RAD50 and *RAD51* Define Two Pathways That Collaborate to Maintain Telomeres in the Absence of Telomerase

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ABSTRACT

Telomere length is maintained by the *de novo* addition of telomere repeats by telomerase, yet recombination can elongate telomeres in the absence of telomerase. When the yeast telomerase RNA component, *TLC1*, is deleted, telomeres shorten and most cells die. However, gene conversion mediated by the *RAD52* pathway allows telomere lengthening in rare survivor cells. To further investigate the role of recombination in telomere maintenance, we assayed telomere length and the ability to generate survivors in several isogenic DNA recombination mutants, including *rad50*, *rad51*, *rad52*, *rad54*, *rad57*, *xrs2*, and *mre11*. The *rad51*, *rad52*, *rad54*, and *rad57* mutations increased the rate of cell death in the absence of *TLC1*. In contrast, although the *rad50*, *xrs2*, and *mre11* strains initially had short telomeres, double mutants with *tlc1* did not affect the rate of cell death, and survivors were generated at later times than *tlc1* alone. While none of the double mutants of recombination genes and *tlc1* (except *rad52 tlc1*) blocked the ability to generate survivors, a *rad50 rad51 tlc1* triple mutant did not allow the generation of survivors. Thus *RAD50* and *RAD51* define two separate pathways that collaborate to allow cells to survive in the absence of telomerase.

TELOMERES ensure chromosome stability by protecting chromosome ends from fusions, recombination, and degradation (reviewed in Greider 1996). In wild-type yeast *Saccharomyces cerevisiae*, the repeated telomere sequence contains ~300 bp of $d(C_{1\text{-}3}A)/d(G_{1\text{-}3}T)$ sequence. This telomere repeat sequence is bound by Rap1p and other telomere-binding proteins that participate in telomere length homeostasis (reviewed in Zakian 1996). Telomeres are elongated by telomerase, a ribonucleoprotein polymerase that contains an essential RNA and protein component(s) (reviewed in Greider 1996; Nugent and Lundblad 1998). When the gene for either the RNA component (*TLC1*) or a protein component (*EST2*) of yeast telomerase is deleted, cells exhibit progressive telomere shortening and ultimately cell death (Singer and Gottschling 1994; Landvay *et al.* 1996; Lingner *et al.* 1997). However, "survivors" that have rearranged and amplified telomere regions appear in late passage cultures. These survivors are dependent on the *RAD52*-mediated yeast recombination system (Lundblad and Blackburn 1993; Lendvay *et al.* 1996).

Recombination has been thought to play a role at telomeres for many years (reviewed in Blackburn and Szostak 1984; Zakian 1989). The repetitive nature of telomeric and subtelomeric sequences makes them a good target for recombination (Horowitz and Haber 1984; Louis and Haber 1990a). Indeed, a recombination model for telomere maintenance based on a gene conversion-type mechanism was proposed before telomerase activity was identified (Bernards *et al.* 1983; Walmsley *et al.* 1984). The ability of linear plasmids to exchange foreign telomere repeats also suggested that recombination might play a role in telomere maintenance (Pluta and Zakian 1989). The requirement for *RAD52* in the generation of survivors in late passage *tlc1* and in *est1*, *est2*, *est3*, and *est4* yeast cultures also suggested a role for recombination in telomere length maintenance (Lundblad and Blackburn 1993; Lendvay *et al.* 1996). Recently, mutations in the genes encoding the yeast Ku proteins *HDF1* and *HDF2* (also known as *YKU70* and *YKU80*) as well as *RAD50*, *XRS2*, and *MRE11* were shown to affect telomere length maintenance (Boulton and Jackson 1996, 1998; Porter *et al.* 1996; Kironmai and Muniyappa 1997; Gravel *et al.* 1998; Nugent *et al.* 1998; Polotnianka *et al.* 1998), which suggests that more than one recombination pathway may play a role in telomere maintenance.

Genes in the yeast *RAD52* epistasis group (*RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *XRS2*, *MRE11*) are involved in both mitotic and meiotic recombination in *S. cerevisiae* (reviewed in Petes *et al.* 1991; Haber 1998). They can be divided into three subgroups on the basis of their mutant phenotypes. *RAD52* appears

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to be essential for all forms of mitotic homologous recombination, which include both spontaneous and double-strand break (DSB)-induced gene conversion, single-strand annealing between directly repeated sequences, and break-induced replication (Rudin *et al.* 1989; McDonald and Rothstein 1994; Rattray and Symington 1994, 1995; Sugawara *et al.* 1995; Malkova *et al.* 1996a). In contrast, although *rad51*, *rad54*, *rad55*, and *rad57* mutants are defective in repair of chromosomal DSBs by gene conversion, these mutations do not prevent such repair in plasmid substrates with an apparently less constrained chromatin structure (Sugawara *et al.* 1995). Moreover, these four genes are also not required for single-strand annealing or for break-induced replication (McDonald and Rothstein 1994; Ivanov *et al.* 1996; Malkova *et al.* 1996a; L. Signon and J. E. Haber, unpublished results). A third group, *RAD50*, *MRE11*, and *XRS2* has a much less profound role in homologous recombination (Ivanov *et al.* 1994; Malkova *et al.* 1996b; Tsubouchi and Ogawa 1998), but mutations in these three genes severely impair nonhomologous DNA end-joining (Ogawa *et al.* 1995; Moore and Haber 1996). The division of these proteins into subgroups reflects their underlying biochemical associations. Rad51p, Rad52p, Rad54p, Rad55p, and Rad57p proteins exhibit a variety of interactions (Donovan *et al.* 1994; Hays *et al.* 1995; Jiang *et al.* 1996; Clever *et al.* 1997; Sung 1997). Rad50p, Xrs2p, and Mre11p proteins interact to form a separate complex (Johzuka and Ogawa 1995), which has been associated with exonucleolytic activity both *in vivo* and *in vitro* (Ivanov *et al.* 1994; Furuse *et al.* 1998; Tsubouchi and Ogawa 1998; Usui *et al.* 1998; Moreau *et al.* 1999).

