Recombination Can either Help Maintain Very Short Telomeres or Generate Longer Telomeres in Yeast Cells with Weak Telomerase Activity†

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Yeast mutants lacking telomerase are able to elongate their telomeres through processes involving homologous recombination. In this study, we investigated telomeric recombination in several mutants that normally maintain very short telomeres due to the presence of a partially functional telomerase. The abnormal colony morphology present in some mutants was correlated with especially short average telomere length and with a requirement for RAD52 for indefinite growth. Better-growing derivatives of some of the mutants were occasionally observed and were found to have substantially elongated telomeres. These telomeres were composed of alternating patterns of mutationally tagged telomeric repeats and wild-type repeats, an outcome consistent with amplification occurring via recombination rather than telomerase. Our results suggest that recombination at telomeres can produce two distinct outcomes in the mutants we studied. In occasional cells, recombination generates substantially longer telomeres, apparently through the roll-and-spread mechanism. However, in most cells, recombination appears limited to helping to maintain very short telomeres. The latter outcome likely represents a simplified form of recombinational telomere maintenance that is independent of the generation and copying of telomeric circles.

Telomeres are important guardians of chromosomal stability. In most human somatic cells, telomeres gradually become shorter due to incomplete replication of chromosomal ends and little or no telomerase activity (31). This can trigger replicative senescence or apoptosis that can serve to reduce the likelihood of cancer formation (26). However, normal cells that bypass cell cycle arrest can further develop into cancers and achieve cellular immortality through the activation of a telomere maintenance pathway (56). While telomere elongation in most cancers is dependent on the enzyme telomerase, a significant minority use a telomerase-independent pathway known as alternative lengthening of telomeres (ALT) (10, 53). Considerable evidence suggests that ALT occurs through homologous recombination (HR). ALT cells display high rates of telomeric recombination including sister chromatid exchange (1, 35) and the persistent presence of telomere-induced DNA damage foci (TIFs) (53). Most ALT cells have ALT-associated promyelocytic leukemia bodies (APBs), which contain telomere-specific binding and recombinational proteins and various forms of telomeric DNA (25, 68). Extrachromosomal telomeric DNA can occur in linear and circular forms (t-circles), which have been suggested to contribute to telomere elongation in cancer cells (8, 24, 43). ALT tumors can sometimes test positive for both telomerase and ALT, but it is not definitively known if both pathways can coexist in individual cells (51, 62).

The budding yeasts Kluyveromyces lactis and Saccharomyces cerevisiae have been used extensively as models for recombinational telomere elongation (RTE) (30, 36, 37, 39). Deletion of telomerase in either species leads to a growth senescence characterized by a decline in the growth rate and increasing irregularity in the colony shape (27, 36, 37, 55). This abnormal colony phenotype correlates with critically short telomeres becoming uncapped and triggering DNA damage responses, including cell cycle arrest. Many cells in late senescent cultures of mutants lacking telomerase are abnormally enlarged and have cell division defects (15, 27). The majority of senescing cells die within 50 to 100 cell divisions, but postsenesce survivors emerge with improved growth rates and telomeres that have been elongated by recombination (36, 37). In S. cerevisiae, telomerase deletion survivors are classified as type I and type II based on their telomere patterns and growth rates. Type I survivors exhibit amplification of subtelomeric Y′ elements and have relatively slower growth and short terminal telomeric tracts (11, 36, 57). Type II survivors, on the other hand, lack subtelomeric amplification, grow relatively rapidly, and have long telomeric repeat tracts. Even though both pathways require RAD52, the requirements for other recombination proteins differ between the two pathways (32, 36, 39, 57). Mutations in proteins that act at telomeres and nontelomeric ends can influence the extent and speed of senescence as well as the structure of the telomeres produced by recombination (5, 18, 29, 48).

Postsenesce survivors generated in K. lactis telomerase deletion mutants (tert-) show moderate recombinational elongation of telomeric tracts and therefore are classified as type II survivors. Considerable evidence, particularly from K. lactis, suggests that type II RTE occurs through a roll-and-spread mechanism whereby a t-circle is used as a template in the rolling-circle copying event to produce a substantially elongated telomere, which in turn becomes a template for break-
induced replication (BIR) events that copy its sequence onto other short telomeres in the cell (34, 44, 45, 58). It has been shown that postsenesence survivors generated in a ter1-Δ mutant in which only terminal telomeric repeats are mutationally tagged repeats often emerge with repeating patterns of wild-type and tagged repeats in their telomeres (45). It has been hypothesized that this happens when a t-circle composed of both repeat types becomes a template in a rolling-circle event that generates the first long telomere. T-circles as small as 100 nucleotides (nt), when transformed into K. lactis cells, have been demonstrated to be incorporated at telomeres as tandem arrays (44, 45). Furthermore, single- and double-stranded telomeric circles as small as ~100 nt/bp have been found in yeast mutants with dysfunctional telomeres (2, 9, 23, 34). Other evidence has verified a key prediction of the roll-and-spread model, namely, that a single telomere can be the source of all elongated telomeric sequences in a postsenescence survivor (58).

Variations of type II RTE that occur in cells other than telomerase deletion mutants have also been documented in yeast. Mutations in the Cdc13/Stn1/Ten1 complex, which binds and protects telomeric single-strand overhangs, can lead to RTE that occurs without senescence and in the presence of telomerase (18–20, 28). The maintenance of telomeres in the stn1-MI mutant of K. lactis has been labeled as type IIR RTE because it produces much longer, “runaway” telomeres than a telomerase deletion mutant (28). Other examples of type IIR RTE were observed in telomerase mutants whose telomeres were composed of certain mutant telomeric repeats that in some cases had a disrupted ability to bind the double-strand telomere binding protein Rap1 (3, 58). T-circles have been shown to be present in cells undergoing type IIR RTE (2, 3), but whether this type of RTE occurs through a roll-and-spread mechanism is currently unknown.

