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Specific DNA Replication Mutations Affect Telomere Length in *Saccharomyces cerevisiae*

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To investigate the relationship between the DNA replication apparatus and the control of telomere length, we examined the effects of several DNA replication mutations on telomere length in *Saccharomyces cerevisiae*. We report that a mutation in the structural gene for the large subunit of DNA replication factor C (*cdc44*/*rfc1*) causes striking increases in telomere length. A similar effect is seen with mutations in only one other DNA replication gene: the structural gene for DNA polymerase α (*cdc17*/*pol1*) (M. J. Carson and L. Hartwell, Cell 42:249–257, 1985). For both genes, the telomere elongation phenotype is allele specific and appears to correlate with the penetrance of the mutations. Furthermore, fluorescence-activated cell sorter analysis reveals that those alleles that cause elongation also exhibit a slowing of DNA replication. To determine whether elongation is mediated by telomerase or by slippage of the DNA polymerase, we created *cdc17-1* mutants carrying deletions of the gene encoding the RNA component of telomerase (*TLC1*). *cdc17-1* strains that would normally undergo telomere elongation failed to do so in the absence of telomerase activity. This result implies that telomere elongation in *cdc17-1* mutants is mediated by the action of telomerase. Since DNA replication involves transfer of the nascent strand from polymerase α to replication factor C (T. Tsurimoto and B. Stillman, J. Biol. Chem. 266:1950–1960, 1991; T. Tsurimoto and B. Stillman, J. Biol. Chem. 266:1961–1968, 1991; S. Waga and B. Stillman, Nature [London] 369:207–212, 1994), one possibility is that this step affects the regulation of telomere length.

Specialized structures called telomeres reside at the ends of eukaryotic chromosomes, where they are essential for ensuring chromosome stability and integrity. Telomeres are deoxyribonucleoprotein complexes consisting of simple tandem DNA repeats plus one or more associated proteins (reviewed in references 3, 17, and 47). The tandem repeats are typically 5- to 8-bp GC-rich sequences, and they form a structural motif that is shared among many species (reviewed in references 3, 17, and 47). In a given cell type, the telomeres usually exhibit a characteristic length; in wild-type *Saccharomyces cerevisiae* strains, the tandem repeats are normally 250 to 350 bp in length (45). The protein component of telomeres is less well characterized, but there are several examples of proteins that appear to bind preferentially to telomeres (13, 16, 33). Most prominent among these in budding-yeast cells are Rap1p, Sir3p, and Sir4p, which also interact at the silent mating-type loci (6, 11, 28, 32, 38).

Because of the unidirectional nature of eukaryotic DNA polymerases, telomeres must be periodically extended by the enzyme telomerase. DNA replication occurs in the 5′ to 3′ direction by a process that requires short RNA fragments to prime the synthesis of a replicating DNA strand (46). These properties lead to an inability to completely finish either lagging-strand (2, 47) or leading-strand (27) replication of telomeric DNA. Thus, in the absence of a unique telomere-extending activity, telomeric DNA is shortened by a few base pairs in each cell cycle. Such shortening is exhibited in many types of mammalian somatic cells, which appear to have no telomerase activity (14, 19, 22; but see also reference 5). Telomerase is a ribonucleoprotein that extends telomeric DNA by using its internal RNA component as a template. This template has recently been cloned from several organisms, including *S. cerevisiae* (4, 15, 18, 31, 39). In *S. cerevisiae*, the RNA template is encoded by the *TLC1* gene. Strains carrying a deletion of *TLC1* show progressive telomere shortening, which ultimately leads to a loss of viability (39).

The control of telomere length is clearly influenced by the structure and copy number of various components of the telomere. Most dramatic is the recent observation of McEachern and Blackburn that certain alterations of the simple sequence present in *Kluyveromyces lactis* telomeres lead to explosive lengthening of the telomeres (31). In *S. cerevisiae*, elevated amounts of the C-terminal half of Rap1p cause telomere extension, and the addition of multiple plasmid-borne telomere sequences causes lengthening (13, 35). In addition, mutations in genes that may encode components of telomerase (*TLC1*, *EST1*) also cause changes in telomere length (29, 39). In light of these various observations, it has recently been proposed that the ability of telomerase to add new telomeric repeats to chromosomal termini is negatively regulated by protein factors that bind to the telomeric repeats (31). Thus, long telomeres would bind more inhibitory proteins, which would decrease the action of telomerase at their ends; short telomeres would bind fewer inhibitory proteins, which would allow telomerase to extend their ends. In this way, a balance between negative regulation and the inherent shortening activity of DNA polymerases would maintain a certain average telomere length.

