3964–3969 *Nucleic Acids Research, 1999, Vol. 27, No. 20*

G-strand overhangs on telomeres in telomerase-deficient mouse cells

Michael T. Hemann[1,2] and Carol W. Greider[2,*]

[1]Predoctoral Training Program in Human Genetics and [2]Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, 617 Hunterian Building, 725 North Wolfe Street, Baltimore, MD 21205, USA

Received July 23, 1999; Revised and Accepted August 18, 1999

ABSTRACT

Telomeres of eukaryotic chromosomes contain 3′ overhangs which are thought to be essential for the maintenance of proper chromosome end structure and function. We examined the requirement for telomerase activity for the generation of these G-strand overhangs in mammalian cells. Using non-denaturing in-gel hybridization to both tissue and cultured cells from mice deficient for the telomerase RNA component, we found that G-strand overhangs exist in the absence of telomerase activity. Quantitation of overhang signal intensity showed no significant reduction in telomerase-deficient cells relative to wild-type. These results support a telomerase-independent mechanism for generating G-strand overhangs.

INTRODUCTION

Telomeres are the dynamic termini of linear chromosomes, capable of repeatedly reforming functional terminal nucleoprotein structures following telomere sequence shortening or elongation. These nucleoprotein structures play essential roles, including the prevention of end-to-end fusions, abnormal recombination and degradation (reviewed in 1). In most eukaryotes, telomeres are comprised of long stretches of tandem short DNA sequences. Mammalian telomeres consist of a variable number of TTAGGG repeats. Human telomeres contain ~1000–2000 repeats, while mouse telomeres contain up to tens of thousands of these repeats (2–4).

The termini of most eukaryotic telomeres include a G-rich strand which extends beyond the DNA duplex to form a single-stranded 3′ overhang. The presence of overhangs is conserved in eukaryotes, but, like telomere length, the exact structure varies between species. Macronuclear chromosomes of the ciliated protozoans, where 3′ overhangs were first identified, have G-strand overhangs specifically maintained at between 12 and 16 bases (5–7). In *Saccharomyces cerevisiae* 3′ overhangs of greater than 30 bases in length appear transiently during S phase (8). It is not known, however, if shorter overhangs are present at other phases of the cell cycle. G-strand overhangs are present throughout the cell cycle in human cells, with an estimated length of between 45 and 275 bp (9–11). These overhangs have been postulated to be involved in telomere functions such as binding end-specific telomere proteins (12,13) preventing end-to-end fusions (14) and promoting proper meiotic and mitotic chromosome segregation (reviewed in 15). The mechanism by which G-overhangs perform these functions is likely dependent upon specific binding proteins and possibly DNA conformation. A number of proteins have been shown to bind G-strand overhangs either *in vivo* or *in vitro*. These include the α and β telomere-binding proteins (TBPs) in *Oxytricha* and *Euplotes* (12,13,16,17), Cdc13p in *Saccharomyces* (18,19) and several hnRNPs in humans and mice (20–22). G-rich single-stranded telomere sequence can assume a number of non-canonical structures *in vitro*, including G quartets and triple helices (reviewed in 23), and they may mediate telomere looping (24). However, it is not clear what role these structures play *in vivo*.

Despite the increasing data on the structure of 3′ overhangs, little is known about the mechanism of overhang generation in mammalian cells. Overhangs are likely to be generated by either elongation of the single-stranded G-strand or degradation of the C-strand. Telomerase is therefore a likely candidate to be involved in creating G-strand overhangs. Telomerase is able to extend 3′ overhangs, preventing progressive telomere shortening which may result from the inability of DNA polymerase to completely replicate linear chromosome ends (25).

Several recent experiments have suggested that telomerase may not be necessary for the generation of G-strand overhangs. In *Saccharomyces*, it has been demonstrated that overhangs are created in the absence of telomerase (26,27). The presence of these overhangs does not, however, preclude a significant contribution by telomerase to the length of normal G-strand overhangs. Additionally, the significant size difference between *Saccharomyces* and mammalian overhangs and the cell cycle dependence of overhangs in *Saccharomyces* suggest that mammalian cells might use a different mechanism to generate 3′ overhangs. G-strand overhangs are also seen in mammalian cells with no detectable telomerase activity (10). However, these cells may still have low levels of telomerase activity or activity may have been transiently expressed.

To examine the role of telomerase in the generation of overhangs in mammalian cells, we used knockout mice which lack telomerase activity (28,29). We undertook a detailed analysis of G-strand overhangs both in liver cells and cultured fibroblasts obtained from these telomerase null mice. Quantitation of overhang signal in telomerase knockout cells compared with

*To whom correspondence should be addressed. Tel: +1 410 614 6506; Fax: +1 410 614 2987; Email: cgreider@jhmi.edu

wild-type cells indicates that telomerase is not primarily responsible for generating G-strand overhangs.

