Telomere fusions caused by mutating the terminal region of telomeric DNA

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Mutations in the template region of a telomerase RNA gene can lead to the corresponding sequence alterations appearing in newly synthesized telomeric repeats. We analyzed a set of mutations in the template region of the telomerase RNA gene (*TER1*) of the budding yeast *Kluyveromyces lactis* that were predicted to lead to synthesis of mutant telomeric repeats with disrupted binding of the telomeric protein Rap1p. We showed previously that mutating the left side of the 12-bp consensus Rap1p binding site led to immediate and severe telomere elongation. Here, we show that, in contrast, mutating either the right side of the site or both sides together leads initially to telomere shortening. On additional passaging, certain mutants of both categories exhibit telomere–telomere fusions. Often, six new Bal-31-resistant, telomere repeat-containing bands appeared, and we infer that each of the six *K. lactis* chromosomes became circularized. These fusions were not stable, appearing occasionally to resolve and then reform. We demonstrate directly that a linear minichromosome introduced into one of the fusion mutant strains circularized by means of end-to-end fusions of the mutant repeat tracts. In contrast to the chromosomal circularization reported previously in *Schizosaccharomyces pombe* mutants defective in telomere maintenance, the *K. lactis* telomere fusions retained their telomeric DNA repeat sequences.

Telomeres in eukaryotic cells are "capped" to prevent them from potentially resembling double-stranded DNA breaks and eliciting an inappropriate DNA damage repair response (for recent reviews, see refs. 1 and 2). The great majority of eukaryotes have telomeres composed of tandem arrays of a short DNA repeat. These repeats serve as binding sites for proteins that contribute to telomere capping. Additionally, telomeric ends cannot be fully replicated by normal DNA polymerases and require a specialized mechanism to prevent sequence loss from ends. Sequence loss at chromosome ends is counteracted by the action of telomerase, a reverse transcriptase that utilizes part of its constituent RNA subunit as a template to synthesize new telomeric repeats. Sequence addition by telomerase is normally regulated such that total telomere size is kept within defined upper and lower limits.

Loss of telomere function is linked to cellular senescence. Many human somatic cell types grown in culture have little or no telomerase activity, and their telomeres steadily shorten, triggering progressive cellular senescence and crisis. Ectopic expression of the catalytic subunit of telomerase can prevent senescence and crisis (4–6). Human cancer cells are effectively immortalized and frequently display high levels of telomerase (3). Telomere function potentially has other impacts on carcinogenesis in humans. Loss of telomere function has also been associated with the occurrence of telomere fusions in precancerous mammalian cells (7, 8), increasing genomic instability, and thereby potentially promoting cancer progression. Indeed, aged telomerase-deficient mice show elevated rates of spontaneous cancers (9).

How telomeres are capped to prevent them from being degraded or fused has not been fully elucidated. In the fission yeast *Schizosaccharomyces pombe*, deletion of either telomerase or the two ATM kinase homologs *tel1*$^+$ and *rad3*$^+$ caused telomere shortening and led to telomere region fusions that circularized the three chromosomes in these haploid cells (10, 11). All of these fusions had completely lost all their telomeric repeat DNA as well as up to a few kilobases of the adjacent subtelomeric regions.

We investigated the effects of altering telomeric Rap1 binding sites in the budding yeast *Kluyveromyces lactis*. Rap1 is an essential protein that binds yeast telomeric DNA and negatively regulates telomere length. Sites for Rap1 binding are conserved among the telomeric sequences of budding yeasts, despite wide interspecies variation in both the size and sequence of unit telomeric repeats (12). By mutating the template region of the gene encoding the RNA subunit of telomerase, we engineered cells with mutant repeats added to the termini of all telomeres. We previously found that certain telomerase template mutations in *K. lactis* and *Saccharomyces cerevisiae* caused telomeres to become elongated (13, 14). The severity of the *in vivo* length regulation defect correlated with the degree of loss of Rap1 binding affinity to the mutant repeats (14, 15). Here, we report that, in *K. lactis*, terminal telomeric repeats containing specific mutations in the Rap1p consensus binding site cause telomere fusions.

Materials and Methods

Strains and Construction of Mutants. All *K. lactis* cells used in this study are derivatives of the haploid 7B520 (16). Growth of yeast cells was carried out on standard media used for *S. cerevisiae*, either yeast extract/peptone/dextrose (YPD) or synthetic defined (SD) supplemented with appropriate nutrients. Yeast strains were passaged by restreaking individual yeast colonies to single cells and allowing them to form new colonies. Mutations in the *TER1* gene were obtained as described (13). The plasmid loop in/loop out procedure that was used results in a precise replacement of the wild-type *TER1* gene of haploid *K. lactis* cells with the introduced mutation at the native chromosomal location.