Although the DNA replication apparatus also clearly plays a role in influencing telomere length, it remains unclear whether the role is direct or indirect. Several years ago, Carson and Hartwell reported that temperature-sensitive DNA polymerase α (*cdc17*/*pol1*) mutants exhibit extremely long telomeres, in

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
CH498	*MAT***a** *ade1 ade2 ura1 lys2 tyr1 gal1 his7*[a]	21
CH526	*MAT***a** *ade1 ade2 ura1 lys2 tyr1 gal1 his7 cdc2*[b]	21
CH532	*MAT***a** *ade1 ade2 ura1 lys2 tyr1 gal1 his7 cdc8*	21
CH533	*MAT***a** *ade1 ade2 ura1 lys2 tyr1 gal1 his7 cdc9*	21
CH545	*MAT***a** *ade1 ade2 ura1 lys2 tyr1 gal1 his7 cdc21*[b]	21
CH552	*MAT***a** *ade1 ade2 ura1 tyr1 gal1 his7 cdc28*	21
CH1806	*MAT***a** *his4-539 lys2-801 ura3-52 CDC44.URA3*[c,d]	23
CH1807	*MAT***a** *his4-539 lys2-801 ura3-52 cdc44-5.URA3*[d]	23
CH1886	*MAT*α *ade2-101 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 CDC44.URA3*[d]	This laboratory
CH2138	*MAT***a** *ura3-52 trp1-289 ade2-101 tyr1 gal2 can1 his pol1-11*	7
CH2139	*MAT***a** *ura3-52 trp1-289 ade2-101 tyr1 gal2 can1 his pol1-12*	7
CH2141	*MAT*α *ura3-52 trp1-289 ade2-101 tyr1 gal2 can1 his pol1-13*	7
CH2142	*MAT*α *ura3-52 trp1-289 ade2-101 tyr1 gal2 can1 his pol1-14*	7
CH2144	*MAT***a** *ura3-52 trp1-289 ade2-101 tyr1 gal2 can1 his pol1-15*	7
CH2145	*MAT***a** *ura3-52 trp1-289 ade2-101 tyr1 gal2 can1 his pol1-16*	7
CH2146	*MAT*α *ura3-52 trp1-289 ade2-101 tyr1 gal2 can1 his pol1-17*	7
CH2147	*MAT*α *ura3-52 trp1-289 ade2-101 tyr1 gal2 can1 his*[e]	7
CH2150	*MAT***a** *ade2 ade1 his7 gal1 lys2 tyr1 ura1 cdc17-2*	21
CH2152	*MAT***a** *his7 ura1 cdc17-1*	L. Hartwell
CH2219	*MAT***a**/α *leu2-Δ1/leu2 his7/his7 ade2-101/ADE2 trp1-Δ1/TRP1 ura1/URA1 ura3-52/URA3 cdc17-1/cdc17-1 tlc1*::*LEU2*[f]/*TLC*[g]	This study
CH2220	*MAT***a**/α *leu2-Δ1/leu2 his7/HIS7 ade2-101/ADE2 lys2-801/LYS2 trp1-Δ1/TRP1 ura3-52/URA3 CDC44.URA3*[d]/*CDC44 tlc1*::*LEU2*[f]/*TLC1*[g]	This study
CH2221	*MAT***a**/α *leu2/leu2 his7/his7 ura1/URA1 ura3-52/URA3 cdc17-1/CDC17 tlc1*::*LEU2*[f]/*TLC1*[g]	This study
CH2247	*MAT***a** *leu2 his7*[g]	This study
CH2248	*MAT*α *ura1 leu2 his7 ura3-52 cdc17-1*[g]	This study
CH2249	*MAT***a** *trp1-Δ1 ade2-101 his7 leu2-Δ1 cdc17-1*[g]	This study

[a] This wild-type strain (A364A) is the strain from which were derived strains CH526, CH532, CH533, CH545, and CH552.
[b] This strain carries one or more additional, unidentified auxotrophies.
[c] This wild-type strain (S288C) is congenic with strain CH1807.
[d] The *URA3* marker in this strain is adjacent to *CDC44* but does not disrupt it.
[e] This wild-type strain (SS111) is congenic with strains CH2138, CH2139, CH2141, CH2142, CH2144, CH2145, and CH2146. These strains all carry an unidentified *his* auxotrophy.
[f] The *LEU2* marker in this strain disrupts a *tlc1* gene with an internal deletion.
[g] This strain is a mixture of S288C and A364A backgrounds.

some cases even at permissive temperature (9). Although they examined other mutations affecting proteins that acted at a similar time in the cell cycle, it was unclear at the time that *CDC17* encoded a protein involved in DNA replication. Therefore, an extensive survey of mutations affecting other DNA replication proteins was not performed. Since the identification of the role of Cdc17p in DNA replication, it has been proposed that the telomere elongation seen in *cdc17*/*pol1* mutants could be derived from either of two causes. First, the mutant polymerase might be susceptible to "slippage" during replication, especially during replication of repetitive sequences like those of telomeres (8, 17). Thus, as the telomeres are replicated, the polymerase might "stutter," leading to multiple repeats of the same sequence and therefore to telomere elongation. Alternatively, the alteration of the DNA replication apparatus may somehow lead to hyperactivity of telomerase.

To understand how telomere length is regulated in *S. cerevisiae*, we have investigated the possible role of the replication apparatus in the control of telomere length. We first examined the effects of several DNA replication mutations on telomere length. In addition to the *cdc17*/*pol1* mutants identified by Carson and Hartwell, we found that only *cdc44*/*rfc1* (large subunit of replication factor C) mutations have a profound effect on telomere length. For both *cdc17*/*pol1* mutants and *cdc44*/*rfc1* mutants, telomere lengthening is allele specific, and elongation correlates with impairment of DNA replication at a given temperature. Using strains deleted for *TLC1* (the structural gene for telomerase RNA), we show that telomere elongation in *cdc17-1* mutants is mediated by the activity of telomerase, not by the action of DNA polymerase α itself.

