

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

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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 nuclease 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 $\sim 350 \pm 75$ 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, telom-

erase-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 ($\sim 10\%$) 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

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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 (Pol δ) 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 (*MAT α MAT α ura3-52/ura3-52 lys2-801 amber/lys2-801 amber ade2-101 ochre/ade2-101 ochre trp1 Δ 63/trp1 Δ 63 his3 Δ 200/his3 Δ 200 leu2- Δ 1/leu2- Δ 1). The yeast strains carrying *tlc1*, *rad52*, *tlc1 rad50*, *tlc1 rad51*, and *tlc1 pol δ* 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 (YEPD) 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-Amp^r* 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. pRS303 was used as a template. The resulting Y'-*HIS3-ori-Amp^r* gene-Y' PCR-amplified fragment was transformed into the YPH501 *tlc1::LEU2 rad50::HIS3/TLC1* strain. The Y'-*HIS3-ori-Amp^r* gene-tagged strains were selected on medium lacking histidine. Tagging of individual telomeres by the *HIS3-ori-Amp^r* gene region was confirmed by Southern blot analysis with a *HIS3* 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 pCRTG₁₋₃550 (42) was cloned into the BstEII-digested Kleon fragment-treated and BglII-digested 4-kb pUG6 plasmid (11), a vector having the *loxP-kan-loxP* marker.*

In vivo telomeric ring-targeting system. The pSH47-containing haploid strains were cultured 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 0.6. The cultures were transformed with the pUG6TG₁₋₃ plasmid as described previously (10). The transformants were then plated on Sc-Ura galactose medium and incubated at 30°C overnight to allow telomeric circle-mediated telomere-telomere recombination to occur. The cells grown overnight were replica plated onto YEPD plates plus 200 μ g of G418/ml for selection and for stopping the CRE expression. Since the telomeric arrays generated by the excised circles can themselves be the targets of Cre, presumably, during the period of Cre induction in galactose medium, there would be both array formation and destruction. Because the cleavage of the arrays would regenerate circles, circles should still be present after Cre expression stops. The *tlc1 pol δ* strain was also cultured at 30°C, its semipermissive temperature. Data shown in Table 1 are the average of three independent transformations for each yeast strain.

Culture condition, DNA preparation, enzyme digestion, gel electrophoresis, and Southern blot analysis. Genomic DNA preparation and Southern blot analysis were performed as previously described (44). To examine the DNA from an individual colony, the colony was expanded in 2 ml of liquid medium to obtain DNA for Southern analysis. The DNA was digested with KpnI, with SacI, or with a mixture of AluI, HaeIII, HinfI, and MspI. When the DNA was examined by two-dimensional electrophoresis, the wild-type culture and *tlc1* culture that was at the dilution point for type II development (40) were expanded in 500 ml of liquid medium to obtain enough DNA for Southern analysis (16). For the *Bal 31* exonuclease digestion experiment, 70 μ g of genomic DNA from G418 survivors was digested with 3 U of *Bal 31* (New England BioLabs) in a 200- μ l final volume. A 50- μ l volume of digested DNAs was removed every 10 min, subjected to

phenol-chloroform extraction and ethanol precipitation, and digested with KpnI and SacI. For pulsed-field gel electrophoresis (PFGE), yeast chromosomal DNA blocks were prepared by mixing equal volumes of yeast cells from stationary-phase cultures with 1% low-melting-point agarose (FMC BioProducts) as described previously (42). PFGE was performed with the dynamically regulated contour-clamped homogeneous electric field CHEF-DR III system (Bio-Rad). Chromosomes were separated on a 1% agarose gel in 0.5 \times Tris-borate-EDTA (TBE) buffer at 14°C for 30 h at 6.0 V/cm (200 V) with a 120° included angle and a 60- to 120-s linear switch time ramp.

