

Telomeric circles are abundant in the *stn1-M1* mutant that maintains its telomeres through recombination

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ABSTRACT

Some human cancers maintain their telomeres using the alternative lengthening of telomeres (ALT) mechanism; a process thought to involve recombination. Different types of recombinational telomere elongation pathways have been identified in yeasts. In senescing yeast telomerase deletion (*ter1-Δ*) mutants with very short telomeres, it has been hypothesized that copying a tiny telomeric circle (t-circle) by a rolling circle mechanism is the key event in telomere elongation. In other cases more closely resembling ALT cells, such as the *stn1-M1* mutant of *Kluyveromyces lactis*, the telomeres appear to be continuously unstable and routinely reach very large sizes. By employing two-dimensional gel electrophoresis and electron microscopy, we show that *stn1-M1* cells contain abundant double stranded t-circles ranging from ~100 to 30 000 bp in size. We also observed small single-stranded t-circles, specifically composed of the G-rich telomeric strand and tailed circles resembling rolling circle replication intermediates. The t-circles most likely arose from recombination events that also resulted in telomere truncations. The findings strengthen the possibility that t-circles contribute to telomere maintenance in *stn1-M1* and ALT cells.

INTRODUCTION

Telomeres protect chromosome ends from nucleolytic degradation, end-to-end fusion and other processes that

could compromise genome integrity (1). They are composed of tandem short DNA repeats and the specialized proteins that bind them (2). Telomeres are normally maintained by the enzyme telomerase that compensates for gradual sequence loss due to incomplete DNA replication by adding telomeric repeats onto chromosome termini. In most human somatic cells, telomerase activity is very low (3,4). This leads to gradual telomere shortening which, in turn, can trigger replicative senescence, a process where a cell with critically short telomeres permanently exits from the cycle of division (5,6). In contrast, the great majority of cancers are able to maintain their telomere lengths indefinitely. In most cases, this occurs because of an up-regulation of telomerase activity (7). However, some cancers maintain their telomere lengths through a telomerase-independent process termed alternative lengthening of telomeres (ALT) (8). The telomeres in ALT cells are highly heterogeneous, often extremely long and appear to be maintained through homologous recombination (9).

Much of what is known about recombinational telomere elongation (RTE) comes from studies in yeast, particularly *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Yeast mutants lacking telomerase undergo growth senescence and most cells eventually die (10,11). The cells that survive senescence are found to have lengthened telomeres through a process dependent upon *RAD52* and other genes involved in homologous recombination (12–14). Recombination in and near telomeres is greatly increased when the telomeres become short (15,16). Work in both *K. lactis* and *S. cerevisiae* has suggested that RTE lengthens telomeric repeat arrays (Type II RTE) through a ‘roll and spread’ mechanism (15,17–19). According to this model, a small duplex DNA circle consisting of telomeric repeats (t-circle), formed by recombination in cells with

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critically short telomeres, is used as a template for extending at least one telomere, through a rolling circle copying event. Once one long telomere is formed, other telomeres become extended by copying its sequence. Appreciable evidence supports this model. We have shown that the sequence of a single long telomere is preferentially copied to all other telomeres during survivor formation (15). Cells transformed with telomeric circles (t-circles) routinely acquire telomeres extended by tandem copies of the sequence of the transformed circle (17,18). In addition, t-circles are abundant in at least some types of cells with dysfunctional telomeres including human ALT cells and a *K. lactis* mutant with altered telomeric repeats (20–22). For recent reviews of RTE and t-circles see references 23 and 24.

It has recently been shown that certain *K. lactis* mutant cells display a form of RTE that is distinct from the RTE that occurs in *ter1-Δ* mutants. The *stn1-M1* mutant causes an amino acid substitution in Stn1, a protein that forms a complex with Cdc13 and Ten1 in *S. cerevisiae*. Stn1 was shown to bind G-rich telomeric substrates as well as to provide an essential protective function at the telomeres (25,26). This mutation displays moderate growth defects and leads to the rapid formation of very long and heterogeneous telomeres. Unlike a *ter1-Δ* mutant that appears to have a telomere-capping defect only when telomeres become very short, the *stn1-M1* mutant has a continuous capping defect that is independent of telomere length. Other examples of this type of RTE, now known as Type IIR (runaway) RTE, have been seen in *K. lactis* telomerase RNA mutants, which generate mutant telomeric repeats (15). The most notable of these appear to be due to alterations in the telomeric repeat that reduce the binding affinity of the double strand telomere-binding protein Rap1 (27). Certain *S. cerevisiae* *cdc13* and *stn1* mutants provide similar examples of RTE independent of the length of the telomeres (28–30). The features of yeast Type IIR RTE are particularly similar to those observed in human ALT cells. Learning more about how telomere maintenance occurs in *stn1-M1* cells is, therefore, of considerable interest. Whether t-circles contribute to either the formation or the maintenance of the long telomeres of Type IIR RTE or of ALT cells is currently unknown. In this study, we show that a broad range of sizes of t-circles is produced in the *stn1-M1* mutant.

