Dna2 Is Involved in CA Strand Resection and Nascent Lagging Strand Completion at Native Yeast Telomeres*

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Post-replication telomere end processing involves both extension by telomerase and resection to produce 3'-GT-overhangs that extend beyond the complementary 5'-CA-rich strand. Resection must be carefully controlled to maintain telomere length. At short de novo telomeres generated artificially by HO endonuclease in the G2 phase, we show that dna2-defective strains are impaired in both telomere elongation and sequential 5'-CA resection. At native telomeres in dna2 mutants, GT-overhangs do clearly elongate during late S phase but are shorter than in wild type, suggesting a role for Dna2 in 5'-CA resection but also indicating significant redundancy with other nucleases. Surprisingly, elimination of Mre11 nuclease or Exo1, which are complementary to Dna2 in resection of internal double strand breaks, does not lead to further shortening of GT-overhangs in dna2 mutants. A second step in end processing involves filling in of the CA-strand to maintain appropriate telomere length. We show that Dna2 is required for normal telomeric CA-strand fill-in. Yeast dna2 mutants, like mutants in DNA ligase 1 (cdc9), accumulate low molecular weight, nascent lagging strand DNA replication intermediates at telomeres. Based on this and other results, we propose that FEN1 is not sufficient and that either Dna2 or Exo1 is required to supplement FEN1 in maturing lagging strands at telomeres. Telomeres may be among the subset of genomic locations where Dna2 helicase/nuclease is essential for the two-nuclease pathway of primer processing on lagging strands.

Yeast telomeres end in ∼300 bp of short repeated sequences that are GT-rich in the 3'-terminating strand and CA-rich in the 5'-terminating strand (1, 2). Yeast telomeres replicate in late S/G2, from late-firing subtelomeric origins of replication (autonomously replicating sequence). Because the replication fork most frequently proceeds outward toward the end of the chromosome, the nascent telomere GT-strand is synthesized by the leading strand polymerase, and the CA-strand is synthesized by the lagging strand polymerase. In yeast, as in many organisms, the telomeres are not fully duplex but have single-stranded GT-overhangs. Yeast GT-overhangs are about 12–14 nucleotides long in G2, and double in length during S phase (3, 4). Overhangs occur on both leading and lagging strands (5–7), but it is not known if they are generated in the same way on both ends. Synthesis of the CA-strand by the lagging strand polymerase is predicted to leave a short unreplicated segment. This could either be due to RNA primer removal or to the uncoupling of the leading and lagging strands, i.e. absence of the replisome (8), resulting in a single-stranded 3'-GT-overhang. Nucleolytic resection of the CA-strand might also contribute to the lagging strand 5'-GT-overhang. The end replicated by the leading strand assembly, however, is expected to have a blunt end and would require 5'- to 3'-nucleolytic resection to create a 3'-GT-overhang, suggesting that the overhangs arise due to resection (9). A number of helicases and nucleases are known to participate in these processes, but the precise pathways are still unclear.

The GT-overhangs are extended by telomerase, which, in effect, prevents erosion of the chromosome end (10). How the number of repeats added by telomerase is regulated and how the complementary lagging strand is synthesized and matured, referred to as CA-strand fill-in, are not completely understood. Also poorly understood is how aberrant recombination and checkpoint activation is prevented. Understanding regulation of telomerase and the DNA damage checkpoint, respectively, requires understanding the roles of numerous proteins, including Mre11, Rad50, Tel1, Xrs2, Dna2, Est1, Est3, Cdc13, Stn1, Ten1, Pif1, Rap1, Rif1, Rif2, RPA, Ku70/Ku80, and the entire lagging strand replication machinery (11–22).

Dna2 is a 5'- to 3'-helicase specific for forked substrates and a 5'- to 3'- and 3'- to 5'-exo/endonuclease (23–27). Dna2 and FEN1 are assumed to work together in the “two-nuclease” pathway of RNA primer removal during Okazaki fragment processing (22, 28–41). A third nuclease, Exo1, can substitute for FEN1 or Dna2 in some instances. It is not clear where in the genome the two-nuclease pathway is required. However, telomeres are one possibility, because replication forks are known to stall within the duplex GT-rich repeats, indicating they pose difficulties for the processive replisome and because bulk Dna2 localizes to telomeres from yeast to mammals (42–44).

Background: DNA2 helicase/nuclease participates in telomere maintenance by unknown mechanisms.

Results: dna2 mutants show slightly shortened telomeric GT-overhangs and accumulate small, nascent lagging strand DNA chains at telomeres.

Conclusion: Unlike at DSBs, additional nucleases largely compensate for the simultaneous loss of Dna2 and Mre11 in producing GT.

Significance: FEN1, Dna2, or Exo1 is necessary to mature lagging strands at telomeres.

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Dna2 has a second critical function, namely in homology-directed recombination. In conjunction with the Sgs1 RecQ helicase, Dna2 catalyzes long range resection of the 5’ strand at intra-chromosomal DSBs² to initiate homology-directed recombination and checkpoint activation (45–47). Exo1 acts redundantly with Dna2 in long range resection. Interestingly, Mre11 nucleases also function in resection and becomes essential for DSB repair in the absence of Dna2 and vice versa (46, 48).

FEN1 has also recently been detected by ChIP assays at an HO-induced DSB (in Schizosaccharomyces pombe) (49), and over- expression of FEN1 suppresses the x-ray sensitivity of dna2-1 mutants (50). It is striking that the same nucleases used in Okazaki fragment processing also function in DSB resection.

Dna2 associates in bulk with telomeres during G₁, dissociates from telomeres during S phase, moving to many other chromosomal sites, and then reassociates with telomeres at late S/G₂ phase. Just as in S phase, Dna2 is also displaced from telomeres when cells undergo bleomycin-induced DSB DNA damage (42). This release may facilitate the recruitment of Dna2 for resection and activation of the Mec1 checkpoint kinase (51, 52).

Mre11 also functions in multiple capacities at telomeres. De Novo Telomere Addition—The de novo telomere addition and resection assays were carried out as described previously (13, 21, 42). Strains UCC5913 and UCC5913 dna2-1 were grown in SC-lysine media overnight, collected by centrifugation, resuspended in YP-2.5% raffinose media, and grown to a cell density of 10⁷ cells/ml at 23 °C. Nocodazole (10 µg/ml) was added and incubation continued until 95% of the cells appeared as dumbbells. Cells were collected by centrifugation, resuspended in YP-3% galactose, and incubated at the appropriate temperature. Chromosomal DNA was isolated on Qiagen P20 columns. For telomere addition assays, the DNA was digested with SpeI, electrophoresed on a 1% agarose gel, blotted by capillary transfer onto GeneScreen Plus with 0.1 M NaOH, and hybridized with the ADE2 proximal probe. The ADE2 probe was synthesized as a PCR product created with oligonucleotides ADE2-5’- and ADE2-3’. The distal probe was synthesized as a PCR product with oligonucleotides ADH-HO and NotI-ADH.

