Differential Processing of Leading- and Lagging-Strand Ends at *Saccharomyces cerevisiae* Telomeres Revealed by the Absence of Rad27p Nuclease

Julie Parenteau and Raymund J. Wellinger

Département de Microbiologie et Infectiologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada

Manuscript received May 28, 2002

Accepted for publication September 16, 2002

**ABSTRACT**

*Saccharomyces cerevisiae* strains lacking the Rad27p nuclease, a homolog of the mammalian FEN-1 protein, display an accumulation of extensive single-stranded G-tails at telomeres. Furthermore, the lengths of telomeric repeats become very heterogeneous. These phenotypes could be the result of aberrant Okazaki fragment processing of the G-rich strand, elongation of the G-rich strand by telomerase, or an abnormally high activity of the nucleolytic activities required to process leading-strand ends. To distinguish among these possibilities, we analyzed strains carrying a deletion of the *RAD27* gene and also lacking genes required for *in vivo* telomerase activity. The results show that double-mutant strains died more rapidly than strains lacking only telomerase components. Furthermore, in such strains there is a significant reduction in the signals for G-tails as compared to those detected in *rad27Δ* cells. The results from studies of the replication intermediates of a linear plasmid in *rad27Δ* cells are consistent with the idea that only one end of the plasmid acquires extensive G-tails, presumably the end made by lagging-strand synthesis. These data further support the notion that chromosome ends have differential requirements for end processing, depending on whether the ends were replicated by leading- or lagging-strand synthesis.

Telomeres, the complex nucleoprotein structures at the ends of eukaryotic chromosomes, are essential for chromosome integrity: they protect chromosome ends from degradation and random fusion events (McClintock 1941; Sandell and Zakian 1993), and they ensure the complete replication of the chromosomal DNA (Watson 1972; Greider 1996; Zakian 1996). Telomeric DNA is composed of short, direct repeats in the majority of eukaryotic species (Wellinger and Sen 1997). For example, vertebrate telomeric repeats can be abbreviated as C1–3A/TG1–3 (Shampay et al. 1984; Moysz et al. 1988). Virtually all telomeric-repeat sequences are similar in that they contain clusters of G residues in the strand running 5′–3′ from the centromere toward the physical end of the DNA molecule (the G-rich strand; Wellinger and Sen 1997). Most of the telomeric-repeat DNA is duplex and, in general, at the very ends of the chromosomes the G-rich strand protrudes into the G-rich strand by a variable number of nucleotides, depending on the species. For vertebrate chromosomes, this G-rich strand overhang (hereafter called G-tail) may span 50–150 bases (Makarov et al. 1997; McCelligot and Wellinger 1997; Wright et al. 1997). In certain ciliate species, G-tails are much shorter (14–16 bases) and in yeast, a similarly short overhang is suspected to be present (Wellinger and Sen 1997). However, the length of the G-tails is subject to variation during the cell cycle. For example, yeast telomeres acquire a transient ≥30-base G-tail specifically during S-phase, when the telomeres are replicated (Wellinger et al. 1993b, 1996).

Although there is mounting evidence that conventional DNA replication and the specialized replication to maintain telomeric repeats are interrelated, little is known about mechanistic details of how this coordination is achieved (Price 1997; Diele and Gottschling 1999; Adams Martin et al. 2000). All conventional DNA polymerases need a primer, usually in the form of RNA, and synthesize DNA in the 5′ → 3′ direction (reviewed in Waga and Stillman 1998). Given these properties, conventional replication is expected to leave at least primer-sized gaps on the 5′ end of the new strands that were made by lagging-strand synthesis, but also to be able to completely copy the G-rich strands on the leading-strand ends (Olovnikov 1973; reviewed in Zakian 1995). The incurring losses of terminal sequences are counteracted by telomerase, a telomere-specific reverse transcriptase, which uses its RNA component as a template for addition of new telomeric repeats of the G-rich strand onto chromosome ends (reviewed in Greider 1995; Lingner and Cech 1998; Nakamura and Cech 1998). Thus, the particularities of chromosome end replication entail a specialized enzyme for new leading-strand synthesis (telomerase) and for continued lagging-strand synthesis and a mechanism to convert the
blunt ends created at the leading-strand ends into ends with a G-tail. Previous evidence suggested a model in which a strand-specific 5′-3′ exonuclease generated a G-tail, at least at the leading-strand ends (Wellinger et al. 1996; Makarov et al. 1997), and this exonuclease could be associated directly with the DNA replication machinery (Dionne and Wellinger 1998). In light of all of the above, it is not surprising that some components of the conventional replication machinery appear to be involved in the control of telomere length (Carson and Hartwell 1985; Adams and Holm 1996; Diede and Gottschling 1999; Parenteau and Wellinger 1999; Adams Martin et al. 2000). Among them is the yeast Rad27p protein, a homolog of the mammalian FEN-1 protein (flap endonuclease 1; Harrington and Lieber 1994a,b). Deletion of RAD27 in yeast results in several distinct phenotypes, including a temperature-sensitive growth defect (Reagan et al. 1995; Sommers et al. 1995), an elevated spontaneous mutation rate (Tishkoff et al. 1997), high levels of instability of micro- and minisatellite sequences (Johnson et al. 1995; Freudenreich et al. 1998; Koroska et al. 1998; Schweitzer and Livingston 1998; Spiro et al. 1999), and sensitivity to alkylating agents like methyl methanesulfonate (Reagan et al. 1995). These identified phenotypes suggest functions for Rad27p in DNA replication and repair (Tishkoff et al. 1997; Koroska et al. 1998), and in particular, they are consistent with a proposed role of Rad27p in the removal of RNA primers during the processing of Okazaki fragments (Bae et al. 2001). Moreover, yeast cells carrying a deletion of RAD27 also display a high degree of telomeric-repeat instability (Parenteau and Wellinger 1999). However, at the telomeres, not only overall telomere repeat length, but also the particular DNA end structure is affected (Parenteau and Wellinger 1999). When cultures are incubated at the nonpermissive temperature for rad27Δ cells, an appearance of an abnormally large amount of G-tails can be detected (Parenteau and Wellinger 1999). Since the CnA strand at the end of chromosomes is synthesized by lagging-strand synthesis (reviewed in Price 1997), these results suggested a specific defect for synthesizing the C-rich strand in rad27Δ cells. Alternatively, it remained possible that the generation of the excess G-tails in rad27Δ cells was due to an interference with the coordination of telomerase activity with the lagging-strand machinery or an abnormally high activity of the nucleolytic activities required to process leading-strand ends.