MATERIALS AND METHODS

Yeast strains

The strain 7B520 (*ura3-1 his2-2 trp1*) was described previously (31). The *stn1-M1* and *stn1-M1 ter1-Δ* strains used here were also described previously (32). All strains were routinely grown at 30°C.

DNA isolation

Genomic DNA used to generate the telomere restriction fragments separated by one- or two-dimensional (1D or 2D) gel electrophoresis was isolated from 96 (1.5 ml) YPD liquid overnight cultures. Low molecular weight

extrachromosomal telomeric DNA for electron microscopy examination was treated with RNA at 37°C for an hour and then isolated by running uncut genomic DNA on 0.8% agarose gels at 90V for 60 min. DNA migrating between 500 and 3500 bp linear markers was excised from the gel and electro-eluted onto 12000 to 14000 MWCO Spectra/Por dialysis tubing (Spectrum Laboratories Incorporated, Ranch Dominguez, CA). Electro-eluted DNA was subsequently concentrated using microcon YM-10 spin columns as directed by manufacturer (Amicon Bioseparations, Raleigh, NC). High molecular weight DNA for electron microscopy (EM) examination was attained by spheroplasting and isolating nuclei as described previously (33) with the following modification: the lytic enzyme used was 100 μg/ml Zymolyase 100T (Seikagaku).

Southern and in-gel hybridizations

For 1D gel electrophoresis, *EcoRI* (NEB Beverly, MA) digested or undigested genomic DNA was separated on a 0.8% SeaKem LE agarose gel (Lonza, Rockland Inc., Rockland, ME). For 2D gel analysis of low molecular weight DNA, uncut, RNase-treated genomic DNA was separated in a 4% NuSieve 3:1 agarose gel initially containing 0.6 μg/ml chloroquine (Lonza, Rockland, ME) as previously described (20). In the above experiments, the gels were blotted onto Hybond N+ membrane and probed with either K1ac1-25 G-strand telomeric probe (5'-ACGGATTTGATTAGGTATGTGGTGT-3') or the K1ac-25-1 C-strand telomeric probe (3'-ACACCACATA CCTAATCAAATCCGT-5'). All hybridizations were carried out in the presence of 500 mM Na₂HPO₄ and 7% sodium dodecyl sulfate (SDS) and the washes were done in 100 mM Na₂HPO₄ and 2% SDS. For 2D gel electrophoresis of high molecular weight DNA, *EcoRI*-digested DNA was separated along with 50 ng of circularized *HindIII* λ fragments, as described previously (33). The gel was blotted, probed with ³²P-labelled *K. lactis* telomeric C-strand and then subsequently probed with ³²P-labelled *HindIII* λ fragments.

Electron microscopy

Low molecular weight gel-extracted DNA from *stn1-M1* and *stn1-M1 ter1-Δ* cells was incubated with 20 μg/ml T4 gene 32 protein (gift of Nancy Nossal, NIH, Bethesda, MD) for 5 min in a buffer containing 10 mM HEPES pH 7.5 and 1 mM EDTA. The samples were treated with 0.6% glutaraldehyde on ice for 10 min and chromatographed over a 2.5 ml BioGel A-1.5M column (Bio-Rad, Hercules, CA). Fractions containing DNA and DNA-protein complexes were prepared for EM by absorption onto negatively charged carbon-coated grids in the presence of spermidine followed by dehydration through a series of graded ethanol washes, air drying and rotary shadowcasting with tungsten at 1 × 10⁻⁶ Torr (33,34). For examination of high molecular weight telomeric DNA, isolated genomic DNA was digested with *AluI*, *HpaII* and *NlaIII* (NEB Beverly, MA), at enzyme concentration of 1 U/mg for 2 h, and then supplemented with an equal amount of each enzyme for an additional 2 h.

The telomere restriction fragments were then separated by size exclusion chromatography and the eluted fractions monitored for DNA concentration and telomeric DNA abundance. Telomere-enriched fractions were prepared for EM by surface spreading on a denatured protein film (33,35). Samples were examined on an FEI Tecnai 12 instrument (Hillsboro, OR). Images were captured using a Gatan Ultrascan US4000SP digital camera (Gatan, Pleasanton, CA) and molecule dimensions determined using Gatan Digital Micrograph 3.0 software. Images for publication were captured on sheet film, and digitized using ACT-1 software (Nikon, Tokyo, Japan) and a Nikon SMZ1000 stereoscope. Brightness and contrast were adjusted using Adobe Photoshop (Adobe Systems, San Jose, CA).

