Dynamics of Telomeric DNA Turnover in Yeast

Michael J. McEachern,*,[1] Dana Hager Underwood* and Elizabeth H. Blackburn†

**Department of Genetics, Life Sciences Building, University of Georgia, Athens, Georgia 30602-7223 and †Department of Biochemistry, University of California, San Francisco, California 94143-0414*

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ABSTRACT

Telomerase adds telomeric DNA repeats to telomeric termini using a sequence within its RNA subunit as a template. We characterized two mutations in the *Kluyveromyces lactis* telomerase RNA gene (*TER1*) template. Each initially produced normally regulated telomeres. One mutation, *ter1*-AA, had a cryptic defect in length regulation that was apparent only if the mutant gene was transformed into a *TER1* deletion strain to permit extensive replacement of basal wild-type repeats with mutant repeats. This mutant differs from previously studied delayed elongation mutants in a number of properties. The second mutation, *TER1-Bcl*, which generates a *Bcl*I restriction site in newly synthesized telomeric repeats, was indistinguishable from wild type in all phenotypes assayed: cell growth, telomere length, and *in vivo* telomerase fidelity. *TER1-Bcl* cells demonstrated that the outer halves of the telomeric repeat tracts turn over within a few hundred cell divisions, while the innermost few repeats typically resisted turnover for at least 3000 cell divisions. Similarly deep but incomplete turnover was also observed in two other *TER1* template mutants with highly elongated telomeres. These results indicate that most DNA turnover in functionally normal telomeres is due to gradual replicative sequence loss and additions by telomerase but that there are other processes that also contribute to turnover.

TELOMERES, the protein and DNA complexes at the ends of eukaryotic chromosomes, function to protect chromosome ends from terminal sequence losses and fusions and also appear to be important for meiotic chromosome segregation (reviewed in Zakian 1995; Lingner and Cech 1998; McEachern *et al.* 2000b). Telomeric DNA in the great majority of eukaryotic species is composed of tandem repeats of 5- to 26-bp sequence units. These repeats contain binding sites for proteins required for telomere function. Because DNA polymerases cannot fully replicate DNA ends, telomeres use specialized mechanisms to ensure their complete replication. In eukaryotic cells with telomeres composed of short tandem repeats, this involves the enzyme telomerase.

Telomerase is a reverse transcriptase composed of both protein and RNA subunits. Part of the RNA subunit serves as a template for synthesis of telomeric repeat units. Telomerase binds to 3′ single strand tails of telomeric DNA, partly through base pairing interactions involving nucleotides in the templating domain of the telomerase RNA and partly through other interactions of telomerase with the 3′ overhang of the telomeric DNA (Lee and Blackburn 1993; Prescott and Blackburn 1997a). Without telomerase activity, the telomeres of dividing yeast and mammalian cells gradually shorten. In yeast cells, this leads to telomere shortening, loss of telomere function, and eventual death of the great majority of cells (Lundblad and Szostak 1989; Singer and Gottschling 1994; McEachern and Blackburn 1995). Many human somatic cells naturally lack or have low levels of telomerase activity. In contrast, most human cancers express high levels of telomerase (Kim *et al.* 1994). This has led to the suggestion that telomere shortening in somatic cell lineages may be an adaptation that limits the proliferative capacity of developing cancers. All immortal human cell lines appear to have an active pathway of telomere maintenance, through either the presence of telomerase activity or, less frequently, the less understood telomerase-independent pathway termed alternative lengthening of telomeres (ALT; Reddel *et al.* 1997). Yeast cells lacking telomerase that survive beyond the initial phase of growth senescence emerge with telomeres that have been elongated through mechanisms involving *RAD52*-dependent homologous recombination (Lundblad and Blackburn 1993; McEachern and Blackburn 1996; Teng and Zakian 1999).

Telomerase RNA genes are particularly useful for studying telomere function because mutating the template region allows experimental alteration of the telomeric sequences. Typically, such mutated sequences cause net telomere lengthening or shortening (Yu *et al.* 1990; Singer and Gottschling 1994; McEachern and Blackburn 1995; Prescott and Blackburn 1997b, 2000) and, in some cases, telomeric fusions, aberrant chromosome segregation, and nuclear division (Yu *et al.* 1990; Kirk *et al.* 1997; Smith and Blackburn 1999; McEachern *et al.* 2000a). Certain altered template sequences in Tetrahymena and the yeast *Saccharomyces cerevisiae* also cause

[1]*Corresponding author:* Department of Genetics, Life Sciences Bldg., University of Georgia, Athens, GA 30602-7223.
E-mail: mjm@arches.uga.edu

misincorporation of bases as well as other defects in the enzymatic activities of telomerase *in vitro* and *in vivo* (Yu and Blackburn 1991; Gilley *et al.* 1995; Gilley and Blackburn 1996; Prescott and Blackburn 1997b).

The majority of single or double template base substitutions in *Kluyveromyces lactis* led to an abnormal telomere length phenotype soon after replacement of the wild-type *TER1* gene with a mutant *TER1* gene carrying the mutant template (McEachern and Blackburn 1995). For two such mutants (*ter1-Acc* and *ter1-Bsi*) the degree of immediate telomere lengthening correlated with the reduction in Rap1p binding affinity *in vitro* (Krauskopf and Blackburn 1996). Rap1p regulates telomere length in *S. cerevisiae* and *K. lactis* (Conrad *et al.* 1990; Lustig *et al.* 1990; Krauskopf and Blackburn 1996). Two other *K. lactis TER1* template mutants, *ter1-Bgl* and *ter1-Kpn*, which initially had short telomeres, produced greatly elongated telomeres only after a lag period of >100 cell divisions (McEachern and Blackburn 1995), although the repeats made by *ter1-Bgl* and *ter1-Kpn* did not display weakened affinity to Rap1 *in vitro* (Krauskopf and Blackburn 1996). During the lag period, the mutant telomeric repeats were confined to the outer tips of telomeres; only when most of the basal wild-type repeats had been replaced with mutant repeats did telomeres elongate and lose length control. Such extensive replacement could occur either through gradual turnover over prolonged cell passaging or by transforming the mutant *TER1* gene directly into a *TER1* deletion strain with very short telomeres.

A phenotypically silent telomerase *TER1-Bcl* template mutation has been a valuable tool for studying functions of both telomeres and telomerase (Krauskopf and Blackburn 1998; Roy *et al.* 1998; Smith and Blackburn 1999; Tzfati *et al.* 2000; McEachern and Iyer 2001). This class of mutant may not exist for most species as typical very short telomeric repeats must bind multiple different telomeric proteins and may have little or no tolerance for mutational change. The unusually long (25 bp) telomeric repeats of *K. lactis* (McEachern and Blackburn 1994) are likely to tolerate mutations better than the short repeats in most other species.

Here we report a detailed examination of two *K. lactis* telomerase RNA template mutations, *TER1-Bcl* and *ter1*-AA, that each initially produce normal-length telomeres. The *TER1-Bcl* mutant generates a restriction site in newly synthesized telomeric repeats, allowing repeat incorporation to be monitored. We investigated long-term telomeric repeat turnover both in *TER1-Bcl* cells and in the immediate elongation mutants *ter1-Acc* and *ter1-Bsi*. We concluded that the *TER1-Bcl* mutant behaves indistinguishably from wild type. In both normal-length and elongated telomeres, telomeric turnover was found to involve all but the innermost few repeats. We propose that normal telomeric turnover occurs primarily by gradual replacement involving replicative sequence losses and telomerase action in small increments, but that other processes are also involved.

