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Permalink
https://escholarship.org/uc/item/08r4w3h5

Journal
The Journal of cell biology, 145(2)

ISSN
0021-9525

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Publication Date
1999-04-01

DOI
10.1083/jcb.145.2.203

Peer reviewed
Uncapping and Deregulation of Telomeres Lead to Detrimental Cellular Consequences in Yeast

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Abstract. Telomeres are the protein–nucleic acid structures at the ends of eukaryote chromosomes. Tandem repeats of telomeric DNA are templated by the RNA component (TER1) of the ribonucleoprotein telomerase. These repeats are bound by telomere binding proteins, which are thought to interact with other factors to create a higher-order cap complex that stabilizes the chromosome end. In the budding yeast Kluyveromyces lactis, the incorporation of certain mutant DNA sequences into telomeres leads to uncapping of telomeres, manifested by dramatic telomere elongation and increased length heterogeneity (telomere deregulation). Here we show that telomere deregulation leads to enlarged, misshapen “monster” cells with increased DNA content and apparent defects in cell division. However, such deregulated telomeres became stabilized at their elongated lengths upon addition of only a few functionally wild-type telomeric repeats to their ends, after which the frequency of monster cells decreased to wild-type levels. These results provide evidence for the importance of the most terminal repeats at the telomere in maintaining the cap complex essential for normal telomere function. Analysis of uncapped and capped telomeres also show that it is the deregulation resulting from telomere uncapping, rather than excessive telomere length per se, that is associated with DNA aberrations and morphological defects.

Key words: telomere • telomerase • chromosome aberrations • cell division • Kluyveromyces lactis

Telomeres function to protect chromosomes from incomplete replication (Watson, 1972; Olovnikov, 1973), end-to-end fusions (McClintock, 1941; van Steensel et al., 1998), and loss (Kyrion et al., 1992; Sandell and Zakian, 1993). The G-rich strand of telomeric DNA is templated and synthesized by the cellular ribonucleoprotein telomerase (Greider, 1995). Telomeric DNA is bound by proteins, forming a chromosome-protective cap (reviewed in Grunstein, 1997). It was shown previously that mutation of the Tetrahymena thermophila telomerase RNA gene (TER) results in the addition of mutant telomeric DNA to the chromosome termini, and dramatic cellular phenotypes (Yu et al., 1990; Lee et al., 1993; Romero and Blackburn, 1995). These include blockage of cells in late anaphase with failed chromosomal separation and somatic nuclei containing much greater than normal DNA content (Yu et al., 1990; Lee et al., 1993), suggesting that proper telomere function is important for completing nuclear division and mitosis (Kirk et al., 1997). Cells lacking either the TER gene or telomerase activity experience progressive telomere shortening with each cell division (Lundblad and Szostak, 1989; Counter et al., 1992; Singer and Gottschling, 1994; McEachern and Blackburn, 1995; Bodnar et al., 1998) until telomeric DNA is reduced below a critical length, resulting in chromosome instability and failure of cells to proliferate. These results highlight the requirement for both the presence of telomeres and a minimal telomere length in order to form a functional telomeric cap complex.

In most species, including budding yeasts, telomere length is normally maintained within a narrow size range that is species specific (Walsmey and Petes, 1985; Henderson and Petes, 1992). Although the telomeric “set length” within a yeast strain may be affected by temperature (McEachern and Hicks, 1993), carbon-source, or growth conditions (Blackburn, E.H., unpublished results), length regulation is a robust process that keeps the length of a given telomere within a relatively homogenous, tightly regulated range. In the budding yeasts Saccharomyces cerevisiae and Kluyveromyces lactis, telomere length has been experimentally manipulated by mutation of either telomeric DNA itself (via mutagenesis of the telomerase RNA gene template sequence), the telomerase enzyme, or the protein factors associated with the telomere (Prescott and Blackburn, 1997; Roy et al., 1999). In budding yeasts, the repressor–activator protein (Rap1p) binds duplex telomeric DNA repeats (Berman et al., 1986; Buchman et al., 1988).
A I K . lacticis haploid strains were derived from the parental haploid strain K7B520 that has been previously described (M. E. Achern and Blackburn, 1995). K7B520 was transformed with up to 10–50 ng/μl of pTER-BX plasmid, a Y15 derivative containing the wild-type TER1 gene with described template mutations (Fig. 1a). Cells were electroporated (2.5 kV, 200 μF, 25 μF) and plated on medium lacking uracil and containing 1 M sorbitol. After 2 d, transformants were restreaked onto 5-fluoroorotic acid–containing medium and resistant colonies were screened for the desired gene mutations. At least three independent loop-outs were cultured for each strain. We similarly recapped Acc, Bgl, and Kpn strains with a phenotypically silent marked telomere in combination with a COOH-terminally deleted RAP1 allele experience rapid telomere elongation and colony inviability like that in the Acc mutant (K rauskopf and Blackburn, 1996, 1998). A ddiction of wild-type repeats to the terminal mutant telomeric termini (recapping) converts the telomeric DNA to an unregulated smear to a regulated pattern of discrete bands when visualized by Southern blot analysis. We similarly recapped Acc, Bgl, and Kpn strains with a phenotypically silent marked telomeric repeat to investigate how a wild-type–like distal end affects these mutant telomeres and the cellular phenotypes.

