*s are highly variable in length, ~150 nt in ciliates, ~450 nt in vertebrates and ~1300 nt in budding yeasts. Despite its variation in size, the secondary structures of *TER*s are conserved

among species. All *TER*s of ciliates, vertebrates, and yeasts contain a pseudoknot domain, which contains the RNA template for the reverse transcription, and 5' template boundary elements (TBE) that define where polymerization stops and translocation occurs (32, 34, 49, 125, 238). For yeast *TER* (*TLC1* in *S. cerevisiae*, and *TER1* in *K. lactis*), in addition to the potential pseudoknot structure required for binding yeast *TERT* protein Est2p, two other stem-loop structures were identified for binding with telomerase-associated proteins Est1p and Ku (167, 188, 217-218). The Est1p-Tlc1p and Ku-Tlc1p interaction allow the recruitment of telomerase to telomeres (188, 201).

The single-stranded telomere DNA substrate interacts with telomerase through the thumb domain of *TERT* and reverse transcription is initiated, which is carried out in the catalytic site located at the palm domain (64). After addition of each nucleotide, there will be a small translocation of the catalytic site along the template. When the reaction reaches the end of the template, defined by the template boundary element, the RNA template will dissociate from the telomeric DNA transiently and translocate to the distal region of telomeric DNA, realigning with the primer 3' end (42). During dissociation and translocation, the TEN domain, associating weakly with the DNA, will serve as the "anchor" for the telomerase (94). The repeating cycles of synthesis, dissociation, and translocation of telomerase lead to the processive addition of telomeric repeats (71).

Recent experiments have suggested that in addition to telomere synthesis, telomerases can exhibit other activities, such as affecting cellular transformation (1, 66, 202), cell proliferation (196), cell survival (59, 117), and chromatin regulation (137).

TELOMERES, REPLICATIVE SENESCENCE AND CANCER

In the 1960s, Leonard Hayflick and Paul Moorhead found that human fibroblasts in culture could not divide more than ~50 times. This was termed the Hayflick limit (83). Studies of telomeres have revealed that shortened telomeres have something to do with this limited capacity for cell divisions.

Although expressed at a high level in human embryonic stem cells and some proliferative reproductive cells as well as in some rapidly dividing pluripotent stem cells, telomerase activity is greatly reduced or transcriptionally silenced in most human adult tissues. This causes the shortening of telomeres in these tissues during cell divisions. When telomeres reach a critical minimal length, a DNA damage signal is triggered and sensed by the MRN complex, which activates a signaling transduction cascade involving ATM, ATR, CHK1 (checkpoint1), and CHK2 (checkpoint2) that ultimately activates p53 and pRB (the Retinoblastoma tumor suppressor protein) to elicit the senescence response (81). At this stage, cell volumes increase and the cells lose their original shape, accompanied by changes in nuclear structure, gene expression and metabolism (22, 158, 195). This form of senescence, caused by the continual replication in culture is termed replicative senescence to distinguish it from senescence that occurs in response to various stresses (called stress-induced senescence) (128). In some cases, cells can continue to divide if senescence is bypassed by inactivating the p53 and pRB pathways, with the result that telomeres continue to shorten until cell death arise from chromosome fusions and genomic catastrophe, an outcome termed “crisis” (190). It has been suggested that shortened telomeres would cause senescence and crisis; however, several recent studies argue that it is not the actual shortened telomere length but the abnormal “uncapping” of the telomere, possibly as a result of telomere shortening, that activates the senescence and crisis response (14-15). First, overexpression of TRF2 in human fibroblasts showed accelerated telomere shortening, even

though cells underwent the same number of cell divisions before the senescence began (101). Second, disruption of the telomere binding protein TRF2 activates the senescence response, even when the telomeres are longer than those of control cells (24, 100, 114, 197, 223).

When the p53 pathway is intact, shortened telomeres serve as potent tumor suppressors by activating replicative senescence to prevent tumor formation. However, a dysfunctional p53 pathway will result in an environment allowing cells to proliferate and to tolerate the genomic damage, which will eventually lead to cancer (See Figure 1.2). Experiments showed that in contrast to G5/G6 (5^{th}/6^{th} generation) mice with a *mTERC*-/- background, which causes high rates of aging-related phenotypes, that correlated with shortened telomeres (116), the G5/G6 mice with *mTERC-/- p53-/-* background showed dramatic restoration of cellular proliferation, and reduction of apoptosis in many tissues (39). However, these mice also showed significant acceleration of tumor formation (2) and displayed the non-reciprocal translocation of chromosomes, the hallmark of genome instability due to telomere dysfunction that leads to telomere fusion and breakage-fusion-bridge cycles (2). Therefore, shortened telomeres, combined with an impaired p53-dependent DNA break repair pathway, leads to the accelerated carcinogenesis.

Studies have shown that most human cancers activate telomerase at some point during tumorigenesis to maintain their telomeres and that this event will immortalize the cells (44, 105). Telomerase activity is largely regulated at the level of transcription of the *TERT* gene (17). The *TERT* promoter is controlled in some contexts by the c-Myc oncogene (70, 109), which may be activated during tumor development. Studies have found that the relationship between activation of telomerase and promotion of tumorigenesis is complex. Indeed, if telomerase is activated in cells that have already entered the breakage-fusion-bridge cycles, telomeres will be stabilized in

the presence of ongoing genome instability from broken chromosomes that will advance the cells towards malignancy. However, if telomerase is activated before the cells enter the breakage-fusion-bridge cycles, telomeres are stabilized and not processed by the non-homologous end joining (NHEJ) machinery. In this case, telomerase will prevent genomic instability and suppress tumor formation (3).

ALTERNATIVE LENGTHENING OF TELOMERES

In another 5-10% of human cancer cells, there is no detectable telomerase activity, demonstrating that telomeres are maintained by a telomerase-independent mechanism, which is referred to as “alternative lengthening of telomeres” (ALT) (20). ALT occurs in ~50% of osteosarcomas (19, 86, 185, 220), 30% of soft tissue sarcomas (43, 77, 86, 98, 138, 148, 233), 25% of glioblastoma multiforme (78, 86), and 10% of neuroblastomas (165). ALT is more abundant in sarcomas of mesenchymal origin, but also occurs rarely in carcinomas of epithelial origin (87). These observations may reflect tighter regulation of telomerase in the tissues of mesenchymal origin (86-87). Similar to telomerase-positive cancers, ALT cancers are associated with mutations of p53 (88), which seems to be important for unblocking homologous recombination between telomeres (62) and allowing the ALT cells to continue to proliferate in the presence of telomere damage signals (28, 148, 187, 200, 219).

One hallmark of ALT cancers is that telomeres are heterogeneous and long, spanning from <2 kb to >50 kb (87, 95, 155). Another characteristic phenotype of ALT is the presence of a specific subcellular organelle called ALT-associated PML (promyelocytic leukemia) bodies (APB) (236), whose composition includes telomeric DNA, sheltein proteins, and many other proteins involved in DNA repair, replication and recombination such as MRN and the SMC5/6

complex (87, 96-97, 172, 236). Several studies have suggested that APBs may be the sites of ALT activity, because live-cell imaging showed that telomeres associate and dissociate with APBs in a dynamic manner (147). Also, APBs are enriched during the G2 phase, when homologous recombination is most active and ALT activity may occur (74, 230). Experiments showed that shelterin proteins TRF1, TRF2, TIN2, and RAP1, the PML protein, the MRN complex and the SMC5/6 complex are all required for APBs' formation (97, 172).

Although not fully understood, the mechanism of telomere maintenance in ALT cells is suggested to be recombination-dependent. First, a DNA tag sequence inserted in the telomere was shown to be copied onto other telomeres in ALT cells, but not in telomerase-positive cells (54). Second, highly elevated post-replicative telomere exchange occurs in ALT cells compared to non-ALT cells (8, 129). Third, in ALT cells, the extrachromosomal DNA circles composed of telomeric repeats, called t-circles, are present (26-27, 57, 225, 239-240). T-circles are also present in the cells with mutations in telomeric proteins, TRF2, and POT1A (one isoform of POT1 in mice) in a NBS- and XRCC3-dependent manner (225, 230), suggesting that t-circles are generated by improper resolution of the t-loop junction by homologous recombination (21, 133). In addition, C-rich t-circles have been shown to be a specific marker for ALT (85). Their abundance is correlated with the abundance of C-strand overhangs in ALT cells (163). Meanwhile, the elevated telomeric recombination of ALT cells is reflected by the presence of the structure called the t-complex, which is proposed to consist of entangled recombination intermediates of telomeres (157).

Telomerase-independent telomere maintenance mechanisms have been found to be the normal mode of telomere mainenance in some species. For example, the telomeres in the fruit fly *Drosophila melanogaster* are composed primarily of two types of non-LTR retrotransposons,

HeT-A and TART, which can be transposed onto the very termini of chromosomes occasionally to counteract the gradual loss of the terminal sequences (10, 102, 120, 149, 214, 222). Chromosomal telomeres in the midge *Chironomus* (41, 184), the mosquito *Anopheles gambiae* (11), and the plant *Allium cepa* (170), as well as mitochondrial telomeres in cilates (152) and some yeasts (211), are likely elongated by recombination.

RECOMBINATIONAL TELOMERE ELONGATION IN YEAST

The mechanism of how telomeres are maintained through recombination has been extensively studied in budding yeasts. In *S. cerevisiae*, shortened telomeres caused by deletion of telomerase subunits *EST1*, *EST2*, *EST3*, or *TLC1* will lead to replicative senescence (4, 119, 132, 194). The defective growth capacity of yeast cells during senescence correlates with shortening of telomeres in the cells, which are normally a few hundred base pairs and shortened at 3-5 bp/cell division in the absence of telomerase (132, 142, 194). The poorest growth point closely correlates with telomeres at their shortest size (usually below 100 bp) (142). Senescence becomes prominant when telomeres are below a certain size threshold, such that an initially longer telomeres will delay the onset of senescence while a shorter telomere will accelerate senescence (126, 140, 198). The cell growth arrest depends on the DNA damage checkpoint proteins, *MEC1*, *DDC2*, *MEC3*, and *RAD24* (4, 55). Although most highly senescent cells do not survive, occasional yeast cells can restore their growth capacity by lengthening their telomeres using homologous recombination, a process referred to as “recombinational telomere elongation (RTE)” (115, 131, 140). RTE in yeast is a process similar to ALT in human cells. The emerging surviving cells are called post-senescence survivors. These cells depend on homologous recombination because deletion of *RAD52* will eliminate virtually all the survivors (131, 140). There are two types of post-senescence survivors existing in *S. cerevisiae* (143). Type I survivors

are characterized by the large scale amplification of subtelomeric Y' elements with little extension of telomeric repeats (131). This process requires *RAD51*, *RAD54*, *RAD55*, and *RAD57* (115, 209), suggesting that they are formed by the canonical *RAD51*-mediated strand invasion pathway. On the other hand, type II survivors feature the amplification (lengthening) of most telomeric sequences with little or no Y' element amplification (131). This process depends on *RAD50*, *RAD59*, and *SGS1* (a helicase with homology to the human BLM protein) (37, 40, 115, 208-209, 216), suggesting that their formation involves strand-displacement, resection, and single-stranded annealing. The growth of type I survivors is slower than that of the type II survivors and consequently, even though they arise less frequently than the type I survivors, the type II survivors can outgrow the type I survivors in long term culture, especially when grown in liquid medium (143).

In *K. lactis*, because of the lack of subtelomeric blocks of repeated sequences, there are no type I survivors and only type II post-senescence survivors emerge (140). The genetic requirement for *K. lactis* type II survivors seems to be different from this *S. cerevisiae* counterpart, since deletion of genes in both the *RAD51* pathway and the *RAD50* pathway appears to be needed to appreciably disrupt survivor formation (Basenko and McEachern, manuscript in preparation). Also, deletion of *RAD52*, despite greatly reducing survivor formation, does not eliminate survivors completely (140).

ROLL AND SPREAD MODEL OF RTE

Several models have been proposed for RTE, including intertelomeric recombination, intratelomeric recombination, and rolling-circle DNA amplification (143). Several lines of evidence support a mechanism involving both rolling-circle DNA amplification and

intertelomeric recombination, called the roll-and-spread model, which states that a t-circle could be generated when telomeres are shortened to a critical minimal size, and then the t-circle will be utilized as the template to elongate one shortened telomere, followed by the rest of the short telomeres copying the sequence from the elongated telomeres through a break-induced replication-like intertelomeric recombination mechanism (143, 159) (Figure 1.3). One form of evidence for this model is that t-circles can be visualized by electron microscopy from a *K. lactis* mutant (*ter1-16T*) with long telomeres (75). These t-circles include both double-stranded and single-stranded circles of telomeric DNA that are formed in a *RAD52*-dependent manner. Circles down to ~100 bp/nt has been observed. A second type of evidence for the roll-and-spread model is that, after being transformed with a DNA circle containing both telomeric repeats and a selectable marker *URA3*, the telomeres in senescencing *ter1*-Δ cells acquired tandem arrays of *URA3*-telomere units, indicating that circles could be used to amplify telomeres in cells with shortened telomeres (159-160). Experiments that transformed mixtures of two types of *URA3*-telomere circles differing by only a single restriction site suggested that a single transforming t-circle molecule was responsible for all integrated copies (sometimes >100) of the integrated sequence of the circle (160). Third, when the post-senescence survivors are generated from a precursor with both basal wild type repeats and terminal mutationally-tagged repeats (Bcl repeats; carrying a phenotypically silent base change that generates a *Bcl*I restriction site), some survivors were found to contain most or all of their telomeres containing the same simple repeating pattern of wild type and Bcl repeats. This result, suggested that a circular DNA composed of both kinds of repeats could be formed and used during RTE and was the ultimate source of the sequence of most or all lengthened telomeres (160). An additional result directly demonstrated that the sequence from one telomere could be spread to all others during survivor formation. This

experiment was done by introducing a single telomere with totally mutationally-tagged Bcl telomeric repeats into *K. lactis* cells prior to the deletion of telomerase and showing that the sequence of the Bcl telomere spread to all the telomeres in the eventual survivor (213). The spreading occurred in an all-or-none fashion with ~10% of survivors derived from cells containing a normal length Bcl telomere. This frequency is consistent with one out of the twelve telomeres being randomly chosen to be amplified and spread to all the other telomeres. Additionally, when an extra long single telomere with only Bcl repeats was used in the same experiment, (which mimics a transient stage where a single long telomere formed as predicted by the roll-and-spread model), the Bcl sequences were found to be spread to all the telomeres in ~95% clones. Both of these outcomes are consistent with predictions of the roll-and-spread model (213). Studies of type II RTE in *S. cerevisiae* have also suggested that it involves a roll-and-spread mechanism (124).

TYPE IIR RTE AND *STN1-M1* MUTANT

In addition to the shortened telomeres resulting from telomerase mutations, mutations affecting telomere capping proteins can also initiate RTE. For example, in a *cdc13-1 mec3* double mutant, cells display senescent growth and then generate survivors (67). Only type II RTE survivors were found in this mutant, and their formation required *RAD50* and *RAD52* but not *RAD51* (67). These data suggested that RTE can be initiated when telomeres are at or near normal length but have lost this normal protective structure (143). Another interesting example is exhibited by the *K. lactis stn1-M1* mutant, which has a point mutation in the single-stranded telomeric DNA binding protein Stn1 that results in dysfunctional telomere capping (93). The *stn1-M1* mutant displays a chronic growth problem resembling a moderately senescent state, and contains heterogeneous, very long telomeres maintained in a telomerase-independent manner.

These traits are very similar to those in the human ALT cells. The RTE in *stn1-M1* is designated as "type IIR" for the "runaway" telomeres to distinguish from the much more modest telomere elongation in type II *ter1*-Δ post-senescence survivors (93) (Figure 1.4). Telomeres in *stn1-M1* cells are defective in capping, because they have long single-stranded G tails, highly elevated recombination near telomeres, and rapid telomere shortening upon introducing the wild type *STN1* gene (93). Recent work also showed that t-circles are abundant in *stn1-M1* cells (5). Other examples of type IIR RTE have subsequently been seen in *ter1-Δ* mutants that maintain certain mutant telomeric repeats (7, 213). In each of these cases, cells again display a chronic modest growth phenotype and very long and heterogeneous telomeres. The mutant repeats that induce this phenotype have additional defects at being able to negatively regulate sequence addition by telomerase (7, 221). This suggests that the same capping defect may render telomeres to be simultaneously prone to elongation by both telomerase and recombination.

FOCUS OF THIS STUDY

The *stn1-M1* mutant, which displays many similarities with human ALT cells, provides a good model system to study the recombinatioal telomere maintenance mechanism. The studies in this work suggest that a mechanism involving rolling circle and intertelomeric recombination occur during the establishment and maintenance of long telomeres in *stn1-M1* cells. The evidence further suggests that telomeres in *stn1-M1* cells undergo dramatically increased recombination compared to those in the wild type cells.

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Table 1.1. Organisms and their telomeric repeat sequence

Organism Name	**Telomeric repeat sequence**	**Reference**
Oxytricha nova	TTTTGGGG	(107)
Tetrahymena thermophila	TTGGGG	(16)
Saccharomyces cerevisiae	$TG_{2–3}(TG)_{1–6}$	(141, 189)
Schizosaccharomyces pombe	G2–8TTAC(A)	(99, 156)
Kluyveromyces lactis	ACGGATTTGATTAGGTATGTGGTGT	(141)
Candida albicans	ACGGATGTCTAACTTCTTGGTGT	(141)
Caenorhabditis elegans	TTAGGC	(23)
Arabidopsis thaliana	TTTAGGG	(177)
Homo sapiens	TTAGGG	(153)

Fig. 1.1. Telomere and telomeric proteins in budding yeast.

This schematic illustrates the DNA-protein structure of telomeres in budding yeast. The single-stranded telomeric DNA is bound by Cdc13, which can associate with Stn1 and Ten1 to form a CST complex to negatively regulate telomere elongation by competing with telomerase (Est1/2/3/Tlc1) for binding with Cdc13. CST complex also contribute to protecting the telomeric ends by forming the capping structure. The double-stranded telomeric DNA (double- line white boxes) is directly bound by Rap1, which can interact with Sir2/3/4 and Rif1/2. Other protein complexes such as the Ku70/80 complex, MRX (Mre11-Rad50-Xrs2) complex are also associated with telomeres and play important functions in telomere protection and length regulation.

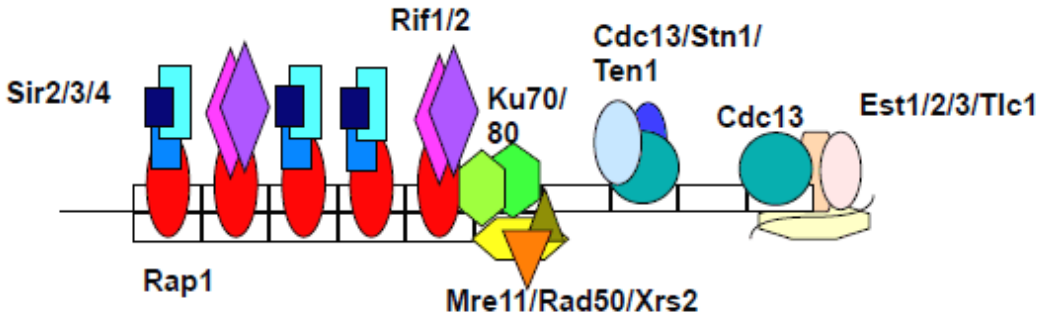

Rif1/2
Cdc13/Stn1/
Ten1
Sir2/3/4
Ku70/
80
Cdc13
Est1/2/3/Tlc1
Rap1
Mre11/Rad50/Xrs2

Fig. 1.2. Telomere length shortening in human somatic cells and maintenance in immortalized and cancer cells.

The graph illustrates how telomere length (on Y-axis) of human somatic cells decreases over successive cell divisions (population doubling on X-axis). The shortening telomeres will trigger replicative senescence at a certain length. Inactivation of p53/Rb allows cells to continue to divide and to enter into “crisis”, when chromosomal instability, genetic catastrophe and eventually, cell death happen. Activating telomerase maintenance mechanisms (TMM) including telomerase and ALT will restore telomere function. If TMM is activated before the genetic catastrophe occurs, the cells will be immortalized without being malignant, while if TMM is activated after the genetic catastrophe occurs, the cells may be cancer-prone.

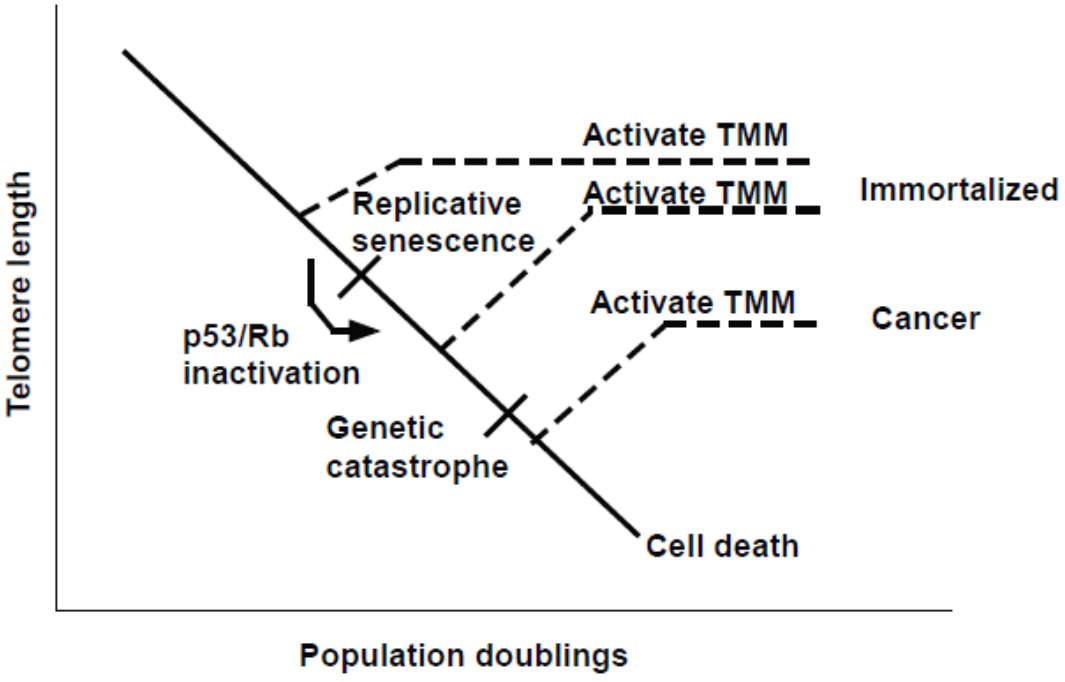

Telomere length
Activate TMM
Activate TMM
Immortalized
Replicative
senescence
Activate TMM
Cancer
p53/Rb
inactivation
Genetic
catastrophe
Cell death
Population doublings

Fig. 1.3. The Roll-and-Spread model

Telomeres in a *ter1-Δ* mutant are shortening during cell divisions. A telomere reaching a critical minimal size could trigger the cell growth arrest and initiate recombination in telomeric and sometimes subtelomeric regions. Telomeric recombination can produce telomeric circles (t-circles) at a low frequency. T-circles could be utilized as templates to generate the first long telomere, and the sequence of this elongated telomere will be copied by other short telomeres through break-induced replication (BIR)-like gene conversion to generate multiple long telomeres. Figure is adapted from (143).

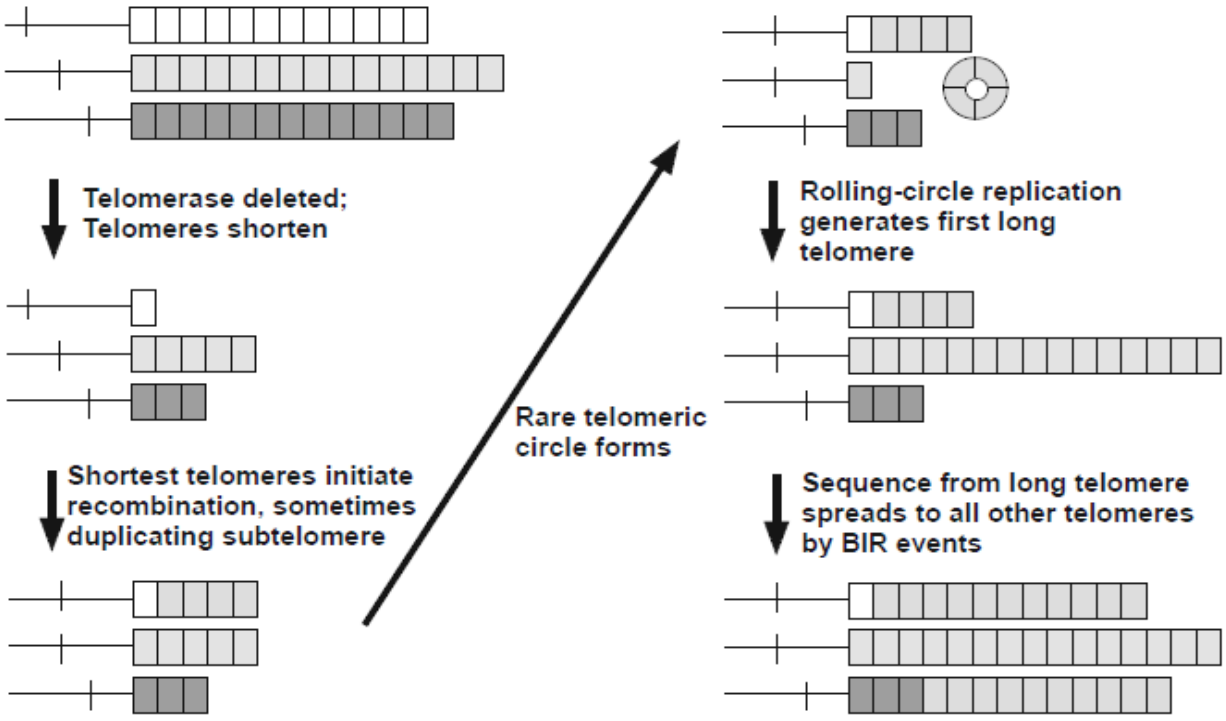
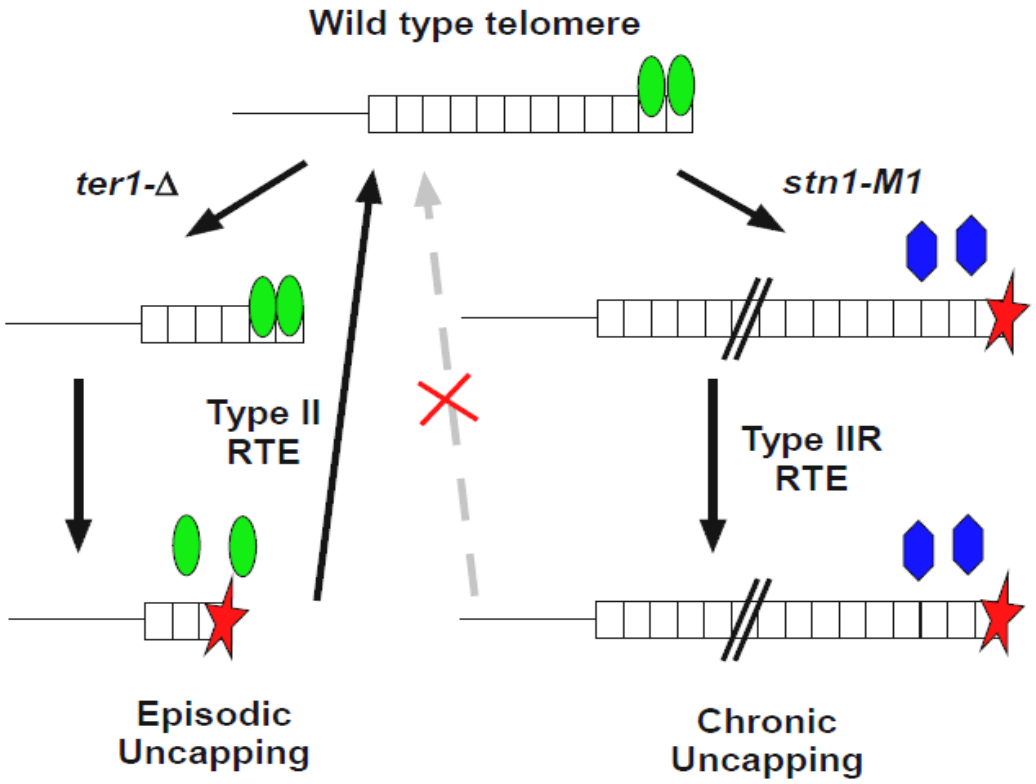

Telomerase deleted;
Telomeres shorten
Shortest telomeres initiate
recombination, sometimes
duplicating subtelomere
Rare telomeric
circle forms
Rolling-circle replication
generates first long
telomere
Sequence from long telomere
spreads to all other telomeres
by BIR events

Fig. 1.4. Differences between type II RTE and type IIR RTE

Type II RTE occurring in a *ter1-Δ* mutant is initiated when the shortening telomeres reach critical minimal length and presumably lack of enough space for adequate binding of telomeric proteins, which generates uncapped (non-protective) telomeres. Once telomeres are elongated to above ~100 bp, telomeres become resistant to further recombination. Therefore, telomeres in *ter1-Δ* cells are episodically uncapped. Type IIR RTE occurring in *stn1-M1* mutant is initiated because Stn1p is defective (perhaps due to a defect in binding telomeres), which generates chronic uncapped telomeres. Therefore, no matter how long the telomeres are that are produced by RTE, the chronically uncapped telomere state will make it continuously subject to recombination and the RTE will be going on independent of telomere length.