Hybridizations. The wild-type *K. lactis* telomeric oligonucleotide (Klac1–25) used in hybridizations has been described (13). This probe was used at 50°C for the hybridizations shown in Figs. 2*A*, 3, and 4 and at 45–48°C for the hybridization in Fig. 2*C*. The wild-type telomeric probe used in Fig. 2*B* is KLAC 11–6: TTAGGTATGTGGTGTACGGAT. It was used at 52°C. The oligonucleotide probe used to detect BsF repeats in Fig. 2*A* was KLBSF (GTGGCGTAGTACGG), which was used at 40°C. The oligonucleotide used to detect both wild-type and BsF repeats in Fig. 2*A* was KLWT (GATTAGGTATGTGG), which was used at 42°C. Hybridizations were carried out with a sodium phos-

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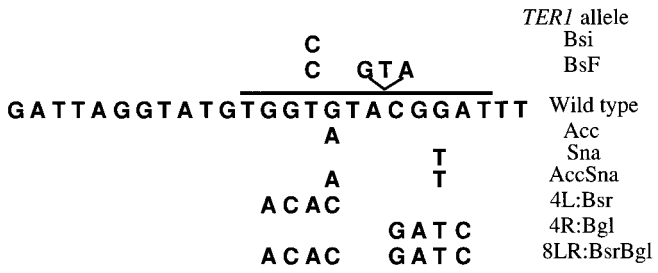

Fig. 1. *TER1* template mutations used in this study. Shown is the 25-nt sequence of the *K. lactis* telomeric repeat (the strand and permutation that is synthesized by telomerase). The overlined nucleotides represent the Rap1 binding site. The BsF mutation was generated from a partial filling in the overhang of the *Bsi*WI site present in the *ter1*-Bsi mutant (13). All other alleles have names that include the restriction enzyme site(s) produced by the mutation. The G to A change of *ter1-AccSna* produces an *Acc*I site whereas the G to T change produces a *Sna*BI site. The latter change, by itself, constitutes the *ter1*-Sna allele.

phate/SDS hybridization buffer (17). Subtelomeric probes were hybridized at 65°C.

Bal31 Nuclease Sensitivity. Bal31 digestions of genomic DNA isolated from yeast cells were done as described (18), using a limiting amount of Bal31 exonuclease and sampling at different time points. Samples were then digested with *Eco*RI before electrophoresis and Southern blotting.

Linear Minichromosome. Construction and use of the pHisLin1 *K. lactis* minichromosome vector will be described elsewhere (D. Underwood, M.M., and E.B., unpublished data). The vector carries *Escherichia coli* plasmid sequences as well as an *S. cerevisiae HIS3* gene (which functions as a selectable marker in *K. lactis*), a *K. lactis* centromere, a *K. lactis* autonomously replicating sequence (ARS), and two appropriately positioned and oriented *K. lactis* telomeres. After a restriction digestion to remove sequences between the ends of the two telomeres, the DNA was electroporated into *K. lactis* cells as a linear molecule.

Results

We analyzed the effects of mutating telomeres in haploid *K. lactis* yeast cells, in which a tandem array of 10–20 perfectly repeated 25-bp telomeric repeats is located at each end of the six chromosomes (20). We constructed a set of 1- to 8-base *ter1* template sequence mutations in conserved positions of the Rap1p binding consensus site located within the telomeric repeat (Fig. 1). We previously showed that, in *K. lactis*, two *ter1* mutations, *ter1*-Bsi and *ter1*-Acc (Fig. 1), which produced telomeric repeats with base substitutions that reduced Rap1 binding, caused immediate and severe telomere elongation (13, 15). A different phenotype was exhibited by the *ter1*-BsF mutant. This contains, in addition to the same T to C base change as the *ter1*-Bsi mutation, a 3-bp insertion in the Rap1 binding site consensus sequence (Fig. 1). Eight independent *ter1*-BsF mutants were grown for 14 serial streaks, and two others were followed for 60 streaks (each streak is ≈20–25 cell divisions). Their telomeres changed over time, as shown by Southern blotting (Fig. 2). Like the *ter1*-Acc and *ter1*-Bsi mutants studied previously, the newly created *ter1*-BsF mutants showed evidence of immediate telomere elongation as well as degradation. This is most clearly seen with the BsF repeat-specific probe [compare, for example, the smear of telomeric hybridization signal in lanes 1 and 2 of Fig. 2*A* (BsF probe) to wild-type telomeres cut with the same enzyme, shown in Fig. 2*B* (WT lane)]. However, *ter1*-BsF mutants differed from the earlier mutants in at least two