MATERIALS AND METHODS

Yeast strains and media. Yeast strains used in this study are listed in Table 1. These strains were maintained in standard media and under standard growth conditions (37). The strains were constructed by standard genetic techniques (37). The *cdc17-1*/*cdc17-1 tlc1*Δ::*LEU2*/*TLC1* (CH2219), *cdc17-1*/*CDC17 tlc1*Δ::*LEU2*/*TLC1* (CH2221), and *CDC17*/*CDC17 tlc1*Δ::*LEU2*/*TLC1* (CH2220) diploid strains were generated by crossing strains CH2248 and CH2249, CH2247 and CH2248, and CH2247 and CH1886, respectively. The parent diploids were then transformed with the *Xho*I fragment of pBlue61::LEU2, a *TLC1* knockout plasmid obtained from D. Gottschling (39); Southern analysis was used to confirm integration of the *tlc1* deletion (40). As expected, sporulation of integrants revealed 2:2 segregation of *tlc1*Δ::*LEU2*.

Growth conditions. For telomere studies, strains were maintained for many generations of growth. Cultures were grown to stationary phase in yeast extract-peptone-dextrose (YEPD), diluted appropriately (10^3- to 10^4-fold), and used to innoculate fresh YEPD for continued growth. This constituted one "cycle" of growth (9). In all studies, the cells in the first cycle were grown at the permissive temperature, the culture was split, and the cells in subsequent cycles were grown at either permissive or semipermissive temperature. For the *CDC17*/*cdc17-1 TLC1*/*tlc1*Δ sporulation experiment, spores were grown at 24°C for approximately 30 to 40 generations before they were inoculated into YEPD for first-cycle growth.

Southern blot analysis. Genomic DNA was isolated as described previously (34) and digested with *Xho*I to release the terminal telomeric fragments. Telomere-associated Y′ sequences contain a single *Xho*I restriction site proximal to the terminal simple sequence (10, 41). Digestion at this site removes a terminal fragment of telomeric DNA that migrates at approximately 1.1 to 1.4 kb for wild-type cells (10, 41); this fragment includes 250 to 350 bp of simple-sequence telomeric DNA (45). Southern blots of genomic DNA were hybridized with an *Xho*I-*Sal*I fragment of plasmid pJH345 (kindly supplied by Vicki Lundblad), which contains Y′ sequence and poly($G_{1-3}T$). This fragment was labeled with ^{32}P

by random priming (DECAprime; Ambion). Standard techniques were used for gel electrophoresis, DNA transfer, and hybridization (1).

Microfluorometric analysis. *cdc17/pol1* strains were grown to early log phase in liquid YEPD at either 24°C (permissive temperature) or 30°C (semipermissive temperature). *cdc44/rfc1* strains initially were grown only at 30°C, which was believed to be the permissive temperature. After evaluation of the fluorescence-activated cell sorter (FACS) profile, *cdc44/rfc1* cultures were grown at 35°C, which was then determined to be a more realistic permissive temperature. Aliquots were fixed in cold 70% ethanol, diluted, and treated with RNase A (1 mg/ml) overnight. Following staining with 50 μg of propidium iodide per ml, the cells were processed in a Becton Dickinson FACScan apparatus (24). For each DNA histogram, 20,000 cells were counted. For most samples, the flow rate was low to allow smooth data collection. For a few samples (e.g., cdc17-2 in Fig. 3), cell concentrations were sufficiently low that a high flow rate was used. At least two independent scans were obtained for each type of sample. The cell morphology of these log-phase cultures was also examined in a Zeiss phase-contrast microscope.

RESULTS

Only some DNA synthesis mutants exhibit elongated telomeres. To better understand how the replication complex may be involved in telomere length regulation, we examined the telomere length in several mutants known to be defective in DNA synthesis. To examine the role of polymerases, we examined DNA polymerase α (*cdc17/pol1*) and DNA polymerase δ (*cdc2*) mutants; DNA polymerase α mutants had previously been demonstrated to exhibit strikingly elongated telomeres (9). Although Carson and Hartwell (9) attempted to examine functionally related mutants, few were identified at the time of their studies. Therefore, we examined mutants with mutations in DNA polymerase δ (*cdc2*), thymidylate kinase (*cdc8*), DNA ligase (*cdc9*), thymidylate synthetase (*cdc21*), and the large subunit of replication factor C (*cdc44/rfc1*).

For each mutant, we examined telomere length after prolonged growth (multiple "cycles" of growth to stationary phase) at either the permissive or semipermissive temperature. Of the mutants surveyed, only those with *cdc44/rfc1* and *cdc17/pol1* mutations had undergone significant telomere elongation (Fig. 1, lanes 11 to 16) (9); judging from molecular weight standards, we estimate that *cdc44/rfc1* and *cdc17/pol1* mutants have telomeres that average two to four times the length observed in congenic wild-type strains (see Materials and Methods). Although it is not clear whether this represents a new equilibrium length for the telomeres, it is clear that the regulation of telomere length has been dramatically altered in these two mutants. All other mutants examined showed relatively minor or irreproducible variations in average telomere length; such minor variations are common among different yeast strains or among different isolates of the same strain (36). Furthermore, drug-induced defects in DNA metabolism, such as those caused by prolonged exposure to methyl methanesulfonate or hydroxyurea, had no effect on telomere length (data not shown). Thus, telomere elongation does not appear to be a general response to the dysfunction of DNA metabolism. Although our survey was certainly not exhaustive, among the conditions and mutants we examined, telomere lengthening seems to be a specific characteristic of *cdc17/pol1* and *cdc44/rfc1* mutants.