Materials and Methods

Strains

Genomic DNA was prepared from cells grown in yeast extract/peptone/dextrose at 30°C until late log phase. A total of three independent isolates were cultured for each of the uncapped ter1-ACC, ter1-BGL, and ter1-KPN strains. A total of three independent loop-outs were cultured for ter1-BGL recap and ter1-BGL strains. DNA was cut with EcoRI (New England Biolabs Inc.) and the appropriate second restriction enzyme at 37°C overnight.
(47°C for Bcl) and electrophoresed 0.8% agarose, 1× TBE gels at 40 V for 24 h or 1% 0.5× TBE pulsed-field gels at 230 V for 16 h (50 ms pulse time). A first depuration, samples were transferred to Hybond N+ nitrocellulose (Amersham Corp.) and cross-linked with 1200 μl using a Stratalinker 1800 (Stratagene Inc.). H4x2 and 30,000 ungated events were counted in 1 ml PBS. Washed cells were then fixed in 70% ethanol in PBS and diluted to 1 A492 μ/ml (~2 × 107 cells/ml). A 1-ml sample of fixed cells was washed twice with 1 ml PBS-50 g/ml 2,6–diamidinophenylindole (DAPI) and 20 μg of Proteinase K (Boehringer-Mannheim Biochemicals) was added and samples were incubated at 55°C for 1 h. Cells were centrifuged at 5,000 rpm in a microfuge, washed once with 1 ml of PBS, and resuspended in 500 μl of 50 μg/ml propidium iodide (Sigma Chemical Co.) for 1 h at room temperature in the dark. Stained samples were diluted 1:10 in PBS and 30,000 ungated events were counted at 650 nm wavelength on a FacsCalibur® (Becton Dickinson & Co.).

For statistical analyses, the mean of the 2 N peak was measured and the >2.5 N cutoff was calculated using gated plots for each sample. We arbitrarily chose “greater than diploid content” as extending 2.5 N. Statistical t-tests were performed using Microsoft Excel 98. Gated histograms were imported into A dobe Photoshop, where line thickness and gray shade resolution and then cropped in Adobe Photoshop.

**FACS® Analysis**

Triplicate K. lactis cultures were grown to an A600 of 0.5–1. Triplicate cultures from a single stock were used for each uncapped strain, while three independent loop-out strains were used for re-capped and Δter1 strains. Cells were centrifuged at ~5,000 rpm in a clinical centrifuge and washed twice in 1 ml PBS. Washed cells were then fixed in 70% ethanol in PBS and diluted to 1 A492 U/ml (~2 × 107 cells/ml). A 1-ml sample of fixed cells was washed twice with 1 ml PBS. Cells were resuspended in 500 μl PBS + 1 mg/ml RNase A (QIA GEN Inc.) and incubated overnight on a rotating platform at 4°C. A few days later, 20 μg of Proteinase K (Boehringer-Mannheim Biochemicals) was added and samples were incubated at 55°C for 1 h. Cells were centrifuged at 5,000 rpm in a microfuge, washed once with 1 ml of PBS, and resuspended in 500 μl of 50 μg/ml propidium iodide (Sigma Chemical Co.) for 1 h at room temperature in the dark. Stained samples were diluted 1:10 in PBS and 30,000 ungated events were counted at 650 nm wavelength on a FACS Calibur® (Becton Dickinson & Co.).

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**Microscopy**

The same fixed samples used for FACS® analysis (see above) were used for microscopy analyses carried out in parallel. Approximately 1 ml of 70% ethanol/PBS-fixed cells was centrifuged at 5,000 rpm in a microfuge and washed twice with 500 μl of PBS. Cells were stained for 5–15 min at room temperature with 1 μg/ml 2,6-diamidinophenylindole (DAPI) and washed twice with 1 ml of PBS. A total of three isolates of each strain were resuspended in 500 μl of PBS and sonicated for 30–40 s at 30% duty with a sonifier (Branson Ultrasonics Corp.). Microscopy was performed using a Leica DM 1 microscope (Leica Microsystems Inc.) with a 300 dpi color CCD camera. The total number of cells counted for each mutant is noted in Table I. Photographs were scanned at 300 dpi resolution and then cropped in Adobe Photoshop.