MATERIALS AND METHODS

Strains: All strains used in this study are derivatives of *K. lactis* 7B520 (*ura3-1 his2-2 trp1*; Wray *et al.* 1987). The *his2-2* allele is complemented by the *S. cerevisiae HIS3* gene. Use of the term "wild type" refers to this parental strain. Construction of *TER1* template base changes to make the *TER1-Bcl*, *ter1-Acc*, *ter1-Bsi*, and *ter1*-AA mutants was done through a plasmid loop-in, loop-out procedure (McEachern and Blackburn 1995). Construction of $TER1^{ex\Delta}$ mutants was done by transforming a *ter1* deletion allele (lacking ~300 bp of *TER1*, including the template) with pTER-BX-UA containing *URA3* and either *TER1-Bcl* or *ter1*-AA. These plasmids integrated at the *ter1*-Δ allele to form a plasmid loop-in with one functional telomerase RNA gene. Two slightly different types of $TER1^{ex\Delta}$ mutants were utilized in this study. One type, shown as the $ter1\text{-}AA^{ex\Delta}$ clones in Figure 2, were loop-in strains still containing the integrated *URA3* vector flanked by the functional *TER1-Bcl* or *ter1*-AA allele on one side and the nonfunctional *ter1*-Δ allele on the other side. The second type, shown as the $ter1\text{-}AA^{ex\Delta}$ clones in Figure 3, were loop-out clones lacking the integrated *URA3* plasmid vector and the nonfunctional *ter1*-Δ allele.

Long-term passaging of mutant cells was carried out by serial streaking on rich media (YPD plates) at 30°. Strains were streaked twice weekly down to single cells, which grew into colonies. Each streak is estimated to represent 20–25 cell divisions. Transformations of *K. lactis* were performed using procedures and media identical to those used with *S. cerevisiae*.

***K. lactis* linear vectors and cloning telomeres:** Minichromosome vectors were created combining *K. lactis CEN + ARS* vectors pKL313 (*HIS3*) and pKL316 (*URA3*) (Roy *et al.* 1998) with a restriction fragment containing two telomeres oriented head to head that are separated by a *URA3* gene. The two-telomere fragment was created by inserting a *Dpn*I telomere fragment into the *Bam*HI and *Bgl*II sites in the polylinker of pMH3, flanking the *URA3* gene (Hollingsworth and Byers 1989). An *Eco*RI fragment containing the two telomeres was then excised and inserted into the *Eco*RI site of pKL313 and pKL316. The *Dpn*I telomere fragment was derived from a cloned *K. lactis* telomere (McEachern and Blackburn 1994) containing ~12 telomeric repeats. One *Dpn*I site was provided by a polylinker *Bam*HI site next to the end of the telomere and the second *Dpn*I site was provided by a *Bgl*II-linked *Xba*I site present ~120 bp internal from the start of the telomeric repeats. The resulting linear vectors were designated pHISLIN1, pHISLIN2, pURALIN1, and pURALIN2 depending upon the marker present and the orientation of the two-telomere + *URA3* insert. In pHISLIN1 and pURALIN1, the *URA3* gene between the two telomeres is transcribed toward the *Kpn*I site in the polylinker.

To clone mutant telomeres, two techniques were employed. In one, *TER1-Bcl K. lactis* cells were transformed with pHISLIN1 after cleavage with *Bam*HI and *Xho*I to linearize the plasmid and excise the *URA3* gene separating the two telomeres. DNA from a transformant was then isolated, cleaved with *Sma*I, which cleaves off one telomere, treated with T4 polymerase to blunt ends, and circularized with ligase. Plasmid clones were then recovered by transformation into *Escherichia coli*.

In the second telomere cloning protocol, a plasmid containing 0.6 kb of *K. lactis* subtelomeric DNA was transformed into long-term *TER1-Bcl* cells where it integrated next to a telomere. This plasmid, pSubtelHis, was generated by cloning an *Eco*RI-*Xba*I fragment (located in *K. lactis* ~120 bp from the innermost telomeric repeats and homologous to 11 of the 12

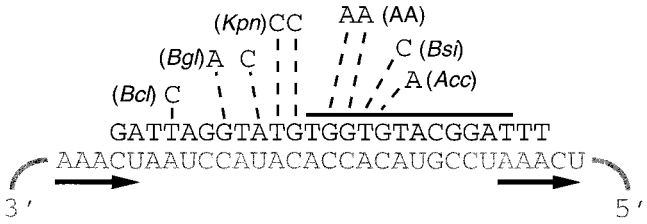

FIGURE 1.—Diagram of the *K. lactis TER1* template region and the position of alterations in four mutants. Shown in gray is the sequence of the 30-nucleotide (nt)-long template region of *TER1*. The arrows indicate the positions of the 5-nt direct repeats. The sequence shown in black is that of the 25-bp telomeric repeat unit of *K. lactis*. The solid bar indicates the position of the Rap1 protein binding site within telomeric sequences.

telomeres) into pRS423 (CHRISTIANSON *et al.* 1992) and allows for the recovery of transformants containing the plasmid integrated into any one of most of the telomeres in the cell. The transforming plasmid either was in circular form or was first linearized within its subtelomeric sequence using *Nco*I. DNA from transformants was isolated, cut with *Xho*I (to cleave the plasmid + telomere away from other chromosomal DNA) treated with T4 polymerase to ensure blunt ends, ligated, and then transformed into *E. coli*.

The permutation of the *K. lactis* telomeric repeat present at the subtelomere/telomere junction is as indicated for the strand running 5′ to 3′ toward the terminus: subtelomere, GGTGTACGGATTTGATT*AGGTATGT. The T residue marked with an asterisk denotes the position of the Bcl mutation. The number of copies of the sequence shown is what was counted as basal wild-type repeats.

Hybridizations: Southern blotting was performed using Hybond N^+ membrane (Amersham, Pharmacia Biotech, Piscataway, NJ). All hybridizations were carried out in Na_2HPO_4 and SDS (CHURCH and GILBERT 1984). Oligonucleotides used as hybridization probes are as follows: Klac 9-22 (GATTAGG TATGTGG), Klac 17-8 (ATGTGGTGTACGGATTT), Klac 1-25 (ACGGATTTGATTAGGTATGTGGTGT), and KLAA (GTATG TAATGTACG). Underlined nucleotides of KLAA show the mutant-specific changes. Other oligonucleotides are perfect matches to wild-type *K. lactis* telomeric repeats. All washes for hybridizations were carried out at the same temperature as hybridizations in Na_2HPO_4 and SDS (200 mM Na^+ and 2% SDS).

