

Limited TTP supply affects telomere length regulation in a telomerase-independent fashion

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Received December 20, 2004; Revised and Accepted January 12, 2005

ABSTRACT

An adequate supply of nucleotides is essential for DNA replication and DNA repair. Moreover, inhibition of TTP synthesis can cause cell death by a poorly characterized mechanism called thymine-less death. In the yeast *Saccharomyces cerevisiae*, the genes encoding thymidylate synthetase (*CDC21*) and thymidylate kinase (*CDC8*) are both essential for *de novo* TTP synthesis. The effects of temperature-sensitive mutations in these genes have been characterized and, curiously, the phenotypes displayed by cells harboring them include shortened telomeric repeat tracts. This finding raised the possibility that the enzyme telomerase is very sensitive to TTP-pools. We tested this possibility *in vivo* by assessing telomerase-dependent extension in situations of lowered TTP supply. The results show that the above-mentioned short telomere phenotype is not a consequence of an inability of telomerase to elongate telomeres when TTP synthesis is impaired. Moreover, this telomere shortening was abolished in cells harboring a mutation in DNA polymerase α . Previously, this same mutation was shown to affect the coordination between conventional replication and telomerase-mediated extension. These results thus re-emphasize the importance of the interplay between conventional replication and telomerase-mediated addition of telomeric repeats in telomere replication.

INTRODUCTION

Telomeres are the specialized complexes at the end of eukaryotic chromosomes, where they ensure chromosome stability and integrity and facilitate completion of DNA replication [for

reviews, see (1,2)]. Telomeric DNA is made up of short direct repeats and an adequate repeat tract is necessary and sufficient for most functions ascribed to telomeres (3,4). Therefore, the regulation of the lengths of these telomeric repeat tracts is crucial for genome integrity. Telomeric repeats usually are short (6–10 nt) and their specific base composition leads to a G-rich strand and a C-rich strand. In all cases examined, the strand with the 3' end at the chromosomal terminus is the G-rich strand and this strand is longer than the C-rich strand making up the 5' end (4). Given this specific sequence orientation, the G-rich strand is always synthesized by leading-strand synthesis and the C-rich strand by lagging-strand synthesis (5). In addition and due to the unidirectional nature of eukaryotic DNA polymerases, the replication of chromosome ends remains incomplete when the last RNA primer for lagging-strand synthesis, essential to prime the synthesis of a replicating DNA strand, is degraded (6,7). Without a mechanism to compensate this loss of chromosomal DNA, telomere sequences are progressively shortened until chromosomes become unstable, causing growth arrest (8,9). In most organisms, telomerase, an RNA-directed DNA polymerase, or reverse transcriptase, extends telomeric DNA by using its internal RNA component as template for addition of new telomeric sequences onto the G-rich strand at chromosome ends (10). Thus, conceptually G-strand extension by telomerase could also be viewed as continued leading-strand synthesis, and there is increasing evidence that this specialized G-strand synthesis at chromosome ends is coordinated with C-strand synthesis, as are the conventional leading-strand and lagging-strand syntheses at internal chromosomal loci.

Telomere length regulation is governed by a variety of proteins and processes. Most of them are thought to act during S-phase, when telomeric repeats are synthesized. In the yeast *Saccharomyces cerevisiae*, for example, elongation of telomeric G-strands by telomerase and C-strands by lagging-strand components is thought to occur in S-phase (11,12). This elongation, however, is negatively regulated by a mechanism involving Rap1p and the association of potential

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Given that telomerase does require TTP for telomeric repeat synthesis, it was thus possible that this enzyme was exquisitely sensitive to TTP supply, and that a lowered capacity of nucleotide synthesis could lead to the telomeric phenotypes observed. Thymidine kinase of herpes simplex virus (HSV-TK), when expressed from appropriate promoters, is active in yeast and can also function as a thymidylate kinase, as it complements a loss of the *CDC8* gene (25). In addition, HSV-TK converts 5-fluoro-2'-deoxyuridine (FUdR) into 5-fluoro-2'-deoxyuridine monophosphate (FdUMP), which covalently binds and inhibits thymidylate synthetase (Cdc21p) (26,27). We used these characteristics of HSV-TK to examine whether telomerase was sensitive to nucleotide supply *in vivo*. Surprisingly, the results suggest that the short telomere phenotype is not a consequence of an inability of telomerase to elongate telomeres in a situation of low dNTP supply. In addition, although increased dUTP pools in such cells can cause UTP utilization for DNA synthesis with subsequent repair, this effect also could be excluded as being responsible for the shortened telomeres. Instead, all the available data are most easily explained by an inability of the conventional replication machinery to complete replication of telomeric repeats and/or of the inability to coordinate the transfer between conventional replication to telomerase-mediated extension.