RESULTS

Detection of t-circles in the *stn1-M1* mutant by 2D gel electrophoresis

The *stn1-M1* mutant generates extremely long telomeres independently of telomerase using Type IIR RTE (Figure 1A) (32). Some of the telomeric hybridization

signal seen in uncut genomic DNA from the *stn1-M1* and *stn1-M1 ter1-Δ* cells (the latter deleted for the telomerase RNA gene) appeared throughout the length of the gel. This indicated that some of the smear of telomeric signal, particularly at low molecular weights, likely represented extrachromosomal telomeric sequences. Similar smears largely composed of double- and single-stranded t-circles that hybridized to telomere probes were seen in the long telomere mutant *ter1-16T* (21). Therefore, we hypothesized that t-circles were being produced in *stn1-M1* cells. As a test of this, we electrophoresed uncut genomic DNA from *stn1-M1* and *stn1-M1 ter1-Δ* cells on 4% agarose 2D chloroquine gels to separate low molecular weight t-circles. Filters blotted from these gels were hybridized to oligonucleotide probes matching sequence from either the G-rich or the C-rich strands of *K. lactis* telomeric DNA (Figure 1B and C). With a G-strand telomeric probe (left panels of Figure 1B and C), we observed similar diagonals of closely spaced diffuse spots in both types of cells. With a C-strand telomeric probe (right panels of Figure 1B and C), we observed the same diagonal plus an additional arc of spots. These results are very similar to the ladders

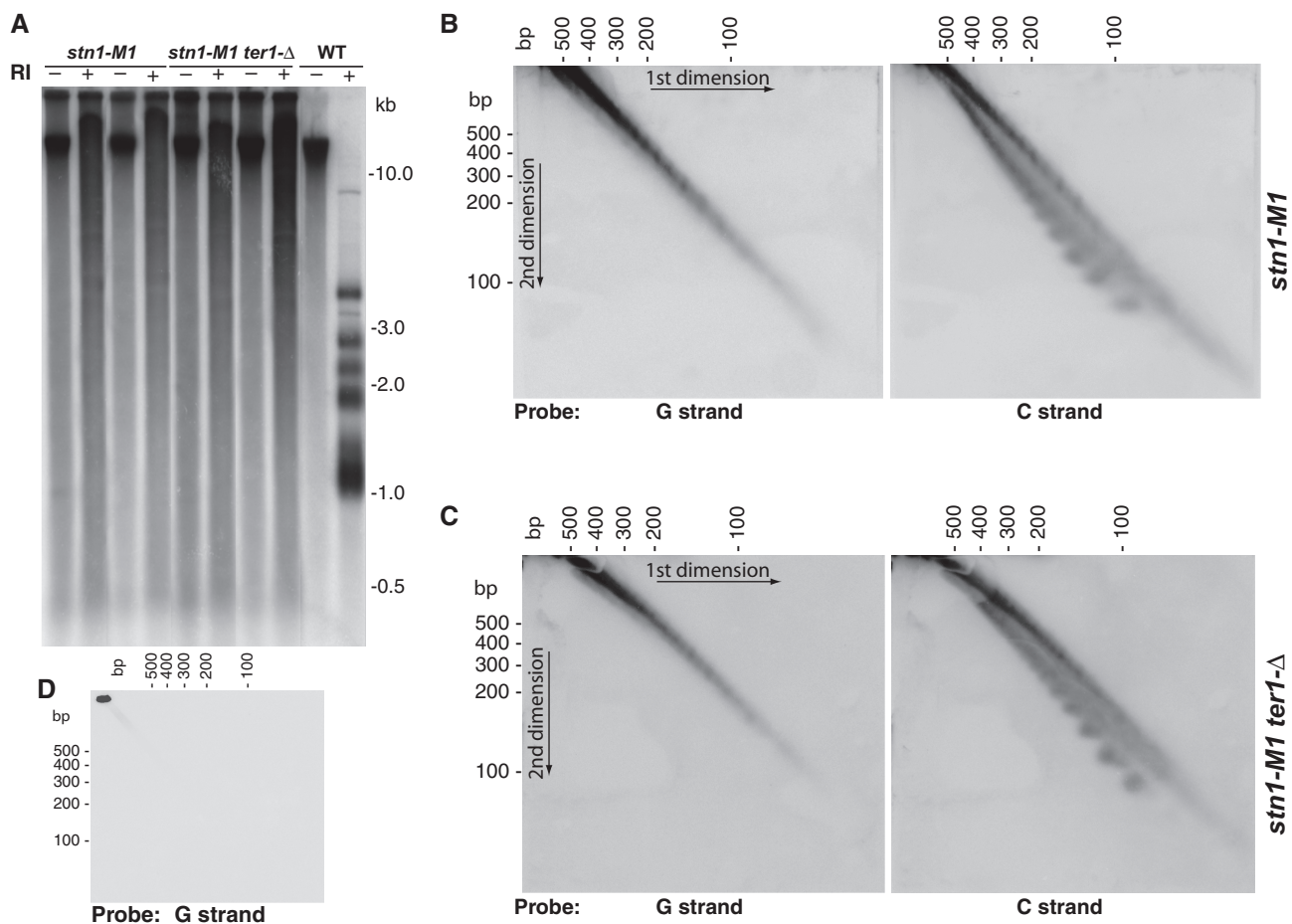


Figure 1. Gel analysis of low molecular weight telomeric DNA from *stn1-M1* and *stn1-M1 ter1-Δ* cells. (A) Southern blot of uncut and *EcoRI*-digested genomic DNA from wild type (strain 7B520), *stn1-M1* and *stn1-M1 ter1-Δ* run on a 1D 0.8% agarose gel and hybridized to a telomeric probe. (B–C) Southern blots of uncut genomic DNA from *stn1-M1* and *stn1-M1 ter1-Δ* run on 2D 4% agarose gels hybridized to either the C-strand or G-strand telomeric probes. (D) Southern blot of uncut genomic DNA from a wild-type control hybridized to G-strand telomeric probe.