Telomere elongation is related to allele-specific perturbations of DNA replication. To examine the specific characteristics that lead to telomere elongation, we examined the telomere phenotype of several alleles of *cdc44/rfc1* and *cdc17/pol1*. For each mutant allele, we attempted to grow the cultures for at least four cycles (approximately 40 generations) at both the permissive and semipermissive temperatures. Because growth at the semipermissive temperature can select against the temperature-sensitive mutants, we checked for reversion of the temperature-sensitive phenotype at the end of the growth regimen. Unfortunately, all the *cdc44/rfc1* alleles showed such a high reversion rate at the semipermissive temperature that it was impossible to ascertain their telomere phenotype at this temperature. Nonetheless, the alleles we examined fell into three classes: (i) six alleles of *cdc44/rfc1* and one allele of *cdc17/pol1* caused no significant telomere lengthening at any testable temperature (data not shown); (ii) one allele of *cdc44/rfc1* (Fig. 1) and five alleles of *cdc17/pol1* caused long telomeres at all temperatures; one of these mutants (*pol1-11*) exhibited additional telomere elongation at the semipermissive temperature (Fig. 2); and (iii) three alleles of *cdc17/pol1* produced telomeres of normal length at the permissive temperature but caused elongated telomeres at the semipermissive temperature (Fig. 2).

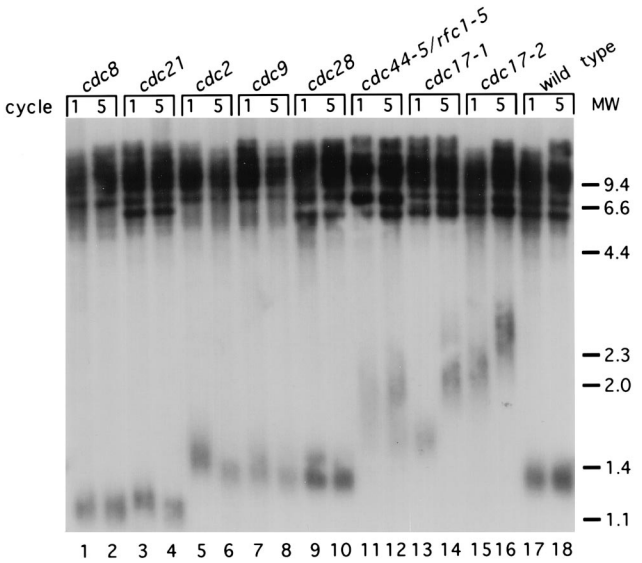

FIG. 1. Analysis of telomere length with seven mutations that affect DNA replication. Telomeric DNA was examined from strains CH498 (wild type), CH526 (*cdc2*; DNA polymerase δ), CH532 (*cdc8*; thymidylate kinase), CH533 (*cdc9*; DNA ligase), CH2152 (*cdc17-1*; DNA polymerase α), CH2150 (*cdc17-2*; DNA polymerase α), CH545 (*cdc21*; thymidylate synthetase), and CH1807 (*cdc44-5*; large subunit of replication factor C). In addition, we examined the telomere length in strain CH552 (*cdc28*; cyclin-dependent kinase), which is not involved in DNA replication. Strains were grown for one cycle of growth at the permissive temperature followed by four cycles of growth at the semipermissive temperature (see Materials and Methods). (For *cdc44*, a high rate of reversion to non-cold sensitivity at the semipermissive temperature required us to keep all cycles of growth at the permissive temperature.) DNA was isolated from samples from cycles 1 (9 to 10 generations of growth) and 5 (45 to 50 generations of growth), digested with *Xho*I, subjected to electrophoresis and Southern blot transfer, and hybridized to a telomere probe.

FACS analysis reveals DNA replication abnormalities in mutants with elongated telomeres. Samples of cells growing at permissive, semipermissive, or restrictive temperatures were prepared for FACS analysis. Invariably, samples exhibiting a normal FACS profile also produced normal-length telomeres; in these cases, the FACS profile showed clearly defined G_1 and G_2 peaks and relatively few cells in the process of DNA replication. In contrast, the samples derived from conditions producing elongated telomeres exhibited an enrichment of cells in the S or G_2 phase (Fig. 3). The most economical interpretation of this result is that whatever is slowing DNA replication in these mutants is also causing telomere elongation. It should be noted, however, that not all conditions that retard DNA replication also cause elongation of telomeres. For example, hydroxyurea treatment causes a profound enrichment of cells in the S phase but produces no discernible change in telomere