RESULTS

Characterization of two telomerase RNA template mutants initially displaying normal telomere length: Figure 1 shows the sequences of the *ter1*-AA and *TER1-Bcl* mutations. The expected sequence change in the mutant AA repeats is located within the conserved binding site of the Rap1 protein (COHN *et al.* 1998), and a duplex telomeric repeat DNA oligonucleotide containing the AA base changes binds Rap1 with reduced affinity *in vitro* (KRAUSKOPF and BLACKBURN 1996). In contrast, the sequence change predicted to be synthesized in Bcl telomeric repeats lies in a region of the repeat that is not conserved among telomeric sequences from a number of related yeasts (COHN *et al.* 1998) and is outside the predicted Rap1 binding site.

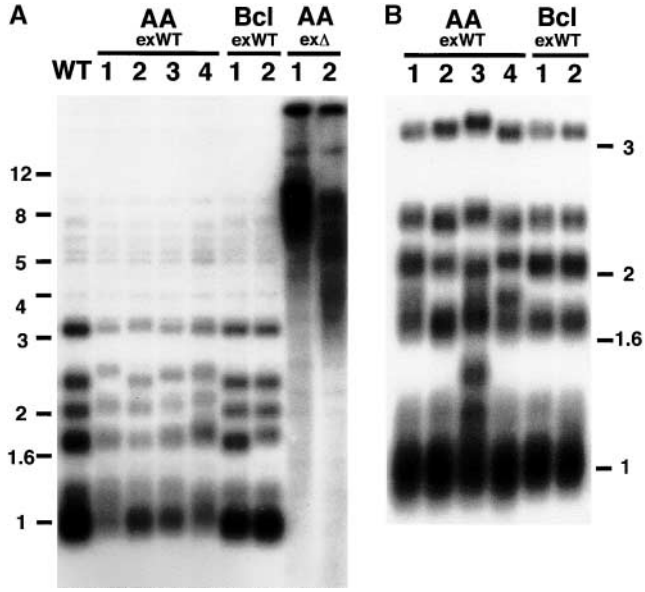

FIGURE 2.—Southern blot showing the telomeric *Eco*RI fragments of *ter1*-AA and *TER1-Bcl* mutants. (A) DNA prepared after long-term passaging of exWT and exΔ (see text for explanation) derivatives of *ter1*-AA and *TER1-Bcl* alongside DNA from a wild-type *K. lactis* (WT). Numbers on top indicate different clonal lineages. The exWT clones were grown for 120 passages and the exΔ clones had been grown for 90 passages. The probe used in A is a telomeric oligonucleotide Klac 19-7 at 38°. The faint bands above 4 kb in size are mostly from nontelomeric sequences due to the relatively low stringency hybridization conditions. One faint band, part of a doublet near 7 kb, is the *TER1 Eco*RI fragment. The relatively weak signal from the $TER1^{exWT}$ cells is due to the poor ability of the oligonucleotide probe used to hybridize to AA repeats. (B) exWT derivatives of *ter1*-AA and *TER1-Bcl* mutants after five streaks. Temporary elongation of certain telomeres is evident in *ter1*-AA clones, particularly clone 3. Size markers (in kilobases) are shown at sides of panels. The probe used is the oligonucleotide Klac 1-25 at 50°.

The *ter1*-AA and *TER1-Bcl* mutants retained normal telomere lengths soon after replacement of the wild-type *TER1* gene (Figure 2). To test whether either mutant underwent delayed telomere elongation, the mutant strains were extensively passaged by serial colony streaking and telomere lengths were monitored periodically. Each mutant was constructed in a genetic background that initially contained a wild-type *TER1* gene and normal-length telomeres, by first integrating ("looping in") a plasmid containing the *TER1* template mutant gene adjacent to the native *TER1* locus, followed by plasmid loop-out and screening for retention of the mutant-template *ter1* allele. Telomeric profiles of clonal lineages of mutants generated in this way (referred to as "exWT") are shown in Figure 2A for four *ter1*-AA^{exWT} and two *TER1-Bcl*^{exWT} clones after 120 consecutive streaks; each streak represented ~20–25 cell divisions. Even after this very long period of growth, the telomeres in *TER1-Bcl* clones remained indistinguishable from wild type, and *ter1*-AA clones showed only slight net elongation.

Thus, in this test, neither mutant behaved like the previously studied delayed elongation *ter1-Bgl* or *ter1-Kpn* mutants, which in comparable experiments eventually exhibited extreme telomere elongation. However, during this prolonged passaging additional subtle changes in length regulation appeared in the *ter1*-AA^{exWT} telomeres. After only 5 streaks, some individual telomeres, particularly in *ter1*-AA^{exWT} clone 3, were appreciably longer than normal (Figure 2B). After 5–10 more streaks, these elongated telomeres had gradually shortened back to normal length. Other sporadic instances of modest temporary telomere elongation occurred over the 120 consecutive streaks of the *ter1*-AA^{exWT} clones. The fluctuations in telomere length, such as those seen in Figures 2B and 3, were distinctly greater than those in either wild-type or *TER1-Bcl*^{exWT} cells and are suggestive of subtly altered telomere function.

In a second type of test of telomere function, the *ter1*-AA and *TER1-Bcl* mutant genes were each transformed into a senescing *TER1* deletion (*ter1*-Δ) strain at the point when telomeres were considerably shortened. We refer to these *TER1* deletion transformed cells as *ter1*-AA$^{ex\Delta}$ and *TER1-Bcl*$^{ex\Delta}$. These "exΔ" strains can exist in two forms, a plasmid loop-in form and a plasmid loop-out form, which appear indistinguishable with regard to telomere function (see MATERIALS AND METHODS for details). It was shown previously that this procedure could shorten the lag before telomeres elongate in the *ter1-Kpn* mutant, apparently because much of the inner tract of basal wild-type repeats was eliminated (McEachern and Blackburn 1995). For each mutant (*ter1*-AA$^{ex\Delta}$ and *TER1-Bcl*$^{ex\Delta}$), two independent transformants were examined. In both *TER1-Bcl*$^{ex\Delta}$ clones the telomere length was wild type and remained so even after 10 additional streaks (data not shown). In contrast, while the telomeres in the *ter1*-AA$^{ex\Delta}$ clones were initially slightly longer than wild type (M. McEachern, T. Fulton and E. Blackburn, data not shown), eventually telomere lengths grew to many times normal length and exhibited the smeared appearance on Southern blots characteristic of telomere length deregulation in other *K. lactis ter1* mutants (McEachern and Blackburn 1995; Smith and Blackburn 1999; Figure 2A). The telomeres in *ter1*-AA$^{ex\Delta}$ clones gradually lengthened over at least 80 streaks (an estimated 1600–2000 cell divisions; data not shown). Unlike some *TER1* template mutations that produce extreme telomere lengthening (McEachern and Blackburn 1995; McEachern and Blackburn 1997; Smith and Blackburn 1999), the *ter1*-AA$^{ex\Delta}$ and *TER1-Bcl*$^{ex\Delta}$ mutants at no stage exhibited abnormal colonies or cell morphology. Also, neither mutant exhibited the fusions between telomeric ends that have been observed in certain other *TER1* template mutants (McEachern *et al.* 2000a).

Four additional clonal *ter1*-AA$^{ex\Delta}$ lineages were analyzed over at least 20 consecutive streaks, beginning shortly after the creation of the strains. Telomere elongation was not always continuous or gradual. In one clonal *ter1*-AA$^{ex\Delta}$ lineage followed for 21 consecutive streaks, overall net telomere length did not increase (average telomere lengths remained roughly double the wild-type length; see Figure 3A), but did fluctuate considerably. Some fluctuations were gradual and occurred over several streaks, with some telomeres gradually shortening as others concurrently gradually lengthened. In other clonal lineages of *ter1*-AA$^{ex\Delta}$ cells, some telomere lengths showed large and rapid increases over shorter growth periods (Figure 3, B and C). In one lineage, within 1–2 streaks

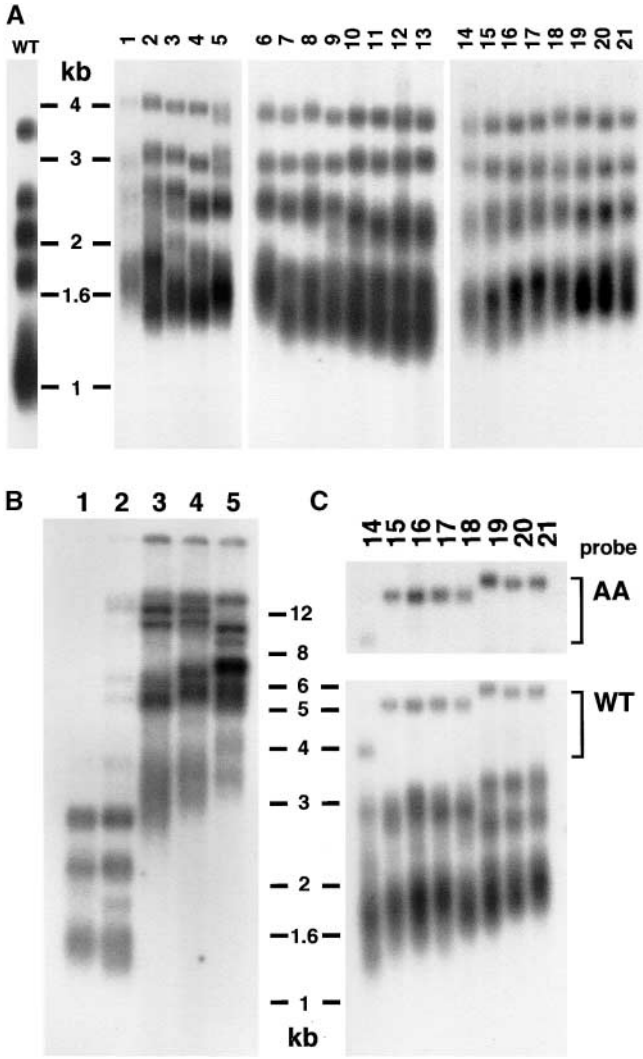

FIGURE 3.—Telomeric length changes in *ter1*-AA$^{ex\Delta}$ cells. Southern blot showing the telomeric *Eco*RI fragments of *ter1*-AA$^{ex\Delta}$ mutants. (A) Fluctuating telomere lengths in DNA prepared from 21 consecutive streaks of a *ter1*-AA$^{ex\Delta}$ clone. DNA from a wild-type *K. lactis* (WT) is shown to the left. B and C show jumps in telomere lengths that occurred in other *ter1*-AA$^{ex\Delta}$ clones. Numbers above lanes refer to the number of streaks cells have gone through from the point the mutant was generated. The bracketed parts of C show the same region of the same filter hybridized with two different probes. The probe used for the upper part of C is the AA repeat-specific oligonucleotide KLAA at 40°. The probe used for all other parts is the telomeric oligonucleotide Klac 9-22 at 40°. Size markers (in kilobases) are shown between panels.

(20–50 cell divisions) all telomeres in the cell grew by ~1.5 kb to over 10 kb (Figure 3B, compare lanes 2 and 3). Later (lanes 4 and 5), the telomeric fragments became relatively stably and uniformly elongated at greater than ~5 kb. In another clonal lineage (Figure 3C), several telomeres (migrating at ~1.2–1.8 kb) gradually lengthened by ~2 bp per cell division. In contrast, the largest telomeric fragment (initially at ~4 kb) showed two large jumps in size (between lanes 14 and 15 and lanes 18 and 19), accompanied by increases in the hybridization to an AA repeat-specific probe but not to a wild-type repeat-specific probe (compare Figure 3C, top, to Figure 3C, bottom). No gradual lengthening was apparent after each jump (see bracketed region in Figure 3C). The largest jump (~1 kb) minimally represents a mean lengthening rate of 40 bp per cell division. These results are consistent with bursts of elongation by the mutant telomerase. Although we have not tested *ter1*-AA, the more severe telomere elongation phenotypes in both immediate and delayed mutants have been shown to be *RAD52* independent (D. Underwood and M. McEachern, unpublished data). This indicates that telomerase and not recombination is responsible for the telomere elongation in both types of mutants. In summary, AA mutant repeats can cause defects in telomere length regulation that can vary stochastically between telomeres in ways that can persist for many cell divisions.

Repeats synthesized by the *TER1-Bcl* telomerase contain only the anticipated sequence change: Mutating the template region of a telomerase RNA gene has been shown to produce the equivalent sequence changes in the telomeric repeats synthesized by the mutant telomerase in ciliated protozoans, yeasts, and mammalian cells (Yu *et al.* 1990; Singer and Gottschling 1994; McEachern and Blackburn 1995; Marusic *et al.* 1997). However, *in vitro* and *in vivo* evidence has also shown that altering the telomerase template sequence can greatly lower the fidelity of the enzyme, leading in some cases to most newly synthesized repeats having nonpredicted sequences (Yu and Blackburn 1991; Gilley *et al.* 1995; McCormick-Graham *et al.* 1997; D. H. Underwood and M. J. McEachern, unpublished data). Therefore, we sequenced the telomeric repeats synthesized by the *TER1-Bcl* mutant by cloned telomeres from mutant cells. A linear minichromosome vector capable of replicating in *K. lactis* (see materials and methods) was introduced into *TER1-Bcl* cells so that repeats synthesized by the mutant *TER1-Bcl* telomerase became added onto the ends of the telomeres of the vector. Total DNA isolated from these yeast cells was treated with *Sma*I to cleave off one telomeric end and then treated with T4 polymerase to create a blunt end on the other telomere. The plasmid DNA was then circularized with ligase and used to transform *E. coli*. The two Bcl telomeres cloned by this method both contained the expected wild-type repeats immediately internal to the predicted mutant repeats. One clone terminated with seven, and the other with four, tandem Bcl repeats, all with the expected single base substitution (see Figure 1A) and no additional alterations. This same result was also observed for five additional telomeres cloned from long-term *TER1-Bcl* cells (see below). Thus, as also reported in another study (Tzfati *et al.* 2000), the Bcl telomerase faithfully synthesized repeats with the predicted mutation. These results, together with the unchanged telomere behavior described above, showed that the *TER1-Bcl* telomerase and Bcl mutant telomeric DNA are functionally equivalent to wild type.

Long-term turnover of telomeric repeats in *TER1-Bcl* cells: The terminal regions of telomeric DNA are highly dynamic, through both elongation by telomerase and shortening due to incomplete replication, and possibly other processes. If telomeric DNA turnover is mediated solely by telomerase, replacement by Bcl repeats is expected to occur progressively from the ends inward. In contrast, recombinational events within telomeric tracts might be expected to cause Bcl repeats to be recombined from outer to inner portions of the telomeric repeat tract. The phenotypically silent *TER1-Bcl* template mutation allowed us to study the dynamics of telomeric repeat turnover in functionally wild-type cells, by following the incorporation of *Bcl*I restriction sites in telomeric repeats. Double digestion with *Eco*RI plus *Bcl*I generates telomeric fragments with the mutant Bcl repeats at the telomeric termini removed (Figure 4C). Telomeres in two clonal lineages of a *TER1-Bcl* mutant were analyzed over 130 consecutive colony streaks (a year of continuous growth, or an estimated ~2600–3250 cell divisions; Figure 4, A and B). The results were very similar for both lineages. As with other *TER1* template mutants (McEachern and Blackburn 1995), Bcl telomeric repeats were present at the first time point after isolation of the mutant strain and, by the 10th streak, had penetrated further into the telomeric repeat arrays. Thereafter, penetration was slower, but detectable as late as the 120th streak. After 130 streaks, an average of ~300 bp of mutant Bcl repeats (12 repeats) were present on each telomere. Size analyses of restriction digests that produced smaller telomeric fragments (our unpublished data) and measurement of hybridization intensities from the filter shown in Figure 4 indicated that after 130 streaks, an average of ~3–4 basal wild-type repeats remained. Consistent with this, seven telomeres cloned from *TER1-Bcl* cells at 120 streaks contained from one to six full-length basal wild-type repeats with all the more distal repeats containing the Bcl mutation (Figure 5A). Thus, on average, ~75–80% of the telomeric repeat array was replaced during the prolonged passaging. This indicates either that telomeres are occasionally truncated to very short lengths or that Bcl repeats are moved internally by a process other than by telomerase addition to ends. One cloned telomere, LTBcl6E2-31, contained only about five complete repeats. Whether this clone represents a rare naturally truncated telomere or a cloning artifact is unclear. A summary of the patterns of turnover in *TER1-Bcl* cells is shown in Figure 5B.

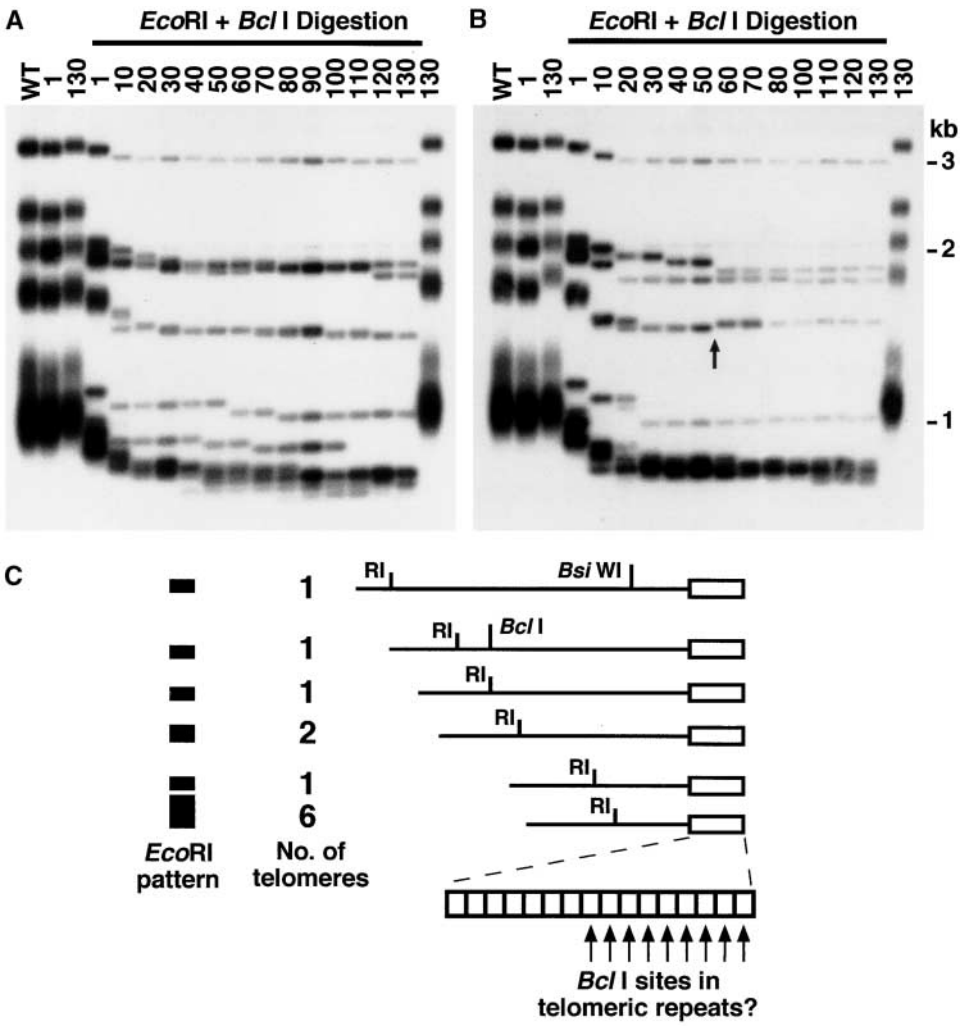

FIGURE 4.—Southern blot examining the extent of penetration of Bcl repeats in a time course of growth of two lineages of *TER1-Bcl*^{exWT} cells (A–B). Controls in the left three lanes and far right lane show *Eco*RI digests of wild-type (WT) or *TER1-Bcl*^{exWT} DNA, showing telomere fragments with all telomeric repeats present. Other lanes (marked by bar) show double digests (*Eco*RI + *Bcl*I) of DNA prepared from the *TER1-Bcl*^{exWT} cells. Numbers above lanes indicate the number of streaks since the isolation of the mutant. Arrow indicates one instance where there was an increase in the size of a *Bcl*I-cleaved telomeric fragment. Note that one telomere, at ~2.4 kb in the *Eco*RI digests, contains a subtelomeric *Bcl*I site and is cut to ~2 kb in size in the absence of Bcl telomeric repeats. This band runs as a doublet with another band. Probe used is the *K. lactis* telomeric oligonucleotide Klac 17-8 at 47°. Size markers are given on the right. (C) A map of the 12 *K. lactis* telomeres with the position of the *Eco*RI site nearest the telomere indicated (RI). Positions of a *Bcl*I site and a *Bsi*WI site within individual telomeric *Eco*RI fragments are also indicated. The diagram illustrates the shortening of telomeric fragments in *Eco*RI + *Bcl*I digests that results from incorporation of Bcl repeats at telomeric termini in *TER1-Bcl* cells.

Two observations on the long-term *TER1-Bcl* lineages are inconsistent with a model in which sequence loss from ends and sequence addition by telomerase are the only mechanisms affecting telomeric structure in the clones we examined. First, in one instance an *Eco*RI + *Bcl*I-cleaved telomeric restriction fragment increased in size between the 50th and 60th streak (arrow in Figure 4B). As this fragment has had all mutant repeats removed by cleavage with *Bcl*I, it must have acquired either additional basal wild-type repeats or additional subtelomeric sequences. Second, this same telomeric band initially existed in the clonal lineage as a doublet representing two *K. lactis* telomeres, but became a singlet between streaks 10 and 20 in one lineage (Figure 4A) and 20 and 30 in the other (Figure 4B and phosphorimaging data not shown). This suggests that one of the telomeres of the doublet underwent a telomeric or subtelomeric gene conversion, events common in a short telomere mutant but rare in wild-type cells (McEACHERN and IYER 2001).

Turnover of telomeric repeats in *TER1* mutants with elongated telomeres: Similar analyses of telomeres over long-term passaging by single-colony streaks were done on mutants with greatly elongated telomeres. In the two previously characterized *TER1* template mutants, *ter1-Bsi* and *ter1-Acc*, telomeres rapidly elongate to many kilobases, beginning immediately after replacement of the wild-type *TER1* gene despite the presence of nearly full-length arrays of basal wild-type repeats (McEACHERN and BLACKBURN 1995).

Repeat turnover in the *ter1-Bsi* and *ter1-Acc* mutants was monitored by restriction digestion, as each mutant telomerase generates a novel restriction site (*Bsi*WI and *Acc*I, respectively) in newly made telomeric repeats. In a clonal *ter1-Bsi* lineage followed over 60 streaks, telomeres remained at several kilobases, but turnover of the basal wild-type repeats continued for up to 50 streaks, at a rate similar to that observed with *TER1-Bcl* strains (Figures 4C and 6 and data not shown). Similar results were obtained with the *ter1-Acc* mutant (our unpublished data), which has even greater telomere elongation than *ter1-Bsi* (McEACHERN and BLACKBURN 1995). We conclude that both functionally wild-type and highly elongated telo-

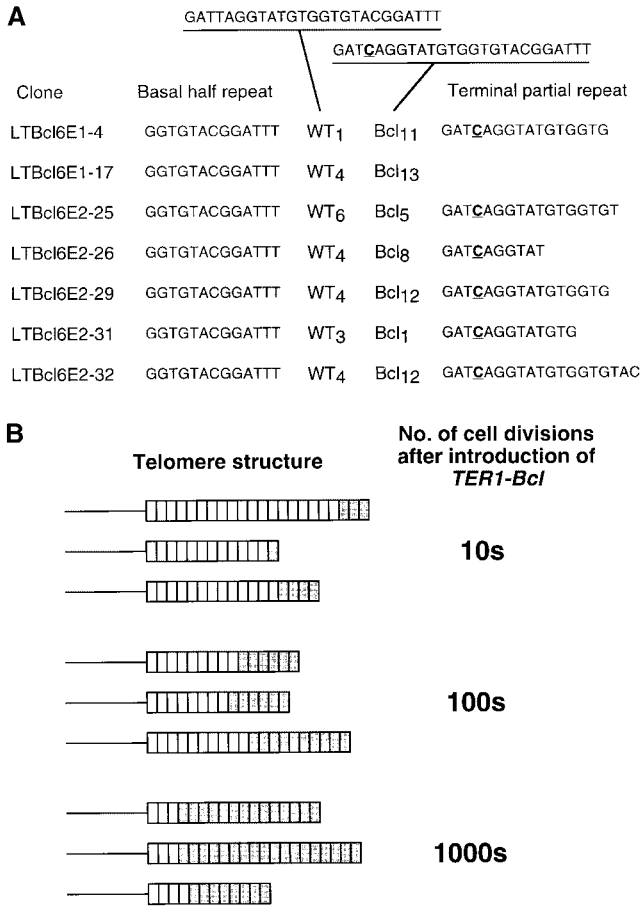

FIGURE 5.—Turnover in long-term *TER1-Bcl* cells. (A) Sequence of seven telomeres cloned from long-term (120 streaks) *TER1-Bcl* mutants. Two clones from lineage 6E1 (Figure 4A) and five from lineage 6E2 (Figure 4B) are shown. Sequences are shown 5′ to 3′ extending to the end of the telomeric sequence homology. Numbers of complete wild-type (WT) and Bcl repeats present in each cloned telomere are indicated. Sequences of each type of repeat are indicated above, with the Bcl mutation shown underlined. (B) Summary of telomeric turnover in the phenotypically wild-type *TER1-Bcl* mutant. Shortly after the wild-type *TER1* gene has been replaced by *TER1-Bcl*, only the terminal few repeats (of ~10–20 total) are Bcl repeats. After a few hundred cell divisions have passed, approximately the outer halves of the telomeres have been replaced. After a few thousand cell divisions, ~80% of the telomeres have been turned over. Open boxes indicate wild-type repeats and shaded boxes indicate Bcl repeats.

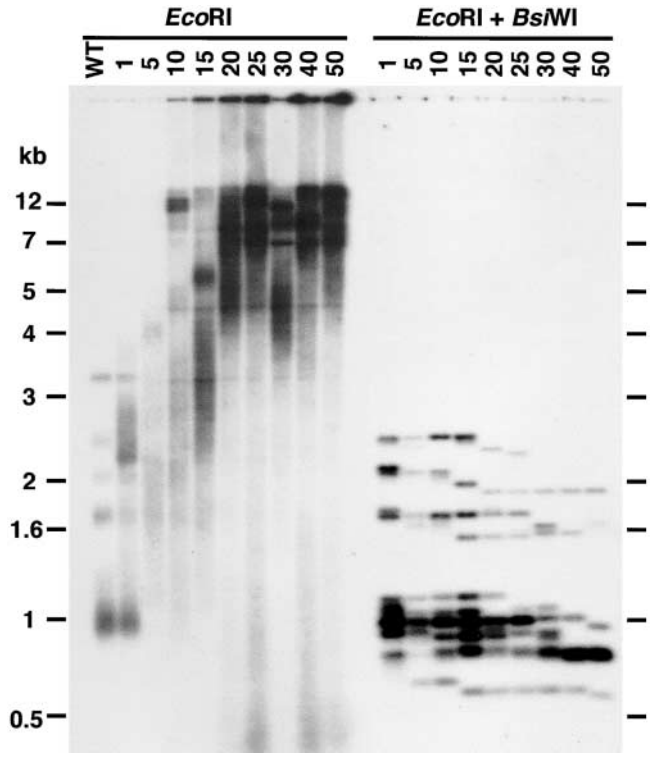

FIGURE 6.—Southern blot examining the extent of penetration of mutant repeats in a time course of growth of *ter1-Bsi* cells. On the left side are *Eco*RI digests of DNAs from wild-type (WT) and *ter1-Bsi* cells from each of multiple time points (indicated by numbers above lanes). These lanes provide an indication of total telomeric fragment lengths. The right side of the figure shows *Eco*RI + *Bsi*WI digests of the DNAs from *ter1-Bsi* cells that show telomeric fragment lengths after removal of all Bsi repeats. Note that the largest telomere in the *Eco*RI digests contains a subtelomeric BsiWI site and is present in the double digests as the fastest migrating band below 1 kb in size (see map of Figure 4C). The probe used is a *K. lactis* telomeric oligonucleotide, Klac 1-25 at 50°. Size markers are shown on both sides.

meres undergo similar patterns of turnover that affect all but the innermost few telomeric repeats.

DISCUSSION

The *ter1*-AA mutation causes a cryptic defect in telomere length regulation: The detailed examination reported here of the *K. lactis TER1-Bcl* and *ter1*-AA mutants, each of which initially produces normal-length telomeres, has uncovered novel features of telomere dynamics. The *ter1*-AA mutant is similar in some respects to the previously described delayed elongation *TER1* template mutants (McEACHERN and BLACKBURN 1995). In those mutants, while telomeres eventually grew very long, they did so only after a protracted latent period during which telomeres were stable and short. The latent period required the presence of internal wild-type repeats to maintain length regulation; only after most wild-type repeats had been replaced by mutant repeats did abrupt lengthening ensue. Similarly, *ter1*-AA mutants generated by transforming a strain initially containing wild-type *TER1* (*ter1*-AA^{exWT}) maintained near-normal telomere length even after thousands of cell divisions. However, transforming the *ter1*-AA gene into senescing *TER1* deletion cells with short telomeres (*ter1*-AA$^{ex\Delta}$) caused considerable telomere lengthening. Because *ter1*-AA^{exWT} telomeres did not lengthen even after thousands of cell divisions, we term *ter1*-AA a cryptic elongation mutant rather than a delayed elongation mutant. These results with *ter1*-AA highlight the importance of the continued presence of sufficient basal wild-type repeats in telomeres in the compensation for defective

telomere function caused by a *TER1* template mutation. This compensation is long lasting in *ter1*-AA^{exWT} cells, apparently because the innermost few wild-type repeats are highly resistant to turnover.

One hypothesis for why telomeres elongate in *ter1*-AA$^{ex\Delta}$ but not *ter1*-AA^{exWT} cells is that in *ter1*-AA$^{ex\Delta}$ cells the elongated telomeres retain fewer basal wild-type telomeric repeats than those of even very long-term *ter1*-AA^{exWT} cells. Alternatively, the physiology of a senescing *TER1* deletion cell could affect telomere function in some ways that allows generation of elongated telomeres by a newly transformed *ter1*-AA gene, in spite of the presence of a number of basal wild-type repeats that, in a *ter1*-AA^{exWT} cell, would be sufficient to block elongation. It is also conceivable that elongation of a small number of telomeres containing very few wild-type repeats somehow induces the elongation of other telomeres containing greater numbers of basal wild-type repeats. This latter possibility could help explain the sudden extensive elongation occurring in some clonal lineages of *ter1*-AA$^{ex\Delta}$ cells, such as the example shown in Figure 3B.

Four distinct differences between *ter1*-AA and the previously identified *ter1-Bgl* and *ter1-Kpn* delayed lengthening mutants (McEachern and Blackburn 1995) suggest that *ter1-AA* represents a distinct type of allele. First, *ter1*-AA cells initially produced normal-length telomeres, while *ter1-Bgl* and *ter1-Kpn* mutants initially produced shorter-than-normal telomeres. Second, the latent period of *ter1*-AA cells before telomere lengthening (>3000 cell divisions), if finite, was minimally several times longer than that of *ter1-Bgl* and *ter1-Kpn* mutants. Third, the telomere lengthening in *ter1*-AA cells was typically more gradual and less extensive than that seen in the other mutants. Finally, the altered telomeric repeats specified by the *ter1*-AA telomerase have a moderately reduced *in vitro* binding affinity for Rap1 protein, while the measured *in vitro* affinity of the mutated repeats of the *ter1-Bgl* and *ter1-Kpn* mutants for Rap1 was normal or slightly elevated (Krauskopf and Blackburn 1996). This is consistent with the fact that the base changes in *ter1*-AA lie within the consensus Rap1p binding site while those of *ter1-Bgl* and *ter1-Kpn* lie outside it (Figure 1). It was shown previously that the degree of immediate telomere elongation caused by the *ter1-Acc* and *ter1-Bsi* mutations *in vivo* correlated with the reduction in binding affinities of Acc and Bsi repeats to Rap1p *in vitro* (Krauskopf and Blackburn 1996). These same studies also found that AA telomeric repeats had a more moderate reduction in the ability to bind Rap1p. The C terminus of Rap1 protein negatively regulates telomere length in both *S. cerevisiae* and *K. lactis* (Conrad *et al.* 1990; Lustig *et al.* 1990; Krauskopf and Blackburn 1996), in part through "counting" the number of Rap1p C termini present at any given telomere (Marcand *et al.* 1997). We propose that the cryptic length regulation defect of *ter1*-AA cells is due to the weakened interaction of Rap1p with AA repeats and that the presence of a minimum number of basal wild-type repeats can prevent telomere elongation from occurring. The properties of *ter1*-AA mutants support the possibility that the delayed elongation phenotypes of *ter1-Bgl* and *ter1-Kpn* stem from an unknown defect in the interaction between the mutant repeats and Rap1p. Possibilities for this defect include effects on the known DNA bending properties of Rap1p (Vignais and Sentenac 1989; Müller *et al.* 1994) or other alteration(s) in the higher-order protein-DNA telomeric complex.

The *TER1-Bcl* allele behaves indistinguishably from wild type: In contrast to the *ter1*-AA mutant, the *TER1-Bcl* mutant was indistinguishable from wild type by several stringent criteria: unchanged telomere length even in very long-term *TER1-Bcl*^{exWT} and *TER1-Bcl*$^{ex\Delta}$ clones, no abnormal colony or cellular phenotypes, no detectable telomeric fusions, and apparently normal telomerase fidelity. In *TER1-Bcl* cells constructed to contain one telomere composed entirely of Bcl repeats (Underwood and McEachern 2001), this telomere also remains wild type in length (our unpublished data), further reinforcing the conclusion that Bcl repeats are completely normal in their length regulation.

A crucial role of telomeres in cells is to prevent chromosome ends from eliciting responses from enzymes involved in the repair of broken DNA ends. Mutational alteration of telomeric sequences might therefore be expected to cause high rates of recombination or end-to-end ligations, two major pathways by which cells are known to repair DNA double-strand breaks. A number of *TER1* template mutants, but not *TER1-Bcl* mutants, are highly prone to one or both of these processes (McEachern *et al.* 2000a; McEachern and Iyer 2001). The fact that telomeres in *TER1-Bcl* mutants cause no abnormal growth phenotype, remain unfused, and undergo normal levels of recombination (McEachern and Iyer 2001) provides strong evidence that telomeres with Bcl repeats provide a wild-type degree of protection for chromosome ends.

Processes besides replicative sequence loss and telomerase-mediated sequence addition at telomeres: The results reported here, together with other short-term studies on altered telomerase template mutants (Yu *et al.* 1990; Singer and Gottschling 1994; McEachern and Blackburn 1995; Prescott and Blackburn 1997b), support the hypothesis that short-term turnover of telomeric repeats is attributable primarily to the combined actions of two processes: sequence addition by telomerase and gradual terminal sequence loss attributable to the failure of DNA polymerases to fully replicate ends. These processes can account for the incorporation of mutant repeats only at telomeric termini soon after replacement of wild-type *TER1* with the phenotypically wild-type *TER1-Bcl*. The relative stability of the more internal part of telomeres was also inferred from work in both *S. cerevisiae* (Wang and Zakian 1990; Forstemann *et al.* 2000) and *Plasmodium falciparum* (Ponzi *et al.* 1992).

We also found evidence for other processes that con-

tribute to telomeric turnover. In wild-type telomeres and also in the very long and deregulated telomeres of *ter1-Bsi* and *ter1-Acc* cells, turnover typically extends into all but the innermost few repeats. During the very protracted growth of *TER1-Bcl* cells, turnover of the original wild-type repeats eventually penetrated into all but the innermost (most basal) 1–5 repeats, *i.e.*, ~125–225 bp further in than the observable lower limit (~10 repeats) of telomere size (see Figure 4). This result cannot be explained solely by the combination of gradual sequence loss and addition by telomerase. If such turnover were the result solely of incomplete replication at DNA ends, failure to elongate the shortest telomeres by telomerase would have to continue for up to 45 consecutive cell divisions, based upon the observed terminal sequence loss rate of 5 bp per cell division in *K. lactis* cells lacking telomerase (McEachern and Blackburn 1995, 1996). Given that short telomeres are the ones most likely to be elongated by telomerase (Marcand *et al.* 1999), it is likely that turnover of the more internal repeats in *TER1-Bcl* cells results from some other, albeit infrequent, process acting at telomeres.

This deep turnover into telomeres must involve both a mechanism for loss of large amounts of telomeric sequence and a mechanism to preserve the sequence of the innermost few repeats. Sizable abrupt shortening of telomeres has been reported previously. During macronuclear development in the ciliated protozoan *Euplotes crassus*, new telomeres are shortened by ~50 bp prior to DNA replication occurring, indicating that nucleolytic cleavage must occur (Vermeesch *et al.* 1993). Dramatic telomere shortening also is observed in *S. cerevisiae* cells constructed to contain some normal-length and some very long telomeres. In this case, long telomeres were sometimes abruptly shortened to wild-type length, a process called telomere rapid deletion (TRD; Li and Lustig 1996). Similarly, telomeres in human cell lines, trypanosomes, and some *K. lactis* mutants with elongated telomeres sometimes undergo deletions too large to be accounted for by gradual replicative sequence loss (Pays *et al.* 1985; Murnane *et al.* 1994; McEachern and Blackburn 1995; Strahl and Blackburn 1996). Multiple mechanisms, including recombination, replicational slippage, and nucleolytic cleavage could potentially produce large deletions of telomeric sequence.

In our mutants, the internal repeats' resistance to turnover could simply be the result of biases in the mechanism generating telomeric deletion. However, it is necessary to explain how long-telomere *ter1-Bsi* and *ter1-Acc* mutants can replace kilobases of telomeric sequence yet specifically retain only their innermost few repeats as wild-type sequences. The mechanism recently suggested to account for TRD could potentially account for our results. It was proposed that the 3′ end of a telomere strand invades into a more internal part of the same telomere and promotes recombinational deletion of the intervening telomeric repeats (Bucholc *et al.* 2001). However, it is not clear whether such a mechanism could be biased to preserve the innermost few repeats.

Another factor that could contribute to the retention of basal wild-type repeats relates to the likelihood that two distinct mechanisms, telomerase and recombinational repair, can repair shortened telomeres. Which mechanism extended a given truncated telomere likely depends upon the number of telomeric repeats. Telomeres retaining enough repeats to efficiently bind proteins required for telomerase function, including Cdc13p and Est1p (Nugent *et al.* 1996; Virta-Pearlman *et al.* 1996), could be readily relengthened by telomerase. In contrast, any telomeres shortened to a greater extent might be less able to bind telomerase and would thus become subject to recombinational repair, the pathway favored in yeast for processing DNA ends lacking telomeric repeats. Also, if the telomere shortening that drives deep turnover occurs as the result of TRD or other recombination events, a newly generated truncated telomere might be better poised to undergo recombinational repair rather than bind telomerase. Some data support the possibility of recombinational repair contributing to telomeric turnover. *K. lactis* telomeres significantly shorter than normal, but still retaining several telomeric repeats, are known to experience greatly elevated gene conversion (McEachern and Iyer 2001). Recombinational repair is also known to be highly efficient in yeast, capable of repairing up to 99% of chromosomes with a double-strand break through gene conversion (Inbar and Kupiec 1999). Severely truncated telomeres, as mimics of double-strand breaks, would presumably be repaired with similar efficiency. Because at least 11 of the 12 *K. lactis* telomeres share substantial subtelomeric homology, a very short or missing telomere could be readily regenerated using a telomere from another chromosome as a template in a gene conversion. Such conversion, initiating as a replication fork that extends to the end of the chromosome has been called break-induced replication or break copy duplication (Malkova *et al.* 1996; Morrow *et al.* 1997; Bosco and Haber 1998). Thus, any greatly shortened telomere could be regenerated by copying another telomere rather than by telomerase, making the innermost wild-type repeats appear resistant to being turned over. Use of another telomere as a template in a gene conversion could explain both the increase in size of the telomeric *Eco*RI + *Bcl*I fragment we observed during passaging of *TER1-Bcl* cells (arrow in Figure 4B) and the apparent loss of one telomere in the doublet of ~2-kb *Eco*RI fragments (Figure 4B).

While the most basal few repeats of *K. lactis* telomeres are generally resistant to turnover even after ~3000 cell divisions, they are unlikely to be stable over an evolutionary time scale. The repeats in basal regions of all the cloned telomeres analyzed to date from a variety of yeast species with different telomeric repeat sequences do not differ in sequence from the rest of the telomere

(McEachern and Blackburn 1994; Cohn *et al.* 1998). Significantly, this has been shown for both *K. lactis* and its close relative *Candida pseudotropicalis*, which has telomeric repeats that differ from those of *K. lactis* at a single position (McEachern and Blackburn 1994; Cohn *et al.* 1998). This suggests that over the very long term, recombination eventually homogenizes all parts of yeast telomeres after the occurrence of a telomerase RNA template mutation. Without exchanges between the inner and outer parts of telomeric repeat arrays, the innermost repeats would be expected to accumulate mutations. Interestingly, in the ciliate *Tetrahymena thermophila*, the innermost tracts of the telomeres of the germline nucleus consist of tandem arrays of a homogeneous repeat variant, TTTGGGG, located next to a shared region of subtelomeric homology (Kirk and Blackburn 1995). This repeat variant differs from the homogeneous TTG GGG repeat sequences known to be templated by the telomerase RNA of this species, suggesting that it is maintained by a nontelomerase-mediated, likely recombinational, mechanism.

In summary, our data suggest that there are multiple mechanisms that contribute to telomeric repeat turnover in *K. lactis* that act with widely different frequencies. Primarily, gradual replicative shortening countered by telomerase addition acts during most or all cell cycles. Second, as identified here, other processes, perhaps including nucleolytic truncation of telomeres, act much less frequently and contribute to turnover of all but the innermost few repeats. Finally, on evolutionary time scales, recombination events may homogenize all repeats of telomeres.

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