De Novo Telomere Resection—For resection assays, DNA was digested with DdeI, electrophoresed on a 1% polyacrylamide gel, and transferred by capillary transfer onto Hybond-N+ membrane. The CA-terminating probe ACCCACCACACACCCCA at 37 °C in SSC, 50% formamide was hybridized with the telomere-specific oligonucleotide probe ACCCCACCACCCCA at 37 °C in 5× SSC, 5× Denhardt’s, 0.01 Na₂P₂O₇, 0.01 Na₂PO₄, 0.5% SDS and washed with 0.2% SSC, 0.2% SDS at 33 °C (62). The same DNAs, 0.1 volume, were also blotted with 0.1 M NaOH and hybridized with 6 × 10⁶ cpm/ml of the CA probe or with 3 ×

The abbreviations used are: DSB, double strand break; G₄, G quadruplexes; Ch, chromosomal region; RI, replication intermediate; pol, polymerase; nt, nucleotide.

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10^5 cpm/ml of a probe completely internal to the Y' element, ATTTCCGCTCAGAAGACCGGGTAAAGGT.

On neutral blots, DNA in the entire lane was quantified using the PhosphorImager and ImageQuant software. For the alkaline loading control blots, only the ~1.2-kb Y' band hybridized to the Y'-specific probe was quantified. The ratio of the neutral counts/alkaline counts was normalized with WT 0-h samples.

Flow Cytometry—Cells were processed for flow cytometry as follows: 5 × 10^6 cells were centrifuged and incubated in 70% ethanol. For staining, cells were centrifuged and resuspended in 50 mM sodium citrate, pH 7.4, containing RNase A (1 mg/ml) for 16 h at 50 °C. Proteinase K (1 μg/ml) was added for 1 h at 50 °C. The cells were sonicated, then incubated with 16 μg/ml propidium iodide, and analyzed with a FACSCalibur from BD Biosciences.

Ookazaki Fragment Analysis at Telomeres and ARS1—Chromosomal DNA was isolated using Qiagen P20 columns. Loading buffer (6× loading buffer: 0.3 M NaOH, 6 mM EDTA, 18% Ficoll, 0.15% bromocresol green, 0.25% xylene cyanol) was added at 1× concentration to each sample. 3 μg of DNA was loaded into each well of a 1% agarose gel prepared in 50 mM NaOH. After electrophoresis, the gel was incubated in 0.1 M NaOH and blotted onto GeneScreen Plus with 0.1 M NaOH. 4 × 10^5 cpm/ml of the GT probe and 10^6 cpm/ml of the ARS1 probe were added to the blots in the previously described hybridization buffer. The blots were hybridized overnight at 37 °C and washed with 0.2% SSC, 0.2% SDS at 30 °C. Lagging strand at ARS1 was GCC ATG CCA AGC CAT CAC ACG GTC TTT TAT, as deduced from Bielinsky and Gerbi (63); the GT telomeric probe was TGGGTGTGGTGTTGTTGTTGGT.

YlpFAT10 Telomere Analysis—The YlpFAT10 parent plasmid was the kind gift of V. Zakian, and linear YlpFAT10-Tel was prepared by in vitro ligation of telomeric repeats onto the ends of linearized YlpFAT10 as described (64). Strains carrying the resulting linear plasmid were characterized exactly as described and then used for telomere analysis, confirming that all detectable YlpFAT10 in each strain was linear (4, 6, 64, 65). To study GT tails on linear YlpFAT10-Tel replicating in vivo, total genomic DNA was run on a 0.8% gel, which allows easy separation of the linear plasmid from the chromosomal DNA (5). In-gel hybridization under neutral conditions was used to detect single-stranded GT tails on YlpFAT10-Tel (5). The gel was then denatured and rehybridized as loading control. Telomere length and telomere/telomere association were estimated by cleaving YlpFAT10 with NsiI and probing with leu2-d, Amp, and/or CA-rich telomere probes, as indicated. Probes used were as follows: AMP4, GCA GGA CCA CTT CTG CGC TCG GCC CTT CCG GCT GCC TGG; LEU2, GAT GAG GCG CTG GGA GCC TCC AAG AAG GCT GAT GCC GT.
RESULTS

Nuclease-defective Dna2 Is Impaired in Elongation of a de Novo Telomere—To examine the roles of nucleases in telomere synthesis, we used a strain in which telomere elongation was measured in G2 phase cells at an HO endonuclease-induced DSB having an 81-bp telomere TG/CA seed sequence inserted centromere-proximal to the HO cut site (Fig. 1A) (21). The inserted TG/CA/HO sequence was flanked on the centromere-proximal side by an exogenous ADE2 gene and on the distal side by an LYS2 gene. Upon HO cutting, a telomerase-dependent pathway efficiently added GT/CA sequences to the telomere seed sequence (Fig. 1B). To monitor extension, we cut chromosomal DNA with SpeI restriction endonuclease and followed the elongation of the SpeI-HO terminal fragment by Southern blotting using a probe recognizing ADE2. We compared WT and the temperature-sensitive lethal dna2-1 strain for the ability to elongate the telomere seed sequence (25). Our experiment was performed under completely nonpermissive conditions for the dna2-1 mutants being studied (37 °C and no HOG1 pathway inducer such as sorbitol or high salt). Telomere extension was seen in WT at 2 h and increased over the time course. Even after 6 h, little elongation is observed in dna2-1 (Fig. 1B, compare lanes 4 and 5 with lanes 9 and 10). We conclude that Dna2 nuclease is required for efficient telomere synthesis, as we showed previously for the dna2-2 mutant (42), although some synthesis eventually occurs in its absence, consistent with the observations of others (58).

Nuclease-defective Dna2 Shows Impaired Resection of 5′-CA Telomeric DNA at the de Novo Telomere, and Telomeric Sequences Delay Resection at a DSB—We used the same strain to determine whether the defect in telomere elongation in the dna2-1 mutant correlated with a defect in 5′- to 3′-CA-strand resection. After induction of the HO break, chromosomal DNA was cut with DdeI, electrophoresed on a denaturing acrylamide gel, and electroblotted. Bands were detected by hybridization with ADE2 strand-specific RNA probes that recognized the CA or GT repeat-containing strand, respectively. The smaller band (287 bp, Fig. 1C and D) results from hybridization to the endogenous chromosomal ade2-101 gene, which is unaffected by HO cutting, and is a loading control. The larger band (300