Wild type telomere
ter1-Δ
stn1-M1
Type II
RTE
Type IIR
RTE
Episodic
Uncapping
Chronic
Uncapping

CHAPTER 2

TELOMERIC CIRCLES AND CONCERTED TELOMERIC AMPLIFICATION IN AN ALT-LIKE YEAST MUTANT.[1]

[1] Jianing Xu and Michael J. McEachern. To be submitted to *MCB*

ABSTRACT

Some cancers utilize the recombination-dependent process of alternative lengthening of telomeres (ALT) to maintain long heterogeneous telomeres. ALT is associated with the generation of telomeric circles (t-circles) whose role in promoting telomere elongation has been suggested but not demonstrated. Here, we present evidence that t-circles can promote recombinational telomere elongation in the ALT-like *stn1-M1* mutant of the yeast *Kluyveromyces lactis*. Transformation of a t-circle into *stn1-M1* cells led to the formation of long tandem arrays of the t-circle's sequence that was incorporated into multiple telomeres. Moreover, the unselected presence of a replicating t-circle at the time of establishment of the ALT-like state efficiently led to similar t-circle-driven telomere elongation. We further show that telomeric DNA in *stn1-M1* cells is subject to rapid variation in its total amount and is apparently mostly extrachromosomal. Tandem arrays derived from t-circles also exhibit very high instability. Novel-sized telomeric repeat blocks arise frequently and sometimes become amplified in a concerted fashion. Based on our results, we propose that new telomeric sequences are often generated in *stn1-M1* cells by rolling circle copying of t-circles and that subsequent stochastic break-induced repair (BIR) events can spread the amplified sequence to multiple other telomeres.

INTRODUCTION

The ability of human cancer cells to grow indefinitely requires maintenance of their telomeres (70). In most human cancers, telomere maintenance is achieved by up-regulating telomerase activity (40). However, 5-10% of human tumors cells maintain their telomeres through a telomerase-independent mechanism, termed Alternative Lengthening of Telomeres (ALT) (for a review, see (14)). A general hallmark of ALT cells is their extremely heterogeneous and often highly elongated telomeres, which span from undetectably short to >50 kb (33, 37, 51,

61). Another characteristic phenotype of ALT is the presence of a specific subcellular organelle called ALT-associated PML (promyelocytic leukemia) bodies (APB) (79), whose composition includes telomeric DNA, telomere-associated proteins and many other proteins involved in DNA repair, replication and recombination (33, 38-39, 64, 79). Several more direct lines of evidence have suggested that telomere maintenance in ALT cells depends on recombination. First, a DNA tag sequence inserted in a telomere was shown to be copied onto other telomeres in ALT cells, but not in telomerase-positive cells (21). Second, there are highly elevated post-replicative telomere exchanges, including sister chromatid exchanges, occurring in ALT cells compared to non-ALT cells (1, 6, 43). Third, ALT cells contain abundant extrachromosomal telomeric circles (t-circles), which are proposed to be generated from resolving the loop structure formed by intratelomeric recombination (11-12, 22, 77, 80-81). T-circles with their C-rich strand and at least partly single- stranded structure have emerged as a particularly reliable indicator of the ALT state (32). Despite these advances, the mechanism of recombination-dependent telomere maintenance in ALT cells remains poorly understood.

The mechanism of recombinational telomere elongation (RTE) has been extensively studied using telomerase deletion mutants in the budding yeasts *Sacchromyces cerevisiae* and *Kluyveromyces lactis*. Such mutants display gradual telomere shortening and growth senescence followed by the *RAD52*-dependent emergence of better growing post-senescence survivors with elongated telomeres (44, 46, 48). Two types of RTE (termed I and II) have been found in *S. cerevisiae*. Type I survivors are characterized by amplification of subtelomeric Y' elements and short telomeric tracts while type II survivors are characterized by long tracts of telomeric repeats (71). The two survivor types differ not only in the end structures they contain, but also in the genes responsible for their formation (16, 19, 41, 74).

Only type II RTE has been found to occur normally in *K. lactis* telomerase RNA deletion mutants (*ter1-Δ*) (46). The telomeric tracts present in *K. lactis* post-senescence survivors are longer than those seen in senescent cells, but typically only hundreds of base pairs in length. Studies of these mutants have suggested that type II RTE involves a roll-and-spread model. According to this model, a tiny t-circle is copied by a rolling-circle mechanism to generate one long telomere, the sequence of which is then spread to all other telomeres by break-induced replication (BIR)-like gene conversion events (30, 54-55, 73). This model was originally suggested by the fact that post-senescence survivors generated in cells with two kinds of telomeric repeats often emerge with a common repeating pattern among most or all telomeres. Transforming DNA circles containing telomeric repeats and a marker gene into *K. lactis* telomerase deletion mutants led to addition of long tandem arrays of the transformed sequence onto telomeric ends. Mixing experiments done with two kinds of such t-circles, differing by just one restriction site, indicated that the integrated long tandem arrays arose from a single transformed molecule (55). T-circles as small as 100 nt have been shown to promote telomere elongation *in vivo* in *K. lactis* (54). Even smaller DNA circles (including t-circles) have been shown to be suitable substrates for rolling circle synthesis *in vitro* (23, 31, 42). T-circles from ~100 bp to >10,000 bp have been shown to be abundant in certain yeast mutants with abnormally long telomeres (3, 30). Important other evidence for the roll and spread model came from the demonstration that the sequence of lengthened telomeres in post-senescence survivors originated from a single telomere source (73).

Work in recent years has expanded our views of the circumstances where RTE can occur and the forms it can take. *K. lactis* mutants with very weak telomerase activity can use recombination to maintain very short telomeres or to occasionally generate survivor-like cells

with all telomeres lengthened, the latter likely via a roll and spread mechanism (2). Combining a *ter1-Δ* mutation with telomeres composed of certain mutant telomeric repeats, including ones defective in binding the double-strand telomere binding protein Rap1, leads to type IIR 'runaway' RTE, which is characterized by an ALT-like state with much longer and more heterogeneous telomeric repeat tracts than is seen in other *ter1-Δ* mutants (4, 73). Rapid formation of long telomeric tracts by RTE occurring without gradual growth senescence was observed when a mutation in *MEC3* was combined with *cdc13-1* and when a deletion of *YKU70* was combined with *cdc13-1* or *tlc1* mutations in *S. cerevisiae* (25, 27, 59). RTE, in at least some cases associated with an abundance of t-circles, is also thought to be responsible for the maintenance of telomeres at the ends of linear mitochondrial DNA in some species of ciliates and yeasts (50, 58, 72).

The initial description of type IIR RTE was made with the *K. lactis stn1-M1* mutant (36), which contains an amino acid substitution in a component of the RPA-like Cdc13/Stn1/Ten1 (CST) complex that binds and protects the 3' single-stranded telomeric overhang [for review see (65)]. Stn1 regulates telomerase addition as well as interacts with Polα/primase (26, 66). The *stn1-M1* mutation exhibits a number of strikingly similar phenotypes with those of human ALT cells. The most obvious of these is the continuous presence of very long and heterogeneous telomeres generated from homologous recombination. As in ALT cells, telomeres in *stn1-M1* cells produce abundant t-circles (3, 12, 77) and are subject to high rates of recombination specifically in or near the telomeres (21, 36). Both *stn1-M1* and ALT cells also lack obvious signs of either growth senescence or survivor formation and appear instead to have chronic but slight growth defects (33, 36, 67). Finally, the presence of telomerase activity does not suppress the phenotypes of either *stn1-M1* or that of most ALT cells (33, 36). The *stn1-M1* mutant is

therefore an excellent potential model to help understand ALT. In this report, we present data that are consistent with t-circles being the key driver of telomeric DNA maintenance in the *stn1-M1* mutant.

RESULTS

The amount of telomeric DNA in *stn1-M1* cells varies during serial passaging.

The pattern of long and heterogeneous telomeres seen in Southern blots of the *stn1-M1* mutant remains generally similar in cell populations followed over extended passaging (36). This was suggested to reflect the presence of a more or less steady state of telomeric DNA that was produced by very high rates of telomeric recombination both extending and deleting telomeric sequences. To further examine the state of telomeric DNA in *stn1-M1* cells, we serially restreaked six independent lineages of *stn1-M1* and four independent lineages of *stn1-M1 ter1-Δ* mutants and measured their relative telomeric DNA amounts at each of four consecutive streaks. Three independent DNA isolations of each overnight cell culture sample were prepared, and the telomeric signal in each was measured relative to that of a wild type control. Graphs showing the results of two representative lineages of each mutant from this analysis are shown in Fig. 2.1. Total telomeric DNA signals in *stn1-M1* and *stn1-M1 ter1-Δ* mutants among the ten sets of samples studied varied from ~8 to ~46 times from that of the wild type strain. Given that the length of the twelve telomeres in a wild type cell average ~500 bp, the total amount of telomeric DNA in the *stn1-M1* and *stn1-M1 ter1-Δ* mutants was measured to be 42-258 kb, consistent with the previous estimation (36). Our results showed that, taken as a group, there was no obvious trend toward gaining or losing telomeric DNA during the brief period of passaging that was examined. However, fluctuations in telomeric DNA amounts of up to two- to three-fold

occurred in some lineages (e.g., *stn1-M1* clone #2, Fig. 2.1). These results, while generally consistent with a steady state model of telomere lengths in *stn1-M1* and *stn1-M1 ter1-Δ* cells, indicate that a net gain or loss in a cell of up to ~100,000 bp of telomeric DNA commonly occurred in the 20-25 cell divisions of a single streak of growth.

A DNA circle containing telomeric repeats can elongate telomeres in *stn1-M1* cells via formation of long tandem arrays.

A prediction from the roll-and-spread model for recombinational telomere elongation (55) is that an exogenously transformed DNA circle containing telomeric repeats could be copied into telomeres as a long tandem array. Such an outcome is routinely observed in *K. lactis* telomerase deletion mutants (54-55). To test whether this is also true in the *stn1-M1* mutant, we transformed the 1.5 kb circle N (54), which is composed of a fragment containing *URA3* and 11.5 *K. lactis* telomeric repeats that have been ligated into a circle, into *stn1-M1* and *stn1-M1 ter1-Δ* mutants (Fig. 2.2A). We analyzed 47 *stn1-M1* transformants and 55 *stn1-M1 ter1-Δ* transformants that grew on uracil-lacking plates by Southern blotting and hybridization to determine their telomere structure. The terminal *Eco*RI fragments were detected using a subtelomeric probe that hybridizes to telomere-adjacent sequences at 11 of the 12 chromosome ends (Fig. 2.2B and E). While untransformed cells displayed a smear of subtelomeric signal in *Eco*RI digests that ran from <2 to >10 kb in size, most transformants in both mutants displayed a greater percentage of the subtelomeric signal running at >10 kb. This indicated that the average length of telomeric ends in transformants was larger than that in untransformed cells. In 34 of the 47 *stn1-M1* transformants and a lower percentage (20 of the 55; p=.006 in unpaired t-test) of *stn1-M1 ter1-Δ* transformants, most of the subtelomeric signal was cleaved to much shorter sizes (much of it 2-4 kb) by digestion with *Eco*RV, which has one cleavage site in the circle N sequence (Fig. 2.2B

and E). This was in sharp contrast to subtelomeric signal from wild type and untransformed *stn1-M1* and *stn1-M1 ter1-Δ* cells, which showed minimal shortening from cleavage by *Eco*RV (Fig. 2.2B and E) (coming from a single *Eco*RI telomeric fragment with a subtelomeric *Eco*RV site). These data suggested that the sequence of circle N had commonly become incorporated into multiple, if not most, telomeres in *stn1-M1* and *stn1-M1 ter1-Δ* transformants. As predicted from this model, *URA3* and telomere probes hybridized to the same elongated subtelomeric *Eco*RI fragments (Fig. 2.2C, D, F and G). *Eco*RV digestion, in contrast, reduced the bulk of *URA3* signal and a substantial part of telomeric signal to a band of ~1.5 kb that was often visible via ethidium bromide staining of total yeast DNA in the gels used for blotting (Fig. 2.2 and data not shown). These results were consistent with transformants commonly carrying a large number of copies of the circle N sequence integrated as tandem arrays at telomeres. Slight variations in the size of the ~1.5 kb band were observed in a few transformants (e.g., clone 3 of *stn1-M1 ter1-Δ* in Fig. 2.2F and G). These likely were due to changes in the number of telomeric repeats present in each block of repeats derived from circle N, a phenomenon seen previously with t-circles transformed into short telomere mutants and thought to be due to recombination that altered the size of the t-circle before its sequence became copied onto telomeres (54). The hybridization of the telomeric probe to the *stn1-M1* and *stn1-M1 ter1-Δ* transformants showed that they commonly retained substantial signal at very long sizes even after cleavage with *Eco*RV. This result suggested that some telomeric repeat tracts much longer than the 11.5 repeat tracts supplied by circle N were likely still present in the transformants.

Cleavage of DNA from circle N transformants with *Eco*RV was expected to remove all but one partial copy of circle N sequence from telomeres. Thus, in clonal isolates such as those examined here, we expected one or more sharp bands of precise sizes that corresponded to

junction fragments (designated as “J” in Fig. 2.2A) containing subtelomeric DNA, some number of telomeric repeats, and ~400 bp of *URA3*. Some such sharp bands, typically ranging from 1.2-3 kb were observed with a subtelomeric probe in many transformants (e.g., clones 2 and 7, Fig. 2.2B). However, much of the subtelomeric signal that was shortened by *Eco*RV cleavage remained smeared in appearance, generally at sizes greater than those of the sharp bands. This result indicated that these fragments, despite not being terminal in location, were nonetheless often heterogeneous in length. We conclude that the number of telomeric repeats that separate subtelomeric DNA from the closest integrated copy of circle N is not fully stable and often becomes heterogeneous in cell populations, presumably due to frequent ongoing recombination events.

An interesting observation with the *stn1-M1 ter1-Δ* transformants was the appearance of sharp bands that hybridized with the subtelomeric probe in some *Eco*RI-digested samples that were not affected by *Eco*RV digestion (e.g., clones 2, 3, 5, 6 and 7, Fig. 2.2E). These *Eco*RV-resistant sharp bands occurred in at least nine *stn1-M1 ter1-Δ* transformants, but only in one *stn1-M1* transformants. As these bands were not detected with *URA3* or telomeric probes, they were likely independent of the sequence derived from circle N and may represent fusions between telomeric ends that have lost all, or most all, of their telomeric repeats. Similar bands were also seen previously during passaging of *stn1-M1* cells (36). The greater frequency of the sharp bands in *stn1-M1* cells lacking telomerase might indicate that telomerase in *stn1-M1* cells can lengthen short telomeres and render them resistant to non-homologous end joining. Telomerase has previously been demonstrated to remain active in *stn1-M1* cells (36).

The copy number of the 1.5-kb *URA3*-telomere units derived from circle N that were present in the transformants was estimated using a *STN1 TER1* strain with a single integrated

copy of *URA3* as a control (49). For 10 *stn1-M1 ter1-Δ* transformants that were measured, the copy number of the *URA3*-telomere insert was estimated to be 3-135 with the mean of 43. For 14 *stn1-M1* transformants measured, the copy number of the *URA3*-telomere insert was estimated to be 6-247 with the mean of 99. The difference in mean copy number between the two mutants was significant (p=.02 in unpaired t-test). The highest copy number we observed was the equivalent of ~4% of total genomic DNA.

The remaining transformants (e.g., clone 1 of Fig. 2.2B-D) did not have a detectable 1.5-kb fragment that hybridized to telomeric and *URA3* probes in *Eco*RI + *Eco*RV-digests and presumably do not have precisely-sized tandem copies of the *URA3*-telomere unit derived from circle N. Among six such examples of *stn1-M1* transformants and 14 examples of *stn1-M1 ter1-Δ* transformants that were examined, we found that the copy number of *URA3* was always below five per cell (data not shown). The *URA3* sequences in most or all of these clones were likely to be incorporated into telomeres, given that *Eco*RV produced at least one sharp band detectable in the subtelomeric hybridization in at least 6 of 13 *stn1-M1* transformants without *URA3* arrays and at least 17 of 25 *stn1-M1 ter1-Δ* transformants without *URA3* arrays (data not shown). These clones presumably represented cases where circle N integrated as single copies or where arrays formed were very short or largely deleted after they formed. The relatively short bands produced by *Eco*RV cleavage in these non-array clones (e.g., clone 1 of Fig. 2.2B and data not shown) suggested that at least some *URA3* sequences reside close to subtelomeric sequence in these transformants.

***URA3*-telomere units are subject to very rapid changes in copy number during serial passaging.**

The heterogeneously sized telomeric fragments and high rate of subtelomeric recombination of *stn1-M1* suggested that telomeres in this mutant were very unstable and undergo high levels of recombination because of this chronic telomere capping defect (36). To study the telomere instability further, 13 independent lineages of *stn1-M1* circle N transformants and 10 independent lineages of *stn1-M1 ter1-Δ* circle N transformants were serially restreaked 4-5 times on rich medium (YPD) plates and analyzed for telomeric structure using Southern blotting and hybridization.

URA3 copy number at each streak of these lineages was measured relative to a known single copy gene control. The results from several of these clones are shown in Fig. 2.3B, and the copy number data for all the clones are shown in Table S2.1. In three control clones of wild type cells that had been transformed with circle N (two of which are shown in Fig. 2.3B), the *URA3* copy number remained essentially stable among all the streaks examined. None showed more than a 20% change in copy number from one streak to the next or from the first streak to the last. Whether the slight differences detected were due to actual copy number changes or to inaccuracies in measurement was not investigated. In contrast, all 23 of the *stn1-M1* and *stn1-M1 ter1-Δ* transformants examined showed *URA3* increases or decreases of copy number of >20% in at least one streak relative to the previous streak, and the majority showed much greater changes (Fig. 2.3B and Table S2.1). Some lineages (e.g., clone 8 of *stn1-M1 ter1-Δ*, and clone 16 of *stn1-M1*, Fig. 2.3B) lost most or all of their initial *URA3* copies during passaging, while others (e.g., clone 41 of *stn1-M1*, Fig. 2.3B) showed pronounced increases in *URA3* copy number. These results clearly show that the *URA3*-telomere arrays in the *stn1-M1* mutants were highly dynamic, with either rapid deletion or amplification events often occurring. Our data also suggested that the absence of telomerase in *stn1-M1* mutants altered the extent or nature of the

instability. Five of ten *stn1-M1 ter1-Δ* clones showed at least a two-fold reduction in *URA3* copy number over the growth course studied while only two of thirteen *stn1-M1 TER1* did (Table S2.1). Furthermore, whereas only one of the eleven *stn1-M1 TER1* clones with an initial *URA3* copy number between 20 and 200 exhibited a drop to 10 or fewer copies at any point of the growth course, four out of five *stn1-M1 ter1-Δ* clones starting in the same copy number range did. This latter difference was statistically significant (p=.006 in Fisher exact test)

One *stn1-M1 TER1* clone (with a starting *URA3* copy number of 6) and three *stn1-M1 ter1-Δ* clones (with starting *URA3* copy numbers of 135, 11 and 45) were observed to have lost all detectable *URA3* copies by the fifth streak (Table S2.1). To further study the potential for the loss of all copies of *URA3* from the telomeres of these transformants, we patched cells from single colonies of *stn1-M1* and *stn1-M1 ter1-Δ* transformants onto 5-FOA medium, which selects for Ura^- cells. Thirteen of the 20 *stn1-M1* transformants and 8 of the 13 *stn1-M1 ter1-Δ* transformants were found to produce 5-FOA resistant colonies. One or two $5\text{-}FOA^r$ clones from each transformant were examined by Southern blotting and hybridization to a *URA3* probe (Fig. 2.4A and data not shown). All 37 of these 5-FOA resistant derivatives of *stn1-M1* transformants (which initially contained 35-136 copies of *URA3*) and of *stn1-M1 ter1-Δ* transformants (which initially contained 8-152 copies of *URA3*) were confirmed to no longer contain any detectable copies of *URA3* (Fig. 2.4A). As with the Circle N transformants examined in Fig. 2.2, the transformants producing 5-FOA resistant colonies had *URA3* copies at most of their telomeres, as judged by their sensitivity to *Eco*RV digestion (Fig. 2.4B). The transformants that did not produce 5-FOA resistant colonies mostly contained >200 copies of *URA3*. These results demonstrate that even copy numbers of the *URA3*-telomere sequence of >100 that are scattered

among multiple telomeres can be completely lost from cells over a period of no more than ~25 cell divisions.

Rapid variation in the length of telomeric repeat tracts within *URA3*-telomere arrays.

The circle N used to transform cells contains a block of ~11.5 repeats in its telomeric sequence. By studying the size of this block of telomeric repeats in the circle N transformants, it was possible to determine whether any changes in its size took place after it entered into *stn1-M1* cells. DNA samples from the circle N transformants of *stn1-M1* and *stn1-M1 ter1-Δ* described above were digested with *Xma*I + *Pst*I to excise the telomeric blocks and an adjacent small section of the *URA3* gene away from the bulk of the flanking *URA3* sequences (Fig. 2.3A). A Southern blot of these samples was then probed with a combination of three labeled oligonucleotides specific to the *URA3* sequence still attached to the telomeric repeats blocks (*URA3*-HX, as indicated by black arrows in Fig. 2.3A). In the two wild type circle N transformants examined, a band of the same size as that in circle N itself was generated and remained the same size in the subsequent streaks (WT Clones 3 and 5, Fig. 2.3C). However, the six *stn1-M1 ter1-Δ* circle transformants and twelve *stn1-M1* circle transformants that were examined displayed a different behavior. As expected, in lineages such as clone 8 of *stn1-M1 ter1-Δ* and clone 16 of *stn1-M1*, where large decreases in *URA3* copy number occurred during passaging, the signal of telomeric bands showed corresponding decreases. More strikingly, while most lineages of the mutants contained primarily a single band of the same size as the fragment in the transforming circle N at the first streak, all the *stn1-M1 ter1-Δ* and *stn1-M1* mutants examined displayed multiple bands in one or more later streaks (Fig. 2.3C and data not shown). These extra bands were generally smaller than the original band, and in some cases (e.g., *stn1-M1* clone 41), the bands were clearly present in a ladder that appeared to vary by ~25 bp

steps. These results indicated that the telomeric blocks present in the tandem *URA3*-telomere arrays often became deleted through loss of unit numbers of telomeric repeats. In a few instances, a large percentage of the telomeric signal changed in size from one streak to the next. For example, in *stn1-M1 TER1* clone 41, from streak 3 to streak 4, the most intense band changed to a size apparently one repeat shorter (Fig. 2.3C). Using PhosphorImager analysis, the band in streak 4, indicated by the white arrow, was estimated as 177 copies (60% of 295 copies) whereas one streak earlier, the same sized band was estimated as being only 39 copies (11% of 359 copies). Another example of this is *stn1-M1 TER1* clone 44, where, between streak 1 and streak 2, with the *URA3* copy number increased from ~30 to ~170, most of the signal from telomeric blocks changed from the original ~300 bp fragment to bands of ~225 bp and ~250 bp. A third example is *stn1-M1 ter1-Δ* clone 6, where between streak 3 and 4, with *URA3* copy number changing from ~80 to ~90, substantial fraction of the signal from telomeric blocks changed to a much smaller fragment (Fig. 2.3C). These results indicate that a high percentage of *URA3*-telomere units derived from circle N, even up to hundreds of copies, could be deleted and replaced by a large number of new copies of a unit with a different number of telomeric repeats in a period of growth of no more than 20-25 cell divisions.

To further study the alteration of size of the telomeric blocks in *URA3*-telomere arrays, we examined seven independent colonies from the 3rd streak of *stn1-M1* clone 31 and ten independent colonies from the 3rd streak of *stn1-M1* clone 35 in the same way (Fig. 2.3D). Although subclones from the same transformant generally bear some resemblance to one another, extensive differences in the banding patterns were evident. For example, at least two of the subclones of *stn1-M1* clone 31, subclone 1 and subclone 5 (Fig. 2.3D), showed prominent smaller bands that had been preferentially amplified from streak 3 to streak 4. Some others

contained bands corresponding to fragments with all numbers of telomeric repeats from one to eleven. In subclone 5 of *stn1-M1* clone 35, a particularly intense band, indicated by a white arrow in Fig. 2.3D, comprising 65 % of the total *URA3*-telomere units, appeared at ~100 bp (4 telomeric repeats) despite this size fragment being faint or undetectable in other subclones. The sudden increase of the abundance of a particular previously faint band here and in the data shown in Fig. 2.3C, suggests that a concerted mechanism for amplifying one particular *URA3*-telomere unit can occur during the maintenance of the long telomere state of *stn1-M1* cells. Whether these newly amplified novel-sized bands reside together in tandem arrays or on a common subset of telomeres is not known.