To distinguish between these possibilities and to further examine the participation of telomerase in the appearance of single-stranded DNA in rad27Δ cells, we have constructed several haploid yeast strains that carry a rad27Δ mutation and mutations in genes implicated in telomerase activity. One of the predictions was that if Rad27p functions in lagging-strand DNA synthesis alone, the combined absence of telomerase activity and Rad27p in yeast cells should result in an accelerated loss of telomeric repeats. Indeed, we observe that cells with a deletion of RAD27 and lacking telomerase components are not able to grow for the same number of generations as cells lacking only telomerase. In addition, the relative signals for G-tails on telomeres derived from the double-mutant strains were significantly higher than those from wild type or telomerase-lacking strains. These data suggest that in telomeric DNA, an absence of Rad27p results primarily in incomplete synthesis of the C-rich strands, generating an excess of G-tails. These G-tails may then be further elongated in a telomerase-dependent fashion. The analyses of replication intermediates of a linear plasmid derived from rad27Δ cells further corroborate this conclusion in that they suggest that excess G-tails occur on only one end of the plasmid, presumably the end replicated by lagging-strand synthesis (Bailey et al. 2001).

MATERIALS AND METHODS

Strains and plasmids: Yeast strains used in this study are listed in Table 1. UCC3535 (gift of M. Singer and D. Gottschling; Wellinger et al. 1996) was transformed with the 2.3-kb EcoRI-SphI fragment of pMRnad27Δ::URA3 (obtained from E. Friedberg and M. Reagan; Reagan et al. 1995) to delete the RAD27 gene. The resulting strain was named RWY20. The haploids JPY204c and JPY206b were obtained after sporulation and dissection of spore tetrads of RWY20. RWY25 diploid strain was obtained by mating JPY204c and JPY206b. RWY26 was generated by transformation of the RWY25 strain with the 2.7-kb BamHI-SphI fragment of pVL152 (Lundblad and Szostak 1989; kindly provided by V. Lundblad) to delete the EST1 gene. The entire coding region of the EST1 gene was replaced by the HIS3 gene in strain RWY25 to yield strain RWY27. The est3Δ deletion fragment was generated by using plasmid pRS305 (Sikorski and Hieter 1989) and the following PCR deletion primers: EST3D-F, 5′-ATGCGGCAGAATTATCTTGGAGTCTCATGCAGGCAACAGAATTGTCTGAGAGTCCACGC-3′ and EST3D-R, 5′-GTCATATAATTATATACAAATGGGAAAAATCTTTAAACGACTGGGCTTTCACA- CCG-3′ (the underlined bases are complementary to pRS plasmids). The resulting colonies of these three transformations were monitored for deletion on one allele by Southern analysis (data not shown). RWY28 was constructed by mating JPY204c with MVL26a (obtained from V. Lundblad; Lendvay et al. 1996). The yeast strains used for two-dimensional agarose gel electrophoresis were SX46A and SX46A rad27Δ::URA3 (a gift from E. Friedberg and M. Reagan; Reagan et al. 1995). The linear plasmid YlpFAT10 was derived from the circular plasmid YEpFAT10 (Conrad et al. 1990) as described previously (Wellinger et al. 1993b).

Yeast cells were transformed by a modification (Gietz et al. 1995) of the lithium acetate method (Ito et al. 1983) and grown in standard yeast media (Zakian and Scott 1982; Rose et al. 1990). Plasmid constructions were propagated in bacteria using standard Escherichia coli strains and growth conditions (Sambrook et al. 1989).

