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A Mutation in the *STN1* Gene Triggers an Alternative Lengthening of Telomere-Like Runaway Recombinational Telomere Elongation and Rapid Deletion in Yeast

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Some human cancer cells achieve immortalization by using a recombinational mechanism termed ALT (alternative lengthening of telomeres). A characteristic feature of ALT cells is the presence of extremely long and heterogeneous telomeres. The molecular mechanism triggering and maintaining this pathway is currently unknown. In *Kluyveromyces lactis*, we have identified a novel allele of the *STN1* gene that produces a runaway ALT-like telomeric phenotype by recombination despite the presence of an active telomerase pathway. Additionally, *stn1-M1* cells are synthetically lethal in combination with *rad52* and display chronic growth and telomere capping defects including extensive 3′ single-stranded telomere DNA and highly elevated subtelomere gene conversion. Strikingly, *stn1-M1* cells undergo a very high rate of *t*elomere *r*apid *d*eletion (TRD) upon reintroduction of *STN1*. Our results suggest that the protein encoded by *STN1*, which protects the terminal 3′ telomere DNA, can regulate both ALT and TRD.

Telomeres are the DNA-protein complexes that protect the ends of linear eukaryotic chromosomes (19, 57). They are normally maintained by the reverse transcriptase telomerase, which utilizes a sequence within its RNA component as a template to add new telomere repeats onto the 3′ end. Telomerase plays a vital role in immortalization of human cancer cells. Since a majority of human somatic cells lack telomerase activity, expression of telomerase in many cells is sufficient to achieve immortalization, one of the characteristic features of cancer cells (5). Thus, chromosome end protection or "capping" and telomere length regulation play an important role in preventing cancer and genomic instability in human cells.

How capping is achieved is not fully clear, but a number of proteins binding to the single- or double-stranded region of the telomeres are known to be involved in regulating this process (9, 19, 42, 57). In the budding yeast *Saccharomyces cerevisiae*, the 3′ telomeric terminus is protected by Stn1p in association with Ten1p and the single-strand telomere binding protein Cdc13p (11, 16, 18, 24, 25). Recruitment of Stn1p to the telomere by fusion of the DNA binding domain of Cdc13p is sufficient to rescue the lethality of the null mutation of *cdc13* (11, 50). Another yeast telomere protein influencing end protection is Rap1p, which binds to duplex telomere sequences (3) and appears to prevent the occurrence of telomere fusions (41). Similarly, in human cells, single-stranded telomere binding protein POT1 and its interacting protein (PTOP/TINT/PIP1), along with duplex telomeric DNA binding proteins TRF1 and TRF2, have been implicated in providing end protection and contributing to telomere length control (1, 4, 35, 57, 67, 68). Additionally, in a variety of organisms the telomeres have been postulated to form a protective structure, where the 3′ end is thought to strand invade into the internal duplex region of the telomere to form a t loop (10, 26, 44, 48, 60, 61). Whether budding yeast telomeres form t loops is still unknown; however, some evidence indicates that they exist as fold-back structures (14).

Defects in capping can lead to a variety of problems including telomere fusions, disrupted telomere length regulation, elevated recombination near telomeres, degradation of the 5′ strand of telomeric DNA, DNA damage checkpoint activation, and premature senescence (2, 19, 42). Many or all of these problems arise from telomeres being recognized as DNA double-strand breaks. In yeast mutants lacking telomerase, telomeres gradually shorten, leading to a gradual decline in growth rate, cell cycle arrest, and cell death from critically short or uncapped telomeres. Rare emergence of postsenescence survivors occurs due to recombinational telomere elongation (RTE) (36, 38, 59). In *S. cerevisiae*, telomere elongation by RTE occurs either by amplification of the telomeric DNA sequences (type II RTE) or by amplification of subtelomeric DNA sequences while maintaining short telomeric terminal tracts (type I RTE) (36, 59). However, only the type II RTE pathway has been shown to occur in *Kluyveromyces lactis* (38). The *K. lactis* survivors are proposed to arise through a "roll-and-spread" mechanism (46). According to this model, a small telomeric DNA circle acts as a template to elongate a single telomere by rolling-circle replication. Subsequently, the spreading of the sequence from this elongated telomere to other telomeres occurs by gene conversion (62). Additional data have shown that telomeric circles transformed into *K. lactis* strains promote RTE and that DNA circles as small as ~100 bp can form in a mutant with long dysfunctional telomeres (26a, 45, 46).

In a minority of human cancer cells, telomerase activity is absent and the telomeres are maintained by the activation of alternative lengthening of telomeres (ALT) (7). ALT is

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thought to involve recombination, as a sequence tag introduced in a single telomere in an ALT cell lineage often became copied to additional telomeres (17). ALT is best characterized, in addition to lack of telomerase activity, by extreme telomere length heterogeneity and the presence of *A*LT-associated *p*romyelocytic leukemia *b*odies (APBs) (28). APBs are intranuclear structures that contain a number of telomere- and recombination-specific proteins including TRF1, TRF2, RAD51, RAD52, and the RAD50/MRE11/NBS1 complex (28, 54). The molecular mechanisms triggering ALT are presently unknown.

In addition to elongating telomeres, recombination has also been shown to trigger sudden deletion of telomere sequences in yeast, a process termed telomere rapid deletion (TRD) (33, 37). Telomere truncations have also been observed in other organisms, such as *Euplotes*, *Tetrahymena*, and *Xenopus*, and in human ALT cells (13, 32, 51, 64). Whether these truncations involve recombination, is currently unknown.

In this study, we have identified a mutation in the *K. lactis STN1* gene manifesting a striking combination of phenotypes including telomere capping defects, very long and heterogeneous ALT-like telomeres produced by recombination, and a rapid shortening of all telomeres upon reintroduction of *STN1*.

MATERIALS AND METHODS

Yeast media and strain construction. All *K. lactis* cells used in this study are derivatives of the wild-type haploid strain 7B520 (*ura3-1 his2-2 trp1*) (66). The *K. lactis* plasmid genomic library used to clone the *STN1* gene was previously described (29).

Serial passaging of all the strains was performed by selecting single colonies and allowing them to grow for 3 days at 30°C on standard rich media containing yeast extract, peptone, and dextrose (YPD). The construction of *stn1-M1 ter1*-Δ and *stn1-M1 TER1-20C*(*ApaL*) double mutants was performed through standard yeast replacement procedures. Replacement of the native *TER1* gene by a *ter1* gene disrupted by the *URA3* construct was done by transplacement as described previously (38). Replacement of the *TER1* gene by *TER1*-20C(*ApaL*) was done by a plasmid "loop in-loop out" procedure described previously (39). Additionally, the *K. lactis rad52* and *ter1*-Δ7:*URA3* deletion alleles, described previously (38), were used for isolating the double-mutant *stn1-M1 ter1*-Δ and *stn1-M1 rad52*Δ strains by mating and tetrad dissection.