Most of the telomeric signal from *stn1-M1* cells does not migrate into a pulsed field gel.

We next examined *stn1-M1* mutants and their transformants with *URA3*-telomeric arrays using pulsed-field gel electrophoresis. Chromosomes from both wild type and the various mutant strains examined were able to enter the gel based upon the ethidium bromide stained image of the gel (Fig. 2.5A) and upon hybridization to a single copy *RAD50* gene probe (Fig. 2.5B). Strikingly, however, 68-77% of signal to a telomeric probe of the *stn1-M1* mutants was trapped in the wells compared to only 30% in the wild type control (Fig. 2.5C). A similar but more limited retention of telomeric signal in the wells of standard gels has been previously seen for restriction-digested DNA from *stn1-M1* cells [(36) and see also Fig. 2.2D and G of this work]. Other telomere-linked sequences also showed preferential retention in wells. A probe made from a subtelomeric sequence adjacent to 11 of 12 telomeres also typically showed 32-69% of its total signal in the wells in the mutants compared to just 19% in the wild type (Fig. 2.5D). Why a single sample, the leftmost *stn1-M1 ter1-Δ* clone, behaved differently is not clear. Finally, a probe made from both *S. cerevisiae URA3* and *K. lactis RAD52* showed weak signal in wells and

hybridization to a single chromosome in the wild type and in the *stn1-M1* mutants that had not been transformed with circle N, a result consistent with detecting just the single copy *RAD52* gene (Fig. 2.5E). However, the same probe hybridized to most or all chromosomes in circle N-transformed strains, consistent with *URA3* being present at most telomeres. 70-85% of the total signal from this probe remained in the wells (Fig. 2.5E).

Overall, these data suggested that majorities of the telomeric repeats and the *URA3*-telomere units in transformed mutants as well as a significant fraction of immediate subtelomeric sequence from *stn1-M1* and *stn1-M1 ter1-Δ* cells were in structures that were detached from chromosomes in our samples and could not migrate into a pulsed-field gel. Such results conceivably could arise from a very high rate of telomeric recombination creating complex tangled molecules that often become detached from chromosomes *in vivo* or *in vitro*. Conformations of DNA previously shown not to enter pulsed field gels include large nicked DNA circles (8), DNA enriched with single-stranded tails or gaps (53) and non-linear DNA such as replication and recombination intermediates (57, 62).

We also noticed that a chromosomal band in at least two of the *stn1-M1* mutant samples had either shifted in size (marked with white arrow in Fig. 2.5C-D) or was missing (marked with asterisk in Fig. 2.5C-D). As the cells used were all haploid, it is likely that this latter event also involved a shift to a new size rather than chromosome loss. Interestingly, each of these cases of altered chromosomes corresponded to chromosome 2, the only chromosome which contains a telomere (the 2R telomere) lacking an R element, a sequence located immediately next to telomeres with homology to all other R elements (56). Because the shared sequence of the R elements provide a backup means to repair deleted telomeres via homologous recombination (49),

the 2R telomere might therefore be expected to be more prone to terminal deletions and rearrangements occurring as a consequence of telomere dysfunction.

A replicating t-circle present at the point of establishment of the *stn1-M1* long telomere state is readily incorporated at telomeres as long tandem arrays.

To test whether t-circles could be used to elongate telomeres in *stn1-M1* cells without selection, we transformed plasmid pCXJ3 (17) that contains a *K. lactis* ARS sequence and the *URA3* gene, as well as circle A, a pCXJ3 derivative containing 11.5 telomeric repeats (Fig. 2.6A) into *stn1-M1* cells complemented with a plasmid (p*STN1*) containing the wild type *STN1* gene. As expected, we found that both pCXJ3 and circle A could be maintained extrachromosomally in these cells as indicated by both the genetic instability of the *URA3* marker and the presence of *URA3*-hybridizing species of DNA running ahead of uncut genomic DNA in Southern blots (data not shown). Plasmid-transformed cells were then streaked onto rich medium plates, and colonies with the rough colony phenotype of *stn1-M1* formed after loss of p*STN1*were identified and studied. 38 independent newly generated *stn1-M1* clones were recovered from the pCXJ3 transformants, and 163 were recovered from circle A transformants. 32 of the 38 clones derived from pCXJ3 transformants and 130 of the 163 clones derived from circle A transformants were completely Ura^- when tested on plates lacking uracil and showed no *URA3* signal in Southern blots (Fig. 2.6B and data not shown) consistent with them having lost circle A. For the 33 Ura^+ newly generated *stn1-M1* clones, 10 of them, including clones 3 and 4 in Fig. 2.6B, showed *URA3* signal in bands which ran ahead of the bulk genomic DNA running at limit mobility in the gel. The bands in these clones migrated at variable positions and did not hybridize with a telomere probe suggesting that *URA3*-containing plasmids remained in these clones but that they had rearranged and no longer had telomeric repeats. These rearrangements appeared dependent

upon the *stn1-M1* mutant state as circle A molecules grown in multiple clones of wild type cells did not show evidence for rearrangements (data not shown). The remaining 23 Ura^+ newly generated *stn1-M1* clones, including clones 5 and 6 in Fig. 2.6B, all showed *URA3* signal, often intense, at limit mobility in the gel, suggesting that the *URA3* sequences from circle A was now incorporated into chromosomes in multiple copies. In marked contrast, none of the 6 Ura^+ *stn1-M1* mutants derived from pCXJ3 transformants showed altered sizes or had *URA3* signal running with genomic DNA (data not shown). Our data therefore indicates that integration into genomic DNA depends on the presence of telomeric DNA in the plasmid.

To examine the structure of telomeres in the 23 *stn1-M1* clones with integrated circle A sequences, DNA from the clones was digested with *Hpa*I and with *Hpa*I + *Xho*I and probed with subtelomeric, *URA3*, and telomeric probes (Fig. 2.6D-F). *Hpa*I does not cleave circle A and releases most telomeric ends in *STN1* cells as 3-4 kb fragments (see WT, P1 and P2 samples in Fig. 2.6D). *Xho*I cleaves once in circle A and cleaves only one of the twelve *Hpa*I telomeric fragments in *STN1* cells. Hybridization using a subtelomeric probe showed that untransformed *stn1-M1* cells (M1 in Fig. 2.6D-F) showed no *URA3* hybridization and little or no sign of shortening of the long smeared fragments visible with telomeric and subtelomeric probes. Ura^- *stn1-M1* derivatives of circle A-transformed cells, including clones 1 and 5 in Fig. 2.6D-F, behaved similarly, consistent with the telomeres in these cells containing no sequence from circle A. In contrast, in the 23 *stn1-M1* clones with integrated *URA3* sequence, including clones 2-4 in Fig. 2.6D-F, most of the subtelomeric signal was cleaved to substantially shorter sizes by digestion with *Xho*I (Fig. 2.6D). These data indicated that the sequence of circle A was present at multiple, if not most, telomeres in these clones. The *URA3* signal in *Hpa*I + *Xho*I digests of these clones was present almost entirely in a band of ~6 kb (Fig. 2.6E) that also hybridized strongly to

a telomeric probe (Fig. 2.6F). These results were consistent with the circle A sequence in these 23 clones being integrated as tandem arrays at telomeres. The copy number of *URA3* was measured in 11 of the clones and estimated to range from 3 to 137, with the mean of 30 (data not shown). Our results indicated that in the absence of selection, DNA circles containing telomeric repeats were incorporated very efficiently as tandem arrays into the telomeres of newly forming *stn1-M1* mutants.

DISCUSSION

The structure of tandem *URA3*-telomere arrays is consistent with being generated by a rolling circle mechanism.

Substantial evidence, particularly from *K. lactis*, now suggests that senescing yeast telomerase deletion mutants undergo RTE by a roll-and-spread mechanism, whereby a small t-circle is copied to make their first elongated telomere, and other telomeres then copy that sequence to make themselves longer (30, 54-55, 71, 73). However, to what extent copying t-circles can contribute to RTE in other circumstances, such as that seen in human ALT cancers, remains unclear. It is therefore critical to understand more ALT-like examples of RTE such as those seen in the *stn1-M1* mutant (36) with its long and heterogeneous telomeres and absence of obvious growth senescence. Our results here demonstrate that *stn1-M1* cells can efficiently incorporate and amplify the sequence of t-circles at their telomeres. As seen previously in *ter1-Δ* cells (55), *URA3*-telomere circles transformed into *stn1-M1* cells lead to long tandem arrays of the circle's sequence present at most chromosome ends. Notably, the blocks of telomeric repeats between the *URA3* genes within the tandem arrays were initially mostly uniform in size and matched the size of the repeat block in the transformed DNA. This result was consistent with the arrays being derived from rolling circle synthesis and is inconsistent with independent

integration of multiple t-circle molecules. Further support for this conclusion comes from results with transforming *URA3*-telomere circles into wild type and *ter1-Δ* cells which showed that the resulting tandem arrays, even when present at all chromosome ends, are derived from a single transforming t-circle molecule (55). The length of the arrays in wild type cells, commonly 10 or more copies of a 1.5 kb *URA3*-telomere unit, may provide an estimate of the length of the rolling circle DNA synthesis that can occur on the transformed t-circle.

How the telomeric tandem arrays derived from *URA3*-telomere circles become spread to most or all chromosome ends is not known but is likely to involve BIR events where uncapped chromosome ends strand invade and copy sequence from other ends. Evidence from *ter1-Δ* post-senescence survivors indicates that telomere copying events can either initiate within telomeric sequences or within subtelomeric sequences (which share sequence among 11 of 12 *K. lactis* telomeres) (49, 73). This is almost certainly true in *stn1-M1* cells as well given the very high rate of BIR-like events affecting subtelomeric regions that have been reported there (36). The number of chromosome ends that acquire *URA3*-telomere tandem arrays after transformation with circle N is undoubtedly influenced by the selection for *URA3*. Because the arrays are highly prone to deletion, having them at multiple telomeres increases the likelihood that they will not be readily lost.

Telomere maintenance in the *stn1-M1* mutant involves frequent and large telomeric deletions.

Increased recombination in and near telomeres is a common problem associated with defects in telomere capping (4, 28-29, 47, 60). However, the extent of telomere instability in *stn1-M1* cells is nonetheless striking. Previously described abnormalities in the mutant include

highly heterogeneous telomeric restriction fragments, high levels of single-stranded telomeric DNA specific to the G-rich strand, high levels of t-circles and a >5000-fold increase in the rate of subtelomeric recombination (3, 36). The molecular basis of the *stn1-M1* telomere capping defect is unclear, however, the amino acid substitution present in the mutant (I79K) resides in a region of the protein thought to be involved in binding the 3' overhang of the telomere (24, 36).

Our results show that the copy number of *URA3*-telomere units fluctuated dramatically, sometimes showing increases or decreases of more than 100 copies (totaling >150,000 bp) within a single passage on plates. Such instability was not observed with *URA3*-telomere arrays present in wild type cells. Our ability to rapidly select for *stn1-M1* cells that had lost all of their 100+ *URA3*-telomere units, despite their being initially present at multiple telomeres, argues that deletions affecting most or all telomeres are extremely common in this mutant. This conclusion is consistent with the past observations of an extremely high loss rate of subtelomeric BIR events in *stn1-M1* (36). It is also consistent with the deep turnover of telomeric repeats reported in a *K. lactis* telomerase deletion mutant that had telomeres composed of repeats with a Rap1 binding deficiency which undergoes a type IIR RTE similar to that of *stn1-M1* (4, 36).

Another sign of extreme telomere instability in the *stn1-M1* mutant was our finding that the majority of its telomeric DNA was unable to enter pulsed field gels. The simplest interpretation of this result is that the bulk of telomeric DNA in *stn1-M1* cells is extrachromosomal at any given time. Although t-circles exist in *stn1-M1* cells (3), they are not abundant enough to account for the extent of retention observed. Extrachromosomal telomeric DNA in a variety of forms has been found to exist in ALT cells, and material designated t-complex, thought to be highly branched recombination intermediates, is known to be unable to enter gels (15, 52). Such structures might be difficult or impossible for cells to effectively

resolve. Removing tangled t-complex from chromosome ends might then require cleaving off most sequence from the involved telomeres, much like removing matted hair requires a haircut.

One possible mechanism for large deletions is telomeric rapid deletion (TRD), which is thought to involve the cleavage of t-loop-like intermediate after strand invasion of a single-stranded telomeric 3' end into a more internal double-stranded part of the same telomere (5, 45, 77). In favor of this model, t-loop-like structures have been observed to be enriched in a *K. lactis* mutant with long dysfunctional telomeres and abundant t-circles (13). Resolution of such looped structures is thought to be a mechanism of producing t-circles (30, 75, 77).

Roll and spread episodes initiated by endogenously produced t-circles could account for concerted amplifications of novel *URA3*-telomere units.

The repeated observation of instances where the predominant form of *URA3*-telomere unit was rapidly and substantially replaced by a new form containing a different number of telomeric repeats argues strongly that the maintenance of telomeres in *stn1-M1* cells includes a mechanism that results in concerted amplification of particular small telomeric regions. We propose that these sequence replacement events are the result of new rolling circle copying events that occurred on *URA3*-telomere circles generated endogenously in the mutant cells. An individual rolling circle copying event, by itself, would probably be insufficient to amplify the *URA3*-telomere to the extent we sometimes observe. We suggest that the largest concerted amplifications are due to episodes where stochastic BIR events happen to copy the first amplified array onto multiple other telomeres. As this would be occurring in the absence of any selective pressure, the extent of spreading to other telomeres would generally be expected to be less than that seen with the original circle N transformations. Because we cannot detect events where existing copies of the predominant form of *URA3*-telomere unit are replaced with freshly

amplified copies of the same unit, it is likely that our results considerably underestimate the occurrence of concerted array amplifications/replacements. A model summarizing the potential role of t-circles in both the initial formation of *URA3*-telomere tandem arrays and their subsequent alterations is shown in Fig.7. Consistent with this model, t-circles of a broad range of sizes were recently shown to be common in *stn1-M1* cells (3). Additionally, *K. lactis ter1-Δ* cells and *stn1-M1* cells with tandem *URA3*-telomere arrays exhibit ladders of extrachromosomal *URA3*-telomere DNAs, presumably including t-circles, in gels ((55); data not shown).

In three of the concerted amplification events we observed (streak 4 of *stn1-M1* clone 41 in Fig. 2.3C, subclone 1 of clone 31 in Fig. 2.3D and subclone 5 of clone 4 in Fig. 2.3D), a single novel-sized fragment of telomeric repeats was markedly amplified. If derived from a t-circle, these amplifications must either have copied a circle composed of a single *URA3*-telomere unit or copied a circle with >1 *URA3*-telomere unit where the telomeric blocks in each unit were the same size. In a fourth example of concerted amplification (streak 2 of *stn1-M1* clone 44 in Fig. 2.3C), two novel-sized telomeric repeat fragments were amplified to similar extents. This might suggest that a circle composed of two *URA3*-telomere units, each with different numbers of telomeric repeats, was used as a template for rolling circle amplification.

How variant *URA3*-telomere units with differing numbers of telomeric repeats arise is not known. It presumably involves recombination events where blocks of repeats between *URA3* sequences are broken and modified prior to reforming. The significance of the blocks tending to lose repeats is also unclear. This could reflect some bias in the mechanism that alters them or it could be due to a selective advantage imparted by the shorter blocks. Conceivably, arrays with smaller blocks of telomeric repeats might be poorer targets for recombination events that cause breakages and deletions.

Although the bulk of our data in this report concern the behavior of *URA3*-telomere arrays, there is excellent reason to believe that these data are informative of the behavior of purely telomeric repeat arrays in *stn1-M1* cells. Our results showing that net changes of many tens of thousands of base pairs in the total amount of telomeric DNA can occur in the period of a single streak (20-25 cell divisions) clearly demonstrate that there is a very high level of instability in telomeric repeat arrays in *stn1-M1*. The greater average length of telomeres containing *URA3*-telomere arrays compared to those of untransformed *stn1-M1* cells (Fig. 2.2B, subtelomeric probe) might actually be an indication that *URA3*-telomere arrays are more stable than tracts of purely telomeric repeats. Conceivably, the more complex sequence of *URA3* compared to the 25 bp repeats of the telomere might act to slow homology searches during recombination.

Establishment and maintenance of type II and type IIR RTE.

The roll-and-spread model was originally proposed to explain the generation of lengthened telomeres in senescing *ter1-Δ* mutants that previously contained only extremely short telomeres (55). The type II RTE in these cells, though also involving elongation of telomeric repeat tracts, is very different from that of the type IIR RTE of *stn1-M1* cells. Because the telomere uncapping that drives it is due to short telomere size (<~100 bp), type II RTE can essentially shut down once telomeres are even moderately elongated. Only after gradual telomere shortening again reduces telomere size to below critical length will recombination again be induced. If senescing *ter1-Δ* cells contain even one telomere that is relatively long, the sequence of that telomere will be copied and spread to all other telomeres, presumably avoiding the need for copying a t-circle (73). As telomere lengths in *ter1-Δ* post-senescence survivors are generally more heterogeneous than those of senescent cells, this may imply that continued

maintenance of lengthened telomeres by type II RTE (after the initial production of survivors) might occur with less use of copying of t-circles than at the initial establishment of post-senescence survivors.

Similarly, the role of t-circles might differ between the establishment and the maintenance phases of the Type IIR RTE that occurs in *stn1-M1* cells. At establishment (i.e., the moment a *stn1-M1* mutant is first generated), there would be no long telomeres present. Rolling circle synthesis copying a t-circle might therefore be an especially effective mechanism in such circumstances to make long tracts of telomeric DNA. Among our results here, only the experiments with circle A involved the study of the establishment stage of the *stn1-M1* phenotype. Strikingly, we found that any newly established *stn1-M1* mutant that retained the full circle A sequence did so by having it integrated as tandem arrays present at most telomeres. This occurred despite the absence of selection for the *URA3* marker carried by circle A. These data demonstrate that t-circles present at the time of establishment of the *stn1-M1* phenotype can be used with considerable efficiency as templates to elongate telomeres. Whether this use occurred during or slightly after establishment cannot be determined, however.

The effect of telomerase on *stn1-M1* cells and *URA3*-telomere tandem arrays.

The initial characterization of *stn1-M1* mutant showed that telomerase is active in mutant cells but that its presence did not grossly affect growth or telomere phenotypes (36). However, here we found evidence suggesting that the presence of telomerase does have some effects on *stn1-M1* mutant cells. First, *stn1-M1 TER1* circle N transformants were more likely to acquire longer telomeres with higher average copy number of *URA3* from the integrated circle N than *stn1-M1 ter1-Δ* transformants. Second, even when comparing clones with a similar initial *URA3*

copy number, *stn1-M1 TER1* transformants were less likely to exhibit net loss of *URA3*-telomere units during the following serial restreaking than were *stn1-M1 ter1-Δ* transformants. Other data of ours argue that presence of telomerase influences *stn1-M1* cells even in the absence of *URA3*-telomere arrays. Past work demonstrated that *stn1-M1* spores produced through meiosis have poor viability (36). Through random spore analysis of a *TER1*/*ter1 STN1*/*stn1-M1* heterozygote, we have shown that *stn1-M1 ter1-Δ* spores are recovered significantly less often than *stn1-M1 TER1* spores (data not shown). This indicates that the absence of telomerase further exacerbates *stn1-M1* spore viability problems. We have also shown that the presence of telomerase lowers the frequency with which mutationally-tagged telomeric repeats present at one telomere are detected to spread to other telomeres during establishment of the *stn1-M1* mutant state (Xu and McEachern, manuscript in preparation).

Exactly how telomerase alters the behavior of *stn1-M1* cells is not clear. Conceivably, it could provide some protection against recombination by simply binding to telomeric ends. Some data in *S. cerevisiae* suggests that telomerase plays a role in protecting telomeres whose capping structures are already defective (76). More likely is the possibility that telomerase affects *stn1-M1* by adding new repeats onto telomeric ends. Lengthening of short telomeric repeat tracts in particular, might be predicted to alter how those tracts behave both with respect to their resistance to initiating recombination and to which recombination pathway they might engage in. The Rad50/Rad59 pathway, for instance, is known to be able to use shorter stretches of homology than the canonical Rad51-dependent pathway of recombination (35). In *stn1-M1* cells with *URA3*-telomere arrays, short telomeric tracts could come from near complete deletions of telomeres or from breaks that occur in the short telomeric repeat blocks within the *URA3*-

telomere arrays. Rad50 and Rad59 have been shown to be important to the recombination events that generate t-circles ((77); E. Basenko and MJM unpublished data).

Significance of our results to ALT cancers.

The phenotypes of *stn1-M1* cells are similar to those of human ATL cancer cells in many ways. In addition to having very long and heterogeneous telomeres maintained by recombination, ALT also contain abundant extrachromosomal telomeric DNA including t-circles (9, 12, 77, 79). Our results would suggest that copying of t-circles is likely to be at least part of the mechanism by which ALT cells maintain elongated telomeres. Consistent with this, depletion of NBS1, a component of the mammalian MRN complex, has been shown to reduce or eliminate t-circle production and lead to shortened telomeres in ALT cells but not in telomerase$^+$ cells (20, 82). These results are consistent with t-circles playing an important, though perhaps not essential, role in telomere maintenance from ALT. We would also predict from our results that the relative extent to which sequence derived from a single t-circle can become amplified will be less in ALT cells than in *K. lactis*. The much greater number of telomeres in humans compared to *K. lactis* would limit the ability of random BIR events to spread sequences from one telomere to a high percentage of other telomeres.

MATERIALS AND METHODS

Strains, plasmid and circles

All *K. lactis* strains used are derivative of wild type (WT) 7B520 (*ura3-1 his2-2 trp1*) strain (78). *K. lactis stn1-M1* and *stn1-M1 ter1-Δ* strains were described previously (36). Circle N that comprises a 11.5 wild type telomeric repeats and 1.2 kb *URA3* gene was described before (54). Plasmid pCXJ3 derivative Circle A was constructed by inserting 11.5 *K. lactis* telomeric

repeats that was obtained as a *Xho*I fragment from pAK25 (49) into an *Sal*I site of pCXJ3 with *URA3* gene transcribed in the same direction as the telomeric repeats. The plasmid p*STN1* was constructed in two steps. First, a 3.4 kb fragment containing the 1.3 kb ORF of the *STN1* gene and 1.6 kb upstream and 0.5 kb downstream sequences was obtained by PCR (forward primer: 5'-ACGAGCTCTGGCAACCCACTTGTGACTA-3', reverse primer: 5'-ACCTCGAGTGCTCAGCCAATTTCTGTTG-3') using the genomic DNA of WT 7B520 strain as the template. Second, the PCR fragment, which contains flanking *Sac*I and *Xho*I sites, was inserted into the *Sac*I and *Xho*I sites in the polylinker of pKL313(*HIS3*) (68) to generate the p*STN1*.

K. lactis transformation was done by electroporation as described for *S. cerevisiae* (7) except for scaling down by 1000-fold. Passaging of cells was carried out by serial streaking of single colonies on rich medium (YPD plates) at 30°C. Strains were streaked every 3 days down to single cells that grew into colonies. Each streak was estimated to be 20-25 cell divisions.

The *TER1*/*ter1-Δ STN1*/*stn1-M1* heterozygote was generated by mating the *stn1-M1 ter1-Δ* and GG1958 (*STN1 TER1 ade2*)(10). Diploid cells were sporulated and analyzed by random spore analysis as described before (10). The *stn1-M1 TER1* and *stn1-M1 ter1-Δ* clones were distinguished based on *TER1* restriction fragment length polymorphisms seen in a Southern blot.

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

K. lactis genomic DNA preparations were done in two ways. The first way yielded the high quality genomic DNA is based on the method of Philippsen, et al (63) except for two aspects: 1) the starting cultures were 1.5 ml instead of 30 ml and the reagents used in the protocol were scaled down proportionally; 2) after step 11, the DNA pellet is dissolved completely in 100 μl of 10 mM Tris-HCl, 1 mM EDTA, followed by adding 1/3 volume of 5 M

Ammonium Acetate pH 4.8 to precipitate proteins on ice for 20 min and repeat the step of 9-11 and follow the protocol to finish. The second, more rapid DNA preparation method was used for screening large number of yeast clones, and is based on the protocol of Hoffman (34) except for that instead of starting with a liquid overnight cultures, the yeast cells were collected by scooping from the surface of solid media and suspended in 0.5 ml water prior to beginning the procedure. This rapid method was only used for the uncut DNA samples isolated for Fig. 2.6B, C.

Yeast genomic DNA (cut or uncut) was run on 0.8% or 3% agarose gels and then transferred onto Hybond N+ membrane. All hybridization were carried out in Na_2HPO_4 and SDS as described (18). The telomeric probe used was Klac 1-25 (5'-ACGGATTTGATTAGGTATGTGGTGT-3') (47). The *URA3*-HX probe was composed of a combination of three oligonucleotides (5'-CTTTCCAATTTTTTTTTTTTCGT-3', 5'-CGTCATTATAGAAATCATTACG-3', 5'-TACGACCGAGATTCCC-3'), which are described in more details in the text. Both the telomeric probe and the *URA3*-HX probe were end labeled with γ-^{32}P-ATP with the temperature of hybridization and washing between 45-50°C. The subtelomeric probe was generated from pKL11-B (Insert of ~1 kb telomeric *Eco*RI-*Sma*I fragment into pBluescript SK-), which was digested with *Xba*I and ligated back together to excise all the telomeric sequence and was then digested by *Eco*RI and *Xba*I to generate a ~600 bp subtelomeric fragment for probe. The *URA3* (from *S. cerevisiae*) and *URA3* (from *S. cerevisiae*) + *RAD52* (from *K. lactis*) probes were described before (49). The *RAD50* gene probe was a ~5.9 kb purified PCR product that contains 3.9 kb ORF of *RAD50* gene from *K. lactis* genomic DNA (forward primer: 5'-AATTTGTGAGTCGGAGGACACT-3', reverse primer: 5'-GTATTGGACATGATGGTGAGCTATT-3'). The *RAD51* gene probe was a ~2.4 kb purified PCR product that contains 1.1 kb ORF of *RAD51* gene from *K. lactis* genomic DNA

(forward primer: 5'-ACGCGGCCGCCTATCGCTGTTTA-3', reverse primer: 5'-ACCTCGACGGACATGCGAGGCTGAT-3'). Subtelomeric, *URA3*, *URA3+RAD52*, *RAD50* and *RAD51* probes were prepared using an NEBlot Kit (NEB). The temperature of hybridization and washing for these probes was 65°C. The membranes were autoradiographed and visualized using a Molecular Dynamics Storm PhosphorImager. The method for the quantitation of *URA3* copy number was described before (49). The quantitation of the relative level of internal telomeric blocks in Fig. 2.3C and D was done by quantitating the signal from each small telomeric band and dividing that by the number of telomeric repeats in each block.