The following probes were used for Southern hybridization: a 690-bp HindIII-PstI fragment of the *Kan^r* gene (Fig. 2B and 3), a 2.4-kb PvuII-SpeI fragment of pUG6 (Fig. 2C), a 377-bp PCR fragment from the 3' end of Y' (Fig. 2D), a 1-kb 5' EcoRI fragment of *PIF1* (Fig. 3B), an EcoRI-digested yeast genomic DNA (Fig. 4B), the 4.5-kb pRS303 plasmid (Fig. 4B), the 3-kb pBSKSII plasmid (Fig. 4B), a 270-bp C₁₋₃A fragment (Fig. 5A and 6B), a 341-bp XhoI-KpnI fragment from the 3' end of Y' (Fig. 5A), a 2.1-kb EcoRI fragment of rRNA (Fig. 5A), the pRS423 containing the plasmid 2 μ m and an autonomously replicating sequence (ARS) (Fig. 5A), and a 586-bp NdeI-NsiI fragment of *HIS3* (Fig. 6A). Probes were randomly labeled with the random prime labeling system (Invitrogen). Data shown here are representatives of two or more experiments from independent spores.

Neutral-neutral two-dimensional agarose gel electrophoresis. Two-dimensional gel electrophoresis was performed according to the method of Brewer and Fangman (2). In the first dimension, samples were separated on 0.7% agarose gels in 1 \times TBE with 0.5 μ g of ethidium bromide/ml at 0.5 to 0.6 V/cm for 20 h. Subsequently, the entire lane was excised and embedded in a 1.3% agarose gel. Separation in the second dimension was at 5 V/cm in 1 \times TBE containing 0.5 μ g of ethidium bromide/ml. After electrophoresis, the DNA was transferred onto nylon membranes and hybridized with the corresponding DNA probes as described above. Imaging and quantification of the data were performed with an Alpha Innotech photodocumentation system. The amount of 2 μ m in each sample was used as a normalization factor for determining the severalfold enhancement of the Y' amount.

The marker-based Y' ring-releasing assay. The wild-type, *tlc1*, and *tlc1 rad50* spores tagged with Y'-*HIS3-ori-Amp^r* gene were used to inoculate cultures. Cultures were inoculated directly from the tetrad master plate into 10 ml of liquid YEPD medium and allowed to grow to stationary phase on a 30°C roller drum. 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 ng of DNA was electroporated into ElecoMax 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, telomeric 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 (Fig. 1A). 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-