Random spore analysis was performed on the spores generated by mating the 7B520 derivative *stn1-M1 ura3 his2 trp1* strain with the UA24B (*rad52 ade2*) strain. Spores created from the diploid cells of this mating were scooped off the sporulation plate and incubated in 200 μl of 100T Zymolyase (concentration of 0.17 mg/ml in 1 M sorbitol) at 37°C for 10 min to digest the ascus sac. The spores were serially diluted in TE (10 mM Tris and 1 mM EDTA) and plated on several synthetic complete (SC) plates lacking uracil and adenine. They were plated to an approximate cell density of ~200 viable cells per plate and allowed to form relatively large colonies at 30°C so that the rough-colony morphology of *stn1-M1* mutants could be easily distinguished. Individual colonies selected for further analysis were digested with EcoRI and hybridized to a *RAD52* gene fragment probe to test for the presence of *stn1-M1 rad52* double mutants.

Southern and nondenaturing in-gel hybridizations. Yeast genomic DNA was isolated from overnight liquid YPD cultures grown at 30°C. Fragments were separated on 0.8% agarose gels and electrophoresed at 25 V for 15 h and then at 35 V for 3 h. They were transferred to HyBond N+ membranes, and hybridization was performed as described previously (12a) at 49°C, with either of two end-labeled telomere probes (G-probe, Klac1-25 [5′ACGGATTTGATTAGGTATGTGGTGT-3′], or C-probe, Klac25-1 [5′-ACACCACATACCTAATCAAATCCGT-3′]). Hybridization of a labeled subtelomere fragment (~0.6-kb EcoRI-XbaI fragment from plasmid pAK25ΔB) using a random priming kit (Stratagene) was also carried out at 65°C as described previously (63). The membranes were autoradiographed using PhosphorImager analysis (Bio-Rad; Molecular Imager). In-gel hybridization experiments were performed as described previously (15) using a telomere oligonucleotide as a probe (Klac25-1). Approximately 3 μg of undigested DNA was electrophoresed through a 0.7% agarose gel and then analyzed using the conditions described previously (63).

Mutagenesis. EMS (ethyl methanesulfonate) mutagenesis was performed using the wild-type haploid strain 7B520 (*ura3-1 his2-2 trp1*). Briefly, 5 ml (10^8 cells/ml) of cells was treated with 50 μl of EMS and incubated for 2 h at 30°C. To obtain a 50% survival rate, 1 ml of the culture was removed after every 30 min and the reaction was inactivated by the addition of 8 ml of 5% sodium thiosulfate. Serial 10-fold dilutions of the cells from the 60-, 90-, and 120-min time points were plated on YPD plates to a density of 200 viable cells per plate and allowed to grow at 30°C for 3 days, so that rough-colony morphology could be easily distinguished.

Subtelomere gene conversion assay. The gene conversion assay was performed according to the protocols described previously (40). Briefly, one of the native telomeres in the *stn1-M1* mutant strain was replaced by transformation with an ~2.0-kb EcoR1 and SacII "STU" (subtelomere, *URA3*) fragment containing the *URA3* gene from *S. cerevisiae* inserted into the subtelomeric sequence of a cloned *K. lactis* telomere. Serially diluted cells of clones containing the "STU" fragment were plated on SC plates lacking uracil, SC plus 5-FOA (5-fluoroorotic acid), and YPD. Measurement of the loss of the *URA3* gene was performed by counting colonies grown on 5-FOA with respect to the total number of colonies grown on YPD and SC plates.

RESULTS

Identification of a *K. lactis* mutant with extremely long telomeres. In order to identify mutants affecting telomere maintenance in *K. lactis*, we performed a screen for haploid mutants exhibiting abnormal colony morphology by EMS mutagenesis. Earlier work demonstrated that a rough-colony phenotype was characteristic of both telomere deletion and runaway elongation mutants of haploid *K. lactis* strains (38, 39). Approximately 10,000 colonies arising from EMS-treated wild-type cells were screened at 30°C, and a total of 345 abnormal colonies were identified. After restreaking on YPD plates to confirm their phenotypes, these mutants were examined for telomere length defects by Southern analysis. Subsequently, we identified 30 mutant candidates exhibiting various degrees of mild telomere length defects and two mutants that exhibited dramatic telomere elongation. The telomere and colony phenotypes of the mutant named M1 are shown in Fig. 1A and will be the focus of this study. This mutant exhibited a heterogeneous telomere repeat signal, ranging from limit mobility (>20 kb) to ~100 bp (Fig. 1B). The presence of a telomere signal below 0.7 kb (the size of the subtelomere DNA in the smallest EcoRI telomere fragment in our wild-type *K. lactis* strain [47]) indicated that a significant amount of telomeric DNA existed in extrachromosomal form. Further passaging of the M1 mutant revealed that the highly elongated telomeres were maintained without apparent change over at least 600 cell divisions (Fig. 1B). A subtelomere probe exhibited a signal migrating from limit mobility to below 1 kb (Fig. 1C). We conclude that telomeres in the M1 mutant are extremely heterogeneous in length, ranging from short to extremely long. In order to estimate the amount of telomeric DNA in the M1 mutant, we quantitatively analyzed the total telomere hybridization signal in the mutant with respect to the wild-type strain by using a PhosphorImager. The results indicated a greater-than-10-fold increase in the number of telomere repeats in the M1 mutant. An interesting observation was the appearance of occasional sharp bands visible with the subtelomeric probe (arrows, Fig. 1C). These may represent unstable telomere-telomere fusions. Similar unstable sharp bands in certain *K. lactis* long *ter1* template mutants have been shown to be telomere fusions (41).

The elongated telomeres in the M1 mutant appear to be composed entirely of telomeric repeats. We next investigated if