Here we show that certain strains of K. lactis with very short telomeres caused by compromised telomerase activity are capable of using recombination for the maintenance of their telomeres. In some cases, this recombination acts simply to help maintain the very short telomeres. However, in some cells of these mutants, recombination can generate substantially longer telomeres. Telomeric repeat expansion experiments suggest that this elongation occurs via a roll-and-spread mechanism.

MATERIALS AND METHODS

Strains and growth conditions. All yeast strains were streaked from a single colony every 3 days and were grown at 30°C on solid rich yeast extract–peptone–dextrose (YPD) medium. As a control strain, we used wild-type K. lactis strain 7B520 (ura3-1 his2-2 trp1) (37, 67). All ter1 mutants are derivatives of 7B520 and were constructed through the plasmid loop-in/loop-out procedure described previously (37, 38). The ter1-28C(Taq), ter1-Dup21-25, ter1-24T(SnaB), and ter1-Bgl(III) mutants have been described previously (40) and are diagramed in Fig. 1. The base change at the −4 position in ter1-Dup21-25 and ter1-Δ-ApaL causes slight telomere shortening by itself (63).

The K. lactis nad5Δ-Δ mutant has been described previously (40). The ter1 nad5Δ-Δ double mutants were generated by mating of single mutant strains and sporulation of the resulting diploids. Matings were done on solid medium containing malt extract. Diploids were grown on selective plates and were sporulated on minimal sporulation medium. Spores were dissected using a Zeiss Axiohot E microscope equipped with a micromanipulator (Carl Zeiss Inc., Thornwood, NY) and were then grown on solid YPD medium containing 1 M sorbitol. The presence of the nad5Δ-Δ allele was confirmed by Southern blotting, while ter1 alleles were confirmed by visualization of a rough-colony morphology (which correlates with short telomeres) and/or by Southern blotting.

RESULTS

Homologous recombination contributes to the viability of some short telomere mutants. Numerous mutations in the K. lactis TER1 gene, including hypomorphic mutations, produced telomeres that stabilized at a length substantially shorter than that of the wild type (40, 60, 63). Some of these mutants...
exhibited colony morphologies ranging from slight to appreciably rough, reminiscent of those of senescing \( \text{ter1} / / H9004 \) cells but without the progressively worsening viability. This suggested that stable short telomeres can sometimes have telomere-capping defects severe enough to cause growth deficiencies. Because highly shortened telomeres in yeast can frequently be repaired through homologous recombination, we sought to examine the role of recombination in the survival of three mutants with stable short telomeres. One of these, the \( \text{ter1-Bgl} \) mutation contains an insertion of GATC and two single-base changes predicted to result in the synthesis of 29-bp telomeric repeats (4 bp longer than wild-type repeats) with ClaI restriction sites (Fig. 1). The \( \text{ter1-\Delta R-Bcl} \) mutant has two template alterations: a deletion of the 5-bp terminal repeat and a phenotypically silent point template mutation that creates a BclI restriction site. The terminal-repeat deletion is predicted to render telomerase unable to carry out the translocation step of telomerase function and thus unable to add more than a single repeat at a time onto a telomeric end. The \( \text{ter1-\Delta1051-1242} \) strain has the silent Bcl template mutation as well as a large deletion near the 3' end of \( \text{TER1} \), which encompasses the region harboring phylogenetically conserved sequences CS5 and CS6, as well as most of CS7 (52, 61). It has been shown previously that while CS5 and CS6 are partially dispensable for telomerase activity, CS7 is critical for Sm protein binding and therefore for the stability of the telomerase enzymatic complex \textit{in vivo} (54, 61).

FIG. 2. Deletion of \( \text{RAD52} \) exacerbates growth defects in some \( \text{ter1} \) mutants with short telomeres. (A) Colony growth of a wild-type control strain (\( \text{TER1 RAD52} \)) and the \( \text{ter1-\Delta1051-1242} \) mutant, shown as examples of normal colony morphology and abnormal rough-colony morphology, respectively. (B) Photographs of clonal passaging of \( \text{ter1-\Delta}, \text{ter1-\Delta R-Bcl}, \) and \( \text{ter1-\Delta1051-1242} \) single mutants and double mutants with \( \text{rad52-\Delta} \) on solid rich YPD medium.
These three mutations were combined via mating and sporulation with a deletion of RAD52, a gene essential to most forms of homologous recombination in yeast. Three to nine independent single and double mutants of each strain were followed over several serial streaks on rich (YPD) medium. The control TER1 rad52-Δ mutants grew somewhat more slowly than TER1 RAD52 strains but produced colonies with completely normal morphology that remained similar in size and appearance over each of the 6 serial streaks (data not shown). These results indicated that, as expected, the deletion of RAD52 itself does not cause a rough-colony phenotype or growth senescence. The control ter1-Δ RAD52 strains senesced and produced better-growing survivors by the 4th or 5th streaks, as has been reported previously (37) (Fig. 2B). In contrast to the ter1-Δ RAD52 strains, all three ter1-Δ rad52-Δ clones produced smaller colonies and a more severe rough-colony phenotype during senescence and became completely unviable by the 3rd or 4th streak (Fig. 2B). This confirmed that in the absence of telomerase, the elongation of short, uncapped telomeres is dependent on RAD52. Deletion of RAD52 in the ter1 mutants with stable short telomeres produced different responses. Each of eight ter1-Bgl(fill) rad52-Δ mutants exhibited almost no change in their very mild rough-colony phenotype over the course of 6 serial restreaks (data not shown). We conclude that recombination does not contribute significantly to the survival of the ter1-Bgl(fill) mutant. In contrast, the growth defects of both the ter1-ΔR-Bcl and ter1-Δ1051-1242 mutants were exacerbated by the deletion of RAD52 (Fig. 2B). Each of three ter1-Δ1051-1242 rad52-Δ mutant clones exhibited senescent-like colony phenotypes that worsened steadily over the streaks examined. Even more pronounced senescence was seen with the ter1-ΔR-Bcl rad52-Δ mutants, where all three clonal lineages ceased growing altogether by the 6th streak (Fig. 2B). During these extended periods of senescence, neither mutant formed the better-growing survivors that occur through recombination in a telomerase deletion mutant (27, 36, 37). These results suggest that homologous recombination is a vital contributor to telomere maintenance in ter1-ΔR-Bcl and ter1-Δ1051-1242 mutants. The lower rate of growth decline in both of these mutants than in a ter1-Δ rad52 mutant is presumably due to the presence of a partially active telomerase. The partial or complete growth dependence of some non-null telomerase mutants on RAD52 strongly suggests that short telomeres in these mutants are frequently repaired and elongated by homologous recombination.