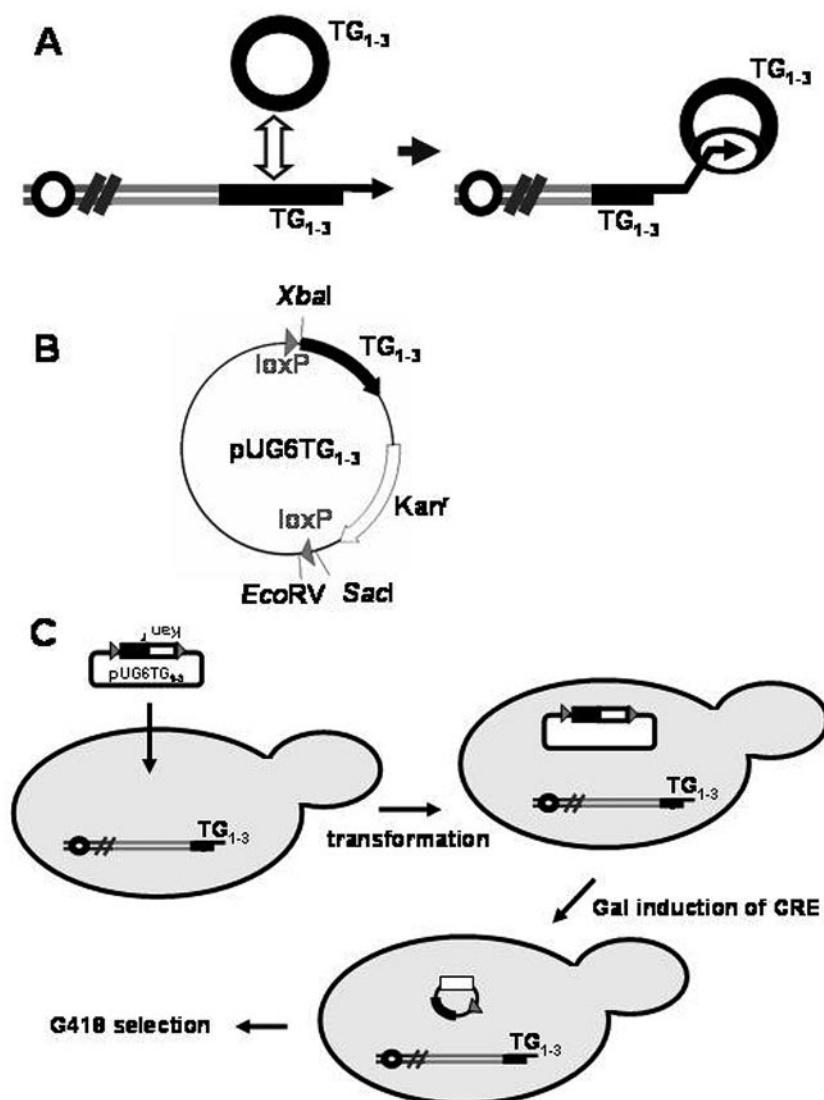


FIG. 1. (A) Schematic of the hypothesis of the initial step of telomere-telomere recombination. The yeast telomere locus consists of tandem telomeric repeats from which circles may be liberated by recombination. The arrows indicate the 3'-end hydroxyl group at telomeres. The liberated telomeric rings 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^r 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.

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 colonies in the wild-type strains 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

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

Strain	Frequency of G418 ^r events ^a	% of circle-mediated telomeric targeting ^b in G418 ^r events (no. of resistant colonies/total no.)	Frequency of circle-mediated telomeric targeting ^d
Wild type	7.90 ± 2.65	100 (32/32)	7.90 ± 2.65 (0.44)
<i>tlc1</i> mutant	18.6 ± 4.34	96.1 (73/76)	17.9 ± 4.17 (1.00)
<i>tlc1 rad50</i> mutant	1.10 ± 0.39	80.3 (41/51)	0.88 ± 0.31 (0.05)
<i>tlc1 rad51</i> mutant	17.0 ± 2.41	85.7 (36/42)	14.6 ± 2.07 (0.82)
<i>tlc1</i> type I	22.1 ± 2.2	100 (39/39)	22.1 ± 2.2 (1.23)
<i>tlc1</i> type II	23.8 ± 9.41	91.3 (42/46)	21.7 ± 8.59 (1.21)
<i>rad52</i> mutant	0.20 ± 0.12	85.7 (6/7)	0.17 ± 0.10 (0.01)
<i>tlc1 polδ</i> mutant	0.00 ± 0.00	NA ^c	NA

^a 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^r 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 *tlc1* strain.

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 events 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 *tlc1* 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. 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 *tlc1* 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.

TG₁₋₃-*kan-loxP* repeats target the physical ends of chromosomes. To obtain a better understanding of the kinds of changes that can occur during the formation of G418-resistant

strains, we used PFGE to determine if the *kan* markers were located exactly on chromosomes in four independent G418-resistant *tlc1* colonies (Fig. 2, survivors 4, 5, 6, and 7). PFGE can separate full-length chromosomes from each other, although certain chromosomes comigrate (XII and IV; VII and XV) and cannot be distinguished. As shown in Fig. 3A, strains 4, 6, and 7 contained one tandem array of G418-resistant markers on chromosomes I, XII or IV, and VIII, respectively. In contrast, strain 5 contains multiple G418-resistant markers on six chromosomes. These multiple arrays could be generated from additional interchromosomal and/or ring-targeting events. All these results support the observation that the TG₁₋₃-*kan-loxP* markers indeed target the yeast chromosomes.

