Extrachromosomal Telomeric Circles Contribute to Rad52-, Rad50-, and Polymerase δ-Mediated Telomere-Telomere Recombination in Saccharomyces cerevisiae

Chi-Ying Lin,1 Hsih-Hsuan Chang,1 Kou-Juey Wu,2 Shun-Fu Tseng,1 Chuan-Chuan Lin,1 Chao-Po Lin,1† and Shu-Chun Teng1*

Department of Microbiology, National Taiwan University College of Medicine,1 and Institute of Biochemistry, National Yang-Ming University,2 Taipei, Taiwan

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Telomere maintenance is required for chromosome stability, and telomeres are typically replicated by the telomerase reverse transcriptase. In both tumor and yeast cells that lack telomerase, telomeres are maintained by an alternative recombination mechanism. By using an in vivo inducible Cre-loxP system to generate and trace the fate of marked telomeric DNA-containing rings, the efficiency of telomere-telomere recombination can be determined quantitatively. We show that the telomeric loci are the primary sites at which a marked telomeric ring-containing DNA is observed among wild-type and surviving cells lacking telomerase. Marked telomeric DNAs can be transferred to telomeres and form tandem arrays through Rad52-, Rad50-, and polymerase δ-mediated recombination. Moreover, increases of extrachromosomal telomeric and Y' rings were observed in telomerase-deficient cells. These results imply that telomeres can use looped-out telomeric rings to promote telomere-telomere recombination in telomerase-deficient Saccharomyces cerevisiae.

Telomeres are dynamic DNA-protein complexes that protect the ends of linear chromosomes, prevent detrimental chromosome rearrangements, and defend against genomic instability and the associated risk of cancer (26, 35, 49). Telomeric DNA is synthesized by the enzyme telomerase (30, 36, 46). In certain human cells, telomerase activity is absent, and telomeres are gradually shortened with successive cell divisions due to incomplete replication, which eventually causes replicative senescence. Once telomeres become sufficiently short, they are thought to lose the ability to protect the ends of the chromosomes from being recognized as broken ends and being subjected to nucleosome digestion and active recombinational repair. Continuous telomere shortening in human fibroblasts leads to chromosome fusions, crisis, and apoptosis (1). Very few human cells can bypass the crisis either through telomerase reactivation or through an alternative recombination pathway for lengthening of telomeres (ALT) (3, 8, 33).

Telomeric DNA in the yeast Saccharomyces cerevisiae consists of ~350 ± 75 bp of TG1–3/C1–3A DNA repeats. Internal to the TG1–3/C1–3A tracts are middle repetitive DNA elements called X and Y' (26, 49). The telomeric TG1–3/C1–3A DNA forms a complex nonnucleosomal chromatin structure. The major component in this complex is a double-stranded, sequence-specific DNA-binding protein–Rap1p complex which includes Rif1p and Rif2p. The copy number of the Rap1p complex negatively regulates telomere length (24, 47).

Even in organisms that normally rely on telomerase, telomerase-independent mechanisms of telomere maintenance exist. Although most cells in S. cerevisiae (22, 37), Kluyveromyces lactis (25), and Schizosaccharomyces pombe (27) that lack the gene for a telomerase component eventually enter cell cycle arrest, survivors arise relatively frequently. In both S. cerevisiae and K. lactis, the generation of survivors requires RAD52-dependent homologous recombination. In S. cerevisiae, the majority of cells that survive in the absence of telomerase activity have multiple tandem copies of the subtelomeric Y' element and very short terminal tracts of TG1–3/C1–3A DNA (22, 42) (type I survivors). In a minor fraction (~10%) of the survivors (type II), the lengths of the telomere sequence are increased variably from several hundred base pairs to 10 kb or longer (42). The generation of type II survivors depends on the presence of Rad50p, Rad59p, and Sgs1p (4, 5, 14, 17, 40), whereas the frequencies of type I and type II formation depend on a number of factors such as strain background (14), cell type, and various genes involved in nonhomologous end joining (21). The structure of type II telomeres in Saccharomyces resembles that of 10 to 15% of human cell lines and tumors that maintain telomeric DNA by the ALT pathway (3, 8, 33).