Particularly short telomeres are associated with the worst colony morphology defects in non-null telomerase mutants. We next examined the telomere lengths of a series of ter1 mutants (diagramed in Fig. 1) that produce stably short telomeres. Using a BsrBI restriction digestion that cleaves only 3 bp internal to the first telomeric repeat of 10 of 12 K. lactis telomeres, we were able to resolve small telomere length differences among these mutants in two independent clones from each mutant (Fig. 3). Each of the seven ter1 mutants examined had telomere lengths that averaged at least 300 bp shorter than the ~500-bp mean size of telomeres in the wild-type control strain. The telomeres in all the mutants examined were present primarily in a series of diffuse bands that were apparently multiples of the 25-bp K. lactis telomeric repeat. Such banding has been reported recently and was interpreted as indicating that K. lactis telomeres do not end at random positions but rather at preferred positions or regions on one or both strands within the 25-bp telomeric repeat (65).

Our results also demonstrated that mutants with the roughest colony morphology, the ter1-ΔL-ApaL and ter1-ΔR-Bcl mutants, also had exceptionally short telomeres, with most of their telomeric signal at the ~100-bp position. The ter1-ΔL-ApaL mutation, like the ter1-ΔR-Bcl mutation, deletes one of the 5-bp terminal repeats of the template (Fig. 1) and probably blocks telomerase from adding more than a single repeat. This may also account for the low colony length and therefore blocks telomerase from adding more than a single repeat.}

**FIG. 3.** The poorest colony morphology correlates with the shortest telomere length. Shown is a Southern blot of genomic DNA, hybridized to a telomeric probe, from the parental wild-type (WT) strain and two independent clones of each mutant (indicated by horizontal lines above lanes). DNA was digested with BsrBI and was resolved on a 3.2% agarose gel.
the ter1-28C(Taq) mutant, that produced near-normal colonies. This may indicate that factors other than telomere length contribute to the tendency of mutant telomeres to promote the formation of abnormal colonies. Alternatively, we cannot dismiss the possibility that telomeres of the ter1-Δ1051-1242 cells that we examined may have been partially elongated by recombination and therefore are not representative of their typical size. Overall, however, our results suggest that especially short telomeres in ter1 mutants, particularly in the absence of appreciable length heterogeneity, are associated with both the rough-colony phenotype and a strong involvement of recombination in telomere repair and cell survival.

RTE can occasionally produce much longer telomeres in the ter1-ΔR-Bcl, ter1-Δ1051-1242, and ter1-Bgl(fill) mutants. During the course of passaging of short-telomere ter1 mutants on solid YPD medium by serial restreaking, it was noticed that some mutants that normally produced abnormally rough colonies, including the ter1-ΔR-Bcl, ter1-Δ1051-1242, and ter1-Bgl(fill) mutants, occasionally produced colonies with a normal, smooth morphology. This was reminiscent of the formation of postsenescence survivors in ter1-Δ cells that utilize RTE for telomere elongation (45). For the ter1-Bgl(fill) mutant, which normally displayed a slight to moderate rough-colony phenotype, three instances of smooth colonies were observed during the course of passaging of four independent clones over more than 20 serial streaks. Two instances of smooth-colony formation occurred 11 streaks apart in one ter1-Bgl(fill) isolate, while the other occurred in a second, independent ter1-Bgl(fill) isolate. We examined these three independent clones with smooth colonies for telomere length at the point of their isolation and at one or more later points during serial restreaking on solid YPD medium. The results of this analysis are shown in Fig. 4A. Whereas the typical ter1-Bgl(fill) isolate (designated “control”) exhibited the expected short telomeres, each of the three smooth-colony derivatives of the ter1-Bgl(fill) mutant were found to have greatly elongated telomeres. The long telomeres did not represent a new set point in telomere length regulation (as can occur with some mutations affecting telomere function) (63). This was evident from the fact that telomere length in these clones greatly decreased over the course of subsequent streaks. Over the course of 16 streaks, clone 1 was observed to return to nearly the typical telomere length of the ter1-Bgl(fill) mutant, while clones 2 and 3 showed large reductions in average telomere size after 4 or 5 additional streaks. Concomitant with this telomere shortening, the colony phenotypes of the clones also gradually returned to the slightly rough phenotype that is typical for the ter1-Bgl(fill) mutant (data not shown).