FIGURE 1. 5′-CA-strand resection and elongation of a de novo telomere are defective in dna2-1 mutants. A, schematic of experimental design (21). Details are found in the text. B, WT and dna2-1 strains were grown in raffinose-containing media, incubated with nocodazole until 95% of the cells were arrested as dumbbells, collected by centrifugation, and resuspended in medium containing galactose to induce the HO endonuclease and nocodazole to maintain cells in G2 phase. Cells were incubated for 0, 1, 2, 4, and 6 h at 37 °C; chromosomal DNA was isolated, cut with SpeI, electrophoresed on an agarose gel, and blotted to GeneScreen Plus, and the blot was hybridized with the ADE2 probe. The band at about 3 kb is the SpeI fragment spanning the ADE2-HO-LYS2 construct. The ~0.7-kb fragment corresponds to the SpeI-HO cut fragment. The band at about 1.6 kb represents the endogenous ade2-101 gene and serves as an internal standard to normalize loadings. These experiments were repeated twice with similar results. C and D, dna2-1 cells are deficient in both 3′-GT extension and 5′-CA-strand resection at de novo telomeres. DNA2 and dna2-1 cells were grown, and the HO cut was introduced as in B. Cells were incubated for 0–4 h at 30 or 35 °C, as indicated. Samples were collected at each time point and harvested, and chromosomal DNA was isolated and cut with DdeI, electrophoresed on a 5% denaturing acrylamide gel, electroblotted onto a nylon membrane, and hybridized with an ADE2 riboprobe recognizing sequences adjacent to the 3′-GT-terminated strand (C). The blot was stripped and then rehybridized to a riboprobe recognizing the 5′-CA-terminated strand (D). The Ddel-HO fragment is 300 nt. The 287-nt band below it is the internal standard corresponding to the endogenous ade2-101 locus. C, 3′-GT extension is inhibited in dna2-1. D, 5′-CA-strand resection is defective. Quantification of 300-bp hybridization intensity (Int300) normalized to the 287-bp loading control (Int287) in D is shown in the graph as the mean ± S.E., n = 2. E, resection of the nontelomeric end at the dna2-1 telomere is delayed. UCC5913-2-1 dna2-1 cells were grown in raffinose-containing media at the permissive temperature, 23 °C, incubated with nocodazole until 95% of the cells were arrested as dumbbells, centrifuged, and resuspended in media containing 3% galactose and nocodazole. After incubation for an additional 0–4 h at 30 °C, nonpermissive for dna2-1 cells, were collected; chromosomal DNA was isolated, cut with SpeI, electrophoresed on a 1% agarose gel, and alkaline-blotted onto GeneScreen Plus. SpeI, instead of DdeI used in C, is used to reveal the distal site. Left panel, blot was hybridized with the centromere-distal probe, a PCR product synthesized by oligonucleotides ADH4-HO and NotI-ADH4. The band disappears as resection occurs past the SpeI site. Right panel, blot was stripped and reprobed with the proximal probe, a PCR product synthesized using oligonucleotides ADE2–3′ and ade2–5′. The upper band in the doublet represents the internal ade2-102 gene, which should not change, and serves as an internal standard. The two SspI-ADE2 bands (proximal) are more difficult to separate than the two Ddel-ADE2 (proximal) bands that are seen in C.
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bp) band appeared only after HO endonuclease induction and represented the DdeI-HO fragment containing the 81-bp telomere seed repeats. When the blot is probed with the riboprobe specific for the 3'-GT-terminating ADE2 strand, the 300-bp band is detected at all time points, indicating that it is not degraded in either WT or dna2-1 (Fig. 1C). The smear of hybridization above the 300-bp band in WT indicated that the GT-terminating strand was elongating and that, on average, 150 nt were added. The smear was absent in the dna2-1 strain, confirming that telomere elongation is defective, consistent with the results shown in Fig. 1B.

When the blot is probed with the riboprobe specific for the 5'-CA-terminating ADE2 strand, to measure resection, the 300-bp band clearly disappears over time in wild-type strains and is barely detectable at 4 h (Fig. 1D). In the dna2-1 mutant, however, the CA-strand is detectable for up to 4 h, showing that resection does occur but at a dramatically reduced rate (Fig. 1D, blot and graph). The defect in resection occurs at 30 and at 35 °C, both of which were completely nonpermissive for dna2-1 growth. The defect in resection of the CA-strand in the dna2-1 mutant is similar to the defect in resection observed in the rad50Δ strain by Diede and Gottschling (13), and we propose that Dna2 participated in efficient CA-strand degradation. Although resection was delayed, some degradation is observed in the dna2-1 strain at 6 h, in keeping with the results of others that a complementary, Exo1-dependent pathway exists (58).

Because it has been reported that telomeric sequences inhibit repair, resection at the centromere-distal side of the HO break, lacking telomere seed sequences, was compared in the same samples where resection on the proximal side, carrying the seed sequence, was assessed. Resection clearly occurred more efficiently on the distal terminus lacking the 81-bp telomeric repeat sequence (Fig. 1E, compare 1- and 2-h time points), demonstrating that the telomeric sequences give rise to a structure that inhibits telomeric end processing in the G2 phase of the cell cycle, as has been shown previously in wild-type cells (21). Similar inhibition of repair of a DSB by telomeric sequences has recently been noted for nonhomologous end joining in G1 phase yeast cells and in mammalian cells (66).

dna2 Mutants Are Defective but Not Blocked in GT-overhang Production in S Phase at Native Telomeres—The de novo telomere is a model for the blunt-ended leading strand telomere. It was generated in G2, after DNA synthesis was complete, and had only a 4-bp 3'-overhang. The de novo telomere sequence used here was 81 bp, about 200 bp shorter than a native telomere. We wondered if Dna2 was also required at native telomeres in S phase, which was when long replication-associated GT-overhangs appeared, and if Dna2 was required for both leading and lagging strands. We therefore monitored the appearance of telomere 3'-GT-overhangs on native chromosomal telomeres in cells undergoing a synchronous cell cycle. For these experiments, we used a dna2Δ mutant, and thus all strains, except the wild-type (WT) control, contain a pif1Δ mutation, which suppresses the lethality of dna2Δ (53).

Cells were arrested with α factor and then released into the cell cycle at 30 °C, a semi-permissive temperature. At various times after release, DNA was purified and cut with Xhol, which cleaves Y'-containing telomeres ~1.2 kb from the end (67), yielding terminal fragments differing in length only by the extent of telomeric tracts (Fig. 2A). Subtelomeric Y' elements were found in about half of all yeast telomeres, depending on the strain. Neutral Southern blotting was performed with a telomere-specific CA-rich oligonucleotide probe to detect single-stranded GT DNA. Fig. 2A shows the time course of appearance of single-stranded GT DNA as G1 cells enter and traverse the S phase after α factor release. The position of the Y'-telomere population was indicated. The hybridization signals extending upward from the ~1.2-kb region represent overhangs on the yeast telomeres that lack Y' elements and therefore have longer heterogeneous Xhol terminal fragments. A loading control is shown (Fig. 2A, DENATURED, which also indicates relative telomere length). In-gel hybridization resulted in large losses of DNA in the critical 1-kb range in our hands, accounting for the use of the more cumbersome but more quantitative blotting methods described here.

In wild-type G1 cells, we observed no long GT-overhangs (0 min) by this method, as shown previously. Unlike the de novo telomere in dna2-1 mutants, elongated 3'-GT-overhangs were detected at 45 min in all strains, including dna2Δ pif1Δ (Fig. 2A, lanes 3, 6, and 9). At this time point, all strains were in S phase as determined by flow cytometry. The apparent delay in the dna2Δ pif1Δ strain was not reproducible (see Fig. 3) and coincided with a delay in the cell cycle (data not shown). The single-stranded DNA is at the terminus and is not internal, because, in a second experiment (Fig. 2B), treatment of samples before electrophoresis with bacterial exonuclease 1, which degrades ssDNA from 3'-OH termini, eliminated the 3'-GT signal in all of the strains at all S phase time points. Quantification (data not shown) by normalizing the signals in Fig. 2, A and B, NATIVE, to the loading controls, DENATURED, indicated that 3'-GT tails appear only moderately, if at all, at reduced levels in dna2Δ pif1Δ compared with pif1Δ strains. From these data, it was clear that Dna2 was not exclusively required for resection, but it was not clear whether the slight reduction was significant.