We previously showed that, prior to the appearance of type II survivors, the average telomere length in a telomerase-deficient culture is very short (40). However, type II lengthening is an abrupt recombinational process instead of a gradual incremental lengthening process (40) (see Fig. 6B). The sudden and dramatic increase in the length of telomere that accompanies the transition to the type II pattern of telomeric DNA is difficult to explain by conventional gene conversion events, because there is no telomere with sufficient length to act as a template for several kilobases of TG1–3 amplification prior to the appearance of type II survivors. Since the type II survivors with the characteristic of long telomeres arise suddenly in a population of telomerase-deficient cells, we speculated that the

* Corresponding author. Mailing address: Department of Microbiology, National Taiwan University, College of Medicine, No. 1, Sec. 1, Jen-Ai Rd., Taipei 10018, Taiwan. Phone: (886) 2-2312-3456, ext. 8282. Fax: (886) 2-2391-5293. E-mail: scteng@ha.mc.ntu.edu.tw.
† Present address: Department of Pharmacology, University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School, Piscataway, N.J.
initial substrates for the type II recombination in *S. cerevisiae* may be the extrachromosomal circles of TG1–3 DNA (40). Recent results by Natarajan and McEachern suggested that transformed circles can lead to the production of extended stretches of telomeric DNA by serving as templates for rolling-circle synthesis in *K. lactis* (29). We show here in vivo that episomal telomeric DNA-containing rings could be generated spontaneously from telomerase-deficient *S. cerevisiae* and that these rings could be the substrates for telomere-telomere recombination. This telomeric circle-derived recombination pathway is mediated by Rad52 as well as Rad50 and is DNA polymerase δ (Polδ) dependent, displaying the same genetic requirement as the type II telomere-telomere recombination.

**MATERIALS AND METHODS**

**Yeast strain and plasmid constructions.** All yeast operations were performed by standard methods. Yeast strains used in this study were derivatives of YPH501 (MATa ura3-52 leu2-3,112 trp1-1 can1-100 ade2-101 och1-1 trp1Δ63/Δ63 his1Δ200 his4Δ200 leu2-3,112/leu2-3,112 Δ1). The yeast strains carrying *tlc1*, *rad52*, *tlc1 rad50*, *tlc1 rad51*, and *tlc1 polδ* were described previously (40, 42, 44). These heterozygous diploids were transformed with pSH47, a *URA3* marker containing Cre recombinase expression plasmid, sporulated, and screened for segregants that were unable to grow at 37°C or for the *URA3*, *HIS3*, and *LEU2* markers by replica plating to selective medium. Spore cells were serially diluted into or restreaked onto yeast extract-peptone-dextrose (YPEP) medium as described previously (44) for making type I and type II survivors. The fragment for tagging the 3′ untranslated region of the Y′ elements with the *HIS3*-ori-Ampr gene region was amplified by PCR with 50-bp Y′ sequences that spanned the stop codon of the Y′ open reading frame 2 at the ends of the primers. pRS850 was used as a template. The resulting Y′-*HIS3*-ori-Ampr gene-Y′ PCR-amplified fragment was transformed into the YPH501 *tlc1*Δ-*LEU2* rad50::HIS3/TLC1 strain. The *Y′-HIS3*-ori-Ampr gene-tagged strains were selected on medium lacking histidine. Tagging of individual telomeres by the *HIS3*-ori-Ampr gene region was confirmed by Southern blot analysis with a Y′ probe as described below. These heterozygous diploids were then sporulated and screened for the *HIS3* and *LEU2* markers by replica plating them onto selective medium. To generate pUG6TG1–3, the *BamHI* and *EcoRV*-digested 550-bp TG1–3 fragment in pCRTG1–3,550 (42) was cloned into the BstEI-digested *KpnI* fragment of pUG6 plasmid (11), a vector having the 2μ orf marker.