**Results**

**DNA Analyses of Strains Containing Uncapped Telomeres**

The telomeric DNA phenotypes of ter1-A cc, ter1-B gl, and ter1-K pn mutants have been reported previously (McEachern and Blackburn, 1995). For the present studies, these findings were confirmed using fresh cultures of the same strains and are summarized in Fig. 1 b. A II three mutations resulted in dramatic telomere deregulation and elongation (Fig. 2 a, lanes 3, 9, and 15, and b, lanes 7, 13, and 20) compared with wild-type (Fig. 2 a, lane 1, and b, lane 1). We define deregulation as the smearing, heterogeneous population of telomeric DNA species identified on these Southern blots. The apparent sizes of the heterogeneous telomeres in these mutant strains ranged from less than the smallest wild-type telomeric restriction fragment to 25 kb. The elongated mutant DNA was largely made up of mutant telomeric DNA repeats, as shown by secondary digestion with each restriction enzyme whose site was cut by telomeric DNA by the mutant telomerase (Fig. 2 a, lanes 4, 10, and 16). A fter cleavage off the mutant repeats, the length of these secondarily digested telomeric fragments reflects the remaining length of the original WT repeat tract that is located internally to the added mutant repeats on the telomere (Fig. 2 c). The size ranges of these internal wild-type repeat tracts were generally similar in all the mutants studied (bracketed area in Fig. 2 a, lanes 1, 4, 7, 10, 13, and 16). While the range of internal telomere sizes in ter1 template mutants was comparable to WT, the individual telomere lengths were slightly shorter than WT after the deregulation following uncapping (Fig. 2 a, compare lane 1 with 4, 10, and 16). In addition, the patterns were different from the WT patterns. This change in the patterns of telomeres has been shown previously to be due to the extensive subtelomeric recombination and has been documented in ter1-A cc and late passage B gl and K pn strains (McEachern and Blackburn, 1995). The 3.5-kb telomeric fragment that lacks subtelomeric homology to the other telomeres (cut by A cc in Fig. 2 a, lanes 4 and 5)

Figure 1. The TER1 template and mutants. (a) The K. lactis telomeric repeat sequence. Grey nucleotides represent the Rap1p binding site. A row indicates base mutations for ter1 mutants, which are named by the unique restriction enzyme site they create in the repeat. (b) Summary of the in vitro Rap1p binding affinity as a percentage of wild-type and the associated telomere length phenotypes of ter1 mutants. Short telomeres are any length shorter than WT. Long telomeres are longer than the longest WT telomere (~3.5 kb).
does not appear to participate in these recombination events (McEachern and Blackburn, 1996). Since the telomeres in these strains are more recombinogenic than WT, individual lines passaged by serial restreaking of single colonies have telomeres patterns that are distinct both from other clonal lineages and the same lineage analyzed at different time points (Fig. 2 a, compare lane 7 with 10, and 13 with 16). In an extreme case, ter1-Bgl recombined all of its homologous subtelomeres into one species (Fig. 2 a, lane 10, lower band). The telomeres of strains that had their TER1 genes deleted for ~50 generations (Δter1) were homogeneous and slightly shorter than WT (Fig. 2 b, lane 5). In the subset of Δter1 cells that survived senescence, the telomere patterns were also significantly altered (Fig. 2 b, lane 6). In summary, while the telomeres of ter1 template mutants were mostly deregulated and elongated,
those of Δter1 survivors were quite short and formed discrete size classes that were regulated. Thus, these short Δter1 telomeres were distinct from the degraded telomeric species observed in the three ter1 template mutant strains.