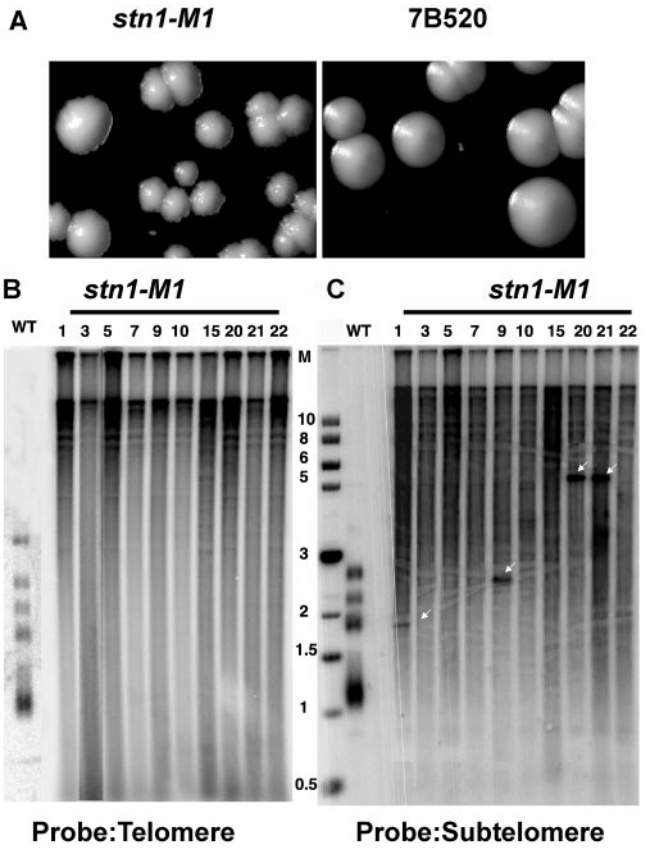

FIG. 1. Identification of a *K. lactis* mutant with extremely long telomeres. (A) The left panel shows the rough-colony morphology of the M1 mutant, while the right panel shows the isogenic 7B520 wild-type strain. (B) Shown is a Southern blot of EcoRI-digested genomic DNA from the M1 mutant hybridized to probe Klac1-25, composed of one (25-nucleotide) repeat of the G-strand *K. lactis* telomeric sequence. The individual clone was passaged for 22 streaks (~440 to 550 cell divisions), and samples were periodically selected for genomic DNA extraction. The wild-type sample is shown overexposed for clarity. (C) The same blot was stripped and reprobed with a subtelomeric probe. Rare sharp bands marked by white arrows may represent fusions between chromosomes with few or no telomeric repeats. Molecular size markers (M), shown in the middle, are in kilobase pairs.

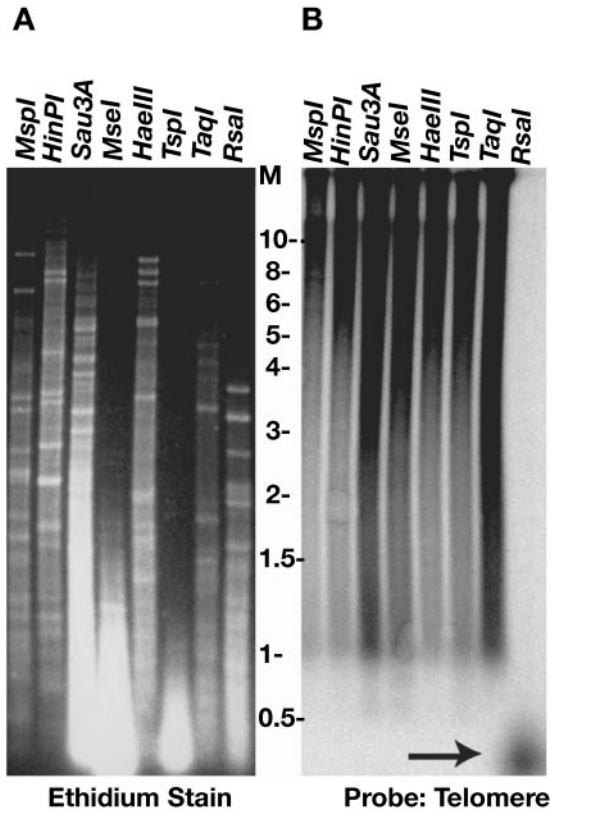

FIG. 2. Telomere elongation in the M1 mutant is exclusively due to additional telomeric repeats. (A) Ethidium bromide-stained gel of genomic DNA from the "M1" mutant digested with eight different restriction enzymes, as labeled. (B) The gel in panel A was hybridized to the Klac1-25 telomere probe. All the telomeres in the lane labeled RsaI have been digested down to less than ~50 bp (arrow), as each *K. lactis* telomeric repeat contains an RsaI site.

the telomere elongation in the M1 mutant was entirely due to amplification of the telomeric repeat sequences. Genomic DNA was extracted and subjected to digestion with several restriction enzymes with 4-bp recognition sequences. Of the enzymes used, only RsaI has a recognition site within the telomeric repeat of *K. lactis* and was expected to cleave away all the telomeric repeats. Southern hybridization using a telomeric probe showed that all digestions except that with RsaI left the long heterogeneous telomeres intact while bulk genomic DNA was cleaved down to small sizes (Fig. 2, compare panels A and B). In contrast, the sample digested with RsaI showed no telomere signal at high molecular weight and only a faint smear that migrated well below 500 bp (Fig. 2B). We conclude that the long heterogeneous telomere fragments in mutant M1 are due to elongated tracts of telomeric repeats. An interesting observation in almost all examined restriction enzyme-digested genomic DNA samples from cells of the M1 mutant was the presence of a substantial telomeric hybridization signal (but not bulk chromosomal DNA) in the wells (Fig. 1 and 2 and data not shown). This telomeric signal in wells is resistant to proteinase K (data not shown) and appears to be a general characteristic of *K. lactis* mutants with extremely long telomeres (39, 63). This suggested that telomeric DNA from M1 cells was often present in a tangled form unable to migrate into the gels. Interestingly, the fraction of telomeric DNA in the well increased in the TaqI digestion, the only digestion performed at 65°C. One possible explanation for this result is that the DNA represents recombination intermediates with complex branched structures and heating at 65°C further promoted the formation of these structures.

***STN1* complements the *M1* mutation in *K. lactis*.** To test if a mutation in a single gene was responsible for the defect seen in the M1 mutant, a segregation analysis was performed. The M1 mutant was mated to a wild-type *K. lactis* haploid strain of the opposite mating type, and the resulting diploids were sporulated. The results showed that only two of the four spores were viable in most of the 15 tetrads dissected. In each of the three tetrads where three spores were viable, the extra spore grew to exhibit the rough-colony morphology characteristic of the M1 mutant. Each of the rough colonies produced as a consequence of sporulation was analyzed for telomere length by Southern analysis and found to contain long and heterogeneous telomeres similar to those

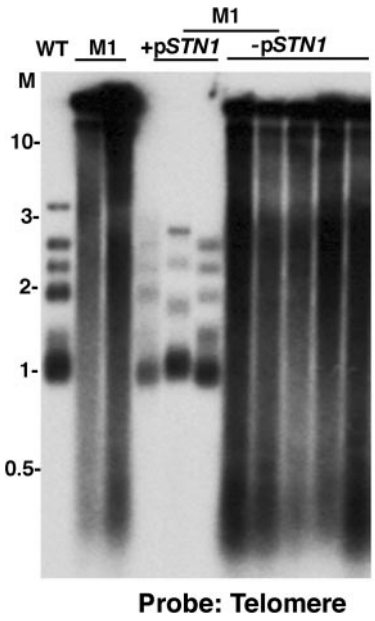

FIG. 3. The long telomeres of the M1 mutant are complemented by the *K. lactis STN1* gene. Shown is a Southern blot of EcoRI-digested *K. lactis* genomic DNA from the M1 mutant hybridized to a telomere probe. The molecular size markers (M) are indicated in kb. The wild-type sample (WT) is shown on the first lane, followed by two passages (one or two streaks) of the M1 mutant. The next three lanes are independent M1 mutant clones shown shortly after transformation with a plasmid containing *STN1* (+p*STN1*). The last five lanes are individual clones of the M1 mutant shortly after losing the *STN1*-containing plasmid (−p*STN1*).