If the lengthening of telomeric sequences from the smooth-colony clones occurred entirely by means of telomerase-mediated DNA synthesis, digestion with ClaI [which cleaves the mutant repeats synthesized by ter1-Bgl(fill) telomerase] should completely remove all of the additional sequence that had been added onto telomeric ends. As shown in the ClaI-digested samples in Fig. 4A, this is not the case. Instead, there are several small bands at the bottom of the gel that hybridize intensely to a wild-type telomeric probe. These bands, shown better resolved on a 6% polyacrylamide gel (Fig. 4A, inset), are apparently composed of small blocks of wild-type repeats that have become amplified in the smooth-colony clones and were not evident in the ter1-Bgl(fill) control. We estimate that the predominant small ClaI fragments produced in the smooth-colony ter1-Bgl(fill) clones are 54 bp, 79 bp, and 104 bp in clones 1 to 3, respectively. These sizes would be expected if one, two, or three consecutive 25-bp wild-type repeats are excised by cleavage within flanking 29-bp repeats (containing ClaI sites) derived from the ter1-Bgl(fill) telomerase (Fig. 4B). Our results indicated that much of the telomere elongation in the ter1-Bgl(fill) mutant is due to amplification of wild-type telomeric repeats rather than to addition of ClaI repeats by telomerase. This amplification of small blocks of wild-type repeats is highly reminiscent of that produced during postsenescence survivor formation in ter1-Δ cells engineered to initially have telomeres composed of basal wild-type repeats and terminal Bel repeats (45). In that case, repeating patterns were proposed to result from the amplification of a telomeric sequence derived from copying a t-circle composed of both wild-type and mutationally tagged repeats. We conclude that although ter1-Bgl(fill) cells do not require recombination to

FIG. 4. Smooth-colony derivatives of the ter1-Bgl(fill) mutant have telomeres lengthened by recombination. (A) Genomic DNA was isolated from three independent clones of the ter1-Bgl(fill) mutant with smooth-colony morphology and a control ter1-Bgl(fill) mutant with the typical slightly rough colony morphology. DNA was digested with EcoRI and also with ClaI (indicated by plus signs above lanes) prior to Southern blotting and hybridization to a telomeric probe. An inset at the bottom right depicts further resolution of bands released by EcoRI and ClaI digestion in the three clones by use of a 6% polyacrylamide gel. (B) Diagram showing a segment of telomeric DNA composed of wild-type (WT) and mutant repeats (shaded and open rectangles, respectively). Cleavage of Bgl(fill) mutant repeats by ClaI (indicated by arrows) excises fragments composed of WT repeats with terminal halves of mutant repeats as well as 29-bp mutant repeat fragments. The latter are not retained on the hybridization membrane.
maintain telomeres, they are capable of occasionally undergoing episodic RTE that can cause most or all telomeres in the cell to become grossly elongated.

The partial or complete dependence of ter1-ΔR-Bcl and ter1-Δ1051-1242 mutants on RAD52 for long-term growth strongly suggested that short telomeres in these mutants are commonly elongated by recombination. As described above, isolates of both of these mutants normally exhibit a steady phenotype of rough colonies when serially passaged on solid YPD medium. However, both mutants also produced occasional large, smooth colonies and colony sectors in streaks. Conceivably, these were clones that had lengthened their telomeres via recombination. The presence of the phenotypically silent Bcl base change in the TER1 template of both mutants allowed for testing for the amplification of wild-type repeats, as was done with the ter1-Bgl(fill) mutant.

The telomeres of several independent clones of the ter1-ΔR-Bcl mutant exhibiting improved colony morphology were investigated using digestion with EcoRI alone or with EcoRI plus BclI (Fig. 5). As anticipated, each of the eight clones displayed telomeres longer than those in typical isolates of rough-colony ter1-ΔR-Bcl clones. In two of five mutants (mutants 3 and 5), BclI digestion cleaved all telomeric fragments and released intensely hybridizing blocks of wild-type telomeric repeats. As with the ter1-Bgl(fill) mutant, this result strongly suggests that RTE involving a t-circle containing both wild-type and mutant Bcl repeats had occurred. Interestingly, the remaining three better-growing clones contained elongated telomeres that were largely or entirely resistant to BclI cleavage. This indicated that telomeric elongation had occurred through the addition of wild-type repeats only, a result consistent with RTE- rather than telomerase-mediated elongation. In a few cases (clones 1, 2, and 4), a faint smear of telomeric signal was visible at or below ~500 bp. These are likely to result from the retention by one or a few telomeres of a Bcl mutant repeat at a near-basal position of telomeres otherwise composed of wild-type repeats. The general lack of Bcl repeats in the smooth-colony subclones of the ter1-ΔR-Bcl mutant probably indicates that prior to RTE, many of the telomeres of this mutant frequently lacked repeats with the Bcl base change. Overall, our results strongly support the belief that the ter1-ΔR-Bcl mutant can frequently generate long telomeres through type II RTE.

Next, we passaged four ter1-ΔR-Bcl smooth-colony isolates for 7 serial streaks, and we examined telomere length and colony growth at each streak (Fig. 6). The EcoRI pattern of telomeres from these mutants indicated that the lengthened telomeres exhibited a steady decline in length over the course of passaging. The decline continued until telomeres reached the very short sizes that characterized the short telomeres of typical ter1-ΔR-Bcl cells. This result is, again, fully expected for clones that had undergone type II RTE. In at least two instances, some secondary telomere elongation events were observed (Fig. 6, clones 5 and 6, arrows). These are likely to represent subsets of cells that had undergone RTE in the cell populations being examined. In both instances, the lengthened telomeres were no longer present in cells of later streaks. This is likely due to the fact that passaging involved streaking to single cells, and a colony with cells containing elongated telomeres at streak 6 was presumably not used for streak 7. The gradual shortening of telomeres in the ter1-ΔR-Bcl clones was accompanied by a gradual decline in the ability of these clones to grow, as indicated by the growth graphs shown above the Southern blots in Fig. 6. Growth was scored using an arbitrary scale developed previously to score senescence in ter1-Δ mutants (37). As shown in Fig. 6, clones started with growth scores of 5 to 4 (slightly rough to normal smooth morphology) but gradually declined until, in three of four cases, they reached scores of ~1.5 (small and very rough colonies), which characterize the growth of typical ter1-ΔR-Bcl cells. We interpret this as indicating that telomere length is the critical determinant for colony growth ability in the ter1-ΔR-Bcl mutant.