MATERIALS AND METHODS

Isolation of liver cells

Whole livers were harvested from age-matched 3-month-old wild-type (C57Bl/6) and mTR KO (C57Bl/6/129) mice. An aliquot of 1 g (approximately one-half) of each liver was homogenized in 5 ml of an ice-cold solution containing 60 mM KCl, 15 mM NaCl, 0.5 mM spermine, 0.15 mM spermidine, 15 mM Tris pH 7.5, 2 mM EDTA, 0.3 M sucrose and 16.7 mM β-mercaptoethanol. The homogenates were layered on top of 7.5 ml of a solution containing 60 mM KCl, 15 mM NaCl, 0.5 mM spermine, 0.15 mM spermidine, 15 mM Tris pH 7.5, 1 mM EDTA, 1.37 M sucrose and 14.4 mM β-mercaptoethanol in 25 × 89 mm ultracentrifuge tubes. The sucrose gradients were centrifuged at 12 000 *g* for 15 min at 4°C. After centrifugation, the supernatent was removed, and the pellet was washed in 10 ml PBSA (Dulbecco's phosphate-buffered saline, without calcium and magnesium). After washing, the cells were pelleted by centrifugation for 5 min at 800 *g* and resuspended in PBSA at a density of 2×10^7 cells/ml.

Isolation and culture of mouse embryonic fibroblasts

Embryos at 13.5 day post-conception were obtained from pregnant wild-type (C57Bl/6/129) and mTR KO (C57Bl/6/129) generation 3 and 4 (G4) females. Mouse embryonic fibroblast (MEF) cell lines were established as described (30). Passage 20 MEFs of each cell line from a 3T3 passaging protocol were trypsinized prior to confluence, washed twice in PBSA and resuspended in PBSA at a density of 1×10^7 cells/ml.

Plug preparation

Liver cells and MEFs were combined with an equal volume of 2% low melting point (LMP) agarose at 40°C and cast into 100 µl plug molds. After casting, plugs were incubated in a lithium dodecyl sulfate (LDS) solution, containing 1% LDS, 100 mM EDTA pH 8.0 and 10 mM Tris pH 8.0 at 37°C for 1 h with constant agitation. The solution was then replaced with fresh solution, and the plugs were incubated overnight at 37°C. This LDS cell lysis protocol does not require the use of a protease, which could potentially inhibit subsequent enzymatic treatment of plug DNA. The plugs were washed twice for 2 h in 20% NDS (2 mM Tris, 6.8 mM *N*-laurylsarcosine and 127 mM EDTA brought to pH 9.5 with NaOH). A high EDTA concentration and pH in the NDS solution were used to prevent nuclease degradation of the plug DNA and non-specific DNA interactions. Plugs were stored at 4°C in 20% NDS.

Pulsed field gel

DNA plugs to be digested were incubated for 1 h in TE, then again for 1 h in fresh TE. The DNA plugs were then incubated for 2 h in 1× *Sau*3A1 restriction enzyme buffer, with the initial enzyme buffer replaced with fresh enzyme buffer after 1 h. Each plug was then placed in 400 µl 1× *Sau*3A1 buffer with 60 U *Sau*3A1 or *Dpn*II and digested for 16 h at 37°C. After 16 h, an additional 20 U of enzyme was added, and the plugs were incubated for 8 h. Plugs were then cut into four approximately equal sized pieces, in order to perform multiple experiments on the same plug preparation. Mung bean nuclease digestion of plugs was performed by first preincubating 1/4 plugs twice for 1 h in 10 µl 1× mung bean nuclease buffer (Life Technologies Inc.) on ice, then adding 39 U of mung bean nuclease (Life Technologies Inc.) to each digestion. Tubes were kept on ice for 15 min, to permit enzyme diffusion through the plug, then incubated at 30°C for 20 min. Reactions were terminated on ice by the addition of 0.01% SDS. Plugs were prepared for electrophoresis by washing for 2 h in 4 ml TE, changing the original TE for fresh TE after 1 h, then for 1 h in gel running buffer. 1/4 plugs were loaded into each well and covered with LMP agarose. Denatured wild-type control DNA was prepared by heat denaturing 1/10 of a *Sau*3A1-digested wild-type plug, adding 40 µl LMP agarose and pipette loading into the well. λ DNA concatamers embedded in agarose (New England Biolabs) were used as molecular weight markers.