Media, senescent phenotype, and survivors: Indicated het-


TABLE 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCC3535</td>
<td>MATα ura3-52/ura3-52 his2-801/his2-801 ade2-101/ade2-101 hisΔ200/hisΔ200 trp1Δ1/trp1Δ1 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 VR-ARDE2Δ T VR-ARDE2Δ T</td>
<td>WELLINGER et al. (1996); M. SINGER and D. GOTTSCHLING (unpublished results)</td>
</tr>
<tr>
<td>RWY20</td>
<td>MATα ura3-52/ura3-52 his2-801/his2-801 ade2-101/ade2-101 hisΔ200/hisΔ200 trp1Δ1/trp1Δ1 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 RAD2Δ7/VR-ARDE2Δ T VR-ARDE2Δ T</td>
<td>This study</td>
</tr>
<tr>
<td>RWY25</td>
<td>MATα ura3-52/ura3-52 his2-801/his2-801 ade2-101/ade2-101 hisΔ200/hisΔ200 trp1Δ1/trp1Δ1 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 RAD2Δ7/VR-ARDE2Δ T VR-ARDE2Δ T</td>
<td>This study</td>
</tr>
<tr>
<td>RWY26</td>
<td>MATα ura3-52/ura3-52 his2-801/his2-801 ade2-101/ade2-101 hisΔ200/hisΔ200 trp1Δ1/trp1Δ1 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 RAD2Δ7/VR-ARDE2Δ T VR-ARDE2Δ T</td>
<td>This study</td>
</tr>
<tr>
<td>RWY27</td>
<td>MATα ura3-52/ura3-52 his2-801/his2-801 ade2-101/ade2-101 hisΔ200/hisΔ200 trp1Δ1/trp1Δ1 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 RAD2Δ7/VR-ARDE2Δ T VR-ARDE2Δ T</td>
<td>This study</td>
</tr>
<tr>
<td>RWY28</td>
<td>MATα ura3-52/ura3-52 his2-801/his2-801 ade2-101/ade2-101 hisΔ200/hisΔ200 trp1Δ1/trp1Δ1 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 RAD2Δ7/VR-ARDE2Δ T VR-ARDE2Δ T</td>
<td>This study</td>
</tr>
<tr>
<td>JPY2046</td>
<td>MATα ura3-52 trp1Δ1/ade2-101 hisΔ200/ade2-101 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 VR-ARDE2Δ T</td>
<td>LENDVAY et al. (1996)</td>
</tr>
<tr>
<td>JPY2066</td>
<td>MATα ura3-52 trp1Δ1/ade2-101 hisΔ200/ade2-101 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 VR-ARDE2Δ T</td>
<td>REAGAN et al. (1995)</td>
</tr>
<tr>
<td>MVL26A</td>
<td>MATα ura3-52 trp1Δ1/ade2-101 hisΔ200/ade2-101 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 VR-ARDE2Δ T</td>
<td>REAGAN et al. (1995)</td>
</tr>
<tr>
<td>SX46A</td>
<td>MATα ura3-52 trp1Δ1/ade2-101 hisΔ200/ade2-101 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 VR-ARDE2Δ T</td>
<td>REAGAN et al. (1995)</td>
</tr>
<tr>
<td>SX46Amtd27Δ::URA3</td>
<td>MATα ade2 his3-52 trp1Δ1/ade2-101 hisΔ200 trp1Δ1 ade2-101 leu2Δ1/leu2Δ1 TCI/TCI Δ5-1 LEU2 VR-ARDE2Δ T</td>
<td>REAGAN et al. (1995)</td>
</tr>
</tbody>
</table>

Erozygous diploids that have the same nesenescent phenotype as a wild-type diploid were grown at 30°C and sporulated at room temperature. After tetrad dissection, only tetratypes were selected for study by growth on SC-Leu (tcl1Δ1:LEU2), SC-His (est1Δ1:His3 and est3α::His3), or SC-Ura (rad27Δ::URA3) plates. The ade122mutant was selected by its senescence phenotype. The apparent senescence phenotype (LUNDBLAD and SZOSTAK 1989) was examined for tetraspore cultures derived from RWY20, RWY26, RWY27, and RWY28 by streaking spore colonies from the dissection plates (~20 generations) onto rich growth medium (YPD) in quarter-plate sectors for 23 generations at 37°C and maintain their telomeres by telomerase-independent mechanisms (see below and LUNDBLAD and BLACKBURN 1993; LENDVAY et al. 1996; LE et al. 1999; TENG and ZAKIAN 1999; TENG et al. 2000; CHEN et al. 2001). To analyze the survivors obtained in our experiments, five more successive restreakings were performed on such healthy looking colonies to obtain colonies of ~100 generations after germination. Single colonies were grown in liquid media at 25°C for 10 additional generations for DNA analysis. DNA isolation: For Figures 2 and 3, yeast cultures were grown in YPD at the permissive temperature for rad27Δ::URA3 strains to midlogarithmic phase [optical density at 660 nm (OD660) = 0.4–0.6] and then shifted to semipermissive temperature (30°C) or to restrictive temperature (37°C) for 12 hr. Total genomic DNA from these cells was isolated using a modified glass bead procedure (HUBERMAN et al. 1987; WELLINGER et al. 1993b). For Figure 4, the SX46A and SX46Amtd27Δ::URA3 cells containing YLPAT10 were grown in SC-Trp medium at 23°C to midlogarithmic phase and shifted to restrictive temperature (37°C) for 8 hr. DNA was isolated by a “Hirt” extraction that enriched for low-molecular-weight DNA (LIVINGSTON and RUTTER 1977). Southern blot analysis, in-gel hybridization, and two-dimensional agarose gel: Agarose gel techniques, Southern blot transfers to nylon membranes, and hybridization conditions were as described previously (WELLINGER et al. 1993b). For the nondenaturing in-gel hybridization, the technique described in DIONNE and WELLINGER (1996) was used. Two-dimensional (2D) agarose gel electrophoresis was carried out essentially as described in BREWER and FAGMAN (1987). Briefly, the first dimension was run at 0.7 V/cm for ~42 hr in 0.37% agarose. For the second dimension, lanes were excised from the first dimension gel, rotated 90°, imbedded in 1.1% agarose containing 1 μg/ml ethidium bromide, and run at 10 V/cm for 4 hr in the presence of 1 μg/ml of ethidium bromide at 4°C. DNA used as probes were pVZ1 (HENIKOFF and EGHTEDARZADEH 1987) and a 22-mer of the sequence 5’-CGCCAGACACACACCGCGACCG-3’ (referred to as CA oligonucleotide; DIONNE and WELLINGER 1996). A heat-denatured 0.6-kb KpnI-KpnI fragment of Y′ sequences (LOUIS and HABER 1990) cloned in the KpnI site of pVZ1 (HENIKOFF and EGHTEDARZADEH 1987) was used as a Y′ control or as a Y′ probe. Single-stranded DNA from pGT75 (WELLINGER et al. 1993b) was obtained by standard procedures using a helper phage (SAMBROOK et al. 1989) and served as the single-stranded G-rich strand.
control DNA (labeled GT). The double-stranded control was obtained by linearization of pMW55 with BamHI. The pMW55 plasmid was made by inserting 55 bp of duplex telomeric-repeat DNA into the EcoRV site of pRS303 (Sikorski and Hieter 1989).