**In vivo telomeric ring-targeting system.** The pSH47-containing haploid strains were cultivated overnight in 50 ml of synthetic complete medium minus uracil (Sc–Ura) to an optical density at 600 nm (OD600) of 1 to 2. The overnight cultures were diluted to 300 ml of yeast extract-peptone-adenine galactose to an OD600 of 0.25 and grown in an incubator at 200 rpm with shaking to an OD600 of 1.2. The cultures were diluted repeatedly 1:10,000 into fresh medium for 48 or 72 h. Genomic DNA from each culture was extracted, and 2.5 mg of DNA was electroporated into *Escherichia coli* DH10B cells (Invitrogen). Data shown in the results are averages of three independent *Escherichia coli* transformations.

**RESULTS**

**In vivo telomeric ring-targeting assay demonstrates that the markers target in tandem the wild-type and *tlc1* yeast genomes.** Due to the repetitive nature of telomeric DNA, telomere circles could be generated by intramolecular recombination (19). We speculated that these extrachromosomal circles of TG1–3 DNA could be the substrate for type II recombination (1). To test this possibility, we generated an in vivo telomeric ring-targeting assay. A marker plasmid, pUG6TG1–3, that contains a 550-bp telomeric repeat and a kanamycin-resistant gene, both flanked by *loxP* sequences, was constructed (Fig. 1B). This plasmid did not contain an ARS element, and therefore it could not replicate extrachromosomally. Additionally, in this plasmid, there is no sequence other than the TG1–3 fragment that is found naturally in the *S. cerevisiae* chromosome. By this design, we reduced the possibility that the plasmid would integrate at sites other than the telomere. This plasmid was then transformed into cultures from Cre recombinase-expressing freshly dissected spores. We expected that a 2.1-kb telomeric DNA-containing marker would be generated in vivo and recombined to telomeres in these yeast cells (Fig. 1C).

To determine if this telomeric circle-mediated telomere-telomere recombination exists, we conducted the in vivo telo-
meric ring-targeting assay with the wild-type and freshly dissected tlc1 cells that existed prior to activation of the alternative recombination pathway. Three independent experiments were conducted, and the frequencies were averaged. As expected, for each microgram of pUG6TG1–3 transformation, 18.6 colonies of G418-resistant tlc1 survivors were recovered. Interestingly, G418-resistant colonies were also recovered from the wild-type strain. Relative to the respective frequency in the tlc1 strains, the frequency of the G418-resistant tlc1 survivors was reduced to 42% (7.90/18.6) (Table 1), suggesting that a more efficient marker targeting occurs in the telomerase-deficient strain. As a control, when a CEN plasmid, pRS314, was used to transform both strains, no difference was observed in the transformation efficiency (data not shown). We checked 76 of the G418-resistant tlc1 survivors by Southern analysis. The genomic DNA was digested with KpnI, which does not cleave within the 2.1-kb TG1–3-kan-loxP marker sequence but cuts at the 3’-most end of the Y’ element (Fig. 2A). The digested DNA was subjected to electrophoresis and analyzed by hybridization to a kan probe (12 samples are shown in Fig. 2B). As expected, heterogeneous signals, a sign of the size of telomeres with major signals being over 2.1 kb, were detected in most survivors (Fig. 2B). This result suggested that kan markers were inserted into the chromosomes of these G418-resistant tlc1 survivors. If circular markers would serve as templates for recombination, tandem copies of the markers might be generated at telomeres before the DNA polymerases fall off. A 1.6- to 2.1-kb band should be detected when a similar