Plugs were run on a 1.2% pulsed field grade agarose (Bio-Rad) gel in 1× TAE. Gels were run using a CHEF-DR II pulsed field gel apparatus (Bio-Rad) for 20 h at a constant 200 V, using ramped pulse times from 1 to 15 s. Running temperature was kept at 14°C.

In-gel hybridization

Following electrophoresis and EtBr staining, gels were dried down on filter paper for 1 h at 50°C. Gels were prehybridized for 1 h at 55°C in 20 mM NaH_2PO_4, 0.1% SDS, 5× Denhardt's reagent and 5× SSC and hybridized with probe for 3 h at 55°C in 5 ml prehybridization solution. $(TTAGGG)_4$, $(CCCTAA)_4$ and $(CA)_{12}$ oligonucleotides were obtained from Operon Technologies Inc. All oligonucleotides used as probes were end-labeled with [γ-^{32}P]ATP and then purified using NAP™5 columns (Pharmacia Biotech). After hybridization, gels were washed three times for 20 min in 4× SSC at room temperature and three times for 20 min in 4× SSC, 0.1% SDS at 57°C. Following sequential native gel hybridizations, dried gels were alkali denatured in 0.6 M NaCl, 0.2 M NaOH for 1 h, neutralized in 1.5 M NaCl, 0.5 M Tris pH 7.4 for 1 h, rinsed in dH_2O for 30 min and reprobed. Image analysis and quantitation was performed using a Fuji Film BAS 1500 Bio-Imaging Analysis System including Image Reader Software v.1.3E. To prevent quantitation of non-specific hybridization near the well, only the signal from DNA that entered the gel was used in our analysis.

RESULTS

Telomeres from mTR$^{-/-}$ mouse livers have 3′ overhangs

If telomerase activity is required for the addition of G-strand overhangs, telomeres from telomerase-deficient mouse cells should lack overhangs. To investigate this possibility, we utilized a recently described telomere non-denaturing in-gel hybridization technique to determine whether cells from telomerase null mTR$^{-/-}$ tissue have 3′ overhangs (26). One advantage of using this technique is that the frequently inefficient depurination and transfer of high molecular weight terminal restriction fragments (TRFs) onto a membrane is not necessary. The other advantage is that a single gel can be probed first under native conditions, then the DNA can be denatured and probed again.

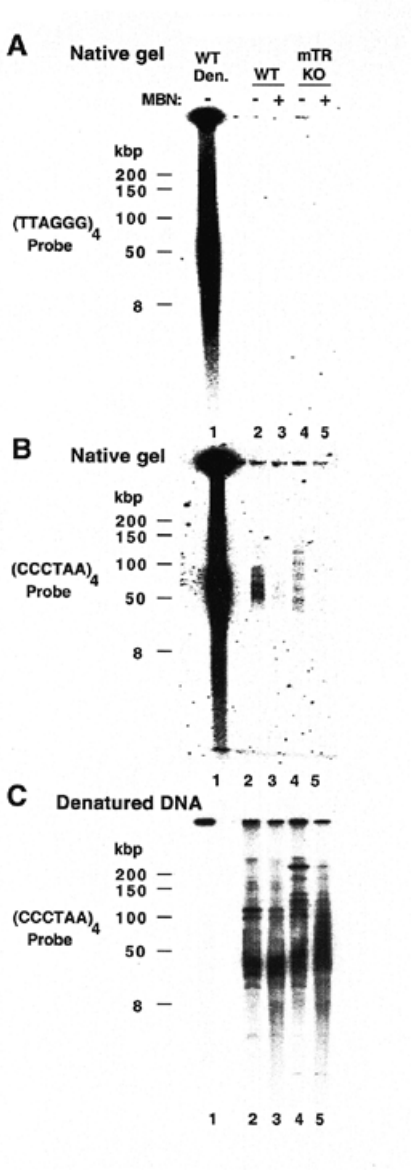

Figure 1. G-strand overhangs are present in the absence of telomerase. End-labeled oligonucleotide in-gel hybridization to *Sau*3A1 digests of agarose-embedded genomic DNA. Wild-type (C57Bl/6) denatured DNA control (lane 1), wild-type (C57Bl/6) (lanes 1 and 2) and mTR$^{-/-}$ (C57Bl/6/129) (lanes 3 and 4) liver DNA plugs with (lanes 2 and 4) and without (lanes 3 and 5) mung bean nuclease. (**A**) Native gel probed with $(TTAGGG)_4$. (**B**) Native gel probed with $(CCCTAA)_4$. (**C**) Denatured DNA probed with $(CCCTAA)_4$.