Quantification for all signals was obtained by storage phosphorimaging with a Storm and ImageQuant software. In the native gel, the integrated volume used comprised the entire lane, background values derived from an area lacking a signal were subtracted, and the resulting value was divided by the corrected volume integrated of the same lane in the denatured gel. For 2D gels, the area described for each experiment was integrated, a background was subtracted, and the resulting value was divided by an internal standard value as indicated. The values obtained for the wild-type strains were set as 1; values of the indicated mutant strains are expressed relative to this wild-type value. Care was taken to ensure that the signals never exceeded the capacity of the storage phosphor plates.

RESULTS

Enhancement of senescence phenotype in rad27 telomerase-minus strains: In vivo telomerase activity in yeast requires at least five genes: TLC1, encoding the RNA component of telomerase, as well as EST1, EST2, EST3, and CDC13 (for review see Nugent and Lundblad 1998; Evans and Lundblad 2000; McEachern et al. 2000). However, in vitro only Est2p and TLC1 are necessary to perform addition of telomeric sequences onto the ends of short DNA primers (Cohn and Blackburn 1995; Lingner et al. 1997), suggesting regulatory and/or coactivator functions for Est1p, Est3p, and Cdc13p (Lingner et al. 1997). Cells lacking TLC1, EST1, EST2, or EST3 or cells containing the cdc13-2 allele progressively lose telomeric sequences from their telomeres at the rate of 3–5 bp/division and die after ~80 generations (Lundblad and Szostak 1989; Lendvay et al. 1996). This delayed cell death phenotype has been called replicative senescence and is caused by the losses of terminal sequences due to telomerase inactivity and thus a lack in the ability to generate leading-strand G-rich telomeric repeats. To examine whether Rad27p contributes to telomeric-repeat maintenance via the genetic pathway defined by telomerase components or via an independent pathway, four diploid strains heterozygous for mutations in RAD27 and mutations in the four genes implicated in telomerase activity were constructed and sporulated, and tetrads were dissected. After tetratypes were identified (see MATERIALS AND METHODS), individual spores of such tetratypes were restreaked at least three times onto YPD plates to compare the senescence phenotype of double-mutant strains with that of each single-mutant strain. As shown in Figure 1 and Table 2, at 23°C, the permissive temperature for cells carrying rad27Δ, the wild type and rad27Δ segregants grow nor-
mally without any senescence phenotype. However, cultures derived from spores with mutations in the genes encoding telomerase components (i.e., tlc1Δ, est1Δ, est3Δ, or cdc13-2Δ) died at an average of 70–85 generations after germination. The double-mutant strains (i.e., strains harboring rad27Δ and one of the above mutations affecting telomerase components) died more rapidly than strains carrying only the mutations in telomerase: our analysis determined senescence to occur in these strains between 35 and 50 generations after germination (Table 2). For example, rad27Δ est1Δ double-mutant cells already yielded colonies of variable sizes with predominantly small colonies upon the first streaking (40G) after the dissection plate (Figure 1A). These small rad27Δ est1Δ colonies did not grow further whereas est1Δ single mutants began to lose viability after only 60 divisions (Figure 1A). We obtained essentially the same results for the rad27Δ cdc13-2Δ double-mutant strain (Figure 1B), as well as for the rad27Δ est3Δ and rad27Δ tlc1Δ strains (Table 2 and data not shown). As anticipated, even in the single telomerase mutants, the number of generations required for the senescence phenotype to appear is variable within a certain range (see the ranges in Table 2). While we find a similar variation for the occurrence of senescence in the double mutants, it is clear that overall senescence onset is earlier. Thus, the rad27Δ mutation caused an accelerated senescence phenotype in strains also lacking components required for in vivo telomerase activity.