3'-GT-overhangs are not detected in telomere blots of wild-type strains in G1, but we noticed a reproducible G1 phase population of long chromosomal 3'-GT tails in the pif1Δ mutants. As reported previously, this G1 population is not apparent in our wild-type control strain (Fig. 2A, compare lane 1 with lane 4 and with lane 7). The detection of single-stranded DNA in G1 may be due to the fact that pif1Δ strains contain inherently longer telomeres than wild type, and telomere length is known to regulate resection (68). Although the GT-overhangs increase over the G1 level during S phase in the pif1Δ mutants (Fig. 2A, lanes 5 and 6), just as they do in wild type, we were concerned that the overhangs observed in dna2Δ pif1Δ might only be found in the absence of Pif1.

To determine whether GT-overhangs arise at native telomeres in the absence of Dna2 when Pif1 helicase was present, we repeated our studies in a different strain background. We used a dna2Δ rad9-null (rad9-320) mutant. We have shown that rad9-320, a rad9 mutation with a stop codon at amino acid 320, which was null for essential Rad9 functions, suppresses the inviability of dna2Δ (69). As shown in Fig. 2, C and D, as in wild type, these strains lack overhangs in G1. Overhangs lengthened in S phase in all strains, including dna2Δ rad9-320. Thus, the...
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**FIGURE 2. Analysis of single-stranded GT-overhangs at native telomeres in dna2Δ strains.** A, schematic of Y' telomere. B, determination of overhang extension in S phase. WT (BY1408), pif1Δ (MB121), and dna2Δ pif1Δ (MB161B) were grown at 30 °C, arrested with α factor, and then released into a synchronous cell cycle. Samples were taken at 0, 30, and 45 min after release. DNA was prepared and cleaved with XhoI to release terminal fragments. The fragments were separated by gel electrophoresis and blotted to GeneScreen Plus under neutral/NATIVE conditions as described under “Experimental Procedures.” The blots were probed with a telomere-specific CA-rich oligonucleotide probe, which detects GT-overhangs. All samples were from the same gel but were rearranged in Photoshop for logical presentation. Notice, for instance, seam between samples 5 and 6 and 6 and 7. DENATURED, the same samples (0.1 volume) were analyzed after alkaline blotting as a loading control for XhoI-cut DNA. Only the terminal −1.2-kb XhoI fragments (i.e. from Y' telomeres) are shown. Gels were not run long enough to determine telomere length differences in the respective strains. These experiments were repeated three times with the same result. (Flow cytometry of these strains is shown in Fig. 3, C and D). B, single-stranded DNA is at the telomere termini and is not internal. Analysis of XhoI-cut DNA from synchronized S phase cells was after cleavage with *Escherichia coli* 3'-exonuclease I. NATIVE, cells were arrested with α factor and released into the cell cycle. S phase samples with GT-overhangs were taken from WT and pif1Δ cells at 35 and 45 min, and samples were taken from dna2Δ pif1Δ at 40 and 55 min to account for the slower progression through the cell cycle of the double mutant. XhoI-digested DNA samples were divided in half, and one-half was treated with bacterial exonuclease 1 as described under “Experimental Procedures.” Samples were then analyzed by neutral blotting as in A (NATIVE). DENATURED, loading control. The same samples (0.1 volume) were analyzed after alkaline blotting. Note that the gel was not run long enough to provide sufficient resolution to estimate telomere length in the mutants, which we have reported previously. C, dna2Δ rad9-320 strains are defective in producing long single-stranded overhangs at native telomeres. The experiment was performed as described in A and B. The NATIVE gel was hybridized to the CA probe. The DENATURED loading control was hybridized to a Y'-specific probe to avoid differences in signal due to different telomere lengths in the various strains. The amount of single-stranded DNA was normalized to the loading control and zero time point. D, quantification of data in C. Note that lanes 10–12 are a repeat of lanes 7 and 8 using a different clone of the same strain and show similar results. E, flow cytometry of samples analyzed in C and D.
absence of Pif1 was not responsible for the appearance of GT-overhangs in dna2Δ pif1Δ. Although overhangs occur in the absence of Dna2, we found a reproducible 2-fold reduction in GT-overhangs in dna2Δ rad9-320 strains compared with wild type or rad9Δ. Also shown is the fact that the rad9Δ and the dna2Δ rad9-null double mutant telomeres, unlike dna2Δ pif1Δ telomeres, are the same length (Fig. 2C, mutant 1, lanes 7–9) or shorter than in WT (Fig. 2C, mutant 2, lanes 10–12), suggesting rad9Δ does not affect telomeres. Although it has never been directly demonstrated, it is generally and reasonably accepted that the reduction in GT-overhang signal corresponded to shorter overhangs and therefore to a defect in resection of the 5′-CA-strand. Therefore, we conclude that Dna2 participates in one resection pathway at native telomeres, just as has been shown for its partner Sgs1 helicase (58), but that other pathways must also exist and can more efficiently compensate for loss of Dna2 at native telomeres than at the short de novo telomere.

Interaction of Dna2 and Mre11 Nuclease at Native Telomeres—Because Mre11 nuclease is required for resection of x-ray-induced DSBs in the absence of Dna2 and vice versa (46), we expected that a dna2Δ mre11-nuclease minus double mutant would have a greater defect in formation of GT-overhangs than dna2Δ. Mre11, the nuclease component of the Mre11 Rad50 Xrs2 (MRX) complex, was one of the nucleases creating 3′-GT-overhangs. MRX deletion mutants have very short telomeres with abnormally short GT-overhangs (5, 12, 60). MRX can support resection in the 5′-GT-overhangs. MRX deletion mutants have very short telomeres with abnormally short GT-overhangs (5, 12, 60). MRX is associated primarily with leading strand telomeres (5, 9, 13). MRX is required for an early step in processing of DNA DSBs at internal chromosomal loci (45, 61). Even though the MRX exonuclease has 3′ to 5′ directionality, rather than the expected 5′ to 3′ activity, MRX can support resection in the 5′ to 3′ direction, either by forming a hairpin and using its associated endonuclease activity or as a bidirectional nuclease reaction involving both MRX endonuclease, exonuclease, and Dna2 or Exo1 5′-exonuclease (70, 71). The N terminus of Mre11 contains the phosphodiesterase active site of both the endonuclease and 3′ to 5′-exonuclease of Mre11 (71–74). Mutations mre11-D56N and mre11-H125N each inactivate both endo- and exonuclease activities and result in a strain with mild sensitivity to DNA-damaging agents but no shortening of the telomere nor defects in the lengthening of 3′-GT-overhangs during S phase (75). This implies that other nucleases, such as Dna2, also participate.

The appearance of 3′-GT-overhangs was investigated in the dna2Δ mre11-nuclease minus pif1Δ mutant. (We have not been able to construct a viable dna2Δ mre11-nuclease minus rad9-320 mutant.) As shown in the NATIVE blot in Fig. 3, 3′-GT-overhangs increase in S phase in two independent dna2Δ mre11-nuclease minus pif1Δ triple mutants (Fig. 3, A, lanes 11 and 12, and B, lanes 6–12), just as they do in wild-type, single, and double mutant strains (Fig. 3A, lanes 1–10). (Flow cytometry profiles are shown in Fig. 3, C and D.) The normalized amounts of S phase-overhangs in dna2Δ mre11-D56N pif1Δ were similar to those in the dna2Δ pif1Δ, and inactivation of Mre11 nuclease did not abolish resection. Although GT-overhangs are detectable in G1 phase, it is significant that the GT-overhang signal increases from G1 into S phase in the dna2Δ mre11-D56N pif1Δ and dna2Δ mre11-H125N pif1Δ strains as it does in wild-type cells (Fig. 3, A, B, and E). When the native blots were hybridized with a probe complementary to the Y′/GT boundary, no signal was observed, showing that the 3′ ssDNA did not extend into the Y′ region (data not shown). Results are quantified in Fig. 3E, verifying that overhangs are not only present in dna2Δ mre11-nuclease minus but that they are longer than in dna2Δ. The presence of 3′-overhangs in a dna2Δ mre11-nuclease-minus strain was completely unexpected, especially because such mutants were completely defective in DSB repair. The differences in Y′-telomere lengths revealed in the alkaline blot of the same samples is a subject for further study (Fig. 3, A and B, DENATURED).