To measure the relative telomeric signal of *stn1-M1* and *stn1-M1 ter1-Δ* lineages, liquid cultures of cell samples were divided into three subsamples of equal volume that underwent DNA prep procedures independently. The *Eco*RI-digested DNA samples from each subsample were loaded at different positions (left, middle or right) of the gels to control the variation of uneven blocking from different positions of the gels. Because of the potential for gene duplication from chromosome rearrangements in the mutants, we used signal level from both *RAD50* and *RAD51* as internal single copy controls. The ratio between *RAD50* and *RAD51* signals were between 0.8 and 1.3 in all the samples we analyzed, consistent with the relative dosage of the two genes remaining unchanged. Telomeric signals of wild type, *stn1-M1* and *stn1-M1 ter1-Δ* strains were estimated by PhosphorImager analysis.

Pulse-field gel electrophoresis

Intact chromosome blocks were prepared as previously described (69). Contour-clamped homogeneous electric field gel electrophoresis (CHEF-PFGE) was carried out in a CHEF Mapper XA Chiller system (Bio-Rad). Chromosomes were separated in 0.8% Certificated Megabase Agarose (Bio-Rad) in 1X TBE (90 mM Tris-borate pH 8, 2 mM EDTA) at 16 °C

using an alternating electric field angle of 120° at 2.7 V cm^{-1} electrode distance with a linear ramp time of field switch of 120 -720 s for 72 h.

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FIGURES

Fig. 2.1. The amount of telomeric DNA in *stn1-M1* and *stn1-M1 ter1-Δ* strains varies during passaging. Cells from two lineages each of the *stn1-M1* and *stn1-M1 ter1-Δ* strains were passaged for 4 streaks on YPD plates. The total telomeric signal was measured on Southern blots from samples at each point. Samples were standardized using the signals from two single copy gene controls. Telomeric signal of the mutants is indicated as a ratio relative to the telomeric signal of a wild type cell culture control.

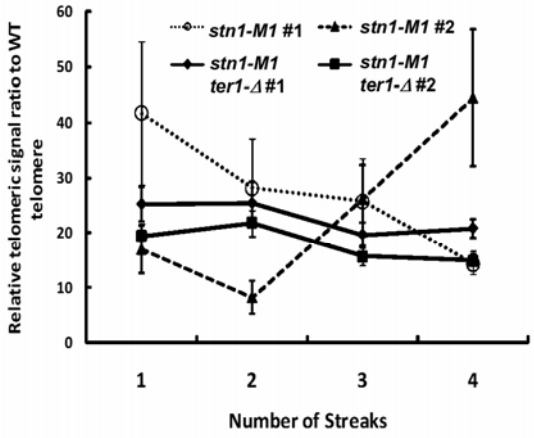
stn1-M1 #1
stn1-M1 #2
stn1-M1 ter1-Δ #1
stn1-M1 ter1-Δ #2
Relative telomeric signal ratio to WT telomere
60
50
40
30
20
10
0
1
2
3
4
Number of Streaks

Fig. 2.2. Long tandem arrays form at telomeres in *stn1-M1* and *stn1-M1 ter1-Δ* cells transformed with a DNA circle containing *URA3* and telomeric repeats. (A) Diagram of the 1.5-kb *URA3*-telomere circle (circle N) used for transformation and the structure of tandem arrays expected to be formed at telomeres from copying the sequence of circle N onto telomeric ends. Gray boxes indicate subtelomeric sequence used as a probe in panel B and E, white boxes indicate blocks of telomeric repeats and black boxes indicate *URA3*. Subtelomeric *Eco*RI sites are ~1 to 3.5 kb from telomeric ends in untransformed *K. lactis* cells. Positions of *Eco*RI sites (RI) and *Eco*RV sites (RV) are indicated. J indicates subtelomere-telomere junction fragments. (B) Southern blot, hybridized with a subtelomeric probe, of *Eco*RI (indicated by "-") and *Eco*RI + *Eco*RV (indicated by "+") digested DNA from seven *stn1-M1* strains transformed with circle N. Untransformed *stn1-M1* and *STN1* wild type (WT) controls are also shown. (C) Same filter as in panel B after stripping and rehybridization with a *URA3* probe. (D) Same filter as in panel B after stripping and rehybridization with a telomeric probe. (E) Southern blot, hybridized with a subtelomeric probe, of *Eco*RI and *Eco*RI + *Eco*RV digested DNA from seven *stn1-M1 ter1-Δ* strains transformed with circle N along with untransformed *stn1-M1 ter1-Δ* and *STN1* wild type (WT). (F) Same filter as in panel E after stripping and rehybridization with a *URA3* probe. (G) Same filter as in panel E after stripping and rehybridization with a telomeric probe.

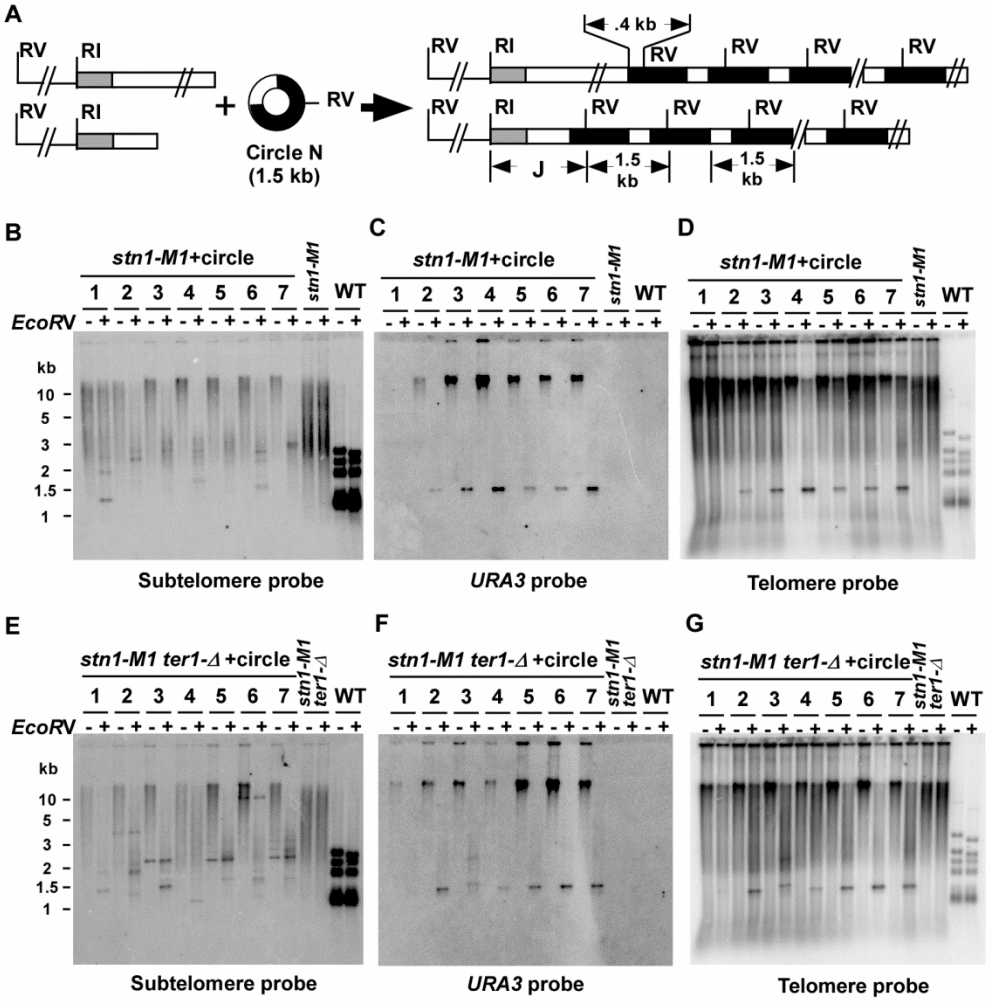
A
RV
RI
RV
RI
Circle N
(1.5 kb)
RV
.4 kb
J
1.5 kb
1.5 kb
B
stn1-M1+circle
1 2 3 4 5 6 7
stn1-M1
WT
EcoRV
kb
10
5
3
2
1.5
1
Subtelomere probe
C
URA3 probe
D
Telomere probe
E
stn1-M1 ter1-Δ +circle
stn1-M1 ter1-Δ
F
G

Fig. 2.3. The *URA3*-telomeric units from circle N-derived tandem arrays undergo rapid changes in both copy number and telomeric repeat number during passaging of *stn1-M1* and *stn1-M1 ter1-Δ* cells. (A) Diagram of a telomere with the circle N-derived tandem arrays of *URA3*-telomeric units (two units of which are shown) in transformed *stn1-M1* and *stn1-M1 ter1-Δ* cells. White boxes indicate telomeric repeats and black boxes indicate *URA3*. Positions of *Xma*I, *Pst*I, *Eco*RI and *Eco*RV sites are indicated. The location of the *URA3*-HX probe used in panels C and D is also indicated. (B) Southern blotting and *URA3* quantitation data from two independent lineages of both wild type (clones 3 and 5) and *stn1-M1 ter1-Δ* (clones 6 and 8), as well as three independent lineages of *stn1-M1* strains (clones 16, 41 and 44), all of which were transformed with circle N. Cells were serially passaged for 4-5 streaks on YPD plates, as indicated. Middle and lower sections of each panel show Southern blots of *Xma*I + *Pst*I digested DNA hybridized to the single copy *RAD52* gene (which is present as two pieces) and the *URA3* gene. Only the largest of the *Xma*I + *Pst*I *URA3* fragments is shown. The graphs show the copy number of *URA3* at the streaks indicated, calculated from the Southern data. (C) *Xma*I + *Pst*I digested DNA from the same samples as in (B) separated on a 3% agarose gel electrophoresis and hybridized with the *URA3*-HX probe. Order of samples is identical to (B) except for the addition of lanes (labeled "C") showing circle N digested with the same enzymes. The sample from streak 4 of *stn1-M1 ter1-Δ* clone 6 was overloaded relative to streaks 1-3 of the same clone. (D) Southern blotting of *Xma*I + *Pst*I digested DNA from multiple subclones from the 4th streak of each of two lineages of *stn1-M1* strains transformed with circle N. DNAs are shown separated on a 3% agarose gel and hybridized with the *URA3*-HX probe. Samples from the 3rd streak are also shown.

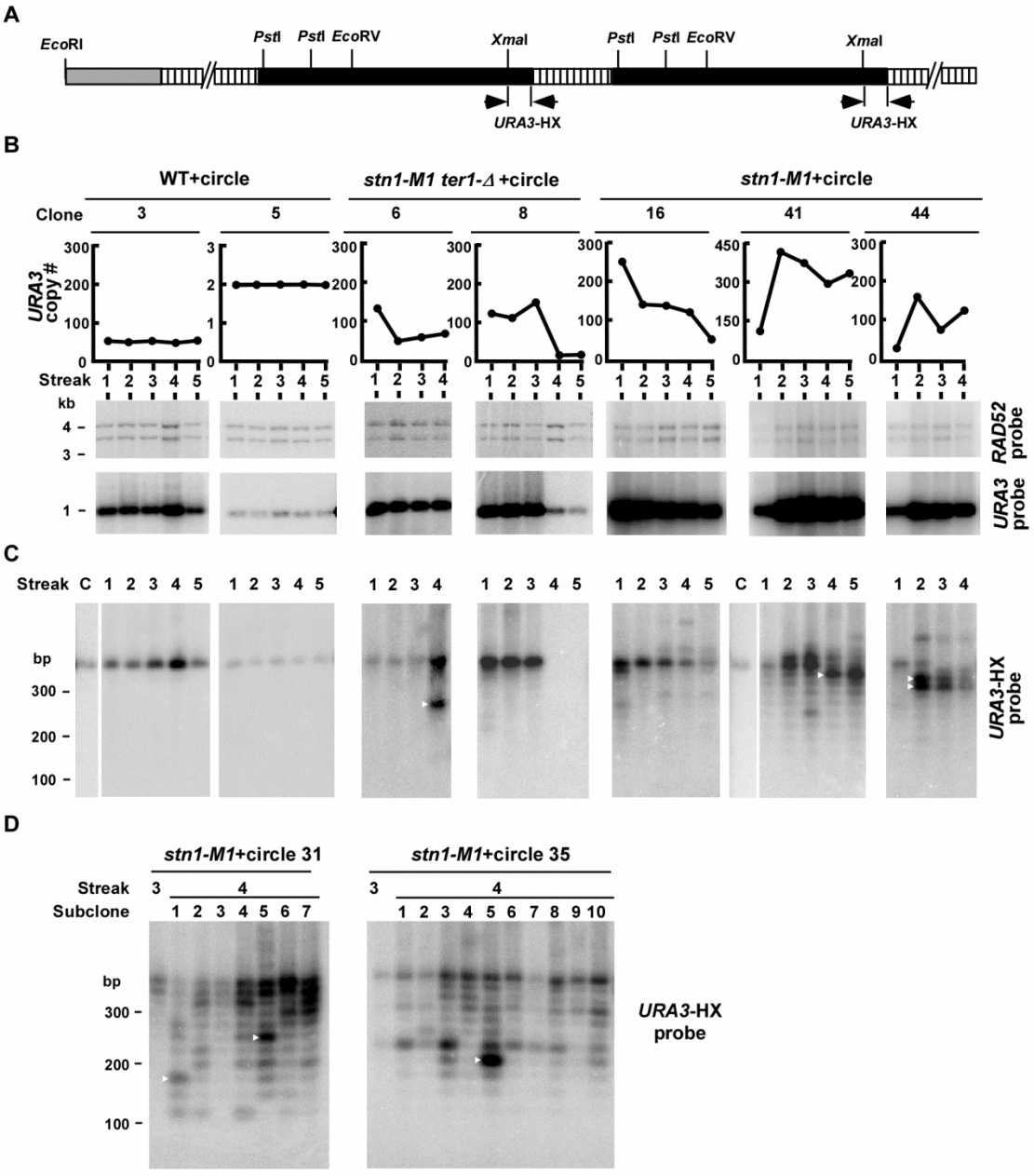
A
EcoRI
PstI PstI EcoRV
XmaI
PstI PstI EcoRV
XmaI
URA3-HX
URA3-HX
B
WT+circle
stn1-M1 ter1-Δ +circle
stn1-M1+circle
Clone 3 5 6 8 16 41 44
URA3 copy#
Streak 1 2 3 4 5
kb
4
3
1
RAD52 probe
URA3 probe
C
Streak C 1 2 3 4 5
bp
300
200
100
URA3-HX probe
D
stn1-M1+circle 31
stn1-M1+circle 35
Streak 3 4
Subclone 1 2 3 4 5 6 7 1 2 3 4 5 6 7 8 9 10
bp
300
200
100
URA3-HX probe

Fig. 2.4. Despite their high copy number, the *URA3* genes in *stn1-M1* and *stn1-M1 ter1-Δ* transformed with circle N could be rapidly and completely lost from cells. (A) Southern blot, hybridized with the *URA3* probe, of uncut DNA from multiple *stn1-M1* and *stn1-M1 ter1-Δ* strains transformed with circle N before (indicated by a B above lanes) and after they were patched on 5-FOA plates (two clones from each, indicated by A1 and A2 above lanes). Quantitation of *URA3* copy number for each clone before plating on 5-FOA was estimated by using the same method as used in Fig. 2.3B and shown below the lanes. (B) Southern blot, hybridized with a subtelomeric probe, of *Eco*RI or *Eco*RI + *Eco*RV digested DNA from the precursor (B) strains in panel A before they were patched on 5-FOA. Untransformed *stn1-M1* and *stn1-M1 ter1-Δ* control strains are also shown. The extent to which bands are cleaved by *Eco*RV provides an indication of the percentage of telomeres containing *URA3* copies prior to plating on 5-FOA.

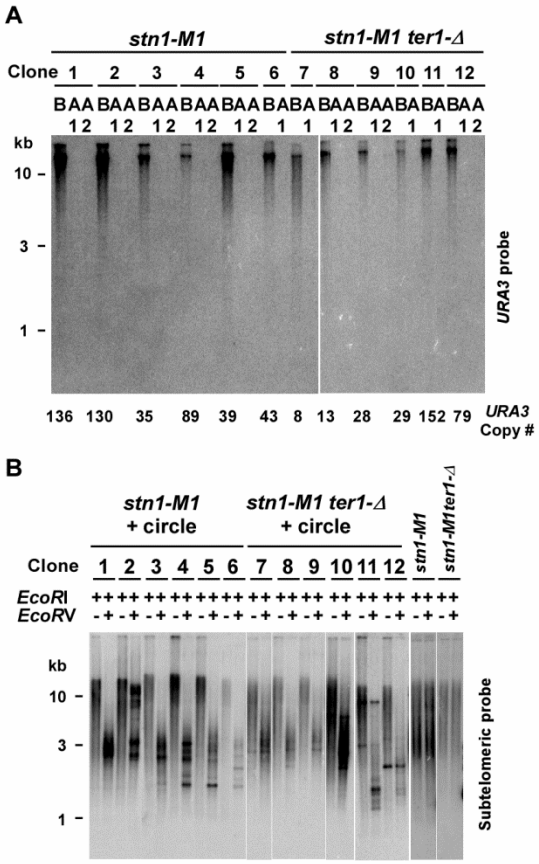
A
stn1-M1
stn1-M1 ter1-Δ
Clone 1 2 3 4 5 6 7 8 9 10 11 12
B A A B A A B A A B A A B A A B A A B A A B A A B A A B A A
1 2 1 2 1 2 1 2 1 2 1 2 1 1 1 2 1 2 1 2 1 1 1 1 2
kb
10
3
1
URA3 probe
136 130 35 89 39 43 8 13 28 29 152 79 URA3 Copy #
B
stn1-M1 + circle
stn1-M1 ter1-Δ + circle
stn1-M1
stn1-M1ter1-Δ
Clone 1 2 3 4 5 6 7 8 9 10 11 12
EcoRI +
EcoRV - + - + - + - + - + - + - + - + - + - + - + - +
kb
10
3
1
Subtelomeric probe

Fig. 2.5. Most telomeric signal of *stn1-M1* and *stn1-M1 ter1-Δ* cells could not enter a pulsed-field gel. (A) Ethidium Bromide stained pulsed-field gel showing chromosomes from wild type *K. lactis* (WT), one clone of *stn1-M1* transformed by circle N (indicated by "+c"), two clones of *stn1-M1 ter1-Δ* transformed by circle N (indicated by "+c") and three clones each of untransformed *stn1-M1* and *stn1-M1 ter1-Δ*. (B) Southern blot showing hybridization using a *RAD50* probe, of the DNA transferred from the agarose gel in panel A. (C) Same filter as in panel B after stripping and rehybridization with a telomeric probe. (D) Same filter as in panel B after stripping and rehybridization with a subtelomeric probe. (E) Same filter as in panel B after stripping and rehybridization with a probe composed of both *URA3* and *RAD52* gene sequences.

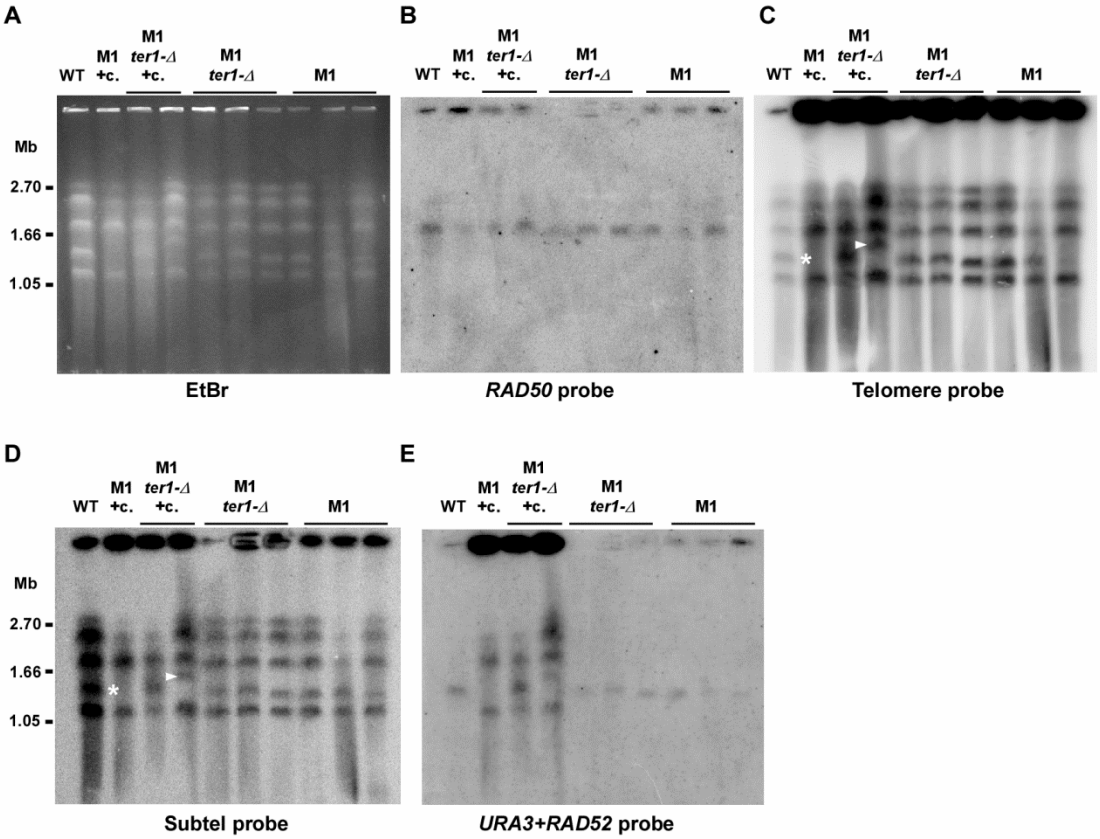
A
B
C
WT
M1 +c.
M1 ter1-Δ +c.
M1 ter1-Δ
M1
Mb
2.70
1.66
1.05
EtBr
RAD50 probe
Telomere probe
D
E
Subtel probe
URA3+RAD52 probe

Fig. 2.6. A replicating plasmid containing telomeric repeats present at the establishment of the *stn1-M1* mutant state becomes incorporated at telomeres as long tandem arrays. (A) Diagram of circle A, a 6.2-kb replicating plasmid containing telomeric repeats as well as the tandem array structure expected if the circle A sequence becomes added onto telomeric ends. Components of circle A including ~11.5 telomeric repeats and an Autonomously Replicating Sequence (ARS) are shown as indicated in the key. Positions of *Hpa*I (H) and *Xho*I (X) sites are indicated. The 1.2 kb of vector sequence from circle A between the basal telomeric repeats and the first *Xho*I site is indicated. (B) Southern blot, hybridized with a *URA3* probe, of undigested DNA from *stn1-M1* clones derived from circle A transformants that had lost complementation by p*STN1*. Clones exhibiting each of three types of outcomes are shown: loss of plasmid (lanes 1-2), retention of extrachromosomal plasmids deleted of telomeric repeats (lanes 3-4) and integration of plasmid into chromosomal DNA (lanes 5-6). (C) Same filter as in panel B after stripping and rehybridization with a telomere probe. (D) Southern blot, hybridized with a subtelomeric probe, of *Hpa*I and *Hpa*I + *Xho*I digested DNA from *stn1-M1* (p*STN1*) circle A transformants (Precursor clones P1 and P2) and *stn1-M1* clones derived from circle A transformants that had lost pSTN1. Clones 1-3 are derived from P1 and clones 4 and 5 are derived from P2. Note that clone designations do not match those in panels B and C. Untransformed wild type *STN1* (WT) and *stn1-M1* control strains are also shown. Also note that one *Hpa*I telomeric fragment in wild type cells has an *Xho*I site in subtelomeric DNA. (E) Same filter as in panel D after stripping and rehybridization with a *URA3* probe. (F) Same filter as in panel E after rehybridization and reprobing with a telomeric probe.

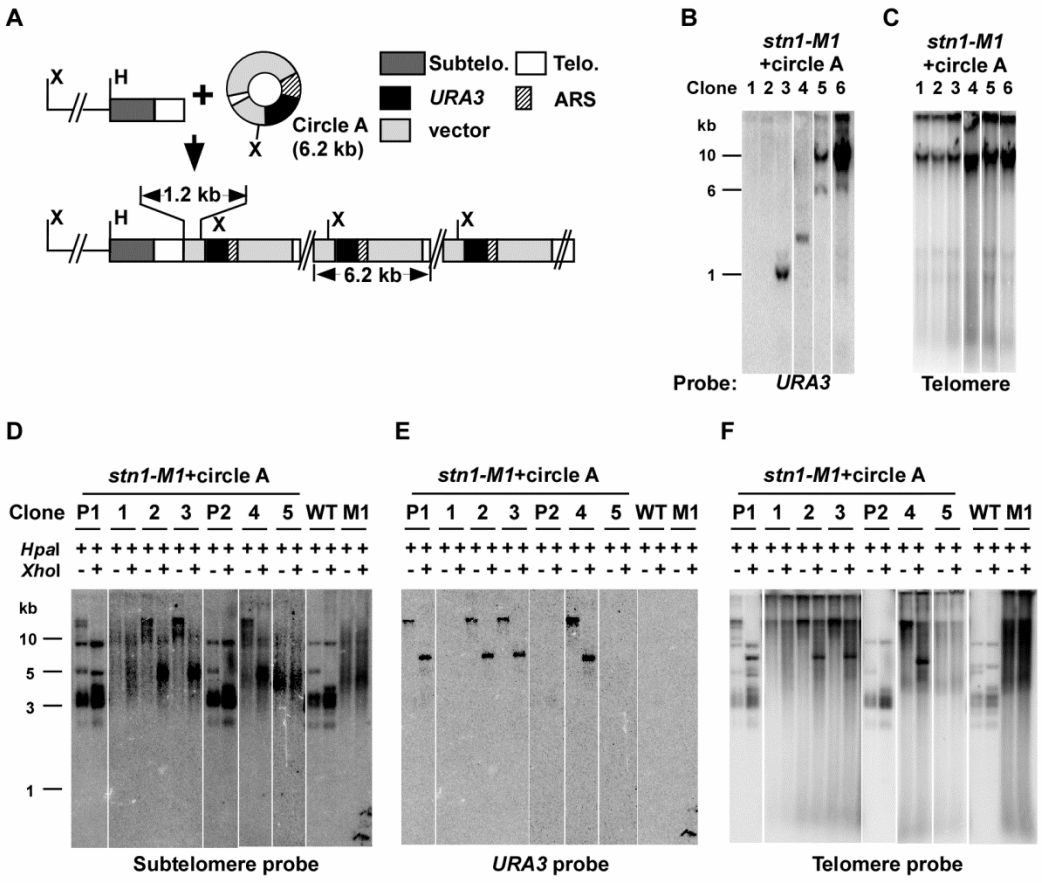
A
X
H
X
Circle A
(6.2 kb)
Subtelo.
Telo.
URA3
ARS
vector
1.2 kb
6.2 kb
B
stn1-M1
+circle A
Clone 1 2 3 4 5 6
kb
10
6
1
Probe: URA3
C
stn1-M1
+circle A
1 2 3 4 5 6
Telomere
D
stn1-M1+circle A
Clone P1 1 2 3 P2 4 5 WT M1
HpaI
XhoI
kb
10
5
3
1
Subtelomere probe
E
stn1-M1+circle A
P1 1 2 3 P2 4 5 WT M1
URA3 probe
F
stn1-M1+circle A
P1 1 2 3 P2 4 5 WT M1
Telomere probe

Fig. 2.7. A model for telomere maintenance in *stn1-M1* cells. (A) Once a *URA3*-telomere circle N is introduced into *stn1-M1* cells, it can be primed by a 3' telomeric end and used as a template for rolling circle replication. Thin lines indicate subtelomeric sequences, white boxes indicate telomeric repeat sequences next to the subtelomeric sequences and terminal telomeric blocks. Black boxes indicate the *S. cerevisiae URA3* gene from the circle N. The yellow box with four dots indicates the block of 11.5 telomeric repeats in circle N. (B) Rolling circle synthesis using the transformed circle N as the template produces a long tandem array of the sequence of the circle N at the end of a telomere. (C) Under selection to maintain *URA3*, the tandem array becomes spread to most other telomeres by more typical BIR events that copy telomeric sequence of one chromosome end onto another. (D) With further cell divisions, the high levels of telomeric recombination break up the uniformly sized telomeric repeat blocks between copies of *URA3*. This leads to accumulating variant blocks with differing numbers of telomeric repeats (indicated by different colored boxes with different numbers of dots). (E-F) If an endogenously produced *URA3*-telomere circle with a variant block of telomeric repeats is produced and used as a template for another round of rolling circle amplification, a tandem array initially containing only the variant block will be added onto a telomere. (G) Because there is no selection for the new array, chance recombination events may delete it, or in some cases, copy it onto some number of other telomeric ends.