FIG. 1. (A) Schematic of the hypothesis of the initial step of telomere-telomere recombination. The yeast telomere locus consists of tandem telomeric repeats from which circles may be liberated by recombination. The arrows indicate the 3’-end hydroxyl group at telomeres. The liberated telomeric rings may be used as templates for telomeric circle-mediated telomere-telomere recombination. (B) Map of the 4.6-kb pUG6TG1–3 plasmid. Black boxes indicate the telomeric repeats, white boxes indicate the Kan’ gene, and gray triangles indicate the loxP sequences. (C) In vivo telomeric ring targeting system. A Cre expression telomeric, kan-marked, ring-releasing yeast strain was generated for the experiments. The TG1–3-kan-loxP ring used for telomeric circle-mediated targeting was released from pUG6TG1–3 under the galactose induction of Cre. The recombinants can be selected on G418 plates.
Southern analysis is conducted using SacI, a restriction enzyme which cleaves once in the 2.1-kb circle (Fig. 2A). The exact size depends on the size of the telomeric tract, ranging from 50 to 550 bp after recombination, because this 550-bp TG1–3 tract may be highly unstable in E. coli and in yeast (28, 42). A band at 1.6 to 2.1 kb was observed in 73 of 76 survivors (Fig. 2B and data not shown). The remaining three showed fixed bands at different sizes (Fig. 2B; lanes 3 and 6, and data not shown). These results implied that the TG1–3-kan-loxP markers in tandem may target the yeast chromosome in tcl1 cells. The actual copy number of segments arrayed in tandem was determined to range from 2 to more than 10 copies (Fig. 2B). As shown in Fig. 2C, four lanes (lanes 4, 5, 7, and 11) in Fig. 2B contained bands at around 4 kb which were generated from ring targeting of pUG6TG1–3 without the Cre-mediated cleavage. These results implied that the TG1–3-kan-loxP markers indeed target the yeast chromosomes.

To determine if sequences from circles associated with telomeres, the same blot was hybridized with a Y′ probe (Fig. 2D). This probe showed a shifting of some subtelomeric signal to the large bands hybridizing to the kan probe with the KpnI digest. To further confirm that the formation of tandem copies of markers in chromosomes depends on the circular element, we tested the ability of formation of tandem copies of markers in chromosomes by using a 2.1-kb TG1–3-kan-loxP linear fragment generated from the XbaI-EcoRV digestion of pUG6TG1–3. This fragment contains only one copy of loxP that is incapable of generating a CRE-mediated ring (Fig. 1B). For each microgram of XbaI-EcoRV-digested pUG6TG1–3 transformation, 0.74 of the colonies of G418-resistant tcl1 survivors were recovered. As expected, none of them (0 of 18) showed any sign of tandem targeting of markers based on the SacI Southern analysis (data not shown), suggesting that a circular structure is required for the formation of tandem copies of telomeric repeats in our assay. KpnI- and SacI-digested Southern blot analysis also demonstrated that the kan markers in all G418-resistant wild-type clones that we examined (32 of 32) were integrated in tandem at the chromosomes (data not shown). This result suggests that telomeric circle-mediated telomere-telomere recombination also occurs in wild-type cells.

**TABLE 1.** pUG6TG1–3 transformation in different genetic backgrounds of yeast strains

| Strain          | Frequency of G418 eventsa | % of circle-mediated telomeric targetingb in G418 events (no. of resistant colonies/total no.) | Frequency of circle-mediated telomeric targetingc |
|-----------------|---------------------------|---------------------------------------------------------------------------------------------|-----------------------------------------------|
| Wild type       | 7.90 ± 2.65               | 100 (32/32)                                                                                  | 7.90 ± 2.65 (0.44)                           |
| tcl1 mutant     | 1.10 ± 0.39               | 85.7 (36/42)                                                                                  | 14.6 ± 2.07 (0.82)                           |
| tcl1 rad50 mutant | 17.0 ± 2.41               | 100 (39/39)                                                                                  | 22.1 ± 2.2 (1.23)                           |
| tcl1 type I     | 22.1 ± 2.2                | 91.3 (42/46)                                                                                  | 21.7 ± 8.50 (1.21)                          |
| tcl1 type II    | 23.8 ± 9.41               | 85.7 (67)                                                                                    | 0.17 ± 0.10 (0.01)                          |
| rad52 mutant    | 0.20 ± 0.12               | NA                                                                                          | NA                                           |
| tcl1 pol6 mutant | 0.00 ± 0.00               | NA                                                                                          | NA                                           |