TRF lengths of most mouse strains vary between 30 and 200 kb and cannot be resolved via conventional electrophoresis (2,31). Cells isolated from age-matched mTR$^{-/-}$ and wild-type mouse livers were embedded in agarose plugs and digested with *Sau*3A1 or *Dpn*II. Plug-embedded digested DNA was then resolved on a pulsed field gel. Following electrophoresis, gels were first analyzed by non-denaturing in-gel hybridization followed by denaturing in-gel hybridization. To control for the possible presence of nicked or denatured DNA, we first probed the gel with an oligonucleotide identical to the overhang sequence. Hybridization of a ^{32}P-end-labeled G-strand oligonucleotide, $(TTAGGG)_4$, to the native gel produced a signal only in the control lane where genomic DNA was denatured before loading onto the gel (Fig. 1A). Subsequent hybridization of a ^{32}P-end-labeled C-strand oligonucleotide, $(CCCTAA)_4$, to the same native gel produced a signal both in the wild-type and mTR KO lanes (Fig. 1B). Treatment with a single-strand-specific DNA nuclease should remove 3′ overhangs. Genomic DNA embedded in plugs pretreated with mung bean nuclease showed no signal when probed with the labeled C-strand oligonucleotide (Fig. 1B). To ensure that the plug DNA was not significantly degraded during mung bean nuclease treatment, we denatured the gel and probed again with the end-labeled C-strand oligonucleotide. Both nuclease-untreated and -treated DNA showed a similar telomere restriction pattern (Fig. 1C). Comparison of the native and denatured DNA (Fig. 1B and C) shows a higher intensity signal in the high molecular weight bands in the denatured gel compared to the native gel. This occurs because, as the amount of double-stranded telomere repeat signal increases, the amount of undenatured overhang sequence remains constant. The variations in TRF lengths seen in the gels is indicative of the significant TRF length heterogeneity that exists between mice (2). Wild-type mice in this study were C57Bl/6J, which show a reasonably discrete pattern of telomere restriction fragment lengths centered around 50 kb. The mTR$^{-/-}$ mice are on a mixed C57Bl/6J and 129/SvJ background (28,29) and the 129/SvJ background have hypervariable TRF lengths (2). The ability to detect a signal with the $(CCCTAA)_4$ probe hybridized to undenatured mTR$^{-/-}$ samples indicates that G-strand overhangs are present in the absence of telomerase activity.

mTR-deficient cultured fibroblasts have 3′ overhangs

To determine whether rapidly dividing cells from mTR$^{-/-}$ mice also contain G-strand overhangs, we assessed the overhang status of actively dividing wild-type and mTR$^{-/-}$ embryonic fibroblasts. We carried out non-denaturing and denaturing in-gel hybridization to *Sau*3A1 digests of genomic DNA derived from mouse embryonic fibroblasts. Hybridization of an end-labeled $(CCCTAA)_4$ oligonucleotide to a native gel produced overhang signals for wild-type fourth generation (G4) mTR$^{-/-}$ fibroblasts and fifth generation (G5) mTR$^{-/-}$ fibroblasts (Fig. 2A). Denaturation of the DNA and rehybridization with the same probe showed the expected identical signal pattern as the overhang signal at significantly greater intensity (Fig. 2B). As shown previously for primary cells, pretreatment with mung bean nuclease or hybridization of non-nuclease-treated fibroblasts with an end-labeled $(TTAGGG)_4$ oligonucleotide probe produced no signal (data not shown). Thus, overhangs are present on telomeres of both wild-type and mTR$^{-/-}$, regardless of the proliferative status of the cells.

G-strand overhang signal intensity is not significantly reduced in mTR-deficient cells

The presence of 3′ overhangs in the absence of telomerase does not, however, preclude a potential role for telomerase in normal G-strand overhang homeostasis. Telomerase may contribute to some extent to the generation of 3′ overhangs. We therefore quantitated the G-strand overhang signal in the presence and absence of telomerase. The relative signal intensity of the end-labeled $(CCCTAA)_4$ oligonucleotide hybridized to 3′ overhangs in wild-type and G4 mTR$^{-/-}$ primary liver cells was