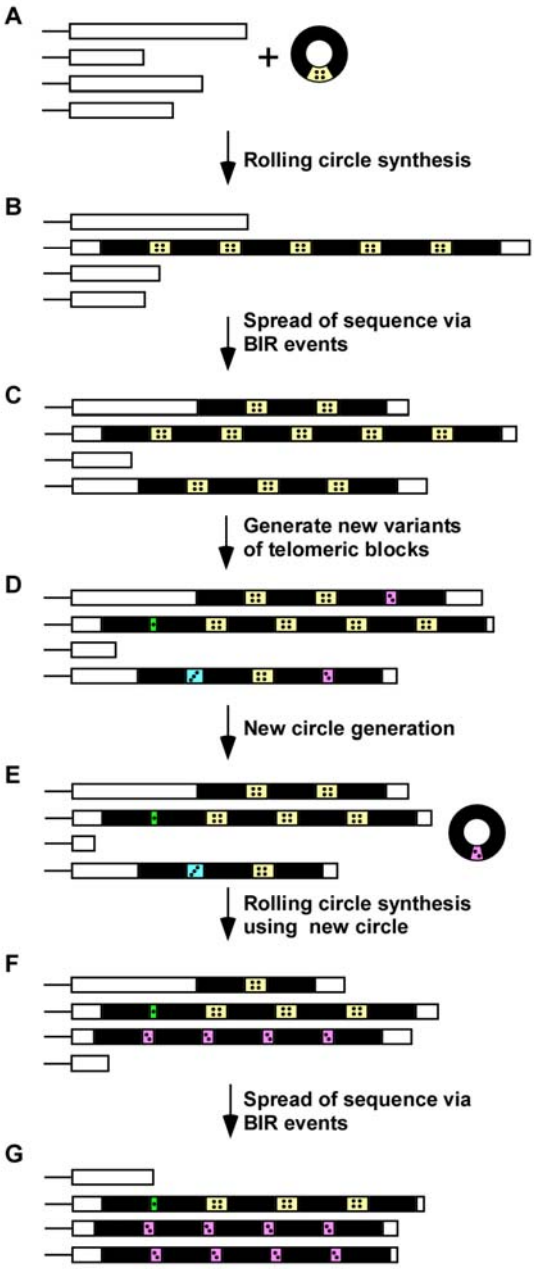
A
Rolling circle synthesis
B
Spread of sequence via
BIR events
C
Generate new variants
of telomeric blocks
D
New circle generation
E
Rolling circle synthesis
using new circle
F
Spread of sequence via
BIR events
G

Table S2.1. *URA3* copy numbers of circle N transformants that were serially restreaked for 4-5 streaks.

The WT, *stn1-M1* and *stn1-M1 ter1-Δ* circle N transformants were serially restreaked on YPD plates, with each streak representing 20-25 cell divisions. The copy number of *URA3* genes are measured as described in the text. (ND=not determined)

Transformants	Lineage	Streaks				
		1	2	3	4	5
WT + circle	3	53	47	47	53	56
	5	2	2	2	2	2
	7	5	4	5	5	5
stn1-M1 TER1 + circle	3	43	88	74	104	70
	4	96	137	129	ND	401
	16	247	147	142	123	48
	22	6	3	0	0	0
	26	89	119	168	165	125
	31	159	290	455	348	
	32	78	338	408	344	536
	35	119	239	124	536	
	40	74	67	67	97	144
	41	104	424	359	295	324
	42	93	64	60	61	204
	43	50	15	5	5	48
	44	28	165	80	121	
stn1-M1 ter1-Δ + circle	4	8	20	7	2	1
	5	45	10	15	38	ND
	6	129	55	80	87	
	8	127	114	169	4	7
	9	135	0	0	ND	0
	11	3	6	3	8	13
	13	11	6	3	0	0
	14	3	5	3	4	7
	15	10	27	7	ND	39
	16	45	12	0	0	0

CHAPTER 3

EVIDENCE FOR THE INVOLVEMENT OF TELOMERIC CIRCLES AND A SINGLE TELOMERE SOURCE DURING THE ESTABLISHMENT OF LONG TELOMERES IN AN ALT-LIKE YEAST MUTANT.[1]

[1] Jianing Xu and Michael J. McEachern. To be submitted to *MCB*

ABSTRACT

Considerable evidence now supports that the moderate telomere lengthening produced by recombinational telomere elongation (RTE) in a *Kluyveromyces lactis* telomerase deletion mutant occurs through a roll-and-spread mechanism (53). However, it is unclear whether a roll-and-spread mechanism can account for other forms of RTE that produce much longer telomeres such as is seen in human alternative lengthening of telomere (ALT) cells or in type IIR "runaway" RTE such as occurs in the *K. lactis stn1-M1* mutant. In this study we have used mutationally tagged telomeres to examine the mechanism of RTE in the *stn1-M1* mutant. Our results suggest that the establishment stage of the mutant state in newly generated *stn1-M1* mutants surprisingly involves a first stage of sudden telomere shortening. Our data also show that, as predicted by the roll-and-spread mechanism, all lengthened telomeres in a cell commonly emerge from a single telomere source. Furthermore, although the presence of telomerase has little effect on the phenotype in later maintenance stages of the mutant, we show that its presence during the establishment stage of the mutant has a large effect on spreading of sequence from a mutationally tagged telomere to other telomeres.

INTRODUCTION

Recombination can maintain telomeres in many situations where telomerase is absent. Natural examples of this include the chromosomal telomeres in the mosquito Anopheles (10, 65) and the mitochondrial telomeres in certain ciliates and yeasts (48, 55, 71). Of particular importance are the 5-10% of human cancer cells where telomerase activity is undetectable and telomeres are maintained by a mechanism termed Alternative Lengthening of Telomeres (ALT) [for a review, see (13)]. ALT cells are characterized by long and heterogeneous telomeres (26, 28, 50, 58) and the presence of ALT-associated PML bodies (APB) that contain telomeric DNA as well as telomeric and recombinational proteins (26, 29-30, 60, 78).

Several lines of evidence suggest that recombination is involved in maintaining telomeres when the long and heterogeneous telomeres already exist in ALT cells. Plasmid tags introduced into a telomere can be duplicated to other telomeres or at the same telomere in ALT cells but not in telomerase positive cells (22, 49). Extrachromosomal telomeric circles (t-circles), likely products of intratelomeric recombination, are abundant in ALT cells (12, 76, 79). Telomeric sister chromatid exchanges (t-SCE) occur at highly elevated rates in ALT cells (2, 8, 39). However, the details of how recombination can establish these long and heterogeneous telomeres from normal-length telomeres in ALT cells are still unknown.

Recombinational telomere elongation (RTE) has been described in yeast mutants lacking telomerase in the species *Sacchromyces cerevisiae* (40), *Kluyveromyces lactis* (43), *Candida albicans* (18) and *Schizosacchromyces pombe* (51). The recombination in these cases is thought to be caused by the shortening telomeres eventually losing part or all of their protective capping function. These mutants commonly display a gradual growth senescence when telomeres are gradually shortening that is followed by the formation of better growing post-senescence survivors with longer telomeres (40, 43, 45). Two types of RTE were initially described in telomerase deletion mutants of *S. cerevisiae*. Both depend upon *RAD52*, suggesting that they require homologous recombination (HR). Type I RTE is characterized by amplification of subtelomeric Y' elements and short tracts of telomeric repeats, and is dependent upon the canonical mitotic HR pathway involving *RAD51*, *RAD54*, *RAD55*, and *RAD57*. Type II RTE is characterized by lengthened tracts of telomeric repeats and is dependent upon a different pathway involving *RAD50, RAD59,* and *SGS1* (14, 19, 34, 69-70, 73). Only type II RTE normally occurs in *K. lactis* telomerase deletion mutants (*ter1-Δ*) (43). Studies, particularly in *K. lactis*, have suggested that type II RTE occurs through a roll-and-spread mechanism, where a t-

circle is used as a template to lengthen one short telomere which in turn can be used as a template to lengthen other telomeres via break-induced replication (BIR) events (25, 52-53, 72). Consistent with this model, post-senescence survivors derived from cells with two kinds of telomeric repeats often contain repeating patterns in most or all lengthened telomeres (53). Additionally, when a DNA circle containing telomeric repeats is transformed into a *K. lactis* telomerase deletion mutant, its sequence becomes efficiently amplified onto telomeric ends as long tandem arrays (53). T-circles are also abundant in yeast mutants with telomere dysfunction (5, 25, 37). Furthermore, sequence from a single telomere is used as the source of all lengthened telomeres in *K. lactis* post-senescence survivors (72). Type II RTE in *S. cerevisiae* has also been suggested to involve rolling circle copying of t-circles (37).

RTE can also be triggered by perturbation of telomeric capping proteins. For example, in *S. cerevisiae*, a *cdc13-1 yku70* mutant can generate type II survivors without a growth senescence (23). In *K. lactis*, telomerase deletion mutants containing telomeric repeats with defects in Rap1 binding develop much longer telomeres than equivalent mutants with only wild type repeats (6, 72). Of particular interest is the *stn1-M1* mutant of *K. lactis* (27). Stn1 is a part of the Cdc13/Stn1/Ten1 (CST) complex that binds to the 3' single-stranded telomeric overhang and protects the telomere termini from degradation and engagement in recombination [for a review see (61)]. Stn1 also regulates telomerase recruitment and telomeric C-strand synthesis, the latter via its interaction with Polα/primase (24, 62). In many ways, the *stn1-M1* mutant displays more similarity to ALT than do *ter1-Δ* mutants. It shares with ALT a steady state of very long and highly heterogeneous telomeres that are produced as soon as the mutation occurs by recombination as well as the immediate presence of chronic but slight growth defects instead of the gradual growth senescence and survivor formation seen in *ter1-Δ* mutants (26-27, 64). Both

ALT cells and *stn1-M1* cells show high levels of telomeres instability including elevated telomere recombination, rapid telomere shortening, and abundant extrachromosomal telomeric DNA including t-circles [(5, 27, 39, 56, 58); Xu and McEachern, submitted]. Finally, the phenotypes of *stn1-M1* and of most ALT cells are not suppressed by telomerase (26-27). While telomeric recombination in *ter1-Δ* cells appears repressed once telomeres are even moderately elongated, the telomere recombination in *stn1-M1* cells is thought to occur at telomeres of all sizes. To distinguish the fundamental differences between telomere capping defects in the two mutants, the RTE in *stn1-M1* mutant was termed type IIR for its "runaway" lengthening characteristics (27). Given its similarities with ALT cells, the *stn1-M1* mutant is therefore an excellent model system to obtain more clues about mechanism to establish long telomeres in ALT cells.

In this work, we utilize mutationally tagged telomeric repeats to study the mechanism of type IIR RTE in *stn1-M1* mutant during the establishment stage where long telomeres are generated from much shorter telomeres. Our results are consistent with predictions of the roll-and-spread model in demonstrating that sequence from one telomere is commonly spread to most or all telomeres of newly formed *stn1-M1* mutants. Our results also suggest that rapid telomere truncations routinely precede the generation of long telomeres and that the presence of telomerase can impact the outcomes observed.

RESULTS

Generating *stn1-M1* mutants from precursors with mutationally tagged telomeric termini.

To study the type IIR RTE that forms highly elongated telomeres in the *stn1-M1* mutant of *K. lactis*, we generated *stn1-M1* mutants from two kinds of precursors with mutationally tagged telomeric repeats. Previously, similar approaches were informative in studying the type II

RTE that forms the more modestly elongated telomeres in *K. lactis* telomerase deletion (*ter1-Δ*) mutants (53, 72). The experimental setup for generating *stn1-M1* cells from the first kind of precursor is diagramed in Fig. 3.1A. An *stn1-M1 ter1-Δ* mutant was first transformed with a plasmid (p*STN1-TER1(ApaL)*) containing both the *STN1* and the *TER1-20C(ApaL)* genes. The *TER1-20C(ApaL)* gene forms a telomerase that adds mutated ApaL repeats onto all telomeric termini. ApaL repeats are phenotypically silent but contain a single base change that both forms an *Apa*LI site and eliminates the native *Rsa*I site (74) (Fig. 3.1A). A transformant was then serially passaged for ten streaks to allow telomeres to shorten to near normal length and to incorporate ApaL repeats at their termini. These passaged cells are referred to as the ApaL precursor cells.

All telomeres in the ApaL precursor cells had acquired ApaL repeats at termini as indicated by their *Eco*RI-digested telomeric fragments being shorter after *Apa*LI digestion (Fig. 3.1B and data not shown). In contrast, telomeric fragments in a wild type control were resistant to *Apa*LI. To estimate the number of ApaL repeats at telomeric termini of ApaL precursors, we digested their DNA by *Bsr*BI +*Apa*LI and *Bsr*BI + *Rsa*I (Fig. 3.1C and data not shown). *Bsr*BI cleaves 3 bp away from 10 of 12 telomeres in *K. lactis* (Fig. 3.1A), and *Apa*LI and *Rsa*I specifically cleave ApaL and wild type (WT) repeats, respectively. From this, we estimated that the terminal ~3-7 repeats of the 400-600 bp total telomeres were composed of ApaL repeats. This was verified by cloning and sequencing two telomeres from an ApaL precursor. One cloned telomere contained 11 basal WT repeats and three terminal ApaL repeats and the other contained ~9 basal WT repeats and three terminal ApaL repeats. To our surprise, clone 2 also contained a 13 bp repeat located between the WT and ApaL repeats (Fig. S3.1A). The sequence of this 13 bp repeat suggested it arose when the terminal bases of a telomere annealed to the middle instead of

to the 3' end of the telomerase RNA template (Fig. S3.1B). This half-sized repeat was not likely formed by the ApaL telomerase because it contained the native *Rsa*I site of WT repeats rather than an *Apa*LI site.

We then selected for newly generated *stn1-M1 ter1-Δ* mutants by plating the ApaL precursors on medium containing 5-flouro-orotic acid (5-FOA) to select for loss of the p*STN1-TER1(ApaL)* plasmid. Because the p*STN1-TER1(ApaL)* became integrated into a chromosome at the *stn1-M1* locus in the ApaL precursor during passaging (data not shown), the rate of recovering *stn1-M1 ter1-Δ* mutants (generated by homologous recombination looping out the plasmid from the chromosome) was relatively low. Because telomerase and the wild type *STN1* gene are deleted at the same time, the generation of long telomeres in the newly formed *stn1-M1 ter1-Δ* cells should depend solely on recombination.

A group of ten newly generated *stn1-M1 ter1-Δ* mutants was initially analyzed. All ten mutant clones showed the long and heterogeneous pattern of telomeres that is the characteristic of the *stn1-M1* phenotype when *Eco*RI digests were observed in a Southern blot (Fig. 3.2A). Telomeric signals in nine of these ten mutants (clones A2-A10, Fig. 3.2A) were not obviously cleaved by *Apa*LI. Hybridization of the same filter to a subtelomeric probe (Fig. 3.2B) showed that most clones had some signal in one or more bands, generally of ~1 kb. This indicates that one or a small number of telomeres retained at least one ApaL repeat near their base. Together, these results indicate that telomeres in these nine mutants were composed almost entirely of WT repeats. Consistent with this interpretation, telomeric signals from these nine mutants were virtually cleaved away by *Rsa*I that specifically cleaves WT repeats (Fig. 3.2A). This result is very surprising, because the *stn1-M1* phenotype forms rapidly without a period of growth

senescence and should therefore not undergo any gradual sequence loss of terminal ApaL repeats before the formation of long telomeres by RTE(27).

Strikingly, the telomeric signal of one *stn1-M1 ter1-Δ* mutant (clone A1) was cleaved into very small fragments by both *Apa*LI and *Rsa*I (Fig. 3.2A). When the same digests were run on a high-percentage agarose gel, the small fragments were observed to be composed largely of a ~125 bp band in the *Apa*LI digests and a ~50 bp band in the *Rsa*I digests (Fig. 3.2C). The former was predicted to contain four WT repeats and two half ApaL repeats and the latter was predicted to contain one ApaL repeat and two half WT repeats. These data suggest that telomeres in this *stn1-M1 ter1-Δ* mutant may contain repeating structures that consist of four WT repeats and one ApaL repeat as the repeating unit. To test this, we cloned and sequenced 38 telomeric fragments from this clone, which were produced by partial *Apa*LI digestion. Although mostly very small *Apa*LI fragments were recovered (Fig. S3.2), the results showed that 19 of 51 (37%) blocks of WT repeats were ~100-125 bp, of which 15 (79%) consisted of three WT repeats and one half WT repeat of the same sequence that was recovered from the ApaL precursor. Although these results rule out the presence of perfectly repeating patterns when DNA was isolated from the A1 mutant, the widespread presence of a particular pattern of repeats could suggest that telomeres with more perfect repeating patterns originally existed but was disrupted by numerous later recombination events.

We next analyzed 83 additional *stn1-M1 ter1-Δ* mutants generated from ApaL precursors. 73 of these clones had telomeric signals that were essentially uncleaved by *Apa*LI but were nearly fully cleaved by *Rsa*I indicating that the lengthened telomeres were composed of virtually all WT repeats (data not shown). However, ten clones had telomeric signals that were cleaved partly or entirely into short fragments by both *Apa*LI and *Rsa*I digestions (Fig. 3.2D, upper

panel). The same digests of these ten mutants were then run on a high-percentage agarose gel to resolve short DNA fragments (Fig. 3.2D, lower panel). Several of these mutants, including B19, B20, C12, C23 and C44 showed favored fragment sizes in both *Apa*LI and *Rsa*I digests (indicated by white arrows) which could be indicative of degraded repeating patterns. In each of these mutants, the most common fragment size of ApaL repeats was smaller than that of WT repeats. The other mutants examined, B8, B12, B16, C4 and C60, sometimes exhibited favored short fragments in *Rsa*I digests but not obviously any in *Apa*LI digests. The average size of the telomeric signal in *Apa*LI digests of these clones tended to migrate at greater average size than that seen in the other clones. In some clones, most notably C4, the *Apa*LI digestion produced ladders of bands that included sizes consistent with the presence of the "half" telomeric repeats as was present in the A1 clone.

Two newly generated *stn1-M1 ter1-Δ* mutants that exhibited the best evidence of repeating patterns (clones A1 and C12) were serially passaged for 5-10 streaks on YPD plates and periodically examined for their telomeric DNA structure by Southern blots of *Apa*LI and *Rsa*I digests run on high-percentage agarose gels (Fig. 3.2E). The initial banding pattern of these mutants became more complicated after passaging. Specifically, the favored fragments of the two mutants in *Apa*LI digests, initially ~110-150 bp, became more variable at later streaks and tended to produce new fragments of larger sizes. This observation supports the idea that telomeres in *stn1-M1* mutants are highly dynamic and are prone to high rates of recombination that can rapidly alter their structure.

The absence of mismatch repair leads to more heterogeneously structured telomeres in newly made *stn1-M1* mutants.

Base mismatches in DNA can reduce the rate of homologous recombination (31). Therefore, the base mismatch between WT and ApaL repeats is likely to interfere with the recombination between telomeres that contain them. To test this, we disrupted the *MSH2* gene (required for mismatch repair) in an ApaL precursor, and generated 17 *stn1-M1 ter1-Δ msh2-Δ* mutants by losing p*STN1-TER1(ApaL)* as described above. Telomeric signals in 5 of these mutants showed little or no cleavage by *Apa*LI (data not shown). However, telomeric signals in the other 12 mutants were cleaved into broad ladders of bands with ~25 bp steps by both *Apa*LI and *Rsa*I (Fig. 3.3A and data not shown). Unlike what was seen in mutants derived from a *MSH2* background, these mutants showed no obvious favored fragments that might have indicated the presence of a degraded pattern. The appreciably higher percentage of *stn1-M1* mutants made in the *msh2-Δ* background that had amplified ApaL repeats might be related to mismatch repair. Alternatively, we cannot rule out the possibility that the increased incorporation of ApaL repeats occurring in the precursor during the additional cell divisions (equivalent to 2-3 streaks) needed to disrupt the *MSH2* gene altered the result.

We speculated that *stn1-M1 ter1-Δ* mutants made in a *msh2* background had sufficiently high levels of telomeric recombination to rapidly break down any repeating structure that might have been formed initially. To test this idea, we attempted to knock out the *MSH2* gene in the A1 clone of *stn1-M1 ter1-Δ* mutant that had highly favored small telomeric fragment in both *Apa*LI and *Rsa*I digests (Fig. 3.2A). Among 72 clones transformed with the knockout cassette, only one had the *MSH2* gene successfully disrupted. This clone was serially passaged on YPD plates for three streaks and its telomeres were examined after each streak by Southern blot (Fig. 3.3B). The same procedure was carried out with a control transformant that still had the intact *MSH2* gene. Upon disruption of *MSH2*, the simple banding patterns of the A1 clone, particularly the

larger fragment in *Apa*LI digests, become distinctly heterogeneous as soon as the samples could be analyzed (Fig. 3.3B). This heterogeneous pattern is similar to those from *stn1-M1 ter1-Δ* mutants generated directly in a *msh2* background shown in Fig. 3.3A. In contrast, the control transformant having the intact *MSH2* exhibited a banding pattern that remained distinctly more stable. This result demonstrates that the elevated telomeric recombination in a *msh2-Δ* background is sufficient enough to rapidly break down a repeating structure that might have been formed initially in telomeres of a newly generated *stn1-M1 ter1-Δ* mutant.

The sequence from a single telomere can be spread to all other telomeres during the establishment of the *stn1-M1 ter1-Δ* state.

RTE in *ter1-Δ* mutants of *K. lactis* regularly involves the spreading of sequence from a single telomere to all other telomeres to generate modest elongated telomeres (72). To test whether the *stn1-M1* mutant can also use this mechanism to generate highly elongated telomeres, we constructed telomeric DNA fragments consisting of the subtelomeric sequence, a *HIS3* marker and Bcl telomeric repeats (Fig. 3.4A). The subtelomeric sequence is shared by 11 of 12 *K. lactis* telomeres allowing the transformed fragments to replace a native telomere through homologous recombination. The Bcl repeats each contain a phenotypically silent base change that generates a *Bcl*I site (47, 66, 75). The DNA fragments containing either ~11 or ~40 Bcl repeats were transformed into *stn1-M1 ter1-Δ* cells containing p*STN1-TER1* integrated at the *stn1-M1* locus (Fig. 3.4A). While the shorter telomeric fragment generated a new telomere of normal length (Fig. 3.4B), the long telomeric fragment generated a telomere substantially longer than those of wild type cells (Fig. 3.4F). The resulting transformants are hereafter referred to as “normal length Bcl precursors” and “long Bcl precursors”, respectively.

Next, we generated 55 clones of *stn1-M1 ter1-Δ* mutants from four normal length Bcl precursors by plating the precursors on 5-FOA-containing medium that selected for the loss of the p*STN1-TER1* plasmid. Telomeric signals in 49 of these clones appear essentially identical in both *Eco*RI and *Eco*RI + *Bcl*I digests (Clones 1, 3, 4 and 5 in Fig. 3.4B and data not shown) which indicated that telomeres in these clones were composed mostly or entirely of WT repeats with very few or no Bcl repeats. Other data were also consistent with this view. The weak signal with a probe specific to Bcl telomeric repeats was not sensitive to *Bcl*I digestion, suggesting that it was due to background hybridization to the heavily amplified wild type repeats (Fig. 3.4C). Additionally, subtelomeric signals were largely resistant to *Bcl*I digestion, except for ~1.5 kb fragments generated in some clones (Clones 2, 4 and 5 in Fig. 3.4D-E) that also hybridized to *HIS3* probe, consistent with them being derived from the original integrated telomere. We classified these 49 clones as having no amplification or spreading of Bcl repeats (Table 3.1). Our results suggest that Bcl repeats were actively avoided as a source of sequence to be amplified during establishing the long telomeres in the *stn1-M1* mutant.

The remaining 6 of 55 clones, including clone 2 in Fig. 3.4B, showed amplification of Bcl repeats estimated to be 3-5 times that present in the single telomere of the precursor strain (Table 3.1 and data not shown). At least two of these clones showed extra copies of the *HIS3* gene (data not shown), suggesting that the modest amplification of Bcl repeats occurred primarily by subtelomeric break-induced replication (BIR) events that produced extra copies of the tagged telomere but without the Bcl repeats otherwise becoming amplified.