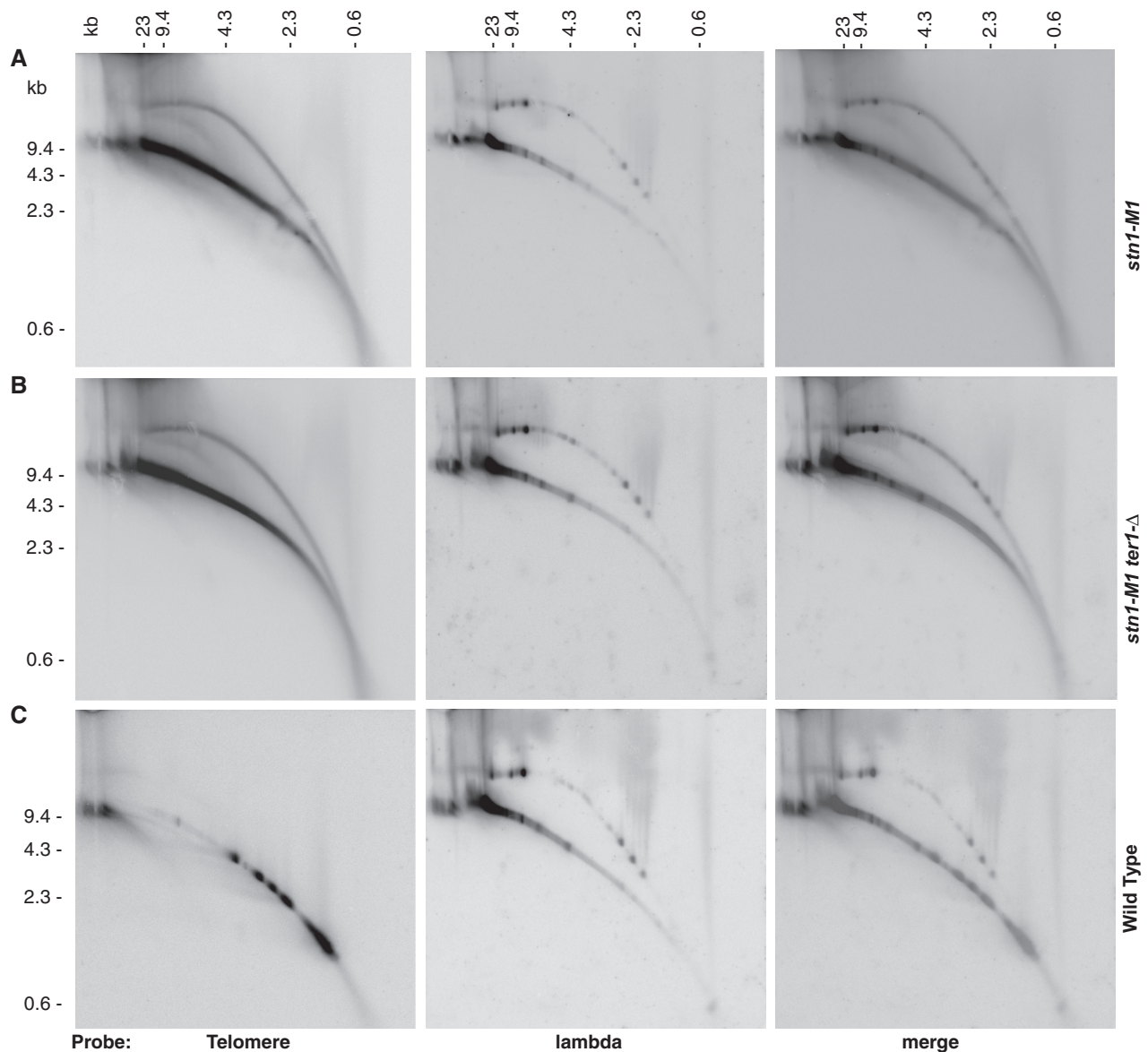


Figure 2. Two-dimensional gel electrophoresis of high molecular weight telomeric DNA from *stn1-M1* and *stn1-M1 ter1-Δ* cells. *EcoRI*-digested DNA (8 μ g) from *stn1-M1* (A), *stn1-M1 ter1-Δ* (B), and wild type (7B520) (C) cells along with 50 ng of ligated phage λ *HindIII* fragments were separated on low percentage 2D agarose gels. The main, lower arc in each panel represents linear DNA while the upper arc present in most panels represents open circular form DNA.

formed from double- and single-stranded t-circles that were observed previously in DNA from *ter1-16T* cells (21). The ladders of spots represented DNA species containing different integral numbers of the 25 bp *K. lactis* telomeric repeat. Our results suggested that *stn1-M1* and *stn1-M1 ter1-Δ* cells, like *ter1-16T*, produce very small double-stranded t-circles as well as very small single-stranded t-circles that were composed specifically of the G-rich strand of telomeric sequence. As expected, wild-type cells produced no observable low molecular weight telomeric spots (Figure 1D).