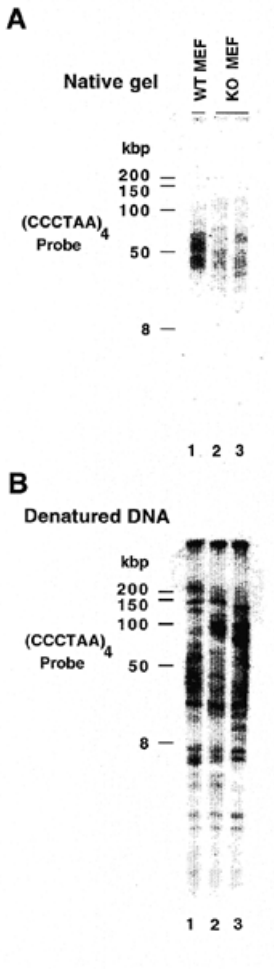

Figure 2. G-strand overhangs are present in actively dividing telomerase null cells. End-labeled oligonucleotide in-gel hybridization to *Sau*3A1 digests of wild-type (C57Bl/6/129) and mTR$^{-/-}$ (C57Bl/6/129) MEFs. Wild-type MEFs (lane 1) and mTR KO generations 4 (lane 2) and 5 MEFs (lane 3). (**A**) Native gel probed with $(CCCTAA)_4$. (**B**) Denatured DNA probed with $(CCCTAA)_4$.

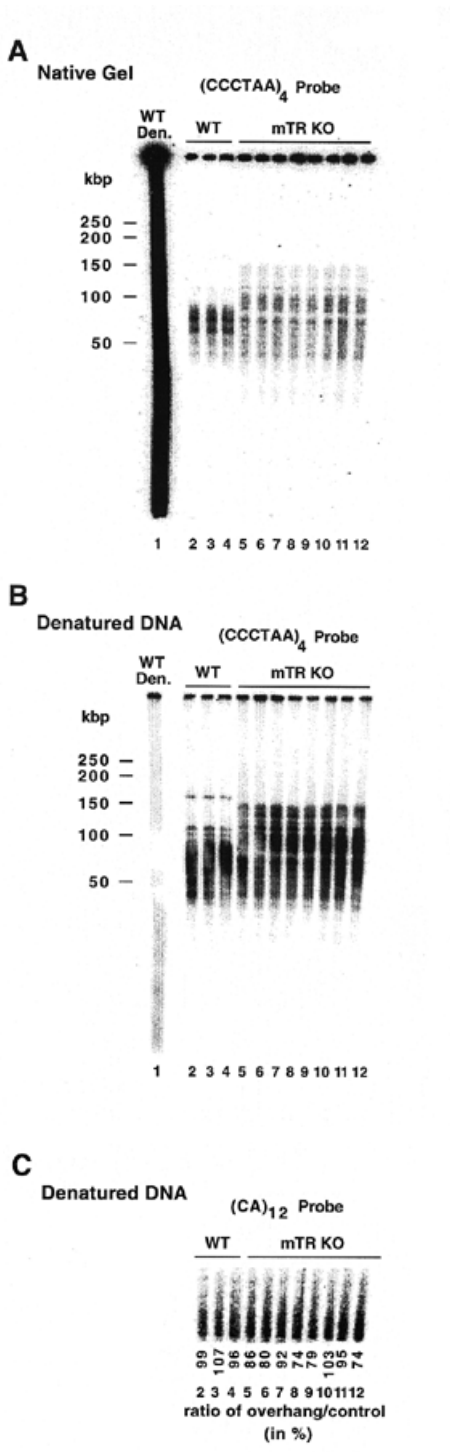

Figure 3. Quantitation of G-strand overhangs. End-labeled oligonucleotide in-gel hybridization to *Sau*3A1 digests of wild-type (C57Bl/6) and mTR$^{-/-}$ (C57Bl/6/129) liver DNA plugs. Wild-type (lanes 2–4) and mTR$^{-/-}$ generation 4 (lanes 5–12) liver DNA plug digests. (**A**) Native gel probed with $(CCCTAA)_4$. (**B**) Denatured DNA probed with $(CCCTAA)_4$. (**C**) Denatured DNA probed with $(CA)_{12}$ and quantitation of relative signal intensity. Values beneath each lane represent the ratio of overhang signal from (A) to control $(CA)_{12}$ signal from (C), normalized to an average wild-type value of 100%. The mean wild-type value is 100%, with a standard deviation of ± 5%. The mean mTR$^{-/-}$ value is 85%, with a standard deviation of ± 11%.