Telomerase is activated in 80–90% of human tumor cells and immortal cell lines and is thought to be required for the long-term growth of these cells (reviewed in Autexier and Greider 1996). However, cell lines and tumors that are telomerase negative have extremely long telomeres, suggestive of a recombination-mediated telomere lengthening (reviewed in Reddel *et al.* 1997). In addition, the ability of telomerase null mouse cells from late generation animals with short telomeres to form tumors may be through a recombination-mediated pathway (Blasco *et al.* 1997). Thus, understanding the role of recombination in telomere maintenance in the absence of telomerase may have implications for tumor growth in cells that lack telomerase or where telomerase has been inhibited.

To understand the role of DNA recombination in telomere maintenance more completely, we examined telomere length in a set of isogenic yeast mutants of the *RAD52* epistasis group in both the absence and presence of the telomerase RNA component *TLC1*. Our results suggest that *RAD50* and *RAD51* define two separate *RAD52*-dependent homologous recombination pathways that collaborate to allow telomere maintenance in the absence of telomerase.

MATERIALS AND METHODS

Yeast strains, plasmids, and methods: The yeast strains used in this study are listed in Table 1. The set of isogenic strains with mutations in different RAD genes has been previously described (Moore and Haber 1996). These strains were maintained in standard growth media and grown at 30°. The *tlc1*::*URA3* disruption plasmid (pLS132A) was created by replacing an *Asp*718-*Nsi*I fragment of the wild-type *TLC1* gene with a *Hin*dIII fragment of the *URA3* gene in pLS131, a pUC119-based plasmid that contains a full-length *TLC1* *Eco*RI-*Xba*I fragment. Yeast strains were constructed by standard genetic manipulations (Rose *et al.* 1988). To construct double or triple mutants, various heterozygous *rad* diploid cells were transformed with an *Eco*RI-*Xba*I fragment of pLS132A to knock out the *TLC1* gene, and Ura$^+$ cells were checked by Southern blot to verify the correct *tlc1*::*URA3* integration. The resulting diploids were sporulated, tetrads were dissected, and spore clones were tested for the appropriate markers (some by Southern analysis). Recombination-deficient cells were also confirmed by their sensitivity to methyl methanesulfonate (MMS).

Cell culture: Cells were passaged in YPD liquid media by serial dilution to examine cell viability and telomere length. When a single spore from a dissection plate was grown to a colony we assumed ~15–20 doublings, depending on the colony size. Cells were then streaked further for single colonies and were estimated to have undergone 20–25 divisions per streakout, depending on colony size. In Figure 4b, a cell spotting assay was used to examine cell viability. Equivalent numbers of cells (measured by hemocytometer) of different genotypes were diluted serially by 10-fold in YPD medium, spotted on a YPD plate, and incubated at 30° for 2 days. To assay cell viability in culture (Figures 2 and 4a), cells were grown to saturation (10^8 cells/ml), and each day were diluted to a concentration of 5×10^5 cells/ml in fresh YPD media and the cell density measured 24 hr later by hemocytometer (Singer and Gottschling 1994). This cycle was repeated for 4–9 days. Using this assay, each day of growth represents up to 10 generations, depending on the cell density of the culture when diluted. In Figure 3, approximate cell doubling numbers were estimated on the basis of actual cell density. At each dilution point, cells were also plated to examine colony size and possible contamination, and some cells were pelleted and frozen for later telomere length analysis.

Telomere length analysis: Yeast genomic DNA was isolated, and ~2–4 μg DNA was digested with *Xho*I or *Pvu*II and separated on a 1% agarose gel. DNA were then transferred to Hybond N$^+$ (Amersham, Piscataway, NJ) membrane, UV cross-linked, and hybridized with a random primed telomeric poly(d[GT/CA]) (Pharmacia, Piscataway, NJ) probe in Church and Gilbert hybridization solution (1 mm EDTA, 0.5 m Na_2HPO_4 pH 7.2, 7% SDS, and 1% BSA) at 60° overnight. The blots were washed once at room temperature and three times at 60° with 2 × SSC and 0.1% SDS and exposed to a Fuji BAS 2000 PhosphorImager screen.