Of the 55 total clones, 41 (including 38 of 49 clones with no amplification and spreading of Bcl repeats) showed no *HIS3* signal (Fig. 3.4E and data not shown). This indicates that these mutants lost the *HIS3*-tagged telomere and probably all the Bcl repeats originally attached to that

telomere. Such frequent loss of the subtelomeric *HIS3* was not surprising as that *stn1-M1* cells showed very high rates of loss of a *URA3* gene inserted at the same subtelomeric location (27). These deletions are likely BIR events that replace one telomere with sequence from another telomere. The same mechanism is likely responsible for occasions in some mutants where the *HIS3*-tagged telomere became duplicated.

Analysis of 76 newly generated clones of *stn1-M1 ter1-Δ* mutants from long Bcl precursors showed somewhat different results (Table 3.1). While 53 clones (70%) showed no amplification or spreading of Bcl repeats, the other 23 clones (30% of the total) did show some degree of amplification and spreading of Bcl repeats. Most notably, telomeric signals in three of these clones, including clones 6 and 13 in Fig. 3.4F, can be completely or nearly completely cleaved away by *Bcl*I digestion, suggesting that telomeres in these clones were composed mostly or entirely of Bcl repeats (Table 3.1). Consistent with this interpretation, the Bcl repeat-specific signals of these three clones were intense in *Eco*RI digests but were eliminated by *Bcl*I digestion (Fig. 3.4G). Furthermore, the long smeared subtelomeric signal of these clones in *Eco*RI digests was largely or entirely cleaved by *Bcl*I into fragments that were generally < 2 kb (Fig. 3.4H). Interestingly, these three clones showed no amplification of the subtelomeric *HIS3* gene (Fig. 3.4I and data not shown), suggesting that the Bcl repeats were amplified by inter-telomeric recombination rather than by subtelomeric duplication.

As indicated in Table 1, 8 of these 76 clones derived from the long Bcl precursor (including clones 1 in Fig. 3.4F-I) were classified as having intermediate amplification and spreading of Bcl repeat based on total telomeric signal that was partially cleaved by *Bcl*I (Fig. 3.4F), Bcl-specific telomeric signal that was at least 8 times that of the precursor (Fig. 3.4G and data not shown), and subtelomeric signal that was substantially cleaved into one or more short

fragments by BclI digestion (Fig. 3.4H). These results suggest that both Bcl and WT repeats were amplified and copied onto multiple telomeres in these mutants. This group of clones had no obvious telomeric fragments of 50-500 bp in *Eco*RI + *Bcl*I digests (Fig. 3.4F and data not shown), suggesting that the amplified Bcl and WT repeats were not generally interspersed closely together. Indeed, the signal from WT repeats that remained after *Bcl*I digestion was generally very long, suggesting that WT repeats in these clones were often in long continuous arrays (Fig. 3.4F and data not shown). Twelve other clones (including clone 4 in Fig. 3.4F-I) were classified as having slight amplification and spreading of Bcl repeats based on lesser degrees of amplification of Bcl repeats and subtelomeric signal that was cleaved by *Bcl*I (Table 3.1).

One clone of the *stn1-M1 ter1-Δ* mutant, clone 15 in Fig. 3.4F-I, showed a unique outcome. It displayed a ~9-10 kb band that hybridized intensely with both telomeric and subtelomeric probes but was resistant to *Bcl*I digestion (Fig. 3.4F and H). These results suggest that this clone contains tandem arrays composed of both WT telomeric repeats and subtelomeric sequences, but not Bcl repeats. *HIS3* was detected in the clone but apparently was not within the amplified fragment (Fig. 3.4I). Conceivably, the putative tandem arrays originated from a native telomere rather than the Bcl telomere. This clone is reminiscent of type I post-senescent survivors in *Sacchromyces cerevisiae*, which are characterized by amplified subtelomeric Y' elements and short tracts of telomeric repeats (41, 70). Although type I-like survivors with alternating telomere and non-telomeric sequences can occur in *K. lactis* cells that are transformed with a circle containing telomeric repeats and the *URA3* gene ((52) and Xu and McEachern, submitted), to our knowledge, this is the first report of RTE amplifying subtelomeric sequences in *K. lactis*.

23 of 76 *stn1-M1 ter1-Δ* mutants (30%) derived from long Bcl precursors had lost *HIS3* signal (Fig. 3.4I and data not shown). All 23 of these clones showed no amplification and spreading of Bcl repeats. This loss rate of *HIS3* was significantly less ($p<0.001$, in Fisher exact test) than that seen in mutants derived from normal Bcl precursors where 41 of 55 clones (75%) showed no *HIS3* signal. This result suggests that a long telomere stabilizes the adjacent subtelomeric sequences from being lost during the establishment of an *stn1-M1 ter1-Δ* mutant.

The absence of mismatch repair facilitates the spreading of sequence from a single tagged telomere to other telomeres.

To test whether the low frequency of spreading Bcl repeats to other telomeres in *stn1-M1 ter1-Δ* mutants was affected by mismatch repair, we constructed normal length Bcl precursor and long Bcl precursor strains containing p*STN1-TER1* and a *msh2-Δ* mutation. After screening for loss of p*STN1-TER1*, we identified 55 *stn1-M1 ter1-Δ msh2-Δ* mutants from three normal length Bcl precursors and 38 mutants from two long Bcl precursors. The results showed that the *msh2-Δ* background permitted a substantially higher frequency of amplification and spreading of Bcl repeats than a *MSH2* background did. Eight of 55 *stn1-M1 ter1-Δ msh2-Δ* mutants (15%) derived from normal length Bcl precursors (including clones 1-2 in Fig. 3.5A-D) showed complete or near complete spreading of Bcl repeats (Table 3.1). Two mutants (4%), including clone 6 in Fig. 3.5A-D, showed intermediate amplification and spreading of Bcl repeats and the remaining mutants (45 of 55; 81%), including clones 5 and 11-13 in Fig. 3.5A-D, showed no amplification (Table 1).

Remarkably, 30 of 38 mutants (78%) derived from long Bcl precursors (including clones 5-7, 9, and 10 in Fig. 3.5E-H) showed complete or near complete spreading of Bcl repeats (Table 3.1). At least 25 of these clones appeared to contain a small percentage of WT repeats

interspersed among the Bcl repeats, as indicated by telomeric signal of <500 bp present in *Eco*RI + *Bcl*I digestion (e.g., clones 5-7, 9, and 10 in Fig. 3.5E). However, as these telomeric signals were not intense, it is likely that the WT repeats were not interspersed throughout the telomeres. Only two clones (6%), including clone 8 and 11 in Fig. 3.5E-H, showed intermediate amplification and spreading of Bcl repeats (Table 3.1). As the disruption of mismatch repair presumably permits Bcl repeats to recombine with WT repeats in an unperturbed fashion, we conclude that the long telomeres formed during the establishment of an *stn1-M1* mutant are commonly derived from a single telomere source.

45 of 55 (82%) *stn1-M1 ter1-Δ msh2-Δ* mutants derived from normal Bcl precursors (Fig. 3.5D and data not shown) and 21 of 38 (55%) mutants derived from long Bcl precursors (Fig. 3.5H and data not shown) had lost the *HIS3* genes. This difference was significant (p=0.0005 in Fisher exact test). Strikingly, many clones that had complete or nearly complete spreading of Bcl repeats (4 of 8 mutants from the normal length Bcl precursor and 14 of 30 from the long Bcl precursor) had lost the *HIS3* genes (clone 5 and 7 in Fig. 5H and data not shown). On the other hand, 4 of 18 clones that had complete or near complete spreading of Bcl repeats contained estimated 5-10 copies of *HIS3* genes (data not shown). These results showed that the spreading of Bcl repeats to all other telomeres could occur either with or without concurrent spreading of the subtelomeric *HIS3* to other telomeres.

The presence of telomerase inhibits the spreading of sequence from a single tagged-telomere to other telomeres.

To test the effect of telomerase on amplification and spreading of Bcl repeats, we constructed normal length Bcl precursors and long Bcl precursors in both *stn1-M1 TER1 MSH2* and *stn1-M1 TER1 msh2-Δ* backgrounds that were complemented by plasmid p*STN1*. These

precursors were similar to those used above except that the subtelomeric marker gene was *URA3* and the complementing plasmid carried *STN1* and *HIS3*. We generated *stn1-M1* mutants from all four precursor types by screening for loss of p*STN1* after streaking onto YPD plates and identifying clones with the rough colony phenotype characteristic of *stn1-M1* mutants. Results from this showed that telomerase significantly inhibited the amplification and spread of Bcl repeats (Fig. 3.6, Fig.S3.3 and Table 3.1). None of 37 *stn1-M1 TER1 MSH2* clones derived from normal-length precursors showed amplification and spreading of Bcl repeats (Fig. S3.3A-D and Table 3.1), and only 1 of 38 *stn1-M1 TER1 MSH2* clones from the long precursors showed detectable amplification and spreading (clone 9 in Fig. S3.3E-H and Table 3.1). The one mutant that did show amplification (an intermediate level) was estimated to contain three copies of *URA3* (data not shown). This may suggest that amplification and spreading of Bcl repeats in this clone occurred primarily through subtelomeric BIR events that produced extra copies of the tagged telomere rather than by telomere-telomere recombination.

Similar outcomes were seen with *stn1-M1 TER1 msh2-Δ* mutants. There, the frequency of amplification and spreading of Bcl repeats was markedly lower than that seen in *stn1-M1 ter1-Δ msh2-Δ* mutants (Table 3.1). None of 28 *stn1-M1 TER1 msh2-Δ* mutants obtained from the long precursors showed complete amplification and spreading of Bcl repeats, and only 7 of 28 mutants showed intermediate or slight amplification. Clone 9 in Fig. 3.6B-E is the sole example of intermediate amplification. Among *stn1-M1 TER1 msh2-Δ* mutants derived from a normal length Bcl precursor, only 3 of 25 mutants, including clones 6 and 7 in Fig. S3.3I-L, showed slight Bcl amplification (Table 3.1).

The subtelomeric *URA3* marker was comparatively stable in the *stn1-M1 TER1* mutants. Only 7 of 67 *stn1-M1 TER1 msh2-Δ* mutants and 0 of 39 *stn1-M1 TER1 MSH2* mutants had lost

URA3 (Fig. S2.3D, H, L, Fig. 3.6E and data not shown). These results suggest that an active telomerase in *stn1-M1* mutants helps to stabilize the adjacent subtelomeric sequences against loss or amplification.

DISCUSSION

Considerable evidence suggests that a roll-and-spread mechanism is involved in generating the moderately elongated telomeres formed in *K. lactis ter1-Δ* post-senescence survivors (reviewed in (45)). Our studies here suggest that this mechanism, involving amplification of sequence from a single t-circle is also involved in establishing the more ALT-like highly elongated telomeres in the *K. lactis stn1-M1* mutant. Some support for the importance of t-circles in *stn1-M1* cells already existed. Previous data had shown that t-circles are abundant in *stn1-M1* cells (5). Additionally, a recent study showed that introducing t-circles into *stn1-M1* cells leads to tandem arrays of the circle's sequence becoming incorporated at multiple telomeres (Xu and McEachern, submitted).

The use of mutationally tagged repeats was critical to earlier studies on how RTE occurs in a *ter1-Δ* mutant (52-53, 72). However, the type II RTE in this mutant is episodic, occurring when telomeres drop below a critical length and essentially shutting off once telomeres are moderately lengthened (43). The transient stability of the lengthened telomeres allowed Southern blots that examined the structure of telomeric DNA from populations of cells to be very informative. We anticipated that the type IIR RTE of the *stn1-M1* mutant, with its apparently continuous high rate of telomeric recombination, would be more problematic to study by this method as any long telomere initially generated would be expected to be unstable and would become altered by further recombination events in many if not most cells of any culture large enough to be studied. The first tagging approach used in this study involved generating *stn1-M1*

MSH2 mutants from precursors with ApaL repeats at all telomeric termini. Among the mutant clones that had both amplified WT and ApaL repeats, roughly half had telomeric blocks of both types of repeats that had single favored sizes. Although other interpretations cannot be ruled out, this result is consistent with a roll-and-spread mechanism that derived all amplified telomeres from the sequence of a single small t-circle if the uniformly repeating patterns predicted from rolling circle copying of a t-circle containing both WT and ApaL repeats had been extensively disrupted by later recombination events. Other results clearly demonstrated that ongoing recombination in *stn1-M1* mutants can disrupt existing signs of repeating patterns. This was most strikingly seen when a *stn1-M1 ter1-Δ MSH2* mutant with a very non-random WT/ApaL repeat pattern had its *MSH2* gene disrupted. As soon as this *msh2-Δ* derivative could be examined, its telomeric repeats had acquired a randomized pattern similar to those seen in *stn1-M1 ter1-Δ* mutants established directly in a *msh2-Δ* background. As discussed further below, loss of mismatch repair is expected to elevate recombination rates between WT and ApaL repeats to levels that would occur if no mismatches were present. Thus, even the complete absence of preferred sizes of blocks of WT and ApaL repeats seen in *stn1-M1 ter1-Δ msh2-Δ* clones is not inconsistent with a single t-circle having been the original source of the amplified telomeres.

A result of particular importance in our study was that Bcl repeats present at a single telomere in a precursor cell were sometimes the source of virtually all of the amplified telomeric DNA sequences in newly generated *stn1-M1* mutants. This effect was most prominent in a strain lacking both telomerase and *MSH2*. This mutant combination is probably the most relevant of those examined that used Bcl repeats to study telomeric recombination. This is because the absence of telomerase assures that all telomeric lengthening is due to recombination while the absence of mismatch repair presumably permits recombination between wild type and Bcl

telomeres to occur at the same frequency as recombination between two wild type telomeres in the same circumstances. In the *ter1-Δ msh2-Δ* strain, the percentage of *stn1-M1* mutants that displayed complete or near complete amplification and spreading of Bcl repeats was 14% in mutants derived from the normal length Bcl precursors and 78% in mutants derived from the long Bcl precursors (Table 1). These results are very similar to those seen in an earlier study with *K. lactis ter1-Δ* mutants, where total spreading of Bcl repeats to all telomeres from a single normal length Bcl telomere and a single long Bcl telomere was measured at 10% and 94%, respectively (72). As was proposed with *ter1-Δ* mutants, we suggest that a roll-and-spread mechanism, where rolling circle amplification of a single t-circle followed by spreading of that sequence to all telomeres by BIR events, can account for these observations. When all twelve telomeres are the same length and each has the same chance to be amplified and spread, the predicted frequency of clones where Bcl repeats have been amplified and spread would be roughly one twelfth. The much greater frequency of spreading of Bcl repeats that is observed from mutants derived from the long Bcl precursors indicates that a longer telomere is better able to promotes its amplification and spreading to other telomeres. The roll-and-spread mechanism predicts that this could occur because the long Bcl telomere is better at forming t-circles and/or can be used directly as a template for lengthening other telomeres. What factors facilitate the spreading of sequence from one telomere to others in newly generated *stn1-M1* mutants are not clear. The fact that all amplified telomeric sequences can be derived from a single telomere suggests that t-circle formation may be limiting. Additionally, it has been reported that multiple double-strand DNA breaks (DSBs) are often recruited to a single Rad52-containing focus for DNA repair in *S. cerevisiae* (38). This phenomenon could potentially facilitate multiple

uncapped telomeres to use a common template when being repaired by homologous recombination.

The amplification and spreading of Bcl repeats in newly generated *stn1-M1* mutants was strongly inhibited by the mismatch repair system. This is not surprising as mismatch repair can inhibit recombination involving sequences with imperfect homology (31). For example, in *S. cerevisiae*, 1% and 6% sequence divergence can reduce mitotic recombination ~20 and ~140 fold, respectively (15). Therefore, the 4% sequence divergence between Bcl repeats and WT repeats in our study may significantly reduce the recombination between the telomeres containing them. Mismatch repair may inhibit recombination by rejecting or processing the heteroduplex formed by strand invasion occurring with homeologous sequences (31). Loss of *MSH2* was previously shown to facilitate the recombination that generates post-senescence survivors in telomerase deletion mutants of *S. cerevisiae* and *K. lactis.* This was attributed to increased recombination between the homeologous telomeric repeats in *S. cerevisiae* and between the homeologous subtelomeric sequences in *K. lactis* (63). Deficiency in mismatch repair can also facilitate ALT-like telomerase-independent telomere elongation in human colon cancer cell lines and in gastric carcinomas (8, 57). Conceivably, this effect stems from the degenerate telomeric repeats that are common in the basal regions of human telomeres (1, 4).

In *stn1-M1* mutants formed in a *ter1-Δ MSH2* background, there was not only a substantial reduction in the frequency of clones exhibiting total spreading of Bcl repeats but also, when spreading was observed, it was far more likely to be partial, accompanied by substantial amplification of WT repeats as well. We suggest that in these clones, formation of a t-circle from the Bcl telomere will occur efficiently (as no mismatches would be present during intratelomeric recombination) but copying sequence from a t-circle composed purely of Bcl

repeats onto any of the 11 wild type telomeres would be impeded. When copying of a Bcl repeat t-circle did occur, the impeded ability of the Bcl repeats to recombine with the resident wild type telomeres could allow time for wild type t-circles to form and also become amplified.

Interestingly, mismatch repair was not an appreciable barrier to recombination between Bcl and WT repeats in *STN1 ter1-Δ* mutants (72). One possibility to account for this is that the extremely short telomeres in senescing cells of these mutants recombine in a different way than the somewhat longer telomeres in newly forming *stn1-M1* mutants. This is supported by the fact that the type II RTE in *S. cerevisiae* depends on a pathway involving Rad50 when occurring at very short telomeres in a senescing telomerase deletion mutant but depends on the more standard Rad51-dependent pathway when occurring at longer telomeres uncapped by defects in telomere capping proteins (23, 69). Also, the Rad51-dependent recombination is inhibited by mismatch repair 13-fold more than Rad50-dependent recombination (68). The formation of post-senescence survivors in *K. lactis STN1 ter1-Δ* mutants has recently been shown to require both the Rad50- and the Rad51- pathways (Basenko and McEachern, unpublished data).

Telomerase is active in *stn1-M1* cells, but its presence does not grossly affect the phenotype of the mutant (27). Recently we showed that telomerase provides a modest enhancement of stability to sequences integrated within telomeres in *stn1-M1* cells (Xu and McEachern, submitted). Our results here demonstrate that the presence of telomerase at the establishment of the *stn1-M1* state can largely inhibit the amplification and spreading of Bcl repeats. While we cannot rule out the possibility that telomerase fundamentally alters the mechanism by which telomeres are maintained in *stn1-M1* cells, we believe this is unlikely. Rather, we suggest that sequence addition by telomerase masks the effects of recombination. In particular, we suggest that the *stn1-M1* mutation, like certain telomeric repeat mutations shown

to produce type IIR RTE, is disrupted not only in telomeres' ability to block recombination but also in their ability to negatively regulate sequence addition by telomerase (6, 72). Consistent with this possibility is our observation that telomerase inhibits the spreading of Bcl repeats not only from a normal length telomere, but also from long Bcl telomere in *stn1-M1* cells. The latter is resistant to telomerase addition in wild type *K. lactis* cells (72). With multiple telomerase-synthesized WT telomeric repeats added onto the ends of both long and normal length Bcl telomeres, t-circles formed from terminal deletions of these telomeres would likely often be composed only of WT repeats. This would of course interfere with the ability of the Bcl repeats to amplify and spread to other telomeres. The relative contribution of recombination and telomerase to new telomeric repeat synthesis in *stn1-M1 TER1* cells is not known. It is reasonable to believe, however, that the contribution of telomerase might be greatest at the earliest stages of formation of the *stn1-M1* state, before recombination has abundant long telomeric sequences available for it to copy.

One unexpected result from our work was that precursor cells with ApaL repeats present at the termini of all telomeric ends produced *stn1-M1* mutants where the ApaL repeats were generally completely absent. This occurred in both *msh2-Δ* and *MSH2* backgrounds and therefore does not appear to require mismatch repair. As the ApaL telomeric base change does not appear to alter telomere function (74), this effect is also not likely to be due to selection against amplification of ApaL repeats. The simplest explanation for this outcome would be that a significant fraction of telomeric termini, at least a third of the ~400-600 bp telomeres by our estimates, is routinely deleted prior to the initiation of recombination events that elongate telomeres when the *stn1-M1* mutant state is established. Telomere shortening also precedes RTE in yeast telomerase deletion mutants. In that case, the shortening occurs very gradually over

many tens of cell divisions from replicative sequence loss (41, 44, 67). However, gradual telomere shortening cannot explain the terminal sequences loss we see in *stn1-M1* cells. Most newly generated spores of the *stn1-M1* mutant die within a few cell divisions (27). This indicates that they experience a severe growth problem immediately after their generation and suggests that the terminal telomeric loss occurs very rapidly. Indeed, the rapid telomeric deletion might help account for the poor viability of *stn1-M1* spores.

A number of mechanisms have been proposed for generating telomeric deletions (for a review see (3)). One well studied mechanism is telomere rapid deletion (TRD) which is thought to be an intratelomeric recombination event that occurs after a telomeric 3' overhang strand invades into the double-stranded region of the same telomere (for a review see (42)). TRD can lead to telomere deletion in wild type yeast cells that contain artificially elongated telomeres and in other circumstances (7, 11, 35). Processes similar to TRD, requiring the recombination protein XRCC3, can lead to shortening of both normal and dysfunctional mammalian telomeres (59, 76). Although TRD has been proposed to generate t-circles (11, 25) and t-circles are abundant in *stn1-M1* cells, we suggest that the initial telomeric deletions, before elongation occurs, do not generally produce t-circles. If they did, ApaL repeats present on the t-circles would be added back to telomeres from BIR events copying those t-circles rather than being lost from the emerging *stn1-M1* mutants.

An obvious question is why telomeric truncations would predominate at the earliest stage of the formation of a mutant that ultimately generates and maintains highly elongated telomeres. At least two possibilities exist. One is that the physiological conditions at the earliest stage of *stn1-M1* mutants, when telomere deletions occur, are different from those at later stages, when telomere elongation predominates. Conceivably, later stages might be influenced by the presence

of chronic DNA damage and react differently to dysfunctional telomeres compared to cells at the earliest stage. This idea is supported by the finding that *S. cerevisiae* telomerase deletion mutants showed expression changes in hundreds of genes during the senescence caused by shorting telomeres (54). Another possibility is that telomeric deletions are always more frequent than recombination events that lengthen telomeres in *stn1-M1* cells. In this scenario, net lengthening might only predominate over shortening once long telomeres or t-circles are present and abundant enough to serve as efficient templates for elongation events. Some observations support this possibility as well. In wild type *K. lactis* cells, deletions from TRD are more frequent than telomere elongation by recombination (7). Also, telomeric deletions are very frequent in *stn1-M1* cells and in other mutants undergoing type IIR RTE ((6) and (Xu and McEachern, submitted)).

Taken together, our results suggest that the establishment of long and heterogeneous telomeres in *stn1-M1* via type IIR RTE may involve the following events. First, upon initiating the *stn1-M1* state, the uncapped telomeres undergo net deletion to generally lose at least one third of the repeats from telomeric termini. Next, an occasional deletion results in the production of a small t-circle that is used as a template to lengthen one or more telomeres. Finally, the initial elongated telomere(s) serve as the templates for lengthening most or all remaining telomeres, generally before other t-circles can form and compete for being copied. Another study of ours (Xu and McEachern, submitted) that examined the stability of tandem arrays at telomeres in *stn1-M1* cells, suggests that once the long telomere state is established, the maintenance of it likely includes secondary formation and copying of t-circles. However, the spreading of sequence from a single source to all telomeres was rare or absent, presumably because cells

already contained many potential templates (both t-circles and linear telomeric tracts) that could be copied to generate long telomeric arrays.

The extent to which our results with *stn1-M1* cells bear on human ALT cells remains to be determined. T-circles are known to be abundant in ALT cells (12), however, there are conflicting data regarding their importance. While Ku mutations inhibit formation of t-circles and block proliferation of ALT cells (36), mutations in Xrcc3 and Nbs1 have been reported to eliminate t-circles without blocking ALT cell growth (20). Our study suggests that it will be important to examine not only the maintenance of long telomeres in established ALT cells but also the role of t-circles during the initial establishment of the ALT cell state. Our data also suggest that a single telomeric template DNA might commonly be the source of elongation of multiple telomeres. Interestingly, multiple telomeres in ALT cells can cluster into single foci which co-localize with PML bodies and may facilitate the recombination between telomeres (21). Our finding that telomerase can have some effects on telomeric recombination in *stn1-M1* cells may have parallels in ALT. Indeed, expression of telomerase was shown to reduce the number of telomeres clustered in single foci in the ALT cells and could therefore decrease the frequency of recombination between telomeres (21). Much further work will be needed to fully understand the mechanisms involved in the establishment and maintenance of ALT.

MATERIALS AND METHODS

Strains and plasmid

All *K. lactis* strains used are derivatives of wild type (WT) 7B520 (77). *K. lactis stn1-M1*, *stn1-M1 ter1-Δ* strains were described previously(27). The precursors of *K. lactis stn1-M1 msh2-Δ*, *stn1-M1 ter1-Δ msh2-Δ* were constructed as follows. The *MSH2* gene was first amplified as a 4.4 kb fragment from genomic DNA of 7B520 cells by PCR (forward primer:

5’AGGGATCCGGGAGGCTCCAATAACAACA3’; reverse primer:5’ACCTCGAGTTGCGAGTGATTCGTTCAAG3’) and cloned into the *Bam*HI and *Xho*I sites of pBluescripts IIKS(-) resulting in p*MSH2*. Then, p*MSH2* was digested by *Bgl*II and *Eco*RI to delete an 891 bp fragment of the ORF of *MSH2,* and a 1.4 kb PCR-amplified (forward primer: 5’AGGCAGATCTGGATGGCGGCGTTAGTATCG3’; reverse primer:5’AGGAATTCCCAGCGACATGGAGGCCCAG3’) fragment of *KANMX* gene from the genomic DNA of SAY557 (32) was inserted into the *Bgl*II + *Eco*RI- digested p*MSH2* to produce the p*MSH2*::*KANMX*. The 3.2 kb disruption cassette containing *MSH2*::*KANMX* was then amplified from p*MSH2*::*KANMX* by PCR (forward primer: 5’ATATTGCAGAGGAGCGAGGA3’; reverse primer: 5’CTTGTACGGACGGGTCATCT3’) and was transformed into either *stn1-M1* cells complemented with p*STN1* or *stn1-M1 ter1-Δ* cells complemented with p*STN1-TER1* or p*STN1-TER1(ApaL).* The knockout of the *MSH2* gene was confirmed by Southern blotting and hybridization to a *MSH2* probe.