We next separated *EcoRI*-digested genomic DNA from *stn1-M1*, *stn1-M1 ter1-Δ* and a wild-type control on low percentage agarose 2D gels (first dimension in 0.6% agarose gel and 1.1% agarose in second dimension) to

test for the possible presence of high molecular weight t-circles. *EcoRI* does not cleave *K. lactis* telomeric DNA but does separate the rest of the genomic DNA into a variety of sizes that can be visualized on gels. *HindIII* fragments of phage λ DNA were ligated into circles and separated along with the *EcoRI*-digested genomic DNA as a circular DNA control. The results of this experiment showed that a significant proportion of signal produced from a telomeric probe in both *stn1-M1* and *stn1-M1 ter1-Δ* cells was present in an arc migrating with the double-stranded relaxed circle controls (Figure 2A and B). This was not observed with DNA from wild-type cells (Figure 2C). We conclude that *stn1-M1* and *stn1-M1 ter1-Δ* cells contain abundant high molecular weight t-circles.

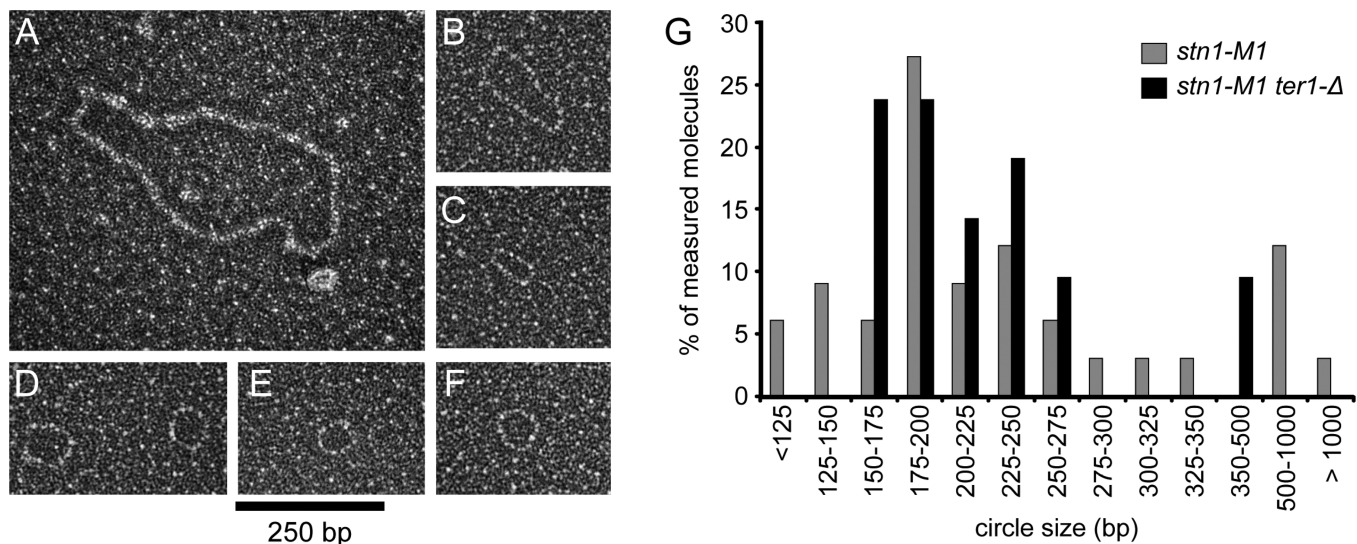


Figure 3. Electron microscopy visualization of low molecular weight DNA circles from *stn1-M1* and *stn1-M1 ter1-Δ* cells. (A–F) Electron micrographs of low molecular weight double-stranded t-circles. Estimated circle sizes are 1320, 428, 191, 216 and 258, 187 and 201 bp, respectively, for A–F. Samples were mounted onto thin carbon foils, shadowcasted with tungsten and are shown in negative contrast. Bar is equivalent to 250 bp. (G) Size distribution of observed DNA circles from *stn1-M1* and *stn1-M1 ter1-Δ*. Measured double-stranded circles observed in low molecular weight extrachromosomal DNA isolated from *stn1-M1* ($n = 33$) and *stn1-M1 ter1-Δ* ($n = 21$) cells.

Visualization of t-circles by electron microscopy

To confirm the presence of small t-circles in *stn1-M1*, we examined extrachromosomal telomeric DNA from *stn1-M1* and *stn1-M1 ter1-Δ* by EM. Undigested samples of *stn1-M1* and *stn1-M1 ter1-Δ* genomic DNA were separated on a 0.8% agarose gel and DNA running between 500 and 3500 bp (relative to linear DNA markers) was extracted. Once purified, this DNA was incubated with T4 gene 32 single strand DNA-binding protein and visualized by EM (Figure 3A–F). In both the *stn1-M1* and *stn1-M1 ter1-Δ* samples, we observed a high percentage of the DNA molecules to be circular. Scoring DNAs by EM, $19.5\% \pm 7.8\%$ were circular in the *stn1-M1* sample and $30.0\% \pm 6.8\%$ were circular in the *stn1-M1 ter1-Δ* sample. Surprisingly, $<2\%$ of the circles were single-stranded as judged by gp32 binding. Double-stranded circles lengths from the *stn1-M1* and *stn1-M1 ter1-Δ* samples were measured and the results are shown in Figure 3G. Nearly all of the circles observed were less than 1 kb and most measured between 175 and 300 bp. This is very similar to the size distribution of t-circles observed by EM in DNA isolated from *ter1-16T* cells using the same protocol (21). The reason why single-stranded circles were much less common in this experiment compared to the 4% agarose 2D gels is not clear.