Additive contribution to the generation of G-tails by Rad27p and telomerase: To establish the contribution of telomerase in the abnormally high levels of G-tails detected in rad27Δ cells grown at the restrictive temperature (Parenteau and Wellinger 1999), terminal restriction fragments derived from individual spores of some of the same tetraploid segregants as above were analyzed. Telomeric-repeat length in yeast can be assessed by digesting genomic DNA with the restriction endonuclease XhoI, which generates diagnostic terminal restriction fragments (TRFs). On DNA derived from wild-type strains, a major band of ~1.3 kb is due to a conserved site in the subtelomeric Y element that is present on most telomeres (V′ TRFs; Chan and Tye 1983). Depending on the strain background, about one-third of the telomeres do not harbor a Y′ element, and the TRFs derived from these telomeres (non-Y′ TRFs) will be longer. In addition, the overall terminal DNA configuration can be determined using a nondenaturating in-gel hybridization technique (Dionne and Wellinger 1996). In this technique, native DNA is hybridized directly to end-labeled oligonucleotides in dried agarose gels, allowing for the detection of single-stranded regions in the DNA. An example of such an analysis of four haploid spores of a tetraploid derived from the RAD27/RAD27 EST1/EST1 heterozygous diploid is shown in Figure 2. Spore cultures of the indicated genotype were pregrown at 23°C and then split and incubated at the indicated temperatures (see materials and methods). Note that rad27Δ strains will grow only a few generations at 37°C and then will stop growing (Reagan et al. 1995). Also, the phenotype in strains also lacking components required for telomerase minus (telomerase minus) (telomerase minus)

<table>
<thead>
<tr>
<th>Genotype of haploid</th>
<th>No. of individual tetratypes analyzed</th>
<th>Average no. of generations on plates (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Double mutants (rad27Δ telomerase minus)</td>
</tr>
<tr>
<td>est1Δ rad27Δ</td>
<td>8</td>
<td>40 (25–50)</td>
</tr>
<tr>
<td>tlc1Δ rad27Δ</td>
<td>10</td>
<td>35 (25–40)</td>
</tr>
<tr>
<td>cdc13-2Δ rad27Δ</td>
<td>9</td>
<td>40 (35–60)</td>
</tr>
<tr>
<td>est3Δ rad27Δ</td>
<td>3</td>
<td>50 (40–60)</td>
</tr>
</tbody>
</table>

Diploids heterozygous for rad27 and a mutation in any of four genes implicated in telomerase activity (i.e., est1, tlc1, cdc13-2Δ, or est3) were sporulated and tetrads were dissected. After tetratypes were identified, the apparent senescence phenotype was examined for individual spore colonies by streaking consecutively on YPD plates (Figure 1).
of the very same gel after DNA denaturation showed about equal amounts of DNA in each lane. In addition and as expected, telomere shortening could be seen in est1Δ strains: the length of Y' TRFs was ~1 kb in strains lacking Est1p, whereas it was ~1.3 kb in the wild type and rad27Δ cells (Figure 2B, arrows). Finally, as reported previously, TRFs became very heterogeneous in both strains lacking Rad27p and incubated at 37°C (Figure 2B, lanes 7 and 13; Parenteau and Wellinger 1999).

The relative signals for single-stranded DNA observed on DNA derived from cells incubated at 23°C and 37°C in the native gels (Figure 2A) were quantified using a PhosphorImager and corrected for DNA loading using the denatured gel (Figure 2B; see Materials and Methods). Compared to the values obtained for the wild-type strain at the same temperature, it is clear that there is significantly more single-stranded G-rich telomeric DNA in rad27Δ strains (Figure 2C). However, we consistently observed ~1.7-fold less signal for G-tails in the rad27Δ est1Δ double-mutant cells as compared to the rad27Δ strains (Figure 2C). For example, the mean values for rad27Δ cells were 12.2 (23°C) and 58 (37°C), whereas those for rad27Δ est1Δ cells were 7.3 (23°C) and 32.1 (37°C). DNA derived from cells with a deletion of EST1 alone scored indistinguishably from wild type at all temperatures. Virtually identical results were obtained with individual spore cultures of a tetratype derived from the RAD27/rad27Δ EST1/est1Δ heterozygous diploid (data not shown). These data suggest that telomerase and the defects of lagging-strand DNA synthesis occurring in yeast cells carrying a deletion of RAD27 each contribute a significant portion to the induction of the extensive G-tails.

Rad27p is not required to produce survivors in telomerase-minus cells: The accelerated death phenotype observed in the rad27Δ telomerase-minus double-mutant

![Figure 2](https://example.com/figure2.png)
strains is reminiscent of the situation observed for \( rad52 \) telomerase-minus double-mutant cells (Lundblad and Blackburn 1993; Lendvay et al. 1996; Le et al. 1999). As mentioned above, cells lacking telomerase activity die after \( \sim 80 \) generations. However, \( RAD52 \)-dependent rearrangements and amplifications of telomere regions can result in the generation of cells that maintain functional telomeres (survivors, Figure 3A; Lundblad and Blackburn 1993; Lendvay et al. 1996; Le et al. 1999; Teng and Zakian 1999; Teng et al. 2000; Chen et al. 2001). Two types of rearrangements that can lead to survivors have been characterized: type I survivors are generated by an amplification of Y′ subtelomeric elements and have very short telomeric-repeat tracts, and type II survivors contain long and very heterogeneous telomeric-repeat lengths (Lundblad and Blackburn 1993; Teng and Zakian 1999). These two types of rearrangements result in characteristic patterns of TRFs derived from DNA of the respective survivors (Figure 3B; compare lane 1, wild-type pattern, with lane 5, survivor type II pattern, and with lane 6, survivor type I pattern). In addition to a general dependence of survivors on \( RAD52 \), type I survivors are dependent on \( RAD51 \), \( RAD54 \), and \( RAD57 \) (Le et al. 1999), whereas type II survivors depend on \( RAD50 \), \( RAD59 \), \( MRE11 \), \( XR52 \), and \( SGS1 \) (Le et al. 1999; Chen et al. 2001; Cohen and Sinclair 2001; Huang et al. 2001). Since \( rad27 \) telomerase-minus mutant cells had the same accelerated death phenotype as \( rad52 \) telomerase-minus cells, we wanted to know whether Rad27p is also required for the mechanisms to generate survivors. Thus, \( Rad27 \) \( TLC1 \), \( rad27 \) \( TLC1 \), \( rad27 \) \( tlc1 \), \( Rad27 \) \( tlc1 \), and \( rad27 \) \( tlc1 \) cells were streaked onto solid media past the senescence point \((\sim 80 \) generations; Figures 1 and 3A), and after \( \sim 160 \) generations, single colonies were grown in liquid media at 23°C for 10 additional generations for DNA analysis. Genomic DNA was extracted and telomeric DNA pattern was assessed by digesting DNA with \( XhoI \). Occasional occurrence of healthy looking colonies (survivors) was very similar in streaks of \( tlc1 \) and \( rad27 \) \( tlc1 \) cells (Figure 3A). Moreover, type I as well as type II survivors can arise in strains lacking Rad27p and telomerase (Figure 3B, lane 8; type I; lane 9; type II). Consequently, Rad27p is not required to produce survivors in yeast cells lacking telomerase activity.