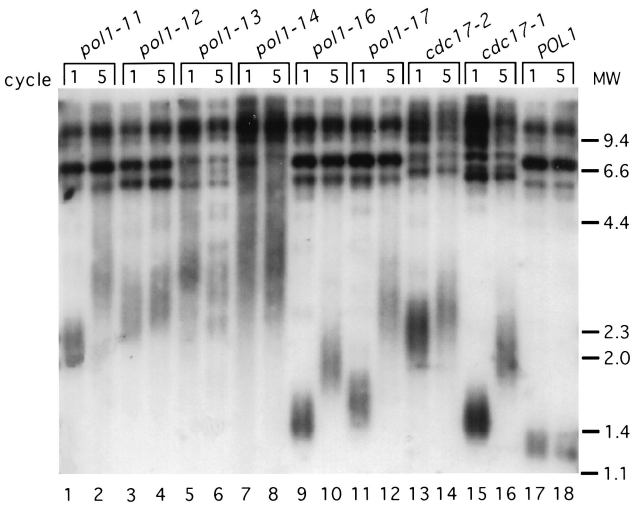

FIG. 2. Allele-specific telomere elongation is caused by *cdc17/pol1* mutations. The telomere length caused by several mutant alleles of DNA polymerase α (*cdc17/pol1*) was analyzed. Telomeric DNA from strains CH2138 (*pol1-11*), CH2139 (*pol1-12*), CH2141 (*pol1-13*), CH2142 (*pol1-14*), CH2145 (*pol1-16*), CH2146 (*pol1-17*), CH2152 (*cdc17-1*), CH2150 (*cdc17-2*), and CH2147 (*CDC17/POL1*) was examined. After one cycle of growth at the permissive temperature (24°C), cells were shifted to 30°C (semipermissive temperature) for all subsequent growth. DNA was isolated from samples from cycle 1 (10 to 12 generations of growth) and cycle 5 (50 to 60 generations of growth), digested with *Xho*I, subjected to electrophoresis and Southern blot transfer, and hybridized to a telomere probe. At the permissive temperature, strains carrying *pol1-11*, *pol1-12*, *pol1-13*, *pol1-14*, and *cdc17-2* mutations exhibit dramatic telomere elongation. In contrast, *pol1-16*, *pol1-17*, and *cdc17-1* strains show relatively normal telomere length at the permissive temperature and exhibit elongation only after prolonged growth at the semipermissive temperature.

length (data not shown). Similarly, some *cdc44/rfc1* and *cdc17/pol1* mutants with normal telomere length nonetheless exhibit an abnormal FACS profile (data not shown). Thus, it appears that a particular type of slowing of DNA replication, not slowing of DNA replication per se, is likely to cause telomere lengthening.

To test the correlation between a normal FACS profile and normal telomere length, we exploited an observation made during the course of the FACS analysis. Although *cdc44/rfc1* mutants exhibit almost normal rates of growth at both 30 and 35°C, their FACS profile shifts markedly from G_2 rich at 30°C to normal at 35°C (Fig. 4A). On the basis of the normal growth rate, we had used 30°C as a permissive temperature for *cdc44/rfc1* mutants, and at this temperature the cells exhibited greatly elongated telomeres. To further examine our previous observation that strains with normal FACS profiles always exhibit normal telomere length, we grew an *rfc1-5* strain for 10 cycles at 35°C and then examined its telomere length. The telomere length indeed began to return gradually to normal under these conditions (Fig. 4B). Using DNA size standards, we estimate that the telomeres shortened at a rate of approximately 4 bp per generation. This rate is in line with estimates of telomere shortening observed in cells in which telomerase is inactive (39). Furthermore, the observation of progressive shortening is consistent with our assumption that the extension of *cdc44/rfc1* telomeres is due to extension of the simple-sequence DNA. Thus, we conclude that by removing the perturbation of DNA synthesis, we have also removed the cause of telomere elongation. Although we cannot say what aspect of aberrant DNA metabolism leads to telomere elongation, it appears that when DNA metabolism is normalized, telomere length simultaneously begins to return to normal.

Telomere elongation is mediated by telomerase in *cdc17/pol1* mutants. Two general models have been proposed for the elongation of telomeres observed in *cdc17/pol1* mutants. On the one hand, the mutant polymerase may be prone to slippage during replication of simple sequence DNA (8, 17); in this model, the extension of telomeres in *cdc17/pol1* mutants would be largely independent of the action of telomerase. In the second model, perturbations of the replication complex lead directly or indirectly to elevated telomerase activity; in this