RESULTS

***Rad50*, *xrs2*, and *mre11* mutants have shortened telomeres while *rad51*, *rad52*, *rad54*, and *rad57* mutant cells do not:** Deletion of the telomerase RNA gene *TLC1* leads to loss of viability only after a phenotypic lag of 40–60 cell divisions in yeast (Singer and Gottschling 1994). Interestingly, *est1 rad52* or *tlc1 rad52* double mutant cells die much more quickly than the *tlc1* or *est1*

TABLE 1

Yeast strains used in this study

Strain	Genotype
JKM111	*MAT***a** Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3*
JKM112	*MAT*α Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3 rad1::LEU2*
JKM114	*MAT*α Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3 rad51::LEU2*
JKM116	*MAT*α Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3 rad50::hisG′*
JKM121	*MAT*α Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3 trp1::hisG′ rad52::TRP1*
JKM126	*MAT*α Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3 trp1::hisG′ rad57::LEU2*
JKM128	*MAT*α Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3 trp1::hisG′ rad55::LEU2*
JKM129	*MAT*α Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3 trp1::hisG′ xrs2::LEU2*
JKM138	*MAT*α Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3 trp1::hisG′ mre11::hisG′*
CSHY76	MAT**a**/α *ade2/ade2 ura3/ura3 leu2/leu2 his3/his3 trp1/trp1 tlc1::LEU2/TLC1 rad52::TRP1/RAD52*
CSHY90	JKM111 × JKM112 *tlc1::URA3/TLC1*
CSHY91	JKM111 × JKM114 *tlc1::URA3/TLC1*
CSHY92	JKM111 × JKM116 *tlc1::URA3/TLC1*
CSHY93	JKM111 × JKM121 *tlc1::URA3/TLC1*
CSHY94	JKM111 × JKM126 *tlc1::URA3/TLC1*
CSHY95	JKM111 × JKM128 *tlc1::URA3/TLC1*
CSHY96	JKM111 × JKM129 *tlc1::URA3/TLC1*
CSHY97	JKM111 × JKM138 *tlc1::URA3/TLC1*
CSHY102	*MAT***a**/α *ade2/ade2 ura3/ura3 leu2/leu2 his3/his3 trp1/trp1 tlc1::LEU2/TLC1*
CSHY106	*MAT***a** Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3 rad50::hisG rad51::LEU2 tlc1::URA3*
ySL43	*MAT*α Δ*hml::ADE1* Δ*hmr::ADE1 ade1 leu2 lys5 ura3 rad50::hisG rad51::LEU2*

single mutants (Lundblad and Blackburn 1993; Lendvay *et al.* 1996). One explanation for the rapid death of *rad52 tlc1* double mutants might be that the telomere length was shorter in *rad52* cells compared to wild type. To investigate this we examined telomere length in a set of isogenic strains that were mutated in genes that are known to be involved in recombination (*RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD57*, *XRS2*, and *MRE11*). For each mutant, telomere length was measured on Southern blots after prolonged cell growth by streaking cells on plates six successive times (Figure 1). Genomic DNA was prepared after one and six streakouts, digested with *Xho*I, and probed with a telomere repeat sequence. The broad band near 1.3 kb is derived from telomeres located adjacent to subtelomeric Y′ elements. The larger bands represent non-Y′-containing telomeres (Louis and Haber 1990b). *rad52* cells did not have shorter telomeres; however, *rad50*, *xrs2*, and *mre11* mutants had significantly shorter telomeres than wild-type telomeres as previously described (Boulton and Jackson 1998; Nugent *et al.* 1998). The other mutants examined, *rad51*, *rad54*, or *rad57*, and *rad1*, had telomere lengths similar to wild-type cells (Figure 1). In some samples telomere length appeared to increase or decrease between the first and sixth streakout (compare bands between 2 kb and 4 kb in Figure 1). These size variations are likely due to telomere length heterogeneity in a population of cells and the founder effect of single-colony analysis. Because a single cell gives rise to a colony, the initial cell might have had a very long telomere within the initial broad distribution. This leads to the initial long telomere length of the resulting colony (Shampay and Blackburn 1988). This effect is not seen when cells are passaged continuously in liquid media (data not shown), because founder effects do not operate under those conditions. To further resolve differences in telomere length, yeast genomic DNA was digested with *Pvu*II that does not cut within the Y′ subtelomeric region and thus allows individual telomeres to be resolved. Again *rad50*, *xrs2*, and *mre11* cells showed distinctly shorter telomeres than wild type (data not shown); similar results were also recently reported by other groups (Kironmai and Muniyappa 1997; Boulton and Jackson 1998; Nugent *et al.* 1998).

Viability declines sooner in double mutants of *tlc1* and *rad51*, *rad52*, *rad54*, and *rad57* than in *tlc1* single mutants: To investigate the role of recombination in telomere maintenance in the absence of telomerase, we constructed a set of isogenic strains deleted for both *TLC1* and *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD57*, *XRS2*, and *MRE11*, respectively. To generate these strains, the *TLC1* gene was deleted from diploids heterozygous for each mutant recombination gene and then double mutants were obtained from meiotic segregants (see materials and methods). Cell viability was examined by growing cells in liquid culture, measuring the density of the culture daily before diluting, and passing to a new culture tube (Singer and Gottschling 1994). We found that *rad51 tlc1*, *rad52 tlc1*, *rad54 tlc1*, and *rad57 tlc1* cells showed more rapid cell death than *tlc1* (Figure