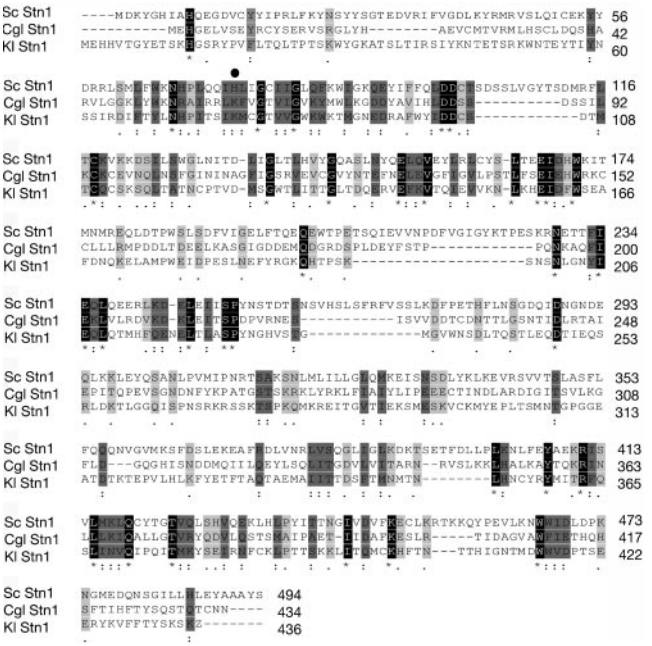

FIG. 4. Sequence analysis of *K. lactis STN1* gene. Shown is an alignment of amino acid sequences of *K. lactis* (Kl) Stn1 with homologues from *S. cerevisiae* (Sc) and *Candida glabrata* (Cgl) (GenBank accession numbers P_38960 and XP_448655, respectively). The protein sequences were aligned in ClustalW using default values. Identical amino acids are shaded in black and marked with a star. Amino acids showing conserved substitutions are marked with two dots and shaded in gray, and semiconserved substitutions are shaded in light gray and marked with a single dot. The dark circle indicates the position of the mutation of the *stn1-M1* mutant.

seen in the original M1 mutant (data not shown). These results support the idea that a single gene was responsible for both the telomeric defect and the rough-colony phenotype of the M1 mutant. Additionally, it also indicated that this mutation was lethal to a high percentage (90%) of spores. The poor viability of *stn1-M1* spores was observed with diploids produced by backcrossing, and its basis remains unknown.

Subsequent efforts focused on complementing the M1 mutation by transforming a *K. lactis* genomic library plasmid into M1 mutant cells. The transformed cells were incubated at 37°C, which was semipermissive to wild-type *K. lactis* and exacerbated the growth defects of the M1 mutant. Approximately 20,000 transformants were visually screened for larger colony size and smoother appearance than the average transformed colony. Seven different transformants that continued to show signs of improved growth and colony morphology after restreaking on YPD plates were then examined for telomere length by Southern analysis. Two of these exhibited a complete loss of the long-telomere phenotype. Telomeres in these transformants remained short after additional passaging for maintenance of the library-derived plasmid (Fig. 3). However, upon plating these strains on plates containing 5-FOA, which selected for the loss of the library plasmid, we observed the highly elongated telomeres and rough-colony phenotypes resembling the original M1 mutant (Fig. 3). Our results suggested that the plasmids present in the transformants were responsible for rescuing the defect of the M1 mutant. After recovery of these complementing plasmids in *Escherichia coli*, both were found to contain ~7.5-kb inserts and had identical restriction fragment patterns upon digestion with RsaI. As expected, retransformation of both the plasmids into the original M1 mutant was found to completely complement the mutation. One of the plasmids, p72, was selected for further sequencing.

To narrow down the complementing region of plasmid p72, an ~2-kb XbaI-BspeI fragment was deleted to create pXB3. pXB3 was also found to fully complement the M1 mutant (data not shown). BLAST analysis of the insert sequence from the pXB3 plasmid revealed two intact open reading frames. One was identified as *RRP8*, encoding an rRNA processing protein. The other was identified as *STN1*, based on 28% identity and 47% similarity of its product to the sequence of the Stn1 protein of *S. cerevisiae* (Fig. 4). Further supporting our finding that the *K. lactis* gene was indeed a homologue of *STN1*, the two genes were not only the best matches to each other in their respective genomes but shared the same neighboring genes, *RRP8* and *PDC2* (data not shown). BLASTP searches using the two sequences revealed related protein products from other yeast species, one of which, from *Candida glabrata*, is shown in Fig. 4.

We used genomic DNA from the M1 mutant and its isogenic parent, 7B520, for PCR amplification of the *K. lactis STN1* gene. Amplified products were purified and sequenced. Analysis revealed a single base substitution resulting in an isoleucine-to-lysine change at amino acid position 79 in the M1

mutant, but not in either the 7B520 parent or the *STN1* gene in plasmid pXB3. The mutation in *stn1-M1* is present in the region of its interaction with Ten1 (C. Nugent, personal communication). From our results we conclude that the phenotypes of the M1 mutant are due to this missense mutation, and we designate this allele *stn1-M1*.

Telomere shortening upon reintroduction of *STN1* is very rapid in *stn1-M1* cells. Previous work on numerous telomerase RNA gene (*TER1*) template mutants has produced extremely elongated telomeres superficially resembling the telomeres in the *stn1-M1* mutant (31, 39, 56, 63). Reintroduction of a wild-type *TER1* gene into these mutants rapidly eliminated the cellular defects and the highly smeared appearance of the telomere fragments but maintained very long telomeres. Only after hundreds of cell divisions did these long telomeres shorten to more-normal lengths. Gradual shortening is expected if telomeric sequence loss occurs primarily from incomplete replication of double-stranded DNA ends. It was therefore surprising that complementation of the *stn1-M1* mutant rapidly shortened all the telomeres to nearly wild-type length without any trace of smears (Fig. 3). To further confirm this rapid sequence loss, we performed mating analysis using *stn1-M1* and *ter1-Acc*(*19A*) strains, the latter a long-telomere *ter1* template mutant capable of synthesizing telomeric repeats defective at binding the Rap1 protein (30, 39). Each of these strains was mated to three different wild-type strains of opposite mating type, and the telomere lengths from the resulting diploids were examined. Similar to previous published work (56), we observed that many telomeres in the *TER1*/*ter1-Acc* (*19A*) diploid cells were very long, similar to those of the *ter1-Acc*(*19A*) parent (Fig. 5A). In striking contrast, the *stn1-M1*/*STN1* diploid cells had only telomeres of nearly normal length. This was true for each of the three independent diploids examined over 125 generations. This result confirmed that the long telomeres of the *stn1-M1* mutant were shortened to nearly normal length within ~30 cell divisions after reintroduction of a single copy of *STN1*.