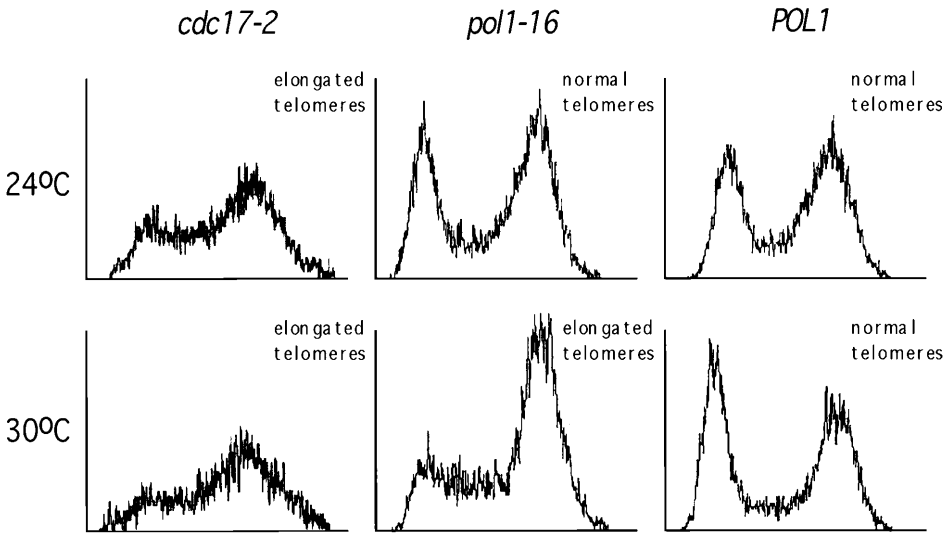

FIG. 3. FACS profiles of strains carrying *cdc17/pol1* alleles that cause different telomere elongation phenotypes. FACS analysis of *cdc17-2*, *pol1-16*, and *POL1* strains is shown. Strains CH2150 (*cdc17-2*), CH2145 (*pol1-16*), and CH2147 (*POL1*) were grown to early log phase in YEPD liquid at either 24 or 30°C. Aliquots of the cultures were collected and prepared for FACS analysis. The FACS profile of strain CH2150 is typical of the class of strains bearing alleles that cause long telomeres at all temperatures. The FACS profile of strain CH2145 is typical of the class of strains bearing alleles that cause telomeres of normal length at the permissive temperature but elongated telomeres at the semipermissive temperature.

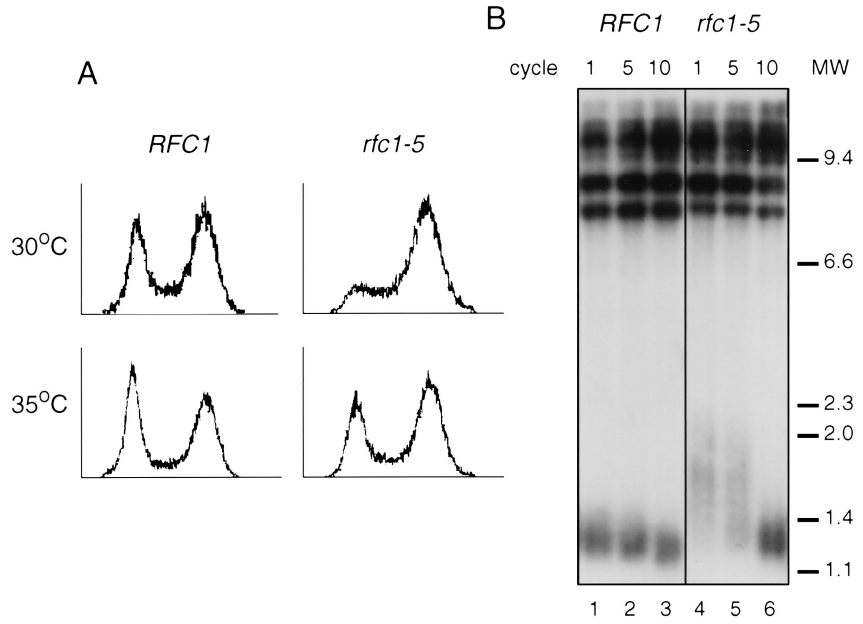

FIG. 4. Telomere length in *rfc1-5* strains gradually returns to normal after prolonged growth at 35°C. (A) FACS analysis of *rfc1-5* and *RFC1* strains at 30 and 35°C. Strains CH1806 (*RFC1*) and CH1807 (*rfc1-5*) were grown to early log phase in YEPD liquid at either 30 or 35°C. Aliquots of the cultures were collected and prepared for FACS analysis. (B) Telomere length analysis of strains CH1806 (*RFC1*) and CH1807 (*rfc1-5*) grown in liquid YEPD for 10 cycles of growth at 35°C. For each strain, DNA was isolated from cycle 1 (10 to 12 generations of growth), cycle 5 (50 to 60 generations of growth), and cycle 10 (100 to 120 generations of growth). The DNA was then digested with *Xho*I, subjected to electrophoresis and Southern blot transfer, and hybridized to a telomere probe. Lane 6 shows that after 10 cycles of growth at 35°C, *rfc1-5* telomeres have shortened to wild-type length. (For comparison, lane 4 shows the elongated telomeres seen in *rfc1-5* at cycle 1.) In control cultures grown at 30°C, the telomere length did not change from its cycle 1 profile in either strain CH1806 or strain CH1807 (data not shown).

model, telomerase activity would be required to produce telomere lengthening.

To distinguish between these two models, we began with cells with normal telomere lengths and examined their ability to elongate their telomeres in the absence of telomerase activity. To inactivate telomerase, diploid strains were transformed with a fragment of plasmid pBlue61::LEU2 that causes the deletion of one copy of the structural gene of the RNA component of telomerase (*TLC1*). Unfortunately, when *CDC44*/*cdc44-5* diploid strains were transformed with this plasmid, the resulting *cdc44-5 tlc1*Δ spores were inviable. Although this result is intriguing because it could indicate an interaction between Cdc44p and telomerase, it precluded this test of the two models for telomere elongation. In contrast, *CDC17*/*cdc17-1 TLC1*/*tlc1*Δ strains produced spores with good viability. After growing the haploid spores under nonelongating conditions (at the permissive temperature), we examined the change in telomere length over four cycles at either the permissive (nonlengthening) or semipermissive (lengthening) temperature.