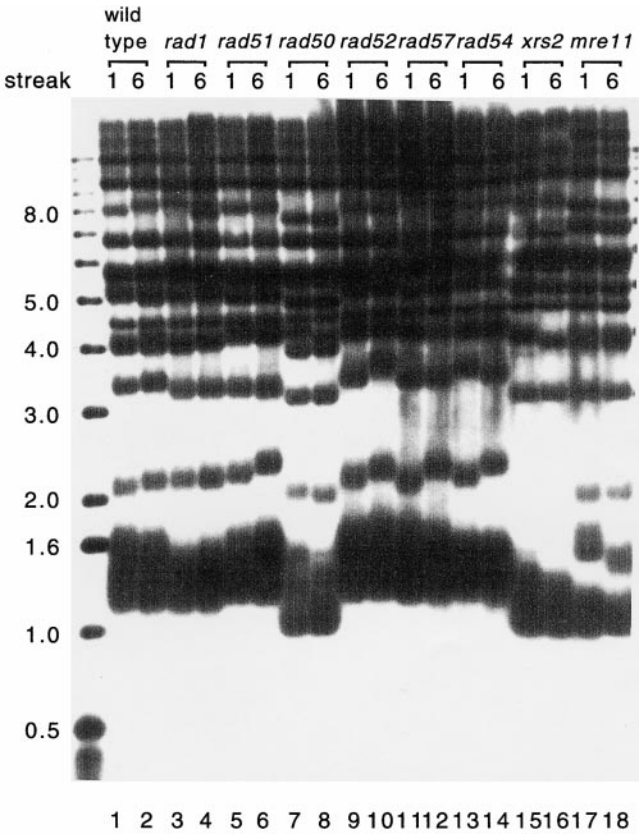

Figure 1.—Telomere length is decreased in *rad50*, *xrs2*, and *mre11*, but not in *rad1*, *rad51*, *rad52*, *rad54*, and *rad57* cells. Southern blots of *Xho*I-digested yeast genomic DNA from cells that were streaked once (odd-numbered lanes) or six times (even-numbered lanes) were hybridized with a telomere-specific probe poly(d[GT/CA]). The exact generation number of the cell divisions before the first streak cells is unknown, as these strains were not freshly derived spore clones. Relevant genotypes of the cells are indicated at the top. The lane on the far left is the ^{32}P-labeled 1-kb DNA molecular mass ladder (BRL).

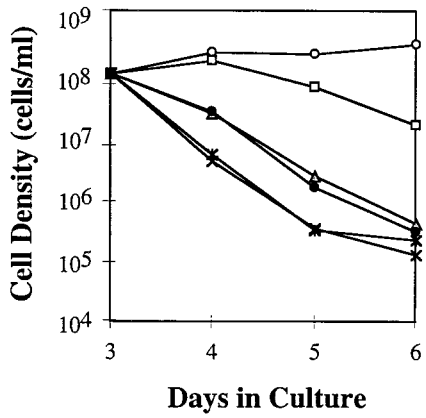

Figure 2.—Double mutants *rad51 tlc*, *rad52 tlc1*, *rad54 tlc1*, and *rad57 tlc1* have a more severe growth defect than *tlc1* alone. Cells were grown in YPD to saturation (1×10^8 cells/ml) and were diluted each day to a concentration of 5×10^5 in fresh YPD media. A hemocytometer was used as a measure of the growth rate and viability of the cells to count the total cell number in the culture each day (Singer and Gottschling 1994; see materials and methods). The cells used in this experiment had been frozen and then revived by streaking on plates before the culturing was begun. Thus the cells had already undergone ~30–35 divisions (equivalent to ~3 days of growth) since isolation as a spore clone (see materials and methods). Thus the X-axis begins at 3 days so that the viability curves are comparable to those shown in Figure 4. The cell density at each time point is plotted for wild type (○), *tlc1* (□), *rad51 tlc1* (△), *rad52 tlc1* (the X), *rad54 tlc1* (*), and *rad57 tlc1* (●).

2). The *rad50 tlc1*, *xrs2 tlc1*, and *mre11 tlc1* cells died with similar kinetics to *tlc1* mutant cells (data not shown). The fact that *rad51 tlc1*, *rad54 tlc1*, and *rad57 tlc1* cells died earlier than *tlc1* cells, like the *rad52 tlc1* mutants, implies a role for these recombination genes in telomere maintenance.

Mutations in *RAD50* group genes retard generation of *tlc1* survivors compared to *tlc1* mutants alone, while mutations in *RAD51* group genes accelerate generation of survivors: It was previously shown that survivors are generated in *tlc1* (and *est1*, *est2*, *est3*, and *est4*) late passage cultures and this process is dependent on the *RAD52* gene product (Lundblad and Blackburn 1993; Singer and Gottschling 1994; Lendvay *et al.* 1996). These survivor cells show significant amplification and rearrangement of both telomeric repeats and subtelomeric regions, and it is presumed that recombination-mediated telomere lengthening allows their sustained growth. We examined the requirement of other *RAD52* epistasis group genes for the generation of *tlc1* late passage survivors. Double mutant *rad50 tlc1*, *rad51 tlc1*, *rad52 tlc1*, *rad54 tlc1*, *rad57 tlc1*, *xrs2 tlc1*, and *mre11 tlc1* cells from freshly dissected spore clones were passaged in liquid culture continuously for >130 generations. Cell aliquots were taken every 15 generations to examine colony size by plating on YPD plates and telomere length was measured on Southern blots (see materials and methods). All double mutants grew well directly after germination and then showed a period of slow growth. Interestingly, after the slow growth period, all cultures except *rad52 tlc1* cells regained a fast growth rate that is indicative of the generation of survivors (data not shown).