We next analyzed the ter1-Δ1051-1242 mutant. Figure 7A and B show the typical short-telomere phenotype of this mutant. Two independent clones are shown at each of two consecutive streaks after growth on solid medium. Telomeric fragments from both clones showed hybridization to a Bcl repeat-specific probe that was stronger at the later streak than at the earlier streak (Fig. 7B). This signal, but not the signal with a wild-type telomeric probe (Fig. 7A), was removed upon digestion with BclII. This indicates that, as expected, the mutant telomerase is functional and has added Bcl telomeric repeats onto telomeric termini.

To address the question of whether RTE also plays a role in the establishment of better-growing colonies in the ter1-Δ1051-1242 mutant, we examined the telomeres of several independent clones with improved colony morphology by Southern blotting; results for three of these are shown in Fig. 7C. EcoRI digestion revealed that in each clone, many telomeres were substantially elongated relative to those of the normal rough-colony form of the same mutant (compare Fig. 7C and A). This
is consistent with the notion that the improvement in colony phenotype is caused by the generation of longer telomeres. Digestion of the same DNAs with EcoRI and BclI showed that in each case, all telomeric fragments in the cell were cut much shorter with BclI. This cleavage also released prominent small fragments that ran near the bottom of the gel. Running these small DNA fragments on a gel with a high percentage of agarose (Fig. 7C, inset) showed that they consisted primarily of a fragment of a single size within a given clone: 50 bp in clone 1, 100 bp in clone 2 and 75 bp in clone 3. In subsequent passaging of the clones, telomeres were observed to begin to shorten (data not shown). We conclude that the elongated telomeres of the ter1-/H9004 clones with improved colony morphology amplified both Bcl and wild-type repeats in a manner consistent with type II RTE.

Assessment of telomere lengths in mutants undergoing RTE in liquid culture. To further assess the similarities between the RTE of ter1-/H9004 cells and that of the mutants examined in this study, we passaged the mutants by serial dilutions in liquid medium. Unlike our passaging on plates, which goes through a bottleneck of a single cell at each streak, culturing in liquid is likely to produce more-heterogeneous populations of cells that will often have mixtures of independent cell lineages in various states of losing and rebuilding their telomeres. Two independent clones each of the ter1-/H9004, ter1-/H9004 R-Bcl, and ter1-Bgl (fill) mutants were inoculated into liquid YPD medium and were passaged serially by 1:1,000 dilutions daily for 20 days, with DNA extracted at each passage.

FIG. 6. Smooth-colony derivatives of the ter1-ΔR-Bcl mutant undergo gradual telomere shortening and return to a rough-colony phenotype with continued passaging. Four clones of ter1-ΔR-Bcl with elongated telomeres were serially passaged over eight streaks. DNA was cut with EcoRI prior to Southern blotting and hybridization to a telomeric probe. Graphs above Southern blots represent colony morphologies. A score of 1 represents the poorest growth with rough colonies, while a score of 4 defines a wild-type-like colony phenotype with completely smooth colonies (37). Arrows in blots represent instances where a sizable increase in the length of one or more telomeres has occurred in a cell population but apparently not in the cell from that streak that became the source of cells for the next streak. Data for the wild-type (WT) parental strain are shown in the panel with clone 4.

FIG. 7. Smooth-colony derivatives of the ter1-Δ051-1242 mutant have telomeres lengthened by recombination. (A) Southern blots of two clones of the ter1-Δ051-1242 mutant with typical rough colonies. Genomic DNA was digested with EcoRI and then also with BclI (indicated by plus signs above the lanes) prior to hybridization with a telomeric oligonucleotide that hybridizes to wild-type (WT) (and mutant) telomeric repeats. The first two streaks of each clone are shown. (B) The filter shown in panel A was rehybridized with the Klac Bcl probe specific to Bcl telomeric repeats. (C) Three better-growing clones with smooth-colony morphology were examined using the same digestions and probe as in panel A. The inset at the bottom shows further resolution of the bands released by digestion with EcoRI and BclI in three clones by use of a 3.2% agarose gel.
different size ranges. Clone 1 had telomeres at passage 1 ranging from very short to wild-type length, while clone 2 started with a major group of telomeres that were all much shorter than those of the wild type. As is typical of \textit{K. lactis} mutants with short telomeres that have been passaged for some time, both \textit{ter1-}\(\Delta\) clones had undergone subtelomeric gene conversions that homogenized subtelomeric polymorphisms, so that fewer than the 5 to 6 different-sized telomeric fragments of the wild type were displayed (40). The notable result with the prolonged passaging is that by dilution 20, the two clones have very similar overall telomere profiles, and the major telomere cluster contains primarily telomeres shorter than wild-type telomeres, but with a tail of signal extending up to 1.5 to 2 kb. This can be interpreted as meaning that the telomeric repeat arrays in postsenescent \textit{ter1-}\(\Delta\) cells can be extended at least occasionally to lengths at least triple the ~500-bp size of wild-type telomeres by RTE. These results provide further evidence that \textit{ter1-}\(\Delta\)R-Bcl cells can undergo a type II RTE that closely resembles that which occurs in \textit{ter1-}\(\Delta\) cells.

The two clones of the \textit{ter1-Bgl(fill)} strain that were passaged in liquid both started with the very short telomeres that typify the normal state of that mutant (Fig. 8). Clone 2, however, was observed to have a rather different profile of telomeric EcoRI fragments at passage 1. While \textit{ter1-Bgl(fill)} clone 1 and all the other mutant clones for which results are shown in Fig. 8 had a major group of telomeric fragments at a size of ~1 kb, \textit{ter1-Bgl(fill)} clone 2 had its most intense band between 1.5 and 2 kb. This difference arose from the occurrence of abundant subtelomeric gene conversions in clone 2 prior to the start of the experiment. The telomere fragment profiles in both \textit{ter1-Bgl(fill)} clones were observed to be stable through 5 passages but showed substantial telomere elongations at passage 10 that persisted until passage 20. The telomere length patterns at passage 20 of \textit{ter1-Bgl(fill)} cells were thus not as uniform as those exhibited by \textit{ter1-}\(\Delta\) or \textit{ter1-}\(\Delta\)R-Bcl cells. This failure of the \textit{ter1-Bgl(fill)} mutant to reach a stable distribution of telomere length in serial liquid cultures probably stems largely from the lower rate of RTE in this mutant, coupled with a presumably insufficiently long experimental growth course. Nonetheless, we conclude that passaging by dilutions in liquid culture provides a promising way to evaluate telomere length profiles in \textit{K. lactis} mutants that undergo RTE.