The long telomeres of the *stn1-M1* strain are generated independently of telomerase. In *S. cerevisiae*, *STN1* has been implicated as a negative regulator of telomerase by preventing Cdc13p-mediated telomerase recruitment (11, 22, 25, 50). Consistent with this, several mutant alleles isolated from different laboratories exhibited elongated telomeres that were formed in a telomerase-dependent manner. We therefore hypothesized that telomere elongation in the *stn1-M1* mutant was mediated by telomerase. To address this, *ter1*-Δ *stn1-M1* double mutants were constructed. Diploid strains constructed by mating between *ter1*-Δ and *stn1-M1* haploid strains were found, as expected, to have telomeres of nearly normal length (data not shown). Tetrads were dissected from three independent diploid strains. Of the 44 dissected spores, we isolated five mutants displaying abnormal colonies and elongated telomeres resembling the haploid *stn1-M1* strain. Southern analysis demonstrated that three of the five mutants were *ter1*-Δ *stn1-M1* double mutants. These mutants did not display the declining growth senescence and survivor colony growth pattern characteristic of *K. lactis ter1*-Δ single mutants; instead they exhibited chronic abnormal growth and colony phenotypes indistinguishable from *stn1-M1* single mutants (data not shown). Consistent with this, no change in telomere length was observed when

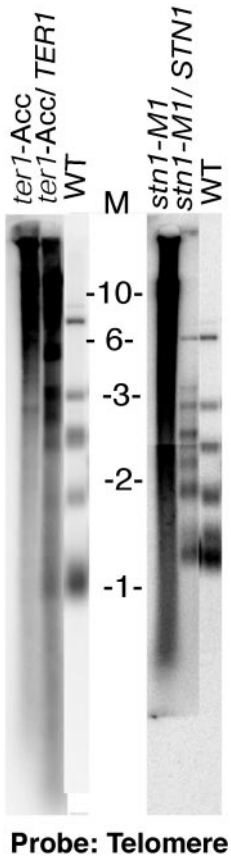

FIG. 5. Immediate shortening of the telomere occurs upon reintroduction of *STN1*. EcoR1-digested genomic DNA of *stn1-M1*/*STN1* and *ter1-Acc*/*TER1* diploids hybridized to a telomere probe. The diploids were generated by mating a wild-type *K. lactis* strain (see Materials and Methods) to the *stn1-M1* strain and to a telomerase *RNA* gene template mutant (*ter1-Acc*) strain. The *stn1-M1*/*STN1* diploid strain exhibits telomeres of approximately wild-type length immediately after mating (within ~30 cell divisions), in contrast to the *ter1-Acc*/*TER1* diploid, which retains long telomeres. Molecular size markers shown in the middle are in kb.

stn1-M1 ter1-Δ cells were passaged over 200 cell divisions. Another *ter1*-Δ *stn1-M1* double mutant was generated by a direct disruption of the *TER1* gene in a *stn1-M1* background (see Materials and Methods). This mutant strain also exhibited highly elongated telomeres and rough-colony morphology consistent with results from the mating analysis (Fig. 6A). From our results, we conclude that the telomere lengthening in the *stn1-M1* mutant was independent of telomerase.

Telomerase is active in the *stn1-M1* mutant. To determine whether telomerase was still active in the *stn1-M1* mutant, the native copy of the telomerase RNA gene *TER1* was replaced with a mutant copy capable of adding telomere repeats with ApaLI restriction sites (*TER1-20C* [*ApaL*]) (63). The *stn1-M1 TER1-20C* (*ApaL*) strain was passaged for ~220 to 275 generations (11 streaks on YPD plates), and genomic DNA from several passages was digested with EcoRI and ApaLI to probe for ApaLI-containing repeats in the long telomeres of the *stn1-M1* mutant strain. Cleavage with ApaLI led to the shortening of telomeric fragments, which increased with progressive

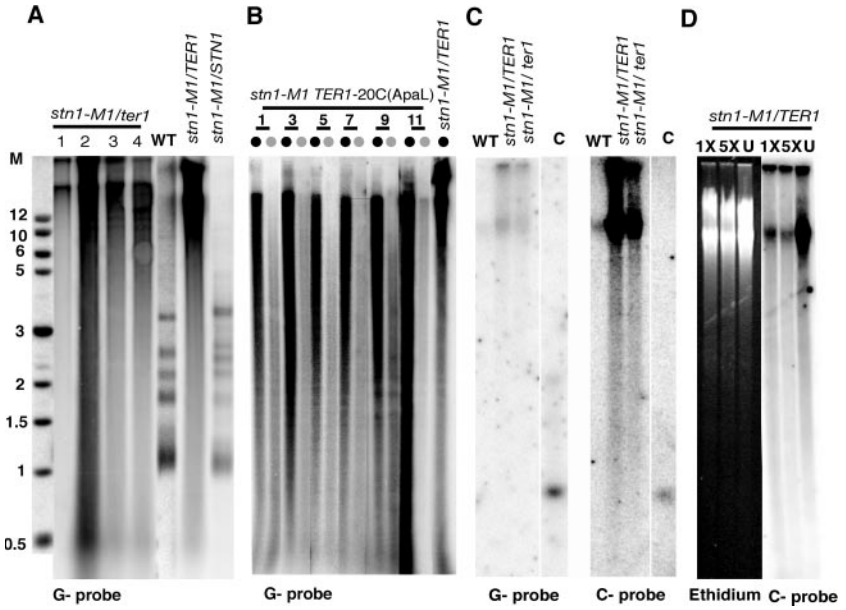

FIG. 6. (A) Telomerase-independent lengthening in *stn1-M1* mutants. Clones marked 1, 2, 3, and 4 are EcoR1-digested genomic DNA of *stn1-M1 ter1*-Δ double mutants hybridized to a telomeric G-strand probe (Klac1-25). The wild-type strain (WT), the *stn1-M1* strain, and the parental *stn1-M1*/*STN1* diploid are also shown. The different intensities of the telomeric signal between the *stn1-M1 ter1*-Δ clones is due to loading differences and possibly also strain differences. Molecular size markers (M) are in kb. (B) Telomerase remains active in *stn1-M1* cells. Shown is a Southern blot of a time course of a clonal lineage of *stn1-M1* containing a silent mutation in the telomerase RNA gene [*TER1-20C* (*ApaL*)]. 1, 3, 5, 7, 9, and 11 represent the numbers of streaks after introduction of the *TER1-20C* (*ApaL*) gene, which generates a telomerase that synthesizes repeats with an ApaLI site. EcoRI (black circles)- and EcoRI-plus-ApaLI (gray circles)-digested genomic DNA of the individual streaks of *stn1-M1 TER1-20C* (*ApaL*) was hybridized to a G-strand telomere probe (Klac1-25). The last lane shows EcoRI-digested genomic DNA from the *stn1-M1 TER1* control. The weaker intensity of the telomeric signal in the EcoRI-plus-ApaL1 lanes results from telomeric repeats with ApaLI sites that are cleaved. (C) Long 3′ overhangs in s*tn1-M1* cells. Shown is a nondenaturing in-gel hybridization of undigested genomic DNA of WT, s*tn1-M1*, and *stn1-M1 ter1*-Δ strains. The left half of the panel has been hybridized to a G-strand telomeric probe, and the right panel shows the same set of samples probed with a C-strand telomeric probe. Lanes marked "C" are loading controls of a denatured plasmid fragment (~3 kb) containing a *K. lactis* telomere. Molecular size markers (M) are in kb. (D) Exonuclease I (ExoI) digest of chromosomal DNA from *stn1-M1* cells. The left half of the panel is the ethidium bromide-stained gel of genomic DNA from *stn1-M1* cells. The lanes marked "1X" and "5X" represent relative amounts of ExoI used to treat genomic DNA from *stn1-M1* cells. The lane marked "U" shows undigested genomic DNA. The right half of the panel represents the gel from the left panel hybridized to the C-strand telomere probe.