**Accumulation of single-stranded DNA in \( rad27 \) occurs at only one end of a linear plasmid:** Rad27p was proposed to be among the candidate exonucleases responsible for the processing of the blunt-ended telomeres left after leading-strand DNA synthesis. Using two-dimensional agarose gel electrophoresis, it was previously shown that the linear plasmid YLPFAT10 can form telomere-telomere interactions that are dependent on the presence of G-rich tails on both ends of the plasmid (Welling and Telomere Replication 1993b, 1996). In this system, circular and branched plasmid replication intermediates yield a characteristic pattern, as outlined in Figure 4, and the circular form of the plasmid (CFP) can be identified by its distinct position in the 2D gel (Welling and Telomere Replication 1993a,b, 1996). Using \( in vitro \)-generated molecules, it was also shown that for up to \( \sim 100 \) nucleotides, the fraction of DNA molecules with telomere-telomere interactions depends on the length of the TG \(_{c,r} \) tails: the longer the G-tails are, the higher the signal of circular plasmids (Welling and Telomere Replication 1996). We used this system to assess whether an absence of Rad27p caused extensive G-tails on one or on both ends of the linear plasmid YLPFAT10 (Figure 4). The prediction was that if the leading- and lagging-strand ends were to acquire the extensive G-tails, the fraction of CFP observed on the 2D gels should be significantly higher in \( rad27 \) cells. However, quantitation of the signals for...
Figure 4.—No significant difference in CFP formation in wild-type and rad27Δ cells. (Top) The migration patterns of replication intermediates of the linear plasmid YLpFAT10 in two-dimensional agarose gels (Wellinger et al. 1993b, 1996). RI1 and RI2 denote the areas used for standardization of the signal obtained for the CFP. 1N indicates the position of the linear, double-stranded, and nonreplicating form of YLpFAT10. (Bottom) DNA of the wild-type or rad27Δ/H9004 cells containing YLpFAT10 and grown at the indicated temperature was analyzed using 2D gels. Arrows indicate the gel migration directions. Randomly labeled pVZ1 plasmid DNA was used as a probe. Molecular size standards are as in Figure 2.

CFP with respect to either the signals for replication intermediates (RI1 and RI2 in Figure 4) or the linear form of the plasmid (1N in Figure 4) showed no significant difference in CFP formation, even if the rad27Δ cells were incubated at 37°C and extensive G-tails were observed on telomeres (Table 3). This result suggests that in rad27Δ cells, the generation of G-tails was confined to one end of the plasmid, which we presume is the lagging-strand end. In addition, if Rad27p was the exonuclease processing the leading-strand ends, its absence could have created a situation in which the leading-strand ends remained blunt ended after replication. In this case, we expected a decrease in the signal for CFP in rad27Δ cells as compared to wild-type cells, since G-tailed ends will not interact with a double-stranded and blunt-ended tract of telomeric repeats, at least in vitro (Wellinger et al. 1996). Within the limits of the experiment, this also was not the case (Table 3). However, all the data are consistent with the hypothesis that chromosome ends in rad27Δ cells are normally processed at one end, presumably the one synthesized by leading-strand DNA synthesis, and abnormally processed at the other end, the one made by lagging-strand DNA synthesis. Hence, Rad27p would not be the 5′-3′ exonuclease responsible for the processing of the blunt end at leading-strand telomeres.

DISCUSSION

Some components of the replication machinery appear to be involved in the control of telomere length. For example, cells carrying temperature-sensitive (Ts) alleles of DNA polymerase α (CDC17/POL1) harbor very long telomeres (Carson and Hartwell 1985; Adams and Holm 1996). In addition, mutations in the gene encoding the large subunit of replication factor C (CDC44/RFC1) and specific mutations in DNA2 result in an elongation of telomeres (Adams and Holm 1996; Formosa and Nittis 1999). Dna2p is an essential yeast replisome replicative endonuclease/helicase that may act together
TABLE 3
Formation of G-tails at only one end in rad27Δ cells grown at the permissive and restrictive temperatures