Recombination may also occur in places other than the telomere in chromosomes. If the marker were at the physical end of a chromosome, it would be sensitive to digestion by the exonuclease *Bal* 31. To address this possibility, genomic DNA was prepared from a G418-resistant event (Fig. 2, survivor 2). This DNA was digested with *Bal* 31, and the samples were removed at 10-min intervals. The DNA was then digested with KpnI and SacI, subjected to electrophoresis, and analyzed by hybridization to a *kan* probe (Fig. 3B). Since the KpnI-digested *kan* hybridizing sequences were found to shorten and the SacI-digested *kan* hybridizing signals were found to decrease as time increased, *kan* sequences should have been near a free end in the chromosomes of the survivor. An internal probe demonstrated that the nontelomeric sequences were not *Bal* 31 sensitive. A 1.6-kb *kan*-containing band which corresponds to the SacI-digested *kan* fragment from the most distal copy of the array was also observed in the strain. Some of the other clones that we examined also had one smaller *kan* fragment that rapidly disappeared, such as the 1.6-kb band shown in Fig. 3B (data not shown). Similar *Bal* 31 digestion results were obtained with five other G418-resistant survivors and wild-type strains (data not shown). These data showed that these telomere-containing rings target telomeres.

Rad52p, Rad50p, and replicative Polδ are involved in telomeric circle-mediated targeting and telomere lengthening. To better understand the effect of proteins involved in telomeric circle-mediated targeting, we began to examine the factors which might be involved in this pathway. We conducted the in