**DISCUSSION**

Our work presented here studied telomere maintenance in \textit{K. lactis} \textit{ter1} mutants with abnormally short telomeres. In all
cases, these mutants retained at least some telomerase activity, as indicated by the fact that none underwent the same growth senescence that is characteristic of a ter1-Δ mutant. In spite of this, however, telomeric recombination was found often to be extensive. In certain mutants, for example, long-term propagation was severely impaired in the absence of the Rad52 protein, presumably because the crippled telomerase was not able to maintain telomeres without aid from recombination. We observed that in at least three mutants, the ter1-ΔR-Bcl, ter1-Δ1051-1242, and ter1-Bgl(fill) mutants, clonal isolates containing telomeres that had been elongated by recombination could be readily found. This could be deduced from the fact that telomere lengthening was accompanied, in part, by amplification of wild-type repeats in cells where telomerase could make only mutationally tagged repeats.

Considerable evidence, particularly from work done with K. lactis, now supports the belief that RTE in yeast cells lacking telomerase can occur through a roll-and-spread mechanism (39, 45). According to this hypothesis, shortened telomeres become prone to greatly increased levels of homologous recombination, some events of which can occasionally produce a small telomeric circle (t-circle). Rolling-circle replication using a t-circle as a template, and perhaps initiated at an invading 3′ telomeric end, is then thought to create a substantially lengthened telomere in a single step. Once cells contain one long telomere, other telomeres can then become elongated by copying its sequence, thereby spreading the sequence present in the original t-circle to most or all telomeres in the cell.

A suite of shared characteristics argue that the elongated telomeres observed in the mutants studied here are produced in a manner that is mechanistically very similar to that observed in K. lactis cells lacking telomerase. In both situations, long telomeres are formed in cells that have very short telomeres as well as abnormal cell and colony morphologies characteristic of cells with unrepaired DNA damage. In both situations, the telomere elongations are closely associated with an improved ability to grow. Additionally, the lengthened telomeres arising in the ter1-Δ mutant and the mutants studied here share a number of characteristics. The extent of lengthening observed is similar; typically, it is limited to hundreds of base pairs. Further, in both situations, RTE is found to lengthen most or all telomeres in the cell. This is consistent with RTE being a concerted process, where once one telomere is elongated, all the other short telomeres are much more likely to be lengthened as well. Previous work has shown that the sequence of a single long telomere present in senescing ter1-Δ cells is copied to all other telomeres at >90% frequency (58). The elongated telomeres are not stable but are instead subject to gradual shortening that can eventually reduce them to very short sizes. Finally, the amplification of interspersed blocks of wild-type repeats, which are often of a single predominant size within the telomeres in a given clone, was remarkably similar between the ter1 mutants studied here and ter1-Δ cells. Such blocks are consistent with the notion that the first telomere-lengthening event involves the copying of a small t-circle composed of both wild-type and mutationally tagged repeats.

The three examples of ter1-Bgl(fill) clones that had acquired elongated telomeres through RTE are exceptional because of the especially large size increases (1 to several kb) in their telomeres. This is superficially similar to the type IIR “run-away” RTE that has been found to occur in the stn1-M1 mutant and certain other K. lactis mutants with abnormal telomere repeats (3, 28, 58). Significantly, the base changes within the Bgl(fill) telomeric repeat map to a short region that, when mutated, can lead to extreme telomere elongation due to either telomerased-mediated or recombinationally mediated elongation (58, 63). This makes it possible, and perhaps likely, that the Bgl(fill) repeat is deficient at blocking telomeric sequences from initiating recombination. However, because the long telomeres in the ter1-Bgl(fill) clones are subject to gradual shortening, we conclude that they are not the product of full-blown type IIR RTE. It should be kept in mind, though, that the telomeres in the long-telomere ter1-Bgl(fill) clones are composed of mixtures of both wild-type and mutant telomeric repeats. Thus, we cannot rule out the possibility that telomeres composed entirely of Bgl(fill) repeats would be capable of undergoing type II RTE. One possible explanation for the long telomeres in the three ter1-Bgl(fill) clones is that capping defects in the Bgl(fill) repeats can occasionally lead to telomeric recombination occurring at slightly greater lengths and producing slightly larger t-circles than would otherwise be the case. Large (1.5-kb) t-circles lead to much greater telomere elongation than do small (100-nt) t-circles (44). It is conceivable that slight differences in the sizes of the very small t-circles thought to trigger type II RTE can cause large differences in how processively those molecules can be copied by a DNA polymerase. Such a phenomenon potentially could explain why type II RTE in S. cerevisiae telomerase deletion mutants produces substantially longer telomeres than those seen in equivalent K. lactis mutants (11, 36, 57). S. cerevisiae telomeric repeats are highly heterogeneous in sequence and might be expected to lead to shortening telomeres that became prone to recombination at heterogeneous sizes. This in turn could lead to the production of t-circles of a larger size distribution than that which occurs in senescing K. lactis ter1-Δ cells.