MATERIALS AND METHODS

Strains, plasmids and other reagents

Yeast strains RWY42-22A (Mata *CDC8*) and RWY42-22B (Mata *cdc8-1*) were obtained after three backcrosses of the starting strain CH532 (Mata *cdc8-1*) (24) with BY4705 (28). Spores with the *ts* allele of *CDC8* (*cdc8-1*) were not able to grow at 30°C while the isogenic *CDC8* spores were. Strain E1000 contains seven copies of the HSV-TK gene integrated at the *URA3* locus (29). Strains Y300 (Mata *RNR*), Y585 (Mata *rnr1-240*) and Y586 (Mata *rnr2-68*) (19), as well as strains RWY12 (Mata *tlc1Δ::LEU2*) (30) and CH2377 (Mata *poll-17*) (16) were described in detail before. In all experiments, experimental strains were compared to isogenic wild-type strains.

Plasmid pRC4-TK⁺, referred to as pTK (2 μm-ARS *TRP1 HIS3 TK amp^R kan^R*), contains a DNA fragment coding for the thymidine kinase (*TK*) of herpes simplex virus (HSV) (25). Plasmid pAZ1 is derived from pRS316 [*ARSH4, CEN3, URA3* (31)] and contains a 5.5 kb genomic DNA fragment spanning the entire *TLC1* locus (32). The pDUT1 plasmid was constructed by PCR amplification of the *DUT1* gene using pDUT1-forw (5'-CGGGATCCCTATGTACATACACACGCACC-3') and pDUT1-Rev (5'-CGGGATCCGCCATGGCTTATGC-ATGCTAC-3') primers, and cloning the resulting fragment into the BamHI site of pRS425 (33). FUdR (Sigma F-0503) was dissolved at 20 mg/ml in deionized water, sterilized by passage through a 0.22 μm filter and kept at -20°C. FUdR was added to a final concentration of 5 or 10 μg/ml to growth media. While 5 μg/ml FUdR extended the cell cycle of HSV-TK+ cells slightly, there was no discernible colony phenotype after 3 days of incubation; 10 μg/ml of FUdR more than doubled cell cycle times and resulted in a clear slow growth phenotype (data not shown). 5FOA plates

consisted of synthetic minimal medium (without tryptophan) supplemented with 0.75 mg/l of 5FOA (American Biorganics Inc.), 0.005% (W/V) uracil, 0.002% (W/V) adenine, 0.03% (W/V) histidine and 0.03% (W/V) tyrosine.

Cloning, yeast transformation and growth analyses

The *E.coli* strain DH5α (was used for all clonings with standard procedures (34). Yeast transformations were performed with a modified LiAc method (35). Strains were grown at specified temperatures and in standard yeast extract-peptone-dextrose (YEPD) or synthetic minimal medium (YC) supplemented with appropriate amino acids (36).

For determination of population doublings (number of generations) in the senescence assays, cells were streaked for single colonies on appropriate selective solid medium and the growth into a colony is assumed to represent about 20 generations. Senescence was then followed on solid medium (YC-trp) with or without FUdR. For the assay in liquid media, RWY12 cells (*tlc1Δ*) containing pAZ1 and pTK plasmids were first plated onto FOA-trp medium with or without FUdR to select for cells that had lost pAZ1 containing the wild-type allele of the *TLC1* gene. These cells were then grown in liquid YC-trp media with or without FUdR. To determine population doublings, cell concentrations were measured by densitometry at 660 nm. Cells were diluted to 5×10^5 cells/ml and regrown to 3×10^7 to 4×10^7 cells/ml, which corresponds to six generations. For *poll-17* experiments, cells precultured for 140 generations on YC-trp with FUdR at 23°C were grown to exponential phase in YC-trp with FUdR at 23°C, and either kept at 23°C or shifted to 30°C for 16 h before telomere length analysis.

Telomere length analyses

Genomic DNA was extracted by a modified glass bead method (36,37): 0.5–1 μg of DNA was digested with the XhoI restriction enzyme and subjected to agarose gel electrophoresis (1% agarose), transferred to a nylon membrane by capillary blotting and hybridized to a 600 bp KpnI restriction fragment derived from pVY'K (30) spanning a distal portion of the subtelomeric Y'-element. Alternatively, a 300 bp fragment containing 280 bp of telomeric repeats derived from pYLPV was used (38). Probes for hybridization were obtained by a random priming labeling procedure (39). Membranes were exposed to Kodak XAR5 X-ray film for appropriate amount of time. The XhoI restriction site in the subtelomeric region is situated at 870 bp from the start of telomeric TG₁₋₃ sequences (40). Hence, the lengths of telomeric repeats were determined by subtracting 870 bp from the lengths of the terminal restriction fragments (TRFs).