* Results demonstrated as the number of G418-resistant colonies from transformation per microgram of pUG6TG1–3 DNA. Experiments using the control plasmid pRS314 showed that all strains exhibited similar efficiencies for plasmid transformation. The calculation is based on three independent measurements. A standard deviation is given.

b The telomeric targeting was determined by Southern analysis using the Kan′ gene fragment as a probe, as described for Fig. 2B and C.

c Not available (NA) because no G418-resistant colony was recovered from this strain.

d The frequency of circle-mediated telomeric targeting was derived by multiplying the values obtained in the previous two columns. Relative frequencies (in parentheses) were calculated by dividing each frequency by that of the tcl1 strain.

% of circle-mediated telomeric targetinga
vivo telomeric ring-targeting assay with yeast cells of several mutation backgrounds. As a control experiment, when a CEN plasmid, pRS314, was used to transform these strains, no difference in transformation efficiency was observed (data not shown). To determine if Rad52, Rad50, and Rad51 proteins may specially govern the formation of ring targeting, we tested their effects in their mutant strains. Since tlc1 rad52 mutants senesced extremely fast, this experiment was limited to a rad52 strain. As shown in Table 1, the telomeric circle-mediated G418-resistant colonies were observed but were almost abolished in the rad52 and tlc1 rad50 strains. Thus, Rad52 and Rad50 functions are involved to generate telomeric circle-mediated targeting. In contrast, tlc1 rad51 strains showed only a slight decrease in telomeric circle-mediated targeting, suggesting that telomeric circle-mediated telomere-telomere recombination does not rely on the strand transfer ability of Rad51p. We previously showed that DNA Polδ is required to generate elongated telomeres and/or subtelomeres during telomeric circle-mediated targeting occurs. The formation of one long telomere is postulated to occur via the gene conversion copying a 2.1-kb (TG13-kan-loxP) circle. The arrows above markers indicate the probe used in panel B. Subtelomeric KpnI sites are located 0.5 kb from the telomeric ends in untransformed yeast cells. Positions of KpnI and SacI sites are indicated. Tandem arrays of TG13-kan-loxP formed at telomeres after targeting are shown here. (B through D) Southern analysis of G418-resistant colonies from the telomeric ring-targeting assay. Equal amounts of genomic DNAs from 12 independent G418-resistant survivors were digested with KpnI and SacI and fractionated in a 0.7% agarose gel. After the survivors were transferred to a nylon filter, the filters were hybridized with a kan probe (B), a pUG6 vector probe (C), and a Y′ 3′-end probe (D), sequentially. Asterisks mark two events (lanes 3 and 6) with only one copy of the marker targeted to the internal regions of chromosomes. Size markers (in kilobases) are shown on the left.