We used FACS® analysis to investigate the cellular DNA content of ter1 template mutant and Δter1 cell populations (Fig. 3). In the WT K. lactis control strain, 13% of the cells contained DNA in excess of diploid content (Fig. 3 a, black histogram). Similarly, 10–16% of early passage (i.e., ~150 generation) ter1-Bgl and ter1-Kpn cells, which have short well-regulated telomeres (Fig. 2 b, lanes 10 and 17), had greater than diploid DNA content (data not shown). While presenescent Δter1 cells had a DNA content profile similar to WT (data not shown), postsenescent Δter1 survivor cultures with short, relatively homogeneous telomeres (Fig. 2 b, lane 6) exhibited a 27% subpopulation of cells with greater than diploid DNA content (Fig. 3 a, dashed histogram). Likewise, ter1-Acc cells, and late passage (>750 generations) ter1-Bgl and late ter1-Kpn cells, which all had deregulated telomeres, showed 27, 35, and 19% subpopulations of cells with greater than diploid DNA content, respectively (Fig. 3 b and c, black histograms, and data not shown). While the Acc and late passage Bgl mutants were significantly different from WT (both P < 0.01), the variability of the WT slightly decreased the significance of the difference from the late passage Kpn mutant (P = 0.06). The increase in DNA content in the ter1 template sequence mutants and Δter1 survivor strains reproducibly coincided with a decreased percentage of cells with 1 N DNA content (Fig. 3, compare 1 N peak in black histograms with gray histograms in a–c). These DNA content changes are unlikely to be explained by an increase in telomeric DNA alone, since the Δter1 survivors had much less telomeric signal than the ter1 mutants but still exhibited an increased DNA content (Figs. 2 b, lane 6, and 3 a, dashed histogram). Furthermore, even assuming that all 12 telomeres in haploid K. lactis lengthened to an average of 100 kb, this would only represent approximately one tenth of the haploid genome size (Seoighe and Wolfe, 1998). Hence, the increased DNA content of these ter1 mutants was likely to have resulted from either endoreduplication and/or defects in chromosome segregation.

**Microscopic Analyses of ter1 Mutants**

To determine whether DNA segregation was affected in ter1 template sequence mutants, we used fluorescence light microscopy and DAPI staining to examine the cellular DNA, and brightfield microscopy to examine overall cell morphology and cell budding indices (Figs. 4–6, and Table I). We predicted that if DNA segregation were affected, then multiple or large DAPI-staining structures should be visible in a single cell body and some percentage of cells might contain little or no DNA. While WT and presenescent Δter1 cells looked indistinguishable (Fig. 4 a and data not shown), postsenescent Δter1 survivors had a 4% population of somewhat enlarged, misshapen cells with abnormally distributed DNA (Table I). These cells also had very degraded cell walls and collapsed buds, as judged by brightfield microscopy (Fig. 4 b, arrowheads).

We found that 10% of ter1-Acc mutant cells had cellular defects. These were distinctly different from, and more severe than, the most extreme morphological defects of postsenescent Δter1 cells. Many ter1-Acc mutant cells had multiple DAPI-staining structures (Fig. 4, d and e, arrows), while others had no brightly staining DAPI structures but did contain large areas of diffuse DAPI staining (data not shown, see similar phenotypes for Bgl and Kpn
mutants in Figs. 5 d and 6 d). These Acc cells often appeared to have budding and division defects. They were frequently grossly enlarged or elongated (Fig. 4, d and e), and some cells formed chains that were resistant to extensive sonication (Fig. 4 e). Other cells were spherical but enlarged to up to five times the diameter of wild-type cells (Fig. 4 d). We use the term “monster cells” generally to describe these phenotypes, with a given cell needing only to exhibit one of these traits to qualify as a monster cell.

The ter1-Bgl and ter1-Kpn mutations also resulted in monster cell phenotypes, but only in cell populations with deregulated, elongated telomeres. Thus, early passage (≈150 generations) ter1-Bgl and ter1-Kpn strains with short, regulated telomeres showed no significant monster cell phenotypes above background levels (Figs. 5 a and 6 a), while isogenic isolates passaged for >750 generations and, with deregulated telomeres, exhibited high levels of severe monster cell phenotypes. The percentages of monster cells in the populations of late passage ter1-Bgl and ter1-Kpn cell strains were 12 and 13%, respectively (Table I). The same monster cell populations also contained either multiple DAPI-staining structures (Figs. 5 c and 6, c and d, arrows) or decondensed DAPI-staining material (Figs. 5 d and 6 d, arrowheads) and apparent budding and division defects similar to the ter1-Acc mutant cells (Figs. 4 e, 5 c, and 6 c).

Our microscopic analyses highlight the differences between the abnormal phenotypes associated with senes-