RESULTS

A low TTP supply results in a shortening of telomeric repeat tracts

Mutations in many genes involved in aspects of DNA replication can alter telomere length regulation (24,41). Somewhat surprisingly, mutations in the *Saccharomyces cerevisiae* genes coding for thymidylate synthetase (*CDC21*) or thymidylate kinase (*CDC8*), two essential genes involved in TTP

synthesis, provoke telomere shortening [(24); see Figure 1C, lanes 1 and 11]. The strains analyzed in these previous experiments harbored temperature-sensitive (*ts*) alleles of the genes (*cdc8-1* and *cdc21-1*), but it was not clear whether the short telomere phenotype was a direct consequence of the lack of TTP or an indirect effect.

Two strategies were used to distinguish between direct and indirect effects. First, if a low TTP level was the cause of shortened telomeres, an increase in TTP supply should restore normal telomere lengths in the mutants. To this end, we introduced a plasmid (pTK) encoding thymidine kinase of herpes simplex virus (HSV-TK) into *cdc8-1* strains. The approach is based on the fact that the kinase activity of HSV-TK can complement the thermosensitivity of mutations in the *CDC8* gene [(25) and see Figure 1B). Telomere lengths in *cdc8-1* cells harboring the pTK plasmid or an empty vector were assessed by Southern hybridization. As expected, telomeres were shorter by about 150 bp, which represents about a 50% decrease in telomeric repeats, in *cdc8-1* cells containing the empty plasmid and they remained short during extended outgrowth (Figure 1C, lanes 1–4). However, upon overexpression of HSV-TK at 23°C, telomere lengths returned to wild-type lengths within 50 generations of growth (Figure 1C, lanes 5–8). In addition, telomere lengths also returned to normal, when the cells were grown at 30°C (Figure 1C, lanes 14–16), a temperature at which cells without the pTK plasmid are unable to grow (Figure 1B, middle panel).

In a second approach, telomeres should shorten even in a wt strain, if TTP supply is reduced in a different fashion. A reduction in TTP synthesis can be achieved by including

FUdR in growth media of cells that also contain the pTK plasmid. In wt yeast cells, FUdR is not converted into FdUMP and addition of it to growth media has no effect on cells. However, the HSV-TK gene product converts FUdR into FdUMP and the latter compound will efficiently inhibit the endogenous thymidylate synthetase [Cdc21p, see Figure 1A and (42)]. Untreated wt cells containing seven copies of the HSV-TK gene inserted into the genome have normal growth characteristics and telomere length [(29); see Figure 2A, lanes 1 and 7). In contrast, when the same cells are treated with FUdR (5 µg/ml), the length of the cell cycle is extended slightly (data not shown), and telomeres shorten over time to about 50% the initial length (shortening of ~150 bp) after about 65 generations (Figure 2A, lanes 2–6). Hence, a specific inhibition of Cdc21p provokes telomere shortening in wt cells.

We conclude that a defect in TTP synthesis leads to a significant shortening of telomeric repeat tracts. However, the results also raised the possibility that a global reduction in dNTP synthesis could affect telomeric repeat synthesis. Growing cells in the presence of hydroxyurea (HU), which inhibits dNTP synthesis by inhibiting the RNR-complex (43), proved confusing. In such cells, checkpoint mechanisms are activated and eventually, certain subunits of the RNR complex can become upregulated (44,45). Hence, such an upregulation of the RNR-complex could offset the effect of the treatment of wild-type cells with HU. Therefore and as an alternative approach, we examined telomere lengths in cells harboring thermosensitive mutations in genes encoding RNR-subunits (19). Clearly and consistent with the hypothesis above,