One conclusion of our work is that the ability of K. lactis cells to undergo type II RTE is linked to the presence of exceptionally short (<~100-bp) telomeres. This is consistent with the past observation that K. lactis telomeres as long as ~100 bp were capable of recombining with other telomeres during ter1-Δ postsenescence survivor formation (58). One interpretation of this is that ~100 bp represents the minimum telomere length that prevents a telomere from initiating recombination. However, it is clear from past work that many ter1 mutants that have not been observed to undergo RTE, such as ter1-28C(Taq) and ter1-Dup21-25 mutants, nonetheless experience highly elevated rates of subtelomeric BIR events as a consequence of telomere dysfunction (40, 46). This leads to the question of how telomere uncapping can lead to widespread BIR events at telomeres without RTE being observed. One possibility is that t-circle formation, presumably the rate-limiting event of RTE, is always far rarer than other recombinational outcomes triggered by telomere uncapping, such as BIR events. In this view, BIR events represent a standard, programmed response of the cell to short telomeres entering a recombinational repair pathway, while RTE from t-circle formation and copying may instead be a rare and stochastic outcome to such repair. Alternatively, or in addition, the uncapping events that trigger RTE could be qualitatively different in some manner from those occurring in cells that undergo BIR.
but not RTE. Conceivably, an altered cellular state associated with severe DNA damage, such as a checkpoint-activated or a damage-adapted state (13), might lead to alterations in the way DNA damage, including uncapped telomeres, is repaired (66). Consistent with this possibility, evidence from *S. cerevisiae* indicates that efficient type II RTE in that organism is dependent on checkpoint sensor proteins (17, 59). It has also been demonstrated that DNA ends with partial telomere function do not elicit repair responses identical to those elicited by nontelomeric ends. For example, senescing telomerase deletion mutants produce a gene expression profile different from that for other DNA damage responses (47). Also, checkpoint signaling at double-strand breaks (DSBs) near telomeric repeats is reduced relative to that at ends without nearby telomeric repeats (41).

Another important inference from our work concerns the nature of the telomeric recombination that occurs in those cells of the *ter1-Δ1051-1242* and *ter1-ΔR-Bcl* mutants that are not lengthening telomeres through recombination but rather are just maintaining short telomeres. Both of these mutants require RAD52 for long-term viability yet can be passaged on plates apparently indefinitely without either net telomere lengthening or net telomere shortening occurring in the bulk of cells. It therefore seems highly probable that most homologous recombination events occurring at telomeres in these mutants involve very modest lengthening events that act to maintain telomeres at short sizes. These HR processes could, in most cells, exclusively involve events where the shortest telomere strands invade and copy sequence from other telomeres only slightly longer than themselves rather than including copying events from t-circles as templates. These processes may be mechanistically similar to the BIR events that can replace subtelomeric sequences, described above. The purely telomeric copying mode of recombination that is independent of t-circles is likely to represent the simplest form of recombinational telomere maintenance. In *K. lactis* *ter1-Δ* mutants, such pure telomere copying either is unable to maintain telomeres by itself or occurs in such poorly growing cells that it is difficult to observe once better-growing post-senescent survivors with substantially elongated telomeres emerge through the roll-and-spread process with its added use of copying of t-circles. Some previous evidence is consistent with the possibility that RTE can occur in the absence of telomeric circles. Senescing *K. lactis* telomerase deletion cells with a single long telomere will specifically copy sequence from that telomere to all other telomeres in the cell (58). Additionally, human ALT cells with knocked down expression of Nbs1 or XRCC3, proteins that appear essential to t-circle formation, do not block continued proliferation of these cells (14).

Our identification of recombinational telomere maintenance that does not involve net elongation of very short telomeres has some important implications. First, it expands the range of phenomena that constitute RTE. Although it would be best described as a form of type II RTE, the maintenance of very short telomeric tracts is reminiscent of type I RTE in *S. cerevisiae*. However, it is not type I RTE, since there is no evidence for amplification of subtelomeric sequences. It could be noted that this also implies that the presence of only short telomeric tracts in *S. cerevisiae* cells using recombination to maintain telomeres is not sufficient to allow the conclusion that type I RTE is occurring. Second, we would predict from our results that the genetic requirements for RTE to occur without net telomere lengthening, and without the need for the formation and use of a t-circle, might be different from those for standard type II RTE. Interestingly, the maintenance of short telomeres by recombination in telomerase-negative mouse cells has recently been reported (42). This implies that RTE in mammalian cells does not necessarily generate the long heterogenous telomeres characteristic of most ALT cancers.

A variety of other examples of telomeric recombination occurring in the presence of telomerase or compromised telomere capping have been documented in recent years. Among the more dramatic are yeast mutants that produce type IIR RTE as a result of defects affecting the function of telomere-capping proteins (21, 28, 49). Similarly, the ALT pathway present in some human cancers, which closely resembles type IIR RTE in yeast, is not typically repressed if telomerase is exogenously expressed (7, 16, 22, 50). Additionally, recombination can also play a role at telomeres in at least some cells with no known telomere dysfunction. In the yeast *Candida albicans*, telomeres become lengthened in a *rad52-Δ* background, suggesting a role for recombination in normal telomere length regulation (12). In *K. lactis*, wild-type telomeres can readily become greatly elongated when transformed with t-circles (45). Recombination has also been shown to cause dramatic shortening of abnormally long telomeres in otherwise wild-type *S. cerevisiae* and *K. lactis* (4, 6, 33). Decreased turnover of internal repeats in a *K. lactis* *rad52-Δ* mutant argues that similar truncations occur even at normal-length telomeres (4).

Since perturbed telomere capping leads to alterations in normal telomere functioning, further investigation of the circumstances under which both telomerase and RTE contribute to telomere maintenance will allow better understanding of the mechanisms underlying carcinogenesis in humans and will potentially help us to further understand the peculiarities of telomere elongation in ALT cancers.

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**REFERENCES**

1. Bailey, S. M., M. A. Brenneman, and E. H. Goodwin. 2004. Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. Nucleic Acids Res. 32:7943–7951.

2. Basenko, E. Y., A. J. Cesare, S. Iyer, J. D. Griffith, and M. J. McEachern. 2010. Telomeric circles are abundant in the snl1-Δ1 mutant that maintains its telomeres through recombination. Nucleic Acids Res. 38:182–189.