Figure 4. (a) DAPI (top) and brightfield (bottom) microscopy for wild-type K. lactis cells. (b) Postsenescent Δter1 survivor strain. (c) Wild-type strains recapped with ter1-Bcl. (d and e) DAPI and brightfield microscopy showing representative ter1-Acc monster cells. Arrows, multiple large DAPI-staining structures within a cell body. (f) ter1-Acc cells recapped with ter1-Bcl. Bars: 10 μM.
cence and monster cells. Although postsenescent Δter1 survivors were phenotypically abnormal, they had irregular, degraded cell walls and collapsed buds unlike those of the ter1 template sequence mutants. Furthermore, postsenescent Δter1 survivors did not exhibit multiple DAPI-staining structures within one cell. Finally, the incidence of monster cells in postsenescent Δter1 populations was at most a third that of other ter1 strains and was considerably more variable between lineages (Table I). In summary, we concluded that telomere uncapping was caused by the Acc, Bgl, or Kpn mutations and resulted in telomere deregulation and elongation. This correlated with a subpopulation of cells containing DNA in excess of diploid amounts and a significantly increased percentage of morphologically aberrant monster cells that were distinct from postsenescent Δter1 survivors. The multiple DAPI-staining structures in all three uncapped ter1 template mutant strains suggested that the cell’s ability to segregate DNA was inversely correlated with the extent of telomere deregulation/elongation.

**Telomere Recapping Restores Length Regulation**

We wished to dissect which property of the uncapped telomeres caused the extreme monster phenotypes described above: deregulation or extreme length. To address this issue, we replaced the mutant ter1 gene in Acc, Bgl, and Kpn strains with a ter1-Bcl allele, which adds phenotypically silent, functionally wild-type, repeats to the telomeric DNA end (McEachern, M.J., and E.H. Blackburn, unpublished...
results; Krauskopf and Blackburn, 1998; Roy et al., 1999). The Bcl repeats contain a BclI restriction enzyme site, so that these added marked repeats can be distinguished from preexisting wild-type or other mutant repeats. The Bcl repeats bind Rap1p normally in vitro and thus were predicted to allow the previously disrupted telomere cap to reform at the distal end of the telomere. In all three mutant ter1 strains studied, recapping with Bcl repeats caused a transition from a deregulated smear of telomeric DNA to a series of discrete, length-regulated but still elongated telomeric bands (Fig. 2b, arrows, compare lanes 7 with 8, 13 with 14, and 20 with 21). This transition occurred within ~50 generations (the earliest time point at which DNA could be analyzed). These reregulated telomeres remained much longer than wild-type (Fig. 2b, lanes 8, 14, and 21), with a significant fraction of the telomere signal still at limit mobility (≥25 kb) for the Bgl and Kpn mutants (Fig. 2b, lanes 14 and 21). Recapping did not significantly change the sizes of the internal wild-type repeat tracts (Fig. 2a, compare lanes 4 with 5, 7 with 8, 10 with 11, 13 with 14, and 16 with 17). Digestion of the cap repeats with BclI revealed that only three to four ter1-Bcl repeats were added to each telomere (Fig. 2b, compare lanes 8 with 9 and 21 with 22). Interestingly, in late ter1-Bgl cells, the Bcl repeats seemed to incorporate further into some late passage telomeres, since digestion of the cap resulted in large decreases in the sizes of some telomere restriction fragments (Fig. 2b, compare lane 14 with 15). The inward migration of these repeats may have been due to faster terminal repeat turnover (Krauskopf and Blackburn, 1998).
or recombination, since isogenic cells passaged for an additional 150 generations exhibited a significantly altered telomere pattern (Fig. 2 b, compare lane 15 with 16).

To determine whether telomeric DNA shortened over all after recapping with Bcl repeats, we performed quantitative analyses of the total telomeric hybridization signal in uncapped and recapped lanes, for all three ter1 template mutants (Fig. 2 b and data not shown). We repeated these analyses using pulse-field gel electrophoresis and compared the total telomeric signals on four chromosomes between uncapped and recapped strains (data not shown). In all cases, there was no significant decrease in telomeric signal after recapping.

In summary, the internal WT repeat tracts of uncapped telomeres in ter1 template mutants shortened only slightly, and were longer than those in post-senescent Δter1 survivors. Recapping added an average of three to four Bcl repeats to the distal tips of telomeres, although in some cases recombination events allowed migration of Bcl repeats further into the telomere. In all cases, however, the recapped ter1 strains regained their ability to regulate telomere length about a new mean size, and the majority of telomeres remained significantly elongated.

**Telomere Recapping Restores Normal Cellular Phenotypes in Mutant ter1 Strains**

The recapped ter1 template mutant strains were examined by FACS® analysis (Fig. 3). After recapping, all three ter1 strains eventually exhibited significantly fewer cells with greater than diploid DNA content. The percentage of recapped ter1-A cc and ter1-Bgl cells with greater than diploid DNA content was the same (7%) as in recapped wild-type cells (Fig. 3, a–c, gray histograms). Interestingly, in ter1-Bgl strains, DNA content did not show an immediate large decrease upon recapping (data not shown). However, ~150 generations after recapping, the fraction of cells with greater than diploid DNA content was reduced to wild-type levels (Fig. 3 c, gray histogram). In contrast, recapped late passage ter1-Kpn strains showed a significant decrease in cells with greater than diploid DNA content as soon as cells could be analyzed (from 19 to 9%; P < 0.001, data not shown).