passaging (Fig. 6B). These results indicated the presence of a functional telomerase in the *stn1-M1* mutant.

***RAD52* is essential for the viability of *stn1-M1* mutants.** To assess the role of recombination in telomere maintenance in the *stn1-M1* mutant, we deleted the *RAD52* gene, which is known to be involved in most homologous recombination events, in the *stn1-M1* background. A haploid *rad52 K. lactis* strain was mated to the *stn1-M1* strain, and the resulting diploids were sporulated. Twenty-three tetrads from six independent diploids were dissected. Of these, 21 tetrads exhibited a ratio of viable to nonviable spores of 2:0, and the other 2 tetrads exhibited a ratio of viable to nonviable spores of 2:1. Visual examination of the nonviable spores revealed growth arrest at the two- to four-cell stage. Southern hybridization analysis confirmed that the two morphologically abnormal clones were *stn1-M1 RAD52* mutants. Thus no viable *stn1-M1 rad52*Δ isolates were identified by this method. We next performed an additional screen for *stn1-M1 rad52*Δ isolates by random spore analysis from the same parental diploids (see Materials and Methods). Of the 64 colonies examined, 8 rough colonies were identified and analyzed by Southern hybridizations. All of these clones proved to be *stn1-M1 RAD52* single mutants. As expected, ~50% of normally growing segregants were *STN1 rad52* mutants. We therefore conclude that the *stn1-M1* mutation is synthetically lethal in combination with the *rad52* gene deletion. In other experiments, we attempted to directly disrupt *RAD52* in mitotically growing *stn1-M1* cells. No *stn1-M1 rad52* double mutants were found from these experiments either.

The *stn1-M1* strain contains long stretches of 3′ G-rich single-stranded DNA. One of the consequences of telomere uncapping in yeast is the presence of extensive 3′ single-stranded overhangs due to excessive degradation of 5′ telomeric DNA (6, 20, 49, 63). We therefore analyzed *stn1-M1* and *ter1*-Δ *stn1-M1* mutants by nondenaturing in-gel hybridizations (15). Undigested genomic DNA from both the strains was electrophoresed on a 0.7% agarose gel. When the gel was hybridized with the C-strand telomere oligonucleotide probe, a very strong signal was observed at limit mobility (>20 kb) and in the wells (Fig. 6C). In contrast, when the same samples were hybridized with the probe derived from the G-strand telomeric DNA, we observed a greatly diminished signal. By equilibrating the signal observed to the control denatured plasmid DNA, we estimate that there is ~20- to 30-fold increase in single-stranded G-strand over single-stranded C-strand telomeric DNA in *stn1-M1* and *stn1-M1 ter1*-Δ cells. The strong signal

TABLE 1. Elevated levels of recombination near telomeres in *stn1-M1* cells[a]

STN1 allele	Gene conversion rate		Relative rate
	Mutation rate ± SD	SE (*n*)	
STN1	$6.70 \times 10^{-6} \pm 2.70 \times 10^{-5}$	7.90×10^{-6} (12)	1.0
stn1-M1	$3.69 \times 10^{-2} \pm 2.54 \times 10^{-2}$	6.87×10^{-3} (15)	~5,500

[a] The subtelomeric gene conversion rates of *stn1-M1* are based on measurements of five independent transformants of the *stn1-M1* mutant containing the *URA3* gene inserted near one telomere (see Materials and Methods). The assay for each clone was performed in triplicate. As controls, the values for the wild-type *STN1* clones have been cited from previously published results (40). The standard error was calculated as the standard deviation divided by the square root of *n*, the number of samples assayed for each strain.

seen with the C-strand telomere probe was largely sensitive to digestion with the 3′-to-5′ single-strand exonuclease ExoI (Fig. 6D). These results are consistent with the *stn1-M1* mutant having a capping defect resulting in large 3′ single-stranded overhangs. The ~30% of the single-stranded G strand that was resistant to both 1× and 5× levels of ExoI presumably represents gapped regions of the telomeric DNA. Whether the signal visible with the G-strand telomere probe was from single-stranded DNA in the *stn1-M1* samples or due to background hybridization to the large amount of double-stranded telomere DNA is unclear.

Telomeres in *stn1-M1* cells exhibit high rates of subtelomeric recombination. We have previously shown that *K. lactis* cells with abnormally short or highly elongated telomeres exhibit highly elevated rates of subtelomeric recombination (40, 63). We hypothesized that *stn1-M1* cells would exhibit elevated recombination rates near the telomeres because of the importance of the *RAD52* gene for their survival. To test this, we performed gene conversion assays to measure the subtelomeric recombination rates (40). Briefly, telomere DNA tagged with a subtelomeric *URA3* marker was transformed into the *stn1-M1* strain, where it replaced a single native telomere. Four transformants that were verified by Southern analysis to contain a single copy of the *URA3* gene were used for the gene conversion assay (see Materials and Methods). The rates of *URA3* loss due to gene conversion were then measured by plating serial dilutions of the cells on media containing 5-FOA as previously described (40). Our results indicate that *URA3* loss was ~5,000-fold elevated relative to a wild-type *STN1* control (Table 1). To date this is the highest rate of subtelomere gene conversion observed among a variety of *K. lactis* mutants exhibiting dysfunctional telomeres (40, 63).