FIG. 2. TG13-kan-loxP markers targeted to chromosomes. (A) Predicted structure of the TG13-kan-loxP markers on chromosomes if telomeric circle-mediated targeting occurs. The formation of one long telomere is postulated to occur via the gene conversion copying a 2.1-kb (TG13-kan-loxP) circle. The arrows above markers indicate the probe used in panel B. Subtelomeric KpnI sites are located 0.5 kb from the telomeric ends in untransformed yeast cells. Positions of KpnI and SacI sites are indicated. Tandem arrays of TG13-kan-loxP formed at telomeres after targeting are shown here. (B through D) Southern analysis of G418-resistant colonies from the telomeric ring-targeting assay. Equal amounts of genomic DNAs from 12 independent G418-resistant survivors were digested with KpnI and SacI and fractionated in a 0.7% agarose gel. After the survivors were transferred to a nylon filter, the filters were hybridized with a kan probe (B), a pUG6 vector probe (C), and a Y′ 3′-end probe (D), sequentially. Asterisks mark two events (lanes 3 and 6) with only one copy of the marker targeted to the internal regions of chromosomes. Size markers (in kilobases) are shown on the left.
lomere-telomere recombination (44). In the partial absence of function of Polδ at its semipermissive temperature, the 2.1-kb telomeric ring should be replicated only incompletely. As expected, no G418 colony was recovered from the tlc1 polδ/H9254 strain (Table 1), suggesting that Polδ/H9254 is required for telomeric circle-mediated targeting. In addition, established type I and type II tlc1 survivors both displayed a 20% higher frequency in telomeric circle-mediated targeting than the tlc1 cells right from sporulation and prior to the survivor formation (Table 1), suggesting that telomeric circle-mediated targeting occurs more frequently in established type I and type II survivors.

Neutral-neutral two-dimensional agarose gel electrophoresis revealed the presence of telomeric rings in telomerase-deficient cells. The technique of two-dimensional electrophoresis has been useful in a variety of versions to analyze replicative intermediates of prokaryotic and eukaryotic organisms (9). To analyze ring structures, ethidium bromide was added to the running buffer. Ethidium bromide intercalates into double-stranded DNA (dsDNA), reducing its mobility in comparison to that of single-stranded DNA and adding the positive superhelical turns into negatively supercoiled circular DNA. The two-dimensional system was relatively insensitive to nicking during the preparation, in comparison to that with alkaline gels. The hybrid of single-stranded DNA–dsDNA, forming more straight lines, was separated from dsDNA, which formed arcs. Circular DNA was easily recognized by the separation of topoisomers or the resulting higher arcs appearance in molecules of lower mobility (7) (Fig. 4A). The assignment of these linear and circular species was confirmed by a comparison of blots with linear and circular controls of plasmid DNAs (Fig. 4B).

To determine if telomeric rings were increased in telomerase-deficient cells, total DNAs were prepared from the wild type and the tlc1 strain that was at the point when the type II survivors were being generated (see Materials and Methods). The samples were then separated by two-dimensional gel electrophoresis. Southern hybridization analysis with a 32P-labeled C1–3A fragment showed a discrete arc of dsDNA linear fragments migrating differently from the main population of chromosomes near the well in wild-type cells (Fig. 5A). In the tlc1...
cells, additional continuous signals corresponding to dsDNA rings and ssDNA-dsDNA hybrids were observed as the arcs above and below the main arc of dsDNA linear fragments, respectively (Fig. 5A and shown enlarged in Fig. 5B). Experimental results after stripping the nylon membrane and reprobing with a Y′ probe showed that there were Y′ rings popping out in both wild-type and tlc1 cells in the upper arc. But the tlc1 cells displayed relatively stronger signals of the Y′ rings (Fig. 5A). The total Y′ copy number was elevated 1.57-fold, and the extrachromosomal Y′ rings were elevated 5.60-fold in tlc1 cells. Other repetitive sequences in chromosomes (rRNA genes) and episomal plasmids (2μm) did not display any major differences with wild-type and tlc1 cells, suggesting that the increase of ring structures is specific to the telomeric regions in telomerase-deficient cells.