<table>
<thead>
<tr>
<th>Strains (temperature)</th>
<th>CFP/RI1</th>
<th>CFP/RI2</th>
<th>CFP/IN</th>
<th>Average ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>rad27Δ (23°)</td>
<td>0.875</td>
<td>0.85</td>
<td>1.105</td>
<td>0.94 ± 0.14</td>
</tr>
<tr>
<td>rad27Δ (37°)</td>
<td>1.18</td>
<td>0.80</td>
<td>0.965</td>
<td>0.98 ± 0.19</td>
</tr>
</tbody>
</table>

The signal in 2D gels for the circular form of the plasmid YLpFAT10 (CFP) was quantified for wild-type and rad27Δ cells. The resulting value was divided by an internal standard value as indicated (i.e., RI1, RI2, or 1N; see Figure 4). The ratios obtained in this manner for the wild-type strain were set as 1; values for rad27Δ strains grown at 23° or 37° are expressed relative to this wild-type value. The average of four measurements for each ratio is presented.

with Rad27p to remove RNA-DNA hybrids during Okazaki fragment maturation (Bae et al. 1998; Parenteau and Wellinger 1999; Bae and Seo 2000).

We have previously shown that, like a strain harboring Ts alleles of the CDC17 gene (pol1-17), strains with a RAD27 deletion display an accumulation of G-tails when grown at a semipermissive temperature (Parenteau and Wellinger 1999; Adams Martin et al. 2000). The generation of these abnormally high levels of G-tails in pol1-17 cells occurs similarly in the presence or absence of telomerase (Adams Martin et al. 2000). This suggests that in this mutant most of the G-tails are the result of a defect in the synthesis of the C-rich strand only, i.e., in the synthesis of lagging-strand DNA, with only a minor contribution of telomerase (Adams Martin et al. 2000). The data presented here strongly suggest that in cells lacking Rad27p and grown at restrictive or semirestrictive temperatures, it is also only the ends replicated by the lagging-strand machinery that are affected (Figure 4 and Table 3). However, the generation of a significant and measurable portion of the increased amounts of G-tails observed in these cells was dependent on telomerase (Figure 2C). These results may reflect the specific and different contributions of these two proteins to lagging-strand synthesis. In the pol1-17 mutants, the lagging-strand synthesis may be inefficient at the very ends of chromosomes. Since DNA polymerase α as well as DNA polymerase β are required in vivo for telomerase-mediated elongation of the 3’ TG1,5 strand (Diehe and Gottschling 1999), and DNA polymerase α appears to at least temporarily interact with components of telomerase (Qi and Zakian 2000), the detected G-tails in pol1-17 mutants would be primarily the result of incomplete C-strand synthesis. Consistent with this hypothesis, the G-tails in the pol1-17 mutants are transient in nature and occur only in S-phase, and chromosome ends display a normal DNA end structure in the next G1 phase (Adams Martin et al. 2000). In the rad27 cells, on the other hand, DNA synthesis on the lagging-strand ends may be initiated properly, but the maturation of the 5’ ends of the Okazaki fragments could be deficient. We envision that the required presence of the DNA polymerase complex for telomerase-mediated elongation at the ends does occur in these strains, yet the absence of Rad27p may cause the newly generated Okazaki fragments to be incomplete or partially degraded (Figure 5). This would explain the significant contribution of both telomerase and deficient C-strand (lagging-strand) synthesis to G-tail generation in these strains (Figure 2). We also attempted to determine whether the abnormally high levels of G-tails observed in rad27 cells were transient, as in pol1-17 cells, or were constitutive. However, despite numerous attempts using different strain backgrounds and various arrest procedures, we were unable to obtain arrested rad27 cells in any phase of the cell cycle (data not shown). While we do not know the precise reason(s) for this effect, we suspect that the high genetic instability and a previously reported link between Rad27p and the regulation of cell-cycle progression (Vallen and Cross 1995) may preclude efficient cell-cycle arrest in such strains.

Consistent with our model described above, the overall length of the telomeric-repeat tracts in rad27 cells becomes very heterogeneous (Parenteau and Wellinger 1999; Figure 2). This heterogeneity would be caused by the combined effects of shortening via the incomplete C-strings and single-stranded DNA breaks and lengthening mediated by telomerase. Such a model would also predict that in the combined absence of telomerase and Rad27p, telomere shortening should be accelerated such that these strains senesce earlier than cells lacking only telomerase. As shown in Figure 1, the results are consistent with this hypothesis: double-mutant cells die ~35–50 generations after germination, while telomerase-lacking cells die after 70–85 generations (see also Table 2). However, this accelerated death phenotype is not specific for rad27Δ telomerase-minus cells. rad52Δ telomerase-minus double mutants display a comparable accelerated senescence (Lundblad and Blackburn 1993; Lendvay et al. 1996; Le et al. 1999). Such rad52Δ telomerase-minus cells have the additional property of being unable to generate survivors, that is, cells that can maintain telomeric repeats in the absence of telomerase (Lundblad and Blackburn 1993; Lend-
telomerase-mediated elongation of the G-rich strand and subsequent failure of maturation of the Okazaki fragment. This would yield very elongated G-tails (right bottom), which would be the combined result of the failure of Okazaki-fragment maturation and telomerase-mediated elongation of the G-rich strand. G, G-rich strand; C, C-rich strand; solid arrows, wild-type Okazaki fragments; stippled arrows, Okazaki fragments in rad27Δ strains; thin lines, template strands; thick lines, newly synthesized strands; dashed line, telomerase-dependent elongation of the G-strand.