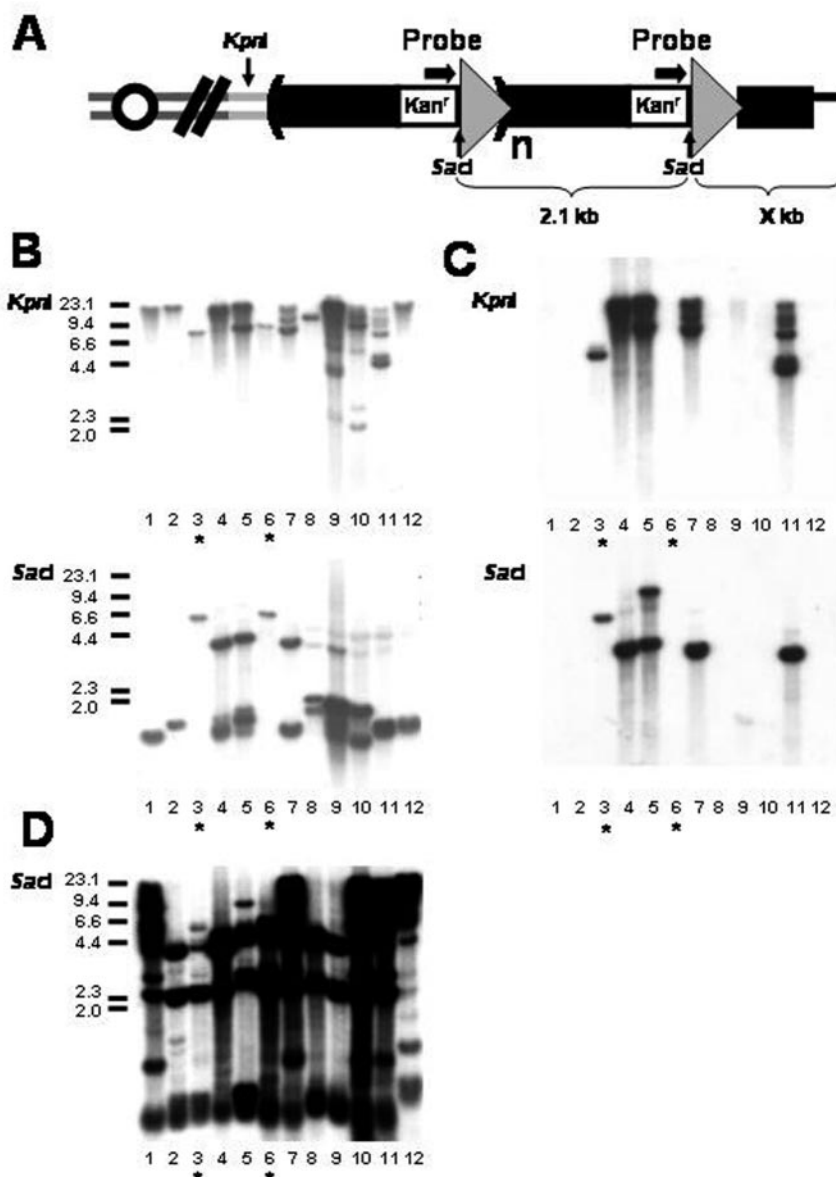


FIG. 2. TG_{1-3} -*kan-loxP* markers targeted to chromosomes. (A) Predicted structure of the TG_{1-3} -*kan-loxP* markers on chromosomes if telomeric circle-mediated targeting occurs. The formation of one long telomere is postulated to occur via the gene conversion copying a 2.1-kb (TG_{1-3} -*kan-loxP*) 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 TG_{1-3} -*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.

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 Pol δ is required to generate elongated telomeres and/or subtelomeres during te-

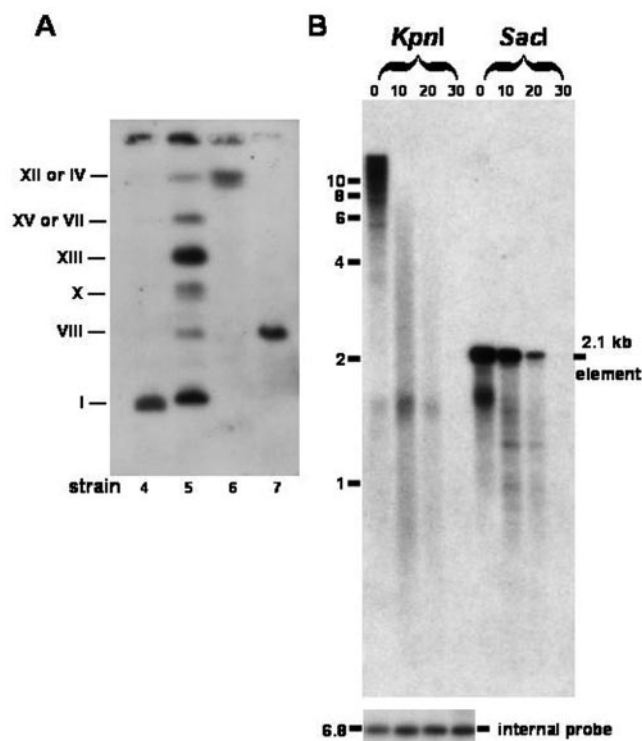


FIG. 3. *kan* sequences targeted to the physical ends of chromosomes. (A) PFGE analysis of G418-resistant survivors from telomerase-deficient *S. cerevisiae*. Chromosomes from four G418-resistant survivors (survivors 4 through 7 in Fig. 2) were separated by PFGE. After the survivors were transferred to a nylon filter, the filter was hybridized with a *kan* probe. Chromosome numbers are shown on the left. (B) The *kan* marker targets telomeres of G418-resistant survivors. Genomic DNA from a G418-resistant *tlc1* survivor was digested with *Bal* 31 exonuclease for increasing lengths of time. Samples were removed at 10-min intervals, subjected to phenol-chloroform extraction and ethanol precipitation, and digested with *KpnI* and *SacI*. Digested DNAs were fractionated in a 0.7% agarose gel and analyzed by Southern hybridization sequentially with a *kan* probe (top) and an internal probe (the *PIF1* gene) (bottom).