To determine if the different rates of cell death in the two subgroups of recombination mutants might be due to a direct effect on telomeres, telomere lengths were examined at several representative passage points (Figure 3). For all double mutants, telomere length initially decreased as the cells were passaged (Figure 3 and data not shown). In the late passage cultures, amplification and rearrangement of the telomeric and subtelomeric region was seen in *rad50 tlc1*, *rad51 tlc1*, *rad54 tlc1*, *rad57 tlc1*, *xrs2 tlc1*, and *mre11 tlc1* cells. New bands with greater intensity of hybridization to the telomeric probe appeared in the late, but not the early passages, similar to the behavior of *est1* and *tlc1* late

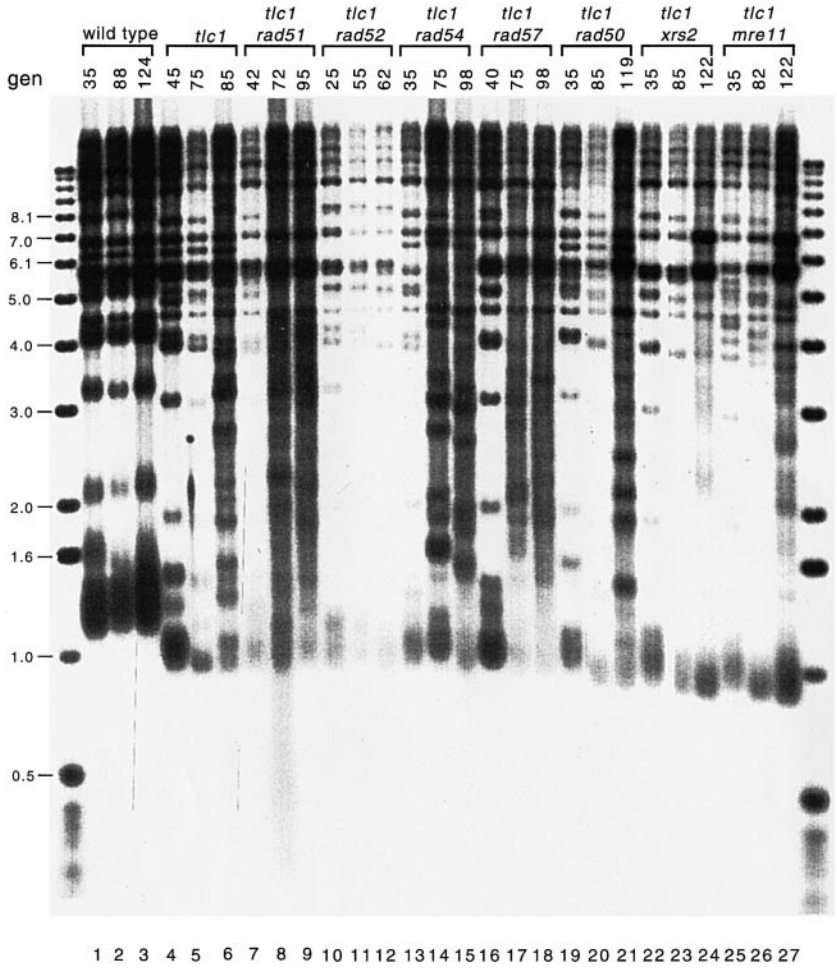

Figure 3.—Telomere length of wild type, *tlc1*, and double mutants *rad51 tlc*, *rad52 tlc1*, *rad54 tlc1*, *rad57 tlc1*, *rad50 tlc1*, *xrs2 tlc1*, and *mre11 tlc1*. Southern blot of *Xho*I-digested yeast genomic DNA from cells that were grown continuously to over 130 generations was hybridized with a telomere-specific probe poly (d[GT/CA]). Equal amounts of DNA were loaded in each lane; however, the signal is stronger in the lanes where survivors were generated because of the amplification in telomere repeats in those lanes. Three representative generations of each cell type are shown. The genotypes and approximate number of cell generations are indicated at the top.

generation survivors (Lundblad and Blackburn 1993; Lendvay *et al.* 1996). The different recombination mutants generated survivors with different kinetics. *rad51 tlc1*, *rad54 tlc1*, and *rad57 tlc1* cells showed aberrant-sized telomere bands indicative of survivors 72–75 cell divisions after germination (Figure 3). This is earlier than *tlc1* single mutant cells, which did not show such aberrant telomere bands until generation 85. In contrast, *rad50 tlc1*, *xrs2 tlc1*, and *mre11 tlc1* generated survivors later than *tlc1* cells (around generation 100), although the major *Xho*I band that represents many telomeres was initially shorter than that in *tlc1* cells (Figure 3, lanes 21, 24, 27, and see Figure 4). Thus the kinetics of generating telomeric amplifications correlated with the initial kinetics of cell death but did not correlate with telomere length in *rad51 tlc1*, *rad54 tlc1*, and *rad57 tlc1* mutants.

As reported previously, *rad52 tlc1* showed continuous telomere shortening (Figure 3, lanes 10–12) until generation 62. No survivors were generated and no evidence of telomeric amplifications or rearrangements was evident. Interestingly, the average telomere length in the last viable passage was longer than in some of the double mutants, such as *xrs2 tlc1* and *mre11 tlc1* (compare shortest telomere in Figure 3, lanes 4–6 with lanes 24 and 27), which again suggests that senescence is not strictly correlated with average telomere length. This is consistent with the observation that certain *tlc1* mutants with shorter telomeres show good viability (Prescott and Blackburn 1997).