Exo1 was also redundant with Dna2 in resection at intra-chromosomal DSBs. We were unable to construct a viable dna2Δ exo1Δ pif1Δ much less a dna2Δ exo1Δ mre11-H125N pif1Δ mutant, presumably because such mutants were inviable, and we could therefore not evaluate GT-overhangs in a triple nuclease-deficient mutant.

Analysis of Telomeres on Linear Plasmids in dna2 mre11 Mutants—To confirm the anticipated ability of dna2Δ and mre11-nuclease minus mutants to produce long 3′-overhangs, we used the linear plasmid YLPFAT10-Tel (64). This short linear plasmid has normal length telomeres, replicates to high copy number, and acquires terminal GT-tails >30 nt in length in late S phase, retaining shorter tails even in G1 phase (4–6, 65). As such, YLPFAT10 offers a higher sensitivity and a higher resolution adjunct to the chromosomal telomere blot data in Fig. 3. We compared telomere overhangs on YLPFAT10 grown in mre11-nuclease minus, dna2Δ, and mre11-nuclease minus dna2Δ double mutant strains. All of these mutants were in a pif1Δ background. Synthetic telomeres were ligated onto YLPFAT10 in vitro; the plasmids were introduced into the various mutant strains, and transformants carrying unit length and linear YLPFAT10-Tel of ~8–9 kb were identified as described previously (data not shown) (6). Fig. 4A shows a schematic of YLPFAT10-Tel. Because YLPFAT10-Tel is greater than 8 kb in length but is well separated from chromosomal DNA upon gel electrophoresis, in-gel hybridization was used to monitor single-stranded telomeric tails (and telomere length) (6). Respective YLPFAT10-Tel transformants were synchronized with α factor (0 min) and released into S phase for 45 min. Plasmid DNA was analyzed at both time points (Fig. 4B, odd-numbered lanes, α factor; even-numbered lanes, S phase). As on chromosomes, single-stranded GT tails arose in pif1Δ strains both in

FIGURE 3. dna2 mre11-nuclease-deficient double mutants are not defective in production of single-stranded DNA in S phase at native telomeres. A, top panel, indicated strains were grown at 30 °C, arrested with α factor, and released into the cell cycle as described under “Experimental Procedures.” Samples were collected at the times indicated, cleaved with XhoI, and analyzed by neutral blotting for single-stranded DNA (NATIVE) and alkaline blotting as a loading control (DENATURED) as described in Fig. 2 and under “Experimental Procedures.” Hybridization was carried out with the same probe for both gels. B, indicated strains were treated as in A, C, and D, flow cytometric analysis of cell cycle progression of strains used in A and B, E, quantification of overhang data, mean ± S.E., n = 3 for WT, dna2Δ pif1Δ, dna2Δ pif1Δ mre11-H125N; n = 2 for pif1Δ dna2Δ mre11-H56N. The amount of single-stranded DNA in each was normalized to the loading control rehybridized with a Y′-specific probe. Normalization was to 0 time.
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**FIGURE 4.** Long GT-overhangs appear in S phase on linear YLpFAT10-Tel in dna2, mre11-nuclease minus, and in dna2 mre11-nuclease minus double mutants. A, schematic map of YLpFAT10-Tel, about 7.5 kb plus the length of the telomeric repeats, B, overhangs on YLpFAT10-Tel. The respective strains carrying linear YLpFAT10 were grown at 30 °C, arrested with α factor, and released into the cell cycle for 45 min. Upper panel, genomic DNA was isolated and separated on a neutral gel. In-gel hybridization with the CA probe hybridizing to GT-overhangs was carried out as described (6). The gel was then soaked in NaOH to denature the DNA and, after neutralization, rehybridized with the CA probe to determine the total GT DNA. Lanes 1 and 2, MRE11 DNA2 pif1Δ, 0 and 45 min after release from α factor; lanes 3 and 4, mre11-H125N pif1Δ, 0 and 45 min; lanes 5 and 6, dna2Δ pif1Δ, 0 and 45 min; lanes 7 and 8, mre11-H125N dna2Δ pif1Δ, 0 and 45 min. C, to estimate telomere length and to monitor telomeric DNA on both ends of the plasmid, the same samples were cut with NsiI and separated by gel electrophoresis. Southern blots were hybridized with the CA oligonucleotide (top panel). The blot was then washed and rehybridized with a LEU2 probe to the 2.5-kb end as loading control, copy number control, and length control (middle panel). Finally, the blot was washed and hybridized with the Amp probe to confirm the 3.5-kb end (bottom panel). Lanes 1 and 2, pif1Δ, 0 and 45 min after release from α factor; lanes 3 and 4, mre11-H125N pif1Δ, 0 and 45 min; lanes 5 and 6, dna2Δ pif1Δ, 0 and 45 min; lanes 7 and 8, mre11-H125N dna2Δ pif1Δ, 0 and 45 min. These experiments were duplicated (using independent transfectants) with the same quantitative results for GT-overhangs, steady-state GT tract length, and steady-state plasmid copy number. D, loading control from chromosomal DNA for C. The blot shown in C was hybridized to a RAD9 probe to normalize for single copy DNA content.

**TABLE 2**

| Strain | Min | Exp. 1 | Exp. 2 |
|--------|-----|--------|--------|
| pif1Δ  | 0   | 1*     | 1      |
| pif1Δ  | 45  | 0.75   | 0.73   |
| pif1Δ mre11-125 | 0   | ND     | 0.70   |
| pif1Δ mre11-125 | 45  | ND     | 0.38   |
| pif1Δ dna2Δ | 0   | 0.53   | 0.06   |
| pif1Δ dna2Δ | 45  | 0.56   | 0.09   |
| pif1Δ dna2Δ mre11-125 | 0   | 0.56   | 0.06   |
| pif1Δ dna2Δ mre11-125 | 45  | 0.49   | 0.06   |

* The phosphorimager counts in the neutral gel (Fig. 4B) were divided by the LEU2 counts or by the Amp counts (Fig. 4C) for each strain. The average of the two results was normalized to the average for the pif1Δ strain at 0 min and are the values reported.

G1 and in S phase. More importantly, single-stranded GT tails arose on YLpFAT10-tel, not only in MRE11 DNA2 wild type (Fig. 4B, lanes 1 and 2) and in the mre11-H125N or dna2 single mutants (Fig. 4B, lanes 3 and 4 and 5 and 6, respectively) but also in dna2 mre11-H125N double mutants (Fig. 4B, lanes 7 and 8). Thus, the overhangs in the dna2 mutant were not arising because Mre11 nuclease is a backup for Dna2 or vice versa. We also noted that just as overhangs at chromosomal telomeres were shorter in dna2Δ rad9Δ than in rad9Δ, plasmid telomere overhangs were shorter in dna2Δ pif1Δ than in pif1Δ. Normalized results of overhang length measurement are presented numerically in Table 2. These observations indicate that resection nucleases indeed interact differently at telomeric repeats than at intra-chromosomal DSBs (Figs. 3, A and B, and 4). These observations suggest that other nucleases, Exo1 or others, are efficient in producing telomere overhangs in the absence of both Dna2 and Mre11.