**In wild-type strains:**

![Diagram of DNA replication in wild-type cells](image)

**In rad27Δ strains:**

![Diagram of DNA replication in rad27Δ cells](image)

The data are most consistent with a defect in rad27Δ cells of the generation and/or processing of lagging strands on telomeric repeats, as described above. On any linear DNA molecule, only one telomere, the one replicated by lagging-strand synthesis, would thus be predicted to be affected by this defect. To test this hypothesis, the replication intermediates (RIs) of a short linear plasmid called YLpFAT10 were analyzed by two-dimensional gel electrophoresis. Extensive analyses of the RIs of this linear plasmid isolated from wild-type cells have shown that replication initiated in the predicted area of the plasmid and that its telomeres acquired long G-tails in late S-phase, as chromosomal telomeres do (Wellinger et al. 1995a,b). Furthermore, the isolated plasmid DNA displayed a cell-cycle-regulated propensity to form a circular DNA held together by noncovalent telomere-telomere interactions. At least in vitro, these end-to-end interactions are dependent on G-tails being present on both ends of the plasmid, and increases in the lengths of G-tails increased the efficiency of circularization (Wellinger et al. 1996). Therefore, we surmised that if the extensive G-tails observed on telomeres in rad27 cells were present on both ends of YLpFAT10, then increased amounts of circularized forms of the plasmid should be recovered. However, the amounts of circular YLpFAT10 DNA recovered from rad27 cells were very comparable to those recovered from a wild-type strain (Figure 4 and Table 3). This inability to observe increased amounts of the circular form was not due to technical limitations of the assay, as increased amounts of circular forms could be recovered from yku70 cells, in which constitutive long G-tails are present on telomeres throughout the cell cycle (Gravel et al. 1998; M. Larrivée and R. J. Wellinger, unpublished data). These results then suggest that on the linear YLpFAT10 molecule, only one of the two telomeres is affected by the absence of Rad27p. This is consistent with our hypothesis of a lagging-strand-specific defect and further suggests that the two ends are processed differently in rad27 cells. While the leading-strand ends may be processed normally, the lagging-strand ends incur losses of the newly synthesized C-rich strand and a telomerase-dependent over-elongation of the G-rich strand, leading to the excessive G-tails. Such a differential requirement of proper processing for the two different types of chromosome ends is not unprecedented: a recent study of mammalian telomeres demonstrated that in the absence of TRF2, a telomeric DNA-binding protein (Broccoli et al. 1997), virtually only leading-strand ends were involved in telomere-telomere fusions with other leading-strand ends. Lagging-strand ends ap-

**Figure 5.**—Model of DNA end structures generated in wild-type and rad27Δ strains. In wild-type strains, the lagging-strand machinery may synthesize the new G-rich strand to very near the end of the chromosome. This could activate telomerase-mediated elongation of the G-rich strand and concomitant synthesis of the C-strand, leading to elongation of both strands. In Rad27p-lacking cells, the generation of Okazaki fragments is impaired and the fragments may be degraded due to a maturation defect. However, attempts at resynthesis may occur near or at the very ends, activating...
pared not to be involved at all in such fusions in this situation (Bailey et al. 2001).

The study presented here thus underscores the differential requirements for telomere maintenance on ends replicated by leading- vs. lagging-strand synthesis. The data demonstrate that interfering with components of the lagging-strand machinery causes defects in telomeric DNA end structures, which can result in increased losses of telomeric repeats. Furthermore, the results suggest that in the particular case of Rad27p, only the ends replicated by lagging-strand synthesis are affected. This implies that the leading-strand ends may be processed normally in the absence of Rad27p and suggests that Rad27p is not part of the nucleases that act on such ends to generate short G-tails. The enzymatic activities responsible for this processing remain enigmatic, even if in one case the MRE11/RAD50/XRS2 complex has been proposed to fulfill this function (Diede and Gottschling 2001). Thus, an absence of proper processing at leading-strand ends can lead to increased telomere-telomere fusions (Bailey et al. 2001), and interfering with the machinery to generate lagging-strand ends can lead to aberrant end structures and telomeric sequence losses.

We thank V. Lundblad, M. Singer, D. Gottschling, E. Friedberg, and M. Reagon for providing strains and plasmids used in these studies. The members of the Wellinger lab are thanked for helpful discussions throughout this project. This research was supported by a grant (no. MOP 12916) from the Canadian Institutes of Health Research (CIHR) to R.J.W. J.P. was a recipient of a studentship of the Fonds pour la Formation des Chercheurs et l’Aide à la Recherche (FCAR) and R.J.W. is a Chercheur National supported by the Fonds de la Recherche en Santé du Québec (FRSQ).

**LITERATURE CITED**


Bae, S. H., E. Choi, K. H. Lee, J. S. Park, S. H. Lee et al., 1998 Dna2 of Saccharomyces cerevisiae possesses a single-stranded DNA-specific endonuclease activity that is able to act on double-stranded DNA in the presence of ATP. J. Biol. Chem. 273: 26880–26890.


Fontosa, T., and T. Nittis, 1999 Dna2 mutant reveals interactions with Dna polymerase α and Cfd4, a Pol α accessory factor, and show that full Dna2 helicase activity is not essential for growth. Genetics 151: 1459–1470.


Kokoska, R. J., L. Stefanovic, H. T. Tran, M. A. Rensick, D. A. Gordon et al., 1998 Destabilization of yeast micro- and minisatellite DNA sequences by mutations affecting a nuclease involved