Mutations in both *RAD50* and *RAD51* block the generation of *tlc1* late passage survivors: *rad51* and *rad50* mutations have different effects on the kinetics of cell death and the appearance of *tlc1*-independent survivors. To test whether these two mutations are part of the same pathway or different pathways in generating survivors, we made a *rad50 rad51 tlc1* triple mutant (see materials and methods). We assayed the growth of the cells in liquid culture assay (Singer and Gottschling 1994) as well as in a cell culture spotting assay (Nugent *et al.* 1998). In the liquid growth assay the triple mutant *rad50 rad51 tlc1* failed to generate survivors (Figure 4A). Each of the double mutants *rad50 tlc1* and *rad51 tlc1* generated survivors with the characteristic kinetics as described above. The *rad50 tlc1* cells generated survivors later than *tlc1* alone and the *rad51 tlc1* cells generated survivors earlier than *tlc1* seen in the previous experiment.

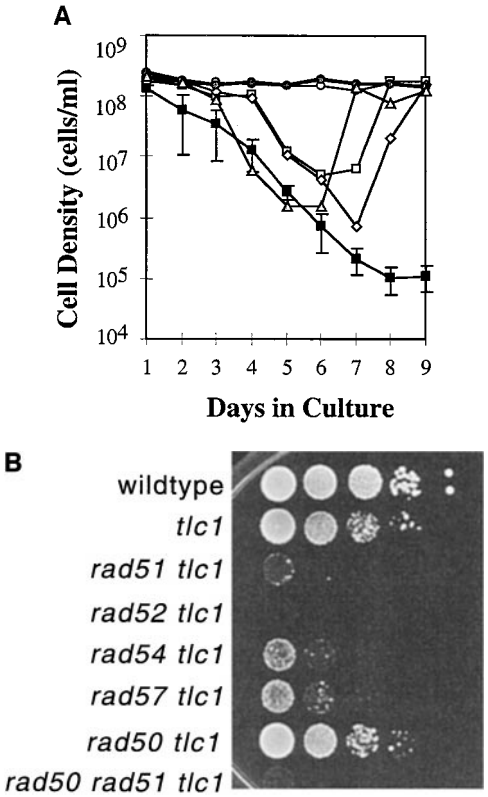

Figure 4.—Survivors are not generated in *rad50 rad51 tlc1* triple mutants. (A) A liquid growth cell viability assay was done as described in Figure 2 to assess the viability of the *rad50 rad51 tlc1* triple mutant compared to double and single mutants. Freshly dissected spore clones were used for this experiment. Four independent *rad50 rad51 tlc1* triple mutants were analyzed. The average and standard deviation is plotted for these. In this experiment only one of each of the other genotypes was assayed, although similar results were obtained in several independent experiments. Of the genotypes assayed here, only the *rad50 rad51 tlc1* triple mutant failed to generate survivors. ○, Wild type; □, *tlc1*; ◇, *rad50 tlc1*; △, *rad51 tlc1*; ●, *rad50 rad51*; ■, *rad50 rad51 tlc1*. (B) Cell viability of triple mutant *rad50 rad51 tlc1* cells compared with several other double mutant combinations. Cells were grown for 60 generations, counted, and diluted to 1×10^7 cells/ml. Serial 10-fold dilutions of each culture were spotted on a YPD plate. The lack of growth in the more dilute samples represents the loss of viability of the culture. The relevant genotypes of the cells are indicated on the left.

As an additional method to evaluate the relative viability of these cultures, we used the cell spotting assay described by Nugent *et al.* (1998). This assay was carried out on cells that had been grown for ~60 generations (similar to days 5–6 in Figure 4A). Tenfold dilutions of each culture were spotted on YEPD plates to determine cell viability. As seen previously, *tlc1* and *rad50 tlc1* were slightly less viable than wild type and *rad51 tlc1*, *rad54 tlc1*, and *rad57 tlc1* showed significantly reduced viability. Both the triple mutant *rad50 rad51 tlc1* and *rad52 tlc1* were inviable after this extent of cell growth (Figure 4B). Telomere length analysis on Southern blots showed no evidence of telomeric and subtelomeric amplification in the *rad50 rad51 tlc1* triple mutant cells (data not shown). The failure to recover survivors with telomeric amplification in the *rad50 rad51 tlc1* triple mutant is similar to what occurs in a *rad52 tlc1* strain. This result suggests that *rad50* and *rad51* mutations affect two different pathways of recombinational maintenance of telomeres.

DISCUSSION

We have analyzed telomere length and cell viability in recombination-deficient strains, which include *rad50*, *rad51*, *rad52*, *rad54*, *rad57*, *xrs2*, and *mre11*, in both the presence and absence of telomerase. These genes can be divided into three groups on the basis of their telomere phenotypes (Table 2). First, *rad50*, *xrs2*, and *mre11* single mutants showed initial telomere shortening followed by stabilization. When combined with *tlc1* mutations, this group generated survivors later than *tlc1* alone. Second, *rad51*, *rad54*, and *rad57* alone had wild-type telomere length, but when combined with *tlc1*, the double mutants died sooner than *tlc1*. Interestingly, survivors appeared at earlier generations than in *tlc1* single mutants. This earlier generation of survivors may be a direct result of the strong selection imposed by the early cell death in this group of mutants. The final group consists of *rad52* and the *rad50 rad51* double mutant; these cells died much earlier than *tlc1* single mutants and failed to generate survivors.