In addition to fully circular DNA molecules, we also visualized a small number of double-stranded DNA circles with tails of varying sizes, examples of which are shown in Figure 4. These structures represented less than 3.5% of the total number of molecules visualized in the *stn1-M1* samples. In a few instances, these molecules were bound by T4 gene 32 protein at the base of the tail indicating the presence of a small region of single-stranded DNA at the circle–tail junction (Figure 4C).

These molecules conceivably represent intermediates in the formation or processing of t-circles, or circles undergoing rolling-circle replication.

To visualize the large t-circles by EM, high molecular weight telomere restriction fragments from *stn1-M1* cells were enriched by gel-exclusion chromatography, as described previously (21,33). Briefly, crude nuclei from *stn1-M1* cells were isolated and total genomic DNA was digested with *AluI*, *HpaII* and *NlaIII* restriction enzymes. This reduces the genomic DNA to very small sizes while leaving telomeric repeat tracts intact. The digested genomic DNA was then separated in a long gel-filtration chromatography column, and the eluted fractions were assayed for total and telomeric DNA content (Figure 5F). The fractions highly enriched for telomeric DNA were concentrated and examined by EM (34). In the telomere-enriched fractions, we observed a large number of t-circles ranging in size from 0.3 to 31 kb (Figure 5A–E). The majority of circles were small, with 73% of the circles from the telomeric-enriched fractions measuring less than 3 kb in total length, and 11.5% measuring over 10 kb ($n = 61$) (Figure 5G). These results are similar to previous experiments in human ALT cells where the bulk of circular molecules were rather small compared to the average telomere size (20).

DISCUSSION

The results presented here demonstrate that a broad size range of t-circles, from ~ 100 to >30 kb, are produced in the *K. lactis stn1-M1* mutant that maintains its telomeres using Type IIR RTE. Both small and large t-circles are formed independently of the presence of telomerase in the mutant cells. As telomeric DNA lacks the ability

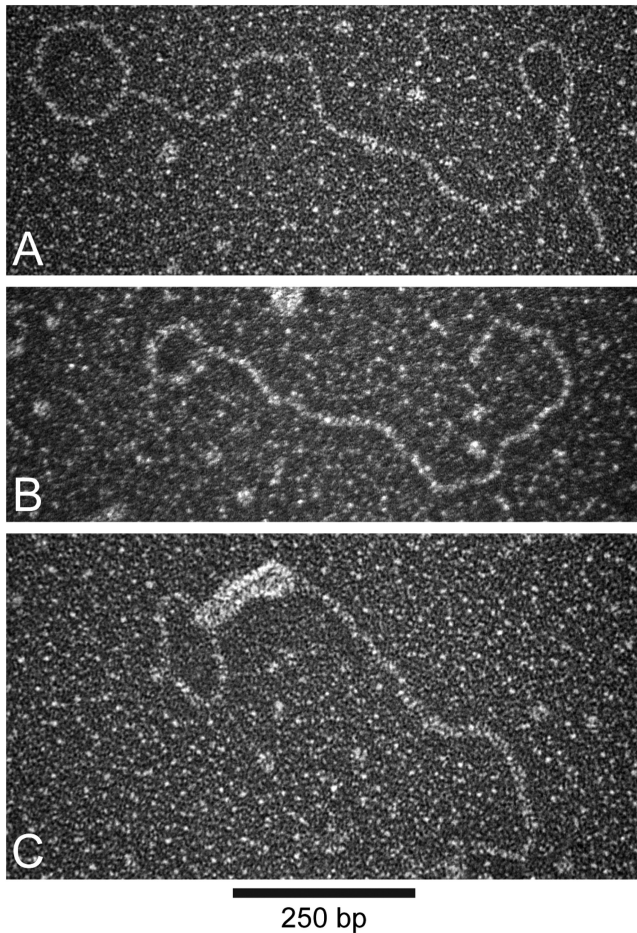


Figure 4. Visualization of tailed circles from *stn1-M1* and *stn1-M1 ter1-Δ* mutant cells. (A–B) Electron micrographs of tailed circle DNA structures. Circular and tail portions of molecules shown in A–B are estimated at 439 and 1361, and 252 and 918 bp, respectively. (C) Electron micrograph of a tailed circle structure with a ds and ss DNA tail. The DNA was incubated with T4 gene 32 protein, glutaraldehyde, crosslinked and prepared for EM as in Figure 3. The loop portion of the molecule shown in C is 400 bp and the total length of the single-stranded and double-stranded tail is equivalent to 796 bp. Bar is equivalent to 250 bp.