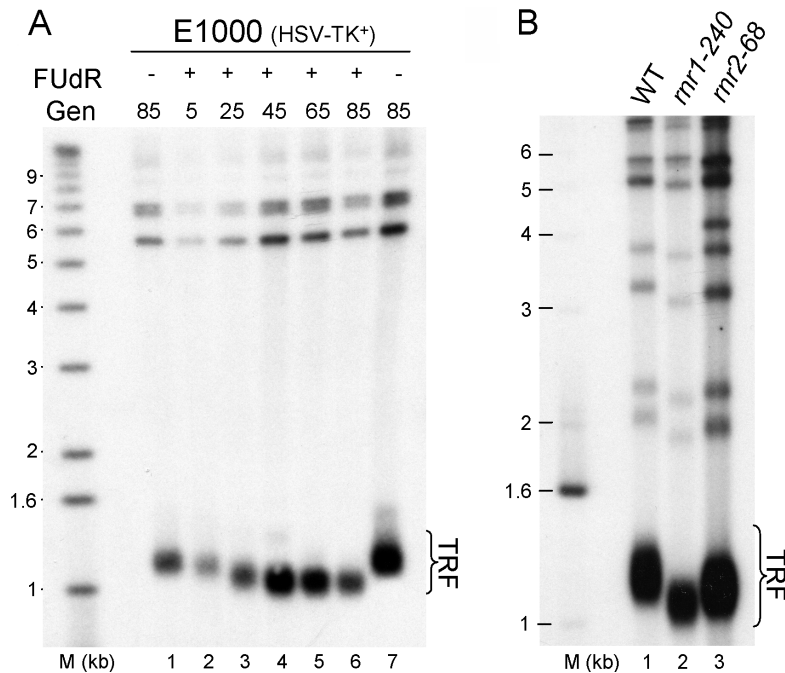


Figure 2. Specific inhibition of thymidylate synthetase in wild-type cells provokes telomere shortening. (A) The yeast strain E1000 contains seven tandem copies of the HSV-TK gene inserted in the genome. Such cells were grown in the presence of 5 µg/ml FUdR (lanes 2–6) or in the absence of FUdR (lanes 1 and 7) for the indicated number of generations at 30°C. Telomere length analysis was performed as in Figure 1. (B) Cells harboring specific mutations in *RNR1* (*rnr1-240*) or *RNR2* (*rnr2-68*) were grown at 23°C, and total genomic DNA was isolated and analyzed for telomere lengths. The probe consisted of a 300 bp fragment of telomeric sequences. M, molecular weight marker; TRF, terminal restriction fragments.

telomeric repeat tracts are shorter in cells that harbor mutations in the *RNR1* or *RNR2* genes when compared to isogenic wt cells (Figure 2B). Thus, lowering general dNTP synthesis by specific mutations in RNR genes can lead to shortened telomeres, suggesting that telomere homeostasis is very sensitive to dNTP supply, and in particular, to TTP supply.

Lowered TTP synthesis accelerates telomere shortening and growth arrest in telomerase-lacking cells

The short telomere phenotype in cells with lowered TTP synthesis could in principle be due to an inefficiency of telomerase-mediated telomere elongation, inhibition of the conventional replication machinery or increased telomere degradation activities. In order to test the first hypothesis, we exploited the fact that cells without telomerase activity can live for about 60 generations before they stop growing (8,9,46). During this outgrowth without telomerase, telomeric repeat tracts shorten by about 3–5 nt per generation. Thus, if lowered TTP synthesis was to affect only the telomerase enzymatic activity, then in the absence of telomerase, the kinetics of telomere shortening and the generations required for growth arrest should not be affected. To test this possibility, we examined cells that had lost the gene for the RNA component of telomerase (*TLC1*) and also contained the pTK plasmid. These cells were then grown in the presence or absence of FUdR and telomere lengths and the onset of population growth arrest were assessed during outgrowth (Figure 3A and B). When grown without FUdR, the telomeres in telomerase-lacking cells shorten at

the expected rate and reached a minimum after about 56 generations (Figure 3A, lane 5). Thereafter, the characteristic Y'-terminal restriction fragments (TRFs) become very heterogeneous and the bands for amplified Y'-elements increase in intensity. These changes in TRF patterns are typical for cell populations that have overcome growth arrest and that maintain their telomeres via telomerase-independent mechanisms (9). The population growth arrest in these cells also occurs around generation 60 to 80, as expected (Figure 3B, left). When these cells were grown in the presence of FUdR, telomere shortening is accelerated, reaching a minimal size after only 38–44 generations of outgrowth (Figure 3A, lanes 9–11). The resulting estimated shortening rate corresponded to 7–8 nt per generation, thus about two times the one observed in non-treated cells. Consistent with this observation, population growth arrest occurs earlier with very few cells being able to grow for more than 40 generations (Figure 3B, right).

These results are inconsistent with the possibility that the lowered nucleotide supply affects primarily the telomerase enzyme and suggest that the cause for the shortened telomeres lies elsewhere.

Telomere elongation by telomerase is not affected by a defect in TTP synthesis

Due to the absence of telomerase in the above experiment, the ability of this enzyme to add telomeric sequences in a situation where TTP synthesis is affected was not tested directly. In order to evaluate the ability of telomerase to elongate telomeres at low TTP supply, we examined telomere elongation