Marker-based ring-releasing assay showed that telomeric Y′ rings are released from chromosomes during the formation of type II survivors. To test the possibility that episomal telomeric-Y′ rings are induced during the survivor formation in telomerase-deficient cells, a more sensitive assay to quantitatively detect telomeric-Y′ rings was developed. We first tagged the 3′ end of Y′ elements with a HIS3 ori-Ampr gene marker (Fig. 6A). Two independent Y′-HIS3 ori-Ampr gene-tagged tlc1 spores were used to inoculate liquid cultures. In the liquid culture assay (40), when cultures starting from freshly dissected spores were repeatedly diluted 1:10,000 at 48-h intervals, type II-like dramatic telomere lengthening could be observed after several dilutions. The genomic DNA from each culture was extracted and subjected to Southern analysis. In the Southern blot analysis, the genomic DNA was digested with a mixture of four restriction enzymes to very small fragments. The telomere sequences, which were not cut by these enzymes, remained relatively large. As shown in Fig. 6B, two independent Y′-HIS3 ori-Ampr gene-tagged tlc1 spores, a and b, revealed a type II pattern developing at the fourth and the fifth dilutions, respectively. This observation was not unexpected because individual telomeres of telomerase-deficient cells are not static and the turning point for the formation of survivors is dynamic. The genomic DNA at each time point was then electroporated into E. coli. As shown in Fig. 6C, dramatic increases in the transformation efficiency were observed at the fourth and the fifth dilutions of spores a and b, respectively. In contrast, DNAs from the wild-type and tlc1 rad50 tagged spores showed no increase in transformation efficiency throughout eight dilutions. Additionally, plasmid DNAs from these E. coli transformants were recovered and confirmed to be the Y′ HIS3 ori-Ampr gene rings by restriction digestion and Southern analysis (data not shown). These results demonstrated that telomeric Y′ rings released from chromosomes are increased during or immediately after the formation of type II survivors.

FIG. 5. Detection of extrachromosomal circular telomeres in telomerase-deficient S. cerevisiae. (A) Two-dimensional gel analysis of DNA from cells lacking telomerase. DNA was isolated from wild-type (WT) and tlc1 cells at the stage when survival were being generated. Similar amounts of DNA were separated on a two-dimensional gel. The blot was first hybridized with a C1–3A probe to detect telomeric rings and was hybridized sequentially with Y′, rRNA genes (labeled rDNA), or ARS and 2μm probes with exposure times of equal lengths. An arrow indicates extrachromosomal rings of telomeres. Size markers (in kilobases) in the first dimension are shown on the top. (B) The enlarged telomere signal of tlc1 cells.

DISCUSSION

In this paper, we tested the possibility of telomeric circles being the template for telomere-telomere recombination in S. cerevisiae. An in vivo Cre-loxP system was used to generate telomeric rings in yeast cells. Qualitative and quantitative results presented in Fig. 2 and 3 and Table 1 suggest a rolling-circle replication model. Although our observations could also be explained by multiple exchanges with an extrachromosomal element, the fact that type II lengthening is abrupt rather than gradual (40) argues against this possibility. We provide two lines of evidence suggesting that telomeric circles exist in S. cerevisiae, especially at the point of occurrence of survivors. First, the increase in the telomeric TG1,3 rings and Y′ rings in tlc1 cells is detected by two-dimensional gels. Second, these Y′ rings can be quantified and confirmed in E. coli by a marker-based system. However, our assays cannot distinguish whether these telomeric (Fig. 5) and Y′ (Fig. 6) rings were generated during or immediately after the formation of survivors. Interestingly, this pathway is favored but does not occur exclusively in telomerase-deficient cells (Table 1). Moreover, established type I and type II survivors displayed higher frequency for telomeric circle-mediated targeting than tlc1 cells prior to the occurrence of survivors (Table 1), suggesting that telomeric
circle-mediated targeting occurs more easily in established survivors. These data also indicate that there is no difference in ring targeting between type I and type II survivors once the alternative recombination pathway is activated and established.