By the criteria of DAPI staining and brightfield microscopic analyses, the nuclear and cell morphologies of recapped ter1-A cc and late passage ter1-Kpn strains were indistinguishable from wild-type (Figs. 4 f and 6 e, and Table I), even though their telomeres remained very long. The early passage recapped ter1-Bgl and ter1-Kpn strains also had DNA contents and percentages of monster cells comparable to wild-type (data not shown, Figs. 5 b and 6 b, and Table I). Immediately after recapping, the late passage ter1-Bgl strain still exhibited a 9% subpopulation of monster cells (Table I). Qualitatively, these recapped ter1-Bgl cells were not as large or grotesquely malformed as the uncapped ter1-Bgl monster cells (Fig. 5, compare c and d with e). However, ~150 generations after recapping, the percentage of monster cells returned to wild-type levels (Fig. 5 f, and Table I) even though the telomeres in these cells appeared qualitatively similar to those immediately after recapping (Fig. 2 b, compare lanes 15 with 16). Thus, while Southern blot analyses showed that ter1-Bgl repeats had been physically added to the distal ends of telomeres within 50 generations, it took additional time for late passage ter1-Bgl mutants to establish a cell population with functional telomeric caps.

**Discussion**

The DNA–protein complex at the end of telomeres is thought to be important for their chromosome-protective functions. When this distal cap complex is disrupted by adding mutant repeats or shortening the existing chromosome beyond a critical length, the chromosome becomes uncapped and subject to damage. Uncapping can be defined as the loss of end protection and results in either net telomere shortening or lengthening, increased recombination in telomeric regions, and/or the loss of regulation about a mean telomere length. Here we have addressed two questions related to telomere length regulation in K. lactis. First, what are the cellular phenotypic consequences of uncapped telomeres in ter1 template sequence mutants and

### Table I. TER1 and ter1 Budding Indices

|          | Unbudded | Small budded | Large budded | Monster | No. cells |
|----------|----------|--------------|--------------|---------|-----------|
| WT       | 22 ± 7   | 32 ± 2       | 46 ± 8       | 0       | 1,555     |
| recapped | 19 ± 1   | 29 ± 0.6     | 52 ± 0.5     | 0.3 ± 0.6 | 299       |
| Δter1 survivor | 11 ± 4 | 28 ± 3       | 57 ± 2       | 4 ± 3   | 529       |
| ter1-Δcc | 15 ± 0.9 | 18 ± 0.7     | 57 ± 0.1     | 10 ± 0.1 | 701       |
| recapped | 23 ± 2   | 29 ± 5       | 47 ± 3       | 0       | 301       |
| ter1-Bgl | ~150 gen | 18 ± 3       | 29 ± 3       | 49 ± 3  | 3 ± 0.7   | 709       |
|          | recapped | 14 ± 0.7     | 33 ± 1       | 52 ± 1  | 0.3 ± 0.5 | 308       |
|          | >750 gen | 16 ± 4       | 21 ± 0.4     | 51 ± 4  | 12 ± 1    | 703       |
|          | recapped | 8 ± 2        | 29 ± 2       | 54 ± 2  | 9 ± 0.5   | 302       |
|          | recapped +150 gen | 27 ± 3 | 28 ± 3 | 44 ± 2 | 0.9 ± 0.8 | 1,153     |
| ter1-Kpn | ~150 gen | 18 ± 2       | 27 ± 2       | 53 ± 2  | 3 ± 0.2   | 708       |
|          | recapped | 16 ± 3       | 30 ± 0.4     | 53 ± 2  | 0.7 ± 0.6 | 304       |
|          | >750 gen | 14 ± 2       | 20 ± 0.9     | 53 ± 2  | 13 ± 0.9  | 943       |
|          | recapped | 21 ± 1       | 28 ± 2       | 51 ± 0.8| 0.7 ± 0.6 | 302       |

Budding indices of wild-type and ter1 K. lactis strains. Standard deviations are shown. Monster cell populations are in bolded text. gen, generations.
postsenescent Δter1 survivor strains? Second, upon finding that cells respond poorly to telomere uncapping, we asked whether it is the resulting telomere deregulation, as opposed to elongation per se, that is correlated with the observed phenotypes.