The plasmid p*STN1* was constructed in following two steps. First, a 3.4 kb fragment containing the 1.3 kb ORF of the *STN1* gene and 1.6 kb upstream and 0.5 kb downstream sequences was obtained by PCR (forward primer: 5’ACGAGCTCTGGCAACCCACTTGTGACTA3’; reverse primer: 5’ACCTCGAGTGCTCAGCCAATTTCTGTTG3’) using the genomic DNA of the 7B520 strain as the template. Second, the PCR fragment, which contains flanking *Sac*I and *Xho*I sites, was inserted into the polylinker *Sac*I and *Xho*I sites of pKL313(*HIS3*) (66) to generate the p*STN1*. Plasmids p*STN1-TER1* and p*STN1-TER1(ApaL)* were constructed as follows. First, the *STN1* gene was cloned as a 3.4 kb fragment from genomic DNA of 7B520 cells by PCR (forward primer: 5’ACGAGCTCTGGCAACCCACTTGTGACTA3’; reverse primer:

5’ACGGTACCTGCTCAGCCAATTTCTGTTG3’) into the *Sac*I and *Kpn*I sites of pCXJ18 (16) to result in plasmid pCXJ18-*STN1*. The 2.6 kb *TER1* or *TER1(ApaL)* gene fragments flanked by *Xba*I and *Kpn*I sites were obtained from pJR31(72), and pJR31 derivative that contained a mutation in the template of *TER1* changing T20 to C to create an *Apa*LI restriction site by oligonucleotide mediated site-directed mutagenesis as described elsewhere (33). Subsequently, the 2.6 kb *TER1* or *TER1(ApaL)* fragment was cloned into the pCXJ18-*STN1* to generate p*STN1-TER1* or p*STN1-TER1(ApaL)*.

The *URA3*-tagged single Bcl telomeres containing ~11 and ~40 Bcl-telomeric repeats were described before (72). The *HIS3*-tagged single Bcl telomeres were based on the *URA3*-tagged single Bcl telomeres, which were cleaved by *Pst*I and *Nru*I to excise the *URA3* gene and replace it with a 1.2 kb *HIS3* fragment amplified from pKL313 (66) by PCR (forward primer: 5’ACAGTGCTGCAGCGGCATCAGAGCAGATTGTA3’; reverse primer: 5’ACTGAGTCGCGATCTGTGCGGTATTTCACACC3’). Either *URA3* or *HIS3*-tagged single Bcl telomeres were transformed into *stn1-M1* cells with a *MSH2* or a *msh2-Δ* genetic background complemented by p*STN1*, or *stn1-M1 ter1-Δ* cells with a *MSH2* or a *msh2-Δ* genetic background complemented by p*STN1-TER1* respectively. Ura^+ or His^+ colonies were then examined by Southern blotting to confirm that *URA3* or *HIS3*-tagged single Bcl telomeres had replaced a single native telomere by subtelomeric recombination.

K. lactis transformation was done by electroporations as described for *S. cerevisiae* (9). Passaging of complemented cells was carried out by serial streaking of single colonies on rich medium (YPD plates) at 30°C. Strains were streaked every 3 days down to single cells that grew into colonies. Each streak was estimated to be 20-25 cell divisions.

Telomere cloning and sequencing

The yeast genomic DNA sample from clone A1 in Fig. 3.2A was partially digested with *Apa*LI. This was terminated by an equal volume of 12.5 mM EDTA added to the digestion reaction. The *Apa*LI partially-digested DNA was ligated with the *Apa*LI digested pACYC177 plasmid, and transformed into DH5α cells. The clones with telomeric fragments were confirmed by a Southern blot hybridized to telomeric probe. Positive clones were then sequenced.

Southern hybridizations

Yeast genomic DNA samples digested with restriction enzymes were run on 0.8% or 3% agarose gels and then transferred onto Hybond N+ membrane in 0.4 M NaOH. All hybridization were carried out in Na_2HPO_4 and SDS as described (17). The γ-^{32}P-labeled telomeric probe is Klac 1-25 (44) The temperature of hybridization and washing for this probe was between 45-50° C. The γ-^{32}P-labeled Bcl probe is KTelBcl (GATCAGGTATGTGG), described before (66) The temperature of hybridization and washing is 40 °C and 34-36°C respectively. The subtelomeric probe was generated from pKL11-B (Insert of ~1 kb telomeric *Eco*RI-*Sma*I fragment into pBluescript SK-), which was digested with *Xba*I and ligated back together to excise all the telomeric sequence and was then digested by *Eco*RI and *Xba*I to generate a ~600 bp subtelomeric fragment for probe. The *URA3* probe was described before (46). The *RAD50* gene probe was the purified PCR product from *K. lactis* genomic DNA (Forward primer: 5'GATAGGTCTACCGCGACCAA3'; Reverse primer: 5'GCGTAAGAGGACGCATTCAT3'). Subtelomeric, *URA3*, and *RAD50* probes were prepared using a random priming kit (NEB). Temperature of hybridization and washing for these probes was 65°C. The membranes were autoradiographed and visualized using a Molecular Dynamics Storm PhosphorImager.

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FIGURES

Fig. 3.1. Use of mutationally-tagged repeats at telomeric termini to study RTE in *stn1-M1* cells. (A) Experiment outline. After transformation of p*STN1-TER1(ApaL)* into *stn1-M1 ter1-Δ* strains, followed by serial passaging for 10 streaks, the ApaL precursor was generated that contains telomeres composed of terminal ApaL repeats (gray boxes) and internal WT repeats (white boxes). The sequence of a WT repeat as well as the ApaL mutation are shown. The positions of *Apa*LI and *Rsa*I restriction sites in the repeats and the subtelomeric *Bsr*BI(B) site are indicated. Upon loss of p*STN1-TER1(ApaL),* newly generated *stn1-M1 ter1-Δ* mutants with long telomeres will be recovered. Four possible outcomes for the telomeres in these mutants are illustrated; 1. Uniformly repeating pattern of the two repeat types, 2. Randomly mixed repeats, 3. Pure ApaL repeats and 4. Pure WT repeats. (B) Southern blot, hybridized with a telomere probe, of *Eco*RI (indicated by "-") and *Eco*RI + *Apa*LI (indicated by "+") digested DNA from *stn1-M1* (M1), WT and ApaL precursor (Pr). (C), *Bsr*BI, *Bsr*BI + *Apa*LI and *Bsr*BI + *Rsa*I-digested DNA from WT and ApaL precursor (Pr) separated on a 3% agarose gel electrophoresis and hybridized with the telomere probe.

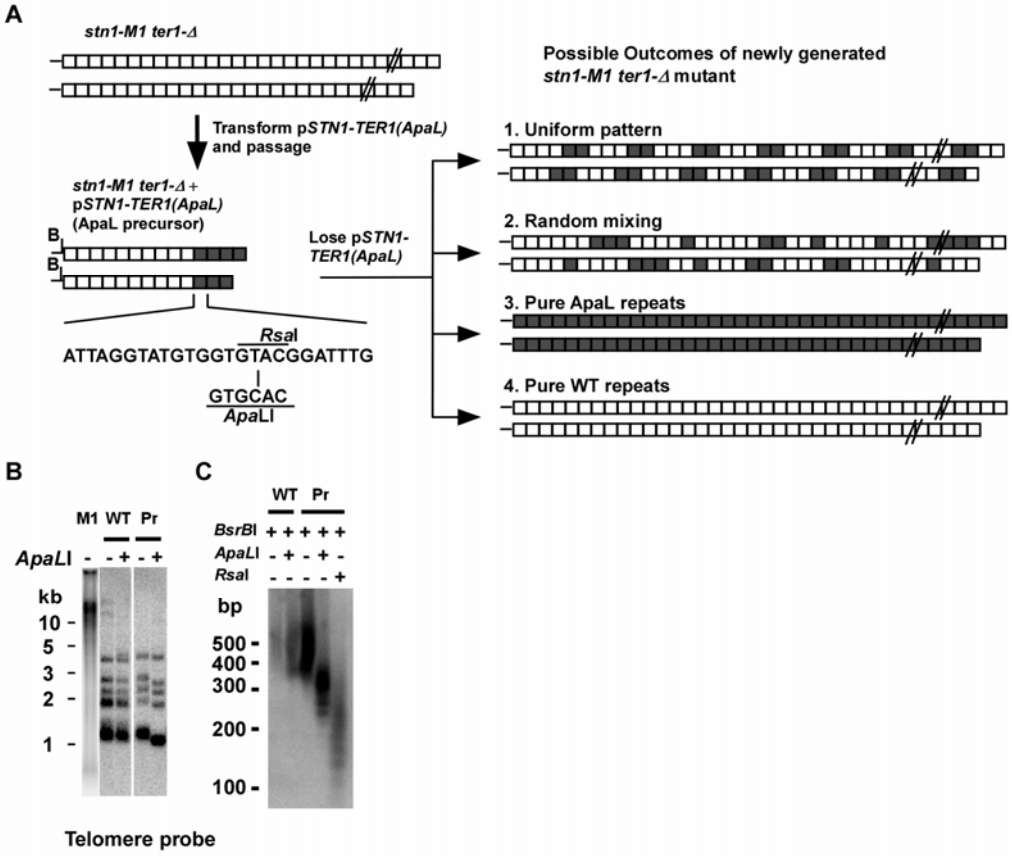

A
stn1-M1 ter1-Δ
Transform pSTN1-TER1(ApaL) and passage
stn1-M1 ter1-Δ + pSTN1-TER1(ApaL) (ApaL precursor)
B
B
Lose pSTN1-TER1(ApaL)
RsaI
ATTAGGTATGTGGTGTACGGATTTG
GTGCAC
ApaLI
Possible Outcomes of newly generated stn1-M1 ter1-Δ mutant
1. Uniform pattern
2. Random mixing
3. Pure ApaL repeats
4. Pure WT repeats
B
M1 WT Pr
ApaLI - - + - +
kb
10
5
3
2
1
C
WT Pr
BsrBI + + + + +
ApaLI - + - + -
RsaI - - - - +
bp
500
400
300
200
100
Telomere probe

Fig. 3.2. Repeating structure can be formed in telomeres of some newly generated *stn1-M1 ter1-Δ* mutant. (A) Southern blot, hybridized with a telomere probe, of *Eco*RI (1st lane of every clone), *Eco*RI + *Apa*LI (2nd lane of every clone) and *Eco*RI + *Rsa*I (3rd lane of every clone) digested DNA from 10 newly generated *stn1-M1 ter1-Δ* mutants generated from the ApaL precursor. (B) Same filter as in panel A after stripping and rehybridization with a subtelomeric probe. (C) *Eco*RI (1st lane), *Eco*RI + *Apa*LI (2nd lane) and *Eco*RI + *Rsa*I (3rd lane) digested DNA from A1 clone in panel A separated on a 3% agarose gel electrophoresis and hybridized with the telomere probe. (D) Southern blot, hybridized with a telomere probe, of *Eco*RI (1st lane of every clone), *Eco*RI + *Apa*LI (2nd lane of every clone) and *Eco*RI + *Rsa*I (3rd lane of every clone) digested DNA from 10 additional newly generated *stn1-M1 ter1-Δ* mutants separated by electrophoreses on a 0.8% (Upper panel) and a 3% (Bottom panel) agarose gel. (E) Southern blot, hybridized with a telomere probe, of *Eco*RI (1st lane of every clone), *Eco*RI + *Apa*LI (2nd lane of every clone) and *Eco*RI + *Rsa*I (3rd lane of every clone) digested DNA from A1 in panel A and C12 in panel D passaged for multiple streaks on YPD medium.

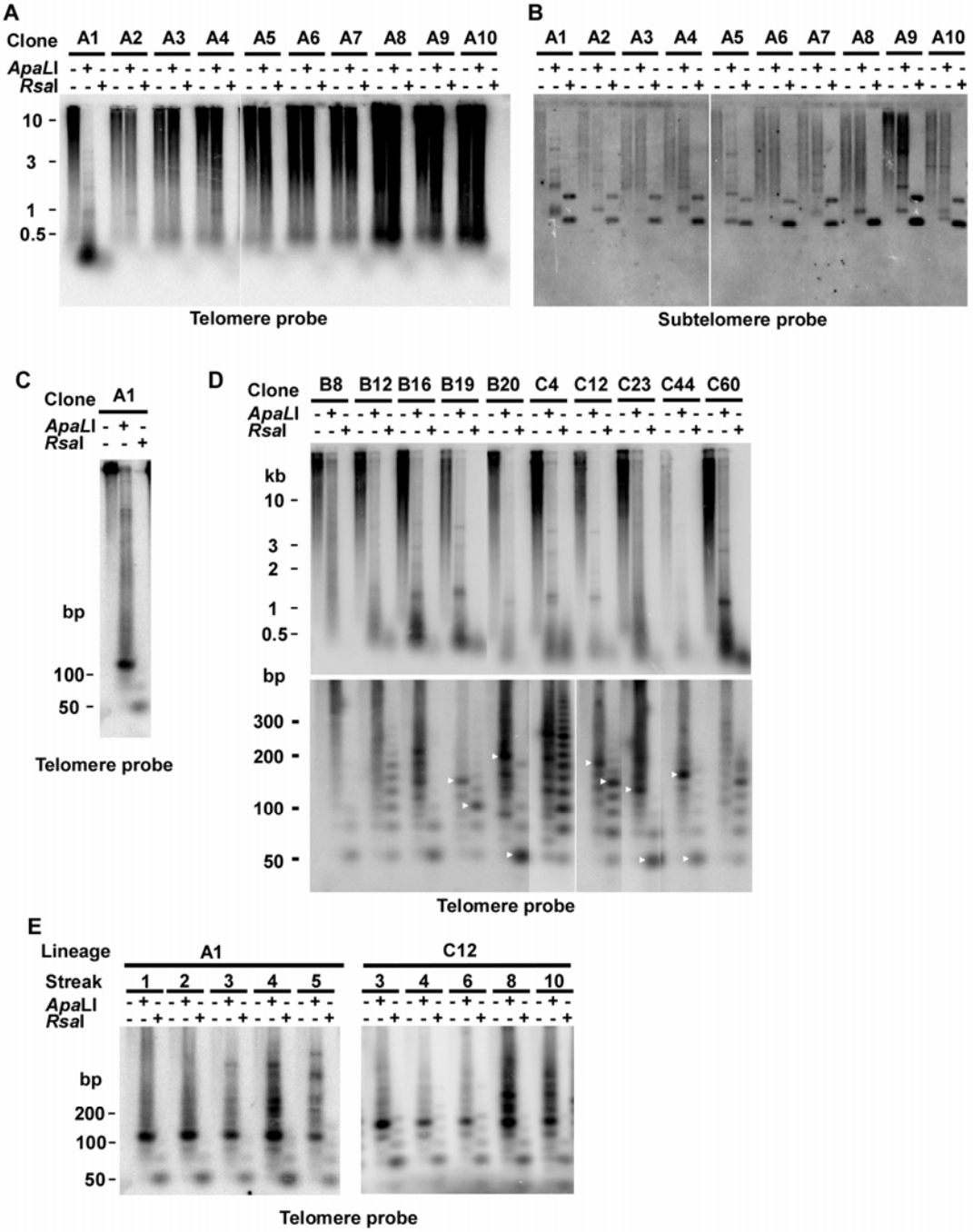

A
Clone A1 A2 A3 A4 A5 A6 A7 A8 A9 A10
ApaLI
RsaI
10
3
1
0.5
Telomere probe
B
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10
Subtelomere probe
C
Clone A1
ApaLI
RsaI
bp
100
50
Telomere probe
D
Clone B8 B12 B16 B19 B20 C4 C12 C23 C44 C60
ApaLI
RsaI
kb
10
3
2
1
0.5
bp
300
200
100
50
Telomere probe
E
Lineage A1 C12
Streak 1 2 3 4 5 3 4 6 8 10
ApaLI
RsaI
bp
200
100
50
Telomere probe

Fig. 3.3. The effect of mismatch repair deficiency on telomeric structure in *stn1-M1* mutants derived from the ApaL precursor. (A) Southern blot, hybridized with a telomere probe, of *Eco*RI (1st lane of every clone), *Eco*RI + *Apa*LI (2nd lane of every clone) and *Eco*RI + *Rsa*I (3rd lane of every clone) digested DNA from nine newly generated *stn1-M1 ter1-Δ* mutants in a *msh2-Δ* background separated on a 3% agarose gel. (B) Southern blot, hybridized with a telomere probe, of *Eco*RI (1st lane of every clone), *Eco*RI + *Apa*LI (2nd lane of every clone) and *Eco*RI + *Rsa*I (3rd lane of every clone) digested DNA from the A1 clone (in Fig. 3.2A) with (A1 *msh2-Δ*) or without (A1 *MSH2*) successful deletion of *MSH2*. Each clone is shown at each of 3 serial streaks. The DNA was separated on a 3% agarose gel. The A1 clone from Fig. 3.2A is also shown.

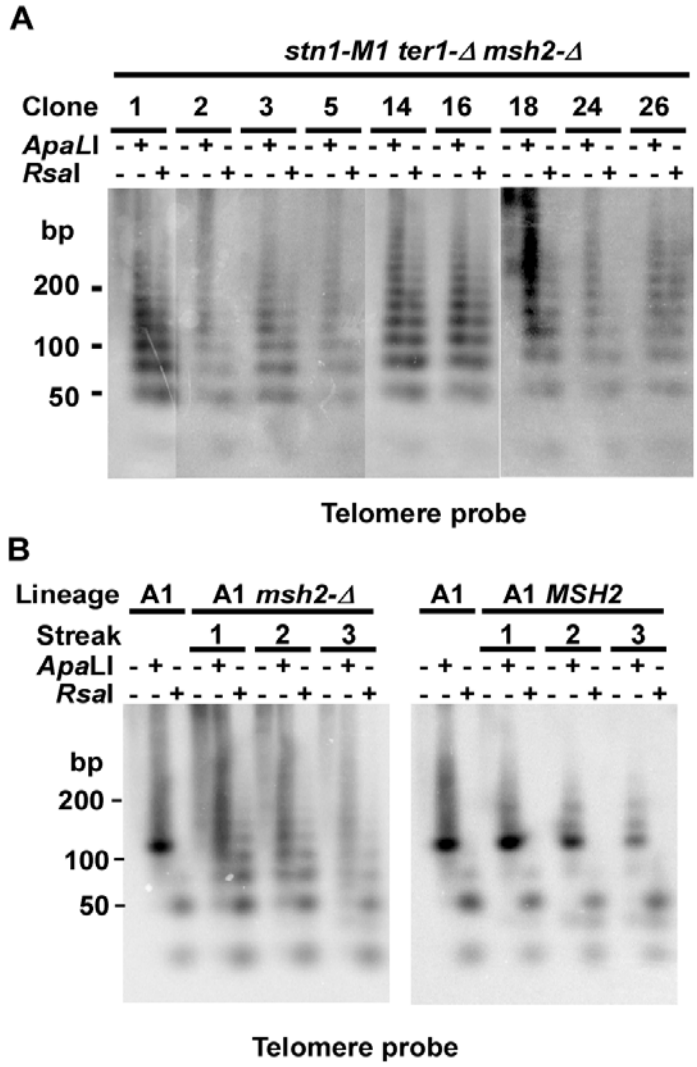

A
stn1-M1 ter1-Δ msh2-Δ
Clone 1 2 3 5 14 16 18 24 26
ApaLI
RsaI
bp
200
100
50
Telomere probe
B
Lineage A1 A1 msh2-Δ A1 A1 MSH2
Streak 1 2 3 1 2 3
ApaLI
RsaI
bp
200
100
50
Telomere probe

Fig. 3.4. Spreading of a telomeric sequence form a single telomere source during RTE in *stn1-M1 ter1-Δ* cells. (A) Experimental outline showing generation of *stn1-M1 ter1-Δ* mutants from *STN1 TER1* precursors containing a single telomere of either normal length or ~2X longer than normal length that are composed of Bcl repeats. The sequence of the Bcl repeat is indicated. (B) Southern blot, hybridized with a telomere probe, of *Eco*RI (indicated by "-") and *Eco*RI + *Bcl*I (indicated by "+") digested DNA from 5 newly generated *stn1-M1 ter1-Δ* clones from normal length Bcl precursors as well as from one normal length Bcl precursor *STN1 TER1* control. (C-E) Same filter as in panel B after stripping and rehybridization with a Bcl telomere probe, subtelomeric probe and *HIS3* probe, respectively. (F) Southern blot, hybridized with a telomere probe, of *Eco*RI (indicated by "-") and *Eco*RI + *Bcl*I (indicated by "+") digested DNA from 8 newly generated *stn1-M1 ter1-Δ* clones from long Bcl precursors as well as from one long Bcl precursor *STN1 TER1* control. (G-I) Same filter as in panel F after stripping and rehybridization with a Bcl telomere probe, subtelomeric probe and *HIS3* probe, respectively.

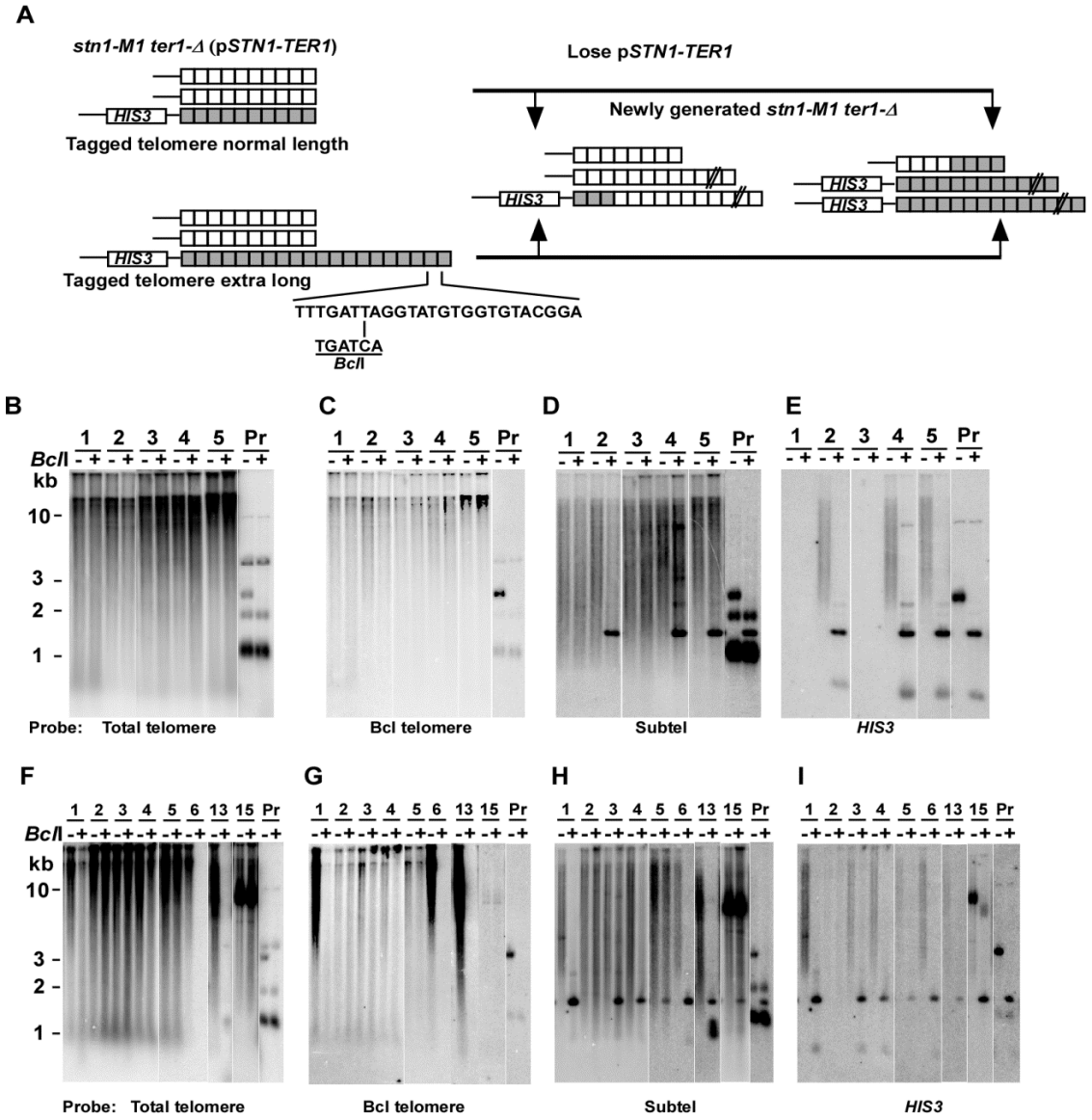

A
stn1-M1 ter1-Δ (pSTN1-TER1)
HIS3
Tagged telomere normal length
Lose pSTN1-TER1
Newly generated stn1-M1 ter1-Δ
HIS3
HIS3
HIS3
HIS3
Tagged telomere extra long
TTTGATTAGGTATGTGGTGTACGGA
TGATCA
BclI
B
C
D
E
1 2 3 4 5 Pr
BclI - + - + - + - + - + - +
kb
10
3
2
1
Probe: Total telomere
Bcl telomere
Subtel
HIS3
F
G
H
I
1 2 3 4 5 6 13 15 Pr
BclI - + - + - + - + - + - + - + - + - +
kb
10
3
2
1
Probe: Total telomere
Bcl telomere
Subtel
HIS3

Fig. 3.5. Spreading of a telomeric sequence from a single telomere source during RTE in *stn1-M1 ter1-Δ msh2-Δ* cells. (A) Southern blot, hybridized with a telomere probe, of *Eco*RI (indicated by "-") and *Eco*RI + *Bcl*I (indicated by "+") digested DNA from 7 newly generated *stn1-M1 ter1-Δ msh2-Δ* clones from normal length Bcl precursors in a *msh2-Δ* background as well as one normal length Bcl *STN1 TER1 msh2-Δ* precursor control. (B-D) Same filter as in panel A after stripping and rehybridization with a Bcl telomere probe, subtelomeric probe and *HIS3* probe, respectively. (E) Southern blot, hybridized with a telomere probe, of *Eco*RI (indicated by "-") and *Eco*RI + *Bcl*I (indicated by "+") digested DNA from 8 newly generated *stn1-M1 ter1-Δ msh2-Δ* clones from long Bcl precursors in a *msh2-Δ* background as well as one long Bcl *STN1 TER1msh2-Δ* control. (F-H) Same filter as in panel E after stripping and rehybridization with a Bcl telomere probe, subtelomeric probe and *HIS3* probe, respectively.