Mutant *cdc17-1* cells fail to elongate their telomeres in the absence of telomerase, even at semipermissive temperatures. As expected, the control cultures of *cdc17-1 TLC1* strains maintained normal telomere length over several cycles of growth at the permissive temperature (Fig. 5A). As was also anticipated, *cdc17-1 tlc1*Δ strains show gradual shortening of the telomere length at the permissive temperature (Fig. 5A). Most striking is the difference between *cdc17-1 TLC1* and *cdc17-1 tlc1*Δ telomeres in cells grown under elongating conditions (i.e., the semipermissive temperature) (Fig. 5B). Whereas *cdc17-1 TLC1* strains undergo telomere elongation as usual, *cdc17-1 tlc1*Δ strains not only fail to elongate but actually exhibit some telomere shortening. This result demonstrates that telomerase activity is required for telomere lengthening in *cdc17-1* mutants and suggests that polymerase slippage must not play a major role in the observed elongation.

To determine whether polymerase slippage might be contributing in a small way to telomere lengthening, we compared the telomere lengths of *cdc17-1 tlc1*Δ and *CDC17 tlc1*Δ mutants. If telomeres are extended by polymerase slippage at the semipermissive temperature in *cdc17-1* mutants, the rate at which telomere shortening occurs in *cdc17-1 tlc1*Δ double mutants should be noticeably reduced compared with that in *CDC17 tlc1*Δ cells. At the permissive temperature, the *cdc17-1 tlc1*Δ and *CDC17 tlc1*Δ spores exhibited similar degrees of shortening, as expected (approximately 4 bp per generation [Fig. 5A]). Although not identical, telomere shortening of *cdc17-1 tlc1*Δ and *CDC17 tlc1*Δ spores at the semipermissive temperature was also very similar (approximately 3 bp per generation for each strain), indicating that polymerase slippage plays at most a minor role in telomere elongation (Fig. 5B). Thus, we conclude that the telomere elongation observed in *cdc17-1* mutants is caused predominantly by the action of telomerase.

DISCUSSION

Our results show that although perturbations of DNA replication can cause elongated telomeres, not all of them do so. Only specific mutations of DNA polymerase α and replication factor C cause dramatic lengthening of the telomeres, whereas other mutant alleles fail to cause significant elongation. Although our survey was certainly not exhaustive, we found that little effect on telomere length was caused by mutations in other DNA replication genes or by chemical treatments that affect DNA metabolism. The results of FACS analysis suggest that telomere elongation is caused by alterations in the process of DNA synthesis. The behavior of *cdc17*/*pol1* mutants in the

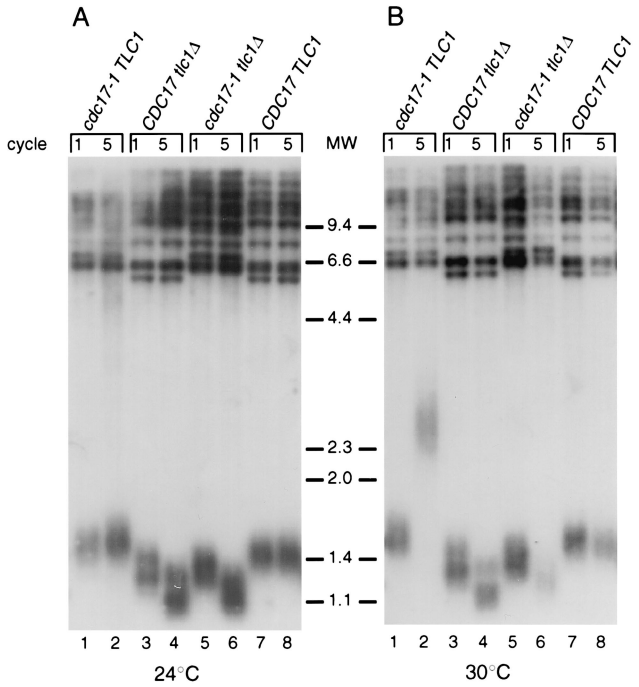

FIG. 5. Telomere lengths of haploid strains derived from a *cdc17-1*/*CDC17 tlc1*Δ/*TLC1* diploid strain. Telomere lengths of sporulation products from a *cdc17-1*/*CDC17 tlc1*Δ/*TLC1* diploid strain (CH2221) were examined by Southern blot analysis. A single tetratype tetrad produced *cdc17-1 TLC1*, *CDC17 tlc1*Δ *cdc17-1 tlc1*Δ, and *CDC17 TLC1* spores. These spores were grown for one cycle (9 to 10 generations of growth) at the permissive temperature followed by four more cycles (36 to 40 generations of growth) at either the permissive or semipermissive temperature. DNA was isolated from samples from cycles 1 and 5, digested with *Xho*I, subjected to electrophoresis and Southern blot transfer, and hybridized to a telomere probe. (A) Telomere profiles from strains grown for five cycles at the permissive temperature (24°C). As expected, telomeres in the *cdc17 TLC1* strain remained normal in length. Telomere shortening was seen in *CDC17 tlc1*Δ and *cdc17-1 tlc1*Δ strains. (B) Telomere profiles from strains grown for five cycles at the semipermissive temperature (30°C). As expected, the *cdc17-1 TLC1* strain shows dramatic telomere elongation after prolonged growth at 30°C. Telomeres in the *CDC17 tlc1*Δ strain and the *cdc17-1 tlc1*Δ strain showed shortening to similar extents.

absence of telomerase function reveals that elongation is actually mediated by the action of telomerase, not by aberrant activity of the replication complex itself.