lomere-telomere recombination (44). In the partial absence of function of Pol δ 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 pol\delta* strain (Table 1), suggesting that Pol δ 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

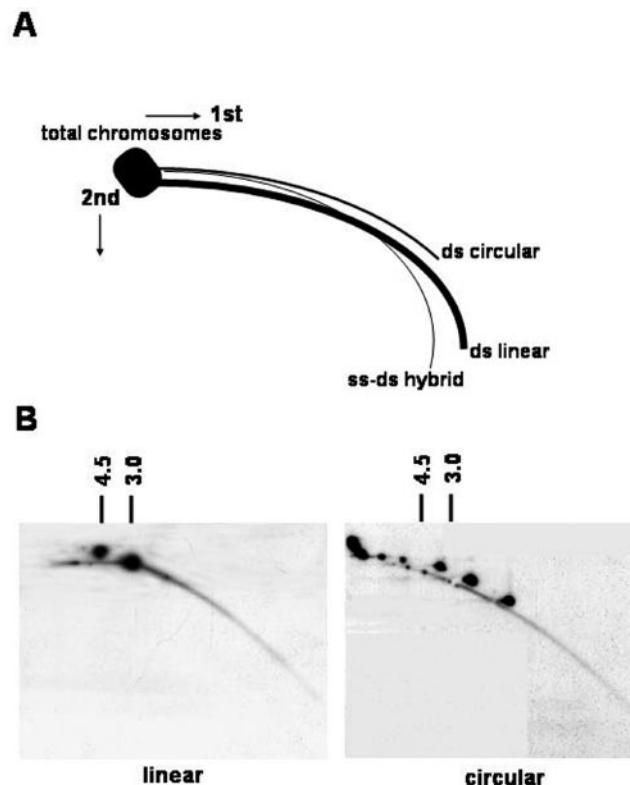


FIG. 4. Detection of extrachromosomal circular molecules by two-dimensional gel analysis. (A) Diagram of two-dimensional gel electrophoretic patterns of genomic DNA generated by populations of linear and circular molecules heterogeneous in size (adapted from previous studies [7]). Each arc consists of molecules sharing the same structure but having different masses. (B) Two-dimensional gel analysis of DNA from plasmid DNAs and yeast genomic DNAs. The *Hin*II-digested linear yeast genomic DNA samples, mixed with either *SacI*-digested pRS303 (4.5-kb) and pBSKS (3.0-kb) plasmids (left panel) or undigested plasmid circles (right panel), were separated on a two-dimensional gel. The blot was hybridized with combined probes containing yeast genomic DNA, pRS303, and pBSKS. The multiple spots of circles reflect the different topologies as well as sizes of these plasmids.

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 ^{32}P -labeled C_{1-3}A 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*