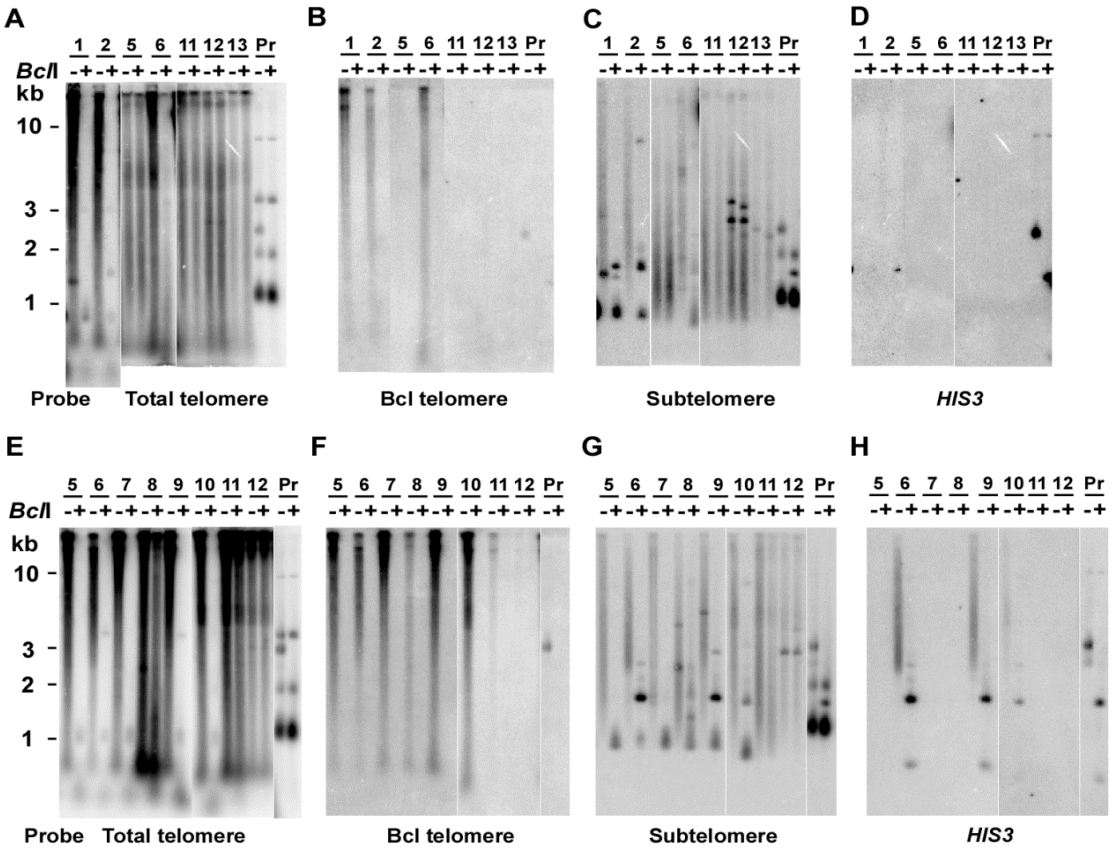

A
B
C
D
1 2 5 6 11 12 13 Pr
-+-+-+-+-+-+-+-+
BclI
kb
10
3
2
1
Probe
Total telomere
Bcl telomere
Subtelomere
HIS3
E
F
G
H
5 6 7 8 9 10 11 12 Pr
-+-+-+-+-+-+-+-+-+
BclI
kb
10
3
2
1
Probe
Total telomere
Bcl telomere
Subtelomere
HIS3

Fig. 3.6. Spreading of a telomeric sequence form a single telomere source during RTE in *stn1-M1 TER1 msh2-Δ* cells. (A) Experimental outline. (B) Southern blot, hybridized with a telomere probe, of *Eco*RI (indicated by "-") and *Eco*RI + *Bcl*I (indicated by "+") digested DNA from six newly generated *stn1-M1 TER1 msh2-Δ* clones from long Bcl precursors in a *TER1 msh2-Δ* background as well as one normal length Bcl *STN1 TER1 msh2-Δ* precursor control. (C-E) Same filter as in panel B after stripping and rehybridization with a Bcl telomere probe, subtelomeric probe and *URA3* probe, respectively.

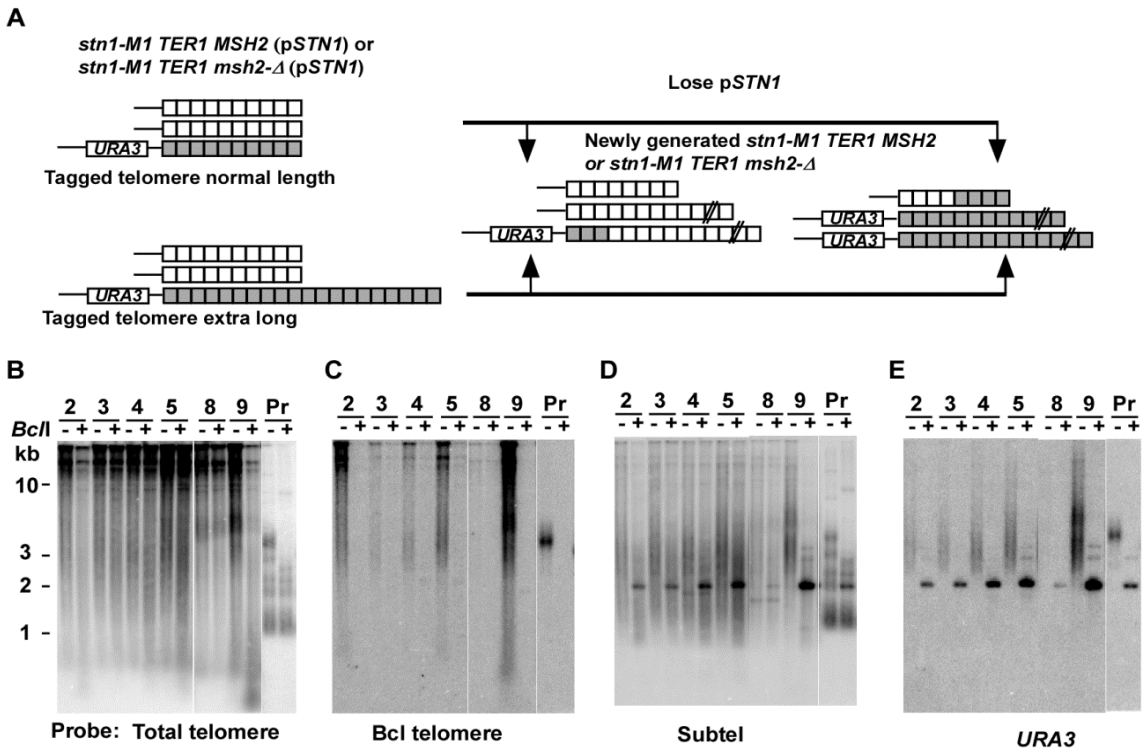
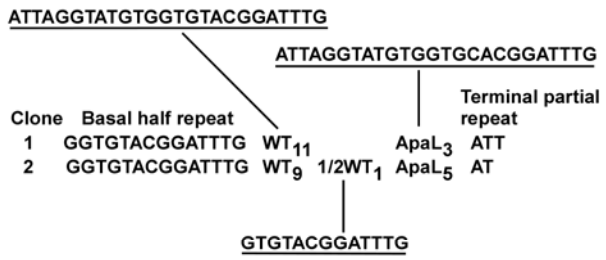

A
stn1-M1 TER1 MSH2 (pSTN1) or
stn1-M1 TER1 msh2-Δ (pSTN1)
URA3
Tagged telomere normal length
URA3
Tagged telomere extra long
Lose pSTN1
Newly generated stn1-M1 TER1 MSH2
or stn1-M1 TER1 msh2-Δ
URA3
URA3
URA3
B
C
D
E
2 3 4 5 8 9 Pr
- + - + - + - + - + - + - +
BclI
kb
10
3
2
1
Probe: Total telomere
Bcl telomere
SubtelI
URA3

Fig. S3.1. The structure of two telomeres cloned from ApaL precursors. (A) Sequences of two cloned telomeres from ApaL precursors. The sequences of WT repeats (denoted as WT), ApaL repeats (denoted as ApaL) and half WT repeats (denoted as 1/2WT) are shown. The number of repeats is shown in the subscript. (B) The potential mechanism to generate the half WT repeat. While the normal translocation of telomerase leads to the synthesis of a whole repeat, the abnormal translocation of telomerase due to the misalignment between RNA template (bottom strand) and telomeric DNA (upper strand) could generate the half WT repeat. The sequence of the repeat that could be synthesized in each case is shown underlined.

A


ATTAGGTATGTGGTGTACGGATTTG
ATTAGGTATGTGGTGCACGGATTTG
Terminal partial repeat
Clone Basal half repeat
1 GGTGTACGGATTTG WT11 ApaL3 ATT
2 GGTGTACGGATTTG WT9 1/2WT1 ApaL5 AT
GTGTACGGATTTG

B

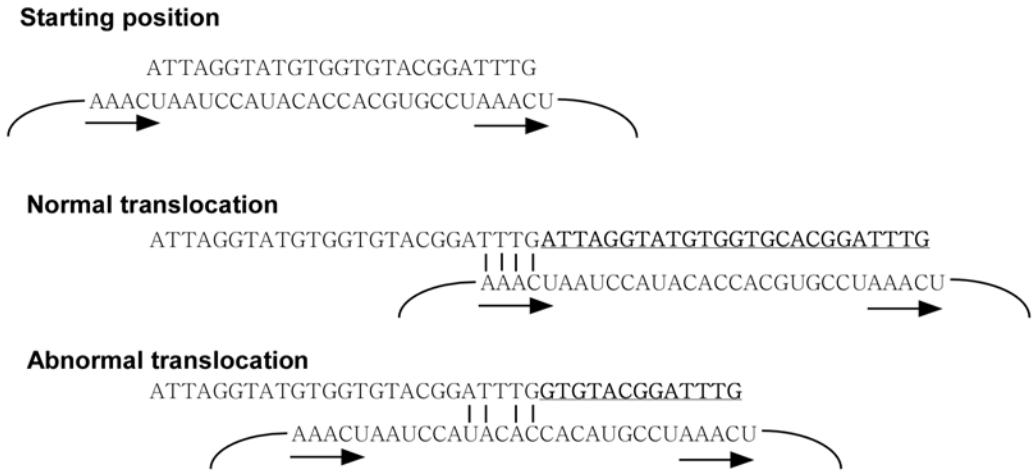

Starting position
ATTAGGTATGTGGTGTACGGATTTG
AAACUAAUCCAUACACCACGUGCCUAAACU
Normal translocation
ATTAGGTATGTGGTGTACGGATTTGATTAGGTATGTGGTGCACGGATTTG
AAACUAAUCCAUACACCACGUGCCUAAACU
Abnormal translocation
ATTAGGTATGTGGTGTACGGATTTGGTGTACGGATTTG
AAACUAAUCCAUACACCACAUGCCUAAACU

Fig. S3.2. Sequences of telomeric fragments cloned from the A1 clone. Shown are diagrams of 38 telomeric fragments cloned from *Apa*LI partially-digested DNA from the A1 clone. The sequences and names of different boxes illustrated in the patterns are shown. The number of clones recovered with the same DNA sequence is indicated at left.

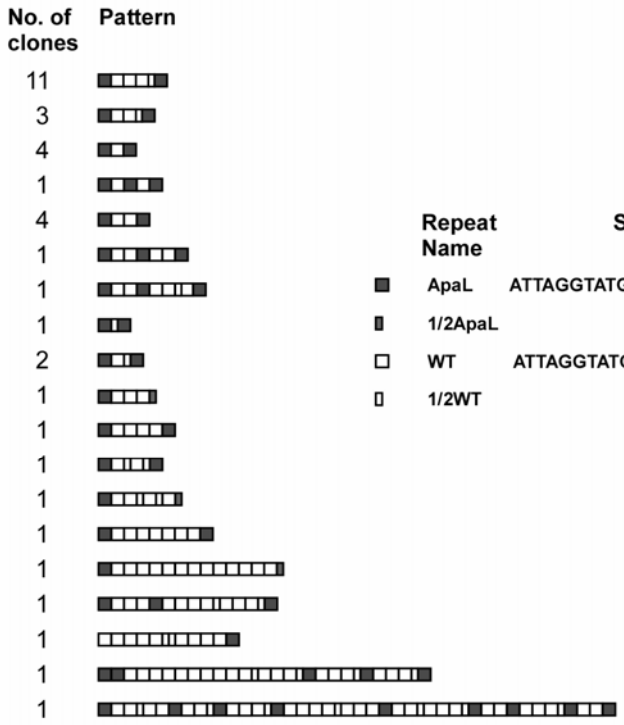

No. of clones
Pattern
11
3
4
1
4
1
1
1
2
1
1
1
1
1
1
1
1
1
Repeat Name
Sequence
ApaL
ATTAGGTATGTGGTGCACGGATTTG
1/2ApaL
GTGCACGGATTTG
WT
ATTAGGTATGTGGTGTACGGATTTG
1/2WT
GTGTACGGATTTG

Fig. S3.3. Spreading of a telomeric sequence form a single telomere source during RTE in *stn1-M1 TER1* cells. (A) Southern blot, hybridized with a telomere probe, of *Eco*RI (indicated by "-") and *Eco*RI + *Bcl*I (indicated by "+") digested DNA from nine newly generated *stn1-M1 TER1 MSH2* clones from normal Bcl precursors in a *TER1 MSH2* background as well as one normal length Bcl *TER1 MSH2* precursor. (B-D) Same filter as in panel A after stripping and rehybridization with a Bcl telomere probe, subtelomeric probe and *URA3* probe, respectively. (E) Southern blot, hybridized with a telomere probe, of *Eco*RI (indicated by "-") and *Eco*RI + *Bcl*I (indicated by "+") digested DNA from 10 newly generated *stn1-M1 TER1 MSH2* clones from long Bcl precursors in a *TER1 MSH2* background. (F-H) Same filter as in panel E after stripping and rehybridization with a Bcl telomere probe, subtelomeric probe and *URA3* probe respectively. (I) Southern blot, hybridized with a telomere probe, of *Eco*RI (indicated by "-") and *Eco*RI + *Bcl*I (indicated by "+") digested DNA from seven newly generated *stn1-M1 TER1 msh2-Δ* clones from normal Bcl precursors in a *TER1 msh2-Δ* background as well as 2 normal length Bcl *TER1 msh2-Δ* precursor controls and an *stn1-M1* mutant (M1) control. (J-L) Same filter as in panel I after stripping and rehybridization with a Bcl telomere probe, subtelomeric probe and *URA3* probe respectively.

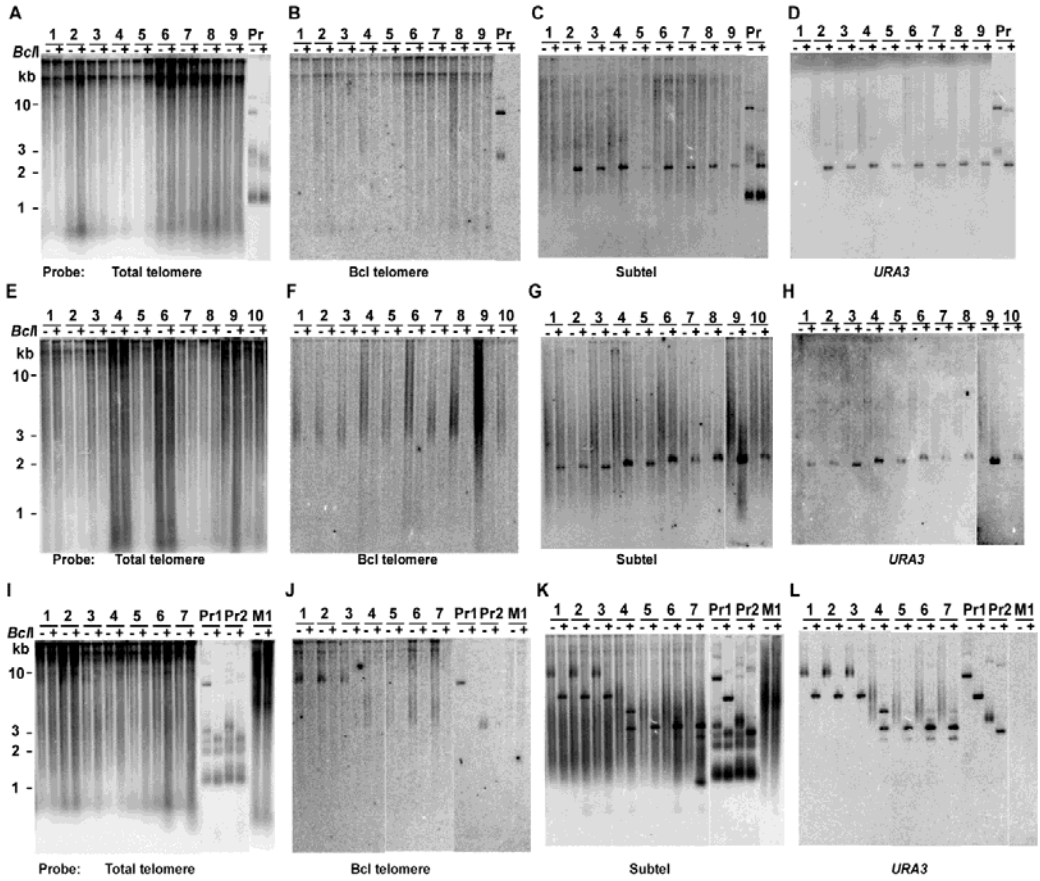

A
B
C
D
E
F
G
H
I
J
K
L
BclI
kb
10
3
2
1
Pr
Pr1
Pr2
M1
Probe: Total telomere
Bcl telomere
Subtel
URA3

Table 3.1. Frequency of spreading of sequence from a single tagged telomere to other telomeres in *K. lactis stn1-M1* mutants with different backgrounds.

Strain back-ground	Tagged Bcl telomere	Number of clones				
		Amplification and spreading of Bcl repeats				Total
		None * (%)	Slight** (%)	Intermediate *** (%)	Complete/Near Complete **** (%)	
stn1-M1 ter1-Δ MSH2	Normal	49(89)	6(11)	0(0)	0(0)	55
	Long	53(70)	12(16)	8(10)	3(4)	76
stn1-M1 ter1-Δ msh2-Δ	Normal	45(81)	0(0)	2(4)	8(15)	55
	Long	6(16)	0(0)	2(6)	30(78)	38
stn1-M1 TER1 MSH2	Normal	37(100)	0(0)	0(0)	0(0)	37
	Long	37(97)	0(0)	1(3)	0(0)	38
stn1-M1 TER1 msh2-Δ	Normal	22(88)	3(12)	0(0)	0(0)	25
	Long	21(75)	6(21)	1(4)	0(0)	28

* None: defined by 1) total *Eco*RI-digested telomeric signal is reduced no more than 1.5 fold by additional *Bcl*I digestion; 2) Bcl specific telomeric signal is less than 8 fold more than that of "precursor"; 3) no significant reduction of *Eco*RI-digested subtelomeric signal by additional *Bcl*I digestion.
** Slight: defined by 1) total *Eco*RI-digested telomeric signal is reduced no more than 1.5 fold by additional *Bcl*I digestion; 2) Bcl specific telomeric signal is less than 8 fold more than that of "precursor"; 3) significant reduction of *Eco*RI-digested subtelomeric signal by additional *Bcl*I digestion.
***Intermediate: defined by 1) total *Eco*RI-digested telomeric signal is reduced no more than 1.5 fold by additional *Bcl*I digestion; 2) Bcl specific telomeric signal is 8 fold or more higher than that of "precursor"; 3) significant reduction of *Eco*RI-digested subtelomeric signal by additional *Bcl*I digestion.
****Complete/Near Complete: defined by 1) total *Eco*RI- digested telomeric signal is reduced at least 3 fold by additional *Bcl*I digestion; 2) Bcl specific telomeric signal is 8 fold or more than that of "precursors"; 3) significant reduction of *Eco*RI-digested subtelomeric signal by additional *Bcl*I digestion.

CHAPTER 4

CONCLUSIONS AND PERSPECTIVES

Our studies show that during the maintenance of the *stn1-M1* state, telomeres are subject to constant and sometimes drastic changes because of the elevated level of homologous recombination. Gaining or losing sequences equivalent to 2-6% of the total genome is frequently observed. Sequences are often gained by the concerted amplification, consistent with the possibility that *de novo* t-circles can be produced and used to amplify telomeric sequences. We also found that telomeres in *stn1-M1* cells could form entangled structures presumably resulting from complex replication or recombination intermediates that could not enter the pulsed field gels.

Our studies also unveiled the mechanisms of telomere dynamics in the establishment and maintenance stages of the *stn1-M1* mutant of *K. lactis*. The studies show that sequences from a single telomere could spread to all other telomeres during the establishment of the *stn1-M1* mutant state, presumably through rolling circle synthesis and/or break-induced replication (BIR)-like gene conversion events. The studies suggest that a rapid telomere truncation event without generating t-circles that are used to elongate telomeres occurs before the elongation events.

One question regarding the entangled telomeric structures is: do they exist in the extrachromosomal form *in vivo*, or do they just become extrachromosomal during the *in vitro* treatment prior to pulsed field gel electrophoresis? To test this idea, it would be useful to use fluorescent *in situ* hybridization to observe the telomeric DNA in *stn-M1* cells. If the entangled structures indeed exist in extrachromosomal forms *in vivo*, it may be interesting to investigate the

mechanism generating this extrachromosomal DNA. Presumably, the mechanical force generated by pulling of two sister chromosomes towards different dividing cells would tend to pull apart and potentially break the entangled telomeres, which are the potential common fragile sites (14). In addition, some enzymatic-based mechanism could also be triggered to carry out the separation via nucleolytic cleavage. The branched DNA and Holliday junction resolvases Mus81-Mms4 and Yen1 may be good candidates to test (4). One prediction from the hypothesis about resolving the entangled telomeric structures that lead to deletion of multiple telomeric sequences is the unequal separation of total telomeric DNA to the two divided cells. Conceivably, mother yeast cells might retain appreciably more telomere entanglements than daughter cells. To test this hypothesis, one could examine the total telomeric DNA distribution in the two divided cells by fluorescence in situ hybridization. Additionally, since whether the entangled structure is correlated with the elongated telomeres is not clear, and we may analyze other mutants with elongated telomeres to check whether the entangled telomeric structure exists.

One open question regarding the spreading of sequences from one telomere to others is how it happens. One possibility is that BIR events copy an initial existing elongated telomere to all other telomeres. BIR is found to depend on Pol32 in *S. cerevisiae* (10), and if this is also the case in *K. lactis*, it would be interesting to test whether the extent of telomere elongation would be affected by deletion of Pol32 in an *stn1-M1* mutant. Alternatively, the spreading of sequences could be generated from multiple rounds of rolling circle synthesis copying either the same t-circle or different t-circles with the same structure. This model is favored given the following facts. First, there is significant amount of G-rich single-stranded telomeric DNA in *stn1-M1* cells (6), which would cause elongated telomeres to lack templates for being copied by short telomeres. Second, the elongated telomeres appear to often be in entangled structures formed by

their engaging in recombination or replication intermediates (Xu and McEachern, in preparation), which might prevent recombination events from effectively elongate the short telomeres to the great extents that are observed. Actually, our results show that long *URA3*-telomere tandem arrays could be formed from a transformed DNA circle in the *stn1-M1* cells where long telomeres composed of pure telomeric repeats already exist, suggesting that different t-circles could be used to elongate telomeres. To further test this idea, it would be useful to transform two kinds of DNA circles into *stn1-M1* cells, as performed with *ter1-Δ* mutants (11), and test whether both circles can be used to elongate telomeres in the same cells.

Another question regarding the telomere elongation process is how t-circles are generated. One prevailing model for t-circle generation is telomere rapid deletion (9), where cleavage of the t-loop structure is proposed to lead to both the shortened telomeres and t-circles. However, the frequency of t-circles generated by this kind of event should be very low given the following facts. TRD events occur at around 4×10^{-3} per cell division in mitotically growing wild type *K. lactis* cells (1), and wild type *K. lactis* cells are effective at using transformed t-circles to form long telomeres (11). However, the incidence of telomere elongation in wild type cells containing long telomere is considerably rarer (1). If TRD events are efficient at producing t-circles, we should expect a much higher frequency of telomere elongation in wild type cells. We propose that t-circles could be derived from the linear extrachromosomal telomeric DNA produced by TRD events. Conceivably, circularization of the linear extrachromosomal telomeric DNA by either single-strand annealing. It should be noted that t-circles could be generated in different pathways in different genetic background. Studies in our lab have shown that the abundance of t-circles is reduced by deletion of *RAD50* in *K. lactis* mutants that contain highly elongated telomeres (Basenko and McEachern, unpublished data). Other studies have shown that XRCC3

is important for t-circle generation in a $TRF^{\Delta B}$ mutant of mammalian cells (18) but not in Werner Syndrome cells with the same mutation (8). Further studies may be needed to address these conflicts.

What causes the phenotype of the *stn1-M1* mutant is not clear. However, based on sequence comparison, the mutation in *stn1-M1* is located at a conserved position within the putative OB domain in the N-terminal region of Stn1p (2) that is proposed to affect DNA binding and is thought to interact with Ten1 (16). Although the C-terminal domain of Stn1, which interacts with Cdc13 (12), is not affected, whether a DNA binding defect of Stn1p in *stn1-M1* cells would affect its binding to Cdc13 and therefore affect the negative regulation of telomerase is unknown. Our data may suggest that telomerase in *stn1-M1* may be not appropriately regulated, which is reflected by the fact that the presence of telomerase can prevent the spreading of Bcl repeats from one telomere - even one of longer size than the other wild type telomeres - to all others. One possibility is that unregulated telomerase activity caused by the *stn1-M1* mutation could act on all telomeric ends and add wild type repeats, which could act to prevent the internal Bcl repeats from spreading. Alternatively, the telomerase could bind to the end of telomeres and block recombination from happening at the telomeric ends. Indeed, telomerase is found to perform some capping functions when the normal capping structure of telomeres is defective (17). It would be interesting to see whether the polymerization activity of telomerase is necessary for the prevention of spreading Bcl repeats by carrying out the experiment using the potential telomerase mutant without polymerization activity.

The CST complex also regulates the Polα-Primase complex through interactions between Cdc13 and the Pol1 subunit of the Polα-Primase complex (13, 15) and interaction between Stn1 and the Pol12 subunit of the complex (3). Whether the *stn1-M1* mutation would cause a defect

in the recruitment of Polα-Primase complex and therefore cause the elevated amount of single stranded telomeric DNA is not clear. To test this hypothesis, it would be interesting to check whether a double mutant of *stn1-M1* and Pol12 would yield a synergistic effect or not; alternatively, one could test whether fusion of Polα-Primase complex to Cdc13 could decrease the amount of the single-stranded telomeric DNA in the *stn1-M1* mutant.

Homologous recombination is essential to the viability of the *stn1-M1* mutants since deletion of *RAD52* causes lethality of *stn1-M1* spores, even in the presence of telomerase (6). This suggests that the telomeres synthesized by telomerase may not be enough to rescue the mutant without homologous recombination. To further test this idea, it would be interesting to investigate whether overexpression of telomerases could rescue the *stn1-M1 rad52* mutant. Alternatively, homologous recombination could carry out some essential function other than synthesizing new telomeric sequences. One such event could be replication fork restart. Collapsed replication forks were suggested to exist at eroded telomeres (7), and it would be interesting to test whether overexpression of the factors facilitating replication fork restart would rescue the *stn1-M1 rad52* mutant. One such factor is the helicase Rrm3. In *S. cerevisiae*, this helicase could promote the replication fork progression through telomeric and subtelomeric regions (5).

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