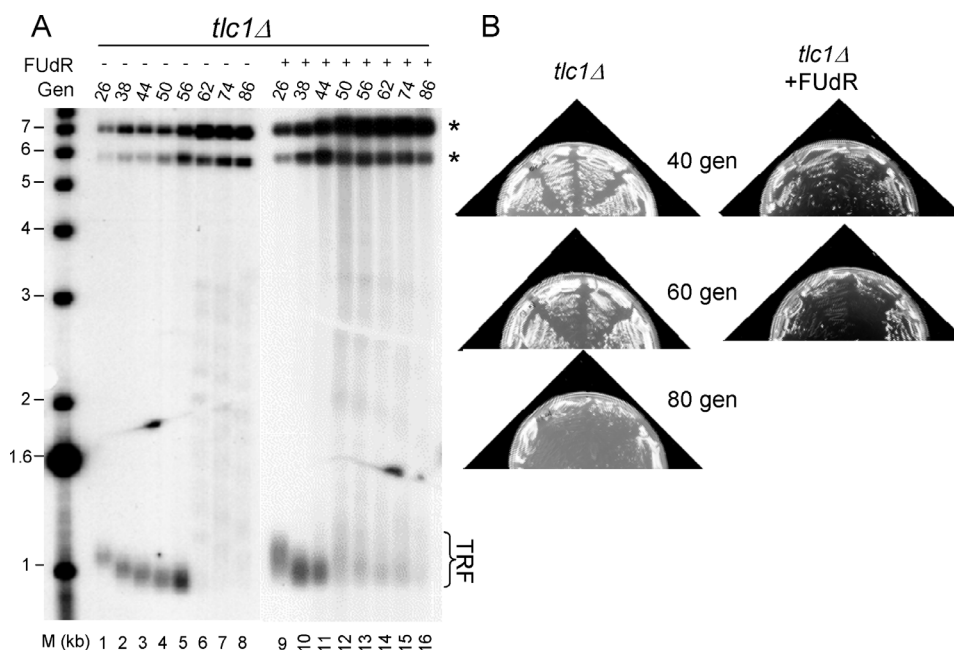


Figure 3. Accelerated telomere shortening and senescence at low TTP supply. Initially, cells carry a deletion of the *TLC1* gene at the genomic locus but are kept alive with a plasmid-borne copy of *TLC1* (pAZ1, *URA3*). These cells also carry a plasmid from which the HSV-TK gene is expressed (pTK, *TRP1*). These cells were then plated onto 5-FOA-Trp plates to select for cells that have lost pAZ1 but kept the pTK plasmid. Telomere length assays (A) and senescence assays (B) were then performed on these cells either with or without FUdR in the media. (A) Telomere length determination in telomerase-lacking cells grown for the indicated number of generations in liquid media with or without FUdR. TRF, terminal restriction fragments. The bands identified by asterisks represent subtelomeric Y'-elements. Note the increase in the intensity of these bands at the time point when the characteristic Y'-TRF is lost (an indication of survivors taking over the culture). (B) Appearance of cell growth on solid media without (left) or with (right) FUdR in the plates. Gen, number of generations.

in strains harboring a thermosensitive allele of DNA-polymerase α (*poll-17*). This *poll-17* mutation induces an over-extension of telomeric repeats, an effect that has been ascribed to a loss of the coordination between polymerase α and telomerase (16). Even at permissive temperature (23°C), cells harboring the *poll-17* allele display somewhat longer telomeres and when shifted to a semi-restrictive temperature (30°C), telomeres lengthen rapidly and in a telomerase-dependent fashion (16). Thus, we tested whether this temperature-inducible lengthening is affected by a lowered TTP supply (Figure 4). Cells unaffected by the presence of FUDR displayed the elongated telomeres at 23°C and over-elongated them upon incubation at 30°C, as expected (Figure 4, lanes 1 and 2). The same cells containing the pTK plasmid and thereby being affected in their ability to synthesize TTP displayed a very similar response in terms of telomere lengthening (Figure 4, lanes 3 and 4). Furthermore, in *poll-17* mutants, telomeric repeat tracts become extremely long upon outgrowth of these cells at the semi-restrictive temperature of 30°C (16). Again, there was no difference in this over-extension of telomeres, whether or not the cells were affected in their TTP synthesis (data not shown). Telomeres also over-elongated in *rif1* Δ cells exposed

to FUDR and containing the pTK plasmid when compared to identically treated wt cells (data not shown). However, the final lengths of telomeres in such treated *rif1* Δ cells did not reach those observed in untreated *rif1* Δ cells. These results can be viewed as consistent with the results obtained with the *poll-17* allele above, although other interpretations are possible for this particular case.

Taken together, these data strongly suggest that the ability of telomerase to add telomeric sequences is not or only in a minor way affected by a lowered TTP synthesis, and that the inhibition of TTP synthesis induced by FUDR and the pTK plasmid do affect telomere replication via the conventional replication machinery or increased telomere degradation activities associated with DNA repair (see below).