We noticed a difference in intensity of the GT hybridization among the strains. This difference was also observed after denaturation of the gel and rehybridization with the CA probe. The difference in hybridization was not accounted for by YLpFAT10-tel copy number, because hybridization with YLpFAT10-tel internal LEU2 or ampicillin probes did not differ to the same degree, and loading of chromosomal DNA was approximately equivalent among the strains (Fig. 4, C and D). These results suggested that the difference in intensity of bands in Fig. 4 reflects the amount of GT/CA tract present on the plasmids, i.e. the equilibrium telomere length attained in each mutant. To estimate total GT tract length directly, the same DNA samples were treated with NsiI, which produces one terminal ~2.5-kb YLpFAT10 fragment containing the leu2-d gene and a second terminal ~3.5-kb fragment carrying the Amp resistance gene (see Fig. 4A). After gel electrophoresis and Southern blotting, both the ~2.5- and ~3.5-kb fragments reveal terminal GT tracts (Fig. 4B, CA probe). The length of these restriction fragments differ in plasmids from the different strains, indicating the difference in the steady-state length of the telomeres (Fig. 4B, LEU2 and Amp4 probes). pif1Δ dna2Δ had shorter telomeres than pif1Δ, as in Fig. 3 and as we have shown previously. pif1Δ dna2Δ mre11-H125N also carried shorter telomeres than pif1Δ. The YLpFAT10 telomeres appear very long (>1 kb) compared with the native Y′ telomeres (<0.5 kb) (compare Figs. 4C with 3, A or B, DENATURED). This significant derepression of YLpFAT10 telomere length may suggest that the high density of nucleosomes at the Y′ regions negatively regulates telomere length (76).

GT-overhangs Also Accumulate in Mutants Lacking Both Dna2 and Exo1—At de novo telomeres, Exo1 (yeast exonuclease 1) can compensate for loss of Dna2 in long range resection (58), but this has not been investigated at native telomeres. Because dna2Δ exo1Δ is inviable even in pif1 or rad9 backgrounds, we used dna2-2 mutants for this experiment. We chose dna2-2, because dna2-2, like dna2-1 (Fig. 1), is defective in de novo telomere elongation (42), but dna2-2 exo1Δ mutants grow much better than dna2-1 exo1Δ mutants. To ensure good
growth, we propagated dna2-2 exo1Δ on medium or high osmotic strength 0.5 M sorbitol (77), which induces Hog1 stress response kinase that stabilizes replication forks experiencing replication stress (78). Suppression of growth defects by sorbitol was not complete, because dna2-2 exo1Δ cells were temperature-sensitive (37 °C) for growth on sorbitol-containing medium. All strains were nevertheless shifted to glucose upon shift to 37 °C. GT-overhangs were difficult to detect in asynchronously cycling wild-type cells but accumulated in temperature-sensitive cdc13-1 mutants when they were shifted to 37 °C, where uncapping occurred due to the temperature-sensitive single-stranded GT-DNA-binding protein, Cdc13. Therefore, GT-overhangs of wild type and cdc13-1 were compared, as negative and positive controls, to GT-overhangs in the dna2-2 exo1Δ mutant (Fig. 5A). When WT-, dna2-2, or exo1Δ strains were shifted to 37 °C, there was no or little accumulation of long single-stranded GT-overhangs (Fig. 5A, lanes 4–11). When dna2-2 exo1Δ double mutants were incubated at 37 °C, however, an increase in single-stranded GT-overhangs was observed (Fig. 5, A, lanes 15 and 16, and B, loading controls, and C, quantification). A role in resection should have led to a decrease rather than an increase in GT-overhang. The increase in GT-overhangs is similar to what was observed in rad27Δ mutants, lacking FEN1, where long single-stranded G tails accumulate even though FEN1 is not required for telomere overhang generation by resection (79). Given the role of FEN1 in Okazaki fragment maturation, our results suggested to us that GT-overhangs might increase in dna2-2 exo1Δ mutants due to a failure of the lagging strand replication apparatus to complete the CA-rich, discontinuously replicated strand (CA-strand fill-in). The dna2-2 exo1Δ telomeres are also significantly longer than those in wild type (Fig. 5B). This phenotype is also similar to that seen in polα mutants (80, 81) and therefore could be due either to a defect in joining of replication intermediates or a defect in fill-in synthesis by polymerase.

**Dna2 Is Required for Efficient Nascent Strand Maturation at Native Telomeres**—Single-stranded DNA can in principle occur at telomeres due to a failure to complete replication of the lagging strand template (either before or after telomerase action) or due to the uncontrolled resection of ends at uncapped telomeres. The unexpected appearance of extended DNA overhangs at native telomeres in dna2-2 exo1Δ mutants prompted us to look directly for enhanced accumulation of lagging strand-specific, low molecular weight, nascent replication intermediates (RIs) at telomeres in dna2 mutants. To establish that we could see nascent intermediates likely to reflect inhibition of lagging strand processing, we used a cdc9-1 and a cdc9-1 rad9Δ double mutant. The rad9Δ checkpoint mutation allows for more complete replication of the genome in S phase and was used to ensure that telomeres, which replicate late in S phase, actually underwent DNA replication (82, 83). DNA was isolated from cdc9-1 or cdc9-1 rad9Δ cells that had been synchronized with α factor, released at 36 °C, and then allowed to pass through S phase at the nonpermissive temperature. We used alkaline gels and Southern blotting to identify replicative intermediates at telomeres, using a GT-rich telomere-specific probe.
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We then carried out the same experiment in dna2-1 and dna2-1 rad9Δ strains. dna2-1 contained a P504S mutation in the nuclease domain that severely inhibited both the intrinsic nuclease and the helicase/ATPase and is temperature-sensitive for growth even at 28 °C. Cells were released from G1, arrest into S phase at 36 °C for 55 min. To quantify the efficiency of converting the RIs into chromosomal sized DNA, the counts in DNA that were 2.5 kb or smaller in size were determined using the PhosphorImager and ImageQuant software. This size range was chosen because it is even smaller than the size reported previously for nascent DNA in a similar analysis of RIs at ARS1 in the presence of hydroxyurea (84). To normalize results in the different strains, the RIs in each lane were divided by the counts from the chromosomal region (Ch) of the lane, and the RI/Ch ratio is reported below each lane. (Similar relative amounts were obtained if RIs smaller than 5 kb were quantified.) Wild-type strains efficiently ligated nascent chains at ARS1, although the efficiency of ligation in this experiment appeared reduced at the telomere. The accumulation of RIs at telomeres in dna2-1 and dna2-1 rad9Δ mutants at telomeres was slightly, although reproducibly, greater than wild-type cells (Fig. 6A, lanes 1, 6, and 11). At ARS1, dna2-1 mutants were nearly as proficient as wild-type cells at converting RIs into chromosomal sized fragments. The ARS1 hybridization establishes that the single-stranded DNA observed at telomeres was not due to DNA degradation during sample preparation.