This is the first detailed report in yeast of the cellular morphological consequences caused by telomere uncapping. Telomere uncapping in ter1 template sequence mutants was correlated with a greater than diploid DNA content, aberrant nuclear morphologies, and apparent cell division defects. We conclude that it is the deregulation of telomeres resulting from uncapping, rather than their elongation, that is associated with these phenotypes. The addition of a few wild-type-like repeats to the extreme terminus of the elongated mutant ter1 telomeres allowed strains to regain their ability to regulate telomeres, even though the telomeres were up to 100× longer than wild type. Interestingly, the ter1-Bgl mutant telomeres were not fully capped at first and Bcl repeats migrated further into the telomeres than in other mutants. This may have been due to continued degradative shortening of the telomeres followed by de novo Bcl addition or recombination of the Bcl cap with the internal tracts. However, after being recapped for ~150 generations, Bgl mutant strains behaved similarly to the A cc and K pn mutants. Hence, telomere recapping eventually caused the DNA content and cellular morphology to return to normal in all three ter1 mutants.

The mechanism by which the deregulation of uncapped telomeres leads to monster cell formation in K. lactis is not known. While general genomic instability and consequent misregulation of gene expression may result in monster cells, the addition of a wild-type telomeric cap is sufficient for recovery of the cell population. In S. cerevisiae, senescing cells show increased chromosome loss (Landblad and Szostak, 1989). Likewise, elongated, poorly regulated telomeres can increase chromosome loss rates (Kyrion et al., 1992). Telomere uncapping can lead to either telomere shortening (∆ter1) or deregulation/elongation (ter1 template mutants); we have shown here that each has distinct telomere and monster cell phenotypes. The ∆ter1 survivors had cell walls that appeared degraded and they did not show multiple DAPI-staining structures in one cell body. On the other hand, monster cells of ter1 template sequence mutants had healthy-looking cell walls, decondensed chromatin, often up to 10 nucleus-sized DAPI-staining objects in a single cell body, with frequently no DNA in the adjacent body. Evidence supporting DNA segregation or replication defects includes the observation that the DNA content of cultures with elongated, uncapped telomeres was greatly increased. Taken together with the observation of cells with either increased DAPI-staining or no staining and the morphological results, these results strongly suggest that deregulated telomeres can cause DNA missegregation.

A Model for the Effects of Deregulated, Uncapped Telomeres on Chromosome Segregation and Cell Morphology

We propose the following model for how uncapped telomeres may negatively affect cells (Fig. 7). While deletion of ter1 results in telomere shortening until the cap is lost, addition of certain mutant repeats can disrupt the cap without telomere shortening. Mutant repeats that cannot bind Rap1p (i.e., A cc) result in immediate telomere uncapping, while mutant repeats that retain Rap1p binding (Bgl and K pn) do not result in immediate uncapping. The effects of the Bgl and K pn mutations accumulate over time (McAchin and Blackburn, 1995) until some as yet undefined change(s) in the properties of the Rap1p-nucleated complex on the mutant telomeric DNA prevents functional end protection. Uncapped telomeres may over elongate by telomerase-mediated or recombination pathways at this point (McAchin and Blackburn, 1996; K rauskopf and Blackburn, 1998). Such telomeres are also subject to degradation, as shown by the smear of telomeric signal migrating faster than wild-type telomeres (Fig. 2 b, lanes 7, 13, and 20). Uncapped, elongated telomeres may be recognized as damage, causing cell cycle delay or accidental repair/telomeric fusion, resulting in dicentric chromosome formation. Individual chromosomes or whole genomes may be lost or missegregated. This genomic instability results in further negative phenotypic consequences for the cell. Once polyploidy or aneuploidy occurs, strong selec-
tion pressures exist for the healthiest cells, suggesting why the majority of cells in a population are not visually aberrant. However, microcolony analyses of phenotypically wild-type ter1-A cc mutant cells revealed that they continually give rise to subpopulations of monster cells (data not shown).

Recapping reverses the phenotypic effects of telomere deregulation. Reestablishment of a functional cap may occur immediately for the population, as in the cases of the recapped ter1-A cc and ter1-K pn strains, or be slower, as in the case of the late passage ter1-B gl mutant. We propose that recapping involves reforming a stable DNA–protein complex at the telomere ends, preventing these chromosomes from becoming deregulated and exerting detrimental effects. Cells with stably capped telomeres are likely to have a substantial growth advantage, and once a ter1 population is recapped the frequency of unstable monster cells decreases as healthy cells take over the population.