determined (Fig. 3A–C). Because telomere length varies between wild-type and mTR-deficient mice, overhang signal intensity could not be accurately quantitated relative to the signal intensity after denaturation of the DNA. Rather, we quantitated overhang signal intensity relative to a probe specific for mouse microsatellite repeats. Denaturation of the DNA after overhang detection and rehybridization with a ^{32}P-end-labeled $(CA)_{12}$ oligonucleotide resulted in a microsatellite repeat signal running at ~4 kb. Multiple DNA preparations in agarose plugs from the same mouse were run together to ensure the reproducibility of the results. Quantitation of three wild-type overhang signals and eight G4 mTR$^{-/-}$ overhang signals relative to the microsatellite repeat signals in the same lanes revealed an average mTR-deficient signal intensity of 85 ± 11% relative to wild-type (Fig. 3C). When the experiment was repeated with cells from a different G4 mTR$^{-/-}$ mouse, the resulting ratio of mTR-deficient overhang signal to wild-type overhang signal was 89 ± 10% (Table 1). In total, the overhang signals from four different G4 mice were quantitated, with an

average signal intensity of 87.8 ± 8.4% relative to wild-type (Table 1). While this decrease in signal intensity is not statistically significant, we cannot rule out a small contribution by telomerase to the generation of overhangs in wild-type cells.

Table 1. Relative G-strand overhang signal intensity from different mTR$^{-/-}$ mice

Generation	Mouse no.	Overhang signal intensity (%)[a]
G4	1	85 ± 11
G4	2	90 ± 10
G4	3	78
G4	4	98
G6	1	87 ± 10
G6	2	94
G6	3	79

[a]Overhang signal intensity is quantitated relative to wild-type values on the same gel.

Telomere length decreases progressively in mTR$^{-/-}$ mice with increasing generations in the absence of telomerase (30). We next sought to determine whether G-strand overhang signal intensity also decreases with increasing generations in the absence of telomerase. The relative signal intensity of the end-labeled $(CCCTAA)_4$ oligonucleotide hybridized to 3′ overhangs from wild-type, G4 mTR$^{-/-}$ and sixth generation (G6) mTR$^{-/-}$ mouse tissue was determined (Table 1 and data not shown). In total, the overhang signals from three independent G6 mTR$^{-/-}$ mice were quantitated, with an average signal intensity of 86.7 ± 7.5% relative to wild-type (Table 1). This relative signal intensity is very similar to the 87.8 ± 8.4% obtained for G4 mTR$^{-/-}$ overhangs (Table 1). Thus, overhang signal intensity is apparently unaffected by the continued loss of telomere repeats between G4 and G6 of mTR deficiency. Analysis of maximum G-strand overhang length by primer extension (10), likewise, showed no difference between successive generations in the absence of telomerase (data not shown).

DISCUSSION

Early work studying telomeres in ciliate macronuclei revealed that 3′ overhangs were present on telomeres (5). The subsequent discovery of G-strand overhangs in *Saccharomyces*, humans and mice demonstrates that terminal overhangs are evolutionarily conserved components of eukaryotic chromosomes (8–10,26,27). The precise functions of overhangs are still unclear, but recent evidence suggests that 3′ overhangs may be required for chromosome stability. Human cells transfected with dominant negative mutants of the telomere-binding protein TRF2 show an absence of G-strand overhangs and the rapid accumulation of chromosome fusions and aneuploid cells (14) and induction of the DNA damage response (32). As additional functions are ascribed to terminal overhangs, it becomes increasingly important to understand how they are generated.

There are a number of possible mechanisms for the generation of G-strand overhangs in mammalian cells. One possibility is that overhangs are created only on one chromosome end, with a length dependent on the placement of the terminal RNA primer on the lagging strand during DNA replication (11). Recent experiments demonstrating the critical role of overhangs in maintaining proper chromosome end structure and integrity seem to argue against such an asymmetry at chromosome ends. Another possibility is that overhangs, particularly in the absence of telomerase, may be generated by a gene conversion type of recombination mechanism. However, the extent and efficiency of recombination required to regenerate overhangs on every chromosome end after every round of DNA replication makes this mechanism improbable. Two additional mechanisms for generating G-strand overhangs are elongation of the G-strand by telomerase and/or degradation of the C-strand. Evidence from budding yeast showed that 3′ overhangs are present in the absence of telomerase, but a quantitative difference in overhang signal intensity in the presence and absence of telomerase was not investigated. Experiments using human cells with no detectable telomerase activity showed the presence of overhangs, however, a low or transient level of telomerase activity may have generated these overhangs (9,10).

Our data show that mouse cells lacking the RNA component of telomerase and telomerase activity still have overhangs. The slight, but consistent, reduction in overhang signal intensity in the mTR$^{-/-}$ cells, while not statistically significant, suggests that telomerase might transiently contribute to overhang length at a specific point in the cell cycle. This contribution might, for example, result from the lag between transient G-strand elongation by telomerase and complementary C-strand polymerization.