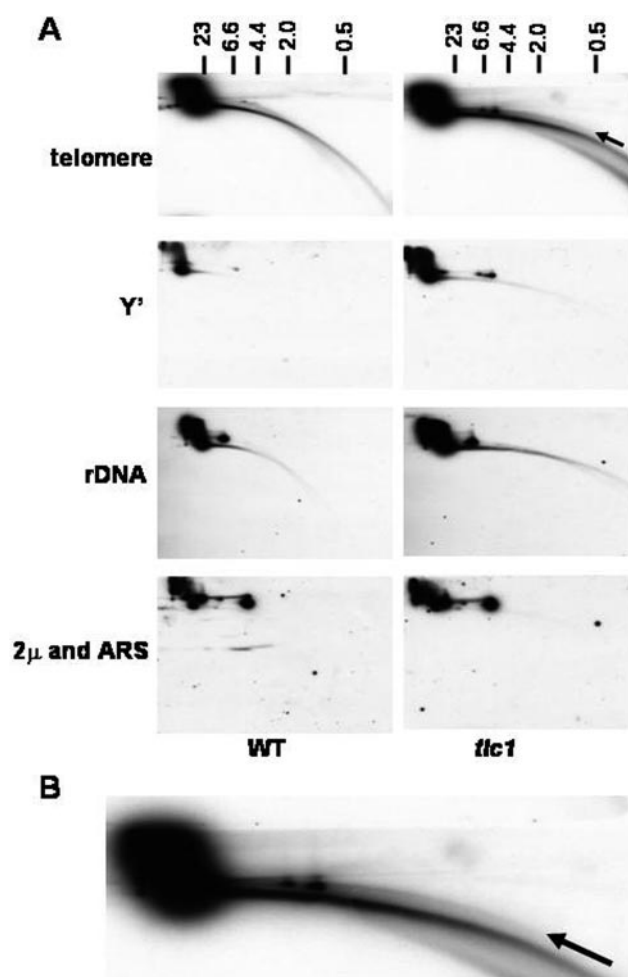


FIG. 5. Detection of extrachromosomal circular telomeres in telomerase-deficient *S. cerevisiae*. (A) Two-dimensional gel analysis of DNA from cells lacking telomerase. DNA was isolated from wild-type (WT) and *tlc1* cells at the stage when survival were being generated. Similar amounts of DNA were separated on a two-dimensional gel. The blot was first hybridized with a $C_{1-3}A$ probe to detect telomeric rings and was hybridized sequentially with Y' , rRNA genes (labeled rDNA), or ARS and 2μ m 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.

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 *tlc* cells. Other repetitive sequences in chromosomes (rRNA genes) and episomal plasmids (2μ m) 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-Amp^r* gene marker (Fig. 6A). Two independent Y' -*HIS3-ori-Amp^r* 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-Amp^r* 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-Amp^r* 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.

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 TG_{1-3} 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