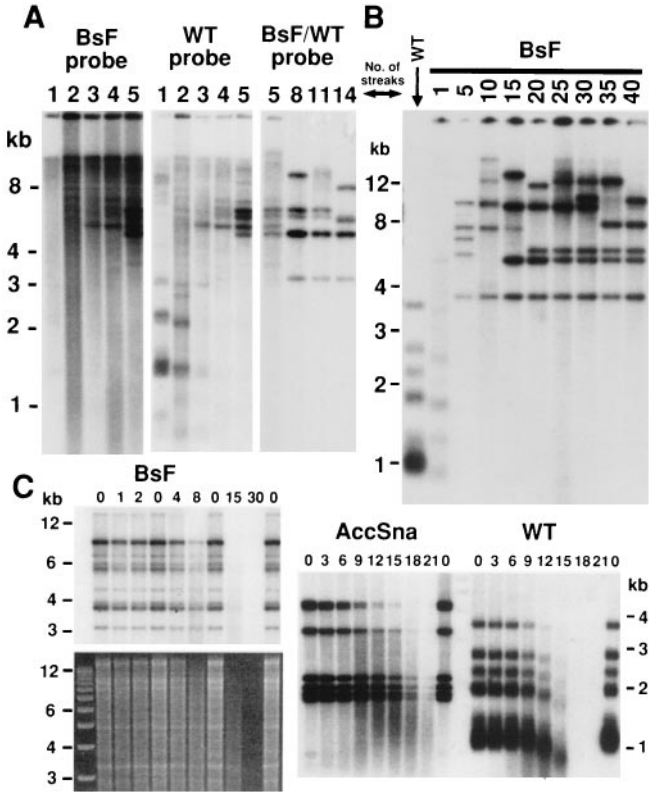

Fig. 2. Telomeric changes in *ter1*-BsF cells. (*A*) Southern blot of *ter1*-BsF mutant followed over 14 serial streaks after its creation (as numbered above lanes). *Eco*RI-digested genomic DNA was first hybridized with a probe specific to BsF mutant repeats (*Left*). After stripping, this filter was rehybridized with a probe specific to wild-type telomeric repeats (*Center*). This detects telomeres that have yet to have BsF repeats added onto them as well as telomeres elongated by the BsF mutant telomerase. Another filter with DNA from streaks 5, 8, 11, and 14 from the same clone probed with an oligonucleotide that binds equally to both wild-type and BsF repeats is shown (*Right*). The telomeric pattern of wild-type cells can be compared from *B*. (*B*) Southern blot showing telomeres of a single clonal lineage of *ter1*-BsF cells followed over 40 serial streaks on rich media. Genomic *K. lactis* DNA was digested with *Eco*RI and probed with a wild-type *K. lactis* telomeric oligonucleotide. DNA from the wild-type parental control is also shown. (*C*) Bal31 exonuclease time course done with DNA from *ter1*-BsF and *ter1*-AccSna mutants after formation of sharp bands carrying telomeric repeats. Numbers above lanes indicate length of Bal31 treatment in minutes. After exonuclease treatment, samples were digested with *Eco*RI before electrophoresis and Southern blotting. Hybridization was with a telomeric probe. Rate of disappearance of telomeric signal paralleled disappearance of total DNA in both mutants as seen on ethidium bromide straining of the gel, whereas disappearance of telomeric signal in wild type preceded complete digestion of total DNA (not shown).

respects. First, early cultures of *ter1*-BsF cells contained a subpopulation of shortened but length-regulated telomeres as well as elongated, highly heterogeneous telomeres. The short telomeres were clearly visualized with a hybridization probe specific to residual wild-type repeats (Fig. 2*A*, lanes 1 and 2, WT probe), but very poorly with the BsF probe, suggesting that they were composed largely of wild-type repeats, whereas the elongated telomeres were composed largely of mutant repeats. This result suggests that mutant repeats are not readily added onto telomeres of wild-type length and sequence, but that once added, loss of length regulation occurs rapidly.

A novel property of the *ter1*-BsF mutant was that, by approximately the fifth streak, the fragments carrying telomeric repeat sequences had shifted from a heterogeneous smear to a collection of several sharp bands of intermediate length (Fig. 2 *A* and