The presence of G-strand overhangs in the absence of telomerase, while not precluding the presence of 3′ overhangs on only one chromosome end, are consistent with a proposed exonuclease- or endonuclease-mediated activity capable of selective degradation of the C-strand. There is considerable evidence from other species that such a nuclease activity exists. For example, in *Saccharomyces* strains deleted for the G-strand-binding protein Cdc13p, chromosome ends show rapid 5′→3′ C-strand degradation (18,19,33). Recent evidence has implicated Rad50p, Mre11p and Xrs2p in telomere maintenance and possibly in C-strand processing (34–36). Mutations in these genes in yeast result in a significantly slower rate of 5′→3′ degradation of single-stranded DNA (37). This is the type of exonuclease activity predicted to be required to form G-strand overhangs, although there is currently some controversy about the role of Rad50p, Mre11p and Xrs2p in telomere maintenance (38–40). Possible other candidates for the regulation of a C-strand nuclease activity in mammalian cells include TRF1, TRF2, various hnRNPs and the catalytic subunit of telomerase, hTERT.

A nuclease-mediated generation of G-strand overhangs has significant bearing on models for both telomere shortening and replication (9,41). Current estimated rates for telomere shortening assume that the amount of sequence lost per round of replication equals the size of the terminal RNA primer or Okazaki fragment (11,42). However, rates of telomere shortening vary significantly between organisms, with yeast chromosomes shortening around 4 bp per cell division (43) and human chromosomes shortening between 50 and 100 bp per cell division (44,45). G-strand overhang size also varies

significantly between species. These differences may reflect actual differences in replication mechanism, or they may reflect differences in the nucleolytic processing of chromosome ends. Current models to calculate sequence loss per cell division make assumptions about the loss of the terminal RNA primer or terminal Okazaki fragment at each cell division (11,42). Given that there may be multiple origins of the G-strand overhang, including C-strand degradation, these models for calculating the rate of sequence loss need to be re-evaluated. The actual terminal sequence loss caused by the inability to fully replicate a linear chromosome may be significantly less than the rate of sequence loss resulting from the nucleolytic degradation of chromosome ends.

Models which assume that telomere end structures are generated directly by telomerase-mediated single-strand DNA elongation must also be re-examined. It has been postulated that, due to the presence of a putative blunt end at one telomere after DNA replication, half of all chromosome ends cannot serve as substrates for telomerase (11,41). The presence of a telomerase-independent nuclease that processes chromosome ends would create a situation where telomerase may never encounter blunt chromosome ends.

ACKNOWLEDGEMENTS

We thank Dr Titia de Lange for helpful discussions and Drs Siyuan Le, Kay Keyer and Stephen Buck for critical reading of the manuscript. M.T.H. was supported by the Predoctoral Training Program in Human Genetics NIH grant GM07814 and this work was supported by NIH grant CA16519 to C.W.G.