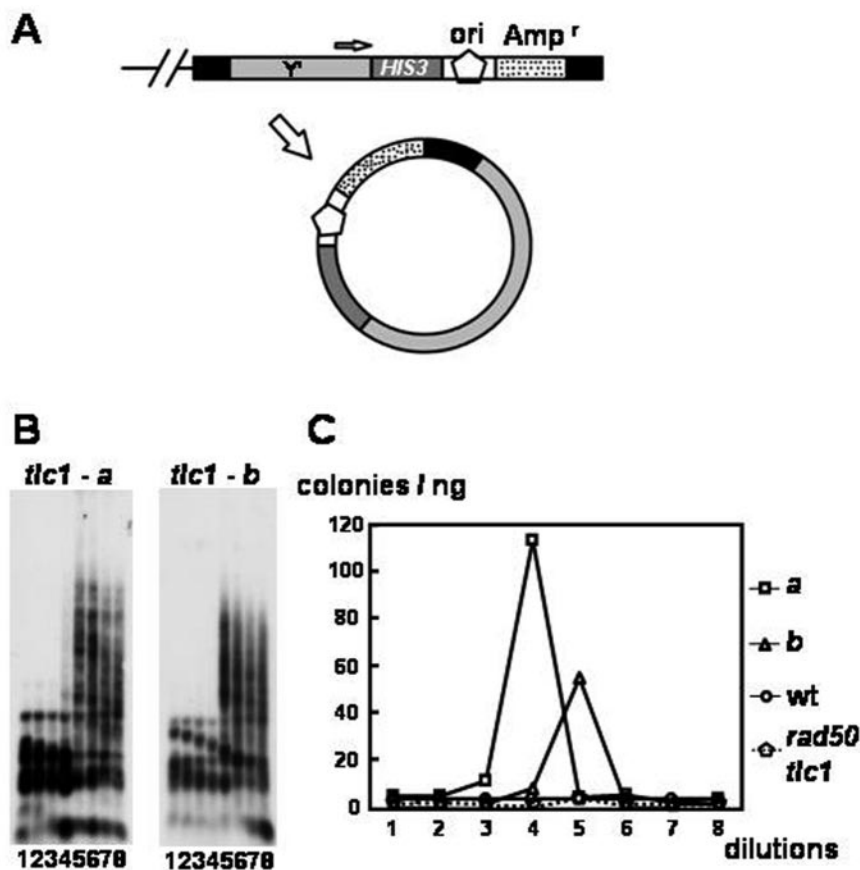


FIG. 6. Increase of extrachromosomal Y' rings in telomerase-deficient *S. cerevisiae*. (A) A marker-based, ring-releasing yeast strain. A $HIS3$ - ori - Amp^r gene marker was tagged at a telomere. We speculate that during telomerase-deficient yeast cell senescence, the whole genome may become more unstable and telomeres may loop out more easily. Black boxes indicate telomeric repeats, dark gray boxes indicate the $HIS3$ gene, light gray boxes indicate the Y' sequence, dotted boxes indicate the Amp^r gene, and pentagons indicate the *E. coli* replication origin. (B) Southern analysis of marked *tlc1* strains. Two independent *tlc1* spores (*tlc1-a* and *tlc1-b*) were subjected to a liquid serial dilution assay (see Materials and Methods). Genomic DNAs from each dilution were digested with a combination of AluI, HaeIII, HinfI, and MspI; fractionated through 1% agarose; and transferred to a nylon filter. The filter was hybridized to the $C_{1-3}A$ probe. (C) Rescue of circular DNA released from telomerase-deficient yeast telomeres. The frequencies of looped-out Y' rings in genomic DNAs were determined by the colony numbers from *E. coli* transformation. wt, wild type.

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 frag-

ments 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

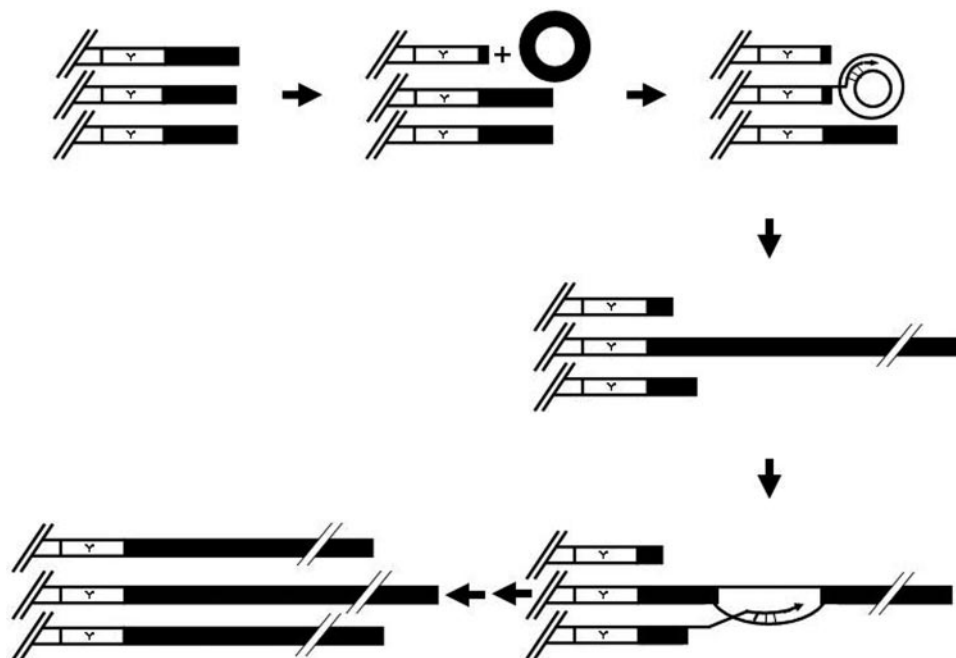


FIG. 7. A model for telomeric circle-mediated telomere-telomere recombination. See text for details.

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 TG_{1-3} sequences in TG_{1-3} -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 TG_{1-3} 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 TG_{1-3} 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 Pol δ -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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