The formation of variable lengths of extrachromosomal circular DNA of telomeric repeats has been shown in human ALT cells (31, 34, 43, 48) and during the development of *Xenopus laevis* (6). The mechanism by which minicircles of telomeres arise may be similar to that involved in the generation of extrachromosomal rRNA gene circles from the cluster of 100 to 200 rRNA gene units repeated in tandem on chromosome XII of *S. cerevisiae*. Horowitz and Haber demonstrated the existence of circles of subtelomeric tandem Y’ elements in the wild-type *S. cerevisiae*. Presumably, they arose from either Y’-Y’ or tract-tract recombination (13). Taken together, the most reasonable interpretation of our results is that, in telomerase-deficient cells, especially at the time for type II survivor development, critically short telomeres increase instability that causes telomeric and subtelomeric fragments to loop out by telomere rapid deletion (TRD)-mediated intrachromosomal recombination (19). Telomere-telomere recombination promoted by telomeric rings was first demonstrated in telomerase-deficient *K. lactis* (29). It was recently reported that DNA circles as short as 18 nucleotides in vitro (20) and 100 nucleotides in vivo (28, 29) can act as catalytic templates for efficient synthesis of long telomeres by DNA polymerase. Additionally, as shown in Fig. 4 and 5, Y’ rings might contribute to type I recombination. However, utilization of Y’ telomere circles to form telomeric tandem arrays might be *RAD50* independent due to the high level of homology provided by the Y’ element.

We demonstrated previously that type I survivors arise by a *RAD51*-dependent process, whereas type II formation is *RAD50* dependent (40). Rad51p, on which type I recombination depends, is a RecA-like, strand transfer protein (39). Our in vivo assay quantitatively showed that telomeric circle-mediated recombination is Rad50p dependent and Rad51 independent. Collectively, creation of this transforming activity in our telomeric ring-targeting assay is recombination dependent and...
it exhibits the same genetic requirement as the type II recombination pathway. The DNA ends are processed to generate 3' overhangs which can invade homologous duplex DNA to initiate gene conversion. Genetic and biochemical evidences indicate that Rad51p acts in concert with Rad54p, Rad55p, and Rad57p. Furthermore, Rad54p is proposed to remodel chromatin to allow Rad55p/Rad57p-enhanced and Rad51p-mediated strand exchange (38). These activities are critical for gene conversion events of regular chromatin structure. Based on our results, the Rad51p-independent extrachromosomal telomeric circle-mediated telomere-telomere recombination indicates that the substrate for these events may not have a regularly packed chromatin structure and might be more like a recombinosome-accessible, Rap1p-protected telomere structure. The Rad50p protein, on which type II survivors and extrachromosomal telomeric circle-mediated telomere-telomere recombination depend, functions in a complex with Xrs2p and Mre11p (18, 45). A potential role for the Rad50/Mre11/Xrs2 complex to process double-strand breaks to single-strand ends (32) could be a critical step for telomeric circle-mediated telomere-telomere recombination. Moreover, RAD51-independent recombination requires much less homology for strand invasion than does RAD51-dependent repair (15). This may explain why Rad50p-dependent recombination machinery is better suited for finding short, sufficiently homologous regions within TG1–3 sequences in TG1–3-containing rings to permit the initiation of recombination.

We previously demonstrated that the type II pathway may be initiated from a single telomere (41). When telomeres become critically short, a single telomere may use a telomeric ring as a template and the 3' hydroxyl group as a primer for telomere-telomere recombination (Fig. 7). We speculate that the initial template for type II recombination is the extrachromosomal circles of TG1–3 DNAs. Due to the repetitive nature of telomeric DNA, telomeric circles could be generated by intramolecular recombination. Additionally, telomeric circles could be generated by intramolecular t-loop formation (23). If a telomere complex invades a TG1–3 circle, a potential rolling-circle replication pathway would allow an abrupt, dramatic, and variable increase in telomere length. Initiation of the first recombinant telomere presumably would be the rate-limiting step in telomere-telomere recombination. This long telomere would preferentially be the template of gene conversion for other critically short telomeres. This result is consistent with the recent finding in K. lactis by the McEachern group (29) suggesting that telomeric rings can promote Rad52-, Rad50-, and Pol6-dependent telomere-telomere recombination in both K. lactis and S. cerevisiae. This mechanism might also be relevant to the ALT pathway as reviewed in reference 12. Whether the formation of extrachromosomal telomeric rings in some ALT-prone tumors would be easier remains to be elucidated.

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