The Importance of the Distal Telomeric Repeats for Cap Formation

The addition of three to four ter1-B cl repeats to the termini of the telomere was sufficient to eventually cap ter1-A cc, B gl, and K pn mutant telomeres. The relatively few B cl repeats that migrated into the B gl telomeres did not appear to have a significant effect on the eventual capping of these telomeres. The telomeres in these recapped strains contain three distinguishable, possibly functional domains: the remaining 250–300-bp internal tract of original wild-type repeats most proximal to the centromere, the adjacent long tract of A cc, B gl, or K pn mutant repeats, which may exceed 25 kb in length, and the (usually) three to four functionally wild-type ter1-B cl repeats at the very terminus of the telomere (Fig. 2 c). Whether the remaining internal wild-type repeats were necessary for the reestablishment of a normal cell population after recapping is unknown. Notably, the total telomeric DNA hybridization signal in elongated ter1 mutants remained unchanged after recapping, providing evidence that recapping is not obligatorily associated with a reduction in mean telomere length. This evidence strongly suggests that it is not telomere length, but the very terminal repeats that are important for preventing monster cell formation.

It is thought that functionally wild-type telomeres assume a higher-order structure nucleated on Rap1p that protects the chromosome end. The COOH terminus of Rap1p interacts with the Sir (Morette et al., 1994) and Rif 1 and 2 (Wotton and Shore, 1997) proteins. Generally, mutations that prevent Rap1p interaction with telomeric DNA (i.e., template mutations), Sirs, and/or Rifs, or COOH-terminal mutations in Rap1p, result in telomere elongation, suggesting that these interactions help stabilize the telomeric complex that regulates telomere length (Kyrion et al., 1992; K rauskopf and Blackburn, 1996). The results reported here also address whether the monster cell phenotypes observed are the pleiotropic effects of changing the amounts of Rap1p or associated factors in the cell. In the case of the A cc mutation, Rap1p binds with significantly lowered affinity in vitro (K rauskopf and Blackburn, 1996), and, therefore, Rap1p occupancy of these repeats in vivo may be low. Although 10-fold less Rap1p is predicted to bind A cc repeats, up to 100× as many repeats may be present at each telomere in ter1-A cc strains. Therefore, the overall Rap1p levels at telomeres may not differ greatly between wild-type and ter1-A cc cells. Nevertheless, the structure of their telomeric complexes are likely distinct. In contrast, both B gl and K pn mutant repeats have normal Rap1p binding affinity in vitro and upon elongation could potentially titrate Rap1p, along with interacting proteins, away from the scores of genes they regulate. Y et the functionally recapped ter1-A cc, ter1-B gl, and ter1-K pn strains all have as much telomeric DNA as uncapped strains and appear as healthy as wild type. Therefore, it is unlikely that titration of Rap1p explains the phenotypes associated with monster cells. It is possible that the uncapped ter1 mutants are unable to regulate the single-stranded ends of the telomere and are therefore unable to regulate length. The A cc, B gl, and K pn mutations may affect the interaction of putative end-binding factors, such as K. lactis homologues of the Cdc13p, Est1p, or Snt1p proteins found in S. cerevisiae (Nugent et al., 1996; V irta-Pearlman et al., 1996; Grandin et al., 1997). If these ter1 mutant repeats were incapable of binding such end factors normally, this could expose the terminal region of the telomere to factors such as recombination-associated activities, including degradation enzymes.

A functional cap complex at the telomere ends appears to be important in other organisms besides budding yeasts. Mutations in the mammalian telomere binding proteins TRF1 and TRF2 have been shown to result in varying degrees of telomere lengthening and chromosome fusions, respectively (Bianchi et al., 1997; van Steensel et al., 1998). In Schizosaccharomyces pombe, the telomere binding protein Taz1p has been shown to be important in telomere length control (Cooper et al., 1997). A dittonally, mutations in Taz1p that result in improper meiotic segregation, defects in telomere clustering, and low spore viability may reflect failure to form a functional cap (Cooper et al., 1998; Nimmo et al., 1998). Understanding the role of capping in telomere function will likely be useful in understanding the roles of telomeres in cell viability and division control.

We thank Mike McE achern for his generous gift of uncapped mutant ter1 template sequence strains, the pTER-BX capping plasmid, and sharing unpublished results, and A nat K rauskopf and M ike McE achern for many fruitful discussions. We also thank Simon Chan, Sandy J ohson, A ndrew M urray, and T hea T isty for critical review of the manuscript, A ndrew M urray’s lab for use of their microscope, and Rudi Grosschedl’s lab for use of their FacsCalibur®.

This work was funded by National Institutes of Health (NIH) grants GM 26259 to E.H. Blackburn, a National Science Foundation Graduate Fellowship to C.D. Smith and NIH training grant T32CA 09270.

Received for publication 25 September 1998 and in revised form 4 March 1999.

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