Preventing dUTP incorporation into telomeric DNA does not re-establish telomere lengths

The above experiments suggest that the lowered TTP supply created in our experimental conditions does not create a situation where TTP becomes limiting for telomerase. Recently, it has been shown that a defect in TTP synthesis can cause an accumulation of intracellular dUTP [(47), see Figure 1A]. A skewed dUTP/TTP ratio can result in the utilization of dUTP instead of TTP during DNA synthesis (48). Uracil misincorporation into genomic DNA is repaired by BER (base excision repair), creating single- and double-strand breaks (48). Hence, it was possible that dUTP misincorporation into telomeric DNA could create telomere shortening via the associated repair processes. dUTPase (Dut1p in *S.cerevisiae*) catalyzes the conversion of dUTP to dUMP, and counteracts dUTP accumulation (Figure 1A), which in the extreme case is lethal for the cells (49). Interestingly and in line with these models, it has been shown that overexpression of Dut1p can counteract the toxic effects of a low TTP supply induced by antifolate drugs, which affect Cdc21p-function in yeast (47). Therefore, if dUTP accumulation and uracil misincorporation was responsible for the observed telomere shortening, the effect should be reversible or at least partially alleviated by overexpression of Dut1p. We examined this possibility by performing the same experiments as above but with cells that overexpress Dut1p (Figure 5). While the overexpression of Dut1p can significantly suppress a slow growth phenotype associated with exposure of TK-containing cells to 10 μ g/ml FUDR (data not shown), it has no influence on telomere length (Figure 5, compare lanes 3 and 6). This result suggests that incorporation and repair of uracil residues in genomic DNA is not the major determinant for telomere shortening at lowered TTP supply.

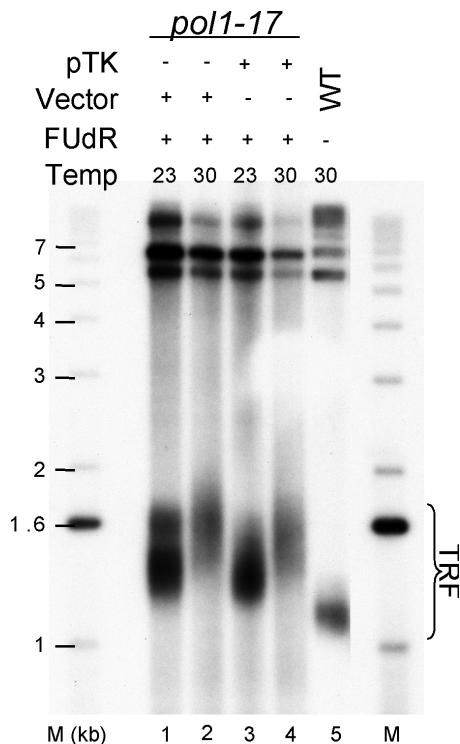


Figure 4. Telomerase-dependent telomere extension is not affected by a lowered TTP supply. *poll-17* cells containing an empty vector (Vector) or a plasmid-borne HSV-TK gene (pTK) were grown for 140 generations at 23°C with 5 μ g/ml FUDR in solid media. After this outgrowth, cells from these plates were transferred to liquid media (YC-trp + FUDR) and allowed to reach exponential growth phase at 23°C. Half of these cultures was then kept at 23°C (lanes 1 and 3) and the other half shifted to 30°C (lanes 2 and 4) for 16 h, followed by telomere length analysis on genomic DNA derived from cells containing the empty vector (lanes 1 and 2) or cells containing the plasmid with the HSV-TK gene (lanes 3 and 4). Lane 5, DNA derived from a wt strain grown in the absence of FUDR at 30°C.

DISCUSSION

Reduced nucleotide pools can provoke telomere shortening

In *S.cerevisiae*, *CDC21* and *CDC8* both are essential genes involved in TTP synthesis. Using two strategies, we show here that TTP synthesis defects are directly responsible for an unexpected phenotype, namely that of cells with lowered TTP supply harboring telomeres that are \sim 150 bp shorter

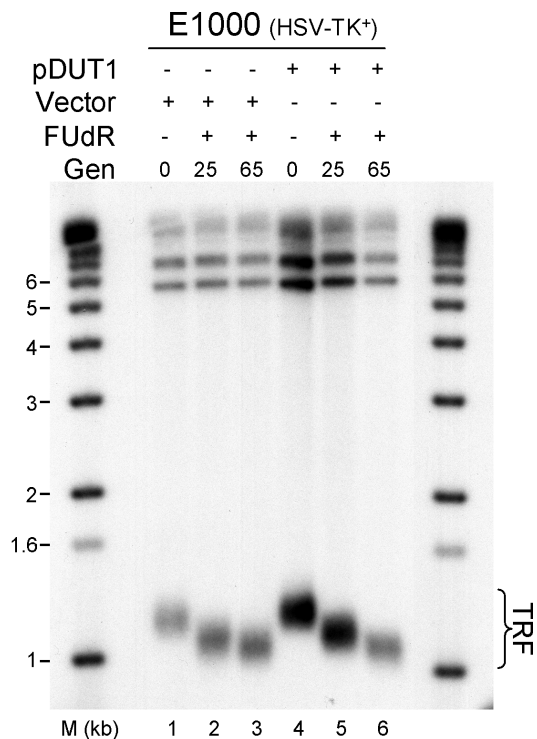


Figure 5. *Dut1p* overexpression does not prevent telomere shortening induced by lowered TTP supply. E1000 cells with integrated copies of HSV-TK either containing an empty vector (Vector, lanes 1–3) or containing an overexpressed plasmid-borne copy of *DUT1* (pDUT1, lanes 4–6) were grown for the indicated numbers of generations with 5 μ g/ml FUdR in the growth media. Telomere length analysis was performed as in Figure 2. M, molecular weight marker; TRF, terminal restriction fragments.