The grouping of these RAD genes into these categories on the basis of telomere phenotypes parallels the grouping on the basis of their recombinational phenotypes and their biochemical properties (reviewed in Kanaar and Hoeijmakers 1997). First, mutations in *RAD50*, *XRS2*, and *MRE11* have similar genetic effects on recombination (Malone *et al.* 1990; Ogawa *et al.* 1995; Ivanov *et al.* 1996). These proteins interact to form a complex (Johzuka and Ogawa 1995; Usui *et al.* 1998) that appears to provide or regulate an exonuclease function (Sugawara and Haber 1992; Sharples and Leach 1995; Ivanov *et al.* 1996; Tsubouchi and Ogawa 1998), but they also act together in other processes that do not appear to rely on exonuclease activity (Alani *et al.* 1990; reviewed in Haber 1998; Tsubouchi and Ogawa 1998; Moreau *et al.* 1999). Second, genetic evidence indicates that the *RAD51*, *RAD54*, *RAD55*, and *RAD57* genes function together in one pathway (Rattray and Symington 1995), and the proteins encoded by these genes interact physically (Hays *et al.* 1995; Jiang *et al.* 1996; Clever *et al.* 1997; Sung 1997). Although Rad52p and Rad51p proteins have been shown to interact both physically and genetically (Shinohara *et al.* 1992; Milne *et al.* 1996), it is clear that their roles in recombination are significantly different (Rattray and Symington 1994; Malkova *et al.* 1996a). There is increasing evidence of a *RAD51*-independent homologous recombination

TABLE 2

Summary of mutant phenotypes

	rad50, xrs2, mre11	*rad51, rad54, rad57*	*rad52*	*rad50, rad51* double mutant
Telomere length	Short	Wild type	Wild type	ND
Kinetics of cell death in *tlc1* background	Not distinguishable from *tlc1*	Earlier than *tlc1*	Earlier than *tlc1*	Earlier than *tlc1*
Kinetics of telomere rearrangements in *tlc1* background	Later than *tlc1*	Earlier than *tlc1*	None	None
Ability to generate *tlc1* survivors	Yes	Yes	No	No

ND, not done.

pathway that requires both *RAD52* and *RAD59* (Bai and Symington 1996).

Multiple recombination pathways play a role in telomere length: Genetic evidence indicates that there are at least three separate pathways involved with homologous recombination in yeast and that the *RAD52* gene is involved in all three pathways (reviewed in Haber 1995; Rattray and Symington 1995; Malkova *et al.* 1996a). Our data suggest that at least two independent recombination pathways are involved in telomere length maintenance. The group of mutants represented by *rad50* had short telomeres and generated survivors late. The fact that survivors were generated at all suggests that another pathway, or gene product, in addition to the *RAD50* pathway plays a role in generating survivors. Similarly, survivors were still generated when mutations of the *RAD51* group genes were combined with *tlc1*. In contrast, neither *rad52 tlc1* nor *rad50 rad51 tlc1* triple mutants generated survivors. One interpretation of these results is that two different pathways defined by the *RAD50* and *RAD51* genes are needed to generate survivors and that *RAD52* plays an essential role in both pathways.

There are two additional interpretations of the result that *rad50 rad51 tlc1* triple mutants fail to generate survivors. One is that the non-homologous-end-joining (NHEJ) and homologous recombination are both needed for the generation of survivors. However, because *RAD52* affects multiple homologous recombination pathways but has little or no effect on NHEJ pathways (reviewed in Petes *et al.* 1991; Kramer *et al.* 1994; Moore and Haber 1996; Tsukamoto *et al.* 1996), it is hard to argue that *rad52 tlc1* cells are deficient in both NHEJ and homologous recombination.

Another possible interpretation of the triple mutant phenotype is that *RAD50* and *RAD51* are in the same homologous recombination pathway but neither deletion mutant alone inactivates the pathway completely; only the double mutant completely knocks out the pathway. However, results from earlier experiments support the notion that *RAD50* and *RAD51* define two alternative homologous recombination pathways. First, a *rad51* mutation reduces spontaneous recombination between heteroalleles as much as 10-fold, while a *rad50* mutation increases recombination by about the same extent (Ivanov *et al.* 1994; Rattray and Symington 1995). Second, for interchromosomal gene conversion induced by a DSB, *RAD51* is essential, while the absence of *RAD50* has only a small effect on the efficiency of repair, although the kinetics of repair are delayed (Ivanov *et al.* 1994; Malkova *et al.* 1996b). Third, the discovery that *RAD59* defines a *RAD51*-independent, but *RAD52*-dependent pathway of recombination (Bai and Symington 1996) argues that there are indeed multiple homologous repair pathways. Finally, although a *rad51* mutation prevents gene conversion, it still can carry out break-induced replication, where one end of a broken chromosome invades a homolog and promotes replication all the way to the end of the chromosomal template (Malkova *et al.* 1996a; Morrow *et al.* 1997; Bosco and Haber 1998). Thus, we interpret the lack of survivors in the *rad50 rad51 tlc1* triple mutant as the elimination of two separate *RAD52*-dependent pathways, one requiring *RAD51* and the other requiring *RAD50*. Although the data cited above support a model where *RAD52* plays a central role, at the present time we cannot exclude a model in which the lack of survivors in the *rad50 rad51 tlc1* triple mutant is independent of the lack of survivors in *rad52 tlc1*.