3. Bechard, L. H., et al. 2009. Mutant telomeric repeats in yeast can disrupt the negative regulation of recombination-mediated telomere maintenance and create an Alternative Lengthening of Telomeres-like phenotype. Mol. Cell. Biol. 29:626–639.

4. Bechard, L. H., N. Jamieson, and M. J. McEachern. 2011. Recombination can cause telomere elongations as well as truncations deep within telomeres in wild-type *Kruyveromyces lactis* cells. Eukaryot. Cell 10:226–236.

5. Bertuch, A. A., and V. Landthaler. 2004. Exol contributes to telomere maintenance in both telomerase-proficient and telomerase-deficient *Saccharomyces cerevisiae*. Genetics 166:1651–1659.

6. Bucholc, M., Y. Park, and A. J. Lustig. 2001. Intrachromatid excision of telomeric DNA as a mechanism for telomere size control in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 21:6559–6573.

7. Cerone, M. A., J. A. Londono-Vallejo, and S. Bacchetti. 2001. Telomere...
maintenance by telomerase and by recombination can coexist in human cells. Hum. Mol. Genet. 10:1945–1952.

8. Cesare, A. J., and J. D. Griffith. 2004. Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops. Mol. Cell. Biol. 24:9996–9997.

9. Cesare, A. J., C. Groff-Vindman, S. A. Compton, M. J. McEachern, and J. D. Griffith. 2008. Telomere loops and homologous recombination-dependent telomeric circles in a Kluyveromyces lactis telomere mutant strain. Mol. Cell. Biol. 28:26–29.

10. Cesare, A. J., and R. R. Reddel. 2010. Alternative lengthening of telomeres: models, mechanisms and implications. Nat. Rev. Genet. 11:319–330.

11. Chen, Q., A. Lipman, and C. W. Greider. 2005. Two survivor pathways that allow growth in the absence of telomere are generated by distinct telomere recombination events. Mol. Cell. Biol. 25:1819–1827.

12. Ciudad, T., et al. 2004. Homologous recombination in Candida albicans: role of CArad52p in DNA repair, integration of linear DNA fragments and telomere length. Mol. Microbiol. 55:1177–1194.

13. Clemenson, C., and M. Marsolier-Kergoat. 2009. DNA damage checkpoint inactivation: adaptation and recovery. DNA Repair 8:1101–1109.

14. Compton, S. A., J. Choi, A. J. Cesare, S. Ozug, and J. D. Griffith. 2007. Xrc3 and Nbs1 are required for the production of extrachromosomal telomeric circles in human alternative lengthening of telomere cells. Cancer Res. 67:1513–1519.

15. Enomoto, S., L. Glowczewski, J. Lew-Smith, and G. J. Berman. 2004. Telomere cap components influence the rate of senescence in telomerase-deficient yeast cells. Mol. Cell. Biol. 24:837–845.

16. Ford, L. P., et al. 2007. Telomerase can inhibit the recombination-based path of telomere maintenance in human cells. J. Biol. Chem. 276:32198–32203.

17. Grandin, N., and M. Charbonneau. 2007. Mre11, a non-essential DNA repair protein, is required for telomere end protection following loss of capping by Cdc13, Yku or telomerase. Mol. Genom. Genomics 277:665–699.

18. Grandin, N., and M. Charbonneau. 2003. The Rad51 pathway of telomerase-independent maintenance of telomeres can amplify T_{\gamma}, sequences in yku and cdc13 mutants of Saccharomyces cerevisiae. Mol. Cell. Biol. 23:3721–3734.

19. Grandin, N., C. Damon, and M. Charbonneau. 2001. Cdc13 prevents telomere uncapping and Rad50-dependent homologous recombination. EMBO J. 20:6127–6139.

20. Grandin, N., C. Damon, and M. Charbonneau. 2001. Ten1 functions in telomere end protection and length regulation in association with Smit1 and Cdc13. EMBO J. 20:1173–1183.

21. Grandin, N., S. I. Reed, and M. Charbonneau. 1997. Snt1, a new Saccharomyces cerevisiae protein, is implicated in telomere size regulation in association with Cdc13. Genes Dev. 11:512–527.

22. Gronbely, J. V., M. Kulp-McEliece, and D. Broccoli. 2001. Effects of recombination on telomerase activity on telomere maintenance by the alternative lengthening of telomeres (ALT) pathway. Hum. Mol. Genet. 10:1953–1961.

23. Groff-Vindman, C., A. J. Cesare, S. Natarajan, J. D. Griffith, and M. J. McEachern. 2005. Recombination at long mutant telomeres produces tiny DNA circles in Kluyveromyces lactis. Mol. Cell. Biol. 25:4406–4412.

24. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

25. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

26. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

27. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

28. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

29. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

30. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

31. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

32. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

33. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

34. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

35. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.

36. Hentze, G. D., et al. 2009. DNA C-circles are specific and quantifiable models, mechanisms and implications. Nat. Rev. Genet. 10:177–180.
65. Wang, Z., L. Guo, L. Chen, and M. J. McEachern. 2009. Evidence for an additional base-pairing element between the telomeric repeat and the telomerase RNA template in *Kluyveromyces lactis* and other yeasts. Mol. Cell. Biol. 29:5389–5398.

66. Wellinger, R. J. 2010. When the caps fall off: responses to telomere uncapping in yeast. FEBS Lett. 584:3734–3740.

67. Wray, L. V., Jr., M. M. Witte, R. C. Dickson, and M. I. Riley. 1987. Characterization of a positive regulatory gene, *LAC9*, that controls induction of the lactose-galactose regulon of *Kluyveromyces lactis*: structural and functional relationships to *GAL4* of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 7:1111–1121.

68. Yeager, T. R., et al. 1999. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res. 59:4175–4179.