than those seen in wt cells. First, the short telomere phenotype of a *cdc8-1* strain can be suppressed by overexpressing HSV-TK (Figure 1C), as is the thermosensitive growth defect of *cdc8-1* mutants [(25), Figure 1B). Consistent with this idea, overexpression of Rnr1p in *cdc21-1*-cells re-establishes telomere length (data not shown). Second, a similar short telomere phenotype could be reproduced in wild-type cells by expressing HSV-TK and including FUdR in the growth media (Figure 2A). However, qualitatively the overall telomeric DNA end-structure, as assessed by native in-gel hybridization was not altered in *cdc21* mutant cells (data not shown). If a lowered TTP supply can cause telomere shortening, one would expect that inhibition of global dNTP synthesis should also cause this phenotype. Indeed, strains harboring mutations in RNR genes (*rnr1-240* and *rnr2-68*) do harbor shorter telomeres as compared to isogenic wild-type cells (Figure 2B). Therefore, a lowered dNTP supply can also lead to an alteration in the regulation of telomere length. We conclude that situations that lead to an inability to upregulate dNTP pools, and in particular to provide an adequate TTP supply, lead to defects in telomere length regulation.

Several and not mutually exclusive hypotheses can be formulated to explain the short telomere phenotype at low TTP supply. These include, but are not limited to (i) TTP becomes limiting for the telomerase enzyme; (ii) repair of telomeric DNA results in shorter telomeres instead of repaired telomeres; (iii) exaggerated telomere degradation by

exonucleases; (iv) the coordinated elongation of telomeres by semi-conservative replication and telomerase is affected.

Telomerase itself appears unaffected by the lowered TTP supply in our conditions

Since telomerase synthesizes the G-rich telomeric strand, our first hypothesis to explain the shortened telomere phenotype in cells with lowered TTP supply was that telomerase is very sensitive to nucleotide concentrations. If indeed this was the case and limited telomerase activity was the most important cause for telomere shortening, the dynamics of telomere attrition in the absence of telomerase should not change when TTP supply is lowered. This is not the case. Telomere attrition clearly is accelerated in this situation (Figure 3A). Similarly, telomere attrition is also accelerated in FUdR-treated versus untreated cells upon loss of the *TEL1* gene (data not shown).

These data show that at least part of the telomere-shortening induced at low TTP levels is due to telomerase-independent mechanisms. In order to verify whether the telomerase enzyme itself is sensitive to the low TTP pools caused in our experimental setting, we resorted to assay telomerase-mediated extension of telomeres in cells harboring a mutation in the gene encoding DNA-polymerase α (e.g. *poll-17*). Telomere lengthening in such cells occurs rapidly upon shift of cells to the semi-restrictive temperature and is not at all affected by a low TTP supply (Figure 4). It could be argued that the telomerase enzyme is temporarily inhibited by the low TTP supply, when exposed to the condition initially, but subsequently be upregulated to counteract the inhibition. However, if that was the case, one would expect the short telomere phenotype seen in wild-type, HSV-TK⁺-cells treated with FUdR to be transient as well, which is not the case (Figure 2A). The fact that telomere elongation in treated versus untreated cells with the *poll-17* mutation was indistinguishable indicates that the defect in the coordination of telomere end-replication is epistatic to defect causing telomere shortening induced by FUdR, reinforcing the idea that the short telomeres observed at low TTP pools are not a consequence of limiting nucleotide supply for the elongating activities at the end, i.e. telomerase. Taken together, these data strongly suggest that the lowered intracellular TTP supply generated by the experimental conditions used here do not affect the ability of telomerase to synthesize telomeric repeats. Thus, while the lowered TTP pools in these cells affect telomeric DNA metabolism, they are not yet limiting for the telomerase enzyme.