Mechanism of recombination-mediated telomere elongation: Recombination-mediated telomere elongation likely occurs by a mechanism similar to break-induced replication. If a chromosome end is lost or severely shortened, the broken chromosome will only have sequences on one side of the break that are homologous to any template from which it can be repaired; thus conventional gene conversion mechanisms will not allow repair (reviewed in Szostak *et al.* 1983). A recombination-dependent replication mechanism, however, can accomplish such repair (Malkova *et al.* 1996a; Morrow *et al.* 1997; Bosco and Haber 1998). In wild-type cells, an HO-induced DSB is nearly always repaired,

most often by a gene conversion process in which the intact homologous chromosome is used as a template to copy sequences around the break. In a *rad52* diploid, >99% of the cells lose the broken chromosome. In contrast, in a *rad51* strain, half of the cells are able to repair the DSB. However, the repair events are not conventional gene conversions; instead, the broken chromosome end proximal to the DSB apparently invades the intact homologous chromosome and replicates all the sequences (100 kb or more) to the end of the chromosome. This *RAD52*-dependent break-induced replication mechanism (Malkova *et al.* 1996a) is analogous to the break-copy repair mechanism proposed by Morrow *et al.* (1997). Recent studies have found that this type of replication is efficient in wild-type cells in circumstances where gap repair cannot occur, such as when the end of a chromosome has been lost (Bosco and Haber 1998). This mechanism is similar to earlier proposals for the repair and maintenance of telomere ends (Bernards *et al.* 1983; Walmsley *et al.* 1984; Dunn *et al.* 1985). The fact that break-copy repair events can apparently occur after several cell cycles (Malkova *et al.* 1996a) suggests that chromosomes lacking telomeres in yeast might be stable through several rounds of replication, as has been previously proposed (Sandell and Zakian 1993). Thus the evidence suggests that break-induced replication is the mechanism that elongates telomeres in the absence of telomerase.

***rad51, rad52, rad54,* and *rad57* mutations accelerate the death of *tlc1* mutants:** We began this analysis with the observation that the *rad52 tlc1* double mutant dies at earlier generations than mutants in *tlc1* alone. We hypothesized that if death is simply due to telomere loss, the *rad52 tlc1* double mutant might lose telomeres at a faster rate. We saw no evidence of this in Southern blot analysis. The double mutants for *rad51 tlc1*, *rad54 tlc1*, and *rad57 tlc1* also showed a faster rate of death than *tlc1*, and yet they also showed no increase in the rate of average telomere shortening. Because our method measures the average length of telomeres, we cannot rule out a subtle effect on a subset of chromosome ends that is not evident in the Southern blot analysis.

If there is no increased rate of telomere shortening, why do the double mutants *rad51 tlc1*, *rad54 tlc1*, and *rad57 tlc1* die sooner? Additive effects of independent pathways that both affect chromosome stability may play a role. There is an accelerated rate of chromosome loss in *rad51*, *rad52*, and *rad54* mutant cells (reviewed in Friedberg 1988) and this rate is further increased by X-irradiation, which leads to an increased rate of death (Mortimer *et al.* 1981). Assuming that *tlc1* cells die, at least in part, from chromosome loss, increasing the chromosome loss rate by combining two mutations, both of which cause loss, may lead to more rapid cell death. Alternatively, DNA repair mechanisms that can normally maintain viability in the face of DNA damage may be overwhelmed when faced with too much damage.

***RAD50, XRS2,* and *MRE11* play a role in telomere maintenance even in the presence of telomerase:** The fact that telomeres are shorter in *rad50*, *xrs2*, *mre11*, *hdf1*, and *hdf2* mutants suggests that these genes are involved in telomere maintenance (Porter *et al.* 1996; Kironmai and Muniyappa 1997; Boulton and Jackson 1998; Nugent *et al.* 1998). The *RAD50*, *XRS2*, and *MRE11* genes are involved in both homologous recombination and DNA end-joining (reviewed in Moore and Haber 1996; Kanaar and Hoeijmakers 1997; Tsukamoto *et al.* 1997; Tsukamoto and Ikeda 1998). Mutations in *HDF1* and *HDF2* confer an altered chromosome end structure, which suggests that the Ku proteins function in telomere end maintenance (Gravel *et al.* 1998; Polotnianka *et al.* 1998). Double mutants of *rad50 tlc1*, *xrs2 tlc1*, and *mre11 tlc1* generated survivors after more cell divisions than double mutants of *tlc1* with *rad51*, *rad52*, *rad54*, and *rad57*. This may be analogous to the mechanism by which *rad50* and *xrs2* mutants delay but do not prevent mating-type switching in yeast (Ivanov *et al.* 1994). Perhaps unprocessed ends are not able to take part in a normal recombination process at telomeres, or, as our evidence now suggests, the absence of a *RAD50*-dependent homologous recombination pathway blocks telomere gene conversion (break-induced replication) at normal telomeres even in the presence of telomerase.

The analysis of telomere length in recombination-deficient mutants presented in this article suggests a role for DNA recombination in normal telomere maintenance in yeast. Recombination has been implicated in telomere length maintenance under special circumstances in human cells (Bryan *et al.* 1995, 1997). Some of the *RAD52* group genes have homologues in higher eukaryotes, including human (reviewed in Ivanov and Haber 1997; Kanaar and Hoeijmakers 1997). Thus it will soon be possible to test whether DNA recombination plays a similar role in mammalian telomere length maintenance.

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