REFERENCES

1. Greider,C.W. (1996) *Annu. Rev. Biochem.*, **65**, 337–365.
2. Kipling,D. and Cooke,H.J. (1990) *Nature*, **347**, 400–402.
3. Lansdorp,P.M., Verwoerd,N.P., van de Rijke,F.M., Dragowska,V., Little,M.T., Dirks,R.W., Raap,A.K. and Tanke,H.J. (1996) *Hum. Mol. Genet.*, **5**, 685–691.
4. Zijlmans,J.M., Martens,U.M., Poon,S.S., Raap,A.K., Tanke,H.J., Ward,R.K. and Lansdorp,P.M. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 7423–7428.
5. Klobutcher,L.A., Swanton,M.T., Donini,P. and Prescott,D.M. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 3015–3019.
6. Pluta,A.F., Kaine,B.P. and Spear,B.B. (1982) *Nucleic Acids Res.*, **10**, 8145–8154.
7. Henderson,E.R. and Blackburn,E.H. (1989) *Mol. Cell. Biol.*, **9**, 345–348.
8. Wellinger,R.J., Wolf,A.J. and Zakian,V.A. (1993) *Cell*, **72**, 51–60.
9. Makarov,V.L., Hirose,Y. and Langmore,J.P. (1997) *Cell*, **88**, 657–666.
10. McElligott,R. and Wellinger,R.J. (1997) *EMBO J.*, **16**, 3705–3714.
11. Wright,W.E., Tesmer,V.M., Huffman,K.E., Levene,S.D. and Shay,J.W. (1997) *Genes Dev.*, **11**, 2801–2809.
12. Gottschling,D.E. and Zakian,V.A. (1986) *Cell*, **47**, 195–205.
13. Gray,J.T., Celander,D.W., Price,C.M. and Cech,T.R. (1991) *Cell*, **67**, 807–814.
14. van Steensel,B., Smogorzewska,A. and de Lange,T. (1998) *Cell*, **92**, 401–413.
15. Dernburg,A.F., Sedat,J.W., Cande,W.Z. and Bass,H.W. (1995) In Blackburn,E. and Greider,C. (eds), *Telomeres*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 295–338.
16. Price,C.M. (1990) *Mol. Cell. Biol.*, **10**, 3421–3431.
17. Wang,W., Skopp,R., Scofield,M. and Price,C. (1992) *Nucleic Acids Res.*, **20**, 6621–6629.
18. Nugent,C.I., Hughes,T.R., Lue,N.F. and Lundblad,V. (1996) *Science*, **274**, 249–252.
19. Lin,J.J. and Zakian,V.A. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 13760–13765.
20. McKay,S.J. and Cooke,H. (1992) *Nucleic Acids Res.*, **20**, 6461–6464.
21. Ishikawa,F., Matunis,M.J., Dreyfuss,G. and Cech,T.R. (1993) *Mol. Cell. Biol.*, **13**, 4301–4310.
22. LaBranche,H., Dupuis,S., Ben-David,Y., Bani,M.R., Wellinger,R.J. and Chabot,B. (1998) *Nature Genet.*, **19**, 199–202.
23. Henderson,E. (1995) In Blackburn,E. and Greider,C. (eds), *Telomeres*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 11–34.
24. Griffith,J.D., Comeau,L., Rosenfield,S., Stansel,R.M., Bianchi,A., Moss,H. and de Lange,T. (1999) *Cell*, **97**, 503–514.
25. Greider,C.W. and Blackburn,E.H. (1985) *Cell*, **43**, 405–413.
26. Dionne,I. and Wellinger,R.J. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 13902–13907.
27. Wellinger,R.J., Ethier,K., Labrecque,P. and Zakian,V.A. (1996) *Cell*, **85**, 423–433.
28. Blasco,M.A., Lee,H.W., Hande,M.P., Samper,E., Lansdorp,P.M., DePinho,R.A. and Greider,C.W. (1997) *Cell*, **91**, 25–34.
29. Lee,H.W., Blasco,M.A., Gottlieb,G.J., Horner,J.W., Greider,C.W. and DePinho,R.A. (1998) *Nature*, **392**, 569–574.
30. Blasco,M.A., Lee,H.W., Rizen,M., Hanahan,D., DePinho,R. and Greider,C.W. (1997) *Ciba Found. Symp.*, **211**, 160–170.
31. Starling,J.A., Maule,J., Hastie,N.D. and Allshire,R.C. (1990) *Nucleic Acids Res.*, **18**, 6881–6888.
32. Karlseder,J., Broccoli,D., Dai,Y., Hardy,S. and de Lange,T. (1999) *Science*, **283**, 1321–1325.
33. Garvik,B., Carson,M. and Hartwell,L. (1995) *Mol. Cell. Biol.*, **15**, 6128–6138.
34. Usui,T., Ohta,T., Oshiumi,H., Tomizawa,J., Ogawa,H. and Ogawa,T. (1998) *Cell*, **95**, 705–716.
35. Haber,J.E. (1998) *Cell*, **95**, 583–586.
36. Le,S., Moore,J.K., Haber,J.E. and Greider,C.W. (1999) *Genetics*, **152**, 143–152.
37. Haber,J.E. (1995) *Bioessays*, **17**, 609–620.
38. Chamankhah,M. and Xiao,W. (1999) *Nucleic Acids Res.*, **27**, 2072–2079.
39. Moreau,S., Ferguson,J.R. and Symington,L.S. (1999) *Mol. Cell. Biol.*, **19**, 556–566.
40. Paull,T.T. and Gellert,M. (1999) *Genes Dev.*, **13**, 1276–1288.
41. Lingner,J., Cooper,J.P. and Cech,T.R. (1995) *Science*, **269**, 1533–1534.
42. Levy,M.Z., Allsopp,R.C., Futcher,A.B., Greider,C.W. and Harley,C.B. (1992) *J. Mol. Biol.*, **225**, 951–960.
43. Lundblad,V. and Szostak,J.W. (1989) *Cell*, **57**, 633–643.
44. Harley,C.B., Futcher,A.B. and Greider,C.W. (1990) *Nature*, **345**, 458–460.
45. Allsopp,R.C., Vaziri,H., Patterson,C., Goldstein,S., Younglai,E.V., Futcher,A.B., Greider,C.W. and Harley,C.B. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 10114–10118.