No evidence of excessive dUTP utilization

As another reason of the short telomeres in cells with lowered TTP supply, we considered the possibility that increased levels of dUTP could lead to the utilization of dUTP for DNA synthesis, which has been shown to occur in certain conditions of Cdc21p-inhibition (47). The misincorporation of uracil residues into DNA is repaired by base excision repair, which can create temporary single-strand DNA breaks. In conditions of continued low TTP supply, the repair machinery may actually use again dUTP for repair and the successive repair with high levels of single-strand DNA breaks is thought to be responsible for eventual cell death in extreme cases of a lack of TTP (thymine-less death) (48). At telomeres, such

repair of misincorporated uracil residues could result in shorter telomeres, since single-strand breaks of the G-rich strand may not be recoverable or lead to degradation of the C-rich strand. It has also been shown that overexpression of dUTPase in yeast can reduce elevated intracellular level of dUTP, thereby preventing dUTP incorporation into DNA and allowing faster growth and less mortality in cells with low TTP supply (47). However, even though dUTPase overexpression did indeed allow faster growth of cells exposed to FUDR in our experimental setting (data not shown), the short telomere phenotype was not relieved (Figure 5). Hence, repair of misincorporated uracil into telomeric DNA appears not to contribute significantly to the short telomere phenotype.

Other reasons for short telomeres at low TTP supply

In theory, telomere shortening could also be the result of increased shortening activities, such as brought about by exonucleases (50), for which there could be enhanced access to telomeres in our experimental conditions. While our data do not exclude this possibility, the fact that telomeres do not shorten in *poll-17*-cells (Figure 4) argues against this possibility. All of these considerations suggest that the defect leading to shortened telomeres in conditions of limiting nucleotide supply has to be associated with the replication fork itself. Leading-strand synthesis on telomeric repeats will require adequate supply of TTP, since the newly synthesized strand is relatively rich in T residues. This is not the case for lagging-strand synthesis; the newly synthesized lagging-strand on telomeric DNA in fact is devoid of T-residues. It is therefore possible that due to the limited supply of TTP for leading-strand synthesis, the fork may slow down or stochastically stall in the double-stranded portion of the telomeric repeats. Such stalled replication forks may cause single-strand breaks, which on telomeres could lead to shortening of the repeats. Stalled forks were already reported to occur near telomeric repeats and their abundance is increased in strains lacking the Rrm3p helicase (51). Intriguingly though, in that particular case telomeres do lengthen. In addition, when DNA derived from cells with lowered TTP supply was analyzed by two-dimensional gel-electrophoresis, we could not find evidence for stalled forks near telomeres (data not shown). A more satisfying hypothesis explaining all the data would imply that due to a differential requirement of leading- versus lagging-strand synthesis for nucleotides, normal coordination of the synthesis of the two strands may be hampered on telomeric DNA in conditions of lowered TTP supply. As a consequence, the transition to telomerase-mediated elongation may also become affected. The only situation where such coordination defects are suppressed occurs in cells harboring the *poll-17* mutation. While the precise reasons for the over-extension of telomeres in *poll-17* cells remain unknown, uncoordinated synthesis of the two strands is the best hypothesis (12,16). Thus, affecting coordinated DNA synthesis at the fork by lowering nucleotide supply would have no effect in this particular situation, where uncoordinated telomeric repeat synthesis of the two strands is allowed by the *poll-17* allele.

Taken together, our results suggest that it is the coordinated replication of both strands of telomeric DNA which is exquisitely sensitive to alterations in nucleotide pools. An inability

to supply adequate dNTPs, in our case TTP, may result in a strand-specific slow-down of DNA synthesis followed by a decreased ability to perform coupled repeat extension. The net result is a decrease in the average length of telomeric repeat tracts. This hypothesis may also explain why a number of mutations in other genes governing replication or replication checkpoint functions lead to shortened telomeres (23,52,53). For example, mutations in the gene encoding DNA polymerase ϵ cause a similar telomere shortening effect as reported here (17), and inhibition of nucleotide synthesis by HU in cells harboring mutations in *RAD53* frequently results in hemi-replicated molecules (54). This indicates that situations leading to fork instability (55–57) may also cause telomeric repeat shortening. Consistent with this interpretation, constitutive upregulation of nucleotide pools that can suppress the lethality of deletions of the *RAD53* or *MEC1* genes (20,58) also restore normal telomere length in cells carrying hypomorphic mutations of these genes (23,59). Thus, we speculate that the short telomeres observed in cells harboring *cdc21-1* or *cdc8-1* alleles ultimately are a consequence of destabilized replication forks induced by unbalanced nucleotide pools.

ACKNOWLEDGEMENTS

We thank R. Sclafani, E. Schwob, D. Gottschling, S. Elledge and C. Holm for plasmids and yeast strains used in this study. Special thanks to Andrea Moore who performed preliminary work for this study and D. Hunting and M. Chakhparonian for insightful discussions on nucleotide synthesis. The members of our lab are also thanked for their continued productive suggestions. This work was supported by a grant of the Canadian Institutes for Health Research (CIHR, grant MOP12616). R.J.W. is a Chercheur National of the Fonds de Recherche en Santé du Québec (FRSQ). Funding to pay the Open Access publication charges for this article was provided by the Canadian Institutes for Health Research.

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