Most of the observed allele specificity of telomere elongation probably derives from the penetrance of each of the mutations. One possibility is that simply decreasing the overall activity of either protein by a certain percentage could lead directly or indirectly to telomere elongation. This interpretation is consistent with the loose correlation between the proportion of large-budded cells in a growing population and the likelihood that the telomeres will be elongated (data not shown); large-budded cells are often produced when DNA replication is delayed (20, 25). Differences in viability, although minor, also correlate loosely with the propensity to develop long telomeres (data not shown). Although less clear, it is also possible that there are particular functional defects associated with specific alleles. Since neither protein has a solved crystal structure, it is difficult to say whether the alleles causing each type of telomere elongation phenotype lay in a particular domain of the protein; no such clustering can be discerned by examining the locations of the mutations on the primary sequence.

Although we showed that one allele of *cdc44*/*rfc1* causes dramatic telomere lengthening at 30°C, it is unfortunate that we were unable to examine the effects of other *cdc44*/*rfc1* mutations at more restrictive temperatures. When cold-sensitive *cdc44*/*rfc1* cultures were grown at 25°C, the cultures always reverted to temperature resistance by the end of four cycles of growth. This effect is probably a consequence of the high spontaneous mutation rates observed in *cdc44*/*rfc1* mutants (30); growth at the semipermissive temperature provides strong selection for temperature-resistant revertants, which overgrow the culture by the end of the experiment. We could have tried to overcome this difficulty by selecting a temperature-sensitive clone after each cycle of growth, but previous experiments have shown that repeated subcloning of cultures causes a wide variation in the observed telomere length (36). Furthermore, by picking a temperature-sensitive clone, we might have been picking exactly those colonies that were suffering the least effects of the *cdc44*/*rfc1* mutation in question. Thus, it is possible that other *cdc44*/*rfc1* alleles cause telomere elongation that cannot be detected by currently available methods.

Although the effects of *cdc44*/*rfc1* and *cdc17*/*pol1* mutations on telomere length could be distinct, the observation that the *cdc44*/*rfc1* and *cdc17*/*pol1* mutations both cause telomere elongation (9; also see above) reveals that the state of the DNA may have a significant effect on telomerase regulation. Earlier reports have shown that altering the stoichiometry of telomere components can affect telomere length (see, e.g., references 13 and 35). Not surprisingly, other reports have shown that eliminating telomerase activity via a *tlc1* or *est1* mutation causes inexorable shortening of the telomeres (12, 26, 29, 39). Along with the results of Carson and Hartwell (9), our results suggest that the state of the DNA may also be one of the elements that affects telomerase regulation. Perhaps the DNA replication defects caused by *cdc17*/*pol1* and *cdc44*/*rfc1* mutations result in the persistence of gaps or nicks that might diminish the binding of negative regulatory telomere-binding proteins. Failure of such inhibitory proteins to bind to telomere DNA has been proposed as one explanation for the explosive telomere lengthening seen in *K. lactis* telomerase RNA mutants (31). Understanding the specific property of the DNA that affects telomerase activity will provide additional insight into how the cell maintains an equilibrium telomere length.

It is interesting that replication factor C and DNA polymerase α have been postulated to have an intimate interaction during DNA replication. Stillman and coworkers have recently shown that both leading- and lagging-strand DNA synthesis are accomplished by a "polymerase switching" mechanism involving DNA polymerase α and DNA polymerase δ with its associated cofactors, including replication factor C (42–44). In a simian virus 40-based in vitro replication system, priming synthesis is accomplished by the polymerase α-primase complex, which synthesizes a short RNA-DNA primer. Polymerase α-primase is then displaced by the binding of replication factor C to the primer-template junction. Subsequently, replication factor C recruits polymerase δ via its interaction with proliferating-cell nuclear antigen, and replication of a substantial length of DNA proceeds. The observation that replication factor C mediates an exchange involving polymerase α may be significant in light of the fact that mutations in only these two DNA replication proteins have been shown to cause dramatic telomere elongation. The functional proximity between these two proteins, as proposed on the basis of in vitro studies, suggests the possibility that this step in DNA replication is defective in our telomere-elongating mutants.

Regardless of the mechanism by which it is accomplished, it is clear that telomerase activity must be regulated to maintain specific equilibrium telomere lengths under specific conditions

(17). In *K. lactis* telomerase RNA mutants, a signal to the regulatory apparatus must indicate that the telomeres are dangerously short, and telomerase explosively extends them (31). In *RAP1* overexpressors, excess telomere protein leads to an increase in the average telomere length (13). Our studies now suggest that defects in two specific DNA replication proteins cause a perturbation of the regulatory signals such that the equilibrium length of telomeres shifts in a temperature-dependent manner. We hope that these disparate observations will ultimately come together in a coherent understanding of telomere length regulation.

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