DISCUSSION

The *stn1-M1* mutant has a telomere cap defect. The Stn1 protein of *S. cerevisiae* is part of the trimeric complex that binds the single-stranded 3′ telomeric overhangs and is known to be involved both in the protective capping function of telomeres and in regulating telomerase's ability to lengthen telomeres (11, 22, 25, 50). This is the first study of a mutant allele of *STN1* in *K. lactis*. A variety of evidence indicates that the *stn1-M1* mutation causes a defect in the protective capping function of telomeres. The extremely long telomeres of *stn1-M1* cells clearly indicate the presence of a defect in telomere length regulation. The abnormal colony and cellular morphologies of the *stn1-M1* mutant resemble those of early-senescing yeast telomerase deletion mutants as well as certain telomerase *TER1* template mutants with extremely long telomeres (38, 39, 56). This suggests that the telomeres of *stn1-M1* cells often trigger DNA damage checkpoints, similar to senescing cells lacking telomerase (29a). Another sign of a telomere capping defect in *stn1-M1* cells is the long tracts of single-stranded telomeric DNA, specifically of the 3′ G-rich strand, consistent with degradation of the 5′ strand of the telomere. Studies with *S. cerevisiae* have shown that single-strand degradation from the 5′ end occurs at double-strand breaks and at telomeres with certain capping defects (6, 20, 65). As 3′ single-stranded DNA is a potent initiator of homologous recombination, it is not surprising that we also observe highly elevated levels of recombination near the telomeres of *stn1-M1* cells. Additionally, the presence of occasional sharp bands seen with a subtelomere-specific probe (Fig. 1B) might suggest that fusions may sometimes occur in *stn1-M1* mutant cells, perhaps only after the loss of telomeric repeats. Overall, our data suggest that Stn1 protects telomeres against initiating homologous recombination events. This protection might occur directly through the interaction of the Stn1/Cdc13/Ten1 complex with telomeric DNA (18), or it might occur indirectly through the ability of Cdc13 and Stn1 to interact with components of DNA polymerase α/primase, which leads to synthesis of the C-rich strand at 3′ G-strand telomeric overhangs (27, 53). It is possible that the role of Stn1 in protecting against telomeric recombination is mediated largely or entirely in the S/G_2 phases of the cell cycle, as this is when the 3′ overhangs are most pronounced (15). Also, recent evidence suggests that double-strand break repair during S/G_2 is usually mediated by homologous recombination while repair during G_1 is mainly mediated by nonhomologous end joining (19).

Chronic uncapping triggers runaway RTE in *stn1-M1* cells. Although both *stn1-M1* and *ter1*-Δ mutants can maintain telomeres by recombination, they are very different in important ways. A striking difference is in the extent of telomere elongation between *stn1-M1* and *ter1*-Δ survivor cells. *stn1-M1* cells have telomeres that are many kilobases in length, while *ter1*-Δ survivors seldom have telomeres longer than several hundred base pairs (38). Secondly, *stn1-M1* and *ter1*-Δ cells differ in growth characteristics. *ter1*-Δ cells display a progressive growth senescence to a point where most cells are dead or very poorly growing (38). After RTE has lengthened telomeres, growth is improved, with survivors often temporarily displaying growth characteristics indistinguishable from wild-type cells. This recovery is followed by irregular cycles of additional senescence and growth improvement brought on by the fluctuating and often very short lengths of the telomeres. In contrast, *stn1-M1* cells exhibit a chronic growth defect reminiscent of moderately senescent *ter1*-Δ cells. This growth defect is stably maintained between different growth passages over long periods of time. The two mutants also differ in their responses to the deletion of genes involved in homologous recombination. In *stn1-M1* cells, deletion of *RAD52* leads to immediate lethality. In contrast, in a *ter1*-Δ mutant strain, lethality also occurs, but only after >50 cell divisions. Additionally, we have observed that deletion of *RAD59*, a gene homologous to *RAD52*, kills or severely affects the growth of *stn1-M1* cells but does not se-

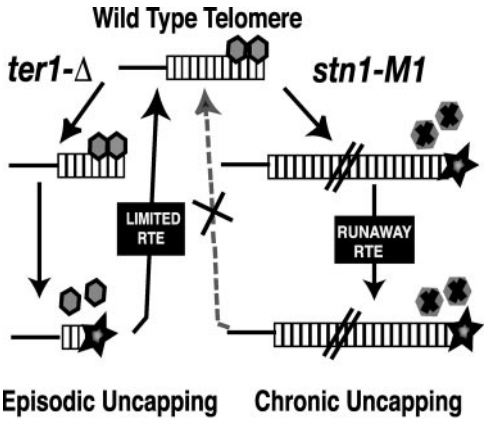

FIG. 7. Two types of RTE in *K. lactis*. (Left) In the absence of telomerase (*ter1*-Δ), a telomere capping defect producing a state prone to initiating recombination (black star) occurs only once telomeres have dropped below ~100 bp in length, presumably due to the inadequate presence of telomere capping proteins (gray hexagons). Once RTE lengthens telomeres above their critical length, they become recapped and resistant to additional elongation by recombination. The capping-defective telomere state, prone to initiating recombination, is thus episodic, and the extent of telomere elongation is limited. (Right) In the *stn1-M1* mutant, a defective capping protein may cause a chronic capping defect that leaves telomeres prone to initiating recombination in a manner largely or entirely independent of telomere length. This produces runaway RTE (type IIR RTE). See text for details.

verely affect the growth or formation of survivors in the *ter1*-Δ background (S. Iyer and M. J. McEachern, unpublished data).

We propose that the differences between *stn1-M1* and *ter1*-Δ cells result from fundamentally different types of telomere capping defects. As shown in the model in Fig. 7, *ter1*-Δ cells can be viewed as having a capping defect that is episodic in nature (Fig. 7, left). Recent work in our laboratory has shown that telomeres in *ter1*-Δ cells are able to recombine only once they have shortened to less than ~4 repeats (100 bp) in size (62). This predicts that, once a telomere is lengthened by RTE to a size appreciably above this length, it effectively becomes recapped and resistant to initiating further recombination events. If all telomeres become lengthened and recapped, the cell can grow normally until one or more telomeres again shorten to below ~100 bp. Telomere length can become kilobases long in "type II" survivors of yeast cells lacking telomerase. However, this is thought to be triggered by rolling-circle replication events that generate a long telomere in a single step (34, 46). In this circumstance, where uncapping is episodic, RTE is inherently self-limiting.

However, we hypothesize that, in *stn1-M1* cells, the capping defect is chronic and partly or entirely independent of the initial length of the telomere (Fig. 7, right). Recombination events that produce abnormally long telomeres in *stn1-M1* cells cannot restore normal telomere functionality due to the continued presence of the dysfunctional Stn1 protein. Thus even the longest telomere in *stn1-M1* cells may be capable of initiating recombinational repair and undergoing RTE.

The RTE-producing moderate elongation of telomere repeat tracts in yeast mutants lacking telomerase has been termed "type II" (12, 59). Since the telomere elongation of *stn1-M1* cells appears to consist only of elongated telomeric repeat tracts, it could also be described as type II RTE. However, given the fundamental differences between RTE in *stn1-M1* and *ter1*-Δ cells, especially in terms of telomere length, we propose that RTE observed in *stn1-M1* cells be called type IIR for producing runaway telomere elongation. We would define type IIR RTE as extreme telomere elongation by recombination resulting from a chronic telomere capping defect that is largely or entirely independent of telomere length. By this definition, the moderate telomere elongation caused by recombination in *S. cerevisiae cdc13-1* cells under certain conditions might qualify as a less extreme example of type IIR RTE (21, 23) because telomeres of nearly normal size can become elongated by recombination. Also, as discussed below, the telomere maintenance in human ALT cells is certainly a candidate for being type IIR RTE.