Because Dna2 is partially functional in dna2-1 mutants, which might account for the high level of residual RI maturation, we next assayed the ability of dna2Δ strains to join nascent fragments at telomeres and ARS1, using the dna2Δ rad9-320 strain employed in the experiment in Fig. 2. In Fig. 6B, we compared wild type and dna2Δ rad9-320 mutants for nascent fragment maturation. At telomeres, the defect in joining nascent fragments in dna2Δ rad9-320 was almost as severe as in cdc9-1 (compare Fig. 6, A and B). The defect in dna2Δ rad9-320 was slightly less than in cdc9 mutants, as expected because reduction of Dna2 may delay the maturation of RIs into chromosomal sized fragments, but it is not expected to result in fragments as short or long lived as observed in cdc9 mutants, due to redundancy of Dna2 with Exo1 or FEN1. The RI/Ch ratio for dna2Δ strain at telomeres is 0.7. At ARS1, in contrast, the RI/Ch value for dna2Δ drops to 0.02, comparable with WT, which drops to 0.01 (Fig. 6B), suggesting efficient conversion of RIs into chromosomal size fragments at ARS1. When the blots were hybridized to a probe recognizing the repetitive ribosomal ARS, the result was the same as that with ARS1, demonstrating that the defect in Fig. 6B, lane 3, was not due to nonspecific degradation in the sample (data not shown).

These results suggest that Dna2 plays a major role in maturation of RIs at telomeres, even when both FEN1 and Exo1 were present. This is the first demonstration of a locus in vivo where Dna2 and the proposed two-nuclease (Dna2 plus FEN1) pathway of lagging strand maturation were functioning.

DISCUSSION

Our studies suggest that Dna2 participates in both telomeric GT-overhang production and CA-strand fill-in (see model in Fig. 7), consistent with the two major known activities of Dna2 that hybridizes to the CA-rich, discontinuously synthesized DNA. We then reprobed the same blot with a lagging strand oligonucleotide from ARS1, representing an early firing internal origin of replication, for comparison. We observed short lagging strand fragments, both within the telomeric repeats and at ARS1 (Fig. 6A, lanes 2 and 3 and 7 and 8). This method can therefore detect nascent DNA both at telomeres and at internal sites. The RIs at ARS1 are of a discrete size compared with a broader size distribution that is reproducibly observed at telomeres, possibly attributable to the nucleosome-free nature of telomeric repeats (83). The intermediates at ARS1 are the same length as observed previously (84) but are slightly longer than the monomeric Okazaki fragments recently described (83), which is probably explained by the fact that the recent study used a more stringent Cdc9 shutoff, i.e. transcriptional repression and degron induction.

FIGURE 6. Both dna2-1 and dna2Δ rad9-320 mutants are defective in nascent DNA maturation at telomeres. A comparison of nascent telomeric DNA in cdc9-1 and dna2-1 mutants. Wild type, cdc9-1, or cdc9-1 rad9Δ mutants were synchronized with α factor and released into S phase for 45 min (WT) or 60 min (cdc9-1 and cdc9-1 rad9Δ) at 36 °C. Genomic DNA was isolated and subjected to alkaline gel electrophoresis to release newly synthesized DNA. Nascent DNA was identified with a telomeric probe (left panel) or an ARS1 probe (right panel), specific for the lagging strand at ARS1, after Southern blotting. The telomere-specific probe was GT-rich, because the nascent DNA from the lagging strand is CA-rich. Ch marks chromosomal sized DNA, and RI indicates DNA shorter than 3 kb. dna2-1 and dna2-1 rad9Δ mutant strains were released with α factor and grown for 60 min at 36 °C before DNA isolation to allow for their slower cell cycle progression. Blots were hybridized to telomere-specific GT probe (left panel) or ARS1 lagging strand probes (middle and right panels). These experiments were repeated at least once with similar results. The RI/Ch ratio, reported below the blots, was determined as described in the text, and the values reported are the average of several experiments (lane 1, n = 1; lane 2, n = 1; lane 3, n = 2; lane 4, n = 2; lane 5, n = 2. B, dna2Δ mutants are defective in nascent DNA maturation at telomeres. The indicated strains were grown at 30 °C after α factor release but were otherwise treated as in A and probed with a telomeric GT-rich probe. Left panel, lanes 1–3, or an ARS1 lagging strand probe; right panel, lanes 4–6. Results are the average of n = 2.
Telomere 5′ CA resection and Okazaki Fragment Synthesis

A Coupled Leading and Lagging Strand Synthesis

B Leading Strand

C Uncoupled Lagging Strand

D Leading Strand 5′ Resection

E Lagging Strand

F Lagging Strand Primer Initiation

G Lagging Strand OF Synthesis

H Lagging Strand OF Processing 5′ Resection

FIGURE 7. Telomere DNA replication and the proteins involved. A replication fork is initiated in the subtelomeric region creating forks that replicate templates (black lines) outward toward the telomere. The GT-rich strand uses the leading strand polymerase, and the CA-strand uses the lagging strand polymerase. Left leading strand, synthesis by the leading strand polymerase results in a chromosome with a blunt end. This end is analogous to the HO endonuclease cut chromosome in the de novo telomere assay used in this study. The appearance of the normal 3′-GT-overhang on the leading strand therefore requires 5′-to 3′-nuclease processing. Subsequent binding of Cdc13 would allow telomerase binding and synthesis. Nucleases MRX, Sae2, Dna2, and/or Exo1 may be required for efficient 5′-to 3′-CA resection (21, 58, and this work). Right lagging strand, pol δ (lagging strand polymerase) arrives at the telomere significantly later than pol ε (leading strand polymerase) (90). After leading strand replication is completed, the helicase presumably falls off, and the leading and lagging strand syntheses are uncoupled. This supports the idea that a 3′-GT-overhang, having at least the size of an Okazaki fragment, appears on the chromosome synthesized by the lagging strand. Further evidence suggesting that a 3′-GT-overhang arises on the lagging strand during its replication is that in an in vitro SV40 linear DNA replication assay, leading strand synthesis replicates the DNA to the end but lagging strand synthesis leaves a 500-bp single-stranded gap at the end (102). The appearance of RPA at telomeres coincides with the arrival of pol ε suggesting that RPA binds the GT template. In mammalian cells, a special process replaces RPA with telomere-specific DNA-binding proteins that do not activate the checkpoint, and we propose the same occurs in yeast for RPA and Cdc13 (103, 104). These overhangs recruit telomerase, recruit protective telomere capping proteins, such as Cdc13, and themselves can form protective structures such as t-loops (13, 105, 106). 3′-GT tails are the primer for synthesis by telomerase.