to initiate replication, the t-circles present in *stn1-M1* cells must be recent products of recombination rather than heritably replicating episomes. Because *stn1-M1 rad52* cells are inviable (32), it has not been possible to directly demonstrate that homologous recombination is required for formation of t-circles in these cells. However, t-circles in other systems, including *K. lactis ter1-16T* cells, have previously been demonstrated to depend upon Rad52 or other homologous recombination genes (19,21,22,36). The presence of the small t-circles from *stn1-M1* cells as a discrete series of spots on 2D gels, as seen previously with t-circles from the *ter1-16T* mutant (21), strongly favours the idea that these spots are composed of integral numbers of telomeric repeats, as expected for a recombination process dependent upon homology.

We conclude that the t-circle formation in *stn1-M1* cells is linked to the chronic telomere-capping defects of this

mutant. This defect results not only in highly elongated telomeres but also in abnormal cell and colony growth, large 3' telomeric overhangs and greatly increased rates of both subtelomeric recombination and telomeric truncation events (32). Unlike the Type II RTE of *ter1-Δ* mutants, where capping defects occur as a result of telomeres becoming too short, cells undergoing Type IIR RTE have capping defects believed to be independent of telomere length and are results of disruptions in the functioning of telomere proteins. These continuous capping defects presumably underlie the extreme telomere lengths and abundant products of telomeric recombination such as t-circles.

The abundance of t-circles in *stn1-M1* cells is compatible with them playing a role in the RTE that occurs in those cells. Although strong circumstantial evidence favours the hypothesis that small t-circles are often involved in the Type II RTE that occurs in yeast *ter1-Δ* mutants (17,18), there is no evidence to date that t-circles are involved in the Type IIR RTE of *stn1-M1* cells or the apparently similar recombinational telomere maintenance of human ALT cells. The proposed role of t-circles in Type II RTE of *K. lactis ter1-Δ* mutants is in building the first relatively long telomere through a rolling circle copying event in a cell that contains only short telomeres. Once one long telomere is present, t-circles may no longer be necessary, as other telomeres may be lengthened directly by copying the sequence of the long telomere (15). We hypothesize that the most significant possible role for t-circles in Type IIR RTE might be in the initial establishment of the long telomere state (such as would occur in a newly germinated *stn1-M1* spore from a *STN1/stn1-M1* diploid). There, the formation of extremely long telomeres from the normal length telomeres initially present (~500 bp in *K. lactis*) could be accelerated by the rolling circle copying of a t-circle. However, even if not vital to the maintenance of Type IIR RTE, t-circles could play a significant role in telomere elongation due to their high abundance in the cell. It is believed that in *K. lactis ter1-Δ* mutants, the telomere lengthening from Type II RTE (typically hundreds of bp) is thought to be limited by both the rarity of t-circles in those cells and the poorly processive copying of extremely small (~100 bp) t-circles. Many, if not most, of the larger t-circles in *stn1-M1* cells would likely be capable of producing much greater extensions. It is known, e.g. that 1.6 kb *URA3*-telomere circles transformed into *K. lactis* cells routinely produce telomere extensions of >10 000 bp (17,18).

The precise mechanism of t-circle formation in *stn1-M1* cells, or in other cells where they have been observed, is currently unknown. A previously proposed hypothesis is that a t-circle can be formed via a t-loop intermediate whereby the 3'-end of the telomere strand invades a more internal region of the same telomere (21,22). In favour of this possibility, t-loop structures have been observed in both human ALT cells and *K. lactis ter1-16T* cells (20,33). Furthermore, the loop portions of these structures exhibit size distributions similar to those of t-circles observed in the same systems. T-circle formation from t-loops would also be expected to cause a deletion of the telomere involved in the process (37).