The mechanistic similarities and differences between type II RTE in *ter1*-Δ cells and the type IIR RTE of *stn1-M1* cells still remain to be determined. Accumulating evidence indicates that the type II RTE in *ter1*-Δ cells occurs through a roll-and-spread mechanism, whereby the first long telomere is generated by copying a telomeric circle of ~100 bp and all other telomeres are elongated by directly or indirectly copying that telomere (45, 46). It is difficult to predict the role of telomeric circles in type IIR RTE. One possibility is that the long telomeres in *stn1-M1* cells could arise from intertelomeric recombination without the need of DNA circles. However, if relatively large circles are present in *stn1-M1* cells, they could be potent templates for generating long telomeric repeat tracts through rolling-circle events. Therefore a reasonable prediction is that telomeric circles are not essential to type IIR RTE but may be important contributors to it.

An interesting feature of the *stn1-M1* mutant (and perhaps a general characteristic of type IIR RTE) is its apparent ability to undergo RTE in the presence of an active telomerase. A question that then arises is why are *stn1-M1 rad52* cells nonviable, if they can utilize telomerase to maintain chromosome ends? We suggest that the essential role of recombination in *stn1-M1* cells stems from a function that cannot be replaced by telomerase. One possibility is that recombination is needed to repair chromosome ends that have lost all telomeric repeats. The very high loss rate of a subtelomeric *URA3* gene indeed suggests that recombinational repair very frequently involves subtelomeric sequences in *stn1-M1* cells. A second possibility is that recombination is required to antagonize the production of single-stranded telomeric DNA. The accumulation of 5 to 10 kb of single-stranded DNA is thought to be sufficient to arrest yeast cell growth (58). The repair DNA synthesis that is initiated from strand invasion or possibly the strand invasion itself might act to counteract the cell cycle arrest signal produced by single-stranded DNA.

Is *STN1* a regulator of TRD? A striking feature of the *stn1-M1* mutant is the rapid loss of its long telomeres upon reintroduction of *STN1* (Fig. 3 and 5A). This is very different from a number of other instances where abnormally elongated telomeres are introduced into cells that otherwise maintain shorter telomeres (31, 33, 56, 69). In these cases, the long telomeres commonly remain long for protracted periods of growth. However, sudden telomere shortening events attributed to processes other than incomplete replication have been observed in a number of circumstances (37). Especially well documented are TRD events that occur in *S. cerevisiae* (8, 33).

Most TRD events are *RAD52* and *MRE-11* dependent and are thought to be intrastrand recombination events resulting in terminal deletions (37). While its underlying mechanism is unclear, the rapid telomere shortening we report here may represent the most extreme example of TRD reported so far.

Both the presence of very long telomeres formed independently of telomerase and the very high rate of subtelomeric gene conversion indicate that the telomeres in *stn1-M1* mutants undergo very high levels of recombination. It is quite likely that RTE and TRD in *stn1-M1* cells could result from the same recombination pathway initiated at a telomere. Our data suggest that Stn1 acts to inhibit telomeres from engaging in recombination events in general, but also that it differentially affects RTE and TRD. The presence of long telomeres indicates that telomere lengthening predominates over telomere shortening in *stn1-M1* cells. In contrast, reintroduction of *STN1* into *stn1-M1* cells does not simply complement defects caused by the mutant *stn1* gene and freeze telomeres at long sizes as might be expected. Instead, telomere deletion events clearly predominate over RTE events, leading to dramatic telomere shortening of all 12 telomeres. It is not currently known whether these TRD events occur within a single cell division or whether they occur more gradually over many cell divisions.

How Stn1p influences the extensive TRD events observed undoubtedly depends on the mechanism by which it caps and protects telomeres. The Cdc13p/Stn1p/Ten1p complex bound to the single-strand telomeric overhang may prevent the 3′ overhang from engaging in any strand invasion into other telomeric repeats. An additional possibility is that the Stn1 complex may affect the outcome of 3′ overhangs that are already engaged in an intrastrand invasion, perhaps by binding to the displaced DNA loop. This might explain how reintroducing *STN1* into *stn1-M1* cells blocks RTE while continuing to permit TRD events in the cell. However, we cannot yet rule out the possibility that the rapid telomere shortening in *stn1-M1* cells arises due to recombination-independent events.

Parallels between *stn1-M1* and human ALT cells. The phenotypes of *stn1-M1* cells are in many ways strikingly similar to mammalian ALT cells. The most obvious similarity between *stn1-M1* and ALT cells is in the very long and heterogeneous telomeres that are produced by recombination. Also, the RTE pathways observed in both *stn1-M1* and ALT cells are genetically recessive (51, 52). Another similarity of ALT cells and *stn1-M1* cells is that both exhibit a mixture of healthy and senescent cells (28, 55). This is suggestive of both having chronic telomere capping defects that can often trigger growth arrests. Like *stn1-M1* cells, ALT cells appear to have elevated levels of telomere recombination (17). Additionally, the presence of telomeric DNA, telomeric proteins, and a growing list of recombination proteins in ALT-associated promyelocytic leukemia bodies also suggested the importance of recombination in the ALT pathway (28). So far, APBs have not been identified in yeast species. Other similarities between ALT and *stn1-M1* cells are that both are able to produce telomeric circles (t circles) and that RTE can occur in the presence of telomerase (10; S. Iyer, A. Cesare, E. Basenko, J. Griffith, and M. McEachern, unpublished data). Whether t circles contribute to RTE in either case is not known. It is currently unclear whether ALT cells, like *stn1-M1* cells, produce at least some telomeres with long 3′ overhangs. Rapid telomere shortening reminiscent of TRD has also been observed in ALT cells (37, 43, 51, 52). The mechanisms triggering such deletion events in ALT and *stn1-M1* cells still remain to be determined. Thus, both ALT cells and *stn1-M1* cells share many traits consistent with their telomeres having chronic capping defects and being very frequently engaged in recombinational repair.

An obvious possibility suggested by our work is that the phenotype of ALT cells could arise from mutations in genes encoding proteins that are components of the single-stranded DNA binding complex. Although a human homologue of *STN1* has not been yet identified, one of the several proteins interacting with hPOT1 (part of the TRF complex) is a potential candidate for being a functional ortholog of *STN1* (35, 57, 67). Another exciting implication of our results arises from the extreme sensitivity of *stn1-M1* to the absence of *RAD52*. This could imply that ALT-positive cells may be much more sensitive to recombination inhibitors than normal cells. We conclude that *stn1-M1* is an excellent model for understanding how telomere capping defects can trigger both RTE and TRD. Further studies of *stn1-M1* should therefore provide many additional important insights and lead to a better understanding of these mechanisms in yeast and other organisms.

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