indicated by genetic studies (28, 42, 45, 46, 85, 86). Dna2 is known to be required for the two-nuclease, Dna2 plus FEN1, Okazaki fragment processing pathway from genetic and biochemical studies, but this pathway has been proposed to occur only at a subset of sequences in vivo, based largely on in vitro studies of the coordination of pol δ, proliferating cell nuclear antigen, FEN1, and Pif1 (34–40). Now we provide in vivo evidence for the “two-nuclease” model by demonstrating that Dna2 plays a role in RI maturation at telomeres. Thus, even in the presence of FEN1 and Exo1, Dna2 is involved in conversion of RIs to chromosomal length DNA at telomeres. DNA ligase 1, which is required for both the one-nuclease and two-nuclease pathways, is also required for RI joining at telomeres. We were able to detect RIs at the putative lagging strand of the internal early firing origin of replication in the ligase-defective mutant but not in the Dna2 mutants. Although we were surprised to find that single-stranded GT tails accumulate at native telomeres in dna2 exo1 double mutants, this is also consistent with a role in lagging strand maturation because it is the same phenotype as mutants deficient in DNA polymerase α, which initiates Okazaki fragments, or mutants defective in FEN1 (62, 79, 80, 87). We propose that in the absence of faithful lagging strand synthesis and processing, single-stranded DNA accumulates. The question remaining is why the two-nuclease pathway is required, perhaps preferentially, at telomeres. One possibility is that it has to do with telomeric proteins (Pif1, Cdc13, Rap1, Rif1, and Rif2) influencing replication fork stalling in the repeats, inducing long flaps and a requirement for Dna2. A second possibility is that Dna2, which binds G4-containing DNA more avidly than single-stranded DNA, unwinds G4
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DNA, and cleaves G4 DNA, is required to remove inhibitory G4 structures arising either in templates or at stalled forks (43, 54). A more intriguing possibility is that it may be due to the lack of nucleosomes in the repeats, which correlates with the fact (Fig. 6) that the nascent DNA intermediates are longer at telomeres than at ARS1 (this work) or at other internal ARSs (83). Finally, it is possible, as in human cells, that leading and lagging strands become uncoupled before CA-strand fill-in, and therefore fill-in is related to but is not a bona fide Okazaki fragment process (8). Our results show that this process nevertheless requires the same machinery involved in Okazaki fragment processing.

We also verified a significant role for Dna2 in telomere resection at de novo telomeres. We observed a greater defect in both de novo telomere elongation and in resection than did Bonetti et al. (58), however, who saw defects only in dna2 exo1 double mutants. This is most likely explained by the fact that Bonetti et al. (58) used conditions semi-permissive for dna2-1 growth in high salt, whereas our experiments were carried out under fully restrictive conditions. (They needed to do this because dna2-1 exo1Δ double mutants are inviable in standard growth media.) High salt and high osmotic media suppress the growth defects of many replication mutants (21), and the mechanism has recently been traced to induction of the stress-activated protein kinase, Hog1, which phosphorylates components of the replication fork such as Mrc1, stabilizing stressed replication forks (78). Interestingly, dna2-1 hog1Δ strains are inviable at any temperature (88). Previously, Gottschling and co-workers (21) established that pol1 mutants that were defective in telomere elongation at the restrictive temperature were competent under semi-permissive temperature conditions, in keeping with the effects being specific for the penetrance of the mutations; in fact, they were even hyperactive. The differences could also be due to the use of different strains, W303 in Bonetti et al. (58) and S288C in our work. In addition to differences in strain background, the de novo telomere reporter constructs differed, as did the integration of the dna2-1 mutation. Bonetti et al. (58) also failed to detect a resection defect in dna2Δ pif1-1m2. pif1 strains themselves affect telomeres. In addition, they are defective in break-induced replication, which may allow increased resection at the de novo telomere. Their result may mirror our results with native telomeres in dna2Δ pif1Δ, i.e. very little reduction in overhangs (Figs. 2 and 3), and be due to the fact that Exo1 plays a larger role in pif1 mutants (58, 89). In any case, we conclude from the reduction and delay that we see that neither Mre11 nor Exo1 can efficiently compensate for the complete absence of Dna2 at a de novo telomere under normal growth conditions when nuclear Pif1 helicase is present.

More novel is our finding that resection is different at native telomeres (and on linear plasmids with steady-state telomeres) than it is at internal DSBs or at de novo telomeres. Either Dna2 nuclease or Mre11 nuclease is required for resection and repair at a DSB, even in pif1Δ (46). At a de novo telomere both Mre11 and Dna2 nucleases appear to be required for efficient resection. However, at chromosomal telomeres synthesized during S phase, GT-overhangs, and therefore resection, can occur in the absence of both Dna2 and Mre11 nucleases (at least in a pif1Δ strain). The movement of the replication fork through the telomere presumably allows resection in the absence of both Dna2 and Mre11 nucleases. The leading strand polymerase, pol ε, may play a critical role in allowing nucleases to process telomere ends, perhaps by uncoupling leading and lagging strands (90). Sgs1 is a partner of Dna2 in DSB resection, and Sae2 is a partner of Mre11 nuclease. sgs1Δ sae2Δ mutants are defective in GT-strand overhang production (58). The difference between dna2Δ mre11Δ nuclease minus strains and sgs1Δ sae2Δ mutants is significant because it must therefore indicate that another nuclease, perhaps Pso2, can carry out resection and that it is modulated by Sgs1 or MRX/Sae2. Pso2 is a 5′-exonuclease related to Apollo, which interacts with MRN and participates in resection of mammalian telomeres (91, 92).

An unanticipated finding was a reproducible G₁ phase population of long 3′-GT tails at both native telomeres and on YLPFAT10-Tel in the pif1Δ mutant. Pif1 helicase is an inhibitor of telomerase (93), and the G₁ phase overhangs may be due to excessive telomerase elongation. Pif1 has also been demonstrated to participate in Okazaki fragment processing, however, where it can act as an accessory factor for pol δ, displacing the RNA/DNA primer to produce the long RNA/DNA flaps that require the two-nuclease pathway for RNA/DNA removal (39, 40, 53, 94). Another explanation for the appearance of the telomeric overhangs is, accordingly, incomplete synthesis of the CA-rich strand by pol δ and then further extension of the GT-strands by telomerase in the absence of Pif1. Similarly, the long GT tails could arise as a consequence of enhanced telomere repeat addition in the absence of Pif1 inhibition of telomerase during S phase, leaving the lagging strand polymerases unable to keep up. However, enhanced telomerase addition does not necessarily result in greater single-stranded GT tails, because overproduction of Tel1 in a strain with short telomeres stimulates telomere addition and checkpoint activation without an increase in 3′-GT tails (95).

Although the relative contributions of the various nucleases at native telomeres remains to be sorted out, the participation of Dna2 in de novo telomere synthesis and resection in Saccharomyces cerevisiae appears to be conserved at chromosomal telomeres in S. pombe. It is analogous to the requirement for Dna2 for efficient telomerase synthesis and CA-strand degradation in S. pombe (96). In S. pombe, the appearance of 3′-GT tails at telomeres in taz1Δ strains is significantly reduced in a taz1Δ dna2-2C mutant (97). Taz1 is a telomere GT/CA DNA-binding protein, and replication forks stall at telomeres in taz1Δ strains (98, 99). This gives rise to a rapid shortening of telomeres that are similarly rapidly repaired by telomerase-catalyzed synthesis of very long telomeres with extensive single strand overhangs (99). These overhangs are generated by resection and fail to appear in dna2-2C mutants (97). This result with taz1Δ mutants generalizes to telomeres of strains even in the presence of normal Taz1, because the appearance of 3′-GT tails and the binding of telomerase to normal telomeres is also defective in the dna2-2C mutant. The dna2-2C mutation is in the helicase domain, between helicase motifs II and III (85), but because nuclease and helicase are coupled (100), it may also have compromised nuclease activity. Conservation may extend even further, because mouse embryonic fibroblasts defective in DNA2 exhibit telomere replication defects (43). It will be interesting to
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