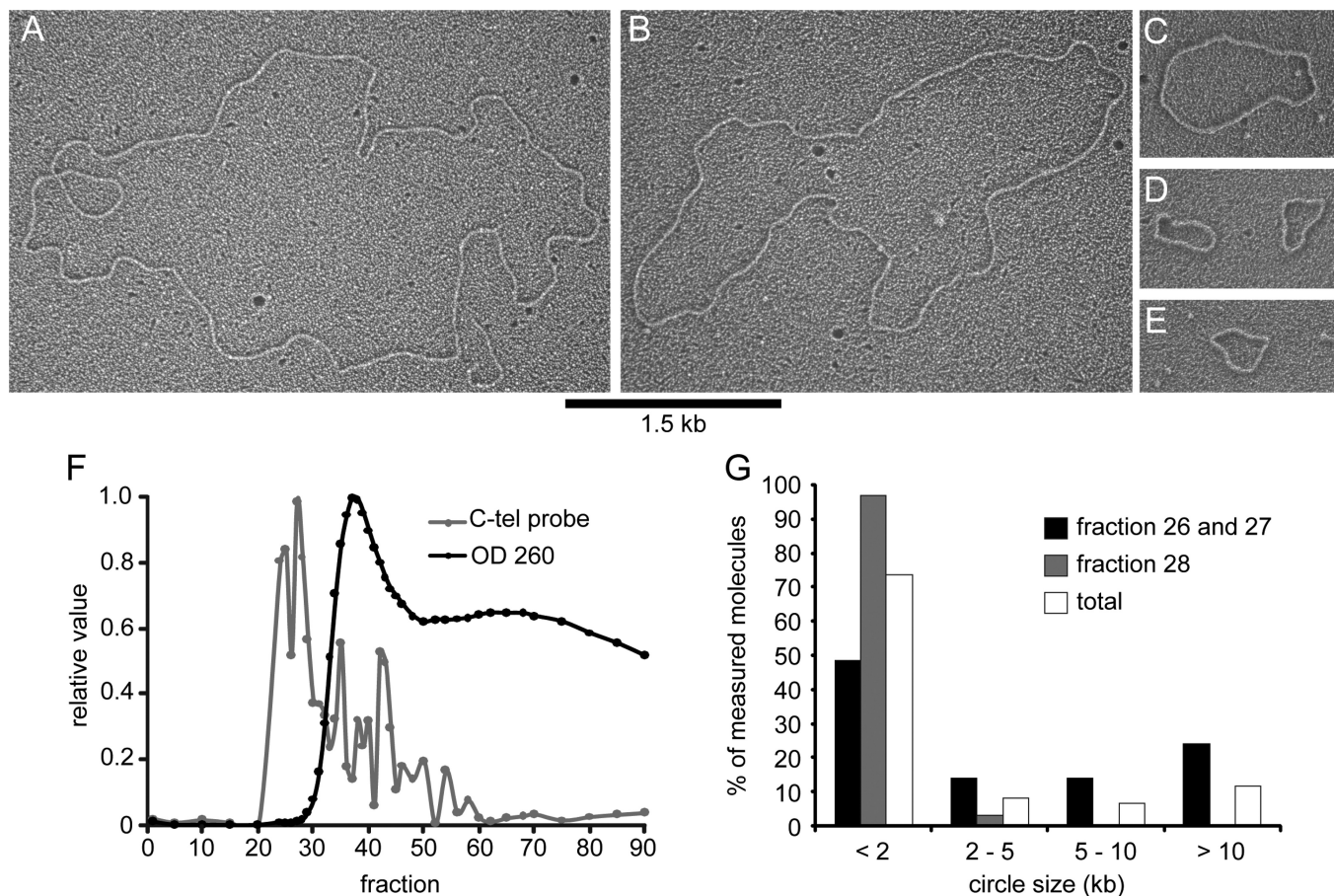


Figure 5. Visualization of DNA circles from high molecular weight telomere-enriched DNA from *stn1-M1*. (A–E) Electron micrographs of DNA circles observed in the telomere-enriched fractions from *stn1-M1*. Circle lengths for the molecules in A–E, respectively, are estimated at 15.7, 12.1, 3.1, 1.2 and 1.2, and 0.9 kb for fractions A–E, respectively. Note that there is also a ~0.8 kb linear fragment in the lower right portion of panel A. Samples were prepared by coating the DNA in denatured cytochrome *c* and are shown in negative contrast. Bar is equivalent to 1.5 kb. (F) Relative DNA abundance and telomeric DNA content of eluted fractions from gel chromatography separation of *AluI*, *HpaII* and *NlaIII* digest of *stn1-M1* genomic DNA. Molecules shown in A–E are from fractions 26 to 28 (F). Size distribution of the measured circles from fractions 26 to 27 ($n = 29$) and 28 ($n = 32$).

Among the extrachromosomal structures observed in this study by EM were a small percentage of tailed circles. These likely represent either t-loops or rolling circle intermediates.

Small single-stranded t-circles, specifically composed of the G-rich strand of telomeric sequence, were previously observed in *ter1-16T* (21) cells where they were approximately as abundant as double-stranded circles, at least for circles <500 bp/nt. Both types of t-circles were absent in *ter1-16T rad52* cells, indicating that they are generated by homologous recombination (21). It was suggested that the processing of a t-loop intermediate to form a t-circle often initially produced a transient t-circle that was partially double stranded and partially single stranded. Further processing then could produce more stable t-circles that were either fully double stranded or fully single stranded. The reason why the relative abundance of single-stranded t-circles varied considerably between the EM and 2D gel analysis remains unclear. Variation in the amount lost during purification of yeast DNA is a possible contributing factor. The relatively small sizes of the single-stranded t-circles likely render them vulnerable to being lost during

alcohol precipitations or dialysis. In addition, we have observed considerable variation in both the total amount of telomeric signal and the amount of small extrachromosomal telomeric DNA from independent isogenic isolates of *stn1-M1* extracted by the same method (J. Xu, S. Iyer, E. Basenko and M. McEachern, unpublished data). It is quite possible that the proportion of single-stranded t-circles may also be naturally variable.

The identification of t-circles in *stn1-M1* cells helps further underscore the similarities between the Type IIR RTE of yeast cells and the ALT phenomenon of some human cancers. Learning more about the mechanism of Type IIR RTE in yeast will certainly help to provide insights into how ALT occurs and what mutations underlie it.

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