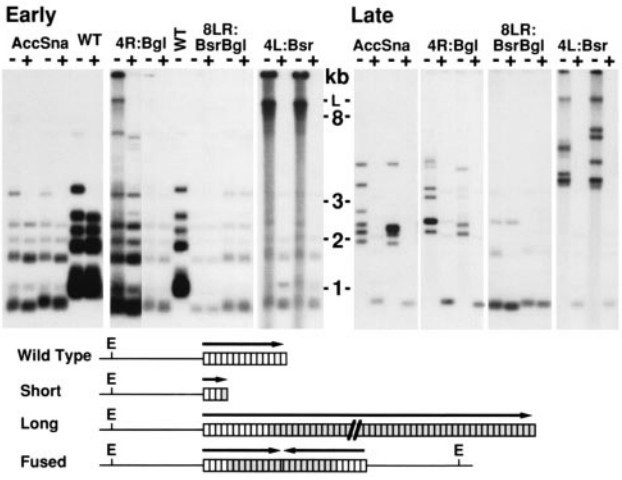

Fig. 3. Telomeric changes in four *TER1* template mutations that disrupt the Rap1 binding site in telomeric repeats. (*Left*) Southern blots of the parental wild-type strain and *ter1* mutants at one streak after their isolation. (*Right*) Southern blots of the same mutants after 20 streaks (except *ter1-AccSna* mutants, which are shown after 32 streaks). Two independent isolates of each mutant are shown. Each isolate is shown digested with *Eco*RI alone (−) or with *Eco*RI plus an enzyme that cleaves off the mutant repeats (+) (*Bgl*II for 8LR:BsrBgl and 4R:Bgl mutants, *Bsr*GI for 4LR:Bsr mutant, and *Acc*I for AccSna mutant). The double digest of the wild-type strain was *Eco*RI plus *Acc*I. Note that *Acc*I cleaves the largest *Eco*RI telomeric fragment at a subtelomeric position. The left clone of ter1–4R:Bgl is shown darkly exposed to show the signal present at limit mobility (''L'') and in the well. Probe used was a telomeric oligonucleotide, Klac1–25. Size markers are shown between panels. Below is a diagram showing the basic structure of telomeres in mutants with short, long, or fused telomeres. White and gray boxes indicate wild-type and mutant telomeric repeats, respectively. Arrows indicate the direction of the telomeric end or, on the fused telomere, the direction of the telomeric ends before fusion. ''E'' indicates the *Eco*RI site nearest the telomere, the actual position of which varies between different telomeres.

B). Hybridization with an oligonucleotide with a sequence common to both wild-type and BsF repeats indicated that, after five streaks, the majority of the telomeric repeats were in these sharp bands (Fig. 2*A*, BsF/WT probe). We tested whether the sharp bands represented fusions between pairs of telomeres by digesting genomic DNA from a *ter1*-BsF mutant with Bal31 exonuclease (Fig. 2*C*). Telomeres differ from nonterminal sequences in two ways in Bal31 digestion experiments: telomeric fragments shorten in unison and digest away more rapidly. We have previously shown that wild-type *K. lactis* telomeres meet both these criteria (Fig. 2*C*, WT) (19). In contrast, in the *ter1*-BsF cells (Fig. 2*C Left*), the sharp bands containing telomeric repeats were not preferentially sensitive to Bal31 by either criterion. This provided strong evidence that the telomeric repeats present in the sharp bands were not chromosome ends but rather represented end-to-end fusions between telomeres. As seen in the long-term passaging of *ter1*-BsF cells (Fig. 2*A*), the pattern of sharp bands was relatively unstable. Loss of a sharp band from one position was typically accompanied by appearance of another sharp band at a new position. However, in at least one case (Fig. 2*B*, 8th, 11th, and 14th streaks, top band), a sharp band was replaced by a diffuse band, which was then lost, and a new sharp band appeared.

A similar appearance of new sharp telomere-hybridizing bands was observed with three of four other *ter1* mutants that disrupt the Rap1 binding site. First, their telomeric patterns shortly after their isolation are shown in Fig. 3 (''Early''). The *ter1*-4L:Bsr mutant, containing a four-base substitution in the left side of the Rap1 site (Fig. 1) showed immediate severe telomere elongation and degradation, like the previously studied *ter1*-Acc and *ter1*-Bsi mutants with single base changes in the same region. However, in the three mutants containing base changes in the right side of the Rap1 site, all or most telomeres were initially shorter than wild type. The *ter1*-Sna mutant, with a single base change in the right side of the Rap1 site (Fig. 1), also produced short telomeres (data not shown). In early cells from the *ter1*-4R:Bgl mutant, some telomeric hybridization signal smeared upward, including DNA accumulating at limit mobility (''L'' in Fig. 3). This suggested that *ter1*-4R:Bgl, like *ter1*-BsF, produces simultaneous telomere shortening and loss of telomere length regulation immediately after replacement of wild-type *TER1*.

After more prolonged passaging (Fig. 3, ''Late''), the AccSna, 4R:Bgl and 4L:Bsr mutants eventually emerged with essentially all telomeric hybridization signal in a series of sharp bands, similar to those observed in the *ter1*-BsF mutant. As before, these bands proved resistant to Bal31 exonuclease (Fig. 2*C*, AccSna; and data not shown), indicating that they were also telomere fusions. This was supported by double digests using *Eco*RI plus the restriction enzyme that cleaved the mutant repeats. Such double digests released restriction fragments that hybridized to a telomeric probe and were similar in size to the wild-type *Eco*RI telomere fragments (correcting for fewer telomeric repeats) (Fig. 3, lanes marked with ''+''; and data not shown). This is the expected result if fusions occurred between two telomeres that contained both wild-type and mutant repeats and had not lost any subtelomeric sequences (see schematic, Fig. 3). In all four independent *ter1*-4L:Bsr mutant clonal lines examined, fusion fragments averaged at least 1 kb longer than the fusions in other mutants, suggesting that they contain larger numbers of mutant telomeric repeats. In sharp contrast to the other mutants shown in Fig. 3, the *ter1*-8LR:BsrBgl mutants produced no telomere fusions; in all three clonal lineages tested, telomeres remained stable at very short lengths even after 20 streaks. Clones of the *ter1*-Sna mutant also displayed no signs of fusions or telomere elongation through at least five streaks, the limit to which they were examined (data not shown).

Sharp, nuclease-resistant bands hybridizing to a telomeric probe were also frequently observed in long-term streaking of the *ter1*-Acc mutant, which initially has only extremely long and degraded telomeres (ref. 13, and data not shown). However, in this mutant, even after extensive passaging, most telomeric signal remained at limit mobility or as a high molecular weight smear, suggesting that free telomeric ends continued to predominate over telomere fusions. High molecular weight telomeric hybridization signal also persisted, although to a lesser extent, in late passage *ter1*-BsF and *ter1*-4L:Bsr cells (Figs. 2*B* and 3, ''Late''). These results suggested that free telomeres persisted, or were regenerated, at a low level in these mutants. The fusion phenotype was recessive, as fusions were not seen in strains carrying single copies of both a wild-type *TER1* and a fusion-forming *ter1* (data not shown).

The patterns of telomere-hybridizing fragments in the telomere fusion mutants were often unstable over time. The degree of instability was similar in the *ter1*-4L:Bsr and *ter1*-BsF mutants (data not shown). The shorter fusion fragments of *ter1*-AccSna were appreciably more stable (Fig. 4 and data not shown), possibly because they had fewer telomeric repeats. Consistent with this interpretation, fusion fragments in *ter1*-BsF and *ter1*-4L:BsrG cells were predominantly smaller than the elongated free telomeric restriction fragments that existed before fusion formation (Figs. 2 and 3).

To test directly whether the telomere fusion mutants caused chromosomes to circularize by telomere–telomere fusions, we used a model minichromosome. We predicted that this minichromosome would become circularized in a telomere fusion mutant (*ter1*-AccSna), but remain linear in wild-type cells. Because the two original terminal telomeric fragments are of

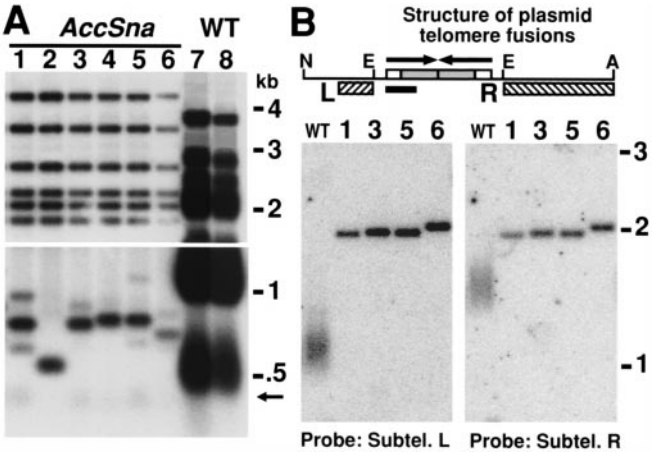

Fig. 4. Formation of telomere fusions after introduction of a linear plasmid. (*A*) A Southern blot hybridized with a telomeric probe of *Eco*RI-digested DNA isolated from the first streak after cells were transformed with the *K. lactis* linear plasmid pHisLin1. The lower part of the gel is shown more darkly exposed. Both telomeres of the linear plasmid run at the same position (≈0.5 kb) in wild-type *TER1* cells (lanes 7 and 8). Six independent small colony transformants of pHisLin1 into *ter1*-AccSna are shown in lanes 1–6. Arrow indicates the position of short, unfused telomeres visible in some of the *ter1*-AccSna transformants. (*B*) Southern blots of *Apa*LI + *Ngo*MIV-digested DNA from some of the same clones after five serial restreaks hybridized with probes unique to either the left or the right subtelomeric region of the transformed linear vector. Numbers above lanes match clone designations from *A*. Diagram above shows the inferred map of a typical telomere fusion. White and gray boxes indicate wild-type and mutant telomeric repeats, respectively. Arrows indicate the direction of the telomeric ends before fusion. Hatched boxes indicate regions used as probes and black bar indicates 200 bp. N, E, and A indicate *Ngo*MIV, *Eco*RI, and *Apa*LI restriction sites.

known size (Fig. 4), circularization would be unambiguously detected from the predicted size range of restriction fragments resulting from their fusion. A linear minichromosome construct (pHisLin1), containing wild-type *K. lactis* telomeres at both ends, was introduced into postfusion *ter1*-AccSna mutant cells, or into control wild-type cells. Introduction of pHisLin1 into both strains produced two size classes of transformant colonies on selection medium. Larger colonies (≈1% of wild-type transformants and ≈10% of *ter1*-AccSna transformants) proved either to lack vector sequences (presumably *HIS3* revertants) or to contain deleted or rearranged forms of the introduced vector (data not shown). Colonies of the more numerous small size class contained the minichromosome. Interestingly, when plated on rich medium not requiring *HIS3* gene expression, these small colonies grew well, suggesting that expression of the *HIS3* marker gene was compromised, perhaps because of its proximity to telomeric repeats, which in *S. cerevisiae* can cause transcriptional silencing (20, 21).

When wild-type cells were transformed, a diffuse new telomere-hybridizing band of ≈0.45–0.7 kb appeared in all eight small colonies analyzed. The size of the new band was that expected for the telomeric fragments from both ends of the minichromosome. This was seen by Southern blotting analysis of *Eco*RI digested whole cell DNA; two examples are shown in Fig. 4, lanes 9 and 10. In contrast, small colonies of *ter1*-AccSna transformants contained a very different group of telomere-hybridizing bands. This was observed in all eight independent colonies analyzed; six are shown in Fig. 4*A*. Directly after their isolation, each clone contained a faint diffuse band at ≈0.35 kb (arrow in Fig. 4). This is the position expected for unfused telomeres that had shortened to the tract length characteristic of early passage *ter1*-AccSna cells (Fig. 3). However, in all clones, most of the minichromosome telomeric signal was in one or more sharp bands between ≈0.55–1.2 kb (Fig. 4*A*, lanes 1–6). These are the predicted results for end-to-end fusions of plasmid telomeres containing variable numbers of telomeric repeats. Because the transformants were analyzed immediately after their isolation, the multiple sharp bands in the samples are likely the results of independent fusion events occurring after transformation but before DNA isolation. Consistent with this interpretation, when clones were grown up from restreaked single cells, they exhibited only one of these sharp bands (Fig. 4*B*, and data not shown). Confirmation of fusions was obtained by hybridizing with probes specific to the sequence adjacent to either the left or right telomere of the linear construct (Fig. 4*B*). In the control wild-type transformants, the left and right minichromosome telomeres were on separate, diffuse fragments (WT lanes, Fig. 4*B*). In contrast, in the *ter1*-AccSna transformants, the left and right telomere probes hybridized to the same longer, sharp fragment (Fig. 4*B*). Finally, without any digestion, the plasmids isolated from *ter1*-AccSna cells ran at a position different from either the linear form of the same plasmid isolated from wild-type cells or chromosomal DNA (data not shown). This result also provided supporting evidence for the formation of circles.

Plasmids containing telomere fusions were recovered from five independent *ter1*-AccSna transformants by transforming uncut DNA purified from whole yeast cells directly into *E. coli*. These circular plasmids had restriction maps consistent with intact recovery of the telomere fusions that existed in the yeast cells (data not shown); specifically, digestion with *Eco*RI plus *Sna*BI (which cleaves the mutant repeats) produced two small fragments, each between 0.2 and 0.4 kb. These matched the sizes expected for the original subtelomeric sequences plus some basal wild-type repeats remaining after the mutant repeats had been cleaved off (data not shown; see diagram in Fig. 4). We estimated that the fusions recovered contain 150–600 bp of mutant repeats. Attempts to sequence across several telomere fusions were unsuccessful but informative. The signal strength of the bands produced in the sequencing reactions dropped sharply at the start of sequence common to both telomeres (subtelomeric sequence ≈120 bp from the start of telomeric repeats). This is the predicted outcome if, on denaturation for sequencing, the inverted repeat structure resulting from fusing the telomeric DNA tracts end-to-end folds into a hairpin that impedes the DNA polymerase used for sequencing. We conclude that the minichromosome-borne telomeres containing basal wild-type repeats and terminal AccSna mutant repeats underwent covalent end-to-end fusions.

The chromosomal telomere fusions in the various *ter1* mutants occurred quite gradually. In the *ter1*-BsF mutant, about five streaks (100–125 cell divisions) occurred before most or all telomeres had fused (Fig. 2 *A* and *B*). After five streaks, most telomeres in the *ter1*-4L:Bsr and *ter1*-4R:Bgl mutants were still unfused, but, by 13 streaks, most were fused (data not shown). In the *ter1*-AccSna mutant, fusions occurred with similar kinetics in one of two clones examined, but not until streaks 20–32 in the other. In contrast, most of the linear minichromosomes were circularized in *ter1*-AccSna cells as soon as DNA could be analyzed (Fig. 4). This more rapid fusion might have been facilitated by the close proximity of the telomeres in the small linear plasmid.

The altered telomeres in all these *ter1* mutants, each of which has a disrupted Rap1 binding site, were associated with colony growth and cell phenotypes. Colonies were rough looking and some or all cells were enlarged, with abnormal morphologies (data not shown). Similar growth defects have been seen in other *ter1* mutants producing extremely elongated telomeres (13, 22, 23) as well as in mutants with very short telomeres (ref. 24, and unpublished observations). Some or all of these growth defects may be attributable to the abnormal or very short

telomeres being mistaken for double-strand breaks and causing cell cycle arrest. In contrast, once all telomeres in these mutants became fused, cells showed little or no mitotic growth abnormalities, although growth remained somewhat slower than wild type (data not shown). A similarly modest effect on cell growth was reported for *S. pombe* cells with circularized chromosomes (10, 11).

Discussion

Here, we report that specific mutations located within the Rap1p binding site of the *K. lactis* telomeric DNA repeats cause high rates of end-to-end fusions between tracts of telomeric DNA. Mutant cells commonly accumulate fusions involving all telomeres in the cell. Many, perhaps all, of the telomere fusions are unstable, appearing to occasionally resolve to free telomeres only to reform later. We also report two phenotypes not seen before in *K. lactis* template mutants: stable but very shortened telomeres, and the temporary coexistence of short telomeres with deregulated, elongated telomeres.

The available evidence is consistent with the telomere fusions in *K. lactis* circularizing the six individual chromosomes. The circularization we observed with a test minichromosome directly supports this possibility. Telomere fusions that circularize essential chromosomes are likely to be selected, because fusing telomeres on two different chromosomes would produce unstable dicentric chromosomes deleterious for cell growth. The gradual accumulation of the telomere fusions in the population of *K. lactis ter1* mutant cells suggests there was ample opportunity for selection of those cells that produced monomeric, monocentric circular chromosomes, even if frequent lethal interchromosomal or interchromatid fusions occurred.

The fusions reported here differ from the situations described in other species. Telomere fusions retaining the telomeric tracts are also caused by overexpression of a dominant negative form of telomere-binding protein TRF2 in human cells (8). The *K. lactis* fusions reported here were produced by abnormal telomeric repeat sequences, which may have affected binding by a yeast functional homolog of TRF2 (see below). However, in the TRF2 mutant human cells, the fusions produced dicentric chromosomes and anaphase bridging, rather than circular chromosomes (8). In the fission yeast *S. pombe*, chromosome end fusions leading to chromosome circularization were produced in mutants that failed to maintain telomeres and showed extreme telomere shortening (10, 11). The molecular nature of the *K. lactis* fused telomeres reported here differ from those in *S. pombe* in at least two respects: the fusions retained telomeric repeats, whereas the *S. pombe* fusions occurred between subtelomeric regions and all telomeric repeats were lost. In addition, the *S. pombe* fusions were typically stable, whereas the *K. lactis* fusions more often were unstable.

Changes in size of telomeric fusion fragments might arise from recombination or replicational slippage. However, the smear of high molecular weight telomeric DNA in mutants with unstable fusions suggests an alternate possibility: that the telomere fusions are occasionally resolved into free telomeres that are then subjected to telomerase addition or truncation by nucleases before refusing. This type of fusion resolution has been previously demonstrated in *S. cerevisiae* cells: two telomeres cloned on a plasmid in head-to-head fashion (essentially an artificial telomere fusion) resolved into free telomeres at a frequency of $\approx 10^{-2}$ per cell division (25). Resolution might occur through the inverted repeat of the telomere fusion, forming a cruciform that is then cleaved by a nuclease. Our results predict that human telomere fusions might also be similarly unstable.

The fusions in *ter1* mutants were not simply correlated with telomere length. Although the long telomeres of immediate lengthening mutants fused, the equally long telomeres of delayed elongation mutants, such as *ter1*-Kpn, did not (ref. 13, and data not shown). Similarly, whereas the short telomere mutants *ter1*-4R:Bgl, *ter1*-4R:Bsr, and *ter1*-AccSna fused, *ter1* strains with comparably short telomeres, including *ter1*-8LR:BsrBgl and senescing *ter1* deletion mutants, did not (Fig. 3; data not shown; and refs. 24 and 26).

The fusion of *K. lactis* telomeres did not require that all repeats of a telomere be mutant, as the fused telomeres still retained basal wild-type repeats to which the terminal mutant repeats had been added. However, the results obtained with the *ter1*-8LR:BsrBgl mutant suggest that a minimum number of mutant repeats needs to be added before a telomere becomes prone to fusion. The *ter1*-8LR:BsrBgl mutation did not cause telomere fusions, although it combined the changes present in the *ter1*-4L:Bsr and *ter1*-4R:Bgl mutations, each of which, by themselves, produced telomere fusions. The *ter1*-8LR:BsrBgl telomerase is active *in vivo*, as indicated by both the stabilization of telomere lengths (albeit at short sizes) and the incorporation of *Bgl*II site(s) at telomeres. However, as judged by digests with and without *Bgl*II (which cleaves mutant repeats; Fig. 3, "Late" panel; compare lowest band in − and + lanes for this mutant), on average, *ter1*-8LR:BsrBgl cells did not contain more than one full mutant repeat (25 bp) per telomere, even after growth for 20 serial streaks. In contrast, all of the *ter1* alleles that caused fusions incorporated mutant repeats onto telomeres to greater extents. The minichromosome fusions occurred between telomeres estimated to contain five or more mutant repeats. Telomere fusions presumably arise through nonhomologous end joining (NHEJ), which is normally used to rejoin double-strand breaks within chromosomes (27). The apparent requirement for greater than one mutant repeat for fusions to occur might reflect NHEJ needing enough sequence free of telomere binding proteins.

Telomere fusion is one manifestation of a telomere capping defect. The fusions reported here contrast sharply with the behavior of telomerase deletion mutants in *K. lactis* and *S. cerevisiae*, which also uncap telomeres as they shorten, leading to growth senescence and greatly increased telomeric recombination rates (refs. 13, 28, and 29; and M.M., S.I., and E.B., unpublished data). Postsenescence survivors of telomerase deletion mutants contain telomeric repeat tracts lengthened by *RAD52*-dependent recombination, but, despite extensive study, they have never shown telomere fusions (24, 30, 31). Thus, the end results of uncapping telomeres strongly depend on the nature of the genetic alteration.

Our data are consistent with the telomere fusions being caused by disruption of binding of a telomeric protein to the most terminal telomeric repeats. Although other possibilities cannot be excluded, our results are consistent with the possibility that disruption of Rap1 binding leads to the observed telomere fusions. None of seven mutations outside the Rap1 binding site that we have analyzed produce fusions (unpublished data). In contrast, all five telomere fusion mutants are predicted to have severely disrupted Rap1p binding to mutant terminal telomeric repeats: four (*ter1*-Acc, *ter1*-AccSna, *ter1*-BsF, and *ter1*-4R:Bg1; Fig. 1) contain at least one specific base change that individually disrupts Rap1 binding *in vitro* (ref. 15; A. Krauskopf and E.B., unpublished data); and at least three of the four base pairs altered in the *ter1*-4L:Bsr mutation are involved in Rap1 binding. In two previously studied *ter1* template mutations, *ter1*-AA and *ter1*-Bsi, the mutant telomeric repeats have only moderately disrupted Rap1 binding *in vitro* (15), but no detectable telomere fusions (refs. 13 and 15; data not shown). Thus, if it is disrupted binding of Rap1 to the terminal repeats that leads to telomere fusions, the disruption may have to be relatively severe.

Yeast telomerase RNA template mutations producing telomeric repeats with weakened Rap1p binding generate varying telomere length outcomes: elongation, shortening (14, 35), and near-normal length (13, 15, 19, 35). As reported here, left side

Rap1 site *ter1*-4L:Bsr and *ter1*-Acc mutants have severely elongated telomeres. This also showed that these mutant repeats are highly competent to be extended by telomerase. This would not be expected if these mutations completely prevented binding by the single-strand telomere binding proteins Est1p or Cdc13p (32–34) or any other protein essential for telomerase action *in vivo*.

In contrast to the left-side mutants, right-side Rap1p site mutants had shortened telomeres. Combining right and left side mutations in cis (*ter1*-8LR:BsrBgl and *ter1*-AccSna mutants) caused telomere shortening to be the dominant phenotype (Fig. 3). Therefore, we propose that the right side of the Rap1 binding site in the telomeric repeat overlaps a second site that functions positively to allow telomerase to act. Loss of Rap1 binding alone (left-side mutation) leads to telomere elongation, whereas interfering with this positive function (right-side mutation) compromises telomerase action, limiting net telomere elongation. The shortened telomeres in right-side mutants could result from defects in telomerase itself. In *Tetrahymena* and *S. cerevisiae* telomerases, certain template mutations interfere with *in vitro* enzymatic function and telomere maintenance *in vivo* (35, 37, 38). However, the *K. lactis* right-side *ter1*-Sna (Fig. 1) has apparently normal *in vitro* telomerase activity (ref. 39; T.B.F. and E.B., unpublished data). Alternatively, these mutant repeats cause telomere shortening by interfering with the binding of the *K. lactis* homologues of Est1p or Cdc13p or other protein(s). By this model, in the short telomere mutants, telomerase addition would not be inhibited until one or more mutant repeats were incorporated onto the telomeric end.

Concurrent disruption of both the ability of telomerase to add new repeats and the ability of those repeats to negatively regulate telomere length might explain the simultaneously existing populations of elongated and shortened telomeres exhibited initially by certain *ter1* and *tlc1* template mutants (this work and ref. 14). A partially crippled telomerase introduced into cells with wild-type telomeres might initially be unable, or poorly able, to add repeats. However, after telomeres shorten from replicative sequence loss, they become more accessible to binding and extension by telomerase (36). Once mutant repeats defective at regulating telomere length were added to a telomere, relatively uncontrolled further addition could ensue. Thus, individual cells might temporarily contain highly elongated telomeres as well as short telomeres that had yet been extended by the mutant telomerase.

Although telomeric DNA sequences are simple tandem repeats, they determine multiple aspects of telomere behavior, including recognition by telomerase, telomere length regulation, and telomere capping properties. Further study of mutants with altered telomeric sequences will be valuable for unraveling the multiple functions of telomeres, and for understanding telomere dysfunction in human cellular